

## REVIEW

# DNA damage response proteins in canine cancer as potential research targets in comparative oncology

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**Abstract**

The DNA damage response (DDR) is a complex signal transduction network that is activated when endogenous or exogenous genotoxins damage or interfere with the replication of genomic DNA. Under such conditions, the DDR promotes DNA repair and ensures accurate replication and division of the genome. High levels of genomic instability are frequently observed in cancers and can stem from germline loss-of-function mutations in certain DDR genes, such as BRCA1, BRCA2, and p53, that form the basis of human cancer predisposition syndromes. In addition, mutation and/or aberrant expression of multiple DDR genes are frequently observed in sporadic human cancers. As a result, the DDR is considered to represent a viable target for cancer therapy in humans and a variety of strategies are under investigation. Cancer is also a significant cause of mortality in dogs, a species that offers certain advantages for experimental oncology. Domestic dogs present numerous inbred lines, many of which display predisposition to specific forms of cancer and the study of which may provide insight into the biological basis of this susceptibility. In addition, clinical trials are possible in dogs and may lead to therapeutic insights that could ultimately be extended to humans. Here we review what is known specifically about the DDR in dogs and discuss how this knowledge could be extended and exploited to advance experimental oncology in this species.

**KEYWORDS**

BRCA1, BRCA2, canine cancer, p53, Rad51, TopBP1

**Abbreviations:** 53BP1, p53-binding protein 1; 9-1-1, Rad9-Rad1-Hus1; ATM, autosomal-recessive ataxia-telangiectasia mutated; ATR, autosomal-recessive ataxia-telangiectasia mutated and Rad3-related; ATRIP, ATR interacting protein; BACH1, BRCA1-associated C-terminal helicase; BCRT, BRCA1 carboxyl terminal; BRC, BRCA2 repeats; BRCA1, breast cancer 1; BRCA2, breast cancer 2; CC3, cleaved caspase 3; CDKs, cyclin-dependent kinases; cDNA, complementary DNA; Chk1, check point kinase 1; Chk2, check point kinase 2; CML, chronic myelogenous leukaemia; CMT, canine mammary tumour; DBD, DNA binding domain in p53; DNA, deoxyribonucleic acid; DNA-PKcs, DNA dependent protein kinases; DDR, DNA damage response; EDD, E3 identified by differential display; GST, specific glutathione-S-transferase;  $\gamma$ H2AX, phosphorylated-gamma histone H2AX; H2AX, histone H2AX; HCC, hepatocellular carcinoma; HR, homologous recombination; IR, irradiation; MDC1, mediator of DNA damage protein 1; MDM2, murine double minute 2; MMR, mismatch repair; MRN, Mre11-Rad50-NBS1; MVC, minute virus of canine; NHEJ, non-homologous end joining; NOS, nitrogen species; p21, cyclin-dependent kinase inhibitor p21; p27, cyclin-dependent kinase inhibitor 1B; p53, tumour protein p53; PALB2, partner and localizer Of BRCA2; PCNA, proliferating cell nuclear antigen; PIKK, phosphatidylinositol 3' kinase-related kinases; PTEN, phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase; Rad51, Rad51 recombinase; RAD9, Rad9 checkpoint clamp component of 9-1-1 clamp; ROS, reactive oxygen species; RPA, replication protein A; SNP, single nucleotide polymorphisms; SSCP, single-strand conformational polymorphism; TopBP1, topoisomerase II $\beta$  binding protein 1; tp53, gen of tumour protein p53; UV, ultraviolet light.

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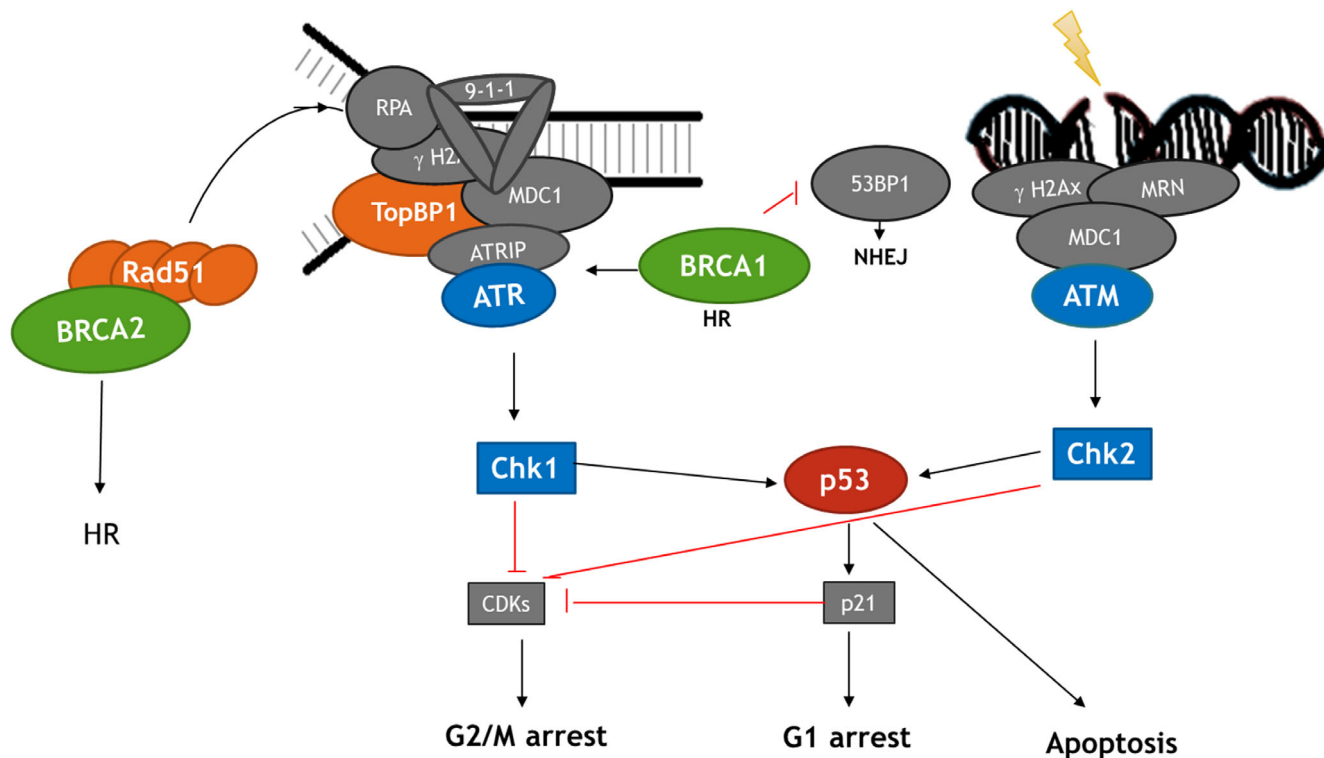
## 1 | INTRODUCTION

### 1.1 | DNA damage response and its role in cancer

The DNA damage response (DDR) is a signalling and effector pathway designed to ensure the genetic stability of eukaryotic cells. It is a complex, multi-faceted process that is activated in response to DNA damage inflicted by both endogenous sources, such as reactive oxygen species (ROS), reactive nitrogen species (NOS), or exogenous sources, such as ultraviolet light (UV) or irradiation (IR).<sup>1</sup> A variety of different mechanisms can contribute to genomic instability.<sup>2</sup> One is enzymatic deamination, which converts 5-methylcytosine to a thymidine residue through removal of the amine group. Replication of the resulting T/G mismatch is mutagenic, and breast and other cancers have been found to exhibit mutation or aberrant expression of cytosine deaminases of the AID/APOBEC families.<sup>3</sup> Another mechanism is microsatellite instability, in which expansions of repeated sequences of DNA occur during replication. Microsatellite instability is linked to mutations in the mismatch repair (MMR) system<sup>4</sup> and are found in hereditary cancers such as Lynch syndrome.<sup>5</sup> Macroscopic chromosome translocations, an abnormal rearrangement of one chromosome segment to another, are frequently observed in lymphoma.<sup>6</sup>

