Revisiting the BRCA-pathway through the lens of replication gap suppression:

"Gaps determine therapy response in BRCA mutant cancer"

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Abstract

The toxic lesion emanating from chemotherapy that targets the DNA was initially debated, but eventually the DNA double strand break (DSB) ultimately prevailed. The reasoning was in part based on the perception that repairing a fractured chromosome necessitated intricate processing or condemned the cell to death. Genetic evidence for the DSB model was also provided by the extreme sensitivity of cells that were deficient in DSB repair. In particular, sensitivity characterized cells harboring mutations in the hereditary breast/ovarian cancer genes, *BRCA1* or *BRCA2*, that function in the repair of DSBs by homologous recombination (HR). Along with functions in HR, BRCA proteins were found to prevent DSBs by protecting stalled replication forks from nuclease degradation. Coming full-circle, *BRCA* mutant cancer cells that gained resistance to genotoxic chemotherapy often displayed restored DNA repair by HR and/or restored fork protection (FP) implicating that the therapy was tolerated when DSB repair was intact or DSBs were prevented. Despite this well-supported paradigm that has been the impetus for targeted cancer therapy, here we argue that the toxic DNA lesion conferring response is instead single stranded DNA (ssDNA) gaps. We discuss the evidence that persistent ssDNA gaps formed in the wake of DNA replication rather than DSBs are responsible for cell killing following treatment with genotoxic chemotherapeutic agents. We also highlight that proteins, such as BRCA1, BRCA2, and RAD51 known for canonical DSB repair also have critical roles in normal replication as well as replication gap suppression (RGS) and repair. We review the literature that supports the idea that widespread gap induction proximal to treatment triggers apoptosis in a process that does not need or stem from DSB induction. Lastly, we discuss the clinical evidence for gaps and how to exploit them to enhance genotoxic chemotherapy response.

Keywords

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1. Overview, taking on the DSB dogma

The concept that DSBs are lethal has been widely described [1,2] and contributed to the idea that DSBs are the killing lesion emanating from genotoxic chemotherapy [3]. While defects in DSB repair (DSBR) often characterize cells with high sensitivity to genotoxic therapy, this correlation may not be causation and requires consideration of other underlying defects. Here, we review this DSB dogma and present a counter proposition that single stranded DNA (ssDNA) is the defining toxic lesion that induces apoptosis without the formation of DSBs. In support of this ssDNA model, we outline the following points. First, while the DSBR defect has been deemed the cornerstone of chemotherapy sensitivity in BRCA deficient cells, these cells also suffer from an inability to suppress and repair replication-associated ssDNA gaps. Second, BRCA deficient cells that acquire resistance display not only restored DSBR, but also replication gap suppression (RGS). Third, in a growing number of cell models, the current DSB model is challenged or requires complexity, whereas gaps readily predict response. Fourth, the predominant lesion commonly induced by a range of genotoxic therapies is ssDNA. Finally, genotoxins induce apoptosis, a cell death response that fragments genomic DNA and could therefore be an indirect source of DSBs. When evaluated in whole, these emerging findings provide sufficient evidence for considering a counter model. Have we distracted ourselves with the DSB and overlooked the core sensitizing lesion?

2. Genotoxic chemotherapy generates a range of lesions, but the DSB is considered the most toxic one

Historically, genotoxic chemotherapy such as ionizing radiation (IR) was found to induce a range of lesions [4]. In addition to DSBs, in which both strands of the DNA phosphodiester backbone are broken, ssDNA breaks (SSBs), nicks, or gaps were identified. Nicks interrupt a single strand of the DNA phosphodiester backbone and maintain clean 3′-hydroxyl ends that enable repair synthesis or ligation, whereas SSBs carry damaged ends that require processing prior to ligation [5]. Larger regions of ssDNA or "gaps" can occur via nuclease extension of nicks/SSBs or when replication undergoes re-priming reactions to skip a replication blocks that lead to daughter strand gaps forming behind the fork as recently reviewed [6,7]. Notably, ssDNA nicks and SSBs vastly outweigh the number of DSBs detected in response to most genotoxic agents often thought to kill via DSBs such as etoposide and IR [3,4,8–10]. Nevertheless, DSBs gained center stage. One argument for the lethality by DSBs derives in part from the impression that DSBs were among the most lethal forms of DNA damage [1,11–13]. Moreover, as compared to other types of DNA damage, DSBs were regarded as intrinsically more difficult to process and repair. Toxicity was also thought to derive from erroneous rejoining of DSBs that compromise genomic integrity as chromosomal material was lost, gained or scrambled. If cell progression was not blocked, following cell division, daughter cells either lacked the requisite genetic material or had extra genomic material incompatible with cell viability. And if cell death was avoided, genomic instability and tumorigenesis would gain a foothold [14]. By contrast, SSBs were considered to have little biologic consequence as far as cell killing because SSBs were

deemed easy to repair using the opposite template. As such, IR is expected to generate an abundance of SSBs but the far fewer DSBs were regarded as the cell lethal event [15].

3. BRCA deficiency is a model of chemotherapy sensitivity and is defective in DNA repair

A resounding finding that validated the presumption that DSBs were the sensitizing lesion stemming from cancer therapies was based on the BRCA model of chemosensitivity. Not only were tumors with mutations in the hereditary breast and ovarian cancer genes *BRCA1* or *BRCA2* hypersensitive to genotoxic chemotherapy [16–18], but also a key feature of BRCA deficiency was the failure to repair DSBs by homologous recombination (HR) [19,20] and reviewed in [21]. Given the BRCA deficiency model linked to DSBs, when these cells were sensitive to genotoxic agents in which the source of the DSB was not entirely clear, it was proposed that the persistence of lesions or their repair intermediates became DSBs when confronted with DNA replication forks [22–24]. This concept was applied to hydrogen peroxide (H_2O_2) that was proposed to kill BRCA deficient cells via SSBs being converting into DSBs [24]. Moreover, camptothecin or etoposide, drugs that "trap" topoisomerase I- or 2-linked cleavage complexes, respectively were proposed during replication to convert to single-ended DSBs [25–27]. Similarly cross-linking agents that induce DNA interstrand crosslinks (ICLs), such as cisplatin were expected to create replication blocking lesions that upon collision with active forks generated DSBs [28]. In some cases, the transition of a stalled fork into a "collapsed" (terminally arrested) broken fork, was associated with structure-specific nucleases that cleave replication intermediates [29–31]. Replication fork breakage was also linked to severe nascent strand degradation [32,33]. This line of reasoning was supported by the finding that replication was required for the maximum toxicity following treatment with genotoxins [34].

The extreme sensitivity of BRCA deficient cells to inhibitors of poly (ADP-ribose) polymerase (PARPi) lead to the initial proposal that PARPi induced SSBs ultimately collide with replication forks to generate DSBs [36,37]. When PARPi was found to "trap" PARP proteins on chromatin, the resulting bulky DNA lesion was predicted to be more cytotoxic than unrepaired SSBs because DNA replication was blocked and therefore stalled forks collapsed into DSBs [38–41]. PARPi was further shown to compromise replication fork stability [42] providing additional evidence for a compromised fork model. When PARPi was shown not to stall or collapse the fork, but rather increase replication [43], the model was further modified. High speed replication was proposed instead to generate DSBs, an accommodation that maintained the framework that DSBs were the toxic lesion necessitating BRCA function in HR [43].

The line of reasoning that "all roads lead to a DSB" at the fork aligned well with the observation that BRCA proteins counter fork collapse. Fork protection (FP) is genetically uncoupled from HR and is a function by which BRCA proteins protect stalled replication forks from undergoing aberrant degradation [44,45]. In the absence of BRCA proteins, stalled forks are over-digested by nucleases such as MRE11 and forks are expected to degrade into DSBs [44–46]. This FP function is shared with other HR pathway proteins such

as RAD51, FANCD2, FANCJ, and RAD51 paralogs [46–52], suggesting that HR and FP are both concerted pathways. The implication also being that sensitivity in tumors with BRCA deficiency is due to defects in HR and/or FP [28]. Accordingly, tumors irrespective of gene mutation that share this sensitivity and potential defects in HR and/or FP were considered to share the BRCA deficiency phenotype, BRCAness [53]. The link of replication to the BRCA proteins as well as genotoxicity furthered the concept that diverse lesions/replication blocks funnel into DSBs during replication and kill cells unless countered by the BRCA-RAD51 pathway (Fig. 1).

