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Participation of DNA repair in the response to 5-fluorouracil

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Abstract

The anti-metabolite 5-fluorouracil (5-FU) is employed clinically to manage solid tumors including colorectal and breast cancer. Intracellular metabolites of 5-FU can exert cytotoxic effects via inhibition of thymidylate synthetase, or through incorporation into RNA and DNA, events that ultimately activate apoptosis. In this review, we cover the current data implicating DNA repair processes in cellular responsiveness to 5-FU treatment. Evidence points to roles for base excision repair (BER) and mismatch repair (MMR). However, mechanistic details remain unexplained, and other pathways have not been exhaustively interrogated. Homologous recombination is of particular interest, because it resolves unrepaired DNA intermediates not properly dealt with by BER or MMR. Furthermore, crosstalk among DNA repair pathways and S-phase checkpoint signaling has not been examined. Ongoing efforts aim to design approaches and reagents that (i) approximate repair capacity and (ii) mediate strategic regulation of DNA repair in order to improve the efficacy of current anti-cancer treatments.

Keywords

colorectal cancer; chemotherapy; DNA damage; base excision repair; mismatch repair; homologous recombination

1. Introduction

Based on the initial observations of Rutman et al. [1] and Heidelberger et al. [2] that rat hepatoma cells utilize uracil to a greater extent in nucleic acid biosynthesis than non-malignant cells, it became of great medical interest to identify uracil analogs that exhibited “selective” anti-cancer activity. Shortly thereafter, the synthesis of 5-fluorouracil (5-FU; Figure 1) [3] and its efficacy as a potential anti-tumor drug was reported [4]. This compound, as well as the nucleoside analog 5-fluoro-2′-deoxyuridine (FdUrd; Figure 1), are part of a class of cytotoxic drugs known as anti-metabolites, which have been integrated into numerous clinical trials and found to exhibit anti-tumor activity in patients. Today, 5-FU is widely used in the treatment of solid tumors, including of the breast, gastrointestinal system (colon, rectum, anus, esophagus, pancreas and stomach), head and neck, and ovary [5]. Most notably, 5-FU is routinely employed in the management of colorectal cancer via one of two FDA-approved first line combinatorial chemotherapy regimes, abbreviated FOLFOX and FOLFIRI, which involve intravenous administration of the fluorinated base analog (Figure 1).

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Despite the recent exciting advances in targeted therapeutics, such as the development of kinase inhibitors and monoclonal antibodies that specifically block the growth of cancer cells (e.g. imatinib mesylate (Gleevec™) or trastuzumab (Herceptin™)), traditional cytotoxics including 5-FU continue to be used in combination chemotherapy, primarily as a means of combating drug-resistant malignant cell populations to which all treatment regimes old and new fall prey [6,7]. The pharmacokinetic profiles and side effects of traditional cytotoxics are also well understood through decades of use. As aspects of cancer management focus on patient quality of life and move towards outpatient treatment paradigms, orally administered anti-cancer drugs are a priority. It is noteworthy in this regard that capecitabine (Xeloda™), which is commonly used to treat breast and colorectal cancer, is an orally available pro-drug of 5-FU [8].

There are multiple pathways involved in the activation and degradation of 5-FU (see Section 2). Depending on the metabolic path, 5-FU and its metabolites can exert anti-proliferative effects through inhibition of thymidylate synthetase (TS) and/or incorporation into RNA and DNA [9]. It remains debated as to the relative contribution of each of these cellular targets to the anti-tumor activity and side effects seen in patients. This review will focus on the DNA repair processes associated with responding to 5-FU in chromosomal DNA, a topic that has received comparatively little attention until recently.

2. 5-FU Metabolism and its Directed Effects

Because 5-FU is a structural analog of uracil and thymine, many of the enzymes that participate in uracil or thymine metabolism also effectively metabolize 5-FU, topics that have been extensively studied and previously reviewed [10]. This brief summary highlights pathways to DNA incorporation.

5-FU and FdUrd rapidly enter cells by a facilitated transmembrane carrier system. Once in the cell, conversion to nucleotides promotes intracellular retention and further metabolism (Figure 2). Uridine phosphorylase and orotate phosphoribosyltransferase convert 5-FU into the ribonucleoside or ribonucleotide, respectively. Thymidine phosphorylase can also salvage 5-FU into the deoxynucleoside, FdUrd. Kinases convert FUrd to FUMP and to FUDP, which provides a branch point in metabolite fate. FUDP is phosphorylated to FUTP, which is a substrate for RNA polymerases. FUDP can also be converted to the deoxynucleotide (FdUDP) by ribonucleotide reductase (RNR). FdUDP is further phosphorylated into FdUTP, which is a substrate for DNA polymerases (discussed in Section 2.1).

The enzyme dUTP pyrophosphatase (dUTPase) performs an absolutely essential function to prevent genomic uracil incorporation by catalyzing the hydrolysis of dUTP into dUMP (Figure 2); *E. coli* and *S. cerevisiae* lacking dUTPase are inviable [11,12]. dUTPase also breaks down FdUTP to FdUMP, which is a noteworthy aspect of 5-FU metabolism. While breaking down FdUTP prevents 5-FU from being incorporated into DNA, it creates FdUMP in the process. FdUMP forms an irreversible ternary complex with TS (a classic biochemistry textbook example of suicide inhibition), the enzyme that converts dUMP to TMP using N_5,N_{10}-methylenetetrahydrofolate as a coenzyme to establish thymidylate nucleotides essential for DNA replication [13]. When FdUrd is used in cell culture experiments, thymidine kinase (TK) efficiently converts FdUrd into FdUMP, driving the generally accepted conclusion that FdUrd primarily exerts its toxicity via TS-directed effects. It is presumed that thymidylate kinase is capable of phosphorylating FdUMP to form FdUDP, which speculatively could counteract the TS inhibitory consequences of FdUMP.

Suicide inhibition of TS by FdUMP also causes a drop in TTP, which has several effects that can influence 5-FU metabolism. First, dUMP accumulates, resulting in a higher intracellular concentration of dUTP and FdUTP, which can overwhelm dUTPase and become available for incorporation into the genome by DNA polymerases. Second, TTP feedback inhibits TK and

allosterically regulates RNR (TTP normally increases dGDP formation and decreases dUDP formation). Loss of this feedback inhibition presumably increases the conversion of FdUrd to FdUMP by TK and the conversion of FUDP to FdUDP by RNR. Thus, there are several interrelated events that can lead to the introduction of 5-FU into DNA (see Section 2.1). We refer readers to other reviews that cover aspects of 5-FU metabolism, including incorporation of 5-FU into RNA and suicide inhibition of TS [9,10,14]. This review will focus on more recent studies that have examined the repair machinery involved in the cellular responses to 5-FU once inserted into DNA.

