Signalling Cell Cycle Arrest and Cell Death through the MMR System

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Abstract

Loss of DNA mismatch repair (MMR) in mammalian cells, as well as having a causative role in cancer, has been linked to resistance to certain DNA damaging agents, including clinically important cytotoxic chemotherapeutics. MMR-deficient cells exhibit defects in G2/M cell cycle arrest and cell killing when treated with these agents. MMR-dependent cell cycle arrest occurs, at least for low doses of alkylating agents, only after the second S-phase following DNA alkylation, suggesting that two rounds of DNA replication are required to generate a checkpoint signal. These results point to an indirect role for MMR proteins in damage signalling where aberrant processing of mismatches leads to the generation of DNA structures (single-strand gaps and/or double strand breaks) that provoke checkpoint activation and cell killing Significantly, recent studies have revealed that the role of MMR proteins in mismatch repair can be uncoupled from the MMR-dependent damage responses. Thus, there is a threshold of expression of MSH2 or MLH1 required for proper checkpoint and cell-death signaling, even though sub-threshold levels are sufficient for fully functional MMR repair activity. Segregation is also revealed through the identification of mutations in *MLH1* or *MSH2* that provide alleles functional in MMR but not DNA damage responses and mutations in *MSH6* that compromise MMR but not apoptotic responses to DNA damaging agents. These studies suggest a direct role for MMR proteins in recognizing and signaling DNA damage responses that is independent of the MMR catalytic repair process. How MMR-dependent G2 arrest may link to cell death remains elusive and we speculate that it is perhaps the resolution of the MMR-dependent G2 cell cycle arrest following DNA damage that is important in terms of cell survival.

1. Introduction.

The DNA mismatch repair system (MMR) repairs base mismatches after DNA replication, inhibits recombination between non-identical DNA sequences and provokes both checkpoint and apoptotic responses following certain types of DNA damage. Defects in MMR are associated with an increased risk of cancer as cells deficient in MMR have a 'mutator phenotype' in which the rate of spontaneous mutation is greatly elevated. The importance of MMR in mutation avoidance is highlighted by the finding that inherited mutations in MMR genes cause Hereditary Non-polyposis colon cancer (HNPCC), while somatic mutations of MMR genes and epigenetic silencing of *MLH1* expression are observed in a significant proportion of sporadic cancers (1) (2). The MMR system also plays a key role in cell killing in response to alkylating agents, the nucleotide analogue 6-thioguanine and the anti-neoplastic drugs cisplatin and carboplatin. MMR-deficient cells are around 100 times more resistant to killing by alkylating agents and are 2-4 fold more resistant to killing by cisplatin.

Much effort has gone towards understanding the mechanisms of MMR, what DNA modifications are recognised by MMR, how MMR system couples to cell killing mechanisms and, more recently, the search has begun to reveal novel therapies that kill tumour cells irrespective of their MMR status or are selective for MMR-deficient cells that are resistant to existing chemotherapies. This review will cover recent advances looking to address the mechanisms involved in MMR-dependent cell cycle checkpoint activation and cytotoxic responses.

The reader is directed to recent papers that provide in-depth analyses of MMR mechanisms and highlight advances in our understanding of the biochemistry of MMR (3,4)

2. Recognition of DNA damage by MMR.

Mismatch recognition is mediated by one of two heterodimers of MutS homologues. hMutS-α (heterodimer of hMSH2 and hMSH6) binds to mismatches and small insertion/deletion loops, whereas hMutS-β (heterodimer of hMSH2 and hMSH3) recognizes larger insertion/deletion loops (2, 5-9). A heterodimer of MutL homologues, hMutL-α (hMLH1 and hPMS2 heterodimer), is also essential for functional MMR, although the exact role it plays in the repair process is unclear.

2a. Alkylating agents and nucleotide analogues.

Cytotoxicity of mono-functional alkylating agents, among them anti-cancer agents such as temozolomide, requires a functional MMR. Mammalian cells proficient in MMR are generally around 100-fold more sensitive to alkylating agents than MMR-deficient counterparts (reviewed in 10,11). The model alkylating agents, *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG), *N*-methyl-*N*-nitrosourea (MNU) and their analogues in clinical use, temozolomide and dacarbazine, produce mainly N7-substituted bases but are cytotoxic as a result of methylation of the O^6 position of guanine in DNA to form O^6-methylguanine (O^6-meG). O^6-meG can be inactivated by the suicide enzyme O^6-meG methyltransferase (MGMT) that catalyses a direct reversal of base methylation (10,12). Resistance to these agents is associated with loss of expression or function of MMR genes, particularly in the absence of MGMT (13,14). The persistence of O^6-meG causes cytotoxicity in an MMR-dependent fashion: in the absence of a functional MMR, DNA damage accumulates but does not trigger cell death. For this reason, the accumulation of O^6-meG in MMR-deficient cells has been named alkylation or methylation 'tolerance', rather than resistance (10). Tolerance to O^6-meG is associated with cross-tolerance to the base analogue and anti-metabolite 6-thioguanine (6-TG). The methyl-donor S-adenosylmethionine

methylates 6-TG to form S^6-methylthioguanine (S^6-meG) in a non-enzymatic reaction, which is incorporated into DNA and is structurally similar to O6-meG (15).

