Adaptive Mutability and Cancer

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Adaptive Mutability is mutations that adjust to fight the therapeutics used to address the initial malignancy. It is a concept that tries to explain how many therapeutics can be vitiated because of the stress placed upon the cancer cells and their resulting propensity to mutate in an adaptive manner. Copyright 2020 Terrence P. McGarty, all rights reserved.

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1 INTRODUCTION

There has been a significant development in cancer therapeutics. For example kinase inhibitors focus on stopping certain pathways which accelerate proliferation and thus lead to metastasis. However, these therapeutics also result in stress on the cells. It is known that stressed cells can often undergo adaptive mutagenesis, namely the very therapeutic process used to mitigate the cancer can itself act as a mechanism to initiate additional genetic alterations resulting in an alternative genetic cancer situation. Adaptive mutagenesis is a well-known process in bacteria, having been studied for almost a century. However the process is less well understood in humans.

1.1 GOOD NEWS BAD NEWS

We know that targeting such things as kinase elements and growth factors can mitigate against cancer proliferation. Yet the cells seem too often adapt and change gene expressions to do work arounds. In a recent Science article Gerlinger has noted:

Mutagenesis can drive carcinogenesis and continue during cancer progression, generating genetic intratumor heterogeneity that enables cancer adaptation through Darwinian evolution. Analyses, such as mutational signature characterization, have revealed specific mutational processes and their temporal activity during carcinogenesis and tumor progression (2). Nevertheless, many of the mechanisms that promote genomic instability in cancer are still enigmatic… Russo et al. reveal that drugs targeting oncogenic epidermal growth factor receptor (EGFR) or BRAF signaling increase mutagenesis in colorectal cancer (CRC) cells, which could drive the acquisition of resistance.

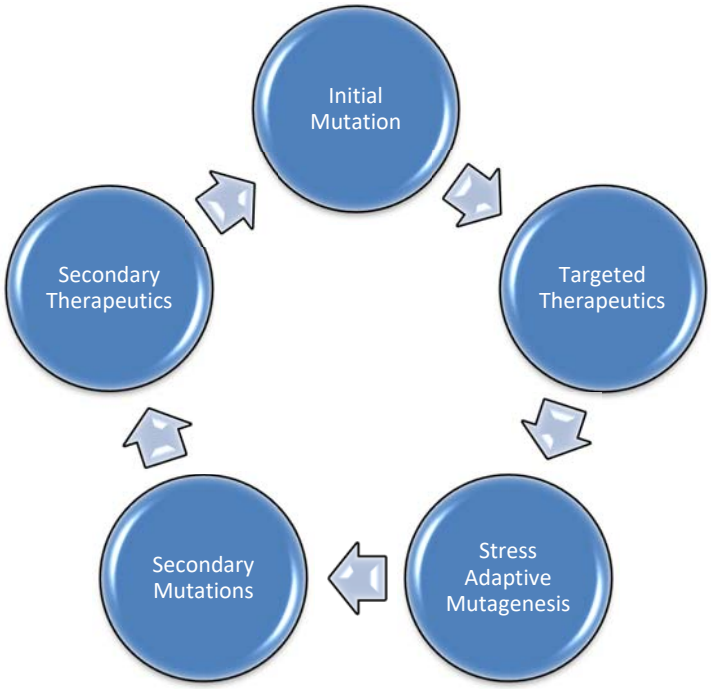
Note that the primary paper referred to above in Science by Russo et al have noted

More than 75 years ago, Luria and Delbrück demonstrated that bacterial resistance to phage viruses was due to random mutations that spontaneously occurred in the absence of selection. Resistance to targeted therapies in human tumors is also widely thought to be due to mutations that exist before treatment. The conventional view is that relapses occur because drug-resistant mutant subclones are present in any detectable metastatic lesion before the initiation of therapy. According to this view, resistance is a fait accompli, and the time to recurrence is merely the interval required for preexisting drug-resistant (mutant) cells to repopulate the lesion.

Here, we explore the hypothesis that resistance to targeted therapies can also be fostered by a transient increase in genomic instability during treatment, leading to de novo mutagenesis. A similar process has been shown to increase the emergence of microbial strains resistant to antibiotics. In a stable microenvironment, the mutation rate of microorganisms is usually low, which precludes the accumulation of deleterious mutations. However, several mechanisms of stress-induced genetic instability and increased mutability, known as stress-induced mutagenesis (SIM), have been described in bacteria and yeast.

1.2 OVERVIEW

In this note we examine in some detail the results and inferences of Russo et al. In our opinion it raises several questions of interest yet the answers seem lacking. The key principle is adaptive mutagenesis, generated under the stress of suppression by the applied targeted therapeutic. The basic principle appears below in the cycle which can be continued through multiple stages.



The authors further make the argument that mTOR activity induces proliferation and in turn there are multiple mis-match repairs, MMR, resulting in effective mutagenesis, and they in turn proliferate in an adaptive manner allowing for the development of new lines resistant to the therapeutic approach.

2 ADAPTIVE MUTABILITY

Mutations occur frequently. Most are filtered out, repaired, or just killed off. However, we generally accept that cancer is the result of a self-sustaining mutation from a cause or causes often unknown. We know that certain cancers, such as thyroid, may be the result of radiation exposure, whereas melanoma is often the result of excess sun exposure. Viruses such as HPV cause cervical cancer and in fact such a cancer cane be prevented via vaccination.

Now Russo et al argue that adaptive mutagenesis is the basis for the loss of targeting of such thinks as kinase inhibitors and growth factor inhibition. However, such is a bit of a stretch if you will see that a clear identification of the process is lacking.

The logic used is as follows (we quote from Gerlinger):

1. *Russo et al. found that human CRC cell lines that were treated with EGFR or BRAF inhibitors down-regulated the expression of high-fidelity DNA repair proteins and increased that of error-prone DNA repair proteins, which may both increase mutation rates.* The issue here is the use of first line therapeutic has a secondary effect of suppressing key DNA repair mechanisms.

2. Specifically mismatch repair, MMR, and homologous repair, HR, were impaired.

3. *Adaptive mutagenesis is a mechanism described in bacteria that increases the mutation rate in response to cell stress. This is triggered by a cell-stress signaling pathway that activates error prone DNA double-strand break repair and it is accompanied by suppression of MMR*. Namely the stress may be reactive oxygen stress, ROS, or the equivalent, and the result is a mis match an from there we get a mutation.

4. *Russo et al. explored whether the mammalian target of rapamycin (mTOR) pathway, a major stress signaling pathway in humans, controls drug induced mutagenesis in cancer cells. mTOR signaling was indeed inactivated by drug treatment, but inhibiting the mTOR pathway alone did not phenocopy the changes in DNA repair protein expression. The trigger of drug-induced mutagenesis in CRC cells is therefore either more complex or different from that in bacteria*. mTOR is a powerful pathway which we shall discuss. However it is not clear that this pathway can alone effect an adaptive mutagenesis.

5. *The contribution of drug-induced mutagenesis to clinically acquired resistance in patients with CRC and other cancer types is now important to assess because this remains unclear for several reasons. Mutational processes differ in the preferred DNA sequence contexts in which they occur and in the genetic variants they generate. MMR deficiency leads to high rates of deletions in nucleotide repeats and to cytosine-to-thymine base changes*. The changes are well known but the changes related to an adaptive path require a more focused driver. Thus what drives the changes, namely selecting those leading to reactivation of aggressive malignancy.

The Russo paper raises questions, presents some answers yet leaves a great dal yet to be explained. We examine adaptive mutagenesis in this section.

2.1 DEFINITION

Let us begin with and attempt to give a definition of adaptive mutation. Before doing so let us examine the two words separately. First adaptive implies that there is some exogeneous element or process which makes the change occur in a manner which is different because of the presence of his exogeneous element. The change is somehow driven by this element. Second, look a mutation. This is a fundamental change in a gene, a change in the underlying DNA and not just some epigenetic modification. Things have been tampered with. Combined the term adaptive mutation is a tampered change in a direction compliant with the presence of the exogeneous agent. Roth et al have discussed[1]:

The term adaptive mutation has been defined as the process by which stresses that are not directly mutagenic activate mechanisms for causing mutations, even in nongrowing cells (stress-induced or stationary-phase mutagenesis). This definition assumes a mechanism that we think is unlikely to exist. In order to consider all explanations of the relevant phenomena, adaptive mutation is defined here as the process by which mutations arise under selective conditions, whether or not mutation rates increase or growth is required. The area discussed here has been reviewed previously from various points of view Darwin suggested that stress might generate the variability upon which natural selection operates.

The classic experiments of Luria & Delbruck and Lederberg demonstrated that some mutations arise without the influence of selective stress. However, the lethal selections they used could not have detected mutations induced by selective conditions. Shapiro, Cairns et al., and Hall pointed out this deficiency and described genetic systems in which selective conditions seemed to increase the mutation rate. The Cairns system has been analyzed in most detail and remains controversial despite this effort.

As Murray noted:

In 1943, it had long been known that bacterial cultures rapidly develop resistance to viral infection. Some biologists argued that viruses directly induced resistance mutations, while others believed the mutations arose spontaneously before exposure to the virus. But when Luria and Delbrück first attempted to distinguish between these two hypotheses, they were frustrated by what appeared to be irritatingly inconsistent mutation rates. Then, after watching a colleague win a jackpot ($3 in dimes!) at a slot machine, Luria realized this inconsistency was telling him something: the number of mutant bacterial colonies present at the end of the experiment depended on when the mutations arose. Mutations arising in earlier generations would be present in many descendent cells (a "jackpot"), whereas mutations occurring in later generations would be present in only a few cells.