2.1 5-FU in genomic DNA

Because 5-FU and uracil metabolism are so intertwined, genomic incorporation of both bases can result from 5-FU treatment (see Figure 2). In particular, dUTP is readily incorporated opposite adenine during DNA replication by a number of polymerases, which do not appear to discriminate between dUTP and TTP. Moreover, DNA polymerase α, the enzyme responsible for synthesis of a chimeric RNA-DNA primer for leading and lagging strand replication, and the DNA repair polymerase, POLβ (see Section 3.1 for additional details), reportedly incorporate FdUTP into DNA opposite adenine with an efficiency similar to dUTP and TTP [15,16]. To our knowledge, neither the processive replicative polymerases (POLδ/ε) nor any of the more specialized DNA polymerases discovered since 1997 have been evaluated for their efficiency to insert FdUTP during DNA synthesis, but undoubtedly, many of them are capable of such activity.

A number of groups have examined incorporation of radiolabeled 5-FU into DNA using 5-FU or FdUrd in a number of cell culture systems [17-26]. Several of the studies detected substantial amounts of genomic 5-FU, although it is not surprising that the amounts varied given the many experimental murine and human cell culture models examined and the multiple metabolic steps required. In some cases, genomic 5-FU was nearly undetectable if FdUrd was used [18,22]. Although earlier studies provide equivocal evidence that genomic 5-FU incorporation contributes to toxicity [17-26], it was pointed out that the rates of incorporation versus the excision efficiency were not determined [10]. Thus, the dynamic interplay between incorporation and DNA repair in dictating the steady state level of base damage was not examined. We discuss next the DNA repair pathways implicated in the recognition of genomic 5-FU.

3. Base Excision Repair and 5-FU Resistance

Base excision repair (BER) copes with specific forms of endogenous DNA damage. In particular, BER is the primary pathway for removing various types of oxidative, alkylative, and spontaneous hydrolytic DNA base and sugar products. The major steps of BER involve the following: (1) removal of a modified or inappropriate base, such as uracil, by a DNA glycosylase, (2) cleavage of the phosphodiester backbone at the resulting AP site by an endonuclease or lyase, (3) clean-up of the 3′ or 5′ terminal end, (4) replacement of the excised nucleotide by a polymerase, and (5) sealing of the final DNA nick by a ligase. The molecular events of BER and the predominant mammalian protein participants are depicted in Figure 3 [27,28].

There are four different known proteins in the human genome with uracil DNA glycosylase (UDG) activity. Note that the abbreviation UDG refers to biochemical activity, i.e. the ability to catalyze cleavage of the N-glycosidic bond, releasing the base from the sugar phosphodiester backbone (Figure 3), whereas abbreviations below refer to specific loci and polypeptides. Biochemical characterization of the four UDGs suggests specialized roles that combat two sources of uracil introduction into the genome, i.e. hydrolytic deamination of cytosine (giving rise to U:G pairs) and incorporation of dUMP during replication (generating U:A pairs),

reviewed elsewhere in detail [29]. Briefly, the *UNG* genetic locus encodes mitochondrial (UNG1) and nuclear (UNG2) forms of UDGs, with UNG2 appearing to account for the bulk of cellular UDG activity [29]. The *SMUG1* genetic locus encodes a glycosylase that has been proposed to serve as a backup for UNG in uracil excision, although SMUG1 also releases a broader range of damaged pyrimidine bases not excised by UNG [30]. TDG (thymine DNA glycosylase) and MBD4 (also known as MED1) appear to counteract cytosine or 5-methyl-cytosine deamination products in double stranded DNA, while TDG can also remove other types of damaged bases, most notably 3,N^4 ethenocytosine [31].

3.1 5-FU excision by DNA glycosylases

The first evidence suggesting a role for BER in the cellular response to 5-FU was reported in 1980 [32]. In this study, both the bacterial and human (presumably UNG2) UDG was found to excise 5-FU, albeit with slightly less efficiency than uracil, from plasmid substrates harboring multiple tritium-labeled base lesions. This in vitro analysis was later confirmed using synthetic oligonucleotide duplexes that contained a single defined, site-specific 5-FU [33]; this study found that both purified *E. coli* and human UDG exhibited a 10 to 18-fold increase in K_M for the 5-FU:A substrate relative to the U:A duplex, with little difference in V_{max}. Since then, purified recombinant MED1 [34], TDG [35] and SMUG1 [30] have also been shown to remove 5-FU from synthetic DNA substrates in vitro. In the case of MBD4, this excision function is specific for 5-FU opposite guanine, a pairing con figuration for which there is no obvious mechanism of formation as will be discussed below (see Section 4.1). TDG is able to remove 5-FU opposite either guanine or adenine, as well as from single-stranded DNA, which stands in surprising contrast to the strict requirement of the enzyme for an opposing guanine when excising uracil or 3,N^4 ethenocytosine [35]. SMUG1 excised 5-FU opposite adenine, but was not tested against other base partners [30]. This study also confirmed that UNG2 displayed a much stronger preference for uracil than 5-FU. Using human cell nuclear extracts and covalently closed circular DNA plasmids, 5-FU:G repair was found to be largely dependent on TDG and UNG2, whereas 5-FU:A pairs were processed mainly by UNG2 [36]. In these experiments, MBD4 and SMUG1 did not detectably contribute to 5-FU removal, yet given the *in vitro* excision activities of the recombinant proteins, could not be excluded from being involved in 5-FU metabolism *in vivo*.

In recent years, studies have begun to examine the biological involvement of specific mammalian BER components in the 5-FU response. In light of the biochemical studies described above, it was natural to suspect UNG. Yet surprisingly, several investigations have concluded using various approaches that UNG does not influence the cytotoxicity of 5-FU [37-39]. In particular, $Ung^{+/+}$ and $Ung^{-/-}$ murine embryonic fibroblasts (MEFs) showed almost no difference in the lethal effects of 5-FU or FdUrd, despite an increased accumulation of uracil in $Ung^{-/-}$ cells [38]. In addition, expression of a protein inhibitor of UNG (i.e. Ugi) in HEK293 cells did not affect the toxicity of 5-FU or FdUrd despite a substantial increase in genomic uracil following treatment [39]. Thus, UNG activity or elevated uracil in DNA does not appear to contribute significantly to cellular sensitivity to 5-FU, although this does not rule out the possible involvement of other UDGs.