The role of MMR in tolerance to alkylating agents can be explained by the recognition and binding of O^6-meG and S^6-meG by hMutSα. Both O^6-meG and S^6-meG can direct mis-incorporation of T during DNA synthesis. Recognition of these mispairs by components of the mismatch repair system leads directly to activation of signalling cascades which lead to a prolonged G2 arrest (16) and eventual cell death, although the mechanism of the resulting cell cycle arrest and cell killing are not fully understood (see sections 3-5).

2b. Cisplatin

Cisplatin [cis-diamminedichloroplatinum (II), CDDP], one of the most commonly used anti-cancer drugs, has also been reported to give rise to lesions that are recognised by, but not processed by, the MMR system (17-19). The purified hMSH2 protein binds to DNA containing cisplatin adducts with high affinity and can specifically recognize DNA containing a single 1,2-d(GpG) adduct (18). The binding of hMSH2 to DNA containing platinum adducts is selective showing high affinity for adducts of clinically effective platinum drugs such as cisplatin but not for trans-platinum adducts (17).

The cytotoxic effect of cisplatin is primarily due to its well-described formation of adducts with DNA (20,21) which leads to replication arrest, cell cycle checkpoint activation and sustained G2 arrest and, if the damage is too severe, cell death. However, it is not clear if MutS-α would gain access to 1,2GpG adducts *in vivo* as other proteins are known to bind to cisplatin adducts with high affinity (20,21) and other DNA repair pathways such as NER and recombinational repair mechanisms, following replication stalling, are primary mechanisms of repairing cisplatin adducts

in DNA. Indeed it can be argued that the 1,3 intra-strand crosslink is the more important therapeutic lesion since it is repaired less efficiently than the 1,2 intrastrand crosslink and its persistence may lead to replication stalling.

Tumour cell lines selected *in vitro* for cisplatin resistance were often found to have lost expression of MLH1 or MSH2 (22-24). Cell lines that have lost expression of MLH1 or MSH2 are around 2-4 fold more resistant to cisplatin (25-30). Low-level resistance is sufficient to allow enrichment of MMR-deficient cells following repeat rounds of cisplatin treatment (29,31) and a reduced response to drug in xenografts models (29,32). Restoration of MMR in drug resistant MMR-deficient cell line models by complementation of the defective gene by chromosome transfer or reversal of epigenetic inactivation restores sensitivity, arguing that the differential sensitivity is due to MMR activity rather than a mutator phenotype allowing accumulation of resistance mutations at loci throughout the genome. Isogenic cell line models using an inducible MLH1 expression system confirms that re-expression of MLH1 confers low-fold sensitivity to cisplatin (30).

A number of reports have suggested that MMR deficiency is associated with clinical outcome to platinum-based chemotherapy. However, most of these studies suffer from small size and lack of statistical power making their interpretation difficult. However, there are now several reports in initially chemosensitive tumour types such as testicular, ovarian and breast cancer that cisplatin or carboplatin based chemotherapy selects for loss of MMR during treatment of patients and is associated with acquired resistance (33,145-146). This emphasises the importance of separating intrinsic markers of tumour prognosis from acquired clinical drug resistance. The predominant mechanism for the loss of MMR in acquired resistance of ovarian tumours appears to

be loss of MLH1 expression due to CpG-island methylation and epigenetic silencing (22,33).

Despite these observations, other reports disputing the involvement of MMR in cisplatin resistance have emerged in the last few years. It has been suggested that the widely used ovarian cancer cell line, A2780, contains a small pre-existing population of cells that do not express MLH1 and also harbour a mutation in p53 and that it is this p53 mutation and not loss of MMR that makes the most significant contribution to cisplatin resistance (34-36). In a similar vein, it is now apparent that a major mechanism of inactivation of MMR during drug selection is CpG island methylation and transcriptional silencing of *MLH1*. However, multiple loci become simultaneously methylated in these drug resistant variants and *MLH1* may be only one of several genes whose inactivation can influence drug sensitivity.

Genetic evidence has also emerged from mice with targeted disruptions in *Msh2* (37) that question the role of MMR in cisplatin resistance. Mouse embryonic stem cells with deficiencies in *Msh2* have similar responses to cisplatin (38). However the exposure of these cells to low doses of cisplatin resulted in a 2-fold difference in survival in clonogenic assays in cells that had lost Msh2 expression and most of the surviving clones maintained this resistance level upon further exposure to the drug (38). These workers extended their analyses to generated ES cells in which *Msh2* could be inactivated and re-activated *de novo* using the *cre-lox* system. Again they found no relationship between cisplatin sensitivity and MMR status of the cells (38). However, these studies are at odds with other reports in mice that demonstrate an association between loss of one or more MMR genes and resistance to cisplatin *in vitro* and *in vivo* (reviewed in 39,40) and so may reflect variations in MMR dependency depending on cell types examined.

2.c Other DNA damaging agents.

The response of cells to other DNA damaging agents has also been reported to be influenced by the MMR status of the cell (reviewed in 11). The isogenic 293T cell line model derived by Jiricny and colleagues, differing only in MLH1 expression (41), was used to test MMR-dependence for sensitivity to IR, topoisomerase inhibitors, cross-linking agents (mitomycin C, psoralin/UV and CCNU), MNNG and cisplatin. Their evidence supports a role for loss of MMR only in resistance to alkylating agents and cisplatin (30).