Luria passed his insight to Delbrück, who worked out the expected statistical distribution of the number of mutant cells per culture. Their data decisively rejected the hypothesis that bacteria became resistant only after being exposed to the virus and strongly supported the prediction that

[1] From the definition section they note: *Adaptive mutation: any process by which fitter mutations arise under selective conditions; may or may not require mutagenesis or growth.*

the phage-resistant mutations had a constant probability of occurring in each cell division. The Luria–Delbrück article had three important impacts beyond its direct conclusion: it showed that elegant statistical analysis could illuminate biological processes that could not be directly observed, it contributed to Luria and Delbrück winning the 1969 Nobel Prize in Medicine or Physiology (shared with Alfred Hershey), and it led, indirectly, to a continuing debate about whether organisms exert physiological control over their mutation rates.

2.2 PROCESS

Foster notes:

Adaptive mutation is defined as a process that, during nonlethal selections, produces mutations that relieve the selective pressure whether or not other, nonselected mutations are also produced. Examples of adaptive mutation or related phenomena have been reported in bacteria and yeast but not yet outside of microorganisms. A decade of research on adaptive mutation has revealed mechanisms that may increase mutation rates under adverse conditions. This article focuses on mechanisms that produce adaptive mutations in one strain of Escherichia coli, FC40. These mechanisms include recombination-induced DNA replication, the placement of genes on a conjugal plasmid, and a transient mutator state. The implications of these various phenomena for adaptive evolution in microorganisms are discussed.

Gerlinger notes:

Adaptive mutagenesis is a mechanism described in bacteria that increases the mutation rate in response to cell stress. This is triggered by a cell-stress signaling pathway that activates error-prone DNA double-strand break repair and it is accompanied by suppression of MMR. Adaptive mutagenesis increases the probability of generating mutations that enable evolutionary adaptation of unicellular organisms to new environments.

On the basis of the pronounced similarities of drug-induced mutagenesis in CRC and adaptive mutagenesis in bacteria, Russo et al. explored whether the mammalian target of rapamycin (mTOR) pathway, a major stress signaling pathway in humans, controls drug-induced mutagenesis in cancer cells. mTOR signaling was indeed inactivated by drug treatment, but inhibiting the mTOR pathway alone did not phenocopy the changes in DNA repair protein expression. The trigger of drug-induced mutagenesis in CRC cells is therefore either more complex or different from that in bacteria.

As Hall has noted:

Adaptive mutations are spontaneous mutations that occur in microorganisms during periods of prolonged stress in non-dividing or very slowly dividing populations and that are specific to the environmental challenge that causes that stress. This article reviews the literature on adaptive mutagenesis since 1993. The evidence that adaptive mutagenesis is both real and general is considered. The most widely used system for studying adaptive mutagenesis, reversion of an F'-borne lacI33 allele, is shown to be a special case that reflects more about F-plasmid biology than about adaptive mutagenesis in general.

New evidence demonstrating that adaptive mutagenesis is, indeed, specific is discussed. A variety of genes whose products affect adaptive mutagenesis are discussed. A model to explain that specificity and new evidence in support of that model are considered, as are potential roles of adaptive mutagenesis in evolution and practical aspects of adaptive mutagenesis.

As Slechta et al have noted:

The term ‘‘adaptive mutation’’ refers here to the process by which selection increases the number of Lac+ revertants. This definition makes no assumptions regarding the role of growth or the contribution of general mutagenesis to reversion. Two aspects of this process are discussed here: reversion and mutagenesis. In this article, ‘‘reversion’’ means mutational correction of a particular lac allele to lac+ under selective conditions defined for this system. By ‘‘mutagenesis,’’ we mean a genome-wide undirected increase in mutation rate occurring during the process of reversion under selection, detected as unselected mutations carried by Lac+ revertants.

2.3 TYPES

Roth et al define three types of adaptive mutagenesis.

1. *Functional Direction of Mutagenesis: This early model proposes that nongrowing cells make only mutations that improve their fitness.*

2. *Generally Hypermutable State: A model proposed by Hall suggests that stress induces a general (undirected, genomewide) hypermutable state (HMS) in a subset (0.1%) of the nongrowing starved population. Cells in the subpopulation ultimately die of lethal mutations unless they first acquire a lac reversion event, which relieves the stress and terminates the HMS. Mutagenesis appears directed to lac because only Lac+ revertants survive hyper mutagenesis.*

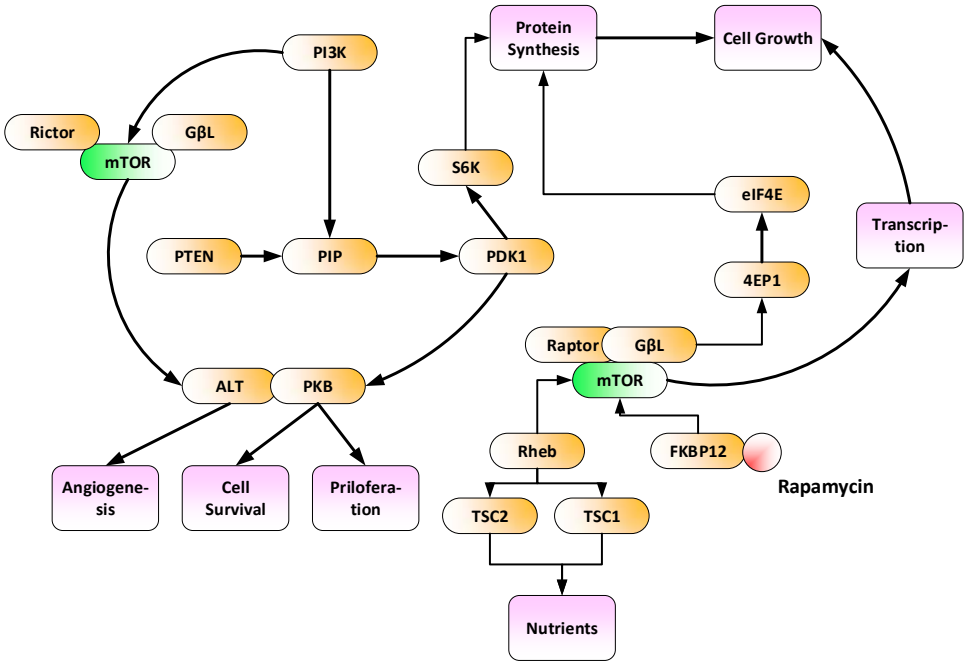
3. *Positional Direction of Hypermutability: This model is a hybrid of the previous two models. It assumes that stress induces DinB in nongrowing cells and mutagenizes any region in which recombinational replication is occurring. Mutagenesis is directed to the F" plasmid, whose conjugation transfer functions (tra) produce DNA ends that stimulate intense recombination between plasmid sequences. Because only the plasmid is mutagenized, associated chromosomal mutations are not expected. The observed lac revertants can be explained by a 100-fold increase in mutation rate on the plasmid (rather than a global 105-fold increase) because the whole population is affected rather than a subpopulation. The genome-wide mutagenesis seen in a minority of revertant clones (10%) is not considered central*

3 mTOR

We consider mTOR as a possible stressor source for adaptive mutagenesis. To do so we examine mTOR and then examine its influence.

3.1 Specifics of Effects

mTOR, the mammalian target of rapamycin, is a gene product (1p36.2) is a protein which acts in a critical manner in interconnecting the genetic circuits in mammals, and especially man. It fundamentally controls glucose transport and protein synthesis. The pathway depicted below is a modification of the graphic from Weinberg (p 785) which shows mTOR in its two modes, one with Raptor assisting and one with Rictor. The Rictor/mTOR mode activates the Akt pathway via the placement of a phosphate and the manages the protein synthesis portion. The inclusion of rapamycin will block the Raptor/mTOR path and reduce the protein synthesis and cell growth portion. The inhibitory effect on Akt/PKB by rapamycin is assumed to be the main factor in its anti-cancer effects.



mTOR plays a significant role in the process of autophagy. We examine its functions and then move to its role in autophagy. The flow shown below demonstrates the position of mTOR. As NCBI notes[2]:

[2] https://www.ncbi.nlm.nih.gov/gene/2475

The protein encoded by this gene belongs to a family of phosphatidylinositol kinase-related kinases. These kinases mediate cellular responses to stresses such as DNA damage and nutrient deprivation. This protein acts as the target for the cell-cycle arrest and immunosuppressive effects of the FKBP12-rapamycin complex[3].