Indeed, work by Barnes and colleagues indicates that the SMUG1 glycosylase, not UNG, functions predominantly in cellular 5-FU repair, despite the fact that these enzymes possess comparable activities on 5-FU:A substrates *in vitro* [37]. In particular, the authors found that genomic 5-FU accumulates specifically in SMUG1-deficient MEFs, but not in $Ung^{-/-}$ MEFs, and that SMUG1-defective cells are uniquely sensitive to 5-FU treatment. One concerning aspect of the study was that the MEFs (regardless of genotype) displayed a greater sensitivity to 5-FU than FdUrd, whereas in nearly all studies using human cell lines FdUrd is at least 10-fold more toxic than 5-FU. That withstanding, the overall picture suggests that the excision

activity of SMUG1, and not UNG, is protective against toxicity caused by genomic 5-FU. The results also imply that SMUG1 upregulation might serve as a means of developing tumor resistance to 5-FU treatment.

Cells deficient in the MBD4 (*a.k.a.* MED1) DNA glycosylase have been reported to be resistant to 5-FU [40,41]. This observation was extended to *in vivo* studies of *Mbd4* -/- mice, in which it was found that apoptosis induced by 5-FU treatment in the small intestine was reduced in knockout animals relative to their wild-type counterparts [41]. This at first glance would appear to run contrary to the hypothesis that 5-FU in DNA is toxic. However, there is a connection between MBD4 and mismatch repair (MMR), a pathway known to promote cell death in response to DNA damage (see Section 4.1), worth emphasizing. Specifically, MBD4 has been shown to interact with MLH1, a key component of MMR [42]. In addition, defects in MMR are associated with hereditary non-polyposis colorectal cancer (HNPCC), i.e. cancers characterized by high microsatellite instability (MSI-H) [43], and mutations in the *MBD4* gene have been reported in human colorectal cancers found to exhibit MSI-H [44-46]. While the links between MMR and genomic 5-FU will be discussed in more detail in Section 4, we note here that a deficiency in MED1 may result in an impaired MMR-dependent cell death response, leading to the observed increased resistance to 5-FU exposure. Since TDG-deficient cells have not yet been reported [31], it is impossible to state with certainty whether this glycosylase influences the cellular response to 5-FU.

3.2 Downstream components of BER

Few studies to date have looked at the involvement of BER components downstream of the DNA glycosylases with regards to 5-FU sensitivity. APE1 is the major abasic endonuclease in mammalian cells, and operates centrally in the BER response after glycosylase-catalyzed base release (Figure 3). APE1 appears to be essential for mammalian cell viability [47,48], yet expressing a dominant-negative APE1 variant (termed ED), which binds with high affinity to substrate DNA and blocks subsequent repair steps, was found to recapitulate the cellular sensitivity to alkylating agents seen with AP endonuclease deficient *E. coli* and *S. cerevisiae* [49]. Moreover, recent work has found that ED expression in Chinese hamster ovary (CHO) cells significantly increases sensitivity to 5-FU (~five-fold) and FdUrd (~thirty-fold), suggesting the formation of an APE1-specific substrate (presumably an AP site), blockage of the normal repair response, and consequent activation of cell death (McNeill and Wilson, manuscript in preparation).

DNA Polymerase β (POLβ) performs two important biochemical functions in mammalian BER, namely nucleotide gap filling and 5′-dRP excision, which immediately follow AP site incision by APE1 (Figure 3). Surprisingly, studies have found that *Pol* $\beta^{-/-}$ MEFs are more resistant to FdUrd (5-FU was not examined) than their wild-type counterparts [50,51]. Furthermore, studies examining CHO cells defective in XRCC1, a protein critical to single strand break (SSB) repair through interactions with POLβ and DNA ligase IIIα, have found no obvious role for this protein in 5-FU [52] or FdUrd resistance (Li and Wyatt, unpublished results). The lack of involvement of POLβ and XRCC1 in 5-FU responsiveness is striking given their prominent role in the later steps of BER. One possible explanation might be altered sub-pathway choice depending on the cell type examined [53]. For example, long-patch BER may complete the steps downstream of APE1 incision, during which POLδ/ε performs nucleotide synthesis, flap endonuclease (FEN-1) removes the nucleotide overhang terminated by the 5′-dRP group, and DNA ligase I seals the nick (Figure 3). Why short-patch BER might be deleterious or dispensable under conditions of TS inhibition and/or 5-FU incorporation is unclear and requires further investigation. Moreover, studies need to more intensively delineate the contribution of 5-FU versus BER intermediates in cell death.

In *S. cerevisiae*, the influence of several BER components in response to 5-FU has been examined [54], although there are a number of biochemical differences in the BER steps between *S. cerevisiae* and mammalian cells to appreciate [55]. *S. cerevisiae* possess a UNG homolog, but lack SMUG1, MBD4, and TDG homologues [56]. *S. cerevisiae* deficient in UNG were more *resistant* to 5-FU than the wild-type strain, suggesting that 5-FU (or uracil) excision and generation of repair intermediates is vital to toxicity in this model system [54]. Conversely, a strain deficient in APN1, the major abasic endonuclease of budding yeast, was exquisitely sensitive to 5-FU compared to a wild-type strain [54], implying that the ability to process AP sites in yeast (and mammalian cells, see above) is crucial for 5-FU resistance. *S. cerevisiae* also lack a paralog of POLβ. It is believed that the 5′-dRP group is instead removed by the 5′-flap endonuclease RAD27 (or FEN1 in mammals) as part of a displaced strand, similar in design to the FEN1-dependent long-patch BER carried out in mammalian cells (Figure 3). Intriguingly, a *rad27* null strain of *S. cerevisiae* was extremely resistant to 5-FU [54], which parallels the observation that *Pol* $\beta^{-/-}$ MEFs are resistant to FdUrd [50]. Collectively, the results examining 5-FU and BER components in *S. cerevisiae* generally mirror those seen in mammalian cells, but such comparisons must be carefully made given the differences in cell death processes between these disparate species.