3. Models of MMR-dependent DNA damage signalling

Models have been proposed to explain how DNA damage recognised by MMR proteins can lead to cell cycle checkpoint activation and cell death. In one model MMR plays an indirect role by initiating futile cycles of DNA repair as damage on the template strand is repeatedly processed (42) leading to the generation of double strand breaks that are cytotoxic. Another model suggests a direct signalling role for MMR proteins i.e. DNA damage is recognised by MMR proteins and, in turn, MMR proteins recruit other proteins that relay a signal that permits activation of one or more cell cycle checkpoints. In this review we will describe the experimental evidence supporting or contradicting these models. In addition we will describe further refinement of these models. For instance, recent evidence favours a direct signalling role for MMR proteins through both p53-dependent and p53-independent pathways (see below). However the details of direct signalling pathways and how they are distinguished from the events of normal mismatch repair are unclear. Further studies favour an indirect role for MMR proteins in damage signalling: aberrant processing of mismatches leads to the generation of DNA structures (single-strand gaps and/or double strand breaks) that provoke checkpoint activation and cell killing (43-45).

How MMR-dependent G2 arrest may link to cell death remains elusive and we speculate that it is perhaps the resolution of the MMR-dependent G2 cell cycle arrest following DNA damage that is important in terms of cell survival.

4. Checkpoint signalling

Cell cycle progression is constantly monitored to ensure that the correct sequence of events in the process of cell division is achieved and that cells with DNA damage do not replicate. Replication and DNA damage checkpoints stop or slow down cell cycle traverse and so re-establish the correct order of cell cycle transitions after repair of the damage. If repair cannot be effected then the cell is committed to die. For each phase of the cell cycle one or more checkpoints have been identified and individual proteins may have overlapping or distinct functions in the different checkpoints and indeed, as is the case for ATR and Chk1, essential roles in normal division cycles (46,47).

The initial activation of the protein kinase cascade in response to DNA damage is not fully understood, although several protein kinases, such as ataxia telangiectasia, mutated (ATM) and ATM- and Rad3-related (ATR), as well as Chk1 and Chk2 are established as principal components involved in sensing and responding to replication stress and DNA damage (48-50). A simplified general model of response to DNA damage is emerging (Figure1). On one hand, arresting DNA replication leads to the generation of single-strand DNA (ssDNA) gaps that lead to the recruitment of the ATR/ATR interacting protein (ATRIP) complex to these gaps, most likely through Replication protein A (RPA) binding to the ssDNA. On the other hand, DNA damage leading to double strand breaks results in direct activation of ATM and subsequent replication fork arrest, thereby leading to ATR activation. These pathways, however, often act in concert depending on the damaging agent and its delivered dose (51).

The main targets of ATM/ATR-dependent checkpoint signalling in G2 are cyclin B1 and cdc25C- key regulators of the mitotic kinase cdc2 (52). The activation of cdc2 at the end of G2 leads to a commitment of the cell to undergo mitosis and inhibition of this kinase following DNA damage plays a key role in the cellular response to genotoxic insults. A recent study identified that there are two distinct G2 checkpoints associated with DNA damage induced by IR (53). One checkpoint is the response to DNA damage in cells that are already in G2 at the time of irradiation and reflects the failure of these cells to progress to mitosis. This checkpoint is rapidly engaged but transient, ATM-dependent and relatively independent of the dose of IR used. By contrast, the later-acting checkpoint is ATM-independent, dose-dependent and represents the accumulation of cells in G2/M that had been in G1 or S-phase at the time of DNA damage. This later checkpoint is typically what is measured by bulk staining of cellular DNA with propidium iodide after DNA damage (53). Importantly, the late G2 checkpoint is not effected by the earlier G2 checkpoint and G2 accumulation following DNA damage occurs in cells that do or do not activate the earlier G2 checkpoint (53).

The cytotoxicity of alkylating agents is associated with a MMR-dependent accumulation of cells in G2 through signaling mechanisms that are not absolutely dependent on wild-type p53 functions (16,28,54,55). Cells defective for MMR do not arrest in G2 following exposure to alkylating agents and are resistant to the cytotoxic effects of these agents. The G2 checkpoint, and sensitivity to alkylating agents, can be restored by transfer of human chromosome 3, the normal location of *MLH1,* or chromosome 2, the normal location of *MSH2*, into cancer cell lines lacking functional MLH1 or MSH2 respectively (56,57).

Recently, the MMR system has been shown to be required for establishing G2 accumulation in response to treatment with low doses of $S_N1$alkylating agents and 6-TG (41,45). Furthermore, low doses of MNNG induce a G2 arrest that is ATR-dependent but not dependent on ATM (45). The cells accumulate in G2 after the second S-phase following treatment. This suggests that two rounds of DNA replication are required for the generation of the checkpoint signal. Both Caffeine and UCN-01, drugs that inhibit ATM/ATR and Chk1 respectively, can abolish the MMR-dependent G2 arrest. However, the effect is only dependent on ATR, not on ATM, as decreasing ATR expression, or that of its downstream partner Chk1 using RNA interference strategies, prevented the G2 arrest. Checkpoint activation was accompanied by the formation of nuclear foci containing ATR, phosphorylated γ-H2AX, RPA and other DNA repair proteins. These foci persist after DNA damage and the authors suggest that they may represent sites of irreparable DNA damage that ultimately signal cell killing (45). This report expands on earlier studies and confirms that cells treated with MNU or MNNG arrest only after the second S-phase following exposure to the drug (41,43).

