PARP and Prostate Cancer

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DNA breaks often lead to Cancer. Double strand breaks are handled by BRCA gene products and Single strand breaks by PARP products. We discuss some recent work on PARP inhibitors focused on PCa.

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1 INTRODUCTION

DNA breaks, whether single strand or double strand are often a hallmark step in the progression of many cancers. The BRCA gene product is key in the mechanism of a double strand break, DSB. Where BRCA1 and BRCA2 are used in the DSB process the product of PARP is essential in the single strand break repair, SSB. The surprising discovery that PARP inhibitors kill BRCA deficient cells very aggressively. Simultaneously the PARP inhibitors have little effect on normal cells. Thus, the repair pathway using PARP, the SSB, which provides a first line defense against aberrant cell growth, the loss of this in normal cells, namely BRCA +/+ or +/- can be worked around. Cells which have lost BRCA defenses are especially sensitive to loss of PARO. Thus, a PARP inhibitor is a putative therapeutic for BRCA deficient cancers. Recent work on prostate cancers. PCa, have demonstrated this fact.

As Karanika et al state:

DNA is continually exposed to various insults causing a range of lesions such as single strand breaks (SSBs), double strand breaks (DSBs), bulky adducts, base mismatches, insertions and deletions and base alkylation.8 Genomic instability refers to a high frequency of alterations within the genome of a cellular lineage.

As genomic instability is deleterious to the organism, normal mammalian cells possess exquisite response mechanisms to avoid accumulation of DNA damage and maintain genomic integrity. These mechanisms are known collectively as the DDR and include detection of DNA damage, accumulation of DNA repair factors and physical repair of the lesion. This critical response program has two very well coordinated functions:

(i) to prevent duplication and partitioning of the lesion into daughter cells and

(ii) to repair the lesion.

The cellular actions that manifest as cell cycle arrest following DNA damage are known as "checkpoint" functions and are considered as a critical part of the DDR.

Depending on the severity of the lesion and the capacity of the DDR system to repair it, cells will resume proliferation, become senescent (a state of irreversible cell cycle arrest), or undergo programmed cell death (apoptosis) to remove damaged DNA from the cellular population.

Inflammation is an important factor in prostate carcinogenesis. Regardless of etiology, inflammation produces cellular and genomic damage, induces secretion of cytokines and growth factors promoting cellular proliferation and angiogenesis and becomes more extensive over the lifetime of the individual.

Inflammatory lesions generate free radicals (e.g., nitric oxides and single oxygen species released from phagocytic inflammatory cells) that cause severe oxidative DNA damage within prostate epithelial cells. These molecular changes result in increased risk of permanent mutations, as the damaged cells may proliferate.

According to the results of a recent study which used an in vitro model of prostate cell inflammation exposure androgen sensitive prostate cancer cells to inflammatory cytokines led to loss of AR and downregulation of p53 signaling. Interestingly, the administration of androgens restored p53/p21 function, inhibiting uncontrolled tumor growth related to DNA damage and genomic instability.

We examine herein some recent work on PARP inhibitors as applied to PCa. As has been shown recently PCa is also related to BRCA mutations and that with BRCA changes then DSB in DNA can be inhibited and malignant proliferations result. We thus:

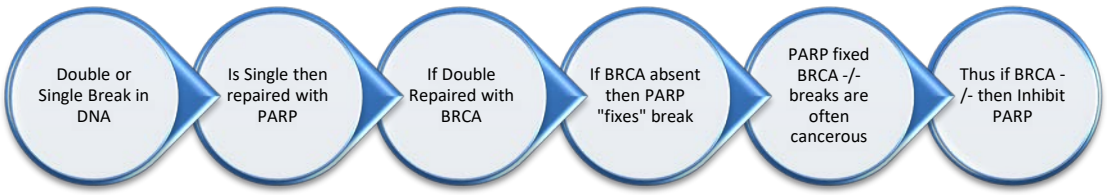
1. Examine PARB and SSB as well as multiple break scenarios.

2. Examine a set of related genes that play a role here including BRCA

3. Examine the therapeutic targets that are available.

4. We conclude with some general observations regarding this as a target.

Fundamentally we build on the paradigm depicted below.



2 PARP AND SSB AND DSB

PARP is the gene whose protein effects the processes in repairing Single Strand DNA breaks. In contrast, BRCA gene products do likewise for Double Strand DNA Breaks. Single strand breaks are much more common that DSB but they also can cause problems.

As Hoeijmakers has noted:

Weakened repair of damaged DNA may be the Achilles' heel of tumors.

Recently, tumors deficient in one of two proteins involved in the repair of double-strand breaks, BRCA1 or BRCA2, were found to be sensitive to inhibitors of PARP, a single-strand–break repair protein.

Antitumor activity of the PARP inhibitor olaparib in carriers of the BRCA mutation has been reported in cases of breast, ovarian, and prostate cancer, and its use may be even more promising for the treatment of early-stage tumors and sporadic cancers with similar defects, and possibly for prevention.

Given the complexity of DNA repair and response systems, there is likely to be further discovery of examples of the selective sensitivity of tumors to specific inhibitors or drugs on the basis of their weakened capacity for repair.

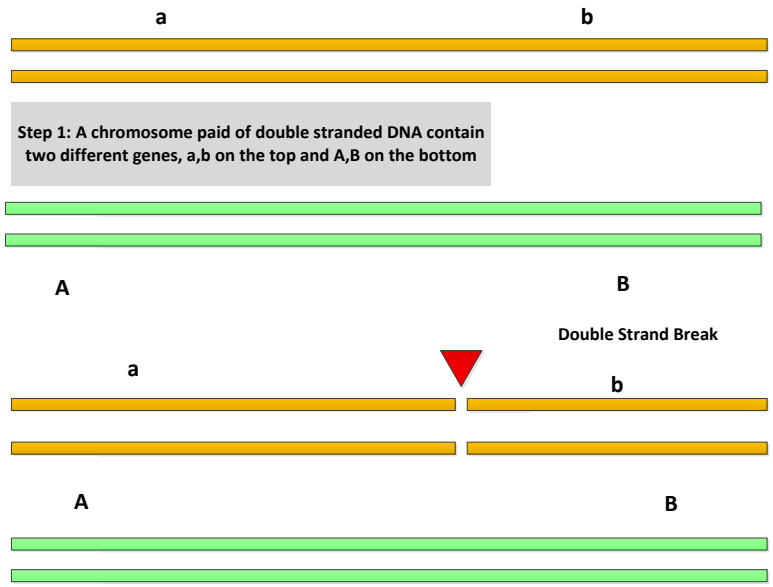
The details of the above-mentioned processes are not completely understood. We have a high level of understanding but like CRISPR Cas9 systems, the actual Cas9 mechanics still require clarification.

2.1 DOUBLE STRANDED BREAKS

Now we ask; how do breaks occur and what fixes them? That is the beginning of gene engineering. We briefly examine homologous repair, a somewhat well understood process, which uses the other chromosome as a template. The use of templates may also be done to insert new genes as well.

1. Let us start with a chromosome pair, one from each parent. We show this below.

2. Now we assume a double strand break, DSB, occurs on the top chromosome pair. We show this below:

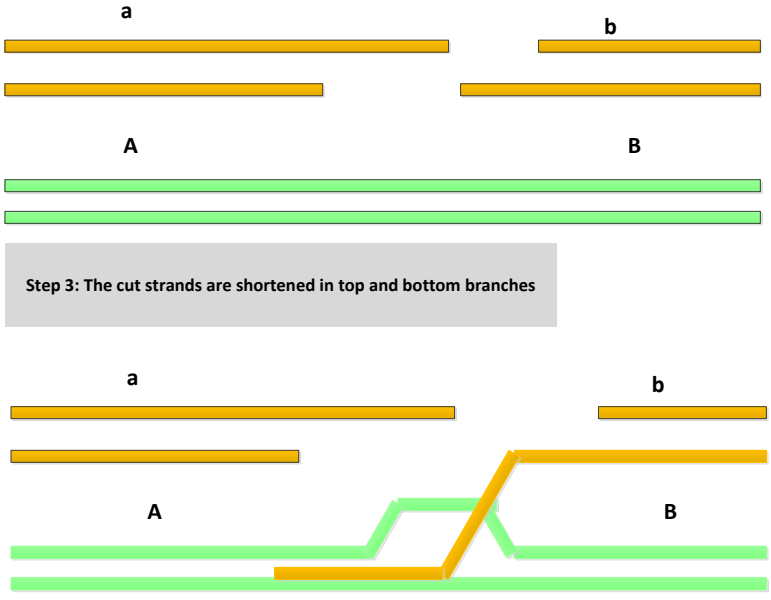


Step 1: A chromosome paid of double stranded DNA contain two different genes, a,b on the top and A,B on the bottom

Step 2: A double strand break occurs on the top DNA. For example we can select where this is with a CRISPR Cas9 approach just described

3. Next, we see a shortening of strands as shown below;

4. Then we see and elongation and use of the strand in the uncut DNA as a template. This can be used for other templates as insertion mechanisms.

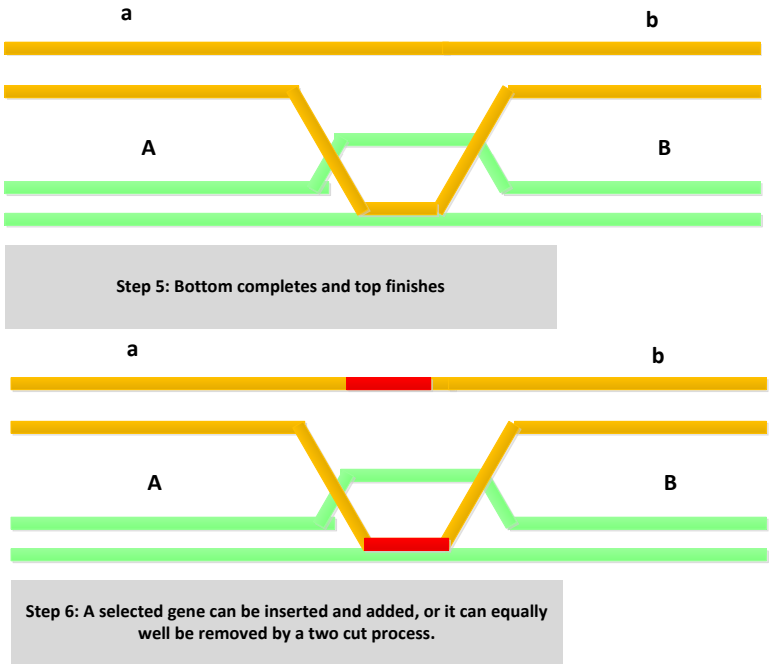


Step 3: The cut strands are shortened in top and bottom branches

Step 4: The bottom branch uses the bottom branch as a renewal template reconstructing the full strand.