Sporadic DNA damage lesions can also arise as a result of errors during DNA replication.<sup>7</sup> Somatic acquired mutations affecting DNA repair and genome stability genes are frequently observed in cancers, and it is considered that they play an important role in the development of the corresponding sporadic cancers.<sup>8,9</sup>

After a DNA damage lesion occurs, a cascade of highly coordinated signalling events begins which activate cell cycle checkpoints, promote DNA repair, and eliminate cells with irreparable lesions via programmed cell death or apoptosis.<sup>10</sup> This cascade (Figure 1) starts with the recognition of damage by the Mre11-Rad50-NBS1 (MRN) complex<sup>11</sup> and Rad9-Rad1-Hus1 (9-1-1) complexes, which act as damage sensors and help in the recruitment of additional DDR proteins to the damage site. These sensor molecules send signals to recruit the phosphatidylinositol 3' kinase-related kinases (PIKK), ataxia-telangiectasia mutated (ATM), ataxia-telangiectasia mutated and Rad3-related (ATR) and/or DNA dependent protein kinases (DNA-PKcs), which then phosphorylate and activate other transducers and effector proteins. One important target of ATM kinase is histone H2AX, which in its phosphorylated form ( $\gamma$ H2AX) serves as a platform for the recruitment of DDR proteins at the damage site.<sup>12</sup> As a result,  $\gamma$ H2AX is a well-known DNA damage marker widely used in DDR research that can be used to monitor and



**FIGURE 1** DDR signalling cascade. When DNA damage occurs, the cascade of DDR proteins starts. First, sensor proteins (MRN and 9-1-1 complexes) recognize the damage and RPA binds the single stranded DNA (ssDNA). ATM/ATR kinases are recruited to DNA damage site by interacting with the sensor proteins. Those kinases immediately phosphorylate histone H2AX at serine 139 ( $\gamma$ H2AX), which helps to attract repair factors. Then, the mediator proteins (BRCA1, MDC1 and TopBP1) stabilize the protein interactions and increase the damage signalling. First, the transducer kinases ATM and ATR activate the effector kinases, Chk1/Chk2, by phosphorylation, which by activation of p53 or inhibition of CDKs induce cell cycle arrest or apoptosis if the damage is irreparable. The mediators BRCA1 and 53BP1 compete to promote different pathways: BRCA1 promotes HR repair pathways, while 53BP1 favours NHEJ pathway. Note: The principal kinases of the DDR are coloured blue. Other DDR proteins discussed at length in this review are depicted in green (BRCA1, BRCA2) or orange (Rad51, TopBP1), while functional components not discussed in detail are grey. Black arrows symbolize activation, and red lines symbolize inhibition

quantify damage lesions and also to detect DNA fragmentation arising from programmed cell death or apoptosis.<sup>13</sup>

During this initial step other important proteins such as p53-binding protein 1 (53BP1), Mediator of DNA Damage Checkpoint protein 1 (MDC1), Topoisomerase II $\beta$  binding protein 1 (TopBP1) and Breast Cancer 1 (BRCA1) amplify the chromatin modifications and help in activation of the checkpoint effector kinases: Checkpoint kinase 1 (Chk1) and Checkpoint kinase 2 (Chk2). These kinases control multiple cell cycle checkpoints and promote DNA repair by modulating the activity of various effectors, such as cyclin-dependent kinases (CDKs), tumour suppressor protein 53 (p53), and Rad51.<sup>14</sup> Chk1 and Chk2 function to induce cell cycle arrest, DNA repair, chromatin assembly, transcriptional and posttranscriptional regulation of gene expression and cell death through apoptosis.<sup>15–21</sup> Other relevant proteins that participate in this cascade are Breast Cancer Associated 2 (BRCA2), whose principal role is to promote DNA repair by homologous recombination (HR),<sup>22</sup> and cyclin/CDK complexes and CDK inhibitors, such as p21 CIP1, whose role is to arrest the damaged cell at different points of the cell cycle.<sup>23,24</sup> Collectively, the DDR/checkpoint system acts to prevent cell division with damaged or partially replicated DNA and to promote accurate repair to enable the damaged cell to survive without permanent genetic damage or mutation. Evidence suggests that these processes play an important role in preventing neoplastic transformation and cell death under conditions of genomic or oncogenic stress.<sup>25</sup> A simplified diagram of the DDR signalling cascade is shown in Figure 1.

## 1.2 | DNA damage response in canine cancers

The DDR system is highly conserved and its organization and functional components are very similar in all mammalian species.<sup>20,21</sup> The similarity between dogs and humans in this context is special because certain important proteins respond in the same way to DNA damage compared to other species.<sup>26</sup> One example is shown in a study where the expression of p53 protein was monitored at various times after DNA damage in different mammalian species.<sup>27</sup> The authors generated human, mouse, dog, monkey, and rat kidney cell lines expressing a fluorescent p53. After irradiation, they observed p53 protein levels to increase and decrease in an oscillatory pattern over a period of 10 h in all species. The oscillatory profile of response of p53 protein was however found to be much slower and more similar between dogs and humans than in rodents.<sup>27</sup>

The literature lacks extensive information on the course of DDR in dogs and details on the role of its individual components. A number of papers describe selected proteins of the DDR pathway in dogs, however there are no studies that document the overall DNA repair process. In addition, although some studies have focused on the description of selected DDR proteins in relation to mutations, changes in the expression level, and their use in comparative oncology, in general they do not consider how knowledge of the canine DDR system could be used to develop new anticancer therapies.<sup>28,29</sup> One study on canine cell lines infected with a canine minute virus (MVC)<sup>30</sup> provides molecular insights into how the DDR functions in dogs. In the case of the DDR induced by MVC, ATM is known to be necessary to induce the G2/M arrest signal.<sup>30</sup>

However, the proximal effector kinase(s) that mediate the cell cycle arrest remains unclear. Another recent study examining MMR deficiency in canine tumours, has shown that oral malignant melanoma and hepatocellular carcinoma (HCC) lack expression of one or more MMR proteins more frequently than other cancer types in dogs.<sup>31</sup> An interesting study showed differential rates of DNA repair rate in embryonic fibroblasts from humans, dogs, mice and rats.<sup>32</sup> As predicted, the DNA repair rate increased in the species with longer life span, suggesting a positive relationship between DNA repair proficiency and longevity. Non-Homologous End Joining (NHEJ) activity in humans and dogs was the highest both in vitro and in vivo, as longer life span may require a more functional DDR to allow cells extended replicative potential.<sup>32</sup>

Some studies investigating DDR proteins in dogs show important similarities in function and expression with their human homologues. A comparison in Table 1 clearly shows that many of the most important proteins involved in the DDR, such as TopBP1, p53, Rad51, BRCA1, BRCA2, Chk1<sup>a</sup>, Chk2<sup>a</sup>, PTEN, PCNA, p21, p27 and Cyclin A, are highly conserved between humans and dogs, indicating that the dogs can be research models for the investigation of DDR phenomena. Combining these discoveries with the fact that humans and dogs have the highest DNA repair rates, emphasizes again the potential of using of dogs as a research model to uncover biological insights that could be translated to humans.

An additional advantage of the dog model is the fact that in both species, humans and dogs, development of tumours is spontaneous, showing higher incidence with age. Heterogeneous course of disease in different patients with an analogous metastatic behaviour, comparable response to antineoplastic therapies, and alteration in DDR pathway are further similarities.<sup>28,63,64</sup> Additionally, over 360 genetic disorders related to cancer were described in dogs, and this constitutes the largest set of natural genetic disorders in a non-human species.<sup>65</sup> These findings clearly justify the need for close cooperation between veterinary and human oncologists in the field of genetic instability and particularly DDR disorders. The increasing availability of a wide panel of various canine cancer cell lines hugely facilitates such studies.<sup>66–69</sup>

Due to significance of DDR disorders in the cancer development in dogs, this review discusses the key components of the DDR, presents their physiological role and the abnormalities associated with these proteins found in canine cancers. We consider findings on the potential causes of genetic instability in canine cancers and indicate potential new research directions. These directions may contribute to the development of new anti-cancer therapies in veterinary medicine, and in view of the potential role of the dog as a model for the study of human diseases, also in human medicine.