It is not clear why cells treated with MNNG do not arrest after the first S-phase following mismatch generation and recognition by MMR system. It has been proposed that mismatch recognition takes place and the resulting processing leads to the generation of intermediate structures, nicks and/or single-strand gaps in the DNA that are not sufficiently frequent and/or sizeable to activate checkpoint pathways. In the subsequent S-phase, these single-stranded gaps opposite the O^6-meG residues would be further processed to generate DSBs leading to collapse of the replication forks and ATR-dependent checkpoint activation. These DSBs would require to be repaired by recombination and could lead to sister chromatid exchanges and other

chromosomal aberrations. Notably, it has been reported that treatment of MMR-proficient cells with methylating agents increases the frequency of SCEs (43,58).

In a follow-on study, Kaina and colleagues suggest that ATM might protect cells from the cytotoxicity of MNNG by permitting efficient repair of secondary damage resulting form MNNG exposure (SCEs and other chromosomal aberrations) that otherwise would promote cell killing (59). The number of chromosomal aberrations detected in the ATM-/- cells was significantly greater than ATM+/+ cells only after the second mitosis after MNNG treatment (59). When this study is viewed in the light of the results of Stojic et al.(45), who found that the downstream target of ATM, Chk2, was activated only 48hr. after MNNG treatment in MMR-competent L-α+ cells, there appears to be a clear interrelationship between MMR/ATR-dependent cell cycle arrest and ATM/Chk2 signalling following treatment with low doses of MNNG or TMZ: such cells can only complete a successful mitosis if the damage provoking G2 arrest is repaired by DSB repair, principally by recombination controlled by ATM (11).

However, the model above is at odds with some key aspects of other earlier studies, one of which described a G2 arrest following the first S-phase after MNNG treatment (60) and another that demonstrated a rapid activation of ATM following MNNG treatment (61). Significantly, the study by Adamson and co-workers used relatively high concentrations of MNNG (10 and 25μm respectively). Jiricny and colleagues have argued that such high concentrations of drug might result in other DNA repair pathways, for example BER, processing alkylation damage leading to rapid activation of ATM/ATR-dependent checkpoint responses (11).

There remains one outstanding discrepancy between the findings of Jiricny and colleagues and a recent study (62). Wang and Qin found that ATR and its interacting

partner, ATRIP, co-immunoprecipitate with MSH2 from HeLa cells and function to regulate the phosphorylation of Chk1 and SMC1 (structural maintenance of chromosome 1) and for activation of an S-phase checkpoint that is independent of ATM (62). Their data support a model where MSH2 and ATR function to regulate signalling pathways in response to MNNG that branch: phosphorylation/activation of Chk1 by ATR requires checkpoint proteins Rad17 and replication protein A and leads to activation of an S-phase checkpoint. The other branch constitutes phosphorylation of SMC1 by ATR, which is independent of both Rad17 and RPA. This demonstrates that the signalling pathway leading to SMC1 phosphorylation is distinct from that mediated by Chk1 with the phosphorylation of SMC1 being required for cellular survival following MNNG treatment but not for checkpoint activation.

A recent report has also highlighted a genetic link between mismatch repair and ATR demonstrating that *ATR* haploinsufficiency results in a high degree of genetic instability and accelerated tumourigensis in cells that are defective for MMR (63). This suggests that monoallelic ATR gene inactivation may be positively selected for during tumour formation as this would drive further genetic instability in a MMR-deficient background (63). This would provide a rationale for the observed ATR mutations in tumours deficient for MMR (64,65). Fang *et al* also reported a constitutive association between ATR-ATRIP and MLH1 and suggest that MutL complexes function as DNA damage sensors or processing factors for coupling damage to ATRIP-ATR-mediated responses and that MMR/ATR interactions may be involved in maintaining the fidelity of recombination (63).

An alternative or additional role for ATM in the MMR-dependent G2 checkpoint has also been postulated. A recent study has demonstrated that the early-acting ATM-dependent G2 checkpoint is lost in MMR-deficient (*Msh2*$^{-/-}$) MEFs when treated with

cisplatin (66). This suggests that Msh2-dependent processes are rapidly engaged to trigger the transient, early G2 checkpoint. These authors propose that this is consistent with post-translational regulation of MMR in the acute response to DNA damage (66). Other reports support this assertion, demonstrating a role for MSH2 in activation of the early G2 checkpoint and recombinational repair following low-dose IR (67), while other reports have provided evidence that the nuclear accumulation and efficient mismatch recognition by MutS-α is regulated by phosphorylation (68,69) and that there is a rapid redistribution of MutL-α to the nucleus following DNA damage (70).

Like the ATM/ATR kinases, activation of stress-activated protein kinases, in particular p38, can also promote G2 arrest by delaying cdc2 activation via p53/p21-dependent and independent mechanisms (71,72, reviewed in 73), most likely through activation of the downstream kinase MAPKAP kinase-2, that these authors suggest may be a 'Chk3' DNA damage effector kinase (74).

A recent study using both pharmacologic and genetic approaches revealed that p38α is necessary for the linkage of methylating agent-induced DNA damage to the G2 arrest in glioma and colon cancer cell lines treated with Temozolomide (75). These authors also demonstrated that processing of O^6-meG lesions by the MMR system was critical for p38α activation in response to methylating agents, because only MMR-proficient cells exhibited Temozolomide-induced p38α activation and G_2 arrest, and only after selective depletion of O^6-meG repair capacity (75). The exact nature of the DNA damage that triggers the biphasic p38 activation following TMZ exposure and the pathways that link this damage to p38 activation remain unclear. Interestingly, p38α appears to act downstream or independently of Chk1 and Chk2 as these checkpoint kinases remained active (phosphorylated) following

pharmacological blockade of p38 or diminution of p38α expression using RNA interference, even though cdc2 inhibition was relieved, most likely through restored cdc25C activity (75). The outcome of p38 blockade was, however, mitotic catastrophe and so it is possible that p38 may have a role in proper recovery from G2 arrest and/or the subsequent mitosis.