5. We see both top and bottom expanding and a crossover occurring.

6. We can see this also as an insertion mechanism.

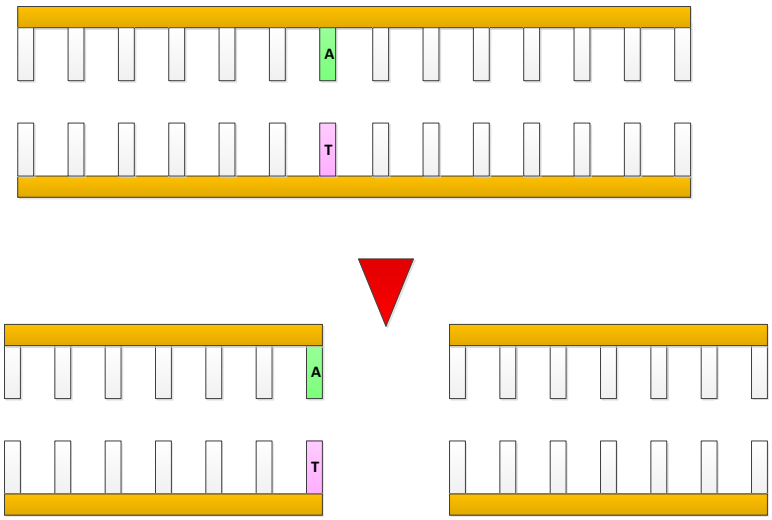


Now this is a simple reconstruction of the process. Details are in Watson et al.

2.2 DSB REPAIR

The genes we have examined play a key role in DNA repair. Although not specifically focused upon, the implications of CHK2 and its control of CDC25C and in turn CDK1 argue for double stranded breaks, DSB, in DNA as a contributing factor. Thus, it is useful to provide a high-level review of what we understand at this time. We also not that with the use of CRISPRs, we have another mechanism for DSBs and that the CRISPR approach may be one where the impact of DSBs and their repair may become ever so much more critical.

There are many ways in which DNA can get distorted but we shall examine only the double stranded breaks, DSB, as possibly one of the most significant. We show this example below where we have a break with no sticky ends, just a clean DSB. This is the most complex to deal with.



The simple break above, this specific DSB, is a cut on opposite sides of the DNA. The specific cause and mechanism of this break may not be fully known or understood. However, the repair mechanisms are somewhat understood.

As Jackson and Bartek have noted:

Key DDR signalling components in mammalian cells are the protein kinases ATM and ATR, which are recruited to and activated by DSBs and replication protein A (RPA)-coated ssDNA, respectively.

Two of the best studied ATM/ATR targets are the protein kinases CHK1 and CHK2 which, together with ATM and ATR, act to reduce cyclin-dependent kinase (CDK) activity by various mechanisms, some of which are mediated by activation of the p53 transcription factor. Inhibition of CDKs slows down or arrests cell-cycle progression at the G1–S, intra-S and G2–M cell-cycle checkpoints, which is thought to increase the time available for DNA repair before replication or mitosis ensues.

In parallel, ATM/ATR signalling enhances repair by inducing DNA-repair proteins transcriptionally or post-transcriptionally; by recruiting repair factors to the damage; and by activating DNA-repair proteins by modulating their phosphorylation, acetylation, ubiquitylation or SUMOylation.

To expand the understanding, we consider what Valerie and Povirk have noted:

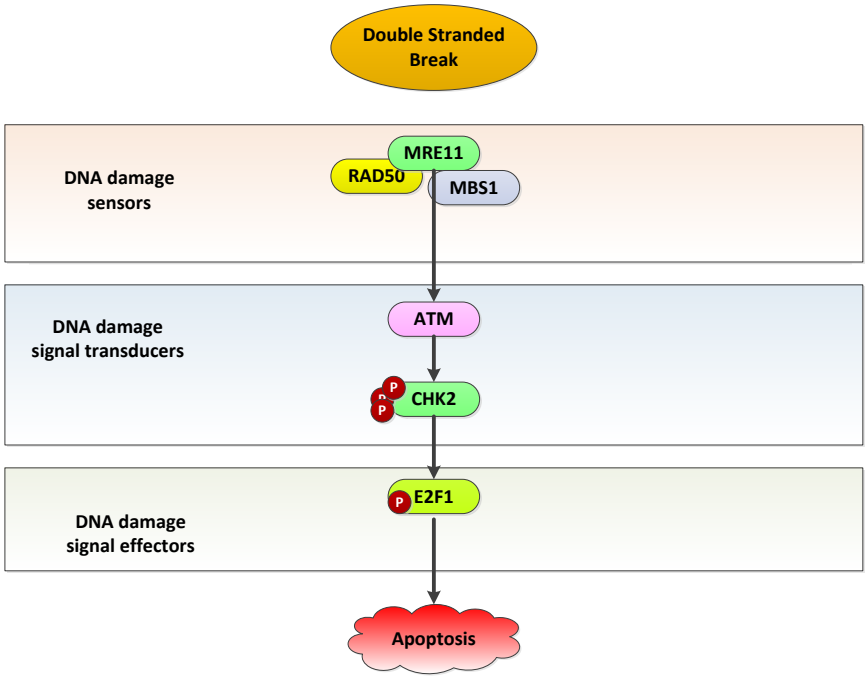
The double-strand break (DSB) is believed to be one of the most severe types of DNA damage, and if left unrepaired is lethal to the cell.

Several different types of repair act on the DSB. The most important in mammalian cells are nonhomologous end-joining (NHEJ) and homologous recombination repair (HRR).

NHEJ is the predominant type of DSB repair in mammalian cells, as opposed to lower eukaryotes, but HRR has recently been implicated in critical cell signaling and regulatory functions that are essential for cell viability.

Whereas NHEJ repair appears constitutive, HRR is regulated by the cell cycle and inducible signal transduction pathways. More is known about the molecular details of NHEJ than HRR in mammalian cells. This review focuses on the mechanisms and regulation of DSB repair in mammalian cells, the signaling pathways that regulate these processes and the potential crosstalk between NHEJ and HRR, and between repair and other stress-induced pathways with emphasis on the regulatory circuitry associated with the ataxia telangiectasia mutated (ATM) protein.

We shall review this in some detail shortly. The above two references lead to the general model depicted below:



There are two methods of repairing DSB, homologous (HEB) and non-homologous (NHEB). As Shrivastav et al state:

NHEJ and HR both contribute to genome stability and both pose risks of large- and small-scale genome rearrangement NHEJ and HR pathways are often described as "error- prone" and "error-free" respectively, but this is an oversimplification. "Clean" DSBs with complementary overhangs, 5' phosphates and 3' hydroxyl groups, such as those produced by nucleases, can be precisely repaired by NHEJ. In yeast and mammalian cells, 25-50% of nuclease DSBs is repaired by precise NHEJ; note that these are minimum estimates because these measurements do not account for multiple cycles of cleavage and precise repair.

When ends cannot be precisely rejoined, NHEJ typically involves alignment of one or a few complementary bases ("microhomology") to direct repair, leading to small deletions and sometimes small insertions. In mammalian cells NHEJ proceeds in a stepwise manner beginning with limited end-processing by the MRE11/RAD50/NBS1 (MRN) complex and perhaps other factors, end-binding by Ku comprising the Ku70 and Ku80 subunits, and recruitment of the DNA-dependent protein kinase catalytic subunit (DNAPKcs), forming the trimeric DNA-PK holoenzyme.

Once bound to broken ends, DNA-PK is activated and it phosphorylates itself and other targets including RPA, WRN, and Artemis; in cells lacking ATM, DNA-PK can also phosphorylate histone H2AX, termed γ-H2AX. In the final step, DNA ligase IV, with its binding partners XRCC4 and XLF (also called Cernunnos), seals the break. The nuclease Artemis helps repair a subset of IR-induced DSBs by NHEJ, and is important for opening hairpins formed during V(D)J recombination.

Ciccia and Elledge have an excellent review article where they also note the likelihood of such damage from various sources. What is striking is the number of lesions per day due to sunlight alone. Compare that to the Hiroshima numbers and one can be surprised.