3.3 BER futile cycling during treatment with TS inhibitors

One of the more interesting aspects regarding the role of BER during TS inhibition is the notion that the repair process acts as an unwitting executioner [57]. Recall that suicide inhibition of TS by FdUMP causes an increase in dUTP, which can become incorporated into DNA during replication. Because BER requires a DNA resynthesis step following uracil excision, elevated dUTP presumably causes reintroduction of uracil into DNA to create a ‘futile cycling’ of attempted repair [58]. Unrepaired BER intermediates, namely abasic sites and SSBs, are known to be toxic and clastogenic DNA lesions, reviewed in [59]. Thus, repetitive uracil excision during TS inhibition is thought to contribute to cellular lethality.

The evidence in favor of BER futile cycling stems in large part from studies examining the crucial roles of dUTPase and dUTP levels in mediating toxicity caused by TS inhibitors. Several studies have established a direct relationship between dUTP pools, the extent of DNA fragmentation, and cytotoxicity [60-64]. DNA strand breaks were measured by pulsed-field electrophoresis or the comet assay, yet these investigations did not explicitly differentiate between SSBs and double strand breaks (DSBs). Following on from the observations of elevated intracellular dUTP levels affecting DNA integrity and cellular viability, a number of studies specifically modulated the levels of dUTPase [65-68]. Interestingly, increasing dUTPase activity only delayed, but did not prevent the lethality of TS inhibitors, implying that cell death does not entirely depend on DNA damage resulting from uracil incorporation [67, 68]. In total, the early studies established important associations between TS inhibition, the formation of DNA strand breaks and lethality, but left unanswered important questions regarding which specific DNA repair pathways and proteins might contribute to the formation or resolution of the strand breaks.

The source of strand breaks observed following treatment with TS inhibitors in mammalian cells was proposed to be BER-mediated, although not experimentally demonstrated to be dependent on specific BER components. BER generates a SSB intermediate, so additional events would be required to create a DSB following uracil incorporation and excision from the daughter strand during replication. In one series of studies, an endonuclease activity was implicated in the production of DNA strand breaks following TS inhibition [69,70]. However, it was not established whether the endonuclease activity was associated with DNA repair or the execution of apoptosis. In addition, the studies that examined DNA strand breaks utilized

anti-folates (e.g. methotrexate, CB3717, raltitrexed) or FdUrd, not 5-FU. Thus, the contribution of genomic 5-FU (its incorporation or excision) was not explicitly examined.

Caradonna and coworkers reported that preventing BER futile cycling from occurring confers resistance to FdUrd [71]. In brief, they found that resistance to FdUrd is observed in certain human cell lines in which the nuclear isoform of UNG (UNG2) is prematurely degraded in S-phase following FdUrd treatment, as opposed to G2 when UNG2 is normally degraded [71]. They speculated that this premature degradation confers resistance to FdUrd by preventing the futile BER response. However, there has been a question regarding the interpretation of the FACS analysis used to conclude that the proposed premature UNG2 degradation actually occurred in S-phase [72]. Nonetheless, Fischer *et al.* showed at least in HeLa cells that siRNA against UNG2 conferred resistance to FdUrd [71], offering evidence in support of the futile cycling model.

The prediction of the BER futile cycling model is that restraining UDG-initiated BER would protect against the toxicity of TS inhibition. However, prior studies with the *Ung* $^{-/-}$ MEFs showed no differential sensitivity to 5-FU or FdUrd (see Section 3.1). Interestingly, these experiments also showed an accumulation of genomic uracil, implying that the accumulation of downstream BER intermediates is responsible for the cytotoxicity of fluoropyrimidines. To our knowledge, no studies have simultaneously manipulated UDG and dUTPase activity in mammalian cells to test whether tolerance of genomic uracil occurs as a means of developing resistance to TS inhibitors. The ‘tolerance’ of genomic 5-FU is a topic touched upon both above and in Section 4.

4. Mismatch Repair and S-phase Checkpoint Signaling pathways

4.1 Mismatch Repair

MMR is responsible for correcting replication errors such as base:base mismatches and polymerase slippage products (i.e. insertion/deletion loops) at nucleotide repeat sequences [56]. As noted above, germ-line mutations in MMR genes have been found to give rise to HNPCC, thus linking a specific repair defect with predisposition to colorectal and other cancers [43]. Biochemically, base:base mismatches are recognized by a heterodimeric protein complex (MSH2:MSH6, also known as MutSα). The second step of MMR involves recognition of the bound MutSα by a second heterodimer (MLH1:PMS2, also known as MutLα). Recruitment of MutLα signals exonucleases to degrade the daughter strand containing the mismatch, and subsequently polymerase and ligase activities to complete repair (Figure 4). Mutations in MLH1 and MSH2 seem to account for the majority of HNPCC cases [43], while silencing of MLH1 by promoter hypermethylation is a frequent event in sporadic colorectal cancer with MSI-H [73,74]. In addition to correcting replication errors, MMR also plays an important role in apoptotic signaling in response to DNA damage [75-77]. Specifically, MMR recognition of damaged DNA can signal to the cell death machinery to trigger apoptosis, so that loss of MMR by genetic or epigenetic means can promote a ‘tolerance’ to DNA damage and resistance to chemotherapeutic DNA damaging agents.

It has been demonstrated in biochemical assays that human MutSα can recognize 5-FU paired opposite guanine but not adenine in DNA [36,78], and 5-FU:G mispairs are efficiently corrected in MMR proficient cell extracts [36]. In a similar vein, MutSα recognizes U:G but not U:A pairs in DNA [78]. Thus, MMR recognition of 5-FU may not be recognition *per se*, but detection of a “mismatch”, *i.e.* a uracil analog paired opposite guanine. The hMSH2:hMSH3 complex (MutSβ), which recognizes insertion/deletion loops, does not recognize 5-FU opposite adenine or guanine [78].