A failure to activate p38 in response to cisplatin treatment correlates with resistance to cisplatin (76,77) and this is consistent with the idea that p38 family members play a role in cisplatin-mediated cell killing. It has also been shown that cisplatin treatment leads to phosphorylation of histone H3 at Ser10 and that this phosphorylation is dependent on p38 activity (78). Phosphorylation of serine 10 of histone H3 is associated with mitotic and meiotic chromosome condensation (79-81). Although the exact role of this histone phosphorylation is not understood, these data suggest that there may be a direct link between H3 Ser10 phosphorylation and cisplatin cytotoxicity.

Another study, examining MMR-dependent cell cycle arrest mediated by 6-TG revealed a role for PKB/Akt, that plays a role in the completion of G2 and M during an unperturbed cell cycle (82), both in overcoming the cell cycle arrest and cell killing associated with 6-TG exposure (83). Three direct targets for PKB that are likely to play a role in the PKB-mediated abrogation of 6-TG induced G2 arrest have been identified. In the first case, BRCA1, which is a substrate for PKB (84), and which is also known to interact with MMR proteins (85,86) and play a key role in G2 arrest following DNA damage (87,88). In the second case, it has been shown that activated PKB can inhibit Chk1 by direct phosphorylation on Ser280 and this impairs Chk1 kinase activity in response to IR or replication stress (treatment with hydroxyurea) (89). It has also been shown that dephosphorylation of an ATR site of Chk1 is

essential for recovery from G2 arrest, at least in *S.pombe* (90), and most likely in human cells.

5. Cell Death Signaling and MMR

Alkylating agents, including those in common use as cancer chemotherapeutic agents, kill cells mainly through apoptosis resulting from the cellular response to 0^6-MeG (43,91). Two opposing DNA repair pathways govern cytotoxicity, one by direct repair of 0^6-MeG via an alkyltransferase, O^6-meG methyltransferase (MGMT) (92) the other via mismatch repair.

As the apoptotic response to alkylating agents is strictly dependent on MMR, it has been argued that recognition or processing of DNA damage by MMR proteins is required for induction of p53 and/or p73 and subsequent apoptotic events. Conversely, loss of expression of MMR leads to tolerance of alkylated DNA and may lead to reduced competence for activation of apoptotic pathways.

The tumour suppressor p53 is rapidly stabilised in MMR-dependent manner in cells following exposure to alkylating agents (17,93,94). However, induction of apoptosis in MMR-proficient cells does not appear to require wild-type p53 function (41,94), although there may be a degree of cell-type specificity in the choice of apoptotic programme utilised in response to 0^6-MeG as other workers have examined 0^6-MeG-dependent apoptosis in rodent cells, CHO cells and normal human lymphocytes and found that the extrinsic 'death receptor' pathway and p53 are crucial components of the apoptotic response to MNNG and Temozolomide (43,95,96).

A recent report sought to address the relative roles of the mitochondrial and death receptor pathways in response to 0^6-methylguanine (97). There was a MutSα-dependent activation of caspases-2, -3, -8, and -9 in response to MNNG exposure. However, using specific caspase inhibitors, they observed only a minimal requirement

for these proteases in the cell death program triggered by 0^6-MeG mispairs which they also reported to be strictly dependent on mitochondrial death signaling but not death receptor signaling. Significantly, overexpression of either Bcl-2 or Bcl-X_l could effectively block apoptosis but could not prevent loss of clonal survival of the cells demonstrating that the cells ultimately do die but not by apoptosis (97). Non-apoptotic, MMR-dependent cell death has also been reported for MNNG (41,45) although the ultimate response to alkylating agents is likely to be complex with the demise of the cell a result of either apoptosis, mitotic catastrophe or senescence-like state and, with high doses of alkylating agents, by a regulated form of necrotic death, which may or may not be MMR-dependent (98,99). So, it appears that mismatch repair status, rather than p53 status, is a strong indicator of the susceptibility of cells to alkylation-induced cell death.

For cisplatin, it has been reported that cells are killed following drug treatment through signalling pathways that are regulated by MMR and p53 acting largely independently to promote cell killing (34, 100,101). A prominent role has been established for an MMR-dependent signalling pathway that requires the tyrosine kinase c-Abl. Cisplatin exposure leads to activation of c-Abl and JNK kinases and resultant stabilisation of the p53 family member p73 in a MMR-dependent manner with subsequent cell death by apoptosis (102). In addition, recent studies have confirmed the importance of p73 for cell killing after DNA damage (103) and in chemoresistance (104).

The p73 gene encodes carboxy-terminal splice variants that are pro-apoptotic isoforms (transactivation-competent; TA) as well as variants that lack the transactivation domain, so called ΔN isoforms, that are anti-apoptotic (105). The ΔN isoforms are thought to act by blocking transactivation of target genes of both TAp73

and p53 (106). All forms of p73 are phosphorylated and stabilised by c-Abl, suggesting that the outcome to c-Abl activation i.e. cell death or survival, might be dictated by the ratio of TAp3/delta-p73 isoforms in the cell (107).