NCBI (via KEGG) notes[4]:

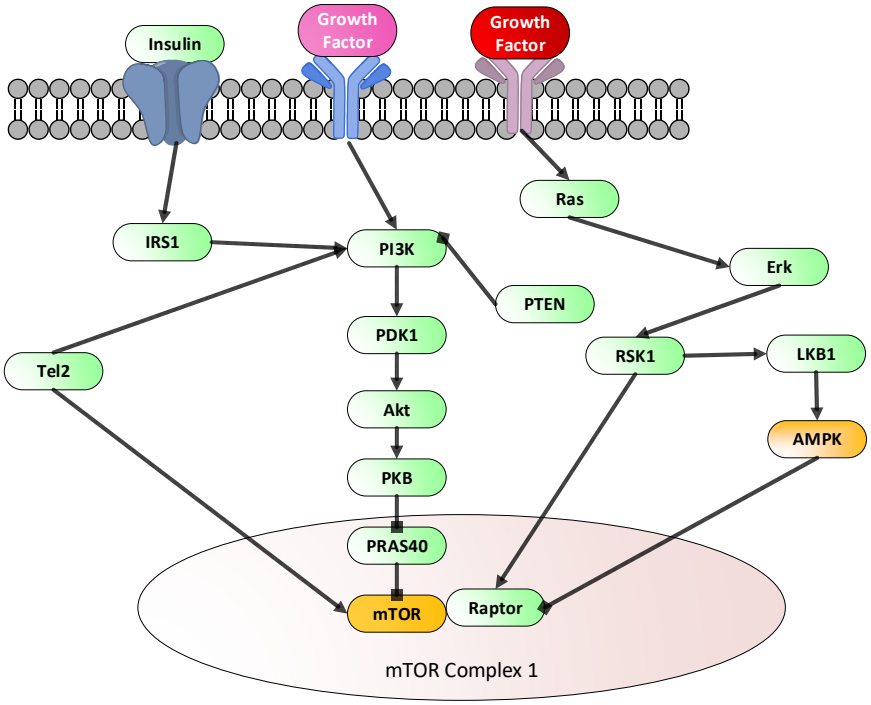
Autophagy (or macroautophagy) is a cellular catabolic pathway involving in protein degradation, organelle turnover, and non-selective breakdown of cytoplasmic components, which is evolutionarily conserved among eukaryotes and exquisitely regulated. This progress initiates with production of the autophagosome, a double-membrane intracellular structure of reticular origin that engulfs cytoplasmic contents and ultimately fuses with lysosomes for cargo degradation. Autophagy is regulated in response to extra- or intracellular stress and signals such as starvation, growth factor deprivation and ER stress. Constitutive level of autophagy plays an important role in cellular homeostasis and maintains quality control of essential cellular components.

The pathways above demonstrate this discussion. mTOR is facilitated by Raptor, Deptor, PRAS40 and mLSTS. This is the core of the mTOR signalling pathway. The ATG genes then facilitate the autophagy process when activated via this mTOR complex. mTOR plays a significant role in cell survival and proliferation.

Currently the pathways which control many cancers are also pathways in which mTOR plays a role. In the following figure above we depict some of these which have obtained significant clinical interest and attention.

[3] From NCBI, *The mammalian target of rapamycin (mTOR) also known as mechanistic target of rapamycin or FK506 binding protein 12-rapamycin associated protein 1 (FRAP1) is a protein which in humans is encoded by the FRAP1 gene. mTOR is a serine/threonine protein kinase that regulates cell growth, cell proliferation, cell motility, cell survival, protein synthesis, and transcription. mTOR belongs to the phosphatidylinositol 3-kinase-related kinase protein family.*

[4] https://www.ncbi.nlm.nih.gov/biosystems/83058?Sel=geneid:2475#show=genes also see the KEGG pathway https://www.kegg.jp/pathway/hsa04140



3.2 FURTHER DETAILS

From Paquette et al:

The mechanistic target of rapamycin (mTOR) is a serine-threonine protein kinase that can be divided into two functionally and biochemically distinct complexes, mTORC1 and mTORC2. Both are implicated in growth factor sensing, but mTORC1 is generally the one associated with cell proliferation and cancer progression when deregulated. Significant progress was made in recent years to understand mTORC1 response to growth factors, such as insulin and insulin-like growth factor. ....

Macroautophagy (referred to as autophagy hereafter), the cellular self-degradation process, plays an important role in energy supply, particularly during development and in response to nutrient stress. It is a process through which cargo is delivered to double-membrane vesicles, termed autophagosomes, which fuse with the lytic compartment and release the inner vesicle into the lumen, leading to the degradation of cell components and the recycling of cellular building blocks.

This intracellular mechanism is conserved in eukaryotes from yeast to complex multicellular organisms, and its dysfunction has been implicated in many human diseases, including myopathy, neurodegeneration, and cancer, as well as resistance to pathogen infection. At the molecular level, autophagy plays a context dependent pro-survival or pro-death role by regulating different signaling pathways, including p53, Bax-interacting factor-1 (Bif-1), Beclin 1 (BECN1), ultraviolet irradiation resistance-associated gene (UVRAG), mTOR, protein kinase B (Akt), B-cell lymphoma 2 (Bcl-2), Ras, and Class I PI3K (PI3KI) in cancer [80]. The focus of this part of the review will be mainly on mTOR pathways; however, these pathways are

interconnected and they can integrate into an autophagy-related cancer network that could ultimately affect the fate of cancer cells.

Among several components involved in the tight regulation of autophagy, mTORC1, but not mTORC2, has been shown to be a key player in coordinating the respective anabolic and catabolic processes in response to environmental and physiological stresses. Studies have shown that mTORC1 inhibition increases autophagy, whereas stimulation of mTORC1 reduces this process. mTORC2 was reported to indirectly suppress autophagy through the activation of mTORC1.

The PI3K signaling axis activates mTORC2, which, in turn, phosphorylates AKT at two different sites, leading to AKT/mTORC1 signaling axis activation. Further studies are required to determine whether there is a direct role for mTORC2 in autophagy regulation. In mammals, and under nutrient-rich conditions, it was reported by three independent groups that mTORC1 controls autophagy through the regulation of a protein complex composed of unc-51-like kinase 1 (ULK1), autophagy-related gene 13 (ATG13), and focal adhesion kinase family-interacting protein of 200 kDa (FIP200) through directly phosphorylating and suppressing this kinase complex required to initiate autophagy.

mTORC1 was reported to directly phosphorylate and suppress this kinase complex required to initiate autophagy.

Conversely, nutrient withdrawal stimulates the ULK1/ATG13/FIP200 complex formation and initiates autophagy via ULK1 auto-phosphorylation and phosphorylation of its binding partners. In line with these findings, rapamycin-induced inhibition of mTORC1 was shown to enhance the kinase activity of ULK1, while mTORC1 activation through Rheb overexpression potently represses ULK1. Subsequent studies further identified Ser758 in the human protein as the major mTORC1-mediated inhibitory phosphorylation site on ULK1, leading to the complex dissociation and autophagy repression.

In addition to phosphorylation of ULK1, mTORC1 was also shown to indirectly inhibit autophagy through the phosphorylation of autophagy/Beclin-1 regulator 1 (AMBRA1), preventing ubiquitination of ULK1 by TNF receptor-associated factor 6, an E3 ubiquitin protein ligase (TRAF6), which, under starvation conditions, causes ULK1 self-association, stabilization, and enhancement of its kinase activity…

3.3 PATHWAY COMPLEXITIES

Looking at the complexity of the mTOR pathway it presents an interesting one for addressing PCa. Kinkaide et al (2008) indicate:

Among the major signaling networks that have been implicated in advanced prostate cancer are the AKT/mammalian target of rapamycin (AKT/mTOR) and MAPK pathways. Indeed, deregulated expression and/or mutations of the phosphate and tensin homolog tumor suppressor gene (PTEN) occur with high frequency in prostate cancer, leading to aberrant activation of AKT kinase activity as well as its downstream effectors, including the mTOR signaling pathway.

In addition, many prostate tumors display deregulated growth factor signaling, which may result in activation of MAPK kinase 1 (MEK) kinase and ultimately ERK MAP.

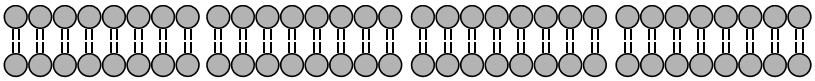
Notably, previous studies have demonstrated that the AKT/mTOR and MAPK signaling pathways are alternatively and/ or coordinately expressed in advanced prostate cancer and function cooperatively to promote tumor growth and the emergence of hormone- refractory disease. These observations formed the basis for our hypothesis that targeting these signaling pathways combinatorially may be effective for inhibiting tumorigenicity and androgen independence in prostate cancer.