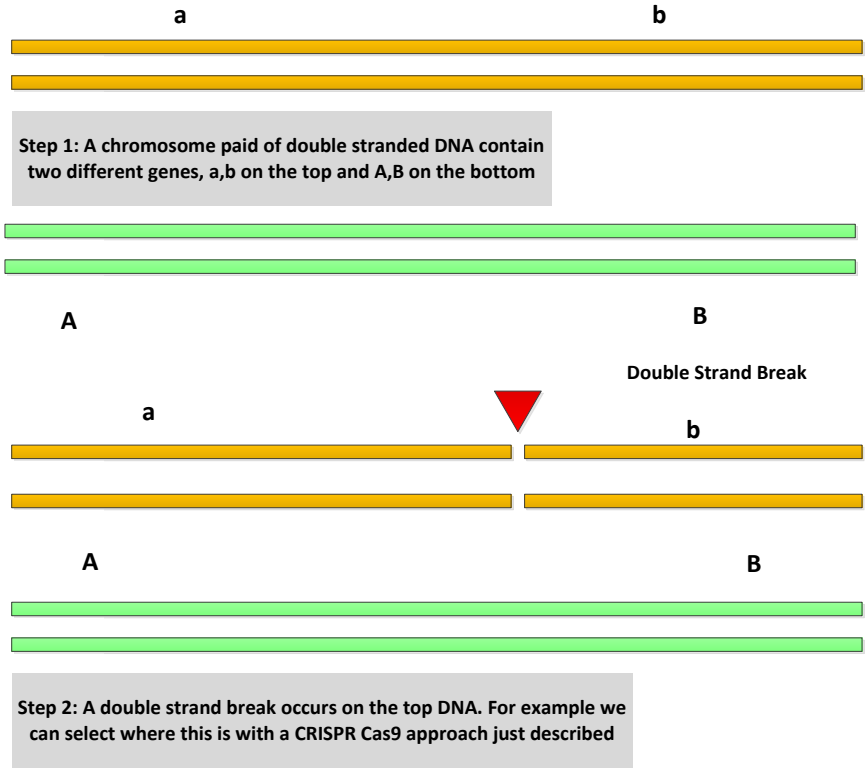
Exogenous DNA Damage	Dose Exposure (mSv)	DNA Lesions Generated	Number Lesions/Cell/Day
Peak hr sunlight	—	Pyrimidine dimers, (6–4) photoproducts	100,000/day
Cigarette smoke	—	aromatic DNA adducts	45–1029
Chest X-rays	0.02	DSBs	0.0008
Dental X-rays	0.005	DSBs	0.0002
Mammography	0.4	DSBs	0.016
Body CT	7	DSBs	0.28
Head CT	2	DSBs	0.08
Coronary angioplasty	22	DSBs	0.88
Tumor PET scan (18F)	10	DSBs	0.4
131I treatment	70–150	DSBs	2.8–6
External beam therapy	1800–2000	DSBs	72–80
Airline travel	0.005/hr	DSBs	0.0002/hr
Space mission (60 days)	50k	DSBs	2
Chernobyl accident	300l	DSBs	12
Hiroshima and Nagasaki atomic bombs	5–4000k	DSBs	0.2–160

2.3 HOMOLOGOUS REPAIR

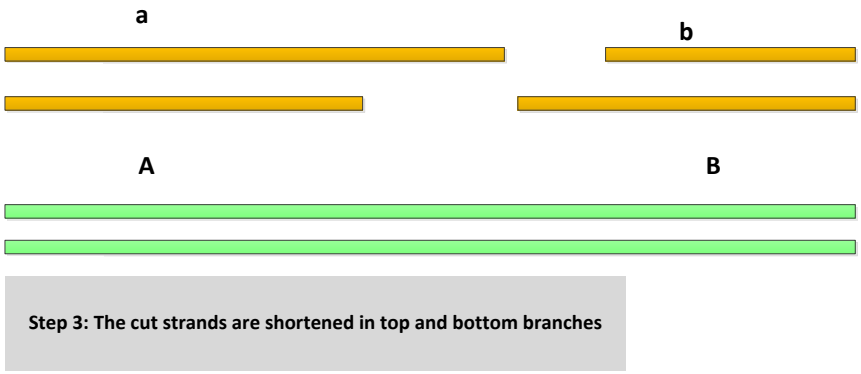
The paper by Chapman et al is a key document presenting many of the details we show herein. We have taken a simplified view so as to focus on the genetic elements of concern herein. Thus, there is a significant amount of complexity left aside.

Homologous repair is one where a DSB uses another comparable chromosome or DNA sequence and uses it for a pallet to compare and restructure the broken DNA. The following Figure depicts this process. We have explained this in the applications to CRISPR editing as well.

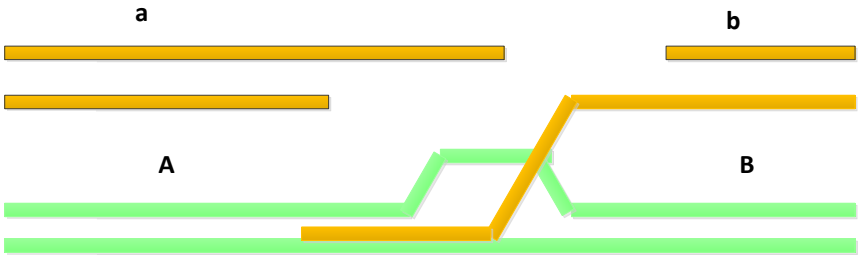
We start with the process and expand it in 3 Figures. The following Figure depicts the beginning.



Now the second phase is shown in the following Figure. Here we show how a sister piece of identical DNA can be used as a repair template. That assumes that such an identical pair exists and is available.

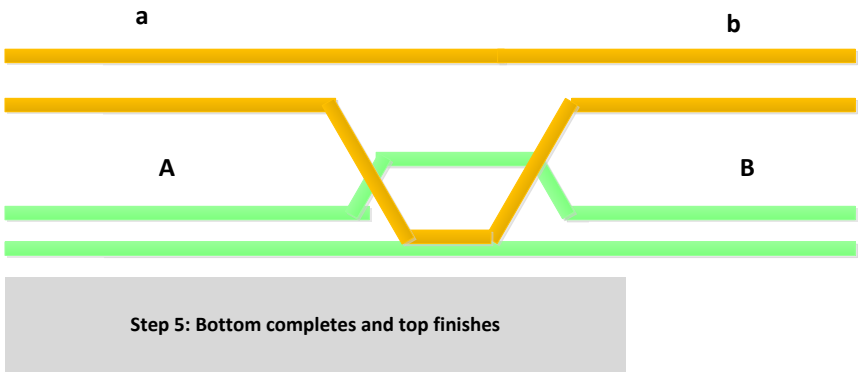


Step 3: The cut strands are shortened in top and bottom branches

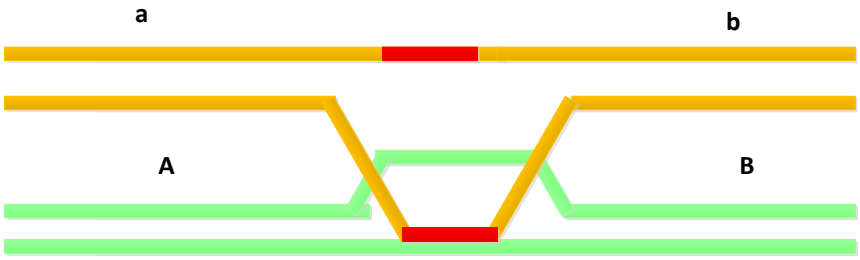


Step 4: The bottom branch uses the bottom branch as a renewal template reconstructing the full strand.

The third step in the process is shown in the Figure below. The sister elements are copied and the final reconstruction is accomplished. Generally, this is a fairly accurate process with reasonably good copying.



Step 5: Bottom completes and top finishes



Step 6: A selected gene can be inserted and added, or it can equally well be removed by a two cut process.

The net result of the Homologous repair is almost in all cases a perfect repair. However as noted is presupposes the existence of a sister pallet and a functioning mechanism.

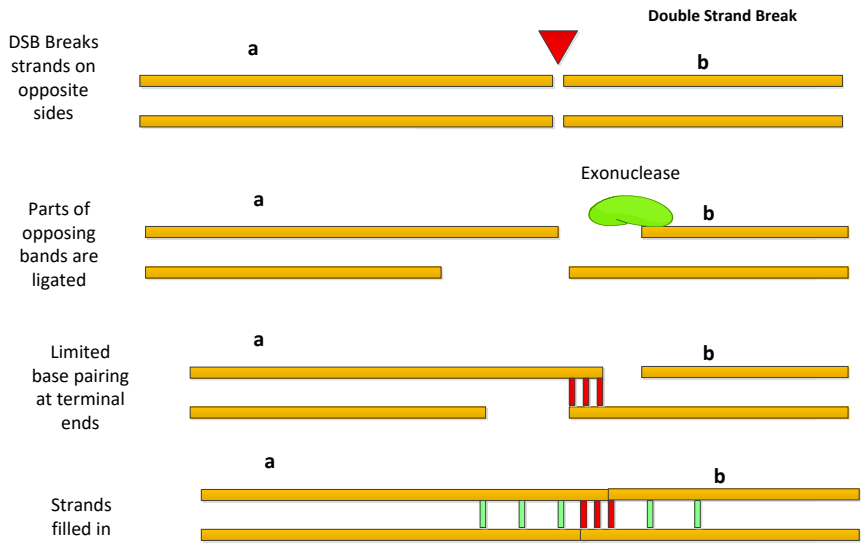
2.4 NON-HOMOLOGOUS

The non-homologous mechanism takes the assumptions of the homologous mechanism away and tries to repair by itself. We demonstrate this process below. Basically, it does the following:

1. With a clear DSB the ends of the opposing sides are ligated on opposing strands opening what may be “sticky” ends. This is done by an exonuclease.

2. The longer ends try to find a match and begin the process of sticking. This is the more difficult phase since the match finding may result in the ligation of bases.

3. Once the base pairs are matched the complete repair is performed in a standard manner.

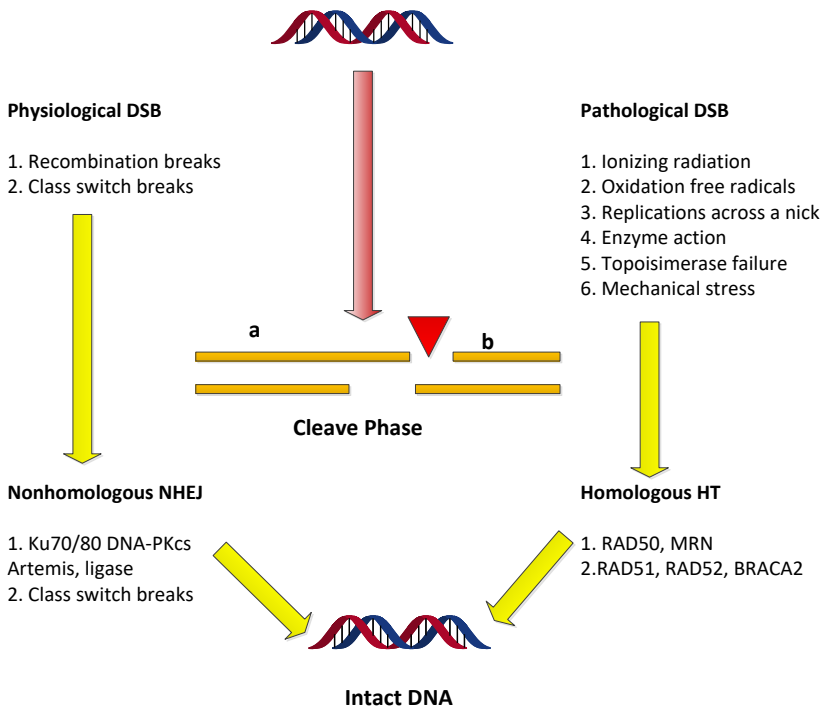


See p 53 Mendelsohn et al

This is not a perfect process. It is prone to a loss of bases and this can create a gene mutation. In fact, this may be one of the most imperfect processes around and could very well be the cause for many malignancies.