A number of studies have reported that cells deficient in MMR components, particularly MSH2 and MLH1, are resistant to 5-FU [78-80], which fits with the model that MMR-dependent recognition of certain forms of DNA damage initiates apoptosis. Genomic 5-FU paired opposite guanine was higher in MMR-deficient cells, implying that the presence of 5-FU was being tolerated due to the loss of MMR [78]. This is parallel to the phenomenon of apoptotic cell death being induced by an MMR-dependent recognition of O^6-methylguanine opposite thymine [59]. However, there is an aspect of the studies of MMR and genomic 5-FU that is ambiguous. Specifically, how do 5-FU:G mispairs occur in chromosomal DNA? U:A pairs arise when dUTP is incorporated by DNA polymerases, while U:G mispairs occur through cytosine deamination [56]. MMR-dependent cell cycle arrest following 5-FU treatment has been found to take place in the first cell cycle [78,81], seemingly requiring that a DNA polymerase insert 5-FU opposite a guanine. However, from the limited biochemical evidence available, FdUTP is incorporated opposite adenine. Future studies aimed at delineating the mechanism of 5-FU:G formation are necessary, perhaps examining whether one of the various DNA polymerases has the capacity to insert 5-FU opposite guanine.

It is important to point out that, depending on the cell model, the MMR-dependent influence on 5-FU toxicity is determined by the duration and dose of the base analog [36,81], suggesting that other consequences of 5-FU treatment contribute to the cell death response. BER status and dUTPase activity were not evaluated in any of the above MMR models, so it is unclear to what extent MMR and BER collectively contribute to 5-FU cellular sensitivity. Similarly, clinical studies examining 5-FU response in MSI-H patients do not provide a clear picture of the specific involvement of MMR. In particular, an early report offered promise that MSI-H patients might selectively benefit from 5-FU treatment [82]. However, other studies have since found that patients with tumors lacking MSI (*i.e.*, MMR proficient) more significantly benefit from 5-FU treatment [83-85], while other studies report no obvious difference in 5-FU response and MSI status [86,87]. BER status was not simultaneously examined in any of these clinical studies.

4.2 Checkpoint Signaling and other repair pathways

There is growing momentum in targeting DNA damage and cell cycle checkpoint signaling pathways as a means of cancer treatment [88,89]. This is relevant for 5-FU therapy, as TS inhibition and incorporation of the fluorinated base into DNA occurs during S-phase. The PI3K-like kinases, ATM (ataxia telangiectasia-mutated) and ATR (ATM-related), are central mediators in the response to DNA damage during S-phase [90], and their protein substrates number over 700 [91]. CHK1 is thought to be an important downstream target of ATR and is phosphorylated by ATR in response to replication stress [90]. There is some evidence suggesting that S-phase checkpoint pathways respond to 5-FU treatment and TS inhibition. For instance, ATR hypomorphic cells are hypersensitive to 5-FU [92]. Furthermore, TS inhibitors induce CHK1 phosphorylation [93] and phospho-CHK1 foci that colocalize with replication protein A [94]. UCN-01 inhibits the CHK1 kinase and has reached clinical trials [89]. Notably, coadministration of UCN-01 with 5-FU increases sensitivity [95], and Chk1 deficiency similarly sensitizes cells to 5-FU [96,97]. A recent report suggests that ATR and CHK1 status influence cellular sensitivity to 5-FU in a manner that is dependent on MMR- or BER-mediated responses, dictated by the drug dose and exposure period [81].

Nucleotide excision repair copes with bulky helix-distorting lesions, such as those generated by ultraviolet light or the crosslinking agent cisplatin [56]. Although it seems unlikely that nucleotide excision repair would participate in a 5-FU response, given its preference for larger DNA adducts, there is evidence that the pathway recognizes and excises more subtle base lesions, such as 8-oxoguanine [98]. To our knowledge, the contribution of NER to 5-FU resistance has not been explored. Repair pathways for DNA DSBs are worth mentioning

because of the studies cited earlier that identified associations between strand breaks and the lethality of TS inhibitors. There are two major repair pathways that respond to DNA DSBs, namely homologous recombination (HR) and non-homologous end-joining (NHEJ). CHO cell lines defective in components of NHEJ (Ku80 and DNA-PKcs), HR (XRCC3, a RAD51 family member), and S-phase checkpoint signaling (XRCC8, an ataxia telangiectasia-like mutant) do not appear to be specifically sensitive to 5-FU treatment [52]. Nonetheless, there appears to be a number of reasons to examine HR more carefully. First, transient depletion of the RAD51 recombinase by siRNA rendered cells sensitive to thymidylate deprivation induced by an anti-folate TS inhibitor [94]. Second, at least for alkylation damage, HR seems to resolve aberrant DNA structures (e.g. DSBs that arise during replication in S-phase) caused by both unrepaired BER intermediates and MMR-dependent recognition of O^6-methylguanine opposite thymine [59]. Considering the evidence for BER and MMR involvement in 5-FU management (see Sections 3 and 4.1), it is reasonable to presume that HR would be involved in a compensatory response to 5-FU-related DNA damage. Third, there are interesting links among S-phase checkpoint signaling and the HR machinery. For example, CHK1 has been reported to be required for HR [99]. Thus, the observed sensitization of cells to 5-FU when CHK1 is deficient also suggests that HR may be required.

Closing Thoughts

5-FU and its pro-drug derivative capecitabine are commonly employed today in the clinic to eradicate or manage various solid tumors, most notably of the colon. Evidence clearly indicates that products of 5-FU metabolism can affect intracellular nucleotide pools, and ultimately lead to the incorporation of “false” bases, i.e. uracil and 5-FU, into genomic DNA. Thus, it is not surprising that recent studies have suggested that DNA damage responses play a key role in dictating cellular responsiveness to 5-FU exposure.

To date, the pathways that appear most relevant in determining 5-FU outcome are BER and MMR (summarized in Table 1). The finding that the latter pathway contributes to 5-FU sensitivity is striking, given that genetic mutations that disrupt MMR capacity predispose for colorectal cancer. As studies are mixed regarding the clinical efficacy of 5-FU treatment as predicted by MMR genotype, it stands to reason that other factors play at minimum equally vital roles in determining individual responsiveness to 5-FU exposure. One such pathway is undoubtedly BER (namely the proteins SMUG1 and the abasic endonuclease, see Section 3), although mechanistic details need to be elucidated. In particular, the relative contribution of genomic uracil, genomic 5-FU, abasic sites, and strand break intermediates to cytotoxicity remains unclear. Furthermore, DNA damage responses including HR have yet to be extensively interrogated (Table 1). While past investigations have largely focused on measuring TS (target), TK (activator) and dihydropyrimidine dehydrogenase (breaks down 5-FU) for predicting tumor response, we suggest that future studies focus on repair potential, e.g. deleterious polymorphisms in BER genes, as markers for forecasting 5-FU outcome.