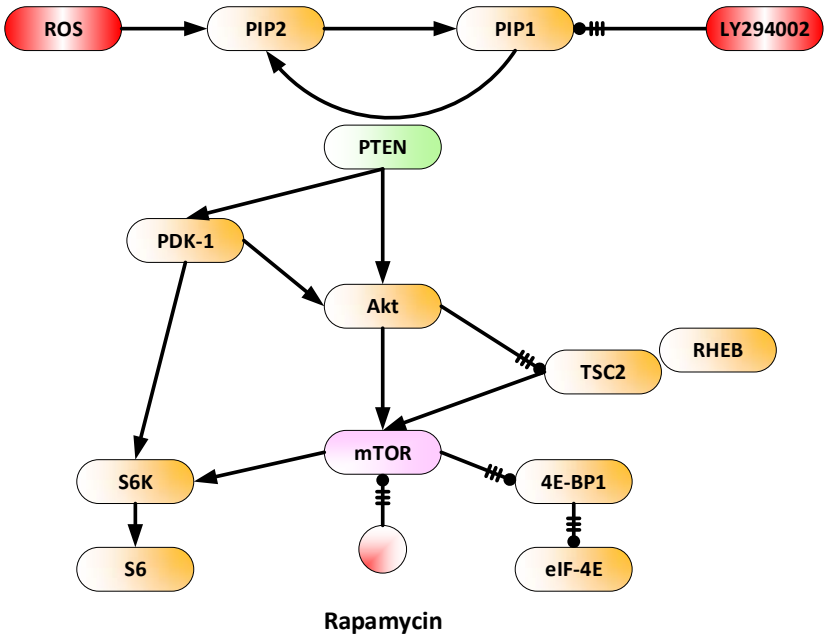
Kinkaide et al also demonstrate the creation of HGPIN via their work. This represents another pathway of HGPIN to PCa. LoPiccolo et al state:

The PI3K/Akt/mTOR pathway is a prototypic survival pathway that is constitutively activated in many types of cancer. Mechanisms for pathway activation include loss of tumor suppressor PTEN function, amplification or mutation of PI3K, amplification or mutation of Akt, activation of growth factor receptors, and exposure to carcinogens. Once activated, signaling through Akt can be propagated to a diverse array of substrates, including mTOR, a key regulator of protein translation. This pathway is an attractive therapeutic target in cancer because it serves as a convergence point for many growth stimuli, and through its downstream substrates, controls cellular processes that contribute to the initiation and maintenance of cancer.

Moreover, activation of the Akt/mTOR pathway confers resistance to many types of cancer therapy, and is a poor prognostic factor for many types of cancers. This review will provide an update on the clinical progress of various agents that target the pathway, such as the Akt inhibitors perifosine and PX-866 and mTOR inhibitors (rapamycin, CCI-779, RAD-001) and discuss strategies to combine these pathway inhibitors with conventional chemotherapy, radiotherapy, as well as newer targeted agents. We (show) how the complex regulation of the PI3K/Akt/mTOR pathway poses practical issues concerning the design of clinical trials, potential toxicities and criteria for patient selection.

LoPiccolo et al show the more simplified pathway as follows:





As we have shown with the more complex Weinberg model, here mTOR and PTEN play a strong role in the overall control. The authors show the points of possible control. The complexity of the pathways will be a challenge. It is less an issue of size complexity than a feedback and instability complexity. E Nelson et al (2007) have demonstrated similar results as well.

Other researchers have also posited other simple models. We demonstrated the one by Hay as has been stated:

The downstream effector of PI3K, Akt, is frequently hyperactivated in human cancers. A critical downstream effector of Akt, which contributes to tumorigenesis, is mTOR. In the PI3K/Akt/mTOR pathway, Akt is flanked by two tumor suppressors: PTEN, acting as a brake upstream of Akt, and TSC1/TSC2 heterodimer, acting as a brake downstream of Akt and upstream of mTOR.

In the absence of the TSC1/TSC2 brake, mTOR activity is unleashed to inhibit Akt via an inhibitory feedback mechanism. Two recent studies used mouse genetics to assess the roles of PTEN and TSC2 in cancer, underscoring the importance of Akt mTOR interplay for cancer progression and therapy.

The argument being made above for the over expression of mTOR and it turn its impact on proliferation is well known. However the impact on mutagenesis begs the question since this link is unverified.

An additional model has been proposed by Baldo et al (2008). The model is consistent with what we have shown previously. Specifically they state:

The process starts when mTOR inhibitors bind to FKBP12 and generate with TORC1 a potent inhibitory complex for the signaling pathway. mTOR inhibitors different from "rapalogs" may act directly on PI3K, thus converting PIP2 to PIP3 and activating Akt.

3.4 CONTROL AND TARGETING

The Baldo et al model is quite similar to the Weinberg model shown initially. It clearly demonstrates the overall controlling influence of mTOR. As Baldo et al state:

There is a great body of evidence supporting consideration of the mTOR signaling system as an important network in cell regulation, differentiation and survival . mTOR is a sensor of mitogen, energy and nutritional levels, acting as a "switch" for cell-cycle progression from phase G1 to phase S.

The antibiotic Rapamycin, a potent mTOR inhibitor, has been known to the National Cancer Institute and recognized for its potential anticancer properties since the 1970s. The observation that cell lines from different cancer types exposed to low doses of Rapamycin underwent cell-cycle arrest in phase G1, provided the basis for considering mTOR as a target for cancer therapy.

Development of mTOR inhibitor compounds has proceeded empirically due to the lack of understanding of the precise molecular targets and the required dose of the new compounds . The development of Rapamycin analogs ("Rapalogs"), but also of other, structurally different, mTOR inhibitors, was directed at the selection of specific cancer type sensitivity and an optimization of pharmaceutical forms.

To give an example, Temsirolimus revealed clinical responses in patients with renal cell carcinoma in advanced stage. Temsirolimus was approved by the FDA on May 2007 for this therapeutic use and is being investigated in clinical trials for other cancer types (breast cancer, lymphoma, renal cancer, glioblastoma); significantly there are a considerable number of clinical studies involving mTOR inhibitors currently active worldwide…

The mTOR pathway controls cell size and cellular proliferation*….nutrient metabolism, mRNA translation and cell survival control. Disruption of TOR leads to early embryonic death in flies and mammalian cells, indicating mTOR plays an important role in regulating cell survival. … deregulation of several mTOR components leads to modified cell proliferation patterns and, on the other, that many mTOR components are deregulated in several human cancers.*

*… **Therefore, inhibition of mTOR leads to slowing or arrest of cells in the G1 phase**. Translational control may have an important role in the balance of cell survival and death, and*

hence for apoptosis. Importantly, components of mTOR are deregulated in some human cancers, for example, breast and colon. Alteration of PI3-K/Akt is frequently observed in head and neck cancer .

Then if we look at mTOR as a necessary element in mitosis, then perhaps there may be a putative link, but it appears to be at best a supportive role. The authors continue:

PTEN, a phosphatase that acts on PIP3 to convert it to PIP2, normally regulates the mTOR pathway negatively, and shows decreased activity in some tumors. A strong relation seems to exist between the sensitivity to the effect of Rapamycin and PTEN loss or deregulation. PTEN is frequently mutated in several cancers and in cancer-like syndromes like Cowden and Proteus syndromes…

Loss of PTEN function can occur in 26-80% of endometrial carcinomas, …recent studies of human prostate cancer have shown that loss of PTEN is strongly associated with more aggressive cancers. The relationship between PTEN status and sensitivity to rapalogs has been questioned by several investigators. Some attention has recently been dedicated to the role of the mTORC2 complex in the mTOR pathway.

In fact this complex, believed until recently to be completely insensitive to the effect of Rapamycin, after long-term exposure to Rapamycin is able to prevent mTOR-mediated Akt phosphorylation and the activation of the mTOR pathway. Another component, the TSC1/TSC2 complex located upstream of mTOR, is predicted to integrate signals derived from nutrients, cellular energy status and hypoxia into a common growth regulatory signal to the mTORC1 complex.

As Easton and Houghton state:

Proteins regulating the mammalian target of rapamycin (mTOR), as well as some of the targets of the mTOR kinase, are overexpressed or mutated in cancer. Rapamycin, the naturally occurring inhibitor of mTOR, along with a number of recently developed ***rapamycin analogs*** *(rapalogs) consisting of synthetically derived compounds containing minor chemical modifications to the parent structure, inhibit the growth of cell lines derived from multiple tumor types in vitro, and tumor models in vivo.*

Results from clinical trials indicate that the rapalogs may be useful for the treatment of subsets of certain types of cancer. The sporadic responses from the initial clinical trials, based on the hypothesis of general translation inhibition of cancer cells are now beginning to be understood owing to a more complete understanding of the dynamics of mTOR regulation and the function of mTOR in the tumor microenvironment. This review will summarize the preclinical and clinical data and recent discoveries of the function of mTOR in cancer and growth regulation.

The above again recapitulates the necessity for supporting mitosis but not compelling it. Finally, as Hsieh et al have noted:

It is unknown whether specialized networks of translationally controlled mRNAs can direct cancer initiation and progression, thereby mirroring cooperativity that has mainly been

observed at the level of transcriptional control. This is an important question, as key oncogenic signalling molecules, such as the mTOR kinase, directly regulate the activity of general translation factors.

Downstream of the phosphatidylinositol-3-OH kinase (PI(3)K)–AKT signalling pathway, mTOR assembles with either raptor or rictor to form two distinct complexes: mTORC1 and mTORC2.

We see mTORC1 and mTORC2 as complexes supporting the mTOR pathway and possible therapeutic targets. The authors continue:

The major regulators of protein synthesis downstream of mTORC1 are 4EBP1 (also called EIF4EBP1) and p70S6K1/2. 4EBP1 negatively regulates eIF4E, a key rate-limiting initiation factor for cap-dependent translation. Phosphorylation of 4EBP1 by mTORC1 leads to its dissociation from eIF4E, allowing translation initiation complex formation at the 59 end of mRNAs5. The mTOR-dependent phosphorylation of p70S6K1/2 also promotes translation initiation as well as elongation.

At a genome-wide level, it remains poorly understood whether and how activation of these regulators of protein synthesis may produce specific changes in gene expression networks that direct cancer development. Here we use a powerful new technology known as ribosome profiling to delineate the translational landscape of the cancer genome at a codon-by-codon resolution upon pharmacological inhibition of mTOR.