Further supporting that genotoxic agents kill via DSB induction, chemotherapy resistance in BRCA cancer was linked to restored DSBR and/or FP [54] implying that the ability to repair or prevent DSBs was essential for cancer cells to survive genotoxicity. In BRCA deficient cancer the most common mechanism of resistance is via secondary mutations in the *BRCA* genes that reinstate the BRCA reading frame, and in many cases full BRCA function [55–57]. In BRCA1 deficient cells, HR was also found to be restored by loss of anti-resection factors such as 53BP1 [58,59]. In BRCA2 deficient cells, in the absence of HR, FP alone was found to be restored and able to confer resistance [60]. Thus, loss of the ability to repair or prevent DSBs through HR and FP, respectively characterized chemosensitive BRCA deficient tumors and HR and/or FP restoration characterized the emergence of chemoresistance (Fig. 2).

4. Could something else confer chemotherapy response?

It seems implausible in the face of all this evidence that alternative mechanisms contribute to or solely cause sensitivity to genotoxic agents. Here, it is important to consider the emergence of nonconforming outlier cases. These models present the argument that HR and/or FP alone may not be sufficient to mediate chemotherapy response to genotoxic agents. For example, initially FP alone was found to confer chemoresistance in BRCA2 deficient cancer [60]. However, with the identification of additional fork degradation factors, it is clear that loss of some but not all fork degradation factors impact the response of BRCA deficient cancers to genotoxic agents [28,54,62,63]. Thus, the FP model was complicated and required concessions. Moreover, there was the unexpected case of a Fanconi anemia (FA) patient cell line, which harbors one mutant RAD51 T131 P allele. These cells displayed the characteristic sensitivity to cisplatin associated with the chromosomal instability disease, but surprisingly these cells were proficient in HR [64]. Thus, the role of HR in chemoresistance was unclear or at minimum the DSB model of chemotherapy response required complexity. Conceivably, HR is not optimal at DSBs associated with forks or HR alone cannot compensate in this model because there are FP defects that generate so many DSBs. However, even when FP is restored in this model sensitivity persisted [62,63]. Thus, it is critical to consider the other possibility, HR and/or FP is not sufficient to confer resistance because DSBs are not the killing lesion emanating from genotoxic chemotherapy. For DSBs to be demoted or possibly irrelevant, several criteria would have to follow. First, BRCA deficiency would cause defects in HR, FP and another function. Second, acquired resistance would restore this distinct function in cells also displaying HR and/or FP. Third, restoration of this distinct function would explain outlier cases in which HR and/or FP were not restored but cells displayed acquired resistance. Specifically, this alternative function

would remain coupled to therapy response whereas HR and FP would in some cases be uncoupled. Fourth, cancer therapies believed to kill via DSBs, would induce a distinct toxic lesion (s). Assuming DSBs are toxic, they would have to be extraneous somehow as would be the case if derived indirectly from genotoxic agents.

5. BRCA deficient cancer cells have defects in replication gap suppression (RGS)

The argument that a loss of distinct function aside from HR and FP confers chemosensitivity in BRCA deficient cells requires identification of another defect. Here, there is a growing body of research indicating that BRCA-RAD51 pathway functions in DNA replication [65] with its deficiency generating replication restraint defects and gap formation [45,47,64,66–73]. Gaps in BRCA deficient *Xenopus* extracts were initially visualized on newly synthesized daughter strands at and behind the replication fork by electron microscopy (EM) [47]. Gaps were also observed in chromosome spreads in BRCA2-deficient cells following stress and speculated to form on the lagging strand upon fork uncoupling [45]. Moreover, gaps are an outcome associated with loss of several Fanconi anemia (FA) proteins including FANCD2 [48], FANCM [74], and the FA associated nuclease, FAN1 [75]. In response to ICLs, FA patient lines accumulate the ssDNA binding protein, RPA [76] consistent with gap formation. In the absence of the BRCA-FA pathway, internal gaps and gaps forming at the fork junction were associated with a failure to restrain replication in response to stress [47,48,63,66,69,77–79]. While details about gap size and relationship to other lesions is still emerging, visualization by EM revealed that in RAD51-depleted extracts internal- and fork associated gaps were generally under 200–300 nucleotides [47]. A recent study identified replication associated daughter-strand gaps in BRCA deficient cells that were independent of DSBs [80].

While it is not entirely clear how BRCA proteins function to suppress gaps, it is foreseeable that canonical functions are relevant to both restrict gap formation during replication and fix gaps in post-replication. In particular, BRCA function in the S phase checkpoint could ensure forks slow properly and limit repriming reactions that generate gaps [77, 81,82]. Consistent with this point, local fork conflicts activate a global replication arrest signal to halt polymerase activity [83,84]. High levels of ssDNA results from either loss of the S phase checkpoint [85–88], or loss of RAD51 [70]. Loss of RAD51, similar to loss of other fork remodeling factors such as HLTF and SMARCAL1 leads to gaps dependent on PRIMPOL [52,70,83,89] suggesting failure to slow and reverse replication forks favors repriming. Given that fork reversal does not require BRCA1 and BRCA2, reviewed in [71] their loss could more indirectly cause gaps due to checkpoint defects that fail to restrain repriming. How repriming is regulated and if coordinated by checkpoint and/or the BRCA-RAD51 pathway remains to be determined.

Moreover, gaps could be minimized by BRCA-RAD51 recombination activity that either promotes fork restart to limit repriming and new origin firing or facilitates gap repair during or in post-replication [68, 90]. Recombination-dependent replication restart initiated by RAD51 filament formation on a gap occurs without induction of a DSB, reviewed

in [91]. As opposed to DSBs thought to form when unrepaired lesions, gaps or nicks collide with the fork [92,93], gaps could directly induce recombination [94]. Indeed, gaps provide excellent substrates for recombination and sister chromatid exchange reactions [95,96]. Recombination also contributes to template switch (TS) that promotes DNA damage tolerance and fork continuation as well as gap repair in post-replication reviewed in [6,7].

Finally, gaps and their expansion could be limited by the BRCA-RAD51 pathway that inhibits nuclease degradation of nascent strands [47,65]. Given that fork stalling is rare in unchallenged cells, repair and protection of nascent strands gaps as opposed to stalled forks could be more central to BRCA-RAD51 function in unperturbed cells [80]. Similar to a stalled fork, however some resection of a gap could be fundamental for RAD51 loading and recombination-based gap repair [91]. Recent findings demonstrate that resection of gaps is required for recombination reactions in post-replicative gap repair [97]. Given that unregulated extensive gap resection could derail gap repair, it will be critical to understand how resection is modulated during and in post-replication.

A direct role for the BRCA-RAD51 pathway in preventing gaps by way of promoting replication proficiency is also developing with rescue of this function in BRCA deficient cells being essential to therapy resistance. Recently, we found that the BRCA-RAD51 pathway functions in lagging strand DNA synthesis akin to PARP1 [62,98]. PARP1 activation normally recruits XRCC1 to promote gap filling reactions that can ensure the completion of lagging strand synthesis as a backup to canonical Okazaki fragment processing (OFP) [98–100]. For example, when canonical OFP is disrupted by FEN1 inhibition, PARylation (PAR) is elevated [98]. Similarly, we observed that BRCA-RAD51 deficient cells have abnormally high PAR as found previously [101]. In fact, BRCA1 deficient cells resemble cells treated with a PARPi, in which PARP1 is aberrantly "trapped" in chromatin [62]. A more focal 53BP1 is also observed in BRCA1 deficient cells [62] consistent with replication issues [102–105]. Even though PARP1 and PAR are elevated, backup OFP fails to engage in BRCA1 deficient cells. Our data indicate that PARP1 is unable to recruit XRCC1 to salvage OFP in BRCA1 deficient cells because it is foiled by the aberrant accumulation of 53BP1. Consistent with this idea, 53BP1 deletion, restores XRCC1 levels along with LIG3 that resuscitates backup OFP and robust replication. Moreover, loss of canonical OFP factors, FEN1 or LIG1 elevates PAR in control cells more than in cells lacking BRCA1 and 53BP1. By contrast, depletion of non-canonical LIG3 elevates PAR in these cells more than in control cells. This shift in lagging strand synthesis in favor of a XRCC1-LIG3 backup mechanism [62] is in accord with BRCA1 and 53BP1 deficient cells being uniquely sensitized to PARPi by LIG3 loss [106].