An emerging interest in the field of DNA repair is the prospect of manipulating damage response systems to either augment cellular resistance (improve repair) or increase cellular sensitivity (inhibit repair) to enhance therapeutic efficacy of the many DNA damaging drugs used in the clinic. Such agents typically cause different types of DNA modifications, thus potentially invoking several DNA repair mechanisms. In this regard, 5-FU is no different (Table 1). As the factors that are most critical in determining 5-FU responsiveness become identified, novel agents can be designed to selectively inactivate or enhance these key components. Future combination therapies can thus be designed with a better knowledge of which DNA repair and signaling response(s) is invoked and should be targeted. Such information will become crucial as new generations of inhibitors enter the clinic and are used in combination therapies with the established chemotherapeutics that damage DNA.

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Abbreviations

5-FU, 5-fluorouracil
FdUrd, 5-fluoro-2′-deoxyuridine
TS, thymidylate synthase
TMP, thymidylate
TTP, thymidine triphosphate
dUMP, deoxyuridylate
dUTP, deoxyuridine triphosphate
TK, thymidine kinase
dUTPase, deoxyuridine triphosphate nucleotidohydrolase
SSB, single strand break
DSB, double strand break
UDG, uracil DNA glycosylase
BER, base excision repair
AP site, abasic site (apurinic/apyrimidinic)
MMR, mismatch repair
HR, homologous recombination
CHO, Chinese hamster ovary

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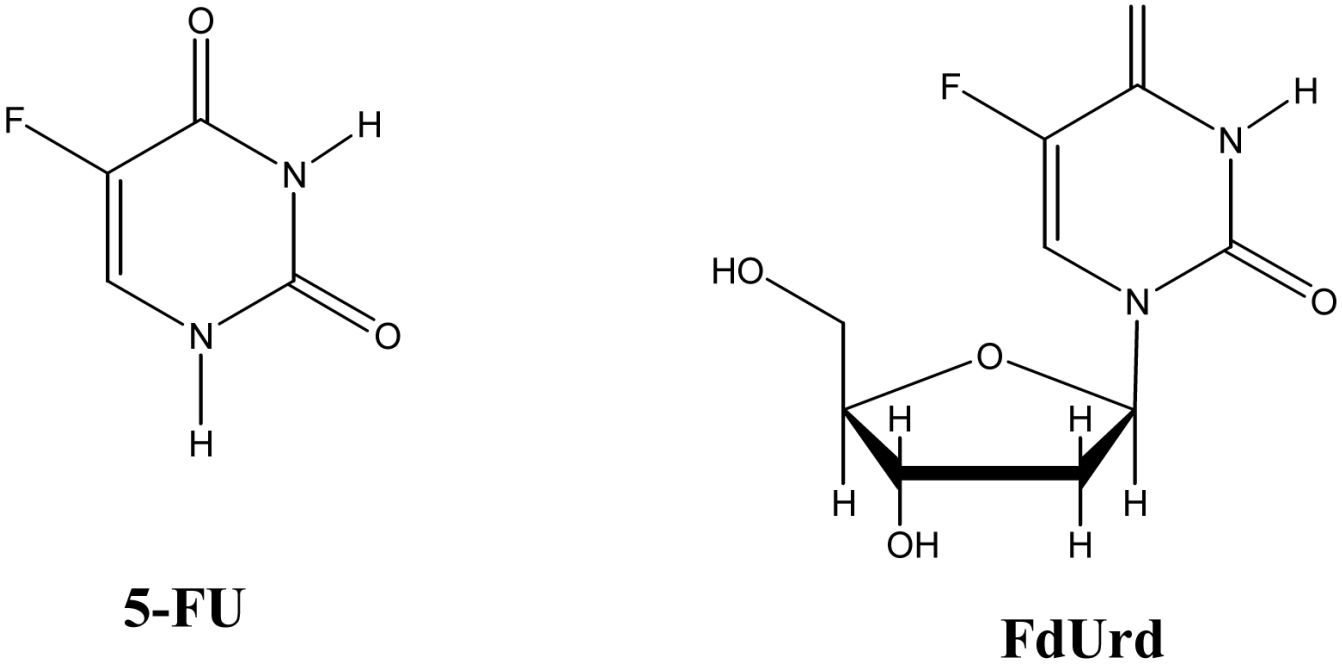

Figure 1. Structure of 5-FU (left) and FdUrd (right)
The fluorine atom at the 5 position of the pyrimidine ring distinguishes 5-FU from uracil (hydrogen at the 5 position) and thymine (methyl group at the 5 position).