The transcription factor E2F1 directly transactivates p73, causing transcription of p53 target genes in a p53-independent manner, and apoptosis. E2F1 is released from pRb during G1 exit, and so the induction of p73 can occur only in early S phase. It will be interesting to see whether c-Abl-induced apoptosis via p73 is dependent on released E2F1, which would explain why Abl induces apoptosis only after pRb hyperphosphorylation in early S phase.

Another report has demonstrated that, in response to cisplatin exposure, PMS2 can directly bind and stabilise p73 and enhances its pro-apoptotic activity, thus providing a direct link between MMR and apoptotic signalling (108). Work from our laboratory, demonstrating a direct interaction between MLH1 and c-Myc, support the suggestion of a direct link between MMR and apoptotic signalling (109). Indeed, p73 and c-Myc have been shown to interact directly (110,111), so it could be argued that p73/Myc and MLH1 may form part of a signaling pathway/module involved in determining cell fate in response to DNA damage.

Although MLH1 expression is required for c-Abl activation and subsequent p73 stabilisation it is not known how MLH1 accomplishes this (112). Recent reports demonstrate that post-translational modifications, including phosphorylation and acetylation, which appear to be tightly coupled with p300-dependent acetylation (113), in which prior c-Abl-mediated phosphorylation is a pre-requisite, enhances p73-dependent transcriptional activation of pro-apoptotic genes. Recent work has established that recognition of c-Abl phosphorylated Y99 of p73 by Pin1, an enzyme that mediates cis/trans isomerisation of proteins at phosphoserine-proline or

phoshothreonine-proline motifs, promotes the conformational changes in p73 that lead to its stabilisation (114). Pin1 has been implicated in the G2-M transition of the cell cycle most likely through its interaction with a number of mitotic phosphoproteins, including Polo-like kinase-1 (Plk1) and cdc25C (115).

Other workers have demonstrated that p73-α is negatively regulated by phosphorylation in G2/M, most likely by cyclin B-cdc2 (116,117). In addition, another report has demonstrated that Chk1, but not Chk2, interacts with and phosphorylates p73- α on ser 47 *in vivo,* and that phosphorylation of p73 by Chk1 is associated with enhancement of p73 transactivation capacity (118,119).

Cell cycle and DNA-damage dependent activation of p73 then appears to be crucial to coupling the G2 checkpoint in response to DNA damage to cell killing. What role, if any, MMR/MLH1 plays in control p73 post-translational modifications in response to alkylating agents and cisplatin merits further investigation.

6. Expression-level effects of MMR proteins and separation-of-function mutants.

While the role of individual protein components in the process of mismatch repair (MMR) has been studied extensively, much less is known about the regulation of MMR, although it does appear to occur mainly at a post-translational level as RNA for MMR components appear to be constitutively expressed throughout the cell cycle. However, recent findings suggest that the established effect of Bcl-2 to stimulate mutagenesis is likely due to the ability of this protein to suppress *MSH2* gene expression by preventing the inactivation of pRB and subsequent release of E2F transcription factors that activate *MSH2* transcription (120). In addition, hypoxia-inducible factor (HIF-1α) can also hinder transcription of *MSH2* and *MSH6* by displacing c-Myc from the promoter of both MMR genes (121), although this study, conducted using HCT116 cells, is at odds with an earlier study in mouse and other

human, tumour-derived cell lines, that reported a specific decrease in MLH1 mRNA only in response to HIF-1α induction (122)

At the level of protein expression, cells lacking one partner in a heteroduplex involved in MMR also express low levels of the other partner, in spite of normal levels of RNA, suggesting that MMR protein stability is coupled tightly to the stability of its cognate partner (41,123-126). Heterodimer formation by MMR subunits also serves to provide an additional level of control as dimerisation of MLH1 and PMS2 appears to regulate the nuclear import of the heterodimer (70).

There is a growing body of experimental evidence indicating that the level of expression of MMR proteins can influence the cellular response to cytotoxic drugs. Cells expressing reduced levels of MLH1 or MSH2 have almost normal levels of MMR activity, do not display microsatellite instability but are more tolerant to DNA damaging compounds. This has important implications for the treatment of cancers that are not defective for MMR but are compromised for MMR-dependent responses following DNA damage.

There is no evidence for haploinsufficiency for MMR repair in humans and tissue from people heterozygous for *MLH1* or *MSH2* does not exhibit MSI (127,128). However, an examination of lymphoid cells from HNPCC patients heterozygous for *MSH2* express around half of the normal level of MSH2 and, while these cells are not significantly compromised for MMR, they are resistant to Temozolomide (129).

Two recent papers also reveal dominant effects of mis-sense mutations in *Msh2* (130) and *Msh6* (131). Cells from mice expressing an Msh2 mis-sense mutation (G674A) were MMR-deficient but retained a normal apoptotic response to DNA damaging agents but the mice were highly cancer prone (130). Similarly, mice with an Msh6 mis-sense mutation (T1217D; M*sh6*^{TD}) are also cancer-prone.

This suggests that defect in MMR alone is sufficient to drive tumourigenesis in these mice, albeit with a delayed tumour onset with respect to the corresponding homozygous-null mice, indicating that the MMR-dependent damage response function could protect from the early occurrence of tumours (130,131).

