THE ROLE OF MISMATCH REPAIR IN MEDIATING CELLULAR SENSITIVITY TO CISPLATIN: THE *ESCHERICHIA COLI* METHYL-DIRECTED REPAIR PARADIGM

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The Role of Mismatch Repair in Mediating Cellular Sensitivity to Cisplatin: the *Escherichia coli* Methyl-Directed Repair Paradigm

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The anticancer drug cisplatin is in widespread use but its mechanism of action is only poorly understood. Moreover, human cancers acquire resistance to the drug, which limits its clinical utility. A paradox in the field is how loss of mismatch DNA repair leads to clinical resistance to this widely used drug.

The phenomenon of cisplatin tolerance in mismatch repair deficient cells was initially discovered in *E. coli*, where methylation deficient *dam* mutants show high sensitivity to cisplatin and *dam* mutants with an additional mutation in either of the mismatch repair genes *mutS* or *mutL* show near wildtype levels of resistance. A prevalent explanation for this observation is the abortive repair model, which proposes that in *dam* mutants, where the strand discrimination signal is lost, mismatch repair attempts futile cycles of repair opposite cisplatin-DNA adducts. Previous findings have supported this model to the extent that MutS, the *E. coli* mismatch recognition protein, specifically recognizes DNA modified with cisplatin. However it has recently been shown that MutS binding to cisplatin adducts may contribute to toxicity by instead preventing the recombinational repair of a cisplatin-modified substrate, and we have previously shown that recombination is an essential mechanism for tolerating cisplatin damage. In the present study, we examined the global transcriptional responses of wildtype, *dam*, *dam mutS*, and *mutS* mutant *E. coli* after treatment with a toxic dose of cisplatin. We also determined any dose-response at the transcriptional level of several SOS response genes and other genes involved in DNA repair by real time RT-PCR. Furthermore, we performed single-cell electrophoresis in order to determine the effect of mismatch repair on the level of double-strand break formation in cisplatin-treated cells. Our results show that Dam-deficient strains exhibit unique gene regulation that may be due to mismatch-repair induced DNA damage in the absence of adenine methylation. In addition, cisplatin treatment induces double-strand break formation and the SOS response in a dose-dependent manner, and both break formation and the SOS response are greatest in the hypersensitive *dam* mutant strain. The higher level of cisplatin-induced double-strand breaks in the *dam* mutant may be dependent on functional mismatch repair.

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Chapter 1: Understanding the basic mechanisms of a successful anticancer agent

Abstract

Approved by the FDA in 1978, cisplatin is currently one of the most widely used anticancer agents (61,326). The drug is particularly effective against testicular germ cell tumors, where cisplatin-based chemotherapy affords cure rates exceeding 90%. Cisplatin's potent toxic effects are believed to be due to its reaction with DNA to form primarily intrastrand crosslinks. These crosslinks induce major structural distortions in DNA including unwinding and bending of the duplex towards the major groove. These structural alterations interfere with essential DNA metabolic processes such as replication and transcription. Furthermore, cisplatin adducts attract cellular proteins with affinity for damaged and/or non-canonical DNA structures, resulting in various effects including repair shielding of the adducts, hijacking of proteins essential for transcription and the repair of other types of DNA damage, and the activation of programmed cell death (apoptosis). Despite the success of cisplatin in the treatment of germ cell tumors, other cancers may be either inherently drug resistant or acquire cisplatin resistance after initial treatment. *In vitro* resistance models show that cells may overcome the toxic effects of cisplatin by several different mechanisms including (1) a reduction the effective intracellular concentration of cisplatin (via reduced uptake or increased efflux of drug or via cellular thiols that inactivate the drug), (2) up-regulation of DNA repair pathways to reduce the number of cisplatin-DNA adducts by excision repair, (3) increased tolerance through the up-regulation of replicative and recombinational bypass mechanisms, and (4) an intrinsic balance of pro- and anti-apoptotic proteins that favor survival over apoptosis. The high sensitivity of testicular germ cell tumors may result from a an inherently reduced DNA repair capability as well as a naturally low threshold for the activation of apoptosis. To mimic the remarkable success of cisplatin in the treatment of testicular germ cell tumors, many second generation platinum compounds have been developed for other tumor types in the hope of overcoming both the dose-limiting toxicity and drug resistance associated with cisplatin treatment.

Chapter 1 Introduction: Understanding the basic mechanisms of a successful anticancer agent

The story of *cis*-diamminedichloroplatinum(II) (cisplatin) marks one of the most remarkable successes in the development of antitumor drugs. Although cisplatin has been known to chemists since 1845 (415), its important biological activity as an anticancer agent was discovered serendipitously only 40 years ago. Experiments designed to study the effects of electric fields on bacteria unexpectedly demonstrated that a chemical agent produced at the platinum electrode inhibited cell division and caused filamentous growth (597,599). Cisplatin, one of the molecules produced, showed antitumor activity in mice and was the first heavy metal to be systematically evaluated for anticancer therapy (187,598). Today, cisplatin is one of the most widely used agents in anticancer therapy (61). This introduction outlines the basic research on cisplatin while focusing on the cellular responses to cisplatin treatment.

1.1 Cisplatin in the clinic

Cisplatin was approved by the FDA in 1978 and has been most successfully used in the treatment of testicular germ cell tumors (TGCTs). TGCTs are the most common tumor type diagnosed in young men between the ages 15 and 34 (58). In 1970, 95% of men with metastatic TGCTs died from the disease (187,452), but today cure rates exceed 90% for all newly diagnosed patients and 70-80% of patients with metastatic disease are cured (58,187,728), making testicular cancer one of the most curable malignancies (Table 1.1). The impressive cure rates of testicular cancer are in large part due to the discovery of cisplatin and the introduction of platinum-based chemotherapies.

TGCTs develop from the germ cells, which give rise to the sperm, and are classified as seminomatous or nonseminomatous tumors. Seminomas account for approximately half of TGCTs and are characterized by homogenous sheets of cells with a clear cytoplasm (452). By contrast, most non-seminomas consist of multiple cell types, including embryonic carcinoma cells—totipotent cells that are capable of differentiating into embryonic and extra-embryonic tissues (58,452). While ~20% of TGCTs contain a mixture of seminomas and non-seminomas, these histopathological types may originate from a common cell (452). Depending on the stage of disease (i.e., stage I, II, or III), men with TGCTs are treated with surgery, radiotherapy or chemotherapy, or a combination thereof. Cisplatin is one the major

chemotherapeutic agents used to treat TCGTs; it was originally used in combination with vinblastine and bleomycin (the agents employed as standard chemotherapy before the advent of cisplatin (187)) in a regimen that cured more than half of the men treated (187). Vinblastine has since been replaced with etoposide, and this new drug combination has resulted in improved response rates and reduced toxicity (452). In addition to TGCTs, cisplatin and its analogs are used to treat ovarian tumors, where platinum-based regimens extend life but unfortunately only rarely cure the disease (192,283). Other tumors for which platinum-based regimens are employed as a first-line therapy include cancers of head and neck, small and non-small cell lung cancer, bladder, cervical, and esophageal cancers (61,579). Regimens that include cisplatin are also used as a second- and third-line therapy against a number of other cancers, such as breast, prostate, and brain tumors (160).

Despite the success of cisplatin in the treatment of TGCTs, the utility of the drug in treating other cancers is limited by two important shortcomings: toxicity and intrinsic or acquired clinical resistance. Cisplatin-induced toxicity, while proving effective in killing cancer cells, is accompanied by harmful side-effects that reduce the dose and duration of platinum-based regimens. Dose-limiting side effects include nephrotoxicity, emetogenesis (vomiting) and various central and peripheral neurotoxicities (422). To protect against unwanted adverse effects, co-administration of thiols such as N-acetylcysteine (NAC) are increasingly used in clinical trials of platinum chemotherapy; NAC can prevent cisplatin-induced ototoxicity, nephrotoxicity, and gastrointestinal toxicity (109,166,519,633). In addition, delivery methods such as polymeric micelles may provide alternatives to standard administration methods that reduce toxicity (701). Reproductive toxicity is also observed with cisplatin treatment; reduction in sperm counts is common, but is generally temporary and reversible (304,744). Importantly, hematologic toxicity is uncommon with cisplatin, which allows the drug to be used in combination regimens with second and third agents that have complementing side effects (422,424). Accordingly cisplatin is widely used in regimens involving multiple other drugs including bleomycin and etoposide mentioned above, paclitaxel, docetaxel, gemcitabine, doxorubicin, vinorelbine, irinotecan, 5-fluorouracil, cyclophosphamide, and ifosfamide (61,470,728) (Table 1.1). In addition to the dose-limiting side effects, clinical resistance presents a major limitation of cisplatin chemotherapy (228,638). Some malignancies, such as colorectal and non-small cell lung cancers, are intrinsically resistant to cisplatin, whereas other cancers, such as ovarian and small-cell lung carcinomas, acquire resistance after initial cisplatin treatment; consequently relapse rates

can be very high (36,503). Platinum analogs and second generation platinum compounds have shown some success in overcoming both the dose-limiting toxicity and clinical resistance associated with cisplatin, and these compounds are briefly discussed in Section 1.5.

Table 1.1 Survival rates for the eleven major cancer types and TGCT

Cancer type	5-year survival rate*	First-line or adjuvant chemotherapies
Pancreatic cancer	1.6%	combination of 5-fluorouracil (5FU), gemcitabine, paclitaxel, and/or cisplatin (692,693)
Lung cancer	1.7%	taxane-cisplatin combination therapy (e.g., paclitaxel or docetaxel combined with cisplatin, carboplatin sometimes substituted for cisplatin); cisplatin or carboplatin with gemcitabine; cisplatin and etoposide (61,115)
Bladder cancer	5.9%	gemcitabine in combination with cisplatin (600)
Colorectal cancer	6.9%	5-FU/leucovorin or capecitabine with either oxaliplatin or irinotecan (368,595)
Cervical cancer	8.6%	cisplatin and 5-FU (61)
Renal cancer	9.2%	gemcitabine-containing combinations for immunotherapy-resistant advanced cancers (574)
Melanoma	15.9%	dacarbazine (DTIC), alone or in combination with BCNU (1,3-bis(2- chloroethyl)-1-nitrosourea) or cisplatin, or in combination with cisplatin and vinblastine; temozolomide (12)
Breast cancer	19.8%	paclitaxel in combination with gemcitabine; tamoxifen (for estrogen positive cancers); 5-FU, anthracyclines (doxorubicin, epirubicin); or the "Dartmouth regimen": DTIC, BCNU, cisplatin, and tamoxifen (21,193,231,437)
Ovarian cancer	23.3%	combination of a platinum (cisplatin or carboplatin) and a taxane (paclitaxel or docetaxel) (10)
Endometrial cancer	26.1%	doxorubicin and cisplatin, topotecan (295)
Prostate cancer	29.8%	one or more of the following: estramustine, cisplatin, etoposide, mitoxantrone, vinblastine, placitaxel, and docetaxel (11)
Testicular germ cell cancer	70-80%	cisplatin, bleomycin, and etoposide (58,187,452)

Table 1.1: *Survival rates for metastatic cancer taken from Masters and Koberle (452) and survival rates listed here may be lower than rates reported elsewhere (10-12). Estimated

incidence and death rates in 2005 for the cancer types listed are available at Cancer Statistics, 2005 <http://CAonline.AmCancerSoc.org>.

1.2 DNA is the ultimate target of cisplatin

Cisplatin is administered to patients intravenously at doses ranging from 20-120 mg/m^2 (58,422). While it is still unclear how the drug enters cells, once inside cells, cisplatin undergoes aquation hydrolysis to form the active compound (Fig. 1.1). The aquated drug reacts with many cellular targets including DNA, RNA, and protein; however it is the reaction of cisplatin with DNA to form cisplatin-DNA adducts that is ultimately responsible for the drug's toxic effects (775). The first line of support for DNA as the key cellular target was the filamentous growth of *E. coli* induced by cisplatin; such filamentous growth was shown to be induced by other DNA-damaging treatments such as hydroxyurea and UV- and ionizing radiation (303,745). In addition, DNA was shown to contain more adducts per molecule compared to RNA and protein (6,546). The most revealing studies, however, showed that DNA repair status altered cellular sensitivity to cisplatin; DNA repair-deficient *E. coli* and human cells were shown to be more sensitive compared to their repair-proficient counterparts, suggesting that differential ability to repair cisplatin-DNA adducts leads to differential sensitivity to the drug (33,173,175,176,636). Later studies correlating the level of cisplatin-DNA adducts with clinical response also point to DNA adducts as the major lesions eliciting toxicity (584,585).