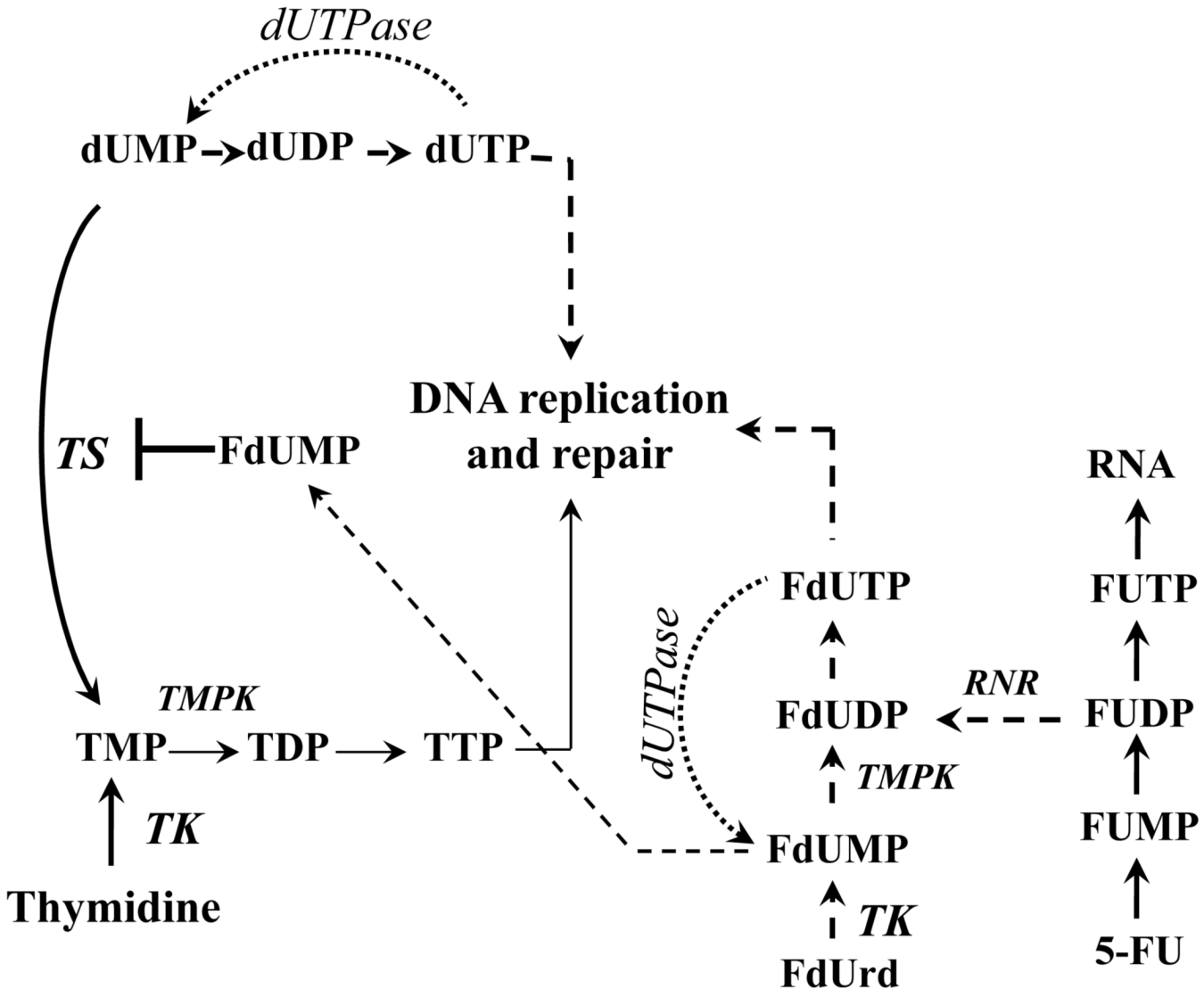

Figure 2. Simplified scheme of 5-FU and FdUrd metabolism

Enzymes are italicized. *TS*, thymidylate synthase provides the only *de novo* source of TMP. *dUTPase*, dUTP nucleotidohydrolase prevents dUTP and FdUTP accumulation. *TK*, thymidine kinase salvages thymidine and FdUrd. FdUMP suicide inhibits *TS*. *TMPK*, thymidylate kinase, phosphorylates TMP and FdUMP. *RNR*, ribonucleotide reductase, converts FUDP and UDP to FdUDP and dUDP, respectively.