The BRCA-RAD51 pathway could normally facilitate lagging strand synthesis by the interaction between RAD51 and polymerase alpha (Polα) [72]. Studies in *Xenopus* extracts indicated that by promoting RAD51 binding to replicating DNA, BRCA2 was able to prevent ssDNA gap accumulation [47,72]. Interestingly, Polα functions in the 53BP1-Shieldin complex [102,107,108]. Thus, in BRCA-RAD51 deficient cells, Polα could be sequestered or blocked by the 53BP1-Shieldin complex. As such, 53BP1 loss could restore Polα-LIG3 dependent-OFP. While lagging strand gaps in BRCA1 deficient cells are suppressed by loss of 53BP1 [62], the BRCA pathway also functions in preventing

gaps during perturbed replication and filling gaps in post-replication suggesting that the gap phenotype in BRCA deficient cells could result from loss of more than one function [63,71].

6. When BRCA deficient cancer gain resistance, RGS is also restored

In addition to the emerging evidence that indicates that gaps are separate lesions behind the fork created by genotoxic chemotherapy and suppressed by the BRCA-RAD51 pathway, RGS is linked to therapy resistance. In particular, ssDNA gaps accurately predict chemo-response and RGS predicts resistance in cell culture, the TCGA patient database, and patient xenografts. Although the molecular triggers are different in the BRCA1 and BRCA2 backgrounds, the fundamentals of the chemoresistance mechanisms appear to be conserved (reduced ssDNA gaps) [62,63] (Fig. 3).

The mechanism for RGS in BRCA deficient cells appears to occur by several mechanisms. As described above, BRCA1 deficiency is buffered by pathways that enhance replication proficiency such as backup lagging strand synthesis [98,109,62]. In BRCA1 deficient cells, our findings indicate that lagging strand gaps due to OFP defects are an intrinsic vulnerability that sensitizes to inhibitors of PARP1, FEN1 or Ligases as well as the alkylating agent, methyl methanesulfonate (MMS), know to induce gaps [10]. As with PARPi, BRCA1 deficient cells gain resistance to these agents upon 53BP1 deletion, furthering the point that resistance is gained by "fixing" of the OFP defect that suppresses gaps [62].

In response to stress induced by a range of agents, it also appears that gap induction is limited by changes in fork dynamics. In particular, when fork responses that slow replication in response to stress are restored in BRCA deficient cells, such as achieved by genetic reversion, gaps are reduced [62,63]. Gaps are also avoided when fork elongation is hyper-restrained or blocked. For example, gaps due to PARPi [62] or loss of the fork remodeler, HLTF [52], are suppressed in FANCJ deficient cells a result that correlates with reduced fork progression. Suggesting hyper-restraint could confer therapy resistance in BRCA-RAD51 deficient cells, a sensitive BRCA1 mutant ovarian cancer cell line, BR5 that gains resistance, BR5-R1 demonstrates a fork hyper-restraint phenotype akin to FANCJ null cells [62]. The hyper-restraint conceivably prevents replication from developing gaps due to OFP defects or repriming reactions that "jump" over obstacles.

Without restored fork restraint in BRCA-deficient cells, gaps are also suppressed when the tolerance pathway of translesion synthesis (TLS) gains access to the replisome to promote continuous replication without gaps [110]. While TLS can function to fill gaps post-replication, reconstitution studies indicate that TLS can operate "on the fly" to maintain replication when the normal replicative polymerases stall at an impediment [111]. Moreover, evidence from cancer cells and genetic models of chemoresistance reveals TLS is aberrantly activated to keep replication moving during stress without gap induction [110]. In particular, CHD4 depletion in BRCA2 deficient cancer elevates TLS and chemoresistance by a mechanism in which replication is further unrestrained but gaps are suppressed [63,112]. TLS suppresses gaps in a pathway distinct from the BRCA-RAD51 pathway [47], implicating that TLS could underlie the mutation signature of BRCA2 mutant

cancer [80,113, 114]. Selectively targeting TLS in cancer holds great promise [115] as its loss resembles premature aging [116] consistent with RGS as a fundamental viability mechanism.

In addition, evidence suggests that RGS that elevates therapy resistance can be achieved when gaps are protected by RPA. Consistent with this mechanism conferring gap suppression, we find that RPA loss by depletion or chemical inhibition augments the synthetic lethality between PARPi and BRCA1 deficiency whereas its overexpression restores PARPi resistance [62]. Notably, high levels of tumor RPA also correlate with poorer clinical outcomes [117], suggesting that cancer cells may gain genotoxic resistance or better fitness by protecting gaps with RPA.

In the absence of the BRCA pathway, it is not clear whether fork restart and RGS by recombination repair or template switch (TS) during or post-replication occurs given these processes involve a homologous donor for strand transfer or template switch reactions that are in part RAD51-dependent [90,91,118]. Suggesting that it is possible in the context of a stalled fork, in BRCA deficient cells, replication forks readily reverse into structures associated with TS and RAD51 can operate in part independent of the BRCA proteins [7,91]. Other mechanisms resolving stalled and reversed forks that would minimize gap induction could also be in play such as resolution by an approaching fork [6]. Understanding how known (Fig. 4) or other RGS mechanisms are regulated will be critical to targeting and developing durable cancer therapies. It is also important to appreciate that some jumping is tolerated and supports cell fitness consistent with a threshold of permissible gaps. Indeed, in BRCA deficient cells, PRIMPOL provides an adaptive response to stress and supports survival [7]. The relative gap threshold could vary from cell to cell and relate to the level of gap protection mediated by ssDNA binding proteins, such as RPA [119].

7. Gaps are distinct from degraded forks

Conceivably gaps seed fork degradation or vice versa and therefore are ultimately the same thing. Inconsistent with this idea, gaps are observed in non-challenged BRCA-RAD51 deficient cells that show high PAR in S phase consistent with OFP defects [62]. Moreover, in response to stress, gaps are observed behind the replication fork - not at the replication fork- are not likely to seed fork degradation or the collapse of forks. Specifically, gaps arise in BRCA deficient cells that fail to properly slow replication in response to stress; as such the ssDNA phenomenon is detected during low dose hydroxyurea (HU) that does not fully deplete nucleotide pools [63]. By contrast, fork degradation is typically studied following high dose HU in which forks are fully arrested [45]. Furthermore, gaps remain detectable after MRE11 inhibition or depletion of the fork remodeler SMARCAL1 [63], which generates the replication fork structure degraded by MRE11 in BRCA2 deficient cells [72,120,121]. These findings suggest that gaps found behind the fork form in nascent DNA prior to remodeling or degradation of replication forks. However, it remains to be determined whether gaps at fork junctions are linked to fork degradation and/or are precursors to internal gaps found distal to the fork.

The BRCA2 mutant V-C8 cell line complemented with BRCA2 S3291A demonstrates that nascent strand degradation does not seed gaps. These cells are HR proficient, but FP deficient [45]. As reported, these cells display nascent strand degradation after high dose HU, but gaps are suppressed in replication tracts during low dose HU or following PARPi and cells are resistant to both agents [45,62,63]. This cell line also indicates that nascent strand degradation is not linked to therapy response as discussed below.

Further separating a stalled fork structure from nascent strand degradation, a recent study found that high dose HU caused both nascent strand degradation and induced reactive oxygen species (ROS). Indicating the ROS was complicit in the degradation, fork stalling alone such as induced by aphidicolin that inhibits the replicative polymerases did not correlate with nascent strand degradation. Moreover, nascent strand degradation following HU was suppressed when ROS was quenched. Instead, ROS was found to activate the signaling kinase, ATM and in turn MRE11 that mediated the nucleolytic resection at replication gaps behind the fork [80].

While gaps in BRCA2 deficient cells in response to low dose HU were not eliminated by MRE11 inhibition, nuclease digestion explains why nascent DNA was largely degraded following extended incubation with HU [63]. Thus, gaps could form as a consequence of OFP defects and/or replication fork repriming that in either case is extended by nucleases [6, 72,89]. However, in either case, the nascent strand degradation occurs at gaps, not at stalled forks per se, consistent with the concept that gaps are a unique structure distinct from stalled forks or DSBs. This conclusion is also supported by findings in yeast cells. Following genotoxic agents, gaps were identified in the wake of replication forks as a separate entity away from replication forks, stalled forks and DSBs [122].