Cisplatin reacts with the N7 atom of purines, with binding to guanine-N7 thermodynamically favored over adenine-N7 (167,586,587). *In vitro* (182,209) and *in vivo* (112,521) studies to identify the major DNA adducts formed by cisplatin have shown that the major adduct is the 1,2-intrastrand crosslink between two adjacent guanines (1,2-d(GpG)), which comprises ~65% of the total adduct spectrum. The intrastrand crosslink between an adjacent guanine and adenine (1,2-d(ApG)) comprises ~25% of the total adduct spectrum while the remaining adducts consist of 1,3-intrastrand crosslinks between two non-adjacent guanines (1,3-d(GpNpG)) and interstrand crosslinks (8-10%) as well as monofunctional adducts to a single guanine (2-3%). The intrastrand crosslinks produce major structural distortions to the DNA, including unwinding and bending of the helix resulting in destabilization of the duplex. The major 1,2-d(GpG) and 1,2-d(ApG) intrastrand crosslinks cause the helix to bend 40-60° toward the major groove and unwind the helix by ~13° (161); the 1,3-d(GpNpG) adduct induces bending by ~35-50° and unwinding by 23°, although different techniques and studies yield different values for bending and unwinding (326,352).

The different DNA-adduct spectrums produced by cisplatin and its therapeutically inactive isomer *trans*-diamminedichloroplatinum(II) (*trans*-DDP or transplatin, Fig. 1.2) provide insight as to the types of adducts that are responsible for eliciting the former drug's toxic effects. Transplatin reacts with the N7 of purines or the N3 of cytosines and forms primarily intrastrand crosslinks between two guanines or a guanine and a cytosine separated by a base (i.e., 1,3-d(GpNpG) or 1,3-d(GpNpC)) as well as interstrand crosslinks between complementary guanine and cytosine residues, with interstrand crosslinks comprising ~20% of the transplatin total adduct spectrum (65,183,184). Although transplatin adducts also generate structural alterations in DNA, this isomer induces alterations that differ somewhat from the alterations produced by cisplatin adducts. For example, the 1,3 transplatin crosslink confers flexibility to the helix, serving as a hinge without directionality (39), whereas the cisplatin intrastrand crosslinks bend the helix towards the major groove. The different adducts and DNA structural alterations formed by the potent anticancer agent cisplatin and its clinically ineffective isomer transplatin most likely result in differential recognition and processing of the adducts by cellular proteins. Indeed, it has been shown that transplatin-modified DNA is more efficiently repaired than DNA modified by cisplatin, which may explain why the latter drug is highly cytotoxic (116,277,565,573). Moreover, the different adducts formed by cisplatin are differentially repaired, with the major 1,2-d(GpG) intrastrand crosslink undergoing excision repair synthesis at 15-20 fold less efficiency than the 1,3-d(GpNpG) adduct (308,492,777).

1.3 Downstream events of cisplatin-DNA adduct formation

The major DNA structural distortions induced by cisplatin lead to important cellular consequences. The distorted structure interferes with essential cellular processes such as DNA replication and transcription. Furthermore, the DNA structural changes caused by cisplatin attract certain nuclear proteins to the adduct site, triggering various events including apoptosis. The following sections highlight the downstream cellular events following cisplatin-DNA adduct formation.

1.3.1 Cellular proteins that recognize cisplatin-modified DNA: implications in repair and repair shielding

Perhaps one of the most significant events following cisplatin-DNA adduct formation is the binding of cellular proteins to the adducts. The first reports of such activity identified proteins with unknown enzymatic activities that bound specifically to cisplatin adducts

(113,172,689). Later it was discovered that proteins with high mobility group (HMG) domains bind specifically to the major d(GpG) and d(ApG) intrastrand crosslinks but not to the minor 1,3-intrastrand d(GpNpG) crosslink (79,563), and a recent study demonstrated the association of native nuclear HMG-box proteins with the major 1,2-dGpG crosslink (784). The HMG domain is a common DNA-binding motif found in proteins belonging to the HMG1/2 superfamily. Proteins of this family may contain multiple HMG domains and may exhibit either structure or sequence specific binding. All HMG-box proteins, however, have a high affinity for distorted DNA structures, including cruciform DNA, which may explain why they recognize and bind to cisplatin-DNA adducts. Table 1.2 lists several HMG domain proteins and their affinity for cisplatin-modified DNA.

Table 1.2 The binding affinity of HMG-box proteins for cisplatin-modified DNA

HMG box protein	Cisplatin substrate*	Binding affinity, K_d	Reference(s)
Structure-specific recognition protein 1 (SSRP1), human	single 1,2-d(GpG)	ND	(79,763)
non-histone, chromatin associated calf HMG1	Globally modified DNA	~0.3 nM	(48,310,698)
non-histone, chromatin associated calf HMG2	Globally modified DNA	~0.2 nM	(48,310)
rat HMG1	single 1,2-d(GpG)	370 nM	(563)
human UBF	single 1,2-d(GpG)	60 pM	(690)
mtTFA	single 1,2-d(GpG)	100 nM	(110)
yeast transcription factor Ixr1	single 1,2-d(GpG)	250 nM	(77,461)
mouse testis specific HMG	single 1,2-d(GpG)	24 nM	(528)
sex determining factor SRY	single 1,2-d(GpG)	120 nM	(691)

Table 1.2: *Sequence context influences the structural alterations induced by cisplatin and may thereby affect the binding affinity of HMG proteins (122,180,564). ND, not determined

The important cellular consequences of HMG-box protein recognition of cisplatin-DNA adducts were first demonstrated *in vivo* in *S. cerevisiae*; a yeast strain with a deletion for the protein Ixr1 (intrastrand crosslink recognition protein 1), which is a member of the HMG-box protein family, was 2-6 fold less sensitive to cisplatin and accumulated one-third as many platinum-DNA lesions as the parental strain expressing Ixr1 (77). Furthermore, this protein bound specifically to DNA modified with cisplatin but not to DNA containing crosslinks formed by the geometric isomer transplatin, and yeast lacking Ixr1 did not show differential sensitivity to transplatin. Additional experiments demonstrated that the

differential effect of Ixr1 on cisplatin sensitivity was significantly decreased in a series of yeast strains deficient in nucleotide excision repair (NER), establishing direct links between protein recognition of the adducts, sensitivity, and DNA repair. It was also later shown that two HMG-box proteins, HMGB1 (HMG-box 1 protein, formerly called HMG1) and the human mitochondrial transcription factor (h-mtTFA), could specifically inhibit the excision repair of the 1,2-d(GpG) crosslink by cell extracts (308). This finding led to the hypothesis that HMG-containing proteins mediate toxicity to cisplatin by blocking the repair of cisplatin-DNA adducts. Since these initial studies, evidence has been presented both *in vitro* and *in vivo* in support of the view that HMG box proteins sensitize cells to cisplatin by shielding cisplatin-DNA adducts from repair (77,409,460,461,547,548,691,776,777).

In addition to HMG-box proteins, many other cellular proteins have been shown to bind cisplatin-modified DNA. Not unexpectedly, many of these proteins are involved DNA damage recognition and play important roles in DNA damage repair pathways. For example, the protein RPA (replication protein A) exhibits a binding preference for cisplatin-damaged DNA over undamaged DNA (548,549,625). RPA is a multiple subunit single-stranded DNA-binding protein that is required for various processes in cellular DNA metabolism including replication, NER, and homologous recombination. RPA also interacts with many proteins including other repair proteins (e.g., XPA, XPG, Rad 51, and Rad52) (138,284,495,673,674) as well as proteins involved in DNA damage signaling and cell cycle control (e.g., p53 (478)). Of relevance to cisplatin-induced toxicity, the relative binding affinities of RPA to the different cisplatin adducts correlate with the efficiency of repair of the adducts; while RPA shows higher binding affinity for the 1,3-d(GpNpG) adduct than for the 1,2-d(GpG) adduct (549), it is the 1,3-intrastrand crosslink that is more efficiently repaired in excision repair assays (308,777). Other NER proteins that show binding affinity for cisplatin-DNA adducts include proteins of the Xeroderma pigmentosum (XP) complementation groups A and E. The proteins of the XP complementation groups, of which there are eight (A through G, and XP-Variant) were initially identified from the cells of XP patients who suffer a hypersensitivity to sunlight and from manifestations such as UV-induced skin lesions and multiple skin cancers (120,405,627). The XPA protein is involved in DNA damage recognition and like RPA was shown to exhibit higher binding affinity for cisplatin-damaged DNA than for undamaged DNA (18,341,393). The recognition of cisplatin damage by XPA may play an important role in clinical responses to cisplatin, as sensitive testicular tumor cell lines express low levels of XPA and show a reduced capacity to repair

cisplatin damage (381,739) and XPA-deficient fibroblasts are hypersensitive to cisplatin treatment (168). Another XP protein thought to be involved in damage recognition, XPE, also recognizes cisplatin-modified DNA (113); the purified protein binds cisplatin adducts but shows no affinity for transplatin adducts (551). The importance of cisplatin damage recognition by NER proteins is underscored by studies correlating NER gene expression and repair capacity with response to cisplatin, with reduced NER capacity leading to hypersensitivity to cisplatin treatment (168,380,381,403,569,739,789) while increased NER confers increased tolerance to the drug (114,146,147,208,663,767).

The damage recognition proteins of DNA repair pathways other than NER also show affinity for cisplatin-damaged DNA. It has recently been shown that the human glycosylase AAG, which is involved in the first step of base excision repair, binds cisplatin-modified DNA in vitro (353). Currently known AAG substrates include alkylated bases such as 3-methyladenine, 3-methylguanine, 7-methylguanine, 1,N^6-ethenoadenine, N^2,3-ethenoguanine as well as the deamination product hypoxanthine and oxidized guanine (7,8-dihydro-8-oxoguanine, or 8-oxoG) (754). Although AAG binding to cisplatin does not lead to excision of the adduct, cisplatin adducts may serve as molecular decoys, luring the enzyme away from its natural damaged-base substrates and thereby reducing the repair of other types of DNA damage (353). Another DNA glycosylase, the *E. coli* MutY and its human homologue hMYH, also bind cisplatin-modified substrates in vitro (Kartalou et al., unpublished results). MutY is a glycosylase that excises adenine opposite oxidized guanine (8-oxoG). Thus MutY serves as a mismatch protein and helps protect the genome against mutations arising from the error-prone bypass of oxidized bases. MutY also shows activity on adenine mispair substrates such as A:G and A:C, albeit with much lower activity (261,654). In addition to binding to cisplatin-DNA adducts in vitro, both bacterial MutY and hMYH retain their ability to excise adenine in the mismatch substrate G:A when the guanine is part of a cisplatin intrastrand crosslink (Kartalou et al., unpublished results). Importantly, cisplatin induces SOS-dependent G to T transversions at the platinated guanine in *E. coli*, suggesting that platinated guanine mispairs with adenine *in vivo* (66,761,762). While both bacterial MutY and hMYH demonstrate excision of adenine opposite guanine when the guanine is platinated in the aforementioned study, the results of Fourrier et al. (223) show that MutY does not display enzymatic activity on adenine opposite platinated guanine, although this data was obtained under different experimental conditions. In addition to DNA glycosylases, damage recognition proteins of the DNA mismatch repair system also

specifically recognize cisplatin-modified DNA. The *E. coli* mismatch recognition protein MutS (91,778) and its human homologue MutSα (179,473,756) both bind the major cisplatin 1,2-d(GpG) adduct with affinities comparable to the binding affinities for a G:T mispair. The binding constant of purified hMSH2 is estimated to be 67 nM ($Kd_{(app)}$) for a 162-bp probe modified with ~6 cisplatin adducts (473). While damage recognition by repair proteins is expected to help protect the cells against the damage, paradoxically mismatch repair proteins sensitize cells to cisplatin damage. The role of mismatch repair in mediating cisplatin-induced toxicity will be discussed in detail in Sections 1.3.4 and 1.4.4 and in the following chapters.