2.5 RELATIONSHIPS

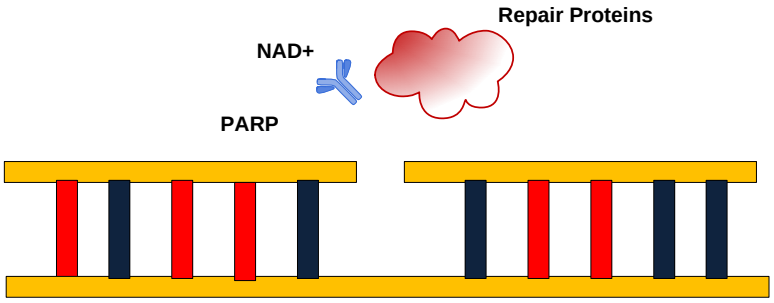
The Figure below depicts the collection of these processes in toto. There are seen to be two classes of DSB. The physiological class tends to lead to non-homologous repair and those of a pathological basis the homologous.



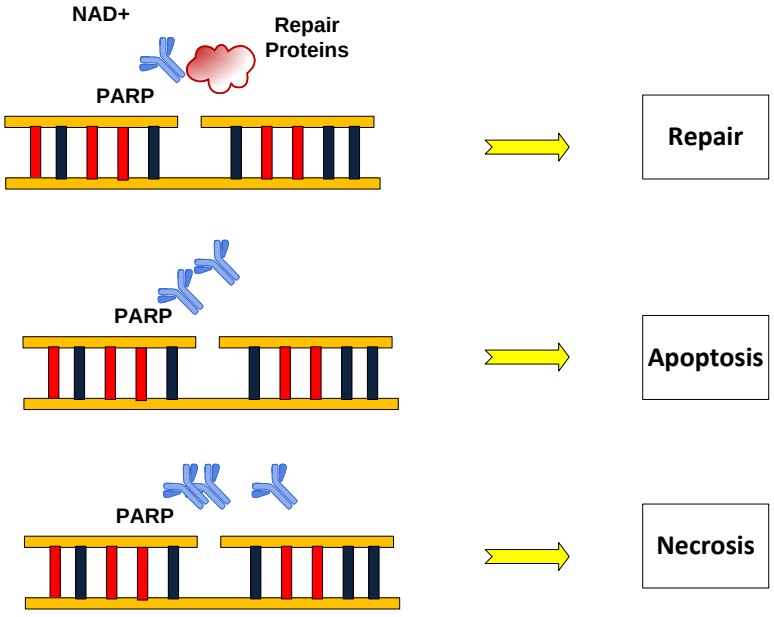
The DSB repair mechanisms are as we have noted prone to mistakes. That is where we also have certain backup mechanisms as p53 and other similar genes. However, if as we have noted the CHK2 process becomes overly active we may have added instability which p53 and its helpers cannot properly control.

2.6 SINGLE STRAND BREAKS

Single strand breaks are more frequent and generally more benign. The repair mechanism is as shown below where PARP protein binds to the repair site along with an NAD+ molecule and attracts a set of repair proteins. The process then rebuilds the break.



This process may proceed benignly or it may result in apoptosis or even necrosis of the cell. We depict this below.



We now examine some of the details of this process. As 32. Torgovnick, and Schumacher note:

Repair Mechanism	Lesion Feature	Genotoxic Source
Base excision repair (BER)	Oxidative lesions	Reactive oxygen species (ROS)
Nucleotide excision repair (NER)	Helix-distorting lesions	UV radiation Trans-lesion synthesis Various Lesions Various sources
Mismatch repair (MMR)	Replication errors	Replication
Single stand break repair (SSBR)	Single strand breaks	Ionizing radiation, ROS
Homologous recombination (HR)	Double-strand breaks	Ionizing radiation, ROS
Non-homologous end joining (NHEJ)	Double-strand breaks	Ionizing radiation, ROS
DNA interstrand crosslink repair pathway	Interstrand crosslinks	Chemotherapy

The authors continue:

Personalized medicine uses targeted therapies on specific patient's cohorts and PARP1 inhibitors represent a new promising class of chemotherapeutic drugs adopted to exclusively disrupt PARP1 function in HR-defective cancers. PARP1 belongs to a family of 17 ADP-ribosyltransferases which utilize nicotinamide adenine dinucleotide (NAD^+) molecules as a substrate to form polymers of ADP ribose units (PAR) on target proteins.

This post-translational modification, known as PARylation, is a reversible fundamental process of the DDR necessary for recruiting to the damaged site PAR-binding factors involved in chromatin architecture and DNA repair.

PARP1 is the most expressed member of the family, it has nuclear localization and it plays a major role in BER by associating with SSBs and recruiting crucial repair proteins like X-ray repair cross-complementing protein 1. In addition, PARP1 is part of the HR and NHEJ machineries thanks to the interactions respectively with E11, RPA, RAD51…
We now focus on the repair mechanisms.

2.6.1 Nucleotide Repair

Basically, a SSB requires a nucleotide repair process. From DeVita et al[1]:

Nucleotide excision repair and base excision repair. Nucleotide excision repair (NER) is activated in response to bulky lesions that are generated, for example, by ultraviolet (UV) irradiation (upper panels).

Global genome repair involves proteins identified by complementation groups in patients with xeroderma pigmentosa (XP proteins). Initial recognition of lesions occurs by a complex

[1] See DeVita, Chapter 3.

containing xeroderma pigmentosa C (XPC). Transcription-coupled repair (TCR) also involves proteins identified by mutation in Cockayne syndrome (CS proteins), and occurs when RNA polymerase II stalls at the site of lesions. Stalled RNA polymerase II recruits Cockayne syndrome B (CSB) to the site of damage. Subsequently, DNA is locally unwound around the injured site by a TFIIH complex containing XPB and XPD.

This process also involves XPG, CSA, and other proteins for TCR. Once unwound, XPA and replication protein A (RPA) contribute to stabilization of an open intermediate and recruitment of the ERCC1 and XPF endonucleases that excise the lesion. Subsequent steps involve DNA synthesis and ligation to complete the repair. In base excision repair (BER) (lower panels), a basic site generated by spontaneous hydrolysis, action of DNA glycosylases, or x-ray-induced single-strand breaks are recognized by the APE1 endonuclease, as well as PARP and XRCC1.

Subsequent repair is influenced by PARP-mediated adenosine diphosphate ribosylation of histones and other proteins, and XRCC1 serves as a scaffold for recruitment of DNA polymerase β and DNA ligase 3. These latter enzymes catalyze nucleotide reinsertion and ligation into the injured strand as part of the short patch repair pathway (major BER pathway).

As Velic et al note when the examine the details of SSB:

All living organisms can suffer from deleterious attacks from extrinsic agents as well as intrinsic sources such as reactive oxygen species. One of the most harmful lesions found in genomic DNA are double-strand breaks (DSBs), whose massive cytotoxicity is the basis for conventional DSB-inducing agents, such as ionizing radiation (IR), radiomimetic drugs and topoisomerase II inhibitors, currently used as treatment of choice for cancer therapy.

Unfortunately, such treatments come with a lack of tumour specificity, resulting in severe side effects that negatively impact on patient's life. Current research efforts are now directed toward identifying small inhibitory molecules targeting specific pathways involved in signalling and repairing DSBs.

These pathways collectively form a complex network, termed the DNA damage response (DDR), evolved to neutralize DNA lesions and prevent transmission of incorrect genetic information to daughter cells during cell division. In normal cells, the DDR can coordinate cell cycle arrest, DNA repair and apoptosis, and consists of three classes of proteins, each exerting critical functions. Sensors detect the presence of DNA lesions, signal transducers generate and amplify the DNA damage signal, and effectors induce DNA repair, cell cycle delay, programmed cell death or senescence.

Cancer cells, for a large part, have compromised DDR pathways, some of them being dependent on a sole back-up pathway for survival. The use of inhibitors against essential components of a back-up pathway, in a synthetic lethal approach, is a promising avenue to specifically eradicate cancer cells. Another inhibitor-based approach to therapy is the potentiation of the effects of DNA-damaging anti-cancer agents, particularly in instances of therapeutic resistance. This review aims to provide a comprehensive view of key inhibitors of DSB DNA damage signalling and repair proteins.

To respond to DNA damage, cells have implemented the DNA Damage Response. DDR is a signal transduction pathway that implicates different actors depending on the type of damage and/or the cell cycle phase.

Firstly, the damage is detected by sensor proteins such as MRN (MRE11-RAD50-NSB1) or PARP1 (Poly(ADP-ribose) Polymerase 1). These sensors recruit the apical kinases Ataxia telangiectasia mutated (ATM) or Rad3-related (ATR).

The signal is further amplified and the response results in repair (following diverse mechanisms), cell cycle arrest and/or apoptosis.

Single-strand breaks are repaired by Single-Strand Break Repair (SSBR—not discussed in this review).

Double-strand breaks are repaired by two main mechanisms: Homologous Recombination (HR) and Non-Homologous End-Joining (NHEJ). HR involves mediators such as BRCA2, RAD51 and PALB2. NHEJ brings into play proteins such as DNA-dependent protein kinase catalytic subunit (DNA-PKcs), Ku70/80, Ligase IV and XRCC4.

During DDR pathway, cells require effectors, activated by ATM and ATR, such as CHeckpoint Kinase 1 (CHK1), Checkpoint Kinase 2 (CHK2) or p53 to arrest the cell cycle or enter in apoptosis.