## 2 | SELECTED DDR PROTEINS AND THEIR POTENTIAL ROLE IN CANINE RESEARCH

### 2.1 | BRCA1 and BRCA2

Inherited germ-line mutations affecting the BRCA1 and BRCA2 genes are associated with the development of familial cancer in women.

**TABLE 1** Summary of similarities and differences in DDR proteins between humans and dogs

Protein	Dogs	Humans
TopBP1	<ul style="list-style-type: none"> <li>Similar function in both species</li> <li>Overexpressed in malignancy</li> <li>Cytoplasmic localization in malignancy<sup>33–35</sup></li> <li>Validated antibodies<sup>33–35</sup></li> </ul>	<ul style="list-style-type: none"> <li>Inhibition of p53 pathways when TopBP1 is overexpressed<sup>36</sup></li> </ul>
p53	<ul style="list-style-type: none"> <li>78.4% shared protein identity</li> <li>Similar function in both species</li> <li>Role in malignant transformation when mutated</li> <li>Highly conserved DNA binding region and C- and N-termini</li> <li>Alteration present in different types of cancers</li> <li>Low-frequency inherited mutations in cancer<sup>37–45</sup></li> <li>Validated antibodies<sup>44,45</sup></li> </ul>	—
Rad51	<ul style="list-style-type: none"> <li>99% gene homology</li> <li>Interaction with BRCA2 and BRCT domains</li> <li>Overexpressed in cancer</li> <li>Presence of polymorphisms in tumour<sup>24,46–49</sup></li> <li>Interaction with BRCA2 C-terminal domain<sup>50</sup></li> <li>Validated primers<sup>48,49</sup></li> </ul>	—
BRCA1	<ul style="list-style-type: none"> <li>84% of gene identity</li> <li>BCR conserved regions share 77% homology</li> <li>Mutations increased risk of mammary tumour</li> <li>Increased malignancy when expression decrease<sup>46,51–53</sup></li> <li>Validated primers<sup>46,53</sup></li> </ul>	—
BRCA2	<ul style="list-style-type: none"> <li>68% of protein homology</li> <li>Mutations increased risk of mammary tumour</li> <li>Increased malignancy when expression increased<sup>46,48,52,54,55</sup></li> <li>Validated primers<sup>46</sup></li> </ul>	<ul style="list-style-type: none"> <li>Its loss triggers p53 mutations<sup>56</sup></li> </ul>
Chk1 <sup>a</sup>	<ul style="list-style-type: none"> <li>Inhibition suppresses proliferation<sup>57</sup></li> </ul>	
Chk2 <sup>a</sup>	<ul style="list-style-type: none"> <li>Mutation in cancer<sup>58</sup></li> </ul>	
PTEN	<ul style="list-style-type: none"> <li>Lack of or reduced expression correlated with malignancy<sup>29,59</sup></li> </ul>	
PCNA	<ul style="list-style-type: none"> <li>Increased in proliferating tumours<sup>60</sup></li> </ul>	
p21	<ul style="list-style-type: none"> <li>Overexpressed in tumour<sup>24,61</sup></li> </ul>	
p27	<ul style="list-style-type: none"> <li>Loss is related to malignancy<sup>24,61,62</sup></li> </ul>	
Cyclin A	<ul style="list-style-type: none"> <li>Overexpression correlated with carcinogenesis<sup>23</sup></li> </ul>	

<sup>a</sup>These proteins have not been deeply studied in dogs. For TopBP1, p53, Rad51, BRCA1, and BRCA2, studies that have validated antibody reagents for protein detection, or PCR primers for mRNA quantification, with appropriate positive and negative controls are indicated.

BRCA1 and BRCA2 are tumour suppressors discovered in the early 1990s as genes conferring breast cancer susceptibility.<sup>70,71</sup> Their principal role is to maintain genomic stability by promoting the error-free repair of DNA double strand breaks (DSBs) by HR.<sup>22</sup> Although BRCA1

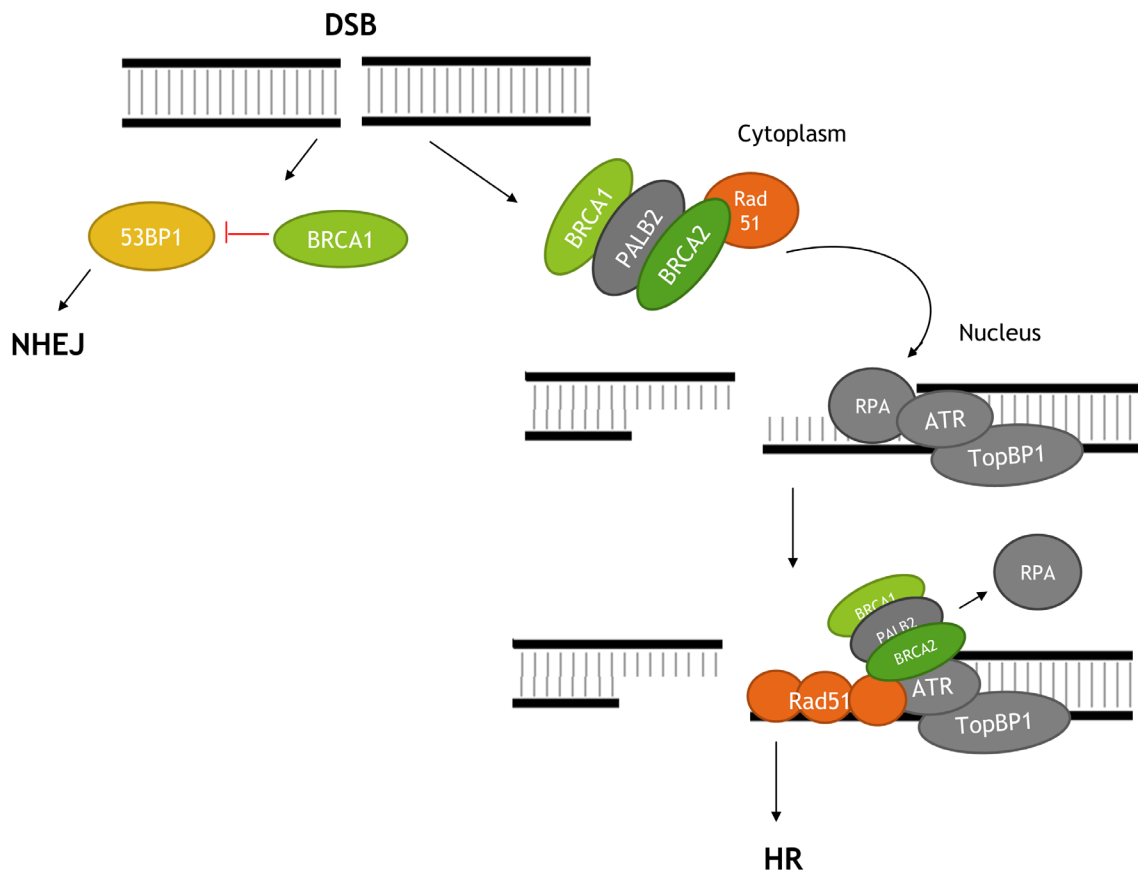
and BRCA2 were initially described as breast cancer predisposition genes, and germ-line mutations in either gene markedly increase the risk of this type of cancer in affected individuals, it is known that inherited or sporadic mutations in BRCA1 and BRCA2 can also contribute to the development of other cancers, for example, ovarian cancer, pancreatic cancer, melanoma, or even prostate and breast cancer in men.<sup>72</sup> BRCA1 and BRCA2 were also reported to play a role in some other genetic syndromes such as Fanconi anaemia.<sup>73</sup>