In addition to the aforementioned repair proteins, still other proteins have demonstrated binding affinity for cisplatin-DNA adducts. These proteins include DNA binding proteins such has Histone H1 (758), TATA binding protein (TBP) (121), and Y-box-binding protein (YB-1) as well as proteins involved in DNA repair and metabolism, which include photolyase, T4 endonuclease VII, and the human Ku autoantigen. The cellular consequences of these protein interactions with cisplatin-modified DNA are reviewed elsewhere (references (326) and (352)). Recently, it was shown that poly(adenosine diphosphate-ribose)polymerase-1 (PARP-1) also binds cisplatin-modified DNA in cells (784), and this finding is consistent with more recent data showing that PARP-1 recognizes distortions in the DNA helical backbone and binds to three- and four-way junctions, hairpins, cruciforms, and stably unpaired duplex regions, all of which induce PARP-1 enzymatic activity (426). PARP-1 catalyzes the successive addition of ADP-ribose to acceptor proteins to form branched polymers (144), and PARP interactions with effectors in DNA repair, recombination, replication, and transcription support a role for poly(ADP-ribosyl)ation in DNA metabolism (414). Cisplatin was shown to increase poly(ADP-ribosyl)ation in treated cells (88,784) and pre-treatment with a PARP-1 inhibitor increases sensitivity to cisplatin in non-small cell lung carcinoma xenografts and cell lines (477). While the latter result suggests that PARP-1 may play a protective role following cisplatin damage, perhaps by facilitating repair of adducts, more work is necessary to determine the role of PARP-1 binding in mediating sensitivity to cisplatin.

1.3.2 Inhibition of replication

Early studies demonstrated that cisplatin-DNA adducts can block DNA polymerases in vitro (266,333). Later studies using SV40 DNA and either HeLa or 293 human cell

extracts or SV40 DNA in monkey CV-1 cells also showed that cisplatin inhibited DNA replication (116,277). Another study showed that both HIV-1 reverse transcriptase (RT) and T7 DNA polymerase strongly paused at one nucleotide preceding the first platinated guanine and at the positions opposite the two platinated guanines of the 1,2-d(GpG) intrastrand crosslink (676). Furthermore, a different study using site-specific adducts in M13 genomes tested the ability of the different cisplatin-adducts to block various polymerases including *E. coli* DNA polymerase I (Klenow fragment), bacteriophage T7 DNA polymerase, bacteriophage T4 DNA polymerase, Taq polymerase, and the major replicative *E. coli* DNA polymerase III holoenzyme (123). This study showed that bypass of the cisplatin adducts occurred approximately 10% of the time and that the major 1,2-d(GpG) intrastrand crosslink is the most inhibitory adduct. In addition, polymerases with low 3' to 5' exonuclease activity were shown to possess greater bypass ability than those polymerases with high 3' to 5' exonuclease activity, suggesting that bypass of cisplatin adducts may lead to mutagenesis (see Section 1.3.5).

Ciccarelli et al. showed that both cisplatin and the trans isomer are equally effective at inhibiting replication when equimolar amounts are bound to SV40 DNA (116). While transplatin is less effective in inducing cell death, other factors must contribute to cisplatin-induced toxicity. One possibility is that cisplatin adducts are retained longer than the transplatin adducts and can therefore persist to block replication, and the less efficient repair of cisplatin versus transplatin adducts (116,277,565,573) is consistent with this possibility. Alternatively, other mechanisms, perhaps in conjunction with replication inhibition, may be important in mediating cisplatin-induced toxicity. The observation that cells deficient in DNA repair die at cisplatin concentrations that do not inhibit DNA replication (659) and the ability of error-prone polymerases to bypass a cisplatin-damaged template (288,455,704,707) provide support for other mechanisms playing a role in cisplatin-induced toxicity.

1.3.3 Inhibition of transcription

Direct inhibition of RNA polymerase progression

In addition to blocking DNA polymerases, cisplatin-DNA adducts have been shown to inhibit transcription by blocking RNA polymerase progression. The first clues for a role of transcription inhibition in cisplatin toxicity came from early studies by Sorenson and Eastman (658-660). Using repair-proficient and -deficient Chinese hamster ovary (CHO) cells, they showed that replication inhibition by cisplatin did not correlate directly with sensitivity to

cisplatin; rather, CHO cells treated with cisplatin continued through S phase (i.e., DNA synthesis continued) to arrest in G2, and the duration of G2 arrest did directly correlate with toxicity (659). Furthermore, repair-deficient CHO cells required lower cisplatin concentrations to arrest in G2 than the repair-proficient cells, indicating that the persistence of cisplatin adducts may interfere with the transcription of genes essential for entry into mitosis. Later studies by Corda et al. (127,128) showed that the major cisplatin adducts could block progression of both wheat germ RNA polymerase II and the *E. coli* RNA polymerase *in vitro*. Moreover, the cisplatin adducts were better inhibitors of RNA polymerase than the transplatin adduct tested, *trans*-d(GpTpG) intrastrand crosslink (129). Other studies in human and hamster cell lines also confirm that cisplatin adducts are more effective blockers of transcription than the transplatin adducts. By transfecting a plasmid containing a β-galactosidase expression vector into NER-proficient and -deficient cell lines, Mello et al. demonstrated that the cisplatin-damaged plasmid inhibited expression of β-gal with greater efficiency than did the trans-DDP-modified plasmid, and this differential inhibition (2-3 fold) was not due to differential repair of the adducts (474). One important result of blocking RNA polymerase progression during transcription is the ubiquitination and degradation of the enzyme; recent studies have shown that cisplatin as well as UV damage induce the ubiquitination and degradation of the RNA polymerase II large subunit (Pol II LS) (71,404,581).

Inhibition via transcription factor hijacking

The studies described above indicate that the presence of cisplatin-DNA adducts can serve as an effective block to RNA polymerase progression and can therefore directly inhibit gene expression. However cisplatin-DNA adducts can also inhibit gene expression indirectly by attracting transcription factors that bind to the adduct site; transcription factor binding to cisplatin adducts diverts these factors away from their normal DNA binding sites, and in such a way cisplatin adducts can act as molecular decoys for essential transcriptional activators, thereby "hijacking" these activators and inhibiting gene expression. Several lines of evidence support transcription factor hijacking of as a potential mechanism in cisplatin toxicity. First, studies have identified the nucleolar transcription factor human upstream binding factor (hUBF) as the protein most strongly attracted to therapeutic platinum-DNA adducts (690,782). This protein, which shows sequence homology to high mobility group box 1 protein (HMGB1) (327), is a critical positive regulator of ribosomal RNA (rRNA) synthesis, and rRNA is essential in protein synthesis for proliferating cells. hUBF binds to a

cisplatin adduct ($K_{d(app)}$ = 60 pM) and to its cognate rRNA promoter sequence ($K_{d(app)}$ = 18 pM) with comparable affinities (690), leading to the proposal that cisplatin adducts may act as molecular decoys for the transcription factor *in vivo*. Importantly, the level of cisplatin adducts required to effectively compete with ribosomal promoters for hUBF binding *in vitro* is well below the levels of adducts found in patients treated with the drug (~ 5 X 10^4 lesions per cell (584)). The hijacking of hUBF was tested in a reconstituted system in which the effect of cisplatin on rRNA gene transcription was determined. Zhai et al. (782) showed that while hUBF could stimulate transcription of an rDNA minigene, an increase in cisplatin adduct/promoter ratio decreased hUBF-activated transcription whereas transplatin adducts had virtually no effect on rRNA transcription level. Taken together the data show that cisplatin adducts specifically inhibit hUBF function by preventing the activator from binding to its normal site on DNA. Other lines of evidence in support of transcription factor hijacking include studies by Jordan and Carmo-Fonseca showing that cisplatin treatment of HeLa cells causes a redistribution of hUBF within the nucleolus, which would be consistent with a hijacking event *in vivo* (345); such a redistribution was not observed following treatment with transplatin. In addition, Vichi et al. showed that the TATA box binding protein TBP/TFIID binds selectively to cisplatin or UV-damaged DNA (713), which extends the hijacking model to transcriptional activators other than hUBF. Lastly, Cullinane et al., using human cell extracts, showed that transcription from an undamaged adenovirus major late promoter fragment was reduced in the presence of increasing amounts of cisplatin damage on an exogenous plasmid, suggesting that cisplatin damage may be trapping an essential factor for transcription initiation (141).

Other consequences of hijacking

As discussed previously, HMG-box protein binding to cisplatin lesions can serve as shields to DNA repair, and as discussed above the affinity for HMG-box proteins for cisplatin adducts may lead to transcription factor hijacking. It is of interest to note that in addition to transcription factor hijacking, HMG-box protein binding to cisplatin adducts may hijack proteins involved in cellular processes other than transcription. For example HMGB1, which exhibits affinity for cisplatin adducts (23,79,355), has recently been shown to act as a cytokine and to possess proinflammatory activity (reviewed in Lotz and Tracey (427)); moreover, a recent report shows that HMGB1 can induce sprouting of endothelial cells *in vitro* (623). HMGB1 can therefore act as an angiogenetic factor in addition to its role as a nuclear transcription factor. Consequently, by the hijacking hypothesis, the binding of

HMGB1 to cisplatin adducts may also eliminate a pro-growth extracellular signal. As will be discussed below, HMG-box protein binding to cisplatin adducts may also interfere with DNA damage response pathways.

1.3.4 Induction of cell cycle arrest and cell death pathways

The formation of cisplatin-DNA adducts and their recognition by cellular proteins ultimately leads to apoptosis and the induction of cell death pathways. Many proteins that bind cisplatin-modified DNA interact with other proteins at sites of DNA damage to form multi-protein complexes that initiate DNA repair, cell cycle arrest, and/or cell death. As mentioned above, the protein RPA binds cisplatin-modified DNA. While this protein is important in NER, RPA is also a phosphorylation target for DNA-dependent protein kinase (DNA-PK) and the ataxia telangiectasia-mutated gene (ATM) protein kinase, and recent observations suggest that RPA phosphorylation status plays a significant modulatory role in the cellular response to DNA damage (reviewed in Binz (49)).

p53 in response to cisplatin damage

RPA interacts with many proteins involved in the DNA damage signaling response network, including p53 (478) The tumor suppressor p53 can activate the transcription of numerous target genes that are involved in the activation of G1 and G2/M cell cycle checkpoint arrest and in the initiation of apoptosis (reviewed in (562,647,719)). One target of p53 transactivation is the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1/SD11}$ (*p21*), which plays a crucial role in DNA repair, cell differentiation, and apoptosis through regulation of the cell cycle, with p21 promoting cell cycle arrest over p53-mediated apoptosis (236). Expression of p53 and p21 was stimulated within 5 to 10 minutes by cisplatin in p53-positive LX-1 small cell lung carcinoma cells (752). Induction of p21 following p53 stabilization in response to cisplatin was also demonstrated in the cisplatin sensitive ovarian carcinoma cell line A2780, and one study showed that the upstream mediators of this response include phosphatidylinositol 3-kinase (PI3-kinase or PI3K, a protein that transmits signals from receptors that promote cell survival (687)) and its downstream targets serine/threonine kinases AKT1 and AKT2 (v-akt murine thymoma viral oncogene homologue), which were required for full induction of p21 following cisplatin treatment (483). However, while suppression of PI3K/AKT signaling inhibited p21 expression, it did not result in alterations in drug sensitivity of A2780 cells or in the expression of the proapoptotic protein BAX following

cisplatin treatment, suggesting that cell death induced by cisplatin may proceed independently of the cell protective effects of PI3K and AKT (483).

In addition to cell cycle-arrest and apoptosis, p53 also plays an important role in NER through the transcriptional regulation of genes involved in the recognition of adducts in genomic DNA. Loss of p53 function results in deficient global genomic repair (a subset of NER that removes lesions from the whole genome) but not of transcription-coupled repair (a subset of NER that removes lesions in the transcribed regions of the genome) of cyclobutane dimers (220,221,265). NER proteins whose expression is regulated by p53-stimulated transcription include p48 (314), the protein product of the *DDB2* (damage-specific DNA binding protein) gene defective in XP group E (XPE) cells, XPC, and GADD45 (growth arrest and DNA damage 45), a DNA damage inducible protein that may facilitate chromatin unwinding in regions of damaged DNA. In addition, the direct binding of p53 to the NER TFIIH factors XPB and XPD inhibits their helicase activities and these interactions may be involved in p53-mediated apoptosis and DNA repair pathways (407,733,734). p53 also interacts with Rad51 *in vitro* and may play additional roles in other DNA repair mechanisms such as recombination repair (81).