A dysregulation of these processes can lead to cell death or, more seriously, to mutagenesis and cancer. When cancer does occur, targeting the principal DDR actors allows the promotion of cell death in order to limit cancer progression. Specifics inhibitors have been designed to target different actors of the DDR pathway. Major inhibitors, discussed in this review, are represented in different boxes in this figure

The last paragraph details the problems that result by a failure of the process. Thus, as Morita et al note:

Eukaryotes have many functional homologues of bacterial BER enzymes, and the mechanism of BER is similar to that of prokaryotes. However, eukaryotes also have specific BER enzymes. To date, poly(ADP-ribose) polymerase (PARP) and X-ray cross-complementing group 1 (XRCC1) have been identified as eukaryotic-specific enzymes. PARP1 uses NAD to add branched ADP-ribose chains to proteins. PARP1 functions as a DNA nick-sensor in DNA repair and as a negative regulator of the activity of Polβ in LP-BER. XRCC1 interacts with DNA ligase III and PARP through its two BRCT domains and with Polβ through an N-terminal domain. XRCC1 also interacts with many other proteins and forms a large DNA repair complex.

We now examine some of the mechanisms of PARP.

2.6.2 Repair Options with PARP

Carriers of germ-line mutations in one allele of the BRCA1 or BRCA2 double-strand–break repair genes are at increased risk for breast or ovarian cancer or for prostate cancer. The tumors in such patients have lost the remaining wildtype allele and are deficient in important branches of the homologous recombination system that repairs double strand breaks and inter strand cross-links). In contrast, normal tissues in these patients, retain one copy of the wild-type allele, which is sufficient to carry out normal double-strand–break repair.

Single strand breaks are an important source of spontaneous double-strand breaks, which occur when single-strand breaks are encountered during replication and then are turned into double-strand breaks.

The last observation is critical. A SSB can readily become the source of a DSB. The authors continue as this moves on to a DSB.

To solve this problem, homologous recombination involving the BRCA1 and BRCA2 proteins allows complex DNA template switching and regression of the arrested replication fork. Different types of spontaneous single-strand breaks, caused by the action of reactive oxygen and nitrogen species, occur at an estimated daily rate of 104 per cell. The majority of clean breaks are quickly repaired by DNA ligases.

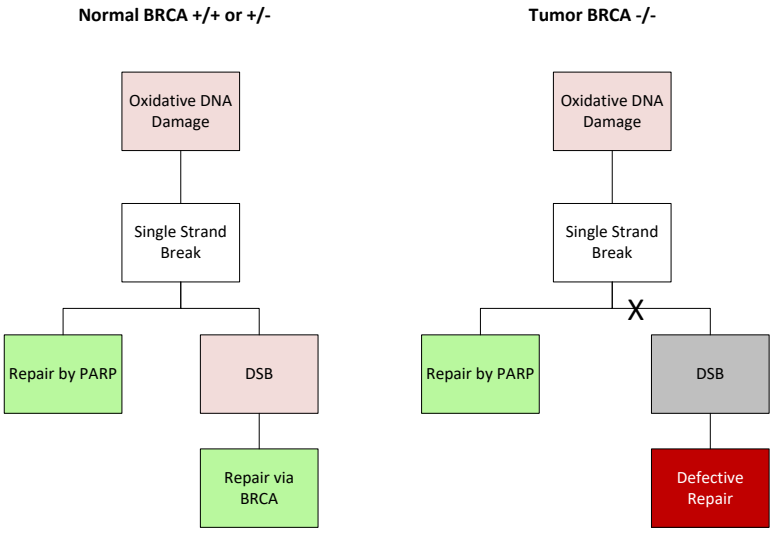
However, when the ends need processing, poly (adenosine diphosphate [ADP]–ribose) polymerase (PARP) is required to engage the mechanism of base-excision repair.

Potent inhibitors of PARP have been identified that greatly increase levels of persisting single-strand breaks.

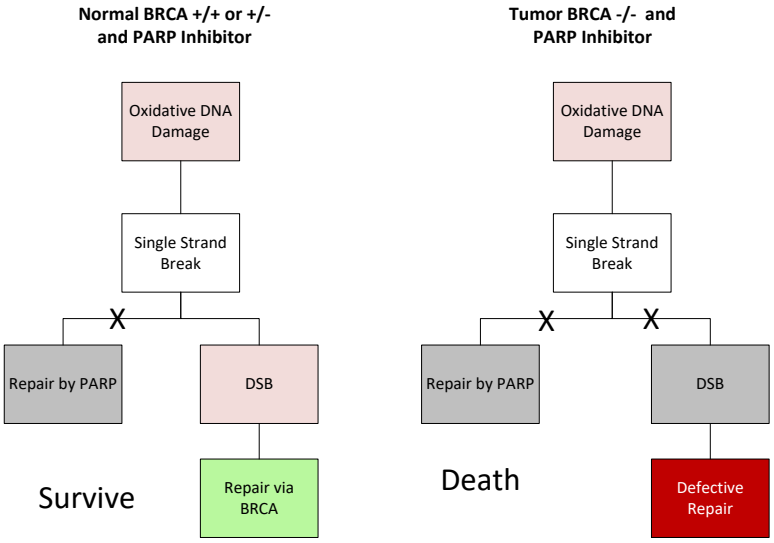
In normal cells from BRCA1 or BRCA2 carriers (which have one intact BRCA allele), the problem of persistent single-strand breaks causing double-strand breaks on replication can still be handled by the homologous recombination machinery when PARP inhibitors are present.

However, in tumor cells lacking both alleles of BRCA1 or BRCA2, this back-up repair solution is missing, and as a consequence, these cells have an exquisite sensitivity to PARP inhibitors, such as olaparib. This principle of synthetic lethality has been used successfully in targeted cancer therapy without clinically significant side effects.

This above process we depict below.



The second phase of this process is depicted below.



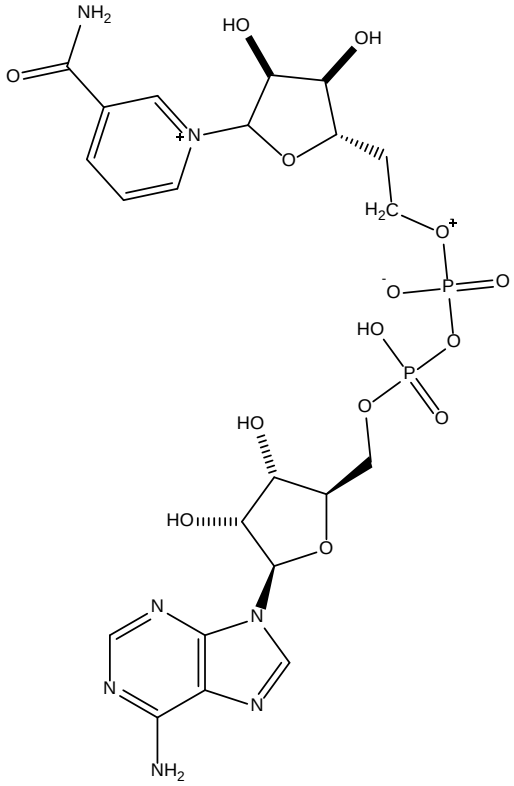
Note that the last step, with a BRCA -/- and PARP inhibitor that we naturally have no repair but the aberrant cell dies off as we discussed earlier.

2.6.3 Chemical Issues

In Kelley and Fishel, pp 116-118, the authors[2] discuss PARP structure in detail. The PARP NAD+ substrate is shown below. There is a break point between the pentose ring and the phenol and the nicotinamide breaks off. This molecule and its functioning is an essential element of the PARP repair mechanism. PARP is inactive until it binds to a SSB via a Zinc finger. It uses the

[2] The authors of this Chapter, Chpt 4 are D'Arcangelo et al.

nicotinamide to rebranch and repair. It is also via this mechanism that we can see that clinically by blocking this we obtain DSB suppression.



2.7 PARP INHIBITORS

Now we consider how to address the inhibition of PARP and thus allow cell death in the event of defective BRCA and DSBs. As McLornan et al note:

PARP1, a nuclear enzyme accounting for 90% of total cellular PARP activity, is dependent on nicotinamide adenine dinucleotide (NAD) to generate poly(ADP-ribose). Multiple protein acceptors, including core histones (e.g., histones 1 and 2b [H1 and H2b], nonhistone chromatin proteins, topoisomerases, and DNA protein kinase), can undergo "PARylation" (post-translational modification involving the covalent attachment of poly[ADP-ribose] to proteins by PARP enzymes) after PARP1 activation.

Collectively, these effects induce chromatin and nucleosome modification and permit assembly of multiprotein complexes required for efficient DNA repair. Knockout models show that PARP1 deficiency is associated with genomic instability and enhanced sensitivity to DNA-damaging agents.

Conversely, overexpression of PARP1 may play a major role in resistance to chemotherapy or radiotherapy. BRCA1 acts as a scaffolding protein for the formation of multifunctional, yet distinct, DNA repair complexes. After ATM or checkpoint kinase 2 (CHK2)–mediated multisite phosphorylation, BRCA1 localizes to DNA-damage sites and heterodimerizes with BARD1 (BRCA1-associated RING domain 1). Colocalization with phosphorylated H2AX (histone H2A histone family member X) at nuclear foci follows, promoting the formation of a multimolecular repair complex.

Both BRCA1 and BRCA2 mutations convey significant lifelong risks of breast and ovarian cancer. 31,32 BRCA1-deficient tumor cells have defects in homologous recombination, cell-cycle checkpoints, and transcriptional regulation.33 Several groups have reported that cells with dysfunctional BRCA1 or BRCA2, in contrast to cells that have normal BRCA function, dramatically enhanced sensitivity to PARP inhibitors, predominantly because of defective homologous recombination

Moreover, a deficiency of other key homologous recombination–related proteins, such as RAD51, ATM, and ATR, can also enhance the efficacy of PARP inhibitors. Early phase studies of AZD2281 (also known as olaparib) involving heavily pretreated patients who had cancers with BRCA1 or BRCA2 mutations showed feasibility, acceptable safety, and modest efficacy. A landmark trial involving heavily pretreated patients who had breast cancer with BRCA1 or BRCA2 mutations revealed a favorable therapeutic index and an objective response rate of 41% among patients who received the maximum tolerated dose of this agent.