Our findings provide genome-wide characterization of translationally controlled mRNAs downstream of oncogenic mTOR signalling and delineate their functional roles in cancer development. Moreover, we determine the efficacy of a novel clinically relevant mTOR inhibitor that we developed, INK128, which specifically targets this cancer program.

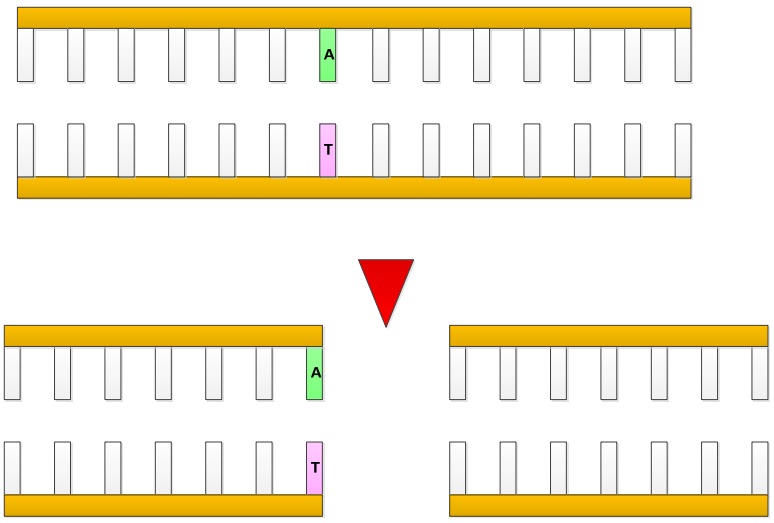
4 MISMATCH REPAIR

Mismatch repair, MMR, is a DNA repair mechanism which attempts to repair DNA when the nucleotide opposite one another are inappropriate. There are many types of breaks, single and double stranded as well as the mis-match ones we discuss here. We first examine the classic double stranded break, DSB.

4.1 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:

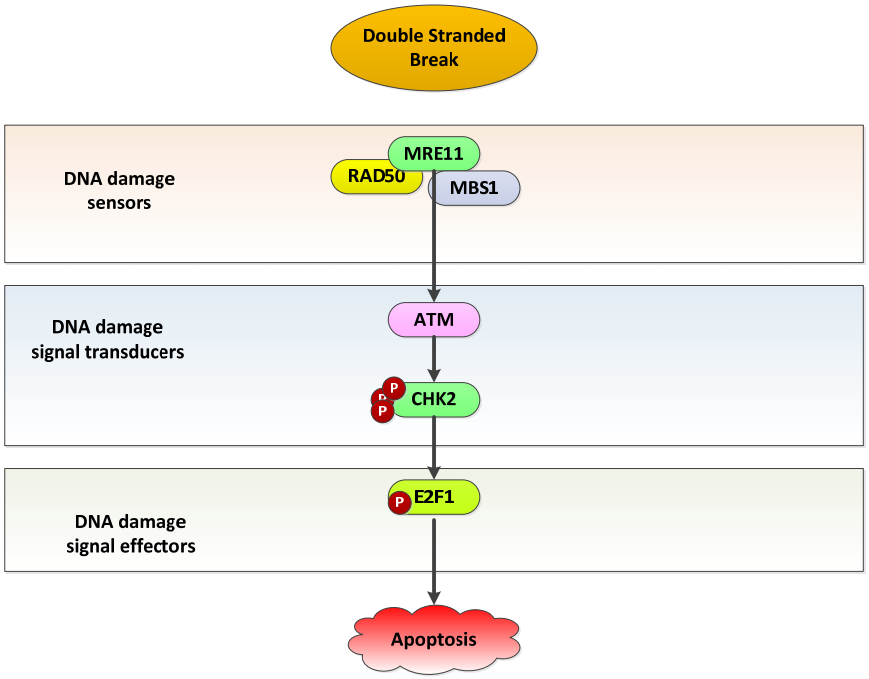
The double-strand break (DSB) is believed to be one of the most severe types of DNA damage, and if left unrepaired is lethal to the cell.

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.

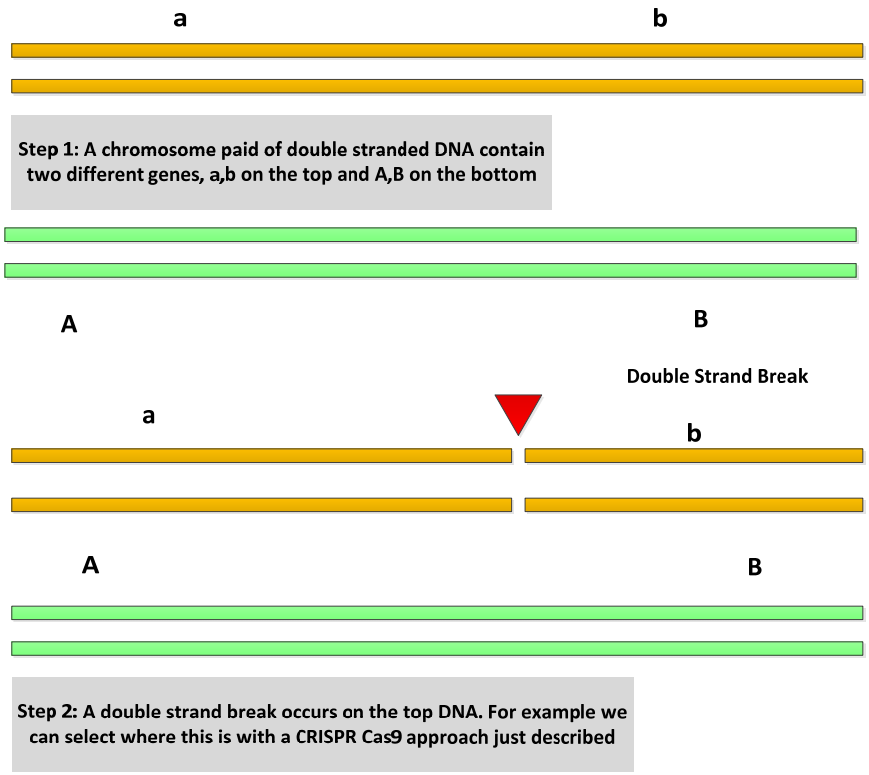
Exogenous DNA Damage	Dose Exposure (mSv)	DNA Lesions Generated	Number Lesions/Cell/Day
Peak hr. sunlight	—	Pyrimidine dimers, (6–4) photoproducts	100,000/day
Cigarette smoke	—	aromatic DNA adducts	45–1029
Chest X-rays	0.02	DSBs	0.0008
Dental X-rays	0.005	DSBs	0.0002
Mammography	0.4	DSBs	0.016
Body CT	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

4.2 HOMOLOGOUS REPAIR

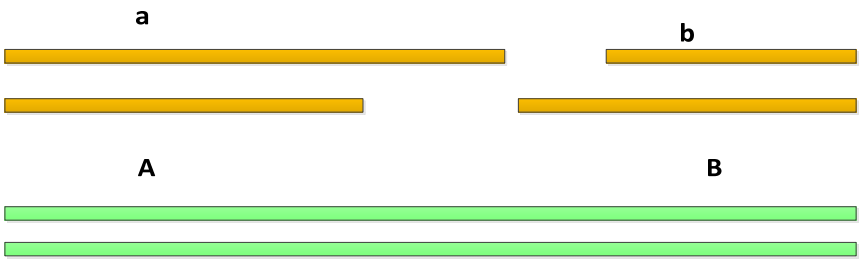
The paper by Chapman et al is a key document presenting many of the details we show herein. We have taken a simplified view so as to focus on the genetic elements of concern herein. Thus, there is a significant amount of complexity left aside.

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.

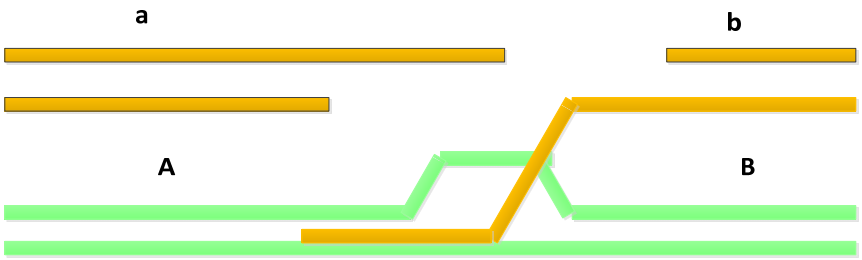
We start with the process and expand it in 3 Figures. The following Figure depicts the beginning.



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.