The human breast cancer associated protein BRCA1 plays a modulatory role in the p53 pathway and may play a role in the response to cisplatin. BRCA1 is a substrate of the ATM kinase and is found associated with multiple other proteins following DNA insult at nuclear foci, the supposed sites of DNA damage. Like p53, BRCA1 facilitates the repair of DNA damage and specifically enhances global genomic repair, though one study showed that BRCA1-stimulated repair is independent of p53 (269). BRCA1 co-localizes with Rad51 in S-phase cells and increased levels of BRCA1 are found in a drug-resistant ovarian cancer subline (312). Increased BRCA1 is associated with more proficient DNA repair and cisplatin resistance, and accordingly inhibition of BRCA1 results in decreased DNA repair, increased sensitivity to cisplatin and enhanced apoptosis (312). BRCA1 and p53 may cooperate to transactivate DNA repair genes as well as *p21* and *GADD45* .

Other proteins in the p53 response pathway have been shown to be involved in cisplatin-induced signaling and apoptosis. Among these proteins is aurora kinase A, which acts upstream of p53 and phosphorylates p53 at Ser315 leading to its ubiquitylation by murine double minute 2 (MDM2) and subsequent proteolytic degradation (361) (see Section 1.4.4 on p53 in cisplatin resistance). Katayama et al. (361) recently showed that inhibition of

aurora kinase A by siRNA leads to decreased phosphorylation of p53 at Ser315 and consequently increased steady-state levels of p53; the effects on p53 stability were accompanied by increased cell-cycle arrest at the G2-M transition as well as increased sensitivity to cisplatin. Conversely, cells stably transfected with aurora kinase A were resistant to cisplatin-induced apoptosis and showed substantially less induction of p53. The apoptotic response regulated by aurora kinase A is mediated by p53, as aurora kinase A expression had no effect on the stability of the p53-related proteins p63 and p73 in untreated and cisplatin-treated cells.

While p53 may play an important role in cisplatin-induced apoptosis, defects in the p53 pathway, either upstream or downstream of p53, may affect sensitivity to cisplatin. For example, a checkpoint kinase upstream of p53 required for p53 transcriptional activation, Chk2, exhibits reduced expression in a majority of invasive germ cell tumors (28). Thus reduced Chk2 activity may result in reduced expression of p53 target genes, including DNA repair genes, thereby making TGCTs more susceptible to DNA damage by cisplatin. Similarly, decreased levels of p21, which promotes survival over p53-induced apoptosis (571,786)), may make TGCTs more susceptible to cisplatin damage, as p21 was rarely detectable in the seminoma and embryonal carcinoma components of the combined germ cell tumors (29).

The AKT pathway

AKT kinase, a substrate of PI3-kinase, acts upstream of p53 and promotes cell survival by down-regulating apoptotic pathways. Interestingly, activation of PI3-kinase and its downstream target, the AKT/PKB serine-threonine kinase, results in phosphorylation of MDM2, an antagonist of p53 that is also induced by p53 (270); phosphorylation of MDM2 by AKT is necessary for translocation of MDM2 from the cytoplasm into the nucleus (459), where the protein can regulate p53. AKT was shown to protect cells from apoptotic death induced by cisplatin (107,148). Dominant-negative AKT sensitizes ovarian cancer cells to cisplatin, but this effect requires functional p53 (225). AKT exerts its pro-survival effects by phosphorylating several mediators of apoptosis, including the X-linked inhibitor of apoptosis (XIAP). Phosphorylation of XIAP by AKT stabilizes XIAP and prevents both auto-ubiquitylation and cisplatin-induced ubiquitylation activities (148). Increased levels of XIAP are associated with decreased cisplatin-induced caspase 3 activity and apoptosis in ovarian cancer cell lines (19,225). AKT also promotes cell survival by inactivating (via

phosphorylation) other apoptotic pathway proteins including Bad, Forkhead proteins, and GSK-3$^{\beta}$ (154). AKT also phosphorylates IκB kinase, which subsequently activates nuclear transcription factor-κB (NF-κB). NF-κB promotes cell survival by stimulating the expression of genes suppressing apoptosis and promoting cell growth (e.g., cyclin D1 and c-Myc) (572) and inhibition of NF-κB is associated with increased sensitivity to cisplatin in both *in vitro* and *in vivo* ovarian cancer models (431). Pommier et al. (572) provide a detailed review of AKT and NF-κB regulation and their roles in apoptotic pathways. In addition, AKT acts upstream of MAPK pathways as discussed below. While the PI3-kinase/AKT cascade triggers pro-survival events, inhibition of this signaling cascade may sensitize ovarian cancer cells to cisplatin (591).

Mismatch repair in cisplatin-induced apoptosis

Cisplatin resistance has been associated with MMR status, where cells deficient in MMR exhibit a higher tolerance to drug treatment compared to their MMR-proficient counterparts (3,4,177,213,216,751). The level of cisplatin resistance afforded by MMR deficiency, though relatively small (up to 2-fold) in *in vitro* systems, is sufficient to produce a large difference in drug responsiveness *in vivo* in tumor model systems (13,212). The available preclinical and clinical data also suggest that tumors with a significant fraction of MMR-deficient cells will exhibit reduced responsiveness to specific drugs including cisplatin (4,112,212,441). In addition to its association to cisplatin resistance, MMR status has also been implicated in the etiology of hereditary nonpolyposis colon cancer (HNPCC); a high proportion of HNPCC patients carry mutations in the MMR genes *hMSH2* and *hMLH1* (82). The influence of MMR deficiency on drug resistance as well as its pathogenetic role in HNPCC may both arise from a role of MMR in triggering apoptosis[♦]. Several studies have shown that MMR status correlates with the ability of cells to undergo apoptosis, with MMR-deficient cells failing to undergo apoptosis following exposure to certain DNA-damaging agents (177,251,311,685). Furthermore, in testicular tissues, where cisplatin-based treatment is most effective, mismatch repair proteins are expressed in high levels (473). Alterations in MMR function may therefore have profound consequences in clinical

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♦ Lin et al. recently demonstrated that an *Msh2*^{G674A} mutation in mice caused DNA repair deficiency that resulted in a strong cancer predisposition while it did not affect the DNA damage response function of Msh2. Although MEFs with this point mutation remained sensitive to cisplatin compared to *Msh2* null MEFs (413), suggesting that mismatch repair function in mutation avoidance rather than DNA damage signaling is involved in tumorigenesis, the growth advantage of the point mutants due to a deficient apoptotic response may have also contributed to tumorigenesis.

resistance to chemotherapeutic agents, and the signaling properties of MMR are discussed in detail elsewhere (38,666).

The mismatch repair proteins MSH2 and MLH1 bind to cisplatin damage (179,213,473) and have also been implicated in the DNA damage response regulatory network. While MMR plays a critical role in mutation avoidance, the mismatch repair proteins MSH2, MSH6, and MLH1 were shown to interact with other DNA damage response proteins, including BRCA1, ATM (a member of the PI3K family), BLM, and the RAD50-MRE11-NBS1 protein complex, to form the complex BASC (BRCA1-associated genome surveillance complex) (736). Potential roles for MMR in DNA damage-induced signaling have been shown by studies demonstrating that p53 phosphorylation on serine residues 15 and 392 following DNA methylation damage is dependent on functional hMutSα and hMutLα (178) and that induction of p53 and apoptosis by MNNG each require functional MutSα in human cells (285). In addition, Brown et al. demonstrated that human tumor cell lines deficient in MSH2 or MLH1 are defective in S-phase checkpoint activation following ionizing radiation (IR), which resulted from deficient ATM phosphorylation of CHK2 (on threonine 68) and subsequent deficiency in CDC25A phosphorylation and degradation; furthermore, MLH1 and MSH2 may directly associate with ATM and CHK2 kinases, respectively, establishing a molecular scaffold for the two kinases that allows ATM to phosphorylate and activate CHK2 in response to IR (76) (Fig. 1.3).

MMR is also implicated in the DNA damage signaling response following cisplatin treatment, and several molecular determinants of MMR-provoked apoptosis in response to cisplatin have been identified. Cisplatin treatment was shown to activate the JNK1 kinase more efficiently in MMR- proficient cells than in -deficient (*hMLH1*$^-$) cells (512,513), and MMR has been shown to be required for the activation of the nuclear non-receptor tyrosine kinase c-Abl in response to cisplatin treatment (251,512,513). Recently it was shown that c-Abl, following MLH1-dependent activation in cisplatin-treated cells, stabilizes the p53-related protein p73; Gong et al. demonstrated the requirement for MLH1 in the activation of c-Abl, which in turn was required for the induction of p73 (251). While this study demonstrated the requirement for MLH1 in activating an apoptotic response involving c-Abl→ p73, the authors showed that MLH1 did not affect the accumulation of p53 in response to cisplatin, although both p53 and p73 pathways contributed to cisplatin toxicity (251). Furthermore, interaction of the mismatch repair protein PMS2 with p73 is enhanced following cisplatin treatment, and

stimulation of p73 apoptotic functions following cisplatin damage requires PMS2 (637). It has also been shown that overexpression of hMSH2 or hMLH1, but not of any of the other MMR proteins, can induce apoptosis in repair proficient and deficient human cell lines (553,785), thus implicating a direct role of these repair proteins in triggering programmed cell death. c-Abl activation in response to another form of DNA damage, ionizing radiation, is dependent on the activation of ATM and ATM phosphorylation of c-Abl (30,628), and although MMR proteins were shown to interact in protein complexes including ATM (736), neither MSH2 nor MLH1 were required for c-Abl activation in response to IR (76). In contrast, MMR is required for c-Abl activation following cisplatin treatment (512,513). While the latter result suggests c-Abl acts downstream in cisplatin-induced signaling, it is also worth noting that the c-Abl DNA-binding domain shows sequence similarity to HMG-box proteins (632,714) and like HMG-box proteins, it recognizes non-canonical DNA structures such as four-way junctions and single-strand loops (714). Therefore c-Abl may recognize cisplatin-modified DNA and may be involved signaling directly, although such binding activity of c-Abl to cisplatin-DNA adducts has not been reported. It is also worth noting that in testis, the tumors of which are extremely sensitive to cisplatin treatment, c-Abl phosphorylation of p73 increases following IR damage (264).

MMR deficiency affords low-level resistance to cisplatin treatment (177,213,216,251,512). Indeed, the effect of acquired resistance due to loss of MMR is more pronounced towards alkylating damage than towards cisplatin damage (up to 100-fold versus ~2-fold difference) (4,311,685). Therefore other mediators of cell death in addition to MMR must be contributing to cisplatin-induced toxicity. Gong et al. (251) showed that both p53 and p73 pathways contribute to cisplatin toxicity. While MutSα may mediate alkylation-induced toxicity in part by a p53-dependent pathway (178,285,685,751), such p53 dependence for MMR-mediated toxicity has not been observed for cisplatin damage. Branch et al. demonstrated in ovarian carcinoma cells that the effects of MMR and p53 on cisplatin cytotoxicity are independent and additive, with p53 status being the major determinant of toxicity (68). In addition, one study has shown that MMR does not mediate cisplatin-induced toxicity in MEFs (119), and MMR did not play a significant role in the acquired drug resistance of A2780 cells (449). Therefore other "sensors" of cisplatin damage, or secondary lesions resulting from ineffective repair of platinated DNA, may trigger programmed cell death following drug treatment. Based on the results of several studies, the role of p53 in apoptosis and toxicity in response to DNA damaging agents varies

greatly depending on the cell type studied ((285,774) and references therein). Zamble et al. demonstrated p53-dependent apoptosis in mouse testicular teratocarcinoma cell lines exposed to cisplatin, although p53 deficiency did not confer resistance to cisplatin treatment as measured by colony formation assays (774). From the evidence available, it is clear that cisplatin treatment induces a complex apoptotic response involving the activation of parallel death response pathways, a MMR-dependent pathway activating p73 and a MMR-independent pathway (via p53). A diagram of the signaling events following DNA damage is shown in Fig. 1.4.