In both rodent and human cell lines engineered to express a MMR protein for which they are defective, albeit at a relatively low level, the resulting cells are MSI stable but are compromised in their responses to DNA damaging drugs. One group has shown, using independent gastric carcinoma cell lines, that microsatellite instability is associated with genetic alterations to MLH1 or MSH2 but not with relatively low levels of expression of these proteins. However, responses to alkylating agents were compromised in the cell lines expressing low levels of MLH1 or MSH2 (132,133). These authors suggested that MMR proteins may function in distinct ways in mismatch repair and responses to alkylating agents. Similarly, human colon cancer cell lines that re-express low levels of ectopic MSH6 corrected MSI but did not restore sensitivity to alkylating agents again suggesting that MMR proteins function beyond the mismatch repair pathway to determine the outcome following DNA damage (134).

Mouse embryonic stem cells engineered to express 10% of the wild-type level of Msh2 are competent for MMR, reverse their mutator phenotype and suppress homologous recombination yet are as tolerant to MNNG as $Msh2^{-/-}$ cells (135).

Methylation tolerance is also associated with a low level of MLH1 expression. Using a derivative of the Human embryonic kidney cell line 293T engineered to express a doxycycline-regulated allele of *MLH1* Jiricny and colleagues demonstrated that low levels of MLH1 could correct MMR in the 293T cell but the cells remained as tolerant to MNNG as the parental line (41).

Another recent report demonstrated that MLH1 D132H variant is associated with susceptibility to sporadic colorectal cancer but these cancers do not display MSI (136). This variant of MLH1 has attenuated ATPase activity and the authors speculate that this may result in uncoupling MMR from apoptosis mediated by MLH1 in response to chemotherapy (136).

The revelation that the MutS-α complex from the *Msh6*^{TD} mice can bind to damaged DNA and mediated apoptotic signalling in response to cisplatin, MNNG and 6-TG exposure supports the 'signalling' model where MutS α is a direct damage sensor and excision and processing of damaged DNA is not required for a MMR-dependent apoptotic signal (131). This is at odds with the experimental evidence reviewed in section 4, which suggests that recognition of DNA damage is not sufficient for cell checkpoint activation and killing. How do we reconcile these apparent discrepancies? One possibility is that MMR-dependent cell killing is biphasic, with an early phase apoptotic response that is not dependent on mismatch processing, and a late-phase mitotic catastrophe that is dependent on prior checkpoint activation and cell cycle arrest. Another possibility, supported by emerging experimental findings, is that MMR proteins function in DNA damage response pathways in addition to MMR (Figure 2) or another possibility is that there may be different processing steps and outcomes from normal mismatch repair compared with repair/processing of DNA damage, even though both are conducted by the MMR system.

The studies described above have important implications for clinical examination of MSI and its use to govern the course of therapy for patients. If MMR capacity can be significantly reduced without affecting MMR efficiency, but does compromise the lethal processing of drug-induced DNA damage, then this may suggest that cancer

cells not displaying MSI may still have a compromised MMR-dependent apoptotic response with implications for the outcome of cancer chemotherapy.

7. But how does G2 arrest relate to cytotoxicity?

Given that the recent findings discussed above have revealed that the G2 arrest in response to alkylating agents is strictly dependent on mismatch repair activity, yet parallel studies have revealed that the role of MMR proteins in the repair process can be uncoupled from the MMR-dependent cell killing response, it is unclear precisely how G2 arrest relates to cytotoxicity. The absence of a simple correlation between the extent and duration of G2 arrest and cell killing by methylating agents suggests that cell cycle arrest reflects the processing by MMR of both lethal and non-lethal DNA damage (55). Both sub-lethal and lethal doses can induce cell cycle checkpoints that are indistinguishable, suggesting again that it is not checkpoint activation *per se* that is important but how the checkpoint is resolved that determines the cell's fate, or more correctly, if the damage sustained can be corrected prior to or just after mitosis in the next G1 phase or is tolerated (i.e. does not provoke mitotic catastrophe) during mitosis and into the next G1 phase.

The possible outcomes following MMR-dependent G2 arrest are complicated. The prolonged arrest is associated with the appearance of cells that display a senescence-like phenotype while another population appears to recover from G2 arrest but undergo mitotic catastrophe. A third subgroup represents cells that successfully resolve G2 arrest and complete mitosis and remain viable (75). Hirose *et al* suggested that inhibiting p38-α may have a dual effect: inhibiting senescence, and therefore permitting apoptosis, and also inhibiting the ability of the cells to recover from an aberrant mitosis (75).

A lot of attention and interest has gone towards unravelling the sequence and details of phosphorylation of protein substrates that activate cell cycle checkpoints. Recently attention has turned to unravelling the mechanisms that control the de-phosphorylation of checkpoint proteins and restarting cell cycle traverse. A number of findings point to Chk1 kinase as a key regulator of checkpoint maintenance and resolution and subsequent mitotic exit (137-141) and recent studies have begun to reveal phosphatases that regulate Chk1 and other checkpoint proteins involved in checkpoint resolution and mitotic progression (90,142-144). Investigating the possible role of MMR proteins in maintenance and resolution of the G2 checkpoint and the subsequent mitosis after recovery from DNA damage may prove fruitful.