The important role of these proteins in genomic stability is related to their influence on DNA repair, replication, and transcription.<sup>56</sup> BRCA1 is a multi-functional protein that plays multiple regulatory roles through interactions with other important components involved in each of these processes. Thus, BRCA1 interacts with different DDR proteins such as TopBP1<sup>33,74</sup> or the MRN complex to impede its binding to DNA, it co-localizes with  $\gamma$ H2AX after DNA damage, controls the activity of c-Abl (a protein kinase involved in apoptosis), and is hyperphosphorylated by DDR kinases in response to DNA damage.<sup>22,56</sup> Interestingly, it was shown to have a role in G2/M checkpoint by activating Chk1.<sup>75</sup> BRCA1 possess a highly conserved RING domain in its structure,<sup>53</sup> through which it gains its E3 ubiquitin ligase activity.<sup>76</sup> By means of its C-terminus, BRCA1 is capable of binding some elements of the transcriptional machinery (RNA polymerase II or helicase A), which explains its role in transcription control.<sup>56</sup>

BRCA2 is a large protein that contains eight clustered BRC motifs that are highly conserved in mammalian species. In fact, the BRC motifs constitute the regions of BRCA2 that are the most highly conserved between humans and dogs, with 70% identity.<sup>77</sup> In addition, sequence analysis of germline BRCA2 mutations associated with familial breast and ovarian cancer showed that these occur most frequently in exon 11 (where the BRC motifs are located).<sup>78</sup>

BRCA1 has two roles in HR repair (Figure 2). First, it promotes end resection at the damage site by inhibiting the activity of 53BP1 and consequently the NHEJ pathway.<sup>74,79</sup> Second, it promotes Rad51 to replace RPA bound to ssDNA generated by strand resection. To do this, BRCA1 acts in conjunction with BRCA2, which binds Rad51 and promotes its translocation from the cytoplasm to the nucleus.<sup>80</sup> BRCA1 forms a complex with PALB2 that acts as a link between BRCA1 and BRCA2, forming the BRCA1-PALB2-BRCA2-Rad51 foci at the sites of DNA damage that are needed to start HR repair.<sup>79</sup>

BRCA2's primary role in HR is through its interaction with Rad51 (more information about this interaction is provided in the section on Rad51), to regulate the subcellular localization of Rad51 (Figure 2).<sup>46,50,80</sup> BRCA2 binds Rad51 from its BRC repeats in exon 11 and from its COOH-terminal domain encoded by exon 27 (both exons are highly conserved regions). The importance of the BRCA2-Rad51 interaction for genomic stability was supported by a study of mutant mice bearing a deletion of BRCA2 in exon 27, which exhibited a high incidence of spontaneous cancer.<sup>81</sup> HR also seems to be controlled by p53 interaction with BRCA2, where p53 binds to the BRCA2-Rad51 complex to repress HR. It is not clear yet if this suppression happens because p53 disrupts formation of the BRCA2-Rad51 complex or because p53 prevents binding of the complex to DNA.<sup>82</sup> It is known that BRCA2 deficiency results in chromosome instability, which is



**FIGURE 2** The role of BRCA1/2 in DNA repair via homologous recombination. After a DNA lesion occurs, sensor proteins attract 53BP1 to start the NHEJ. However, when BRCA1 is recruited to the DNA lesion sites, it inhibits 53BP1-TopBP1 interactions and promotes HR pathway instead. BRCA1 helps in the recruitment of Rad51. Then Rad51 forms a complex with PALB2 and BRCA2, and the complex is relocated from the cytoplasm to the nucleus. There, Rad51 binds DNA lesion site by interacting with RPA and replacing it. Finally, Rad51 forms nucleoprotein filaments on ssDNA that create a more stable platform for the repair machinery

related to the poorly understood role of BRCA2 in stabilization of stalled replication forks.<sup>83</sup>

Studies in which BRCA genes were depleted or produced defective proteins, identified the most important roles of these proteins in DNA repair system. The loss of BRCA1 not only results in defective repair, but it also boosts apoptosis, genetic instability, and tumorigenesis.<sup>84</sup> BRCA2 deficiency results in elevated levels of chromosome breaks, probably due to its role in stabilization of stalled replication forks, but more interesting is that its loss triggers tp53 mutations, which can contribute to cancer progression.<sup>56</sup>

The canine BRCA1 gene was described for the first time in a comparative study in 1996,<sup>53</sup> when BRCA1 genes from human, mouse and dog were sequenced and compared. This revealed an 84% identity of the canine gene with its human homologue.<sup>53</sup> Subsequent sequencing of the canine BRCA2 gene showed a greater homology between humans and dogs than between humans and mice, with a 76% cDNA sequence homology and 68% BRCA2 protein homology.<sup>48</sup> Due to the important role of BRCA1 and BRCA2 in breast cancer in humans, most of the studies in dogs focused on mammary tumours. A study on gene expression showed that decreased BRCA2 levels are related to tumour development.<sup>85</sup> These experiments identified

different splicing variants of BRCA2, one of which could contribute to a reduction in BRCA2 protein levels. This particular splice variant encodes a form of BRCA2 that is unable to interact with a stabilizer protein, DSS1 (deleted in split hand/split foot). Thus, this transcript encodes an unstable and dysfunctional isoform of BRCA2 protein.<sup>85</sup> Further studies of single nucleotide polymorphisms (SNPs) in canine BRCA genes have shown that variants of both BRCA1 and BRCA2 genes can be associated with canine mammary tumour (CMT) development as occurs in humans, whilst other SNPs appear only in tumour samples and never in control normal tissue.<sup>52,86,87</sup>

The canine homologues of BRCA genes are also linked to spontaneous cancer and metastases.<sup>88</sup> Nieto and colleagues<sup>51</sup> analysed BRCA1 expression levels in mammary dysplasia and tumours. They showed that in normal mammary gland the expression of BRCA1 was exclusively nuclear, whereas in a neoplastic gland BRCA1 protein was also observed in the cytoplasm. The relationship between BRCA1 expression and malignancy was also analysed and, as expected, malignancy increased when the levels of BRCA1 decreased.<sup>51</sup> In contrast, another study demonstrated that although BRCA1 levels did not correlate with malignancy, overexpression of RAD51 and BRCA2 was observed in lymph node metastases.<sup>46</sup> The association of high

BRCA2-Rad51 expression with malignancy likely relates to their function in DNA repair by HR.

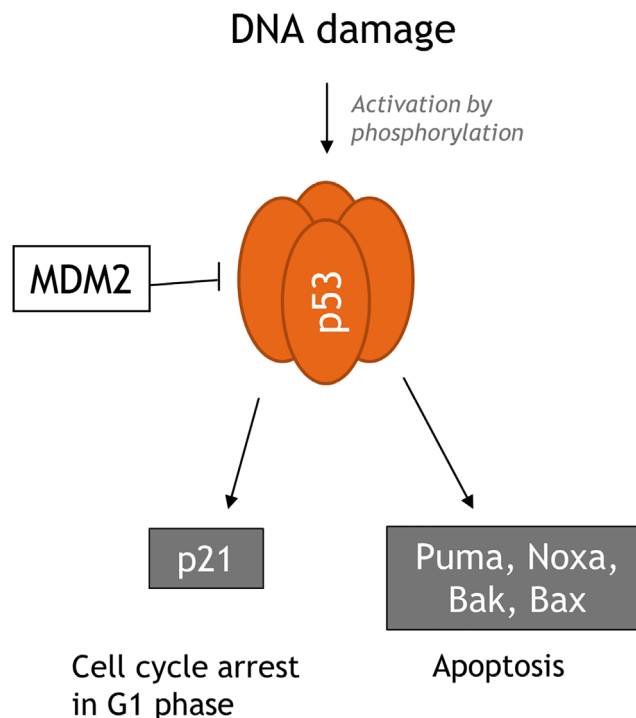
The clinical and molecular similarities existing between the mammary tumours in canines and humans, makes the dog a useful model to study mammary cancer.<sup>89</sup> The observed similarities also apply to the relationship between mammary tumours and DNA damage because mammary tumour development in dogs and women has been associated with deregulation of BRCA1/2 gene function.<sup>85,90,91</sup> As dogs have a long history of inbreeding with low levels of genetic variation, it has been suggested that canine mammary tumour (CMT) development in a single breed should have a more defined homogeneous origin compared with human, which could permit identification of breed specific CMT risk factors.<sup>92</sup> Therefore, the breed predisposition to CMT has been used to study BRCA2 mutations.<sup>80,93</sup> One study conducted in Sweden regarding the English Springer Spaniel breed, which has the highest incidence of CMT in that country, showed that mutations in BRCA1 or BRCA2 increased the risk of the cancer development fourfold. In addition, whereas the frequency of BRCA1 mutations rate seemed to be higher in malignant samples, the frequency of BRCA2 mutations was similar in malignant and benign tumour samples.<sup>52</sup> However, this study did not assess the functional effects of BRCA1/2 gene mutations and further investigations are required to understand the molecular mechanisms responsible for the observed phenomenon in this breed. When considering the aetiology and pathogenesis of CMT, or even genetic/breed predispositions, it must not be forgotten that steroid hormones (oestrogen and progesterone) and their receptors play significant roles in mammary tumour development.<sup>94</sup> As with early pregnancy/oophorectomy in women, early spaying in dogs is linked with lower disease incidence,<sup>95</sup> therefore, any research describing the incidence of CMT must be considered in relation to the current ovariohysterectomy trends in a given country.

## 2.2 | p53

p53 was discovered in 1979 through studies of SV40 antigen T oncoproteins and is widely known as the "guardian of the genome" due to its function in maintaining genomic integrity.<sup>96,97</sup> In its different roles the protein: (a) Acts as a transcription factor for different genes to regulate the cell cycle (Figure 3); (b) Triggers apoptosis in response to oncogene activation or cell proliferation under stress conditions; (c) Silences repetitive sequences of DNA that could be cancer promoters; and (d) Helps in prevention of bacterial and viral infections acting as a part of the innate immune system.<sup>14</sup> It is also involved in anti-angiogenesis and autophagy.<sup>98-101</sup>

The activity of p53 is regulated by multiple post-translational modifications. Site-specific phosphorylation catalysed by multiple protein kinases, including ATM and Chk2, can modulate p53 stability and gene-regulatory functions in diverse ways, depending on the site of modification. p53 is degraded by proteasomes after being ubiquitinated by MDM2, a ubiquitin ligase.<sup>102,103</sup>

After more than 40 years of research on p53, its importance in cancer is clear and a loss of wild type tp53 expression or tumour



**FIGURE 3** Function of p53 in response to DNA damage. After DNA damage p53 is phosphorylated and consequently activated. p53 triggers the cell cycle arrest by inducing expression of p21, which binds and inhibits the activity of cyclin/CDK complexes, such as cyclin E/CDK2 and cyclin A/CDK2 that are necessary for the transition from G1 into S-phase of the cell cycle. p53 can also trigger apoptosis by inducing the expression of other target proteins such as Puma, Noxa, Bax, and Bak, which are pro-apoptotic proteins.<sup>101</sup> A major regulator of p53 activity is a ubiquitin ligase MDM2, which ubiquitinates and targets p53 for rapid degradation via the proteasome in the absence of cellular stress

suppressor function is frequently linked to malignant transformation.<sup>41</sup> In addition, when tp53 is mutated, it can gain oncogenic functions and facilitate cell transformation and tumorigenesis.<sup>38</sup> "Gain of function" mutations can alter p53 molecular functions in complex ways: (a) Through a change of protein function by disruption of protein-protein interactions (e.g., disruption of ATM-MRN complex formation by capturing MRN through interaction with MRE11); (b) By increasing or inhibiting the activity of certain transcription factors (e.g., mutated p53 can bind to p53-related p63/p73 proteins, forming aggregates and preventing their activation in response to DNA damage and consequently suppressing p63/p73-dependent transcription of apoptotic or growth-inhibitory genes such as Bax or p21); (c) Changes in binding properties to other DNA regions not related to gene expression (e.g., matrix elements that participate in chromatin remodelling).<sup>104</sup> Aberrations of p53 expression or function appear in more than 50% of human cancers, and 90% of p53 mutations occur in so called hot-spots, often in the DNA binding domain encoded by exons 5-8.<sup>37</sup>

In veterinary medicine tp53 is an important research topic, and as in humans, it has a role in malignant transformation when mutated.<sup>105</sup>

Veldhoen and Milner isolated the canine version of p53 in 1998 and presented its homology analysis with other mammalian species. Canine p53 protein shares 86.3% homology with feline, 72.3% with murine, and 78.4% with human protein. The central core of the protein, the DNA binding region, together with regions at the C- and N-termini are highly conserved between species<sup>106</sup> and these are also the regions prone to mutations, in both humans and dogs.<sup>39</sup>

Several studies have documented altered p53 expression in different types of veterinary cancers. Takeda et al. analysed mammary tumours, squamous carcinomas and basal cell tumours in dogs and cats documenting p53 protein expression levels by immunohistochemistry.<sup>23,107</sup> Detectable expression of p53 protein by this method was found in 24.6% and 16.3% of mammary tissue samples in dogs and cats respectively, with a higher percentage of expression in the malignant versus benign samples, indicating that p53 overexpression can be related to malignancy. In the case of squamous carcinoma samples, the percentage of cells that expressed p53 was high, 37.5% in dogs and 40% in cats, suggesting an association between high levels of the protein and tumorigenesis.<sup>107</sup> However, no p53 overexpression was detected in the basal cell tumour samples.<sup>23,107</sup>

Another study analysed 170 samples of CMT which were classified into three different groups depending on the malignancy grade.<sup>44</sup> The first analysis was a histological classification. The authors compared the histological grade with the malignancy level of the tumour, observing that higher histological grade was significantly correlated with higher malignancy. Then, sections of each CMT sample were stained with a validated antibody against p53. They observed that p53 was detectably expressed only in a minority of samples, 8 out of 170. A correlation between p53 expression and higher histological grade was observed; but there was no correlation between p53 and malignancy. The conclusion was that p53 expression is related to proliferation of the tumour.<sup>44</sup>

In a more recent study, 35 samples of intestinal cancer were analysed by immunohistochemistry. Histopathology analysis showed that 20 of them were malignant tumours, and the remaining 15 were benign. Interestingly, the malignant samples expressed higher levels of p53 compared to the benign ones. Again, in this study, a validated antibody was used to detect p53, and we can add that we have tested the same antibody and observed p53 expression by western blot in canine cell extracts (BHS and AP, unpublished results). Furthermore, sequencing analysis revealed that 3 of the malignant tissues analysed carried a tp53 mutation.<sup>108</sup>

Another immunohistochemistry analysis performed by Kumaraguruparan and colleagues involved 30 samples of mammary tumours of dogs and humans.<sup>60</sup> The study compared the expression level of p53 and Bcl-2 (an antiapoptotic protein) in tumour and adjacent non-tumour tissues. Both proteins were found to be overexpressed in the tumour as compared with the adjacent normal tissue, 78% and 75% of the samples presented elevated levels of Bcl-2 expression in tumour tissue in humans and dogs respectively, and 75% and 73% of the samples in the case of p53.<sup>60</sup> The authors concluded that the overexpression of both proteins denoted an apoptosis-resistant phenotype in canine and human cells. This finding paved

a path for a concept in which CMT can be used as a model for breast cancer study in humans. A more specific analysis on the mutational status of canine p53 protein and mutations in exons 5–8 of tp53 in 20 mammary canine tumours (12 malignant and 8 benign)<sup>109</sup> confirmed mutations in 33% of the examined tumour samples. The malignant tumours contained four missense and two nonsense mutations, while in the benign tissues two missense and one silent mutation were detected. Five out of six missense mutations were located in the highly conserved regions corresponding to the DNA-binding domain in humans. These regions are often called “hot-spots”. Researchers did a follow-up of the dogs and found that four of them suffered from tumour recurrence after surgery and died. Interestingly, three out of the four deceased dogs exhibited mutant tp53.<sup>109</sup>

Another study examined p53 in multicentric lymphoma in 28 dogs.<sup>110</sup> A total of 19 B-cell and 9 T-cell type lymphomas were assessed, and found to show differential expression of p53, with higher levels in T-cell than B-cell lymphoma as has also been observed in humans. Perhaps surprisingly, levels of p53 expression were similar in all samples within each tumour type, although elevated p53 expression is also rare in human lymphoma.<sup>110</sup> Even though tp53 seems not to be overexpressed in lymphoma, it can be used as a prognostic factor as demonstrated by the genetic study of Koshino et al.<sup>111</sup> They performed a PCR SSPC (single-strand conformational polymorphism) to analyse tp53 mutation in 43 dogs with high-grade lymphoma before and after treatment. They found that only 16% (7 out of 43 dogs) presented a tp53 mutation. After the chemotherapy, 88% of the non-mutation cases responded well to the treatment, versus only 33% of the mutated cases, meaning that tp53 mutations had a negative prognostic significance.

tp53 mutation can also be inherited as in the human multi-cancer susceptibility Li-Fraumeni syndrome.<sup>40</sup> Veldhoen and colleagues investigated tp53 mutations in eight canine lymphomas, comparing tumour and non-tumour tissue. Out of eight dogs only three expressed a mutant tp53, and interestingly one of them presented a heterozygous mutation (tp53+/-) both in the tumour and in normal somatic tissue, indicative of germline transmission. This study was the first evidence of heritable tp53 mutations in dogs.<sup>42</sup> Another study focusing on germline mutations of tp53 involved 10 dogs with cancer. Three of the animals presented a tp53 mutation, including one with heterozygous mutation in normal somatic tissues, confirming heritability of tp53 mutations.<sup>43</sup> Germline mutations of tp53 were also found in multicancer-like syndrome and were suspected to be the cause of the tumour.<sup>58</sup> The mutations analysed in the study were those of tp53 and Chk2, where the latter were associated with failure in G2 arrest and genome instability.

A recent innovative study reanalyzed all the data from the 684 whole genome and exome sequences of canine tumours available to date, in order to quantify tumour mutational burden.<sup>112</sup> The authors performed a comparison between tumour and normal samples from each tumour type analysed, a breed validation in order to detect germline mutations associated to a specific breed, and finally, a human-dog comparison. They observed that there is a relation between somatic mutations and the type of the tumour; T-cell

lymphoma, osteosarcoma, oral melanoma and hemangiosarcoma presented the highest tumour mutational burden. The analysis also showed that the mutation burden was similar among breeds for a given tumour type, meaning that variation is primarily tumour type-specific rather than related to breed. The overall tumour mutational burden values were slightly lower in dogs compared to humans. Another interesting fact is that the tumour mutational burden is correlated with *tp53* mutations. *tp53* mutation have been found in 16.7% of the analysed canine tumours, and it was shown that *tp53* mutations were preferentially present in tumours bearing a relatively higher tumour mutational burden. In the analysis comparing breeds, *tp53* mutations seems to be more frequent in Golden Retrievers, Maltese or Rottweiler, compared to others. However similar oncogenic pathway alterations were observed in canine and human cancers of a given type, suggesting that the evolution of cancer is similar in dogs and humans.<sup>112</sup>

Many immunohistochemistry analyses have been done to study *p53*, but there is the potential for conflict regarding the reliability of this technique when it comes to study of mutations of *tp53*. Strong immuno-expression of *p53* could represent accumulation of non-functional but stable mutant protein, whereas absence of detectable expression could be considered incorrectly as the normal, vanishingly low levels of wildtype *tp53*, when actually *p53* expression is truly absent because of missense mutations.<sup>44</sup> Despite this caveat, the results obtained by immunohistochemistry to detect *tp53* mutations discussed in the following studies,<sup>44,60,110</sup> correlate with results obtained by PCR<sup>108,111</sup> and sequencing analysis.<sup>112</sup> All come to the same conclusion: *p53* mutation can be used as a prognostic factor that its related to proliferation and malignancy of tumours. The novel technique of CRISPR/Cas9 has been used to developed a *tp53* knockout canine cell line,<sup>113</sup> which will mark a milestone in *p53* study in canine cancer. In sum, the results of various studies clearly indicate that *p53* plays a key role in human and animal carcinogenesis. Both the structure and function of this protein (also in its mutated form) showed considerable inter-species homology.

## 2.3 | TopBP1

TopBP1 is a protein with multiple roles in the DDR. It encompasses eight hydrophobic multiple protein–protein interaction domains similar to BRCT (BRCA1 carboxyl terminal), which make it interesting due to the structural similarities to BRCA1. In fact, TopBP1 and BRCA1 share 35% of sequence homology.<sup>33,35</sup> To achieve its diverse functions, TopBP1 interacts with and regulates the activity of a wide range of different DDR proteins (e.g., BRCA1, 53BP1, *p53*, MDC1, ATR, BACH1 (BRCA1-associated C-terminal helicase), RPA/RAD51, RAD9 (Rad9 Checkpoint clamp component of 9-1-1 clamp: Rad9-Hus1-Rad1) by binding through BRCT domains to phosphorylated sites within these partner proteins (Figure 4).<sup>25,36,114,115</sup> The domain of TopBP1 which interacts with and activates ATR (the ATR Activation Domain, AAD) is distinct from the eight BRCTs domains.<sup>116</sup> Importantly, TopBP1 binds not only to multiple interacting partner proteins but also to DNA.<sup>25</sup>

TopBP1 is also involved in HR activity. BRCA2 recruits Rad51 to the RPA-coated ssDNA, but TopBP1 is needed for the formation of Rad51 foci. The mechanism is not clear but available data suggest that BCRT 7/8 domains are essential for filament formation.<sup>117</sup> Another way of HR regulation is through the interaction of TopBP1 with 53BP1 and BRCA1. When both proteins bind to TopBP1, BRCA1 inhibits 53BP1 and resection of DNA ends can occur. In cancer cells without BRCA1, the TopBP1-53BP1 interaction is stabilized and impairs HR, which is likely to result in genomic instability.<sup>74</sup> The authors suggest that the interaction between BRCA1 and TopBP1 is promoted by ATR, which promotes HR under replication stress conditions.

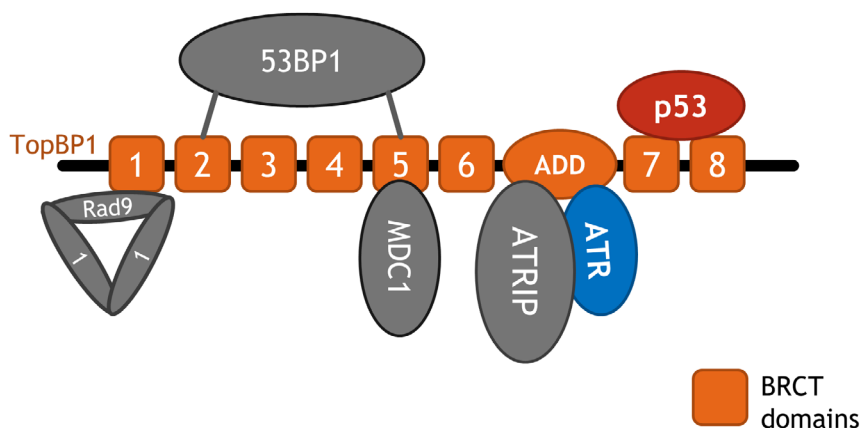
Another interesting function of TopBP1 is the regulation of *p53* protein. The interaction between BRCT 7/8 of TopBP1 with DBD of *p53* inhibits *p53* activation. In this way, *p53* cannot induce cell cycle arrest or apoptosis, and damaged cells continue to survive and replicate with potential for malignant transformation.<sup>36</sup>

Expression of TopBP1 is expected in nucleus, as it is a protein involved in replication and DDR signalling. However, in breast cancer, TopBP1 expression has also been observed in the cytoplasm compared to normal breast tissue.<sup>33,118</sup> A histological study on the expression level of TopBP1 in breast samples from 12 healthy human patients and 61 carcinomas<sup>33</sup> showed that both overexpression and downregulation of the protein were related to cancer. TopBP1 occurred not only in nucleus but also in cytoplasm in cancer cells, suggesting that this mis-localization could be related to malignancy. TopBP1 overexpression also affects *p53* function. When TopBP1 is overexpressed, the G1/S phase checkpoint and apoptosis control mediated by *p53* is inhibited, what promotes cancer development.<sup>36</sup> TopBP1 overexpression could be caused by EDD (E3 identified by differential display) protein, a ubiquitin conjugating the enzyme that controls the localization of TopBP1, which is frequently altered in different types of cancers.<sup>119,120</sup> A schematic description of TopBP1 protein interactions is shown in Figure 4.

The importance of TopBP1 in veterinary medicine is a contemporary and interesting research topic. Immunohistochemistry studies of TopBP1 in dogs and cats with mammary cancer,<sup>34,35</sup> confirmed its expression in all samples but the reaction with the polyclonal antibody against the human protein was much greater in the malignant tissues.<sup>34</sup> As already mentioned, TopBP1 appears in the cytoplasm of malignant but not of normal cells. The pattern of TopBP1 staining in samples for both humans and dogs was similar. In both cases, normal and benign tissues showed nuclear TopBP1, whereas in malignant tissue both nuclear and cytoplasmic staining was observed. In both studies, the incidence of aberrant expression of TopBP1 was found to be statistically significant in malignant samples compared to normal and benign tissue.<sup>33,34</sup> Based on these results, it can be concluded that TopBP1 protein is similar in structure in humans, dogs and cats (cross reaction with human antibody) and probably performs similar functions and undergoes similar changes in canine, feline and human cancers: its overexpression and cytoplasmic localization is related to increased malignancy of mammary tumours.

The role of TopBP1 in canine cancer is probably as significant as in human but thus far relatively little research has been performed on





**FIGURE 4** TopBP1 interactions. TopBP1 contains 8 BRCT domains through which it binds different DDR components, and one AAD through which it interacts with ATR kinase. After DNA damage, MDC1 is recruited to the damage site.<sup>114</sup> It binds  $\gamma$ H2AX at serine 139 and helps in the recruitment of MRN complex.<sup>121</sup> It also binds the 5th BRCT domain of TopBP1, which is recruited to this DNA damage site by interaction with MRN and Rad9.<sup>122</sup> ATR is recruited to the RPA-coated ssDNA, but it is not enough to be activated. TopBP1 interacts with ATR through its ADD domain helping ATR activation in a ATRIP dependent manner.<sup>121,123</sup> This is how TopBP1 participates in the activation of ATR-Chk1 pathway as a response to DNA damage.<sup>124,125</sup> Interestingly, it was shown that 53BP1 can bind TopBP1 along with Rad9, and cooperate in the activation of ATR to control G1/S checkpoint<sup>126</sup>

this subject. Even though TopBP1 has been proved to be related with malignancy, it is not simply a proliferation marker. The previous cited studies compare TopBP1 expression with Ki67,<sup>33,34</sup> which is a nuclear protein frequently used as a marker of proliferation in cancer.<sup>127</sup> Immunohistochemistry staining showed that TopBP1 was expressed in all benign and malignant samples, but only samples expressing Ki67 were proliferating tumours.<sup>33,34</sup> More research is needed both on the structure and function of TopBP1 in animal cancer.

## 2.4 | Rad51

Rad51 is a recombinase of the RecA family of highly conserved proteins that share a common protein fold in their catalytic domain. All members are DNA-dependent ATPases and can create nucleoprotein filaments on DNA that activate the catalytic activity of the DNA bound proteins.<sup>128</sup> Simultaneously, as a component of the DDR, Rad51 plays a central role in HR pathway. The principal function of Rad51 is first to stabilize a DNA chain broken due to direct damage or replication errors, and second, to recognize homologous DNA present in a sister chromatid to start pairing and strand exchange.<sup>128</sup> The requirement for a sister chromatid for DSB repair via HR probably explains why high levels of Rad51 are expressed in cells in the S and S/G2 phases. Rad51 is further stabilized upon formation of the nucleoprotein filament, which can show two conformations (open and closed), making it flexible and dynamic. The regulation of Rad51 activity in DDR is mediated by its interaction with other important proteins of this system. p53, by binding to Rad51, can inhibit strand DNA exchanges and regression of stalled replication forks.<sup>47</sup> BRCA2 is directly related to the regulation and coordination of HR repair mediated by binding Rad51 (Figure 2). Specifically, BRCA2 binds Rad51 through the repeated BRC domains. This way it helps Rad51 both to relocate from the cytoplasm to the nucleus under the conditions of damage and to load Rad51 onto

DNA to form the nucleoprotein filament. During nucleoprotein filament formation BRCA2 helps Rad51 to displace RPA from single stranded DNA.<sup>129,130</sup> Other important interactions of Rad51 involve protein kinases. For example, ATM activates c-Abl which then regulates Rad51 activity. In this context, it is interesting that the oncogenic version of c-Abl, BCR-ABL fusion kinase (expressed in chronic myelogenous leukaemia [CML]) also phosphorylates Rad51.

The level of Rad51 expression is generally higher in human tumour cell lines and in primary tumours than in normal tissues and it is associated with genomic instability and resistance to chemo- and radiotherapies.<sup>131</sup> Also, Rad51 shows sequence polymorphism in different tumours.<sup>132</sup> There are two possible explanations for the elevated Rad51 levels observed in tumour cells. The first is that increased Rad51 expression contributes to malignant transformation, and the second that it reflects the higher proliferation rates of malignant cells, since Rad51 levels are maximal during S and S/G2 under normal conditions.<sup>47</sup>

The canine RAD51 gene cloned and sequenced in 2001 showed a very high homology with human and murine genes.<sup>48</sup> As murine Rad51 was known to interact with BRCA2 through its C-terminus and not only through BRC domains as in humans, the canine Rad51 was tested for its capacity to bind both, the BRC domains and BRCA2 C-terminus. It was found that a deletion of BRCA2 C-terminal domain could increase genomic instability and predispose to cancers in dogs (see BRCA2).<sup>50</sup> This study also demonstrated that Rad51 bound strongly to BRC 1, 2 and 4, moderately strongly to BRC 8, and weakly to BRC 3, 5 and 7, while no interaction was observed with BRC 6 domain. Tests were also performed to check the importance of C-terminus in the strength of Rad51-BRCA2 interaction, where surprisingly, BRC 3, 5 and 8 deletions resulted in stronger binding. Based on these experiments, the authors hypothesized that the strength and correct arrangement of those domains could be related to HR repair

proficiency. In other experiments, the effects of BRC3 and BRC4 polymorphisms on the interaction with Rad51 were investigated. To that end, the BRC domains of BRCA2 gene were sequenced from 236 dogs of five different breeds, three with a high risk of CMT, one with a low risk, and one previously not researched. Two polymorphisms were detected, T1425P and K1435R (described before). The first polymorphism did not appear in Labrador retriever breed, which is interesting, as this breed was the one with low risk of CMT. However, it appeared in 26 other dogs (two were homozygotes). The second polymorphism appeared in all breeds, in 96 dogs (26 homozygotes), but was predominant in Chihuahuas, one of the breed with high risk of CMT.<sup>55,133</sup> Another study analysed the relationship between RAD51 mutations (A209S and T225S) and PALB2 protein. A two-hybrid assay indicated that the interaction of mutant type Rad51 with PALB2 was weaker than that of wild type Rad51. The conclusion was that RAD51 mutations affected oligomerization of the protein and that this could be the reason for its attenuated interaction with PALB2, which could promote cancer in dogs.<sup>49</sup>

Ozmen et al. investigated genetic variations in exons 11 and 27 of BRCA2 gene (a region corresponding to Rad51 binding site), and variations in exons 6 to 9 from RAD51 gene (corresponding to PALB2 and BRCA2 interaction regions) in canine mammary gland. The most prevalent polymorphism found was T1425P in BRC3. This polymorphism was also found by Ochiai et al. and suggested to be associated with CMT.<sup>55</sup> In the second part of the study, a total of nine variations of canine RAD51 gene in exon 7, exon 8, intron 7 and intron 8 were identified. Four of them were non-synonymous and altered the protein sequence. Interestingly, all these potentially significant variations were located in the region binding PALB2 and none in that binding BRCA2.<sup>80,134</sup>

Other genetic variations associated with CMT consist of three single nucleotide polymorphisms (SNP), two in RAD51 gene and one in STK11 (serine/threonine kinase involved in cell growth control). This analysis included 373 dogs of which 212 suffered from CMT. The incidence of the SNPs was as follows: 50.9% in dogs with CMT and 35.4% in healthy individuals for RAD51-SNP1, 42% in CMT and 31.9% in healthy individuals for RAD51-SNP2, and 40.3% in CMT and 26.7% in healthy individuals for STK11. The SNPs in both genes were located in the intronic regions, but neither association was significant enough to provide strong evidence for a causal role in tumour development.<sup>135</sup>

Due to the important functional connections between BRCA2 and Rad51, all studies concerning Rad51 in dogs are carried out in animals suffering from CMT. Some data suggest a possible relation between RAD51 alterations, genomic instability, and predisposition to CMT, but so far RAD51 variations have not been confirmed as a primary cause of cancer.

### 3 | CLINICAL ASPECTS OF DNA DAMAGE RESEARCH IN CANCER IN DOGS

Research on this issue is important both because of the role of DNA damage in the pathogenesis of various types of cancer and because DDR disorders are a potential therapeutic target for cancer therapy.

Examples of how the assessment of DNA damage can help in the clinical treatment of cancer in dogs is shown by the recent results of a number of scientific studies.

It is well known that some dogs breeds are predisposed to lymphoma<sup>136-139</sup> and genetic disease studies in dogs are especially powerful, due to dogs' relative inbreeding and the associated lack of genetic heterogeneity.<sup>140,141</sup> It was hypothesized that lymphoma susceptibility may be associated to breed-related increase in DNA damage and a study to evaluate this hypothesis is the research done by Thamm et al. seeking to explain the cause of the higher incidence of lymphoma in Golden retrievers.<sup>142</sup> The subject of the article is a pilot study on 21 Golden retrievers with lymphoma, 20 age-matched healthy Golden retrievers and 20 age-matched healthy mixed-breed dogs, evaluating DNA repair capability following exposure to either ionizing radiation (IR) or the chemical mutagen bleomycin. The research shows inter-individual variation in DNA repair capacity, evaluated in stimulated canine lymphocytes exposed *in vitro* utilizing the G2 chromosomal radiosensitivity assay to quantify chromatid-type aberrations (gaps and breaks).<sup>143</sup> Surprisingly, the results of the study point to more individual (rather than breed) susceptibility, but at the same time suggests that deficiencies in heritable factors related to DNA repair capabilities may be involved in the development of canine lymphoma. These studies set the stage for larger confirmatory studies, as well as candidate-based approaches to probe specific genetic susceptibility factors.

Another study trying to explain the same genetic/breed predisposition to lymphoma was carried out on boxers.<sup>144</sup> Research aimed to evaluate whether boxer dogs have more endogenous DNA damage in peripheral leukocytes, than age-matched non-boxers, and whether DNA damage is associated with specific Glutathione-S-transferase (GST) alleles. Authors found no difference in leukocyte DNA damage, as measured by the comet assay, between boxers and age-matched non-boxer dogs, nor did they see an association with DNA damage and advancing age within the boxer breed. Observed lack of correlation may be explained by the fact that the experimental set-up was different from that used in the Golden retriever study and relied only on an assessment of spontaneous DNA damage. If the response to induced DNA damage *ex vivo* in boxers versus non-boxers was measured it might possibly uncover DNA repair defects that are masked in a population with heterogeneous exposures.

Another aspect of the clinical use of DNA damage research is shown in the article describing how fish oil supplementation (1000 mg; containing 232 mg EPA and 136 mg DHA), could affect DNA damage in PBMC of healthy dogs.<sup>145</sup> In this study, also no DNA damage was induced, and the percentage of cells bearing spontaneous DNA damage was assessed using comet assay. The study showed that fish oil supplementation not only does not induce DNA damage in PBMC, but actually reduces it. An interesting continuation of this study seems to be to investigate whether a similar effect of fish oil may be observed in conditions of induction of DNA damage in the previously mentioned Golden Retrievers and Boxers. This could be of key importance for an introduction of fish oils as an adjuvant therapy in the treatment of lymphoma in those breeds where the defective

response to DNA damage is considered to be one of the predisposing causes.

The role of diet in the prevention of certain types of cancer has also been shown in a study using the dog as a model for prostate cancer research.<sup>146</sup> Authors presented the first evidence that prostatic DNA damage measured by comet assay may serve as a functional marker of selenium's anticarcinogenic effect on the prostate. Results suggest that measurement of selenium concentration can provide a non-invasive method for titrating and individualizing optimal selenium intake required for prostate cancer protection.

Another example of the potential clinical usefulness of DNA damage assessment may be a study which aimed to determine whether healthy people and dogs in the same households share urinary exposures to potentially mutagenic chemical carcinogens which can lead to the development of urothelial carcinoma.<sup>147</sup> Although voided urothelial cell yields were inadequate to quantify DNA damage, research showed that healthy humans and pet dogs have shared urinary exposures to known mutagenic chemicals, with significantly higher levels in dogs. Correlation studies between mutagenic chemicals found in urine and their effects on DNA damage induction may provide key information on the pathogenesis of urothelial carcinoma in humans and dogs.

Assessment of the DNA damage may also be used in clinical trials to elucidate the mechanism of action of tested agents. Such an application is presented by Dull et al. in their article describing an immunofluorescence assay that distinguishes between apoptosis and drug-induced DSBs by measuring coexpression of  $\gamma$ H2AX and membrane blebbing-associated cleaved caspase 3 (CC3) to indicate apoptosis, and  $\gamma$ H2AX in the absence of CC3 blebbing to indicate drug-induced DNA damage.<sup>13</sup> Because the primary pharmacodynamic endpoint for genotoxic agents is induction of markers of DNA damage repair such assays may have broad clinical and preclinical applicability and be of fundamental importance in the development of new therapies in veterinary oncology.

In veterinary medicine, we have more and more reports on pre-clinical and clinical trials using DNA damage determinations.<sup>148-153</sup> It seems that it is only a matter of time until knowledge about disorders of DNA damage repair in canine cancer cells will be introduced into clinical use to target specific defects with maximally effective therapies. In human medicine, an excellent example of such an approach is the introduction of PARP inhibitors to the treatment of BRCA-dependent breast and ovarian cancer in women.<sup>154-156</sup> Thanks to the dissemination of knowledge about the possibilities of DDR research in dogs, similar targeted therapies could be also introduced in veterinary medicine.

## 4 | CONCLUSIONS AND FUTURE DIRECTIONS

Cancer is a genetic disease linked to genomic instability in the incipient cancer cell. Failures in DDR systems are likely to contribute to cancer development, which makes them an interesting and important

topic in cancer research. The findings we discuss here clearly show similarities between canine and human DDR proteins and justify the use of dogs as valuable models for DDR study. Further studies on the role and significance of key DDR components: BRCA1, BRCA2, p53, TopBP1 and Rad51 proteins in canine tumours will provide the missing information. The importance of such research is significant: if the DDR could be targeted for cancer therapy in dogs, this could aid development of analogous treatments in human oncology.

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## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

## AUTHOR CONTRIBUTIONS

Conceptualization, Aleksandra Pawlak; writing—original draft preparation, Beatriz Hernández-Suárez and Aleksandra Pawlak; writing—review and editing, Aleksandra Pawlak and D.G.; supervision, Aleksandra Pawlak and David A. Gillespie; All authors have read and agreed to the published version of the manuscript.

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