MAPK signaling cascades

As mentioned above, cisplatin induces activation of c-Abl tyrosine kinase, which in turn stabilizes p73 and enhances cisplatin-induced apoptosis. C-Abl is also required for the cisplatin-induced activation of both p38 mitogen-activated protein (MAP) kinase (539) and stress-activated c-Jun N-terminal protein kinase (275,373). These kinases are members of the mitogen-activated protein kinase (MAPK) signaling cascades, of which there are three major groups—extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases/stress-activated protein kinases (JNKs/SAPKs), and p38—, that regulate cell growth, differentiation, survival, and cell death. C-Abl mediates activation of JNK/SAPK in response to cisplatin damage through its phosphorylation of MEK kinase 1 (MEKK-1), an upstream effector of the SEK1 to JNK/SAPK pathway (372). Cisplatin, but not transplatin, was shown to activate JNK and ERK cascades in both the cisplatin-resistant Caov-3 and the cisplatin-sensitive A2780 human ovarian cancer cell lines (275), and cisplatin provoked the activation of SAPK/JNK and p38 kinase dose-dependently in HeLa cells, with significantly lower activation in resistant subclones than in the sensitive parental line (78). Furthermore, one study showed that the differential duration of the activation of MAPK pathways, specifically of JNK and p38, in sensitive and resistant ovarian cancer cells correlated with cisplatin-induced apoptosis, thus demonstrating the important role of the MAPK signaling cascades in cisplatin-induced toxicity (438). Activation of JNK and p38 was associated with the phosphorylation and subsequent activation of c-Jun and induction of an immediate downstream target of c-Jun transcriptional activation and an inducer of apoptosis, the Fas ligand (FasL, Fig. 1.5), as well as with caspase activity and apoptosis. FasL induction was a critical event for cisplatin-induced apoptosis as pre-treatment with a neutralizing anti-FasL antibody inhibited cisplatin-induced apoptosis and resistance to cisplatin correlated with a failure to up-regulate FasL (438). FasL is also regulated by the Forkhead family

transcription factor FKHRL1 in response to cisplatin damage (591). While FKHRL1 induces apoptosis in its unphosphorylated state, cisplatin-induced phosphorylation of FKHRL1 was observed in cisplatin-resistant ovarian cancer cells but not in cisplatin-sensitive cells, and the unphosphorylated form of FKHRL1 was shown to bind the FasL promoter and induce apoptosis. Phosphorylation of FKHRL1 following cisplatin treatment in resistant cells was achieved by AKT kinase, a substrate of PI3-kinase that acts upstream of p53 in promoting survival as described above. The data summarized here thus support an important role of the JNK > c-Jun > FasL > Fas pathway in mediating cisplatin-induced apoptosis.

As mentioned above, in addition to the JNK/SAPK cascade, activation of p38 has been implicated in mediating cisplatin toxicity. P38 activation by cisplatin was shown in Rat1 cells in which expression of c-Myc was deregulated, and c-Myc potentiation of cisplatin-induced p38 activation was associated with Bax activation and the mitochondrial pathway of apoptosis (162,163). Cisplatin also induced c-Myc-dependent activation of mitogen-activated protein kinase kinase (MKK) 3/6 and apoptosis signal-regulating kinase 1 (Ask1), and inhibition of Ask1 blocked cisplatin-induced p38 activation and downstream apoptotic features (162). Another study showed that p38 activity is a major determinant of cisplatin-induced apoptosis in NIH3T3 cells (332), and using specific kinase inhibitors another group showed that cisplatin induced apoptosis through the p38 but not the ERK MAPK pathway (752). The role of p38 in cisplatin sensitivity was shown to be modulated by the AKT2 kinase in ovarian cancer cells; constitutively active AKT2 conferred cisplatin resistance to the cisplatin-sensitive A2780S ovarian cancer cells, whereas PI3K inhibitor or dominant negative AKT2 sensitized A2780S and cisplatin-resistant A2780CP cells to cisplatin-induced apoptosis through regulation of the ASK1/JNK/p38 pathway (771). AKT2 phosphorylates ASK1 at Ser-83 resulting in inhibition of its kinase activity, and accordingly activated AKT2 blocks signaling downstream of ASK1, including activation of JNK and p38 and the conversion of Bax to its active conformation. Thus AKT2 inhibits cisplatin-induced JNK/p38 and Bax activation through phosphorylation of ASK1 and may play an important role in chemoresistance (771). Furthermore, cisplatin-induced Bax conformation change was inhibited by dominant negative forms of JNK and p38, supporting the important role of these kinases in cisplatin-induced apoptosis.

Although the aforementioned studies suggest that p38 and JNK kinases play critical roles in cisplatin-induced signaling pathways and apoptosis, other studies show that the

third MAPK pathway, extracellular signal-regulated kinases (ERKs), also plays an important role in mediating apoptosis in response to cisplatin treatment. Wang et al. (732) demonstrated that cisplatin induced dose- and time-dependent activation of ERK and that this activation was necessary for cisplatin-induced cell death in HeLa cells. Cisplatin-induced apoptosis was associated with cytochrome c release and subsequent caspase-3 activation in HeLa cells, both of which could be prevented by treatment with MEK inhibitors (MEK is upstream of ERK) (732). Another study showed that suppression of the MEK-ERK transduction pathway by a selective inhibitor, 2'-amino-3'-methoxyflavone (PD98059), increased drug resistance of human cervical carcinoma SiHa cells to cisplatin (764). This study also demonstrated the negative regulation of ERK on NF-κB) activation, and NF-κB activation has been shown to inhibit apoptosis (348,709) (although this transcription factor may be involved in positive regulation of human testicular apoptosis (552)). While cisplatin activated nuclear ERK2 and NF-κB in SiHa cells, suppression of the MEK-ERK2 pathway by PD98059 resulted in a further enhancement of cisplatin-induced NF-κB activation. These results suggest that the MEK-ERK signaling pathway plays a role in the chemosensitivity of SiHa cells, and suppression of this pathway increases cisplatin resistance partly via an increase of NF kappa B activation (764). Although p38 was also activated by cisplatin, p38 did not have an effect on NF kappa B activation in this cell type. Another study showed that cisplatin treatment of mice caused phosphorylation of JNK and ERK kinases but not the phosphorylation of p38 in liver tissue (299). Moreover, Arany et al. (17) showed that the three members of the MAPKs—ERK, JNK, and p38—are all activated in cisplatin-treated mice and in an immortalized mouse proximal tubule cell line; however, only inhibition of ERK abolished caspase-3 activation and apoptotic death. This study also showed that cisplatin-induced ERK and caspase-3 activation were epidermal growth factor receptor (EGFR) and c-src dependent as inhibition of these genes inhibited ERK and caspase-3 activation and attenuated apoptotic death.

While activation of the MAP kinases is required for apoptosis in some cell types, one study showed that the MAP kinases may be involved in promoting cell survival following cisplatin treatment. Hayakawa et al. (274) showed that cisplatin, but not transplatin, activated JNK, p38 and ERK and strongly increased ATF2-dependent transcriptional activity in a breast cancer cell line. Activation of JNK but not p38 kinase or ERK kinase was required for the phosphorylation and transcriptional activation of ATF2, and increased ATF2 activity was associated with cisplatin resistance whereas inhibition of JNK and ATF2

sensitized cells to cisplatin. Moreover, the wild-type ATF2-expressing clones exhibited rapid DNA repair after treatment with cisplatin but not transplatin. Conversely, expression of dominant negative ATF2 quantitatively blocked DNA repair. These results indicate that JNK-dependent phosphorylation of ATF2 plays an important role in the drug resistance phenotype likely by mediating enhanced DNA repair (by a p53-independent mechanism). Based on the results presented here, it is clear that the MAPK signaling cascades play an important role in the cellular response to cisplatin (also see section1.4.4).

DNA-PK -dependent signaling in response to cisplatin damage

As mentioned above, mismatch repair proteins, perhaps along with other proteins in the BASC complex, bind to cisplatin-DNA adducts and mediate pathways leading to apoptosis. Another upstream effector of cisplatin-induced apoptosis that binds to cisplatin-damaged DNA is DNA-dependent protein kinase (DNA-PK). DNA-PK is composed of two DNA-binding Ku polypeptides, which are the units that bind both cisplatin-modified DNA as well as double-strand breaks *in vitro* (695,696), and a serine/threonine kinase catalytic subunit, DNA-PK_{cs}, which is a member of the PI3-kinase related family. DNA-PK is essential in recombination and participates in nonhomologous end joining (NHEJ) and V[D]J (variable [division] joining) recombination (202,203) and may also play a role in regulating NER (92,504). The complex is also proposed to have a role in cell signaling after DNA damage and interestingly DNA-PK, like ATM, phosphorylates and activates c-Abl in response to IR (371). Although cisplatin damage has been shown to inhibit DNA-PK activity on duplex DNA *in vitro* (697,699), recent data indicate that this complex may play an important role in intercellular signaling following cisplatin damage. Jensen and Glazer (331) demonstrated that cells deficient in Ku80 or in DNA-PK_{cs} are significantly more resistant to cisplatin compared with their wild-type counterparts. However this resistant phenotype was only observed when the cells were grown at high density (i.e., 70–100% confluency at time of cisplatin exposure, 30,000 cells per cm^2); at relatively low cell densities (500 cells per cm^2), DNA-PK had no effect on cisplatin sensitivity. Both the kinase activity of DNA-PK and direct cell-to cell contact were required for mediating cisplatin sensitivity. While the presence of $Ku80^{+/+}$ cells in contact with $Ku80^{-/-}$ cells during cisplatin exposure decreased the survival of the $Ku80^{-/-}$ cells, this study indicates that DNA-PK is involved in the transmission of a death signal to neighboring cells, and other experiments on gap junction expression and function revealed that this signal is sent via gap junction intercellular communication (GJIC) following cisplatin treatment. The role of DNA-PK in transmitting

death signals is not a general response to DNA damage, as Jensen and Glazer found no differences between Ku80- and GJIC-proficient and -deficient cells treated with UV, mitomycin C, or 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) at high cell density (331). Furthermore, while the cisplatin resistance of MMR-deficient cells occurred at both low and high cell densities, the authors conclude that the MMR-associated damage response pathway is distinct from the density-dependent, DNA-PK- and GJIC-mediated pathway.

Earlier in vitro work showing that the presence of cisplatin adducts inhibits DNA-PK kinase activity (697,699) is not consistent with the Jensen and Glazer study where DNA-PKcs activity is required to transmit the death signal to neighboring cells. Furthermore, DNA-PK mutants were previously shown to be more sensitive to cisplatin (3-4-fold) compared to the wild-type cell line (504) and acquired cisplatin resistance and associated IR-cross resistance was shown to be due to overexpression of the Ku80 subunit resulting in increased Ku-binding activity in resistant cells (227). The discrepancies between the aforementioned studies may be due to cell type-specific effects and/or culture and treatment conditions. However it is of interest to note that in addition to its role in DNA binding, Ku heterodimer is also expressed at the cell surface and may play a direct role in cell-cell adhesion (505). In addition, one study on the cisplatin-sensitive ovarian cell line A2780 and two resistant subclones revealed that the sensitive cell line showed a 20-30% decrease in DNA-PK phosphorylation activity when cells underwent apoptosis whereas the resistant clones displayed 80-90% decrease in activity, with decreased kinase activity associated with proteolytic degradation of DNA-PKcs (280). Therefore the role of Ku and DNA-PK in mediating cisplatin sensitivity requires further study.

Repair shielding and transcription factor hijacking revisited

Proteins involved in DNA repair and the DNA damage signaling network that bind cisplatin-DNA adducts may be inhibited by other adduct-binding proteins that block access to the adduct. For example, in vitro purified replication protein A (RPA), a protein involved DNA damage recognition in NER, binds a cisplatin-modified substrate; however addition of HMGB1 inhibits the cisplatin-DNA binding activity of RPA (548) and may inhibit both repair and regulatory/signaling activities of RPA. Furthermore, while testicular cancer cells are naturally nucleotide excision repair (NER) deficient (380,381,739), repair shielding by HMG-box protein binding to cisplatin adducts may make these cells especially sensitive to the drug. It is of interest to note that in addition to repair shielding by blocking access to the

adducts and to the hijacking of transcription factors of essential growth genes, HMGB1 binding to cisplatin adducts may also interfere directly with the transcription of genes involved in cell cycle regulation, DNA repair, and apoptosis. HMGB1 interacts *in vitro* with p53 and p73 and has been shown to be capable of either enhancing or inhibiting the p73- and p53-dependent transactivation of p53-responsive promoters, including *Bax*, *Mdm2*, and *p21* gene promoters (329,668). Whether HMGB1 stimulated or suppressed transcriptional activity was dependent on the cell type studied. Thus HMGB1 binding to cisplatin-DNA adducts may also contribute to cisplatin sensitivity via p53- and p73-dependent mechanisms as HMGB1 interactions with both p53 and p73 influence the transactivation activity of the latter proteins. However while the interaction of HMGB1 with p53 and p73 may lead to either induction or suppression of apoptotic pathways, it is not clear whether transcription factor hijacking of HMGB1 by cisplatin adducts would contribute to increased or decreased sensitivity to the drug by this mechanism, and more work is necessary to determine if hijacking of HMGB1 alters p53 and/or p73-dependent transcription. The role of HMG-box proteins in repair shielding and hijacking were discussed previously (Sections 1.3.1 and 1.3.3)

1.3.5 Cisplatin-induced mutagenesis

Polymerase beta

As mentioned above, cisplatin adducts are effective inhibitors of DNA polymerase progression; the replicative enzymes pol α, pol δ, and pol ε have been shown to be incapable of replicating past a cisplatin adduct *in vitro* (288,717). However cells have evolved error-prone polymerases than can bypass damage, albeit with low fidelity, in order to cope with and tolerate DNA damage. The cost of this tolerance is the generation of mutations, as these polymerases lack proof-reading activity and can incorporate incorrect nucleotides opposite the damaged template. Both *E. coli* and eukaryotes have different bypass polymerases capable of synthesis past specific lesions (reviewed in Goodman (252)). For example, human DNA polymerase β can bypass cisplatin adducts efficiently and can elongate the product of a stalled polymerase at the site of a cisplatin adduct *in vitro* (288,704). However bypass of cisplatin-adducts by pol β results in mutations, with dTTP being incorporated opposite the 3' platinated guanine residue in the 1,2-d(GpG) intrastrand crosslink at 15-25-fold greater frequency than on an undamaged template (704). Experiments *in vivo* support the *in vitro* data by showing that overexpression of pol β may

contribute to increased spontaneous as well as cisplatin-induced mutagenesis (93,94), the latter which may result from pol β gap filling of excision repair patches (95,534). The effects of pol β, however, have not been tested *in vivo* at normal expression levels.