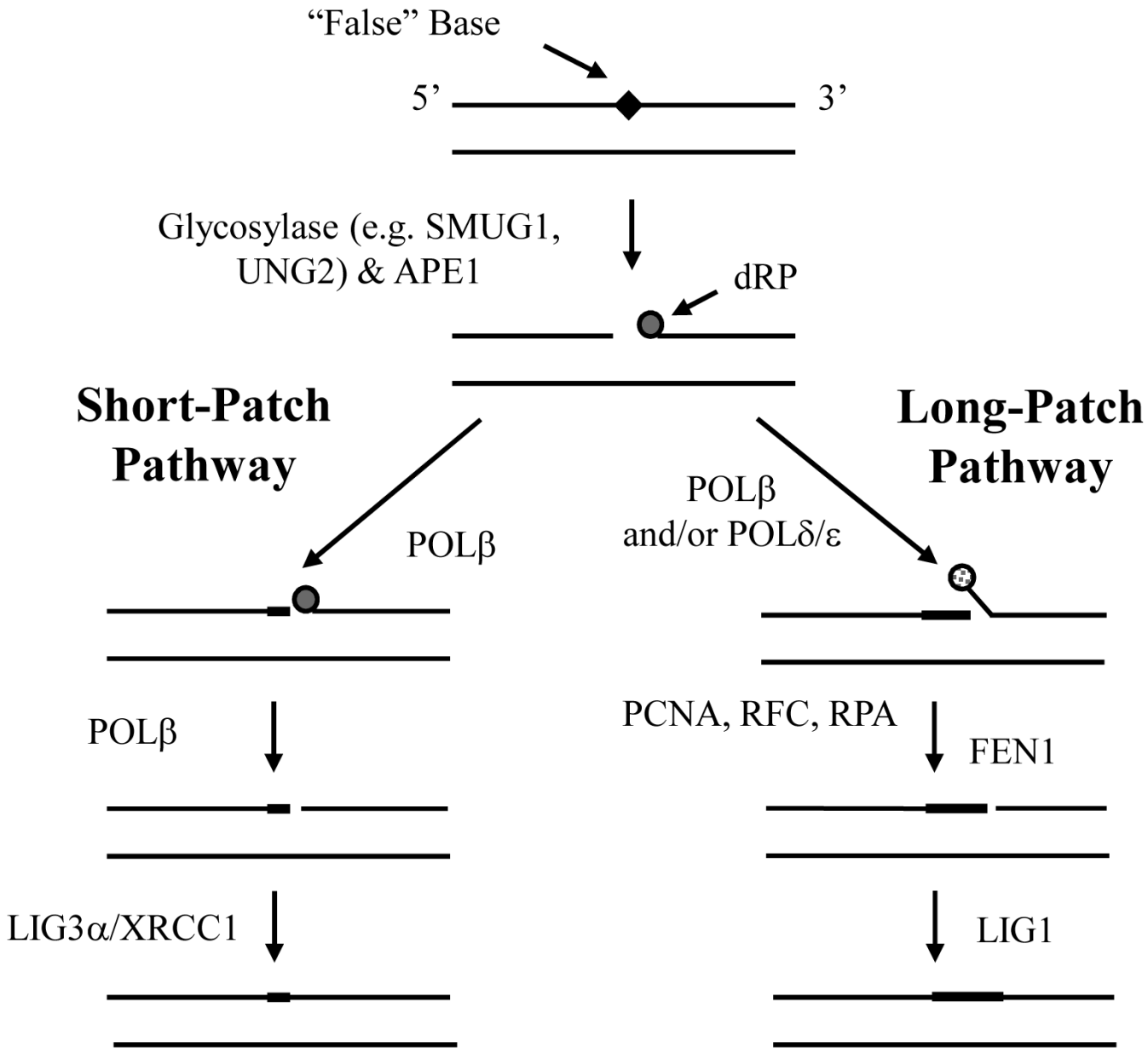

Figure 3. The major enzymatic steps and proteins of mammalian BER

BER is typically initiated by the removal of an inappropriate (e.g. uracil, 8-oxoguanine or certain mismatches) or "false" base, such as 5-fluorouracil, by a lesion specific DNA glycosylase. Following base excision, the resulting abasic site is most often incised by the major AP endonuclease, APE1, to create a strand break with a 5′-deoxyribose phosphate (dRP) residue. At this point, depending on the nature of the 5′-terminal end and other factors (reviewed in [27]), the DNA gap is restored via either short-patch (left) or long-patch (right) BER. In the former situation, the 5′-dRP residue is excised by the lyase activity of DNA POLb and the single nucleotide gap is filled by the same enzyme. Subsequently, a complex of XRCC1 and DNA ligase IIIα (LIG3) seals the remaining nick. In the case of long-patch BER, the 5′-terminal blocking fragment is ultimately removed by a flap endonuclease (FEN1) following strand-displacement synthesis by POLb and/or POLd/e. After excision of the flap DNA structure, the

nick is sealed by DNA LIG1. PCNA, RFC and RPA help facilitate the long-patch repair response. See text for additional details.

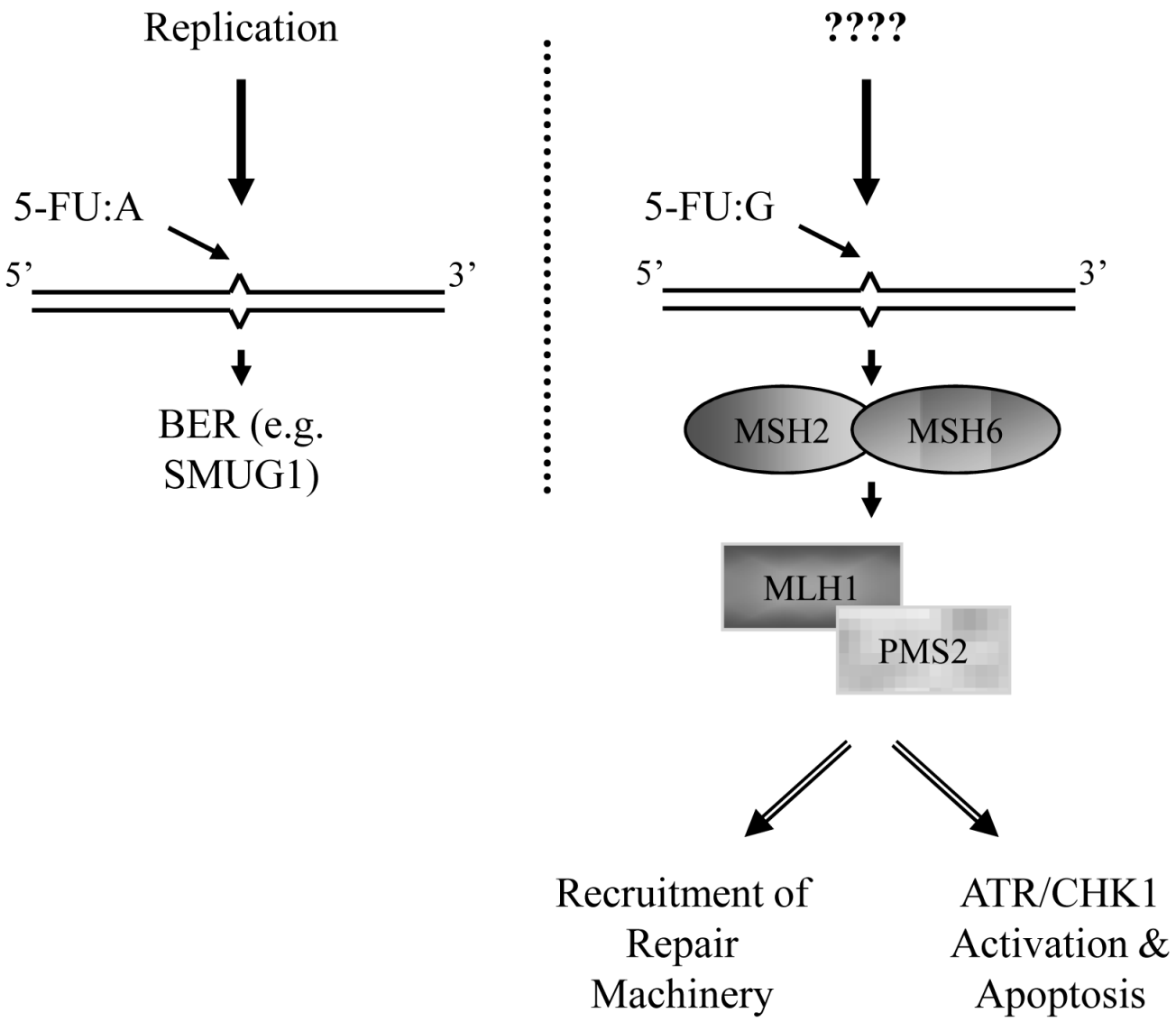

Figure 4. MMR response to genomic 5-FU

5-FU:A pairs that arise upon incorporation of FdUTP into the genome during DNA synthesis are recognized and processed by the BER pathway (left; see text and Figure 3 for details). 5-FU:G pairs, however, are recognized by the MMR MutSa complex comprised of MSH2:MHS6 (right). Subsequently, the MutLα heterdimer made up of MLH1:PMS2 associates and either initiates a repair response or triggers apoptotic signaling through ATR/CHK1 activation. A burning question that remains though is "how do 5-FU:G mispairs arise in chromosomal DNA?" See text for further details.

Table 1

Major DNA Repair Processes and Projected Involvement in 5-FU Response.

DNA Repair Pathway	Primary DNA Substrates	Anticipated Involvement
Direct Reversal		None
MGMT	O6-alkylguanine	
ABH family	N1-alkylpurines	
	N3-alkylpyrimidines	
BER	Small base modifications, abasic sites, SSBs	See Section 3
MMR	Base-base mismatches and small insertion/deletion loops	See Section 4.1
NER	Helix-distorting base adducts	untested, unlikely
Recombination		
NHEJ	DSBs	Not likely
HR	DSBs and collapsed replication forks	See Section 4.2
MGMT = O6-methylguanine DNA methyltransferase		
ABH = AlkB homolog dioxygenase		