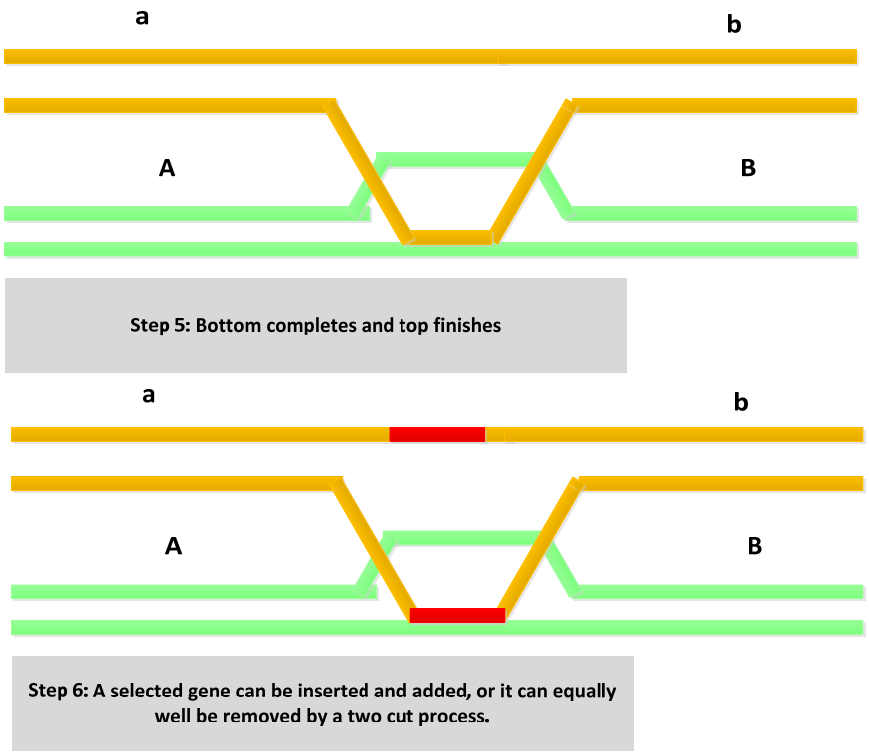


Step 3: The cut strands are shortened in top and bottom branches



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.



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.

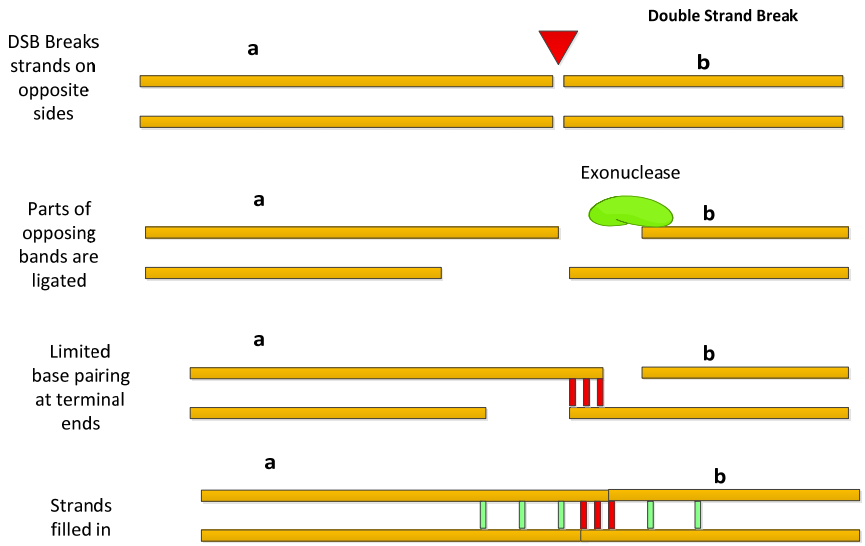
4.3 NON-HOMOLOGOUS

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.

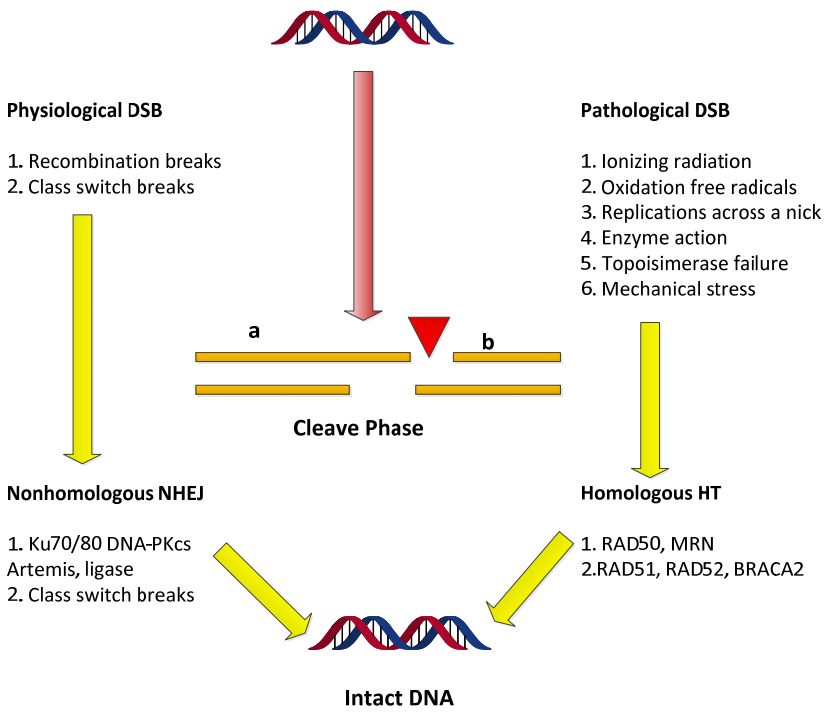


See p 53 Mendelsohn et al

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.

4.4 RELATIONSHIPS

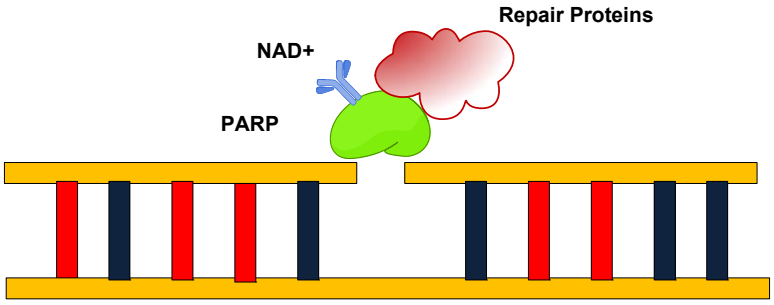
The Figure below depicts the collection of these processes in toto. There are seen to be two classes of DSB. The physiological class tends to lead to non-homologous repair and those of a pathological basis the homologous.



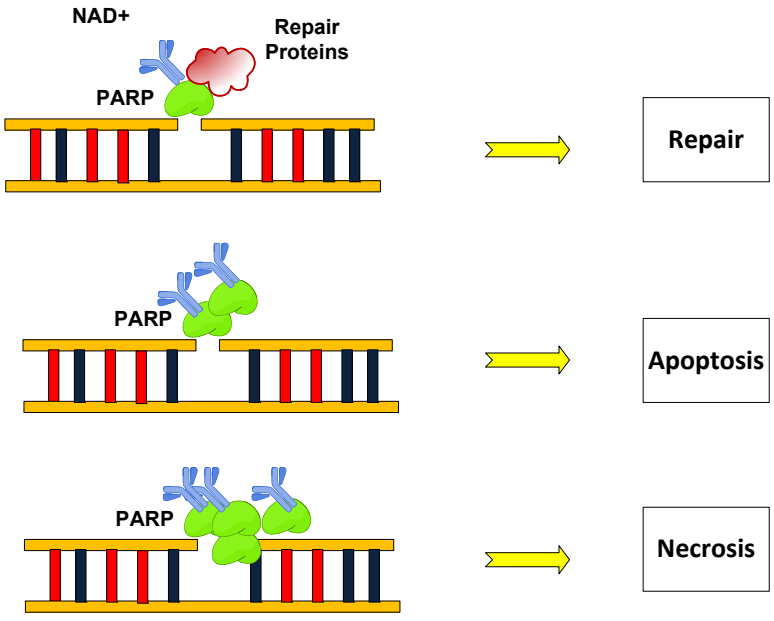
The DSB repair mechanisms are as we have noted prone to mistakes. That is where we also have certain backup mechanisms as p53 and other similar genes. However, if as we have noted the CHK2 process becomes overly active we may have added instability which p53 and its helpers cannot properly control.

4.5 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.



4.6 CATEGORIES

We now examine some of the details of this process. As 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 above shows the categorization of lesions and their sources. Note that the MMR is a replication error and the source is itself replication. The driver of such a process is most likely the mTOR pathway forcing multiple replications and this further enhancing the chance of more errors.

4.7 BASICS

We first present some of the basics. As Hsieh and Zhang have noted:

The general schemes of MMR seem deceptively simple. The newly synthesized strand containing mismatches is targeted for excision followed by high fidelity re-synthesis and ligation. Nevertheless, any scheme must reconcile action at a distance (i.e., the mismatch and a strand-specific signal for excision can be far apart).

MMR in eukaryotes features two families of MMR proteins, heterodimeric homologs of bacterial MutS (MSH) or MutL (MLH).

Early models of E. coli MMR invoke MMR protein-mediated DNA looping between a mismatch and a DNA-methylation excision signal or ATP driven MutS translocation along the DNA. The nucleotide switch model posits the existence of multiple diffusing MSH–MLH clamps.

They give examples of 3' and 5' strand repairs. The 3' strand repair is the simples:

3′ MMR.

(A) MSH (an MMR protein) recognizes a mismatch.

(B) In the sliding clamp model, ATP-dependent binding and nucleotide switching creates MSH sliding clamps that diffuse from the mismatch. The interaction of ATP-bound MLH heterodimers

with MSH sliding clamps and PCNA (proliferating cell nuclear antigen) oriented with respect to 3' termini activates MLH strand-specific nicking. Alternatively, ATP-activated MSH may remain at the mismatch to load MLH and activate nicking.