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Figure 1. A simplified general model of responses to DNA damage. Genotoxic stresses results in the generation of single-stranded gaps in the DNA (e.g. DNA replication arrest) and/or double-strand breaks (DSBs). The appearance of critically-long single strand DNA (ssDNA) gaps leads to the recruitment of the ATR/ATR interacting protein (ATRIP) complex, most likely through Replication protein A (RPA) binding to the ssDNA. On the other hand, DNA damage leading to double strand breaks results in direct activation of ATM through dissociation of inactive dimers via an intra-molecular phosphorylation of Ser1981 of ATM. Checkpoint pathways bifurcate at the level of Chk1 and Chk2 to influence both cell cycle arrest, maintenance and resolution of arrest as well as DNA repair and cell killing. Although presented as parallel and exclusive, these pathways often act in concert depending on the damaging agent and its delivered dose and there is a degree of cross-talk between components of the branches.

Figure 2. MMR-dependent and MMR-independent DNA damage signalling.

Evidence has accumulated demonstrating that G2 arrest and cell killing in response to alkylating agents arises from MMR-dependent processing of mismatched bases to generate ssDNA gaps and DSBs depending on the concentration of the alkylating agent and, perhaps, the duration of exposure to DNA damaging agent. However, recent results have revealed that the role of MMR proteins in mismatch repair can be uncoupled from the MMR-dependent damage responses: there is a threshold of expression of MSH2 or MLH1 required for proper checkpoint and cell-death signaling, even though sub-threshold levels are sufficient for fully functional MMR repair activity. In addition, recent genetic analyses suggest a direct role for MMR proteins in recognizing and signaling DNA damage responses that are independent of the MMR catalytic repair process.

Figure. 1. O'Brien and Brown

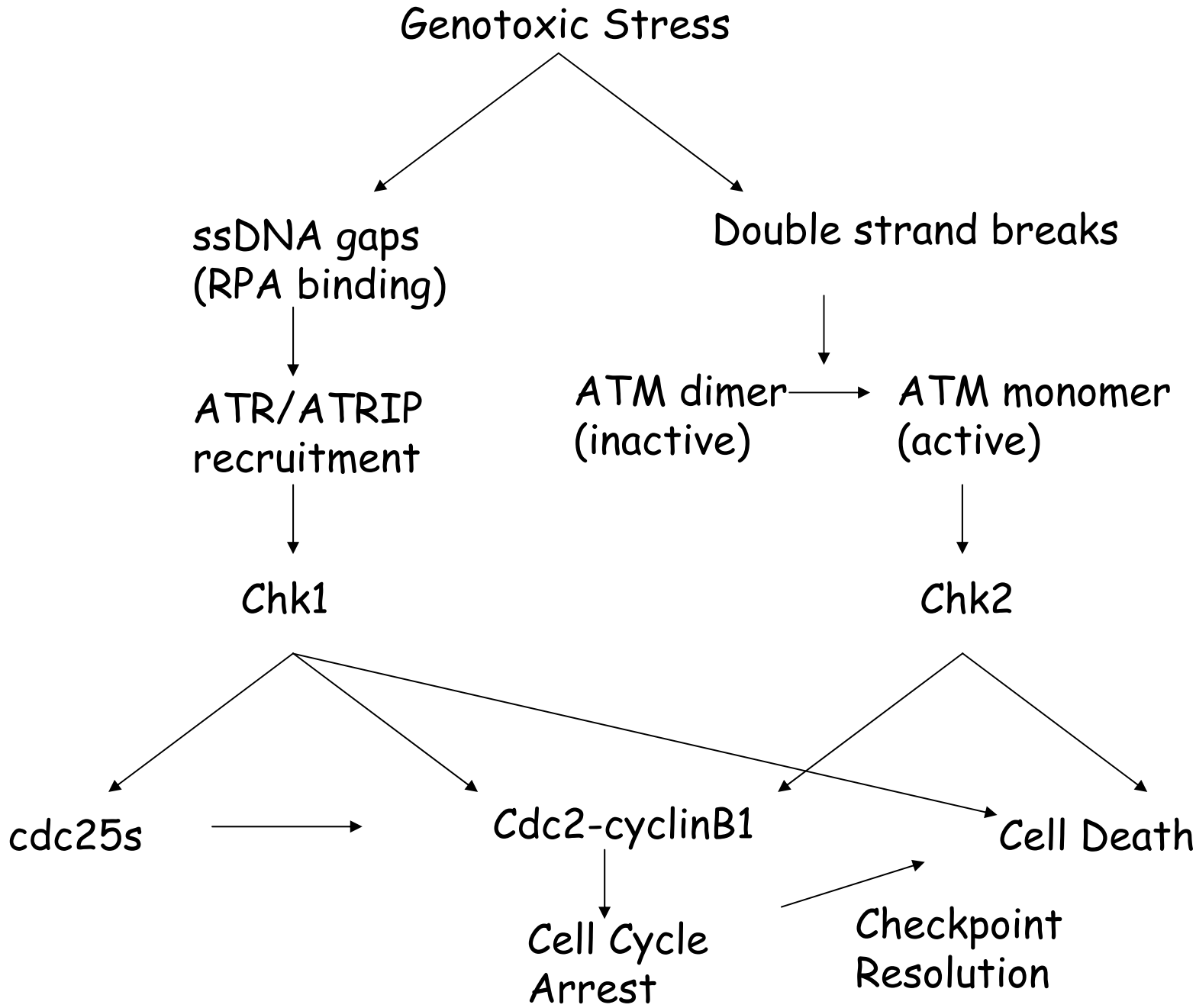

Figure. 2. O'Brien and Brown