Polymerase eta

Another DNA polymerase with the ability to perform translesion synthesis of a cisplatin-damaged substrate is pol η. Pol η is a member of the Y family of bypass polymerases that can temporarily replace the processive replicative polymerase at the site of the adduct and perform efficient translesion synthesis passed the adduct (566). Pol η is encoded by the yeast *Rad30* and the human *XPV* (also called *POLH*) genes and is important in tolerating UV-induced DNA damage (456). Pol η, like pol β, is capable of bypassing cisplatin-DNA adducts *in vitro* (455,707). However pol η is ~150 times more error prone than pol β on an undamaged template, and misincorporation of dTTP opposite the 3' guanine in the 1,2-d(GpG) intrastrand crosslink is 5-10 times greater for pol η than for pol β (31,704,707). Because error-prone bypass of some adducts may require two polymerases—one polymerase for dNTP misinsertion and a second polymerase for extension of the mismatched primer termini—Bassett et al. (31) determined the efficiency of extension for both pol η and pol β of mismatched primer termini opposite the 1,2-d(GpG) adduct. This study found that for pol η, the efficiency of extension of a 3' G:T mismatch for the next two steps is 2.1-2.3 × 10^{-3} on platinum-damaged DNA relative to extension of fully complementary primers on undamaged DNA, while the efficiency of extension of the adduct-containing 5'G:T mismatch is 0.7-1.1 × 10^{-2}. The efficiency of extension of the 3' G:T mismatch for pol β was found to be 1.3-7.2 × 10^{-4} on platinum-damaged DNA. The authors estimate that the overall probability of damage-induced mutations is higher for pol η-catalyzed translesion synthesis (1.8-2.5 × 10^{-4}) than for pol β-catalyzed translesion synthesis (1-10 × 10^{-6}) but concede that an overall error rate of 10^{-4} may be sufficient for accurate bypass of the lesion since a translesion polymerase is only required to insert two nucleotides opposite the adduct and perhaps extend those nucleotides by a single base (31). In addition to base mutations, pol η is more error-prone than pol β with respect to other polymerase errors, namely frameshifts, and has been shown to generate deletions 18-fold more frequently than pol β when replicating an undamaged DNA template (457). Pol η was shown to catalyze -1 frameshift deletions when replicating past the 1,2-d(GpG) adduct *in vitro* with at least 2-fold higher frequency than pol β (32). Taken together, the data show

that both polymerases β and η may play a role in cisplatin-induced mutagenesis, though pol η may be more mutagenic.

Polymerase mu

It has recently been demonstrated that pol μ can efficiently perform translesion synthesis of damaged templates including 8-oxoguanine, an abasic site, 1,N(6)-ethenoadenine, and a cis-syn thymine-thymine dimer (787). Pol μ is a member of the X family of polymerases, which also includes pol β and two recently identified polymerases λ and σ (87). Pol μ has been implicated in nonhomologous end-joining DSB repair (433,788) and was shown to generate a high frequency of -1 deletion products from undamaged DNA templates (788) and -1 and -2 deletion products from damaged templates (787). Pol μ was shown to bypass the 1,2-d(GpG) adduct with an efficiency of 14-35% compared to undamaged DNA, which makes pol μ less efficient than pol η but more efficient than pol β at performing translesion synthesis of cisplatin-adducts under similar reaction conditions (271). Similar to pol η and β, pol μ also displayed misincorporation of dTTP opposite the platinated guanines. Interestingly, pol μ performs better on gapped duplex templates than on primed single-stranded templates, and when tested on gapped DNA templates, bypass of the 1,2-d(GpG) adduct was highly error-prone and resulted in 2, 3, and 4 nucleotide deletions (271). Therefore, in addition to pol β and pol η, pol μ represents another DNA polymerase that may contribute to cisplatin-induced mutagenesis. It is important to note, however, that the above studies were all performed *in vitro*, and the relative contributions of these polymerases to cisplatin-induced mutagenesis *in vivo* have not yet been reported. The DNA polymerases pol κ, which is the human homologue of *E. coli dinB* (pol IV) (242), pol ι, encoded by *Rad30B*, and pol *λ* are all unable to bypass cisplatin-DNA adducts *in vitro* (432,463,527,705).

Replicative bypass and cisplatin-induced mutations in cells

The mutagenetic effects of cisplatin have been extensively characterized in *E. coli*. Cisplatin-induced mutations in *E. coli* require SOS induction and are therefore most likely dependent on the SOS-inducible bypass polymerases Pol II, Pol IV, and Pol V, encoded by the SOS-inducible genes *polB*, *dinB*, and *umuDC*, respectively. Cisplatin induces a 1-6% mutation frequency in SOS-induced cells (89,761). Most of the mutations (>80-90%) are targeted to the 5'-platinated base of the major d(GpG) and d(ApG) adducts (66,89,761), although no sequence-dependent mutational hotspots were detected in the M13mp18 *lacZ'*

gene fragment (762). The mutational spectra reported following cisplatin treatment in SOS induced *E. coli* show that the majority of mutations are G→T (66,761,762) and A→T (89,761) tranversions for the d(GpG) and d(ApG) adducts, respectively, while G→A and A→G transitions were also detected (761,762). Low levels (~10%) of tandem base-pair substitutions occurring at the 5' modified base and the adjacent 5' base were also reported in SOS-induced *E. coli* (89,761). Although the d(GpG) adduct is the most abundant adduct in cisplatin-treated DNA, several studies have shown that the d(ApG) is more mutagenic than the d(GpG) adduct. Yarema et al. showed that the d(ApG) adduct, with a mutation frequency of 6%, is 4-5 fold more mutagenic than the d(GpG) adduct, which displays a mutation frequency of 1.4% in the same experimental system using ssDNA (761). Other studies using adducts in duplex DNA reported lower mutation frequencies, with 0.2 and 1-2% for the d(GpG) and d(ApG) adducts, respectively (66,89). Interestingly, transplatin was shown to be an ineffective mutagen in *E. coli* (34).

Cisplatin-induced mutations have also been reported in Chinese hamster ovary cells, where platinum drugs cause 15-23% deletion mutations as well as base substitutions in the *Aprt* gene (155,156), and in the *SUP4-o* gene of *S. cerevisiae*, where all possible types of base pair substitutions as well as deletions, insertions and double mutations were identified (479). These varied types of mutations may result from other mechanisms of mutagenesis in addition to the error-prone bypass described above. For example, cisplatin-induced strand breaks could result in double-strand break misrejoining and non-homologous repair events leading to large deletions. Indeed, cisplatin resistant ovarian cell lines displayed increased DSB misrejoining activity and deletions of between 134 and 444 base pairs that arose through illegitimate recombination at short repetitive sequences (73). Furthermore, defects in DNA repair, which lead to hypersensitivity to cisplatin, can also lead to increased cisplatin-induced mutations; when a cisplatin-damaged plasmid was transfected into XPC cells, which are defective in the XPC gene, and normal human fibroblasts, an increased mutation frequency was observed in the XPC cells, with most of the mutations consisting of large deletions (106). This result suggests that less repair and the persistence of cisplatin-DNA adducts can lead to a higher mutation frequency, which is not surprising as DNA error-prone polymerases will then have more opportunities to perform translesion synthesis and as the misrepair of secondary damage (i.e., strand breaks) may also result in deletion events. On the flip side, the presence of other cisplatin adduct binding proteins such as HMG-box proteins, may prevent bypass and mutation fixation. For example, HMGB1

binding to cisplatin adducts was shown to inhibit translesion bypass (287,706), which may prevent mutations at the cost of eliciting toxicity. Consistent with the notion that adduct-binding proteins may inhibit bypass and prevent mutation fixation, defects in hMLH1 or hMSH6 (the MMR proteins that bind cisplatin-DNA adducts) were shown to result 2.5-6-fold increased replicative bypass of cisplatin adducts (708), although this observation may result from decreased attempts at mismatch correction as well as from an increased ability to bypass adducts in the absence of MMR adduct-binding proteins. Cisplatin-induced mutagenesis may also be enhanced by the activity of DNA repair proteins. For example in *E. coli*, cisplatin-induced mutagenesis as measured by rifampicin resistance is suppressed in cells that are deficient in the mismatch DNA glycosylase MutY, with *mutY* mutant cells displaying a mutation frequency 25-fold lower than the mutation frequency observed in wild-type cells (Kartalou et al., unpublished results). While the major 1,2-d(GpG) adduct induces mainly SOS-dependent G to T transversions at the 5'-platinated G (66,761,762), both the bacterial and human MutY proteins bind to the major intrastrand crosslink when an A is opposite the 5'-platinated G, and both proteins displayed catalytic activity and introduced incisions on the compound lesion substrate *in vitro* (Kartalou et al., unpublished results). Therefore processing of cisplatin adducts by certain DNA repair proteins designed to protect the genome against mutations may in fact enhance cisplatin-induced mutagenesis.

1.3.6 Other consequences of cisplatin damage

In addition to inhibition of replication and transcription, and the induction of DNA damage signaling and mutagenesis, cisplatin-DNA adducts affect other DNA metabolic processes. For example, telomeres carry potential cisplatin reaction sites in their tandem G-rich repeats (in humans, 5′-TTAGGG-3′ (501)). Telomeres play important roles in protecting chromosome ends from DNA degradation and rearrangements, and telomere length, which shortens with every cell division until it reaches a critical length, is a determinant for the number of divisions a cell undergoes before it reaches senescence and dies. One mechanism for the stabilization of telomere length is the synthesis of the tandem repeats by the ribonucleoprotein telomerase (53,205), and thus telomerase activity may be important for the tumorigenic potential (and immortalization) of cells (255). Telomere length was shown to be degraded in HeLa cells following cisplatin treatment, including with low doses of drug (320). In addition to reacting with telomeric DNA and possibly causing its degradation, cisplatin may also bind to the RNA and/or protein regions of telomerase, and cisplatin, but

not transplatin, was shown to inhibit telomerase activity in testicular cancer cells (83). Thus while cell proliferation depends on telomere length, the effects of cisplatin on telomere maintenance may contribute to drug toxicity.

Cisplatin may also alter chromatin structure by affecting interactions between DNA and nucleosomal proteins including histones, the structural proteins around which DNA is wrapped to form condensed chromatin. Cisplatin treatment may induce crosslinking between nucleosomal proteins and between nucleosomal proteins and DNA (25,210,276,416). Furthermore, an intrastrand crosslink in DNA may itself affect chromatin structure as the 1,3-d(GpTpG) intrastrand crosslink of *cis*-$\{Pt(NH_3)_2\}^{2+}$ alters the translational positioning of DNA and forces opposite rotational settings around the histone octamer core, resulting in asymmetric arrangement of DNA with respect to the core histones (149). As mentioned previously, the linker histone H1 strongly binds cisplatin-modified DNA over transplatin-modified or undamaged DNA *in vitro* (758), and thus interactions between histone proteins and cisplatin-DNA crosslinks may also affect chromatin organization. Studies have also shown that cisplatin treatment affects the post-translational modification of histones. Cisplatin induces phosphorylation of histone H3 at Ser-10 by p38 kinase as well as the hyperacetylation of histone H4 (727). Some of these modifications may be mediated by effectors of apoptosis and may be important for the DNA fragmentation associated with programmed cell death. For example, as mentioned in Section 1.3.1, cisplatin induces poly(ADP-ribosyl)ation of nuclear proteins, and one protein shown to undergo significant poly(ADP-ribosyl)ation is histone H1 (530,602); it has been shown that the poly(ADP-ribosyl)ation of histone H1 correlates with internucleosomal DNA fragmentation during apoptosis (765). .