(C) Excision is EXO1-dependent or -independent, leading to an RPA-coated excision track. An EXO1-independent Pol δ strand displacement pathway is not shown.

(D) Pol δ or e with the aid of PCNA completes gap filling.

(E) DNA ligase I seals the nick.

The 5' is significantly more complex.

4.8 PROBLEMS

The understanding of MMR is not complete. Li notes:

Despite great progress in identifying MMR proteins and genes and application of state-of-the-art biochemical and genetic approaches to analyze the mechanism of MMR in prokaryotic and eukaryotic cells, several key questions about this pathway remain unanswered. One of these questions concerns the mechanism by which MMR proteins facilitate the communication between two physically distant DNA sites: the mismatch and the strand discrimination signal. It is generally agreed that the strand discrimination signal is a strand-specific nick in both prokaryotic and eukaryotic cells, although the source of the nicking activity, at least for the leading strand, is not known in eukaryotic cells.

***Previous studies have proposed several alternative models for this process, which can be classified into "cis-" or "moving" and "trans-" or "stationary" models**.*

***The "stationary" model proposes that interactions among MMR proteins induce DNA bending or looping that brings the two distant sites together, while MutS (or the MSH heterodimers, i.e. MutSα and MutSβ) remains bound at the mismatch. In this model, the MutS (or MSH heterodimers) ATPase activity acts in a proofreading role to verify mismatch binding and authorize the downstream excision**….*

Consistent with this observation, a second study demonstrated that mismatch-provoked excision could be initiated when a biotin-streptavidin blockade was placed between the mismatch and pre-existing nick.

The "cis" or "moving" models suggest that MutS-MutL (or MutSα/β- MutLα) complexes load at a mismatch site and then move away from the site to search for the strand break, where exonucleases can be recruited to initiate excision.

5 APPLICATIONS TO CANCER

We now examine several applications of MMR to cancers. We begin with Hata et al who have noted:

Despite the success of targeted cancer therapies, the duration of clinical response is limited by the inevitable development of acquired drug resistance, as in the case of EGFR mutant non-small cell lung cancers (NSCLC) treated with EGFR inhibitor therapy. Although molecular mechanisms of acquired resistance to EGFR inhibitors have been identified, little is known about how resistant clones evolve during drug therapy. In some cases, clones with clinically validated genetic resistance mechanisms may exist prior to drug exposure and may be selected by treatment.

Alternatively, it has been hypothesized that drug tolerant (or "persister") cells without bona fide resistance mechanisms may survive initial drug treatment by epigenetic adaptations, and undergo further evolution over time to acquire validated genetic resistance mechanisms. Although this would have immediate implications for new therapeutic strategies to prevent resistance, there has not been any direct evidence that drug tolerant cells can undergo such evolution.

As Nickoloff et al have noted:

Defects in DNA repair can result in oncogenic genomic instability. Cancers occurring from DNA repair defects were once thought to be limited to rare inherited mutations (such as BRCA1 or 2). It now appears that a clinically significant fraction of cancers have acquired DNA repair defects. DNA repair pathways operate in related networks, and cancers arising from loss of one DNA repair component typically become addicted to other repair pathways to survive and proliferate. Drug inhibition of the rescue repair pathway prevents the repair-deficient cancer cell from replicating, causing apoptosis (termed synthetic lethality). However, the selective pressure of inhibiting the rescue repair pathway can generate further mutations that confer resistance to the synthetic lethal drugs.

Many such drugs currently in clinical use inhibit PARP1, a repair component to which cancers arising from inherited BRCA1 or 2 mutations become addicted. It is now clear that drugs inducing synthetic lethality may also be therapeutic in cancers with acquired DNA repair defects, which would markedly broaden their applicability beyond treatment of cancers with inherited DNA repair defects. Here we review how each DNA repair pathway can be attacked therapeutically and evaluate DNA repair components as potential drug targets to induce synthetic lethality. Clinical use of drugs targeting DNA repair will markedly increase when functional and genetic loss of repair components are consistently identified. In addition, future therapies will exploit artificial synthetic lethality, where complementary DNA repair pathways are targeted simultaneously in cancers without DNA repair defects.

They continue:

During DNA replication, incorrect nucleotides are occasionally incorporated into the daughter DNA strand, creating mismatched base pairs corrected by MMR; mismatches also form in heteroduplex DNA during HR. MMR comprises four key steps: mismatch recognition, excision of the lesion, DNA synthesis across the SS gap, and ligation. Two heterodimeric proteins recognize the lesion: MutSa (MSH2/6 complex) recognizes short mismatches, and MutSb (MSH2/3 complex) recognizes longer insertion-deletion loops. Binding of either heterodimer recruits the heterodimer MutL (MLH1 and PMS2 complex). MutL recruits Exo1, which excises the mismatched DNA in a reaction enhanced by PARP1 (80). Pol d fills in the gap, and DNA ligase I seals the nick.

Hereditary nonpolyposis colorectal cancer (HPNCC or Lynch syndrome) is an inherited autosomal-dominant disease resulting from defects in MMR proteins, with the majority of mutations affecting MLH1, MSH2, and MSH6. Silencing by somatic methylation of MMR gene promoters also decreases MMR and confers resistance to platinum-based chemotherapy (85). DNA demethylating agents such as 5-azacytidine induce re-expression of MMR components in these cancers, restoring sensitivity to cisplatin or carboplatin

As Torgovnick and Schumacher have noted:

The critical role of mismatch repair (MMR) in tumorigenesis is highlighted by the fact that loss of expression of MMR proteins predispose to colorectal, gastric, endometrial and ovarian cancers and inherited defects in the MMR genes are associated with the most prevalent cancer syndrome in humans, the Lynch syndrome (LS), previously known as hereditary nonpolyposis colorectal cancer.

Moreover, MMR deficiency is present in 15% of all primary cancers. The MMR pathway recognizes base–base mismatches and insertion-deletion loops originating from base misincorporation, tautomeric shifts, slippage of DNA polymerases, damage that acts as mismatch, and recombination duplex. The sequential events in MMR repair comprise damage recognition, excision, and resynthesis steps.

The MutSa and MutSb complexes are the MMR lesion detectors. The first complex is composed by MSH2 and MSH6 and recognizes single base-base mismatches and 1–2 bp IDLs while the second one, formed by the MSH2 and MSH3 proteins, principally find and repair 2–12 bp IDLs. Upon DNA binding, one of the three different heterodimeric complexes MutLa (MLH1-PMS2), MutLb (MLH1-MLH3), and MutLg (MLH1-PMS1) can be recruited to form, with MutS, a ternary structure.

The complex formed with MutLa is the most important in the MMR pathway, is able to translocate in both directions along the damaged area and to recruit proliferating cell nuclear antigen (PCNA), RFC, and EXO1 to perform the excision step. MutLb function is currently unknown whereas MutLg is involved in meiotic recombination.

After damage resection, resynthesis is carried out by DNA polymerase δ and sealing of the nick by DNA ligase I. Being part of the replication fork, the MMR machinery operates mostly in dividing cells, nonetheless few publications report an active presence of MMR in the brain. Mismatch repair dysfunction accounts for the mutator phenotype in which base substitution and

frameshift mutations are highly increased due to microsatellite instability (MSI). Microsatellites are short tandem repeated DNA sequences of 1–4 base nucleotides spread all over the genome. Replication of these repeats has high error risk and when they are present in tumor suppressor genes, a defective repair may have detrimental consequences.

Zhao et al have recounted the issue of MMR in nasophyryngal cancers (also note Doukas et al):

To analyze the mismatch repair (MMR) status and PD-L1 expression in nasopharyngeal carcinoma (NPC), and investigate whether PD-L1 and MMR status could be used as a biomarker for predicting response of immune checkpoint blockades (ICBs) treatment. ....

However, the suitability of MMR status as a biomarker for ICBs in NPC patients was unknown. We determined the MMR status in a cohort of unselected patients using IHC and the same PCR method that was used in the two referenced trials. Our study cohort comprised patients at the First Affiliated Hospital of Xiamen University in Southern China, a region in which NPC is considered endemic. As far as we know, this is the first study to detect the MMR status using both IHC and the MSI Multiplex System in a reasonable sample size of patients with NPC

Significant work has demonstrated MMR in colorectal cancers as Russo et al have noted. Smolarz et al have discussed the implications in breast cancer. Liu et al have done so for gastrointestinal cancers.

6 OBSERVATIONS

The recent Science paper by Russo et al presents an enticing suggestion that adaptive mutagenesis is the basis for the change to an unresponsive set of new cancer cells after initial therapeutics have been applied. However, the paper by Russo et al in our opinion leaves open a number of unanswered questions. It posits such processes as mismatch repair as one of several, it argues that mTOR plays a key role and that ROS elements are a causative factor. Just what the details are seem to be missing. We examine some of there herein.

6.1 CONFLICTS

As we have discussed throughout, the Russo et al argument is that there exists some process which is facilitated by mTOR whereby mutations occur amongst the cancer cells which have been attacked by a therapeutic and the result is via an adaptive mode such that the new cells have the capability to resist the initial therapeutic. Now Roth et al note:

Growth under selection causes new genotypes to predominate in a population. It is difficult to determine whether selection stimulates formation of new mutations or merely allows faster growth of mutants that arise independent of selection. In the practice of microbial genetics, selection is used to detect and enumerate preexisting mutants; stringent conditions prevent growth of the parent and allow only the pre-existing mutants to grow.