The development of PARP inhibitors accelerated rapidly, and several compounds are now being evaluated for the treatment of a range of solid tumors and hematologic cancers.38-44 Given these findings, the use of PARP inhibitors has been explored in tumors lacking alternative functional components of the homologous- recombination pathway, yet it is increasingly apparent that the exact mechanism of action of PARP inhibitors is complex and not yet fully elucidated.

A recent screen of 185 PARP inhibitors showed a lack of specificity for PARP1, with many PARP inhibitors, including promising agents such as AZD2281, rucaparib (also known as AG-014699 or PF-01367338), and veliparib (ABT-888), binding to the catalytic domains of multiple PARPs.45 It is unclear whether this "pan-PARP" inhibition yields additional therapeutic benefits.

This is particularly important, since PARPs may have functional roles other than facilitating DNA repair, such as in telomere maintenance and cellular energetics (e.g., NAD-dependent metabolic reactions.

3 BRCA AND OTHERS

We will now examine some of the genes which are integral in this process. We have discussed such genes as PTEN, AKT, ATR and others elsewhere[3]. From McLornan et al we have the following list:

[3] See McGarty, Prostate Cancer or Melanoma.

Gene	***Location***	***Mass***	***Function***
PARP1	1q41–42	113	Repair of DNA single-strand breaks and double-strand breaks
BRCA1	17q21	207	Repair of DNA double-strand breaks mediated by homologous re-combination, transcriptional regulation, ubiquitination; interacts with key regulators of cell-cycle checkpoints
BRCA2	13q12	384	Repair of DNA double-strand breaks mediated by homologous re-combination, transcriptional regulation, ubiquitination; interacts with key regulators of cell-cycle checkpoints
CDK1	10q21	34	Key regulator of cell-cycle progression
CDK2	12q13	34	Key regulator of cell-cycle progression
ATM	11q22–23	350	ATM-mediated DNA-damage-response pathway and activation of cell-cycle checkpoints
ATR	3q23	301	ATR-mediated DNA-damage sensing and activation of cell-cycle checkpoints
MRE11A	11q21	80	Initial processing of DNA double-strand breaks for both homologous recombination and non-homologous end-joining activation of ATM-mediated checkpoint regulation
RAD50	5q31	154	Initial processing of DNA double-strand breaks for both homologous recombination and non-homologous end-joining activation of ATM-mediated checkpoint regulation
NBN	8q21	85	Initial processing of DNA double-strand breaks for both homologous recombination and non-homologous end-joining activation of ATM-mediated checkpoint regulation
MLH1	3p21.3	84	Mismatch DNA repair
MSH2	2p21	104	Mismatch DNA repair
p53	17p13.1	43.7	Key tumor suppressor and regulator of cell cycle; apoptosis
53bp1	15q15	214	Mediator of DNA-damage response

Gene	***Location***	***Mass***	***Function***
PLK1	16p12	68.2	Serine–threonine protein kinase; essential trigger for G2–M transition
CDK17	12q23	59.6	Serine–threonine protein kinase; histone phosphorylation
AURKA	20q13	45.8	Critical for formation of intact mitotic spindle and chromosomal segregation
KRAS	12p12.1	21.7	Propagation of growth signals in normal homeostasis
MYC	8q24.21	48.8	Transcriptional regulation, cell-cycle progression, apoptosis
MK2	1q32	45.6	Transcriptional regulation, DNA-damage response, cell proliferation
PAK3	Xq23	62.3	Cell migration, cell-cycle regulation, apoptosis
SGK2	20q13.2	47.6	Phosphatidylinositol 3-kinase signaling, cell proliferation
GATA2	3q21.3	50.5	Zinc-finger transcription factor with multiple essential roles
CDC6	17q21.3	62.7	Regulator of DNA replication and cell cycle
4EBP1	8p12	12.6	Mammalian target of rapamycin pathway, protein translation

We now detail some of these key genes as relates to repair.

3.1 BRCA

As NCBI describes BRCA1[4]:

This gene encodes a nuclear phosphoprotein that plays a role in maintaining genomic stability, and it also acts as a tumor suppressor. The encoded protein combines with other tumor suppressors, DNA damage sensors, and signal transducers to form a large multi-subunit protein complex known as the BRCA1-associated genome surveillance complex (BASC). This gene product associates with RNA polymerase II, and through the C-terminal domain, also interacts with histone deacetylase complexes.

This protein thus plays a role in transcription, DNA repair of double-stranded breaks, and recombination. *Mutations in this gene are responsible for approximately 40% of inherited breast cancers and more than 80% of inherited breast and ovarian cancers. Alternative splicing*

[4] https://www.ncbi.nlm.nih.gov/gene/672

plays a role in modulating the subcellular localization and physiological function of this gene. Many alternatively spliced transcript variants, some of which are disease-associated mutations, have been described for this gene, but the full-length natures of only some of these variants has been described.

Likewise, NCBI describes BRCA2[5]:

Inherited mutations in BRCA1 and this gene, BRCA2, confer increased lifetime risk of developing breast or ovarian cancer. Both BRCA1 and BRCA2 are involved in maintenance of genome stability, specifically the ***homologous recombination pathway for double-strand DNA repair.***

The BRCA2 protein contains several copies of a 70 aa motif called the BRC motif, and these motifs mediate binding to the RAD51 recombinase which functions in DNA repair. BRCA2 is considered a tumor suppressor gene, as tumors with BRCA2 mutations generally exhibit loss of heterozygosity (LOH) of the wild-type allele.

We now consider their role in DSB repair. First, we examine the homologous repair path and then the non-homologous. From McLornan et al we have the following:

The classic HR pathway involves the following basic steps. DSBs are recognized by the MRN complex and by checkpoint proteins as previously described. A 5′-3 exonuclease generates 3′ overhangs, which are then coated with replication protein A (RPA). "Mediator" proteins such as BRCA2 or Rad52 then facilitate the recruitment of Rad51-related proteins, which form filaments on the single-stranded DNA, replacing RPA.

A homology search ensues, followed by strand invasion, and DNA synthesis. The links between DNA strands (double Holliday junctions) can be resolved to produce exchange between chromosomes (crossovers) or no exchange (non-crossovers). Enzymes such as the RecQ helicase BLM, in conjunction with topoisomerase IIIα. can resolve these double Holliday junctions. ….

Double-strand break (DSB) repair by homologous recombination and non-homologous end-joining. In homologous recombination, DSBs are recognized by the MRN complex, among other proteins. 5′-3′ Exonuclease activity results in the generation of single-strand overhands that are coated with RPA. Mediator proteins such as BRCA2 and RAD52 stimulate assembly of a RAD51 nucleoprotein filament complex that guides subsequent homology search and strand invasion into the homologous strand (e.g., the identical sister chromatid in late S/G2 and mitosis). Subsequent DNA synthesis and ligation result in the formation of recombination intermediates that contain double Holliday junctions.

These are resolved by resolving enzymes such as the RecQ helicase BLM, in conjunction with topoisomerase 3α. The process of nonhomologous end-joining involves recognition of DSB ends by the Ku70-Ku80 heterodimer, with subsequent recruitment of DNA-PK. DNA ends are then ligated following recruitment of XRCC4 and DNA ligase.

[5] https://www.ncbi.nlm.nih.gov/gene/675

Now we consider the effects of BRCA on this process. As Venkitaraman notes:

Inheritance of one defective copy of either of the two breast-cancer-susceptibility genes, BRCA1 and BRCA2, predisposes individuals to breast, ovarian and other cancers. Both genes encode very large protein products; these bear little resemblance to one another or to other known proteins, and their precise biological functions remain uncertain.

Recent studies reveal that the BRCA proteins are required for maintenance of chromosomal stability in mammalian cells and function in the biological response to DNA damage. The new work suggests that, although the phenotypic consequences of their disruption are similar, BRCA1 and BRCA2 play distinct roles in the mechanisms that lead to the repair of DNA double-strand breaks.

BRCA1 and BRCA2 participate in the biological response to DNA damage. Inevitably, the suggestion that BRCA1 and BRCA2 co-localise BRCA1 1,863 aa RING domain BRCT NLS domains BRCA2 3,418 aa BRC repeats NLSs Fig. 1. Features of the BRCA proteins. The N-terminal RING domain, nuclear localisation signal (NLS) and C-terminal BRCT domains of BRCA1 are shown, as are the eight BRC repeat motifs in BRCA2.

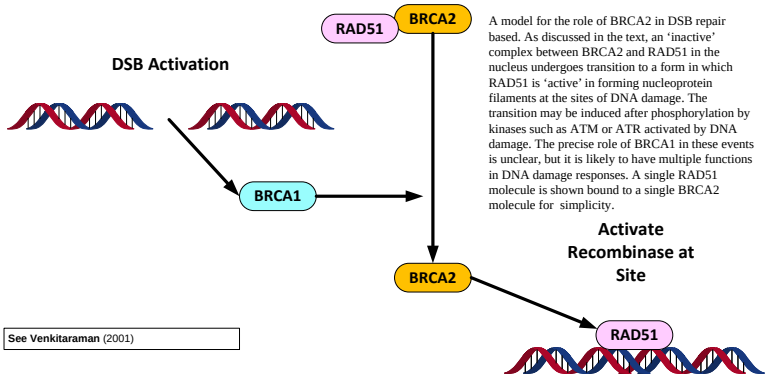
Functions of BRCA1 and BRCA2 in the biological response to DNA damage with RAD51 provoked speculation that they participate in some aspect of the cellular response to DNA damage. Direct evidence for such a function has come from studies on cells that harbor mutations in the breast-cancer-susceptibility genes. The cellular response to DNA damage involves the activation of cell cycle checkpoints and the recruitment of the machinery for DNA repair, processes that are intimately linked to one another.