1.4 Cellular mechanisms of resistance to cisplatin

One of the major limitations of cisplatin chemotherapy is acquired or intrinsic resistance. As mentioned in Section 1.1, certain cancers are inherently resistant to the effects of cisplatin damage, while other cancers acquire drug resistance after an initial response. This acquired resistance may result from the selection of cells during initial drug treatment that carry or develop mechanisms of overcoming or tolerating cisplatin-induced damage; cancers that exhibit intrinsic resistance may already possess such mechanisms. There are several known mechanisms of cellular resistance to cisplatin, which include

reduced intracellular accumulation of cisplatin owing to either decreased uptake or increased efflux of the drug, detoxification of cisplatin by thiol-containing biological molecules, increased tolerance of cisplatin adducts, and enhanced repair of cisplatin-induced genomic damage by DNA repair systems. Cisplatin sensitivity and resistance are also determined by the balance of pro- and anti-apoptotic signals (517,728). Although resistance to cisplatin is multi-factorial in the sense that multiple mechanisms contributing to resistance may be operative within the same tumor (228,458,638), many studies have tried to determine key cellular players in cisplatin resistance in efforts to modulate these resistance mechanisms or in the pursuit of developing new drugs that overcome these mechanisms (see Section 1.5).

1.4.1 Reduced intracellular accumulation of cisplatin

Reduced uptake or increased export of cisplatin may reduce the intracellular concentration of the drug, and intracellular levels of cisplatin have been shown to correlate with cisplatin sensitivity in ovarian cancer cell lines (410,423,482,545) and a human hepatoma cell line (339). Although the mechanism of cisplatin entry into cells is not fully known, recent studies suggest a role for copper transporters in cisplatin transport. The copper transporter Ctr1, for example, was shown to affect intracellular levels of cisplatin in yeast and mouse cells while deletion of Ctr1 results in reduced intracellular accumulation of cisplatin and decreases cellular sensitivity to the drug (321). Furthermore, increased expression of Ctr1 in human ovarian cancer cells leads to increased intracellular accumulation of cisplatin (297). In addition to Ctr1, the copper exporters ATP7A and ATP7B may also modulate sensitivity to cisplatin. These copper exporters exhibit a negative association with cisplatin sensitivity, with increased expression correlating with decreased sensitivity to drug treatment (360,604). Recent data suggests that ATP7A may contribute to cisplatin resistance by sequestering the drug into vesicles (610), thus making potential cellular targets such as DNA inaccessible to the drug. Studies have also shown that cell lines exhibiting resistance to copper also show cross-resistance to cisplatin and visa versa (360,605).

Other membrane proteins may also affect cisplatin uptake and/or efflux. For example, a 48kDa membrane protein is expressed at lower levels in drug resistant human squamous carcinomas cell lines that also exhibit decreased intracellular accumulation of the drug, implicating this protein in cisplatin uptake (43). Conversely, cisplatin resistant murine

lymphoma cells that showed reduced drug accumulation were shown to overexpress a 200 kDa plasma membrane glycoprotein, different from the multidrug resistance-associated P-glycoprotein (170 kDa), suggesting that this larger protein may be involved in cisplatin export (362). In addition to membrane proteins, some evidence suggests that cisplatin accumulation may depend on membrane potential; although cisplatin is not transported by the sodium-potassium pump, the sodium-potassium ATPase inhibitor ouabain inhibits drug uptake (16). Furthermore, because cisplatin uptake cannot be inhibited by structural analogues and cannot be saturated, it has been suggested that drug entry into cells most likely occurs to some extent by passive diffusion (237,306,436). Therefore, although the primary mechanisms of cisplatin uptake and efflux are not known, multiple factors affect the intracellular accumulation of cisplatin and may accordingly mediate sensitivity to the drug.

1.4.2 Inactivation of cisplatin

In addition to reduced uptake or increased efflux, the effective intracellular concentration of cisplatin may also be modulated by cellular proteins that react with and inactivate the drug. Once cisplatin enters cells and undergoes hydrolysis, it can covalently react with thiol-containing compounds, which essentially chelate/quench the drug and reduce the effective intracellular concentration of drug that can react with DNA. The most abundant cellular thiol is the tripeptide glutathione (γ-glutamylcysteinylglycine, GSH), which is present at concentrations 0.5-10 mM inside cells (112). GSH is synthesized in a two-step pathway involving the enzymes γ-glutamylcysteine synthetase (also known as glutamate cysteine ligase), which completes the first and rate-limiting step of peptide bond formation between cysteine and glutamic acid to form γ-glutamylcysteine (γ-GC), and glutathione synthetase, which couples γ-GC with glycine and completes the second step of tripeptide synthesis. The enzyme γ-glutamylcysteine synthetase can be inhibited by D,L-buthionine-*S*,*R*,-sulfoximine (BSO), and this feature of enzyme inhibition has been exploited to determine the effects of limited GSH on cisplatin sensitivity. For example, a cisplatin-resistant murine leukemia L1210 subline has elevated GSH levels compared to its sensitive parent cell line and exposure to BSO reduces GSH levels in the resistant subline to nearly the levels observed in the parent line and abrogates resistance to cisplatin (305). GSH may contribute to cisplatin resistance by facilitating the export of cisplatin once reacting with and inactivating the drug (322). In addition to reducing the intracellular concentration of drug, GSH can react with cisplatin monoadducts in DNA, preventing formation of the crosslink and

thereby reducing the cytotoxic potential of the drug (181). Whether GSH facilitates the export of cisplatin or prevents formation of cytotoxic crosslinks, increased GSH levels have been associated with cisplatin resistance. In studies of ovarian cancer cell lines, GSH levels were shown to be increased in resistant cells (35,248,746,747), with one study showing that a selection for human ovarian drug-resistant cells *in vitro* led to the development of cell lines that showed 30- to 1000-fold increased resistance as well as 13-15-fold increased levels of glutathione as compared with the drug-sensitive parental cells (248). A different study measured intracellular glutathione concentration in human ovarian cell lines and showed that IC50 values for cisplatin and carboplatin correlated with GSH levels (481); however BSO had differential effects on the cell lines, with cellular GSH depletion by BSO increasing cisplatin cytotoxicity in only one of two resistant cell lines. Another study using *in vitro* selection of cisplatin-resistant sublines of human ovarian carcinoma cells showed that GSH levels were only elevated in cells exhibiting high levels of resistance (9-fold and 13-fold) and not in cells that displayed low levels of resistance (2-3-fold) (15). The resistance of both low- and high-resistant sublines, however, could be partially reversed by extended depletion of GSH by BSO treatment. Despite the aforementioned correlative results on GSH levels and cisplatin resistance, other studies have revealed that increased GSH levels is not a consistent characteristic of cisplatin resistant cells. One study showed that GSH levels did not differ between a sensitive human testicular tumor cell line and an *in vitro* derived 5.6-fold drug-resistant subline (364). Another more recent study determined the correlations between intracellular GSH content and drug sensitivity in 14 human cancer cell lines (from non-treated patients) and showed that there was no correlation between GSH content and the growth inhibitory effects of cisplatin and its analogues carboplatin and oxaliplatin (59). Therefore while intracellular glutathione can play a major role in determining cisplatin sensitivity, other factors are clearly involved.

Metallothionein (MT) represents another thiol that may play a role in cisplatin resistance. While one-third of MT's amino acid residues are cysteines that can bind metal ions, this thiol compound plays an important role in the detoxification of heavy metal ions in cells and accordingly confers resistance to cadmium (376). MT reacts with cisplatin at a ratio of 10 +/- 2 Pt(II) per mol of protein (550) and can therefore have a major impact on the concentration of cisplatin available to react with DNA. Human ovarian carcinoma cells that are 3-4-fold resistant to cisplatin showed 9-23-fold elevated levels of MTs (14). However these resistant cells were selected *in vitro* by chronic exposure to $CdCl_2$ and $ZnCl_2$, and

selection with cisplatin did not generate cell lines with elevated MT levels. In another study, tumor cell lines with acquired resistance to cisplatin overexpressed MT, and human carcinoma cells that were chronically exposed to heavy metals maintained high levels of MT and displayed resistance to cisplatin, and cisplatin resistance could be conferred by expression of a vector encoding human metallothionein-IIA (367). MT-conferred drug-resistance is also supported by a recent study that examined the effect of MT on cisplatin resistance in C3H mice inoculated with MBT-2 murine bladder tumor cells. This study showed that reduced MT levels in tumors increased the levels of cisplatin accumulation and the antitumor activity of cisplatin in the tumors (606). Furthermore, overexpression of MT in the ovarian cancer cell line A2780 by stable gene transfection resulted in a protection from the growth-inhibitory effects of cisplatin (7-fold) (289), and a moderate increase in MT content was observed in an *in vitro* derived resistant (5.6-fold) human testicular tumor cell line (364). However as with GSH, higher levels of MT do not always correlate with increased resistance to cisplatin. One study showed that cisplatin resistant human ovarian carcinoma cell lines displayed varying levels of cross-resistance to cadmium and varied expression of MT; furthermore, cisplatin resistance could not be conferred by expression of a metallothionein gene construct in mouse C127 cells, and thus no causal relationship between MT expression and cisplatin resistance could be established (621). In addition, MT content was not a major determinant of tumor sensitivity in a study of ovarian tumor samples from untreated patients and from patients who had undergone chemotherapy (506). Thus although the intracellular levels of thiols such as GSH and MT do not universally correlate with cisplatin sensitivity, they can play a role in cellular sensitivity to cisplatin. Indeed, both GSH and MT may be useful as predictive indicators for clinical response to cisplatin-based chemotherapy in ovarian cancer patients (677).

1.4.3 Repair and bypass of cisplatin-DNA adducts

If cisplatin is not inactivated by thiols, it can react with DNA to form cytotoxic lesions. These lesions can induce apoptosis, as discussed above, if they are not removed by DNA repair proteins, and accordingly DNA repair is a critical determinant of sensitivity to cisplatin. Numerous studies have demonstrated increased removal of cisplatin adducts in cisplatin-resistant ovarian cancer cell lines (35,208,338,340,396,453,454,545,790,790). In addition, Ferry et al. showed that certain NER genes, specifically ERCC1, XPB, XPC, and XPF, are over-expressed in an *in vitro* selected human resistant ovarian cancer cell line. Cisplatin resistance in this subline and other resistant sublines was associated with cross-resistance

to UV as well as increased repair activity (208). Over-expression of the NER genes XPA and XPE, in addition to ERCC1, and associated cisplatin resistance have been shown in other studies of ovarian and other tumor cell lines (75,114,146,147,596,663). While repair capacity is a determinant of cisplatin sensitivity, and while biopsies from untreated ovarian carcinomas reveal as much as a 10-fold difference in the repair efficiency of cisplatin-modified DNA (344), repair capacity may prove useful as predictive marker of clinical response to platinum-based therapy. In support of this idea, patients who were clinically resistant to platinum-based therapy had a 2.6-fold higher expression level of ERCC1 in their tumor tissue (harvested before treatment began) than did patients who responded to that therapy (145). Another study determined the mRNA levels of XPA and ERCC1 in malignant ovarian cancer tissues from patients before administration of platinum-based chemotherapy and correlated the expression level of these NER genes with clinical outcome; this study revealed that greater levels of ERCC1 and XPA mRNA were found in tissues from patients whose tumors were clinically resistant to therapy as compared with tumor tissues from those individuals clinically sensitive to therapy (147). Although alternative splicing of mRNA may inhibit the function of full length gene products, this study also showed that alternative splicing of ERCC1 among the samples was highly variable, with no difference observed between responders and non-responders. However another study showed an inverse correlation between alternative splicing of ERCC1 in a series of human cell lines and tissues and cellular capability to repair cisplatin-modified DNA (767). It is clear from these studies, however, that another mechanism for cisplatin resistance is increased DNA repair capacity, as high expression levels of repair proteins and higher repair capabilities confer resistance to cisplatin and correspond to poor response to cisplatin therapy. Conversely, a deficiency in repair leads to hypersensitivity to cisplatin (168,403,569) and accordingly testicular cancer cells, which are inherently NER deficient (380,381,739), are especially sensitive to cisplatin.