Used in this way, selection detects rare mutations that cause large, easily observable phenotypic changes. In natural populations, selection is imposed on growing cells and can detect the more common mutations that cause small growth improvements. As slightly improved clones expand, they can acquire additional mutational improvements. Selected sequential clonal expansions have huge power to produce new genotypes and have been suggested to underlie tumor progression. We suggest that the adaptive mutation controversy has persisted because the distinction between these two uses of selection has not been appreciated.

If one reads the Luria and Delbruck paper one sees a simple experiment. Namely bacteria are chosen and then attacked by some virus. The bacteria decay but after a while some have mutated and return. The authors present this work in an attempt to estimate the mutation rates of bacteria. The problem is that we know that single cell organisms like bacteria have a high mutation rate and there is arguable some understanding as to its cause. Now arguing that this rate carries over readily to enclosed somatic cancer cells in a human is problematic. In fact one would question the high mutation rate required.

6.2 EPIGENETICS

Epigenetic impacts are generally more readily explainable and more readily ascertained via experimental results. We would argue for a quasi-mutation via epigenetic factors such as methylation of histone suppression. As Charlesworth et al have noted:

If epigenetic changes producing adaptive changes in phenotypes induced by external circumstances were often transmitted to the offspring, this would involve a major change in outlook. The so-called 'central dogma' of molecular biology states that information flows from nucleic acid sequence to protein sequence, and not vice versa. More generally, there is no known mechanism for systematically generating adaptive and heritable DNA sequence variation (see the discussion of 'directed mutation' in 5').

As described above, mechanisms have evolved by which specific kinds of adaptive responses can potentially be transmitted across one or more generations, involving epigenetic marks or the production of small RNA molecules that are transmitted through the germ cells.

If these changes could produce stable adaptive traits in the offspring, and if they occurred sufficiently frequently, such 'Lamarckian' inheritance could play a significant role in phenotypic variation and evolution. However, as noted long ago by Haldane and Muller, such a process is unlikely to be of general importance, because a large body of genetic experiments has established the ineffectiveness of selection on homozygous lines, which lack genetic variation but still show phenotypic variation. In striking contrast, family selection, with no exposure of the selected individuals to the environment in which the trait is favoured, is highly effective.

One of the most spectacular examples of non-genetic phenotypic differences is provided by the sterile worker castes of social insects. Darwin himself pointed out that these could not possibly have evolved by a Lamarckian mechanism, but must be the product of selection on the genotypes of the reproductive individuals to produce workers with phenotypes adapted to different tasks [68]. There is therefore a long-standing and strong empirical basis for rejecting the inheritance of acquired characters as a frequent phenomenon…

Thus the likelihood of epigenetic impacts may be the more likely scenario.

6.3 ROS AND MUTAGENESIS

We have discussed the role of mTOR in the mutagenesis process as well as epigenetic factors. Reactive Oxygen Species also has a putative role. In the extensive work of Halliwell and Gutteridge (pp 245-257, 568-588) we have an extensive discussion of ROS on mutations and cancer progression. Thus it can be strongly argued that the presence or enhancement of ROS can be a primary driver of mutations. This is just a logical argument and at this time not dispositive result in line with Russo et al is available to this author.

6.4 MIRNA

miRNA appear to be pervasive players across the board. Just what they can do in mutagenesis is an open question. We have discussed the miRNA issue extensively elsewhere. Whether miRNAs are drivers of mutagenesis is an open question. Although Mork et al note:

This illustrates that using the protein-driven miRNA–disease associations not only reveals potentially new miRNAs involved in diseases but also provides candidate proteins as molecular

hypotheses underpinning the associations, which can be tested, e.g. through knockdown of the RNA or mutagenesis of the miRNA target region.

6.5 GENE DUPLICATION

Gene duplication is a process whereby new genetic elements are introduced leading to a duplication of a DNA region and effectively become a mutagenesis[5]. There are multiple mechanism for such duplications, namely:

1. Ectopic Recombination: These result from unequal crossing over

2. Replication Slippage: This can reproduce short segments

3. Retrotranposition: sequences of DNA can be transcribed via mRNA, reverse transcriptions, and thus lacking introns, and frequently in regulatory genes.

4. Aneuploidy: This is the creation of an abnormal number of chromosomes

5. Whole Genome Duplication: This is the result of nondisjunction during meiosis

These are all evolutionary events and can be adaptive mutagenesis.

6.6 MISMATCH REPAIR

Mismatch repair, MMR, is one of several DNA problems found in cancers as we have noted. There are a multiple set of suggestions on how to mitigate the issue akin to PARP and BTCA, single and double stand breaks, but none yet in clinical acceptance. As Li notes:

DNA damage accumulates in cells over time as a result of exposure to exogenous chemicals and physical agents (i.e., benzo[a]pyrene, polychlorinated biphenyls, dioxin, cigarette smoke, asbestos, ultraviolet light, radon), as well as endogenous reactive metabolites including reactive oxygen and nitrogen species (ROS and NOS). Another source of DNA damage is errors that occur during normal DNA metabolism or aberrant DNA processing reactions, including DNA replication, recombination, and repair. Nucleotide misincorporation generates DNA base-base mismatches during DNA synthesis at variable rates, depending on many factors, including the specific DNA polymerases.

In general, the replicative DNA polymerases have relatively high replication fidelity, while translesion DNA polymerases, which specifically bypass sites of DNA damage, have lower replication fidelity.

DNA damage, if unrepaired, has the potential to generate mutations in somatic or germline cells, which can alter cellular phenotype and cause dysfunction and disease. To prevent such

[5] See Ditmar and Liberles

deleterious effects and safeguard the integrity of the genome, cells possess multiple mechanisms to repair DNA damage and thus prevent mutations.

*One such system is the critical pathway known as DNA **mismatch repair (MMR)**.*

MMR corrects DNA mismatches generated during DNA replication, thereby preventing mutations from becoming permanent in dividing cells. Because MMR reduces the number of replication-associated errors, defects in MMR increase the spontaneous mutation rate. Inactivation of MMR in human cells is associated with hereditary and sporadic human cancers, and the MMR system is required for cell cycle arrest and/or programmed cell death in response to certain types of DNA damage. Thus, MMR plays a role in the DNA damage response pathway that eliminates severely damaged cells and prevents both mutagenesis in the short term and tumorigenesis in the long term.

6.7 THERAPEUTICS

There are a multiplicity of therapeutic targets for MMR issues. As Nickoloff et al have noted for just one of them:

During DNA replication, incorrect nucleotides are occasionally incorporated into the daughter DNA strand, creating mismatched base pairs corrected by MMR; mismatches also form in heteroduplex DNA during HR. MMR comprises four key steps: mismatch recognition, excision of the lesion, DNA synthesis across the SS gap, and ligation.

Two heterodimeric proteins recognize the lesion: MutSa (MSH2/6 complex) recognizes short mismatches, and MutSb (MSH2/3 complex) recognizes longer insertion-deletion loops.

Binding of either heterodimer recruits the heterodimer MutL (MLH1 and PMS2 complex). MutL recruits Exo1, which excises the mismatched DNA in a reaction enhanced by PARP1. Pol d fills in the gap, and DNA ligase I seals the nick. Hereditary nonpolyposis colorectal cancer (HPNCC or Lynch syndrome) is an inherited autosomal-dominant disease resulting from defects in MMR proteins, with the majority of mutations affecting MLH1, MSH2, and MSH6 (82). Silencing by somatic methylation of MMR gene promoters also decreases MMR (83,84) and confers resistance to platinum-based chemotherapy. DNA demethylating agents such as 5-azacytidine induce re-expression of MMR components in these cancers, restoring sensitivity to cisplatin or carboplatin.

Cancers with MLH1, MSH2, or MSH6 defects display synthetic lethality with therapeutic potential, but it is important to identify the specific MMR deficiency in the tumor as they differ in therapeutic response. For example, MSH2-mutant cancers are sensitive to methotrexate, an antimetabolite that inhibits DNA synthesis, and psoralen, a DNA crosslinking agent, but MLH1-mutant cancers are resistant to both treatments. Unrepaired oxidized nucleotides accumulate upon BER repression or methotrexate treatment, which increases mismatch formation during DNA synthesis, increasing the burden on MMR, and mutagenesis.

These effects are strongly exacerbated in MMR-defective cancers, a dynamic that presents synthetic lethal opportunities. For example, Pol b inhibition is synthetically lethal in MSH2- or MLH1-deficient tumors. Thus, Pol b inhibitors are promising agents for treatment of MMR-deficient cancers, as well as the aforementioned BRCA1/2-mutant cancers. Some MMR-deficient cancers behave like BRCA1/2-mutant cancers, with defects in stressed replication fork repair. MSH3 is critical for loading RAD51 during HR repair, and thus MSH3-deficient cancers are sensitive to PARP1 inhibition.

Clinical trials with PARP1 inhibitors in MSH3-mutant colon cancer are warranted, especially in conjunction with an immune checkpoint inhibitor because genomic instability associated with MMR-deficient colon cancer increases neoantigen production and thus increases the chance of immune recognition.

In Nickoloff et al there are several other suggested therapeutic paths. Some have or are being investigated.

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