8.   RGS is uniquely coupled to chemotherapy response

In support of the framework that replication gaps are the sensitizing lesion as opposed to DSBs, there are several emerging examples. First, the DSB model requires complexity because in some cases, HR proficient [59] or FP proficient [60] cells are as expected chemoresistant, but in other cases HR proficient [64,76], FP proficient [45] or HR and FP proficient [62,63,76] cells are unexpectedly chemosensitive. Thus, HR and FP vary in their relation to PARPi response, a wrinkle requiring the model to include exceptions.

By contrast, the gap model does not require this complexity. It accurately predicts drug response even in the more unexpected cases, in which both HR and FP are proficient (RADX depletion in RAD51 T131 P mutant FA cells) [64,123], but cells are chemosensitive, or in which both HR and FP are deficient (FANCJ K/O cells) but cells are PARPi resistant [62,63]. Gaps also predict drug resistance outcomes in genetic (BRCA1 and 53BP1 deficient cells) and *de novo* models of including cancer cells and patient tumors [62]. In all cases, the sensitive cells have gaps and the resistant cells have RGS. While the restored HR could also be contributing to the resistance in the BRCA1 and 53BP1 deficient cells [58,59], re-sensitization upon LIG3 loss induces gaps, not DSBs [106], reinforcing that gaps align with the response. Moreover, drugs such as an ATR inhibitor that re-sensitize BRCA deficient

cancer cells to PARPi, an outcome proposed to be achieved by HR disruption [124] also restores gaps [110] requiring further consideration of what is causative.

Second, in BRCA2 mutant cancer or FA cells loss of MRE11 or fork remodelers such as SMARCAL1, HLTF, or ZRANB3 reveal that restored FP does not confer resistance [63,76]. Moreover, loss of these genes does not predict poor patient response in BRCA2 mutant ovarian cancer [63] suggesting that in tumors restored FP does not direct therapy response. By contrast, loss of genes such as CHD4, EZH2, or FEN1 that restores FP as well as RGS, elevates chemoresistance and predicts poor patient response [60,63,125,126]. Whether TLS activation is the mechanism conferring resistance in other models of gap suppression (i.e. loss of EZH2 or FEN1) remains to be determined [63]. While possibly distinct, TLS blocks the slowing and reversal of forks that creates the structure degraded in BRCA deficient cells [110,120,121] and, therefore, it follows that when RGS is restored by TLS, FP should also be restored.

The gap model clarifies why high-speed replication as recently proposed [43] does not sensitize cells to PARPi. "Fast forks" due to p21 depletion does not generate gaps and accordingly, cells are not sensitive to PARPi [62]. In summary, gaps are predictive even when HR and FP are not [63]. Said more simply, HR and FP can be uncoupled from therapy response, but gaps remain coupled to therapy response.

Importantly, the gap model also provides clinical insight as to why PARPi has unexpected applications in tumors without the classical BRCAness [127]. Correspondingly, PARPi is synthetic lethal with loss of genes having no corresponding defect in HR or FP [128,129]. Instead, the list of synthetic lethal interactions invokes combined lagging strand gaps as a clear determinant of sensitivity. Not only does PARP1 function at the lagging strand, but also its inhibition sensitizes cells deficient or mutated in OFP such as flap endonuclease I (FEN1), LIG1, XRCC1, or proliferating cell nuclear antigen (PCNA) [98,128,130–132]. Intriguingly, and further raising lagging strand gaps as a cause of toxicity, FEN1 loss is synthetic lethal in BRCA-FA deficient cells [133–135]. In addition, BRCA deficient cells are further sensitized to PARPi when expressing a PCNA mutant that disrupts its ubiquitination at lysine 164 and have a synthetic lethal relationship with loss of USP1, an enzyme that removes this PCNA ubiquitination [132,136,137]. In both cases, ssDNA gaps accumulate which could derive from a catastrophic loss of OFP. These points also align with our finding that BRCA deficient cells have OFP defects akin to PARPi treated cells [98] that we propose underlies the synthetic lethal relationship [62].

9. Cancer therapies generate lots of ssDNA

As described above, initially ssDNA in the form of nicks, SSBs or gaps were considered in the toxicity of genotoxic agents because ssDNA was the predominate lesion identified following diverse chemotherapeutic agents [138,139]. For example, etoposide was found to induce 30-fold more SSBs than DSBs, and ionizing radiation (IR), was found to induce 100 nicks for each DSB [34,9,140,141]. Even when DSBs were considered the major cause of therapy-induced toxicity, it was observed that SSBs outnumbered DSBs that in some cases were barely detectable [138, 142]. Moreover, cisplatin also generates SSBs that are greater

in sensitive cells than resistant cells [143], a clue that ssDNA could mediate toxicity to cisplatin as suggested by our recent findings [63]. Furthermore, cisplatin combined with IR improved responses and accordingly there was a greater induction of SSBs [144]. Notably, the predominance of SSBs following a range of genotoxins was especially clear when sub-lethal cellular doses were analyzed [69].

The idea that persistent nicks, lesions, or trapped protein complexes are further processed to become DSBs or evolve into DSBs at the fork is plausible and provides rational for why HR deficient cells are sensitive, but it is not logical to apply this model conditionally only when a DSB framework is required. The premise that SSBs lead to DSBs is confounded by the finding that drugs or genetic alterations thought to work this way also sensitize HR proficient cells. Notably, MMS, H_2O_2, or loss of the chromatin modulator, ALC1 is cell lethal with BRCA1 or XRCC1 deficiency [24,145,146], the latter with no discernable role in HR. Given that BRCA1 and XRCC1 share gap suppression/repair, but not HR, logically one would conclude that the underlying sensitivity in cells deficient in these proteins derives from unresolved gaps. Instead, the eventual DSB is described as the sensitizing lesion in BRCA deficient cells [24,145,146].

The notion that perturbed replication forks stall or collapse into DSBs is also worth reviewing. The replisome from bacteria to human cells is dynamic with the ability to bypass or jump over lesions/roadblocks [7, 147–150]. Early on, this phenomenon was detected following ultraviolet (UV) radiation that only marginally delayed DNA replication but left gaps in its wake [151–153]. Thus, rather than blocking DNA replication, UV lesions were bypassed leaving gaps on either of the daughter strands [147,154,155]. More recently, DNA interstrand crosslinks (ICLs) were found to be circumvented by a mechanism called replication traverse in which DNA synthesis was initiated just past the ICL [84,156]. In mammalian cells, repriming or traversing has been linked to PRIMPOL [89,157]. PRIMPOL not only enables bypass of bulky adducts but also provides a gap that engages HR in post-replication [97]. In response to agents that do not present a lesion blocking barrier, PRIMPOL is also activated suggesting that repriming is common to many forms of stress, reviewed in [7]. Likewise, it was also uncovered that PARPi did not pause or arrest replication forks, but rather accelerated them [43]. A phenomenon, we find is associated with ssDNA gaps [62]. Thus, we propose disparate lesions/trapped proteins interface with replication and cause a mutual induction of fork skipping/jumping/re-priming of replication that leaves gaps in the wake of replication (Fig. 5).

10. Are DSBs relevant to the response to genotoxic agents?

While the field has "grown up" with the idea that DSBs are toxic and underlie the killing induced by genotoxic chemotherapy at least with respect to BRCA deficiency, it is worth a brief discussion about the universality of this concept. While it is not clear that a non-repairable, or erroneously repaired DSB induced by endonucleases has physiological relevance with respect to genotoxic chemotherapy, it is important to appreciate that findings in such model systems impacted the field. Initial yeast models employing the HO-endonuclease that makes a DSB, indicated that one DSB led to cell death [158], a model in which the DSB is irreparable, and cutting activity is not curtailed. Likewise,

one unrepairable DSB, in a RAD52 null cell, led to cell death, a finding thought to stem from loss of essential genes at the broken chromosome [159]. Moreover, spontaneous sister chromatid breaks if unrepaired in yeast cells were lethal [160]. Without RAD52 or loss of homologous donors, notably a small percent of cells survived through nonhomologous end-joining (NHEJ) mechanisms [161,162], indicating that DSBR was required for survival. One DSB introduced by CRISPR-Cas9 reduced fitness when the checkpoint was induced [163–165], although the gene targeted functions to relocate DSBs confounding interpretation because its loss could also disrupt DSBR [166–168]. Notably, in a range of cancer cell lines, cell fitness was not limited by CRISPR-Cas9 unless extensive cutting occurred consistent with unresolved DSBs being toxic [169]. Thus, a DSB could be lethal, but this is likely contingent on whether the DSB is left unrepaired, is in an essential gene, and/or activates a checkpoint. Thus, it may not be fair to assume that DSBs from genotoxic chemotherapy are toxic if DNA repair is active.

A key issue in judging the role of DSBs in cancer and future cancer following genotoxic chemotherapy [170] is the range of lesions as well as cell death responses emanating from them such that the initiating event and causal relationships are unclear. As discussed above, if repairable, it is not logical that genotoxic therapy induced DSBs would be lethal. Programmed DSBs are essential for V(D)J recombination and these DSBs are resolved by the same pathways as DSBs induced by IR [1]. Moreover, while nucleases degrade reversed forks and have been shown to elicit a MUS81-dependent fork rescue that includes DSB formation in BRCA2-deficient cells [171], nuclease degradation of nascent strands can also be extensive without DSB formation [80,172]. Furthermore, resection of gaps is required for post-replicative gap repair by HR [97]. Thus, degradation occurs on many DNA substrates with and without DSB formation making its relevance to genotoxic therapy unclear.

Most chemotherapies induce apoptosis, a physiological cell death mechanism [173–175]. DNA fragmentation accompanies apoptosis, and it was initially considered as a potential source of drug induced DSBs [4]. Whereas HU doses titrated with cytotoxicity, DSBs were speculated to be indirect and due to apoptosis and its associated nucleases [138, 153]. Notably, ssDNA is associated with the initial response from genotoxic chemotherapy, identifies apoptotic cells, and is itself toxic when delivered to cells [176–183]. Even though ssDNA was found as the initiating lesion, later, the logic was flipped and lesions including DSBs were largely thought to cause apoptosis [35]. To define cause and effect relationships, an important step is to inhibit apoptosis and determine if chemotherapy induced DSBs or other lesions are suppressed, a control rarely done. When done, however there is a striking suppression of DSBs suggesting that DSBs largely result from apoptosis [63,184–186]. Thus, observed DSBs could result from the cell killing mechanism and not directly from the stalled forks that are cleaved or resected into DSBs. In addition to defining how apoptosis is linked to DSB formation, it will be important to understand the kinetics of events emanating from genotoxic chemotherapy. This analysis of kinetics and apoptosis dependence will be essential to address whether agents not initially generating DSBs, such as MMS [10] do so later because SSBs become DSBs at the fork or rather because the widespread SSBs trigger apoptosis especially when SSB repair pathways are blocked, such as PARP1, XRCC1, or Polymerase B [187].

Indeed, another finding that adds to the model that DSBs are the offending lesion in BRCA deficient cells is the high levels of radials and other genomic re-arrangements. These are expected to derive from DSBs that are co-opted by unregulated end joining pathways. There is a well-characterized competition between HR and NHEJ pathways for processing DSBs. BRCA1 promotes HR by restricting anti-resection factors such as 53BP1 that blocks DNA end resection that is required for HR [188]. Thus, with loss of BRCA, NHEJ is more active and drives genomic instability. The question that has yet to be examined is whether these chromosomal phenotypes result from chemotherapy induced DSBs or rather from apoptosis induced DSBs. The drug doses employed to induce these genomic rearrangements are often cell lethal in BRCA deficient cells. Furthermore, BRCA1- defective cells as compared to proficient cells have a greater growth inhibition and apoptosis induction [16,189]. Correspondingly, BRCA1 was found to be critical for normal development and mutant embryos were found to die late in gestation due to apoptosis [190,191]. Acute inactivation of BRCA2 impedes completion of DNA replication which is associated with chromosome segregation defects and cell lethality in non-transformed human cells as well as mouse models [17,73,192].

11. Is ssDNA toxic?

Given that ssDNA forms as part of many biological processes and can be readily repaired [193], gaps emanating from genotoxic agents would be expected to be tolerated unless distinct in some way. Genotoxins could invoke toxicity because gaps develop in genomic regions escaping repair, checkpoint or cell quality control mechanisms. Unlike gaps at a stalled fork, gaps behind the fork could be overlooked or lack coordination with cellular checkpoint responses that elicit a global replication arrest [87]. Furthermore, toxicity could stem from genotoxins directly or indirectly generating gap lengths that exceed RPA protection or the capacity to form secondary structures. Unprotected gaps at some threshold could signal to cell death pathways [181] or be degraded by nucleases and convert into DSBs [87]. Grossly under-replicated gapped DNA could remain until it is too late to either fill-in or repair leading to post-S phase arrest, mitotic cell death, or division without viability due to incomplete or unstable genomes [42,73,119,194]. When cells remain viable, under-replicated regions when segregated can seed chromosomal breakage, rearrangements, and aneuploidy [195]. As discussed, gaps can initiate recombination with the possibility for genomic instability without conversion to and independent of DSBs [5,80,196,197]. In combination with mutagenic- replication mechanisms and -cytidine deaminases, ssDNA can seed clusters of hypermutation [198]. Moreover, ssDNA can induce mutagenesis due to the activity of error-prone TLS polymerases that function during or in post-replication repair. Recent findings from *Xenopus* extracts demonstrate that nicks unload the replisome necessitating replication to resume via the loading of a new helicase therefore revealing that compared to other lesions, nicks are inherently more lethal due to replisome disassembly [199], although the nick in this model further develops into a single-ended DSB intertwining these lesions. Accordingly, ssDNA has the potential to promote deletions, insertions, single nucleotide variants, and cause distinct mutagenic signatures [197]. Therefore, gaps can provoke phenotypes associated with tumor formation. Another consequence of ssDNA induction such as due to FANCD2 loss or virus infection is cellular senescence [48,200].

It is also worth noting that ssDNA blocks transcriptional elongation by RNA polymerase II which is further linked to the induction of apoptosis [201–203]. In summary, depending on the gap threshold, its location, and cellular repair capacity, ssDNA has the potential to promote not only checkpoint responses, but also genomic instability, senescence, or cell toxicity.

Another important question is the relationship of RGS, HR, and FP to cell viability. Viability required HR whereas FP had a minor role in mammary epithelium [73]. However, restored FP was sufficient alone to support viability in BRCA2-deficient mouse embryonic stem cells [60, 204]. The distinction could relate to cell type specificity or additional underlying functional differences not considered. In particular, in these models it will be critical to assess the contribution of RGS for cell viability and address if concurrently restored with either HR or FP. Indeed, a basic problem in suppressing and repairing gaps provides a more direct and logical explanation for why loss of the *BRCA* tumor suppressor genes leads to under replication and proliferation defects as opposed to uninhibited proliferation [42,73,194,205]. Identifying the mechanisms that allow cancer cells to overcome this replication deficit will provide new opportunities for therapeutic intervention.

In summary, genotoxic agents rapidly induce gaps during replication whereas DSBs are more delayed and may depend on apoptosis. Sensitivity is associated with defects in RGS and found in cells that are otherwise HR and FP proficient. Moreover, resistance is associated with restoration of RGS even when cells are HR or FP deficient [63]. These observations suggest a model wherein gaps initiate apoptosis that in turn limits cell survival and generates DSBs as an indirect consequence of genotoxic agents (Fig. 6).

11.1. Limitations of the gap model

One issue in assigning toxicity to a distinct lesion is the fact that genotoxic drug sensitivity is relative. Thus, while gaps may contribute to cell sensitivity, sensitivity could be greater if additional functions such as HR and/or FP were also defective. Developing further separation-of-function models will be fundamental for gaining greater clarity on this issue. Moreover, little is known about the gap parameters that are toxic, such as number, length, distribution, and location. Consequently, the nature of a toxic gap threshold and how it relates to the drug dosing or duration is undetermined. Furthermore, mechanistic understanding of the gap model, while linked to clinical response (i.e. loss of genes leading to RGS predicts poor patient response), largely stems from cell culture and a few patient-tumor samples [62,63]. Thus, clinical validation in mouse models and additional patient samples will be fundamental. Lastly, if agents are found not to generate gaps but are nevertheless toxic to cancer cells with BRCAness, the conclusion that gaps underlie genotoxic agents will also be challenged and require a rethinking.

11.2. Future directions

The gap model, that ssDNA stretches, and not DSBs or replication fork collapse, direct the response from genotoxic agents will ideally be thoroughly tested and provide new insight to the fields of cancer chemotherapy and DNA repair/replication. Gaps as the determinant of genotoxic chemotherapy response will transform how biomarkers are viewed and how drug

combinations are considered. Rather than scoring tumors for the inability to repair/prevent DSBs, we will score the inability to suppress/repair gaps, and a high basal gap level will be predictive of sensitivity or BRCAness. Rather than generating drugs that make DSBs, we will seek agents that maximize gaps. Furthermore, we will pair drugs that induce gaps by distinct mechanisms or that simultaneously limit gap suppression. Indeed, combinations of drugs that induce gaps and block gap suppression by TLS, appear to be more effective than each drug alone [110]. And if gaps are the initiating signal for apoptosis, promotion of RGS or inhibition of apoptosis could serve similar ends and therapies will need to limit these pathways to be durable chemotherapies. If a key physiological difference between tumor and normal cells is gaps, then conventional agents that induce apoptosis will provide precision cancer therapy.

Understanding how gap suppression functions align with other BRCA roles in genome preservation, cell viability, and tumor suppression will be critical future questions. For insight, one can look upstream in the BRCA pathway. The MRN complex, composed of MRE11, RAD50, NBS, activates ATM, which is essential for DSBR. Components of the MRN complex and ATM are highly mutated in human cancer, but surprisingly, ATM was recently revealed to have tumor suppression activity largely uncoupled from its DSBR function. Instead, cancer mutations were linked to defects in ATM checkpoint signaling [206]. Previously, loss of ATM checkpoint function was linked to a phenomenon called radiation resistant DNA synthesis (i.e. replication during stress) and the high sensitivity to IR was proposed to evolve from this checkpoint defect as opposed to defects in DNA repair [207,208]. Moreover, ATM loss creates only a mild HR defect, but robustly sensitizes to PARPi [209], suggesting again that something other than a DSB repair defect and underlying DSBs contributes to PARPi sensitivity. Indeed, ATM loss creates widespread gaps [210] and ATM is activated independent of DSBs, by the gap precursor ROS [211]. These findings suggest that loss of ATM-MRE11 gap prevention/repair could underlie its pathology and seed cancer. An important consideration in this hypothesis, is that a high level of gaps is toxic as demonstrated following oncogene expression that reduces cell fitness [110]. Thus, following oncogene expression or loss of tumor suppressors, such as ATM, gaps could initially serve to restrict cancer. If so, for cancer to ultimately develop, gap suppression should be critical [110]. An interesting supposition is that RGS is also essential for tumors to overcome physiological challenges such as hypoxia, and changes in cell adhesion or tumor microenvironment to metastasize. Consistent with this model, TLS polymerases are elevated by low oxygen and counteract replication stress [80,115].

Collectively, through the lens of replication gaps, one can view genotoxic chemotherapy and the model of sensitivity, BRCA deficiency with predictability of response that underscores gaps as a key feature of BRCAness. By comparison, the DSB model is not fully predictive of tissue culture or patient tumors response to genotoxic chemotherapy. Moreover, adhering to the DSB framework requires greater complexity, concessions, and extensions. Thus, rather than remaining in this box and operating in the confines of its construct, better to challenge its core basis, a process that will ideally transform the understanding of cancer, aging and disease.

Acknowledgments

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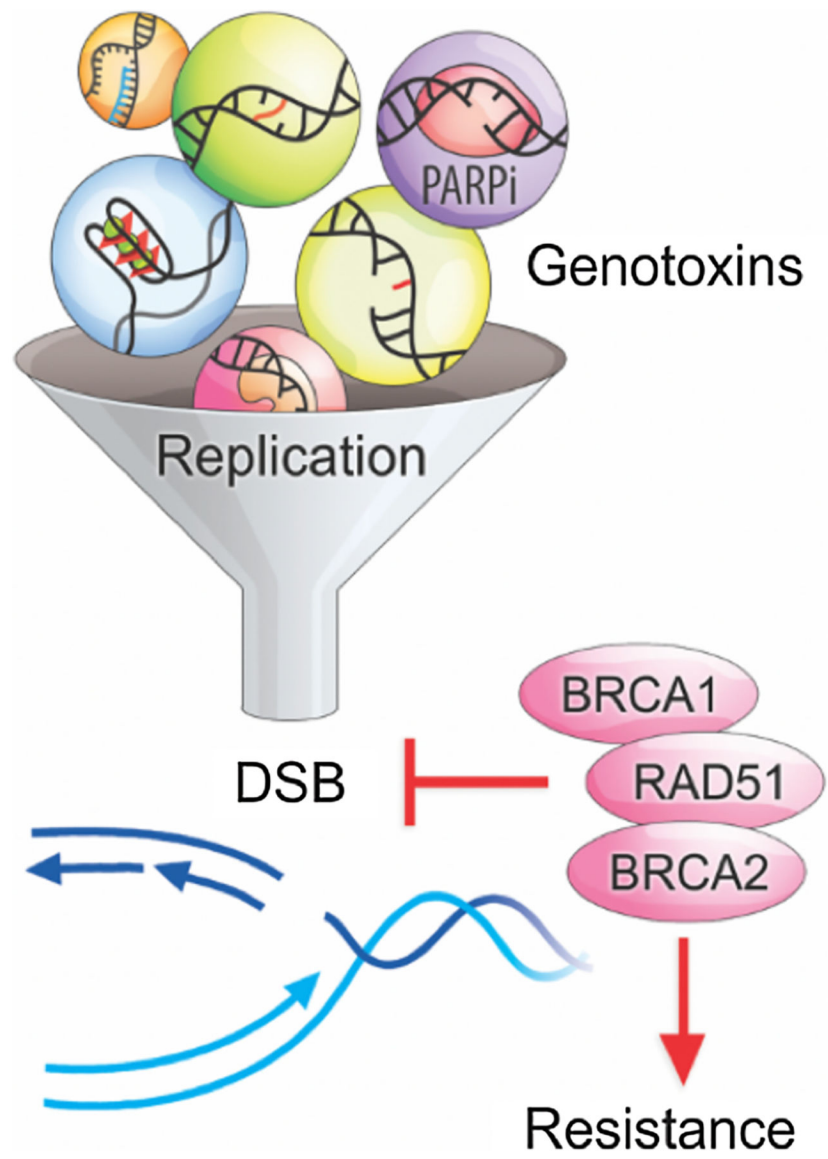
Fig. 1. All roads lead to DSBs.

Model that genotoxins inducing diverse lesions (i.e. crosslinks, trapped PARP or Topo complexes) ultimately convert during DNA replication into DSBs requiring BRCA-RAD51 pathway for resistance.
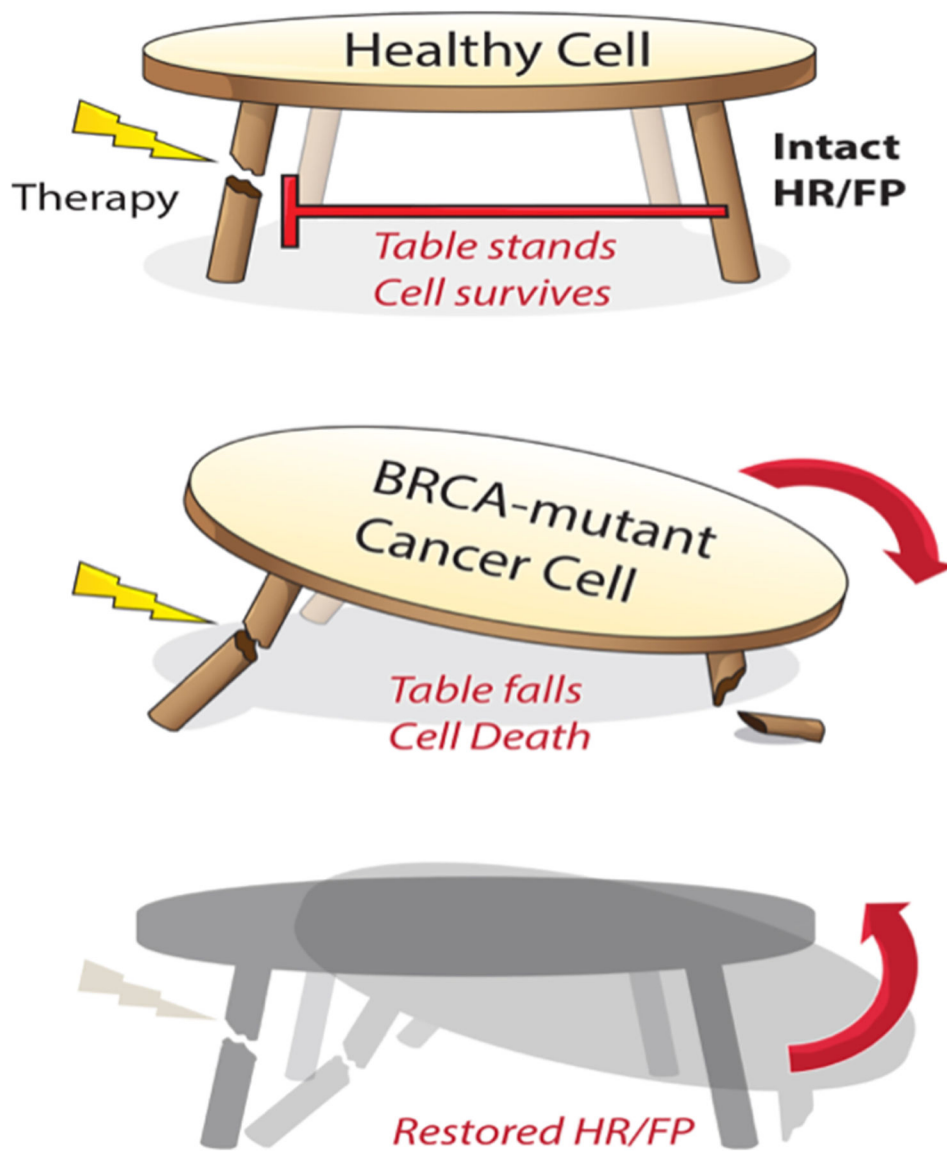
Fig. 2. BRCA deficiency is a model of chemosensitivity supporting the concept that genotoxins induce DSBs.