Studies have also shown that cisplatin-resistant cells exhibit increased repair of coding regions of the genome. Repair of damage in the coding strands of actively transcribed genes is accomplished by a subpathway of NER called transcription-coupled repair (TCR) (reviewed in Hanawalt, (265)). Several studies have shown that cisplatin adducts are removed more efficiently from specific genes—namely dihydrofolate reductase (DHFR), ribosomal RNA (rRNA), multidrug resistance (MDR1), c-myc, and delta-globin genes—compared to non-active genomic regions, although at high levels of damage the differential repair between active and non-coding regions is eliminated

(342,399,580,789,790). One study demonstrated increased gene-specific repair in resistant ovarian cancer cell lines compared to the sensitive parental cells, although this increased repair was limited to interstrand crosslinks and no differential repair of the intrastrand adducts was observed (790).

As with other mechanisms of drug resistance, increased repair is not a universal factor in drug resistance and is not found in all cisplatin-resistant models. Although the case was made above that repair capacity could be a prognostic indicator of clinical response to platinum chemotherapy, one study showed that the DNA repair capacity of protein extracts from different tissues varied significantly but did not directly correlate with the organotrophic toxicity profile of cisplatin (343). In addition, a study of three pairs of cisplatin-sensitive and -resistant cell lines, two derived from TGCT and one from bladder cancer, found no correlation between DNA repair capacity and cisplatin resistance as well as no major differences between the repair of cisplatin damage in transcribed and non-transcribed genes (382). Furthermore, in a study of twelve unrelated human ovarian cancer cell lines derived from patients who were either untreated or treated with platinum-based chemotherapy, only platinum-DNA tolerance correlated strongly with cisplatin sensitivity; intracellular platinum accumulation, glutathione levels, DNA-adduct formation, and DNA-adduct removal did not correlate significantly (337). Adduct tolerance may be achieved by increased bypass of cisplatin-DNA adducts. As discussed above, DNA replicative polymerases stall at cisplatin adducts. The arrested replication complex requires the aid of error-prone translesion polymerases than can synthesize DNA past the damaged template in order to avoid potentially severe consequences such as replication fork collapse and strand-break formation, and such polymerases capable of translesion synthesis past cisplatin adducts include polymerases β, η, and μ (see Section 1.3.5). Therefore increased bypass tolerance of cisplatin-damaged DNA may be a possible mechanism for cisplatin resistance. Overexpression of pol β in CHO cells, for example, was shown to confer resistance to cisplatin (93), and pol β gene amplification has been detected in tumors that are refractory to cisplatin therapy (354,618) and human colon carcinoma cells selected for cisplatin resistance exhibited a 4-5-fold increase in pol β activity (388). In addition, mouse 3T3 cells expressing pol β antisense RNA showed increased sensitivity to cisplatin (300), and down-regulation of pol β by siRNA resulted in increased sensitivity to cisplatin in both HeLa and the relatively resistant ovarian cancer SKOV-3 cell lines (7); however pol β null and siRNA

knock-down mouse embryonic fibroblast cell lines did not exhibit increased sensitivity to cisplatin (525,570,655). Because pol β is involved in repair patch synthesis in BER (222,655), increased expression of pol β may be associated with increased repair rather than increased translesion bypass. Interestingly, extracts from cells overexpressing DNA polymerase β demonstrated a five- to six-fold increase of repair synthesis involving pol β on cisplatin-modified templates compared with control extracts (95). Although dRP lyase activity of pol β was shown to be the rate-determining step in the multi-step BER pathway in vitro (661), it has not been shown that overexpression of pol β alone leads to increased repair *in vivo*. Furthermore, decreased expression of pol β leads to increased sensitivity to cisplatin but not to the nucleic acid antagonist 5-fluorouracil (5-FU) (570); 5-FU, when incorporated into DNA, is a substrate for the base excision repair enzyme, uracil glycosylase, whereas cisplatin has not been shown to be a substrate for glycosylase incision. Therefore the effects of pol β, the major BER polymerase, on cisplatin sensitivity is most likely related to translesion bypass by pol β rather than to excision repair pathways involving pol β. In support of polymerase bypass contributing to cisplatin resistance, yeast strains deficient in polymerase ζ, which has been shown to be capable of bypassing UV thymine-thymine dimmers (401,514), are hypersensitive to cisplatin (750). In addition, platinum-resistant murine leukemia cell lines show a 3-4-fold increase in bypass of platinum-DNA adducts (243) and cisplatin-resistant human ovarian cancer cell lines displayed a 2.3-4.8-fold increase in bypass ability as measured by chain elongation compared to the cisplatin-sensitive parent cell lines (434). The importance of replicative bypass in cisplatin resistance is also demonstrated in a study showing that hMLH1 or hMSH6 defects result in 1.5-4.8-fold increased cisplatin resistance and 2.5-6-fold increased replicative bypass of cisplatin adducts (708).

While tolerance of cisplatin damage by translesion synthesis represents one mechanism of bypass, cells can also tolerate adducts by recombination-dependent bypass. A replication complex arrested at the site of an adduct will collapse, leading to a single-strand gap, if it is not aided by bypass mechanisms such as translesion synthesis. If unrepaired this single gap could result in a more detrimental DSB when the cell undergoes another cycle of replication. This gap, however, can also be "bypassed" and repaired by recombination-dependent pathways of replication fork recovery. The process of fork regression at DNA damage sites in *E. coli* is thought not only to prevent replication fork

degradation, but also to allow other repair mechanisms access to the replication-blocking damage (134). Thus increased strand break repair or recombinational bypass may contribute to cisplatin resistance (refer to Fig. 2.1 in Chapter 2 for strand-break repair pathways in *E. coli*). If the replication fork is not recovered by recombination-dependent pathways, a DSB can occur (B in Fig. 2.1). Thus lethal secondary cisplatin-induced damage requires repair by other DNA repair pathways such as recombination and DSB end-joining pathways. In *E. coli*, DSB repair is accomplished by the RecBCD pathway of recombination repair whereas in mammalian cells, DSB repair requires the Rad family of proteins and may occur by either homologous recombinational repair or by non-homologous end joining (discussed in detail in Chapter 2). *S. cerevisiae* deficient in RAD51 and RAD52 are more sensitive to cisplatin compared to wild-type cells (259). In addition, overexpression of the Rad51 paralog XRCC3 in MCF-7 cells leads to a 2-6-fold increase in cisplatin resistance. While in mammalian cells break repair pathways may be either conservative and relatively error-free or non-conservative, resulting in genomic instability, the use of non-conservative (i.e., low fidelity) pathways may increase sensitivity to the original genotoxic insult; and consequently the use of conservative over non-conservative pathways may contribute to resistance. The importance of DSB repair in determining cisplatin sensitivity is demonstrated by studies showing that the induction of cisplatin resistance is frequently, though not invariably, associated with cross-radioresistance in human cancer cell lines (35,286,428,700), and DSBs are a predominant form of lethal damage induced by irradiation (508). Furthermore, Britten et al. (74) showed that radiation-sensitive and radiation-resistant tumor cells exhibit a differential level of DSB misrejoining activity, with high DSB misrejoining in radiosensitive cells being a result of an increase in non-conservative DSB rejoining activity (perhaps via illegitimate recombination) that results in losses of between 40 and 440 base pairs. A decrease in the fidelity of DSB rejoining may also play a role in cisplatin sensitivity, and in support of this idea, Britten et al. (73) found a significantly lower level of DSB misrejoining activity within nuclear protein extracts from cisplatin-resistant (and radio-resistant) ovarian tumor cell lines than in the nuclear extracts from the cisplatin-sensitive (and radio-sensitive) parental cell lines, with acquired cisplatin resistance associated with a 2-3 fold decrease in the activity of non-conservative DSB rejoining. The mis-rejoining events detected in this study generated deletions between 134 and 444 base pairs, all of which involved recombination at distant short sequence repeats (i.e., micro-homologies).

1.4.4 Regulatory proteins and cellular determinants of apoptosis

p53-mediated apoptosis

Adduct tolerance may result from increased bypass, as described above, or from a defect in damage processing and/or a failure to activate cell death pathways. Such defects may arise from alterations in the expression or function of tumor suppressor genes and oncogenes. The tumor suppressor gene *p53*, for example, plays an important role in delaying cell cycle progression in response DNA damage, thus allowing time for repair; if the level of genomic damage is beyond the repair capacity of the cell or is irreparable, p53 may also initiate apoptosis (reviewed in (562,719)). P53 elicits these effects by acting as a transcription factor for genes involved in DNA damage response signaling including $p21^{CIP1/WAF1}$ (188), which encodes the cyclin-dependent kinase inhibitor p21 with anti-proliferative and anti-apoptotic properties (236,719), *gadd45* (358), which plays a role in the G2 checkpoint response by inhibiting the Cdc2/Cyclin B1 complex (735,783), *Mdm2* (27), which encodes an antagonist of p53 that complexes with p53 to inhibit p53-mediated transactivation (102,493) and to target p53 for ubiquitin degradation (270,298,392), and *Bax* (488), which encodes a Bcl2-related protein that serves as a pro-apoptotic factor by facilitating the release of AIF and cytochrome *c* from the mitochondria, thereby activating the caspase cascade (647). Thus inactive p53 may result in defective cell cycle arrest and DNA repair or failure to activate apoptosis, and likewise alterations in the signaling events upstream in the p53 pathway (e.g., the PI3K/AKT signaling cascade) may also contribute to drug resistance.

Human lymphoma and ovarian carcinoma cell lines expressing mutant *p53* are more resistant to cisplatin compared to cell lines expressing wildtype *p53* and show reduced activation of cell cycle arrest and pro-apoptotic signals (189,196,554). Furthermore, introduction of wildtype p53 in resistant human ovarian and lung cancer cells expressing the mutant protein can sensitize these cells to drug, both *in vitro* (230,657) and in mouse xenograft models (229,375,634,656). Mutations in *p53* have also been found in a proportion of testicular germ cell (302) and ovarian tumor samples (375,615) that failed to respond to cisplatin-based therapy, suggesting that defective *p53* contributes to cisplatin resistance *in vivo* and that treatment may select for resistant *p53* mutant cells. One study showed that in addition to a slightly higher level of mutated *p53* transcripts, a resistant human ovarian carcinoma cell line also displayed higher levels of Mdm2 protein, and thus this study demonstrated two possible mechanisms of p53 inactivation in resistant cells (194).

Furthermore, an *in vitro* study comparing various human cell lines of the National Cancer Institute drug screen, 18 of which express wild-type p53 and 39 of which harbor p53 mutations, the mutant cell lines were significantly more resistant to cisplatin, bleomycin, and 5-fluorouracil than the wild-type cell lines (523). Similarly, gene expression profiling of the NCI's 60 cell lines demonstrated that wild-type p53 positively correlates with sensitivity to cisplatin (712).

As with the other modes of drug resistance, mutated and/or inactivated *p53* is neither necessary nor sufficient for a resistant phenotype. Studies measuring drug-induced apoptosis levels and *p53* status in cell lines derived from human ovarian and testicular cancers—the cancers for which cisplatin is most effective—did not show a correlation between *p53* status and apoptosis following cisplatin treatment (84-86,157). One study compared p53 expression and *p53* mutations in 17 drug-responsive and 18 unresponsive TGCTs; p53 was detected in 59% of the responsive samples and in 83% of the non-responsive tumors (370). *P53* mutations were detected in only 1 of the 17 responsive tumors, while there were no mutations detected in the 18 resistant tumors. In addition, human foreskin fibroblasts with inactivated p53 and *p53* null (*p53*-/-) mouse embryonal fibroblasts are hypersensitive compared to their *p53* wildtype counterparts (273), and likewise sublines of the breast cancer cell line MCF-7 in which p53 function was disrupted exhibited increased sensitivity to cisplatin, which was attributed to their reduced ability to repair cisplatin DNA damage and/or defects in cell cycle checkpoint control (197). Importantly, although MCF-7 cells display normal p53 function, these cells do not readily undergo p53-dependent apoptosis. Thus, perhaps only in cells in which p53 functions in initiating apoptosis does mutated *p53* confer resistance. Moreover, because p53 serves multiple functions and acts upstream of other genes and proteins in the DNA damage response and apoptotic network (it is estimated that p53 activates transcription of 200-300 genes (688)), other proteins in these DNA damage response pathways may be capable of compensating for defective p53. For example, overexpression of BRCA1 may compensate for loss of