Failure to activate these checkpoints or DNA repair following DNA damage manifests as increased sensitivity to genotoxic agents. Indeed, Brca1-deficient and Brca2-deficient murine cells exhibit hypersensitivity to genotoxins such as X-rays, confirming an essential role for the two proteins in the response to DNA damage. The X-ray sensitivity of cells lacking BRCA1 or BRCA2 suggests they have a defect in the repair of DSBs, the major lesion inflicted by ionizing radiation.

Mammalian cells use several mechanisms to repair DSBs, in particular non-homologous end joining (NHEJ) and homologous recombination. NHEJ, which culminates in the ligation of broken DNA fragments without regard to the homology of sequences at their ends, is critically dependent on the DNA-dependent protein kinase (DNA-PK) and its accessory molecules Ku70 and Ku80.

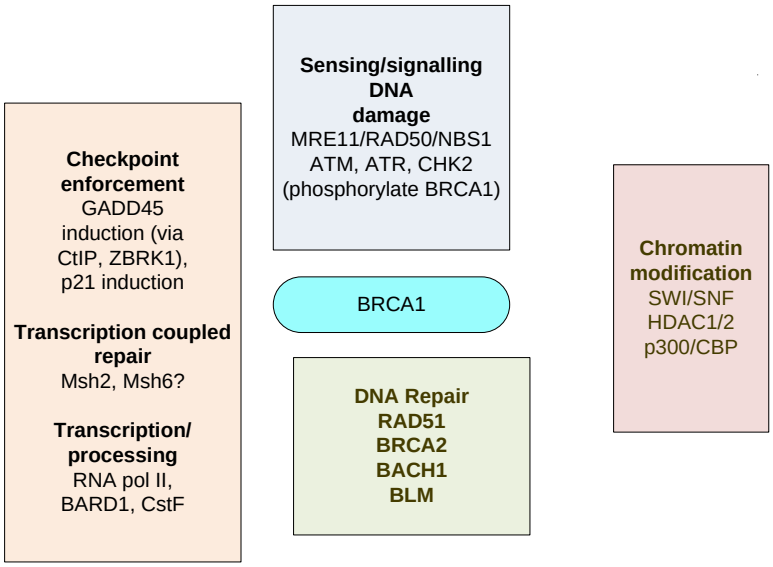
By contrast, DSB repair by homologous recombination is achieved through the exchange of genetic information between the damaged template and a homologous DNA sequence, such as that found on a sister chromatid. Its mechanism in mammalian cells is poorly understood.

We describe this graphically below:



A model for the role of BRCA2 in DSB repair based. As discussed in the text, an 'inactive' complex between BRCA2 and RAD51 in the nucleus undergoes transition to a form in which RAD51 is 'active' in forming nucleoprotein filaments at the sites of DNA damage. The transition may be induced after phosphorylation by kinases such as ATM or ATR activated by DNA damage. The precise role of BRCA1 in these events is unclear, but it is likely to have multiple functions in DNA damage responses. A single RAD51 molecule is shown bound to a single BRCA2 molecule for simplicity.

Finally, the graphic below depicts the various interactions that BRCA1 alone has on the various steps in cell growth integrity.



3.2 P53

The p53 gene has a long history. As NCBI notes[6]:

This gene encodes a tumor suppressor protein containing transcriptional activation, DNA binding, and oligomerization domains. The encoded protein responds to diverse cellular stresses to regulate expression of target genes, thereby inducing cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism.

[6] https://www.ncbi.nlm.nih.gov/gene/7157

Mutations in this gene are associated with a variety of human cancers, including hereditary cancers such as Li-Fraumeni syndrome. Alternative splicing of this gene and the use of alternate promoters result in multiple transcript variants and isoforms. Additional isoforms have also been shown to result from the use of alternate translation initiation codons from identical transcript variants.

As Toledo and Wahl note:

Mutations in TP53, the gene that encodes the tumour suppressor p53, are found in 50% of human cancers, and increased levels of its negative regulators MDM2 and MDM4 (also known as MDMX) downregulate p53 function in many of the rest.

Understanding p53 regulation remains a crucial goal to design broadly applicable anticancer strategies based on this pathway. This Review of in vitro studies, human tumour data and recent mouse models shows that p53 post-translational modifications have modulatory roles, and MDM2 and MDM4 have more profound roles for regulating p53. Importantly, MDM4 emerges as an independent target for drug development, as its inactivation is crucial for full p53 activation.

Overall p53 is a significant and generally well known gene. We have also described it in the pathways above.

3.3 CHK1

From NCBI[7]:

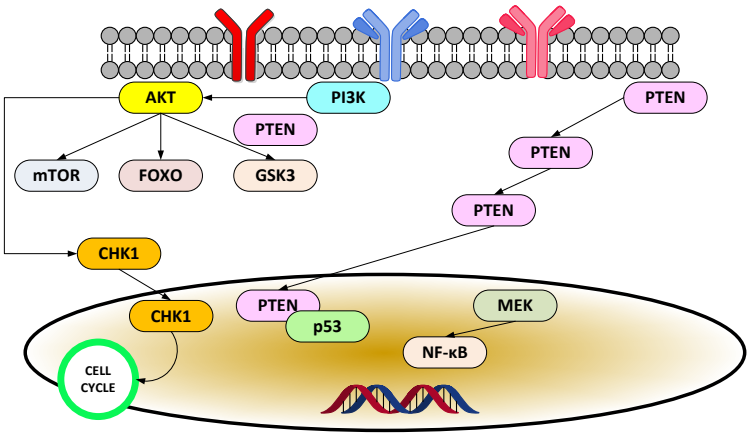
The protein encoded by this gene belongs to the Ser/Thr protein kinase family. It is required for checkpoint mediated cell cycle arrest in response to DNA damage or the presence of un-replicated DNA.

This protein acts to integrate signals from ATM and ATR, two cell cycle proteins involved in DNA damage responses, that also associate with chromatin in meiotic prophase I. Phosphorylation of CDC25A protein phosphatase by this protein is required for cells to delay cell cycle progression in response to double-strand DNA breaks.

The details of the CHK1 pathway are shown below[8]:

[7] https://www.ncbi.nlm.nih.gov/gene/1111

[8] http://www.wikipathways.org/index.php/Pathway:WP707#nogo2



Here we have PI3K driving AKT then CHK1 and finally the cell cycle. The importance of ATM and ATR are shown and as noted they are also key in cell damage, namely DNA damage, responses.

From Karnitz and Zou:

The human ATR gene encodes a kinase that is activated by DNA damage and replication stress as a central transducer of a checkpoint signaling pathway.

Once activated, ATR phosphorylates multiple substrates, including the kinase Chk1, to regulate cell cycle progression, replication fork stability, and DNA repair.

These events promote cell survival during replication stress and in cells with DNA damage.

Accordingly, there has been the tantalizing possibility that ATR inhibitors would be therapeutically useful, especially if they were more effective in tumor versus normal cells.

Indeed, multiple studies have demonstrated that alterations that promote tumorigenesis, such as defects in the ATM-p53 pathway, constitutive oncogene activation, and acquisition of the alternative lengthening of telomeres pathway, render tumor cells sensitive to ATR inhibitor monotherapy and/or increase the synergy between ATR inhibitors and genotoxic chemotherapies. Now, nearly two decades after the discovery of ATR, two highly selective and potent ATR inhibitors, AZD6738 and VX-970, are in early phase clinical trials either as monotherapies or paired with a variety of genotoxic chemotherapies.

Note the above suggestion of an ATR inhibitor. One can somewhat readily develop inhibitors yet the targeting at the best place in a gene signalling domain is essential. We examined PARP inhibitors which are targeting just the gene which effects the problem. Gene inhibitors of actors well above that can result in a multiplicity of unintended consequences. Now from Okada and Mack we have:

Activation of the G2 checkpoint begins with the detection of DNA damage by the sensory molecules ataxia teleangiectasia mutated (ATM) and ATM- and Rad3-related (ATR). Activation

of ATM and ATR in turn activates checkpoint kinase 2 (CHK2) and checkpoint kinase 1 (CHK1), respectively (BOX 1).

These kinases phosphorylate CDC25C, the molecule that is responsible for activating CDK1. Phosphorylated CDC25C associates with the p53 target molecule 14-3-3σ and is exported from the nucleus, resulting in CDK1 inactivation and the establishment of G2 arrest69. p53 sustains G2 arrest through another downstream target molecule, WAF1.

WAF1 binds to CDK1–cyclin complexes and blocks their activation, and also inhibits proliferating- cell nuclear antigen (PCNA) activity. The inhibition or inactivation of any of these G2-checkpoint genes — including those that encode ATR, CHK1, p53, WAF1 and 14-3-3σ 71— results in the death of cells that have sustained DNA damage by mitotic catastrophe. The transduction of genotoxic signalling to CHK1 and CHK2 is complex and involves many molecules that are associated with tumorigenesis.

Histone H2AX, p53- binding protein 1 (53BP1), BRCA1 and Nijmegen breakage syndrome 1 (NBS1) are all targets of ATM and ATR. Phosphorylated H2AX recruits 53BP1, BRCA1, mediator of DNA damage checkpoint protein 1 (MDC1) and the MRE11–RAD50–NBS1 complex to the site of DNA damage. Accordingly, H2AX-deficient cells show a G2-checkpoint defect and genomic instability in response to ionizing radiation.

Similarly, 53BP1 binds to p53, CHK2, structural maintenance of chromosome 1 (SMC1) and BRCA1 at foci that are induced by DNA damage, and mediates downstream DNA-damage signalling. 53BP1-deficient cells also show a G2-checkpoint defect79–81, and 53BP1-deficient mice are cancer-prone82. BRCA1 is necessary for the activation of CHK1, which leads to DNA-damage-induced G2 arrest83, and MDC1 is important for the formation of DNA-damage-induced foci. Cells that lack MDC1 are sensitive to ionizing radiation and show a G2-checkpoint defect

These three genes are reflected as adjuncts to the overall control mechanisms effected by PARP. We have tried to assemble a reasonable understanding of how this control mechanism functions and why the inhibitor is effective.