BRCA deficiency causes defects in both the repair and prevention of DSBs, due to loss of HR and FP, respectively and cells are sensitive to genotoxins thought to make DSBs. Restoration of HR or FP is linked to chemoresistance.
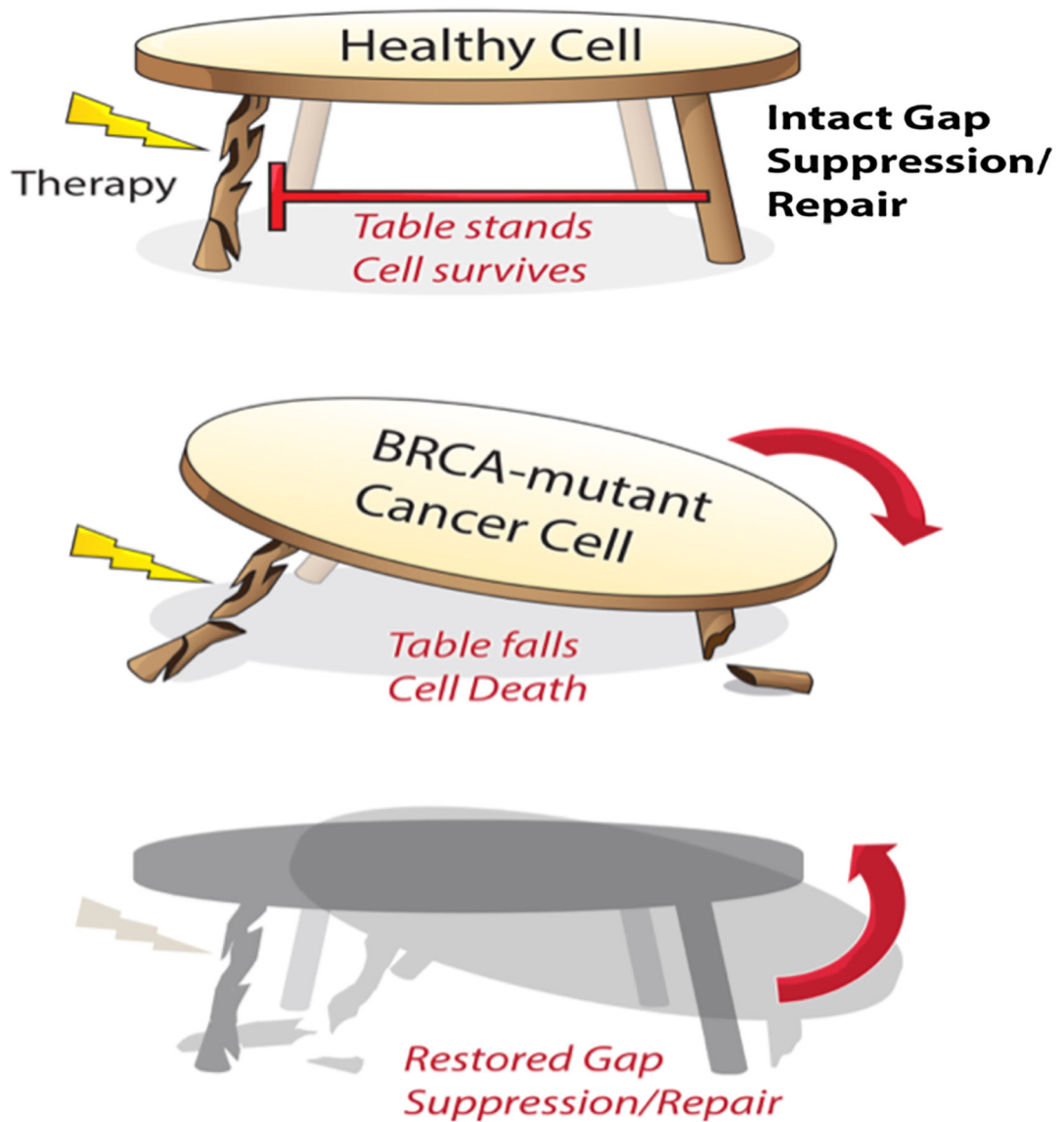
Fig. 3. BRCA deficiency as a model for chemosensitivity due to loss of Gap suppression/repair.
BRCA deficiency causes defects in gap suppression/repair and cells are sensitive to genotoxins without DSB induction. Restoration of Gap suppression/repair is linked to chemoresistance.
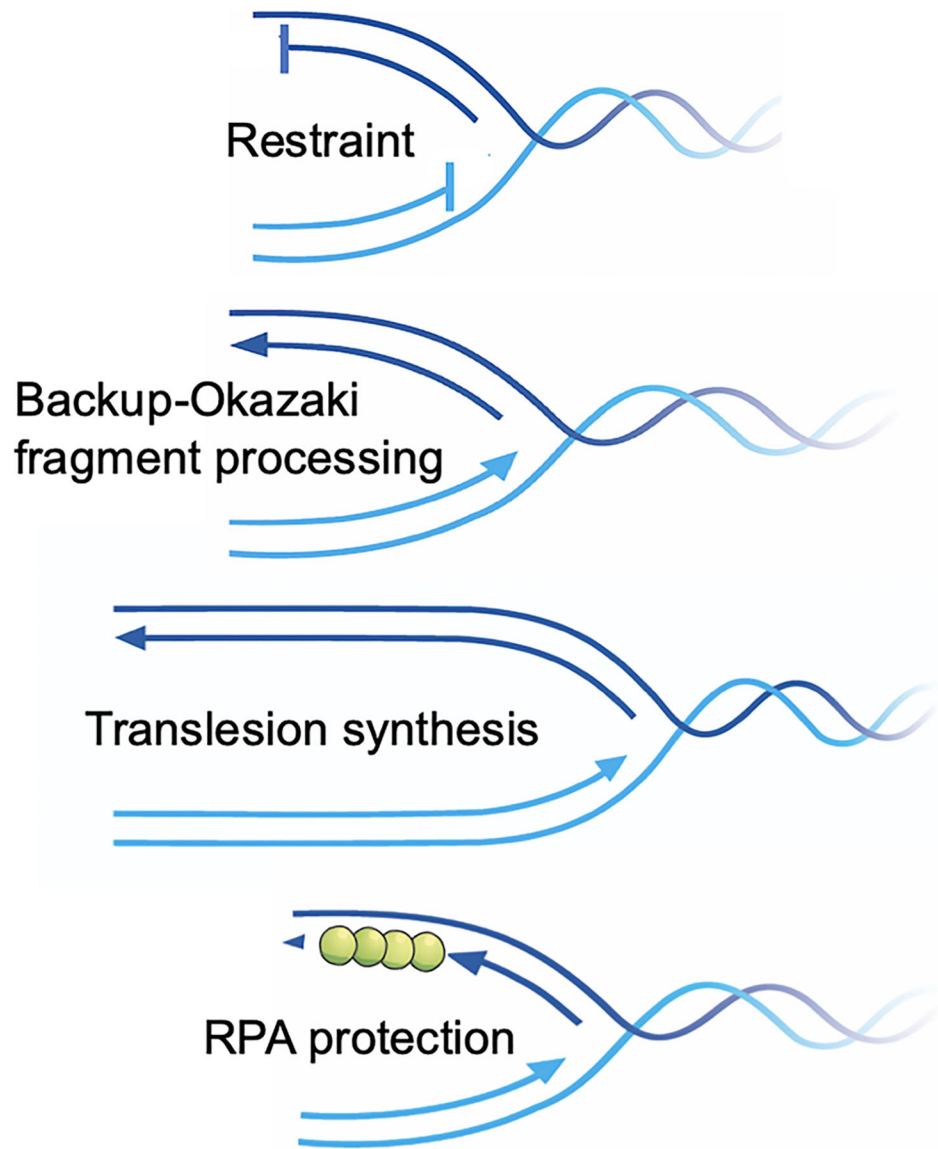
Fig. 4. Mechanisms of RGS in BRCA-deficient cells.
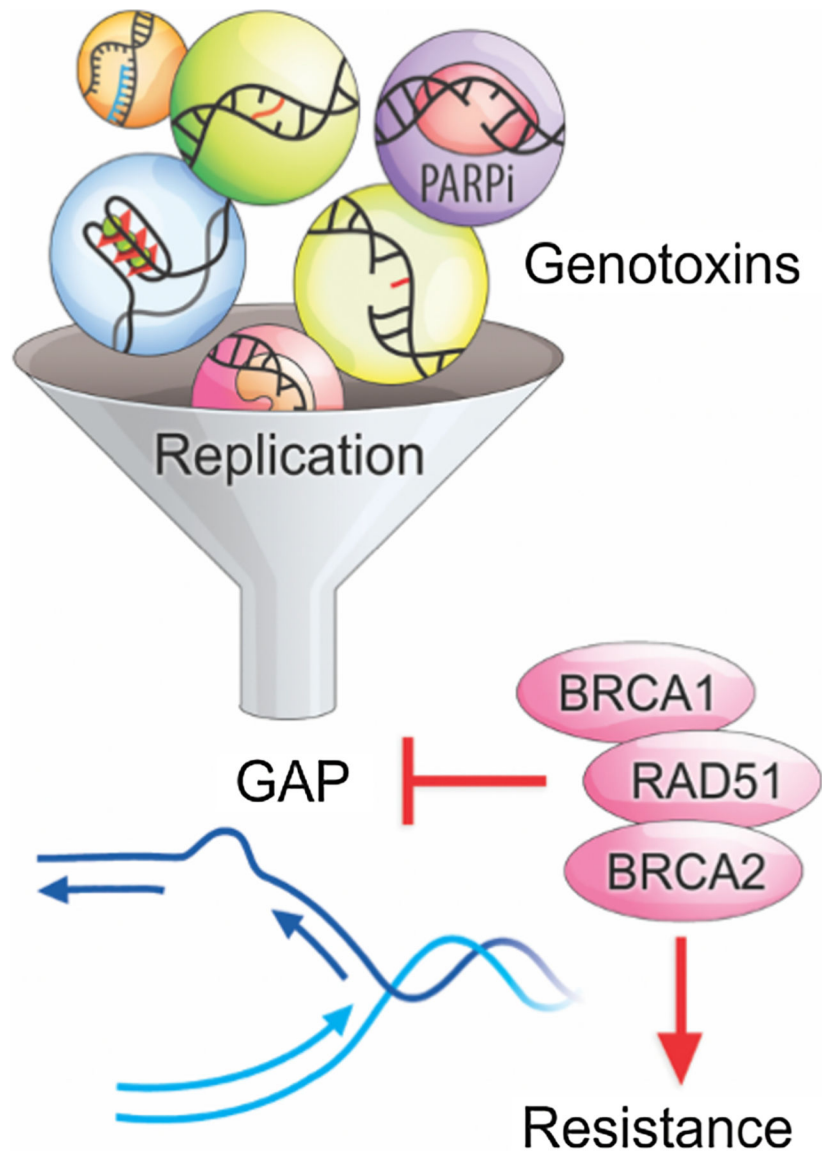Gaps at the fork can be suppressed or protected.

Fig. 5. All roads lead to Gaps.
Model that genotoxins inducing diverse lesions (i.e. crosslinks, trapped PARP or Topo complexes) ultimately convert during DNA replication into gaps requiring BRCA-RAD51 pathway for resistance.
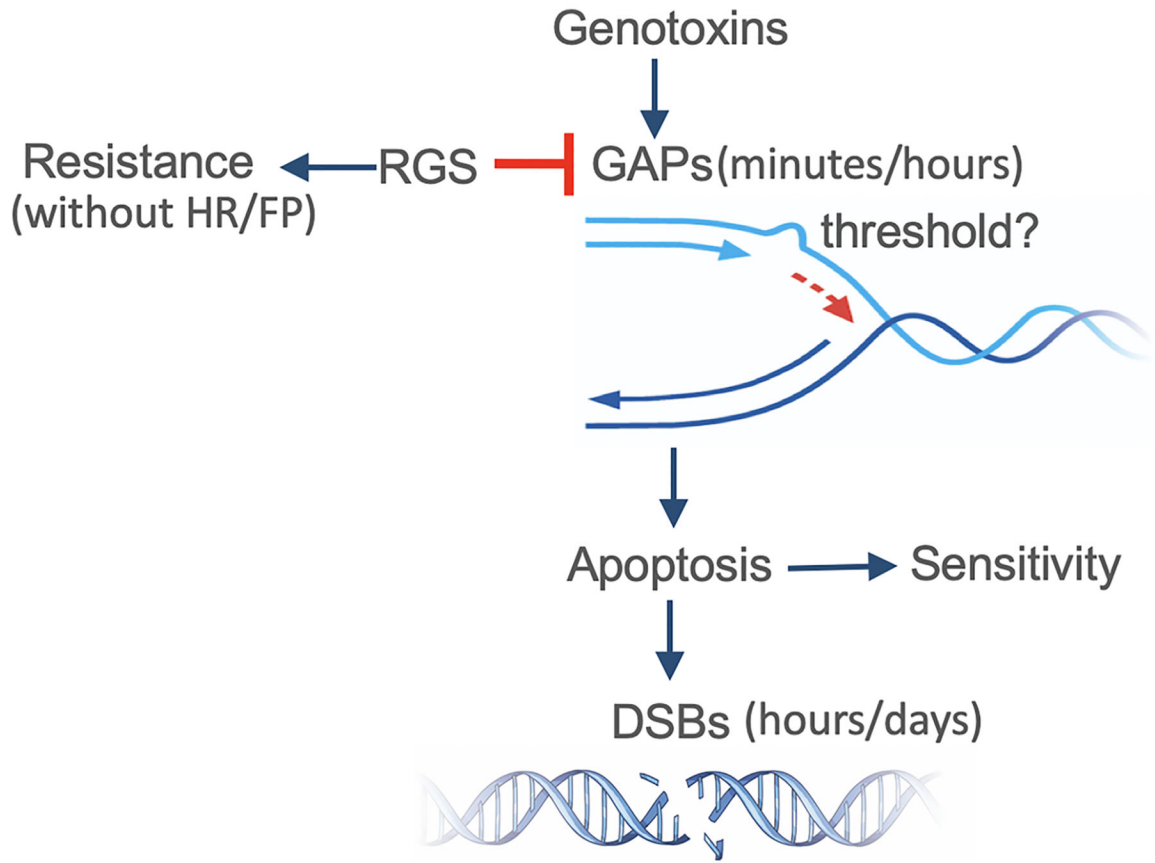
Fig. 6. Model of genotoxic chemotherapy response.
In the aftermath of genotoxic chemotherapy, gaps at a distinct threshold (to be determined) confer sensitivity and RGS confers resistance. Instead, DSB occur later and largely result from apoptosis, explaining how HR and FP are uncoupled from response.