4 THERAPEUTIC PATHWAYS

We will now briefly examine the multiplicity of pathways so as to obtain a holistic understanding of the processes at work. From Fraser et al:

To address the genetic heterogeneity of non-indolent localized prostate cancer, we first comprehensively profiled CNAs in 284 localized prostate adenocarcinomas the profiles recapitulated those previously reported, including recurrent allelic gains of MYC and deletions of PTEN, TP53 and NKX3-1

As Karanika et al stated:

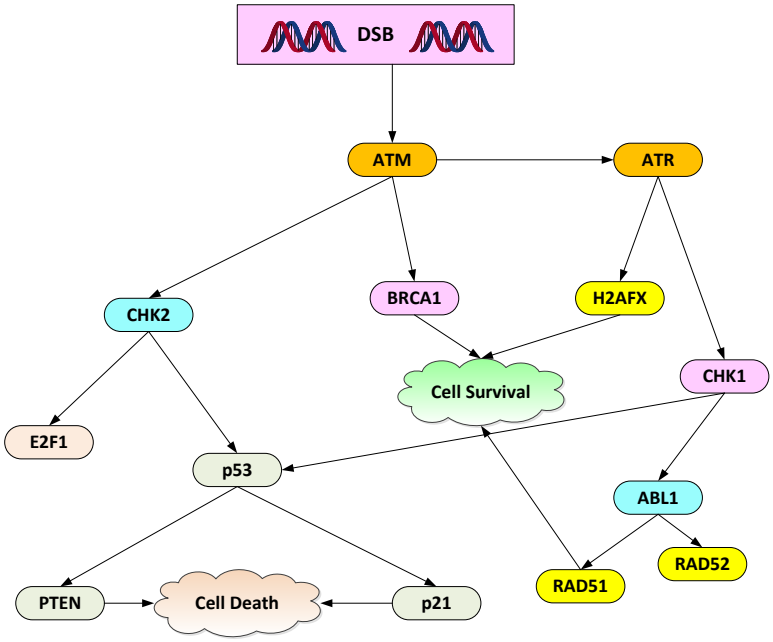
A more recent report suggests that AR inactivation leads to telomere dysfunction, contributing to genomic instability and progression of prostate cancer. Bowen et al. found that the prostate cancer suppressor NKX3.1, which is a target of AR, activates ATM, enhancing the DDR and thus contributing to DNA integrity in prostate epithelial cells. Notably, ATM missense mutations and polymorphisms increase the risk of prostate cancer development.

According to these data, it is conceivable that impaired DDR may promote prostate carcinogenesis while AR may maintain genomic integrity in the earlier stages of the disease through DDR activation, mainly by activating the ATM/Chk2 pathway. Further, it is believed that reactive oxygen species (ROS)–induced unrepaired DNA damage may be one of the main mechanisms related to initiation of this disease. Finally, it has been suggested that mutational or epigenetic inactivation of DDR components is selected for during neoplastic development, allowing malignant progression

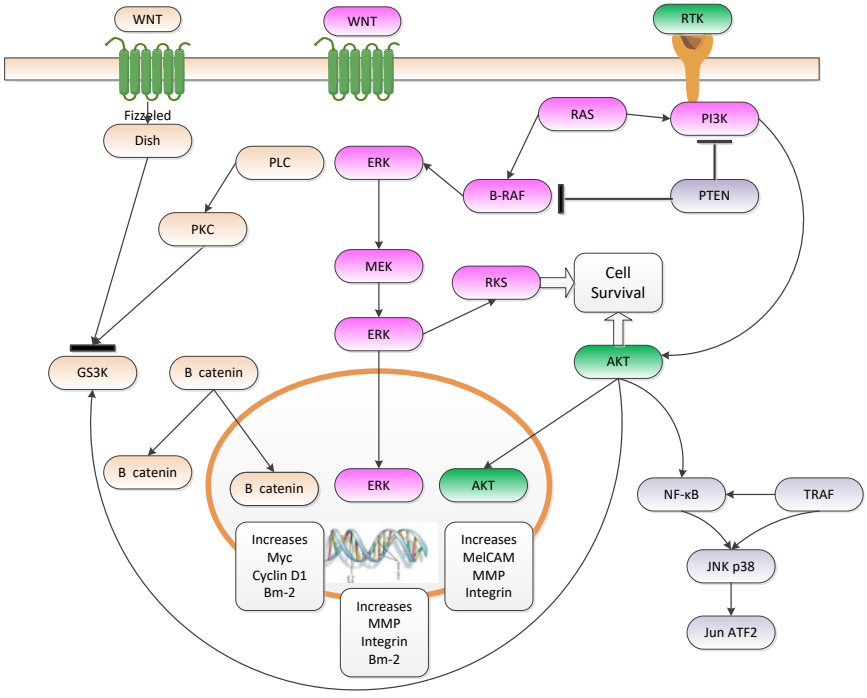
Abate Shen states:

Several tissue-specific regulatory genes have been found to play essential roles in both organogenesis and carcinogenesis. In the prostate, the Nkx3.1 homeobox gene plays an important role in normal differentiation of the prostatic epithelium while its loss of function is an initiating event in prostate carcinogenesis in both mouse models and human patients. Thus, the Nkx3.1 homeobox gene provides a paradigm for understanding the relationship between normal differentiation and cancer, as well as studying the roles of homeobox genes in these processes.

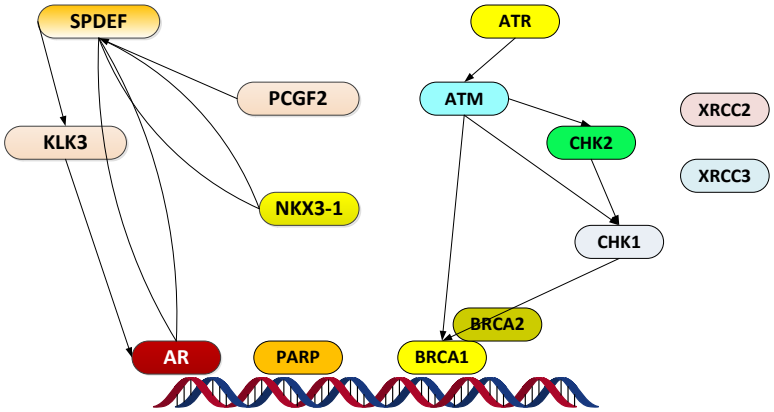
Thus, the network below depicts some of the key elements in pathway dynamics.



The above is a simplified version of the gene elements which are ancillary. A more complete showing is below.



Ultimately, we then must assemble the full complex of genes. This we do below. Here we have PARP as well as the other gene controllers and their effects.


SPDEF
PCGF2
KLK3
NKX3-1
AR
PARP
ATR
ATM
CHK2
CHK1
XRCC2
XRCC3
BRCA2
BRCA1

5 OBSERVATIONS

Cell growth and multiplication is an essential process of organism survival. Accuracy and consistency in DNA is at the heart of that process. Assaults on that process may come from somatic limitations such as BRCA defects or exogenously from assault such as radiation. These defects result in poor DNA repair which in turn results in defective cells, loss of cell reproduction management and a cancerous outcome.

The issue we face are twofold. First, how do we recognize these cells and then attack them which is the essence of the immunological school of thought and the second is the school of thought which says there are defects in certain pathways and we want to remediate them or block them. The latter school is what we are seeing here. In fact, the PARP inhibitor may have been initially counter intuitive. Namely we initially saw PARP as a SSB mechanism and BRCA as a DSB. However, lacking a BRCA then PARP stepped in and kept the process going just not that well. Thus, inhibiting PARP results in death of BRCA deficient DSBs.

As Karanika et al note:

DNA damage is considered one of the most frequent events contributing to development of both hematologic and epithelial neoplasms, including prostate cancer. Mutations and deletions of critical DDR "signaling nodes" regulated by ATM and Chk2 have been shown to increase the risk of prostate cancer development, while p53, an important mediator of DDR, is inactivated in most sporadic prostate cancers. Recent studies provide evidence supporting the role for AR in DDR activation and repair of DNA damage in prostate cancer cell survival through regulation of relevant genetic activities and pathways.

Although there is controversy in the literature, the activation of oncogenic signaling such as Akt and c-Myc has been in general shown to increase DNA damage through replication stress enhancing genomic instability and disease aggressiveness.

In cancer cells bearing defective DDR genes, the remaining active component of DDR becomes critical for tumorigenesis, since further inhibition of DDR may lead to genomic instability incompatible with cancer cell survival. Given a potential for AR to regulate genetic activities and pathways that influence DDR gene, particularly those related to ATM/Chk2 signaling, combination therapy with ADT plus a PARP inhibitor or ADT plus ATR and/or Chk1 inhibitors represents a rational therapeutic strategy.

Combination of a DDR-targeted agent with a cytotoxic DNA-damaging agent (e.g. platinum agents) is another approach to be considered. Clinical trials based on these concepts are critical to determine which agents, conditions, and combinations of agents will benefit patients' quality and longevity of life.

It should also be noted that introducing DDR-targeted agents into clinical management of such a heterogeneous disease as mCRCP is not without potential consequences as evidenced by the emergence of de novo neoplasias following exposure to DNA damaging agents. However, characterizing specific DDR defects in each patient should provide a unique opportunity for

personalized medicine utilizing rational therapy combinations that promote synthetic lethality or synergistic cytotoxicity.

The above is a warning ultimately on the unintended consequences issue. We also know that certain pathway blocking mechanisms have two effects. Fist they often do not last long. Second, they may activate other pathways or enhance them leading to secondary malignancies. This the advantage perhaps of a PARP inhibitor is that it inhibits just at the end of the chain. We think.

As the Cancer Atlas group notes in describing PARP inhibitors on PCa:

Prior data indicate that several DNA repair pathways are disrupted in a subset of prostate cancers. Moreover, the PARP inhibitor olaparib is effective in some patients with prostate cancer. Here, we found inactivation of several DNA repair genes that collectively affected 19% of affected individuals. While we found only one inactivating BRCA1 germline mutation, a frameshift at V923 caused by a 4 bp deletion, BRCA2 inactivation affected 3% of tumors, including both germline and somatic truncating mutations. All six BRCA2 germline mutations were K3326, a C-terminal truncating mutation with debated functional impact but increased prevalence in several tumor types*

Thus, the potential is present but not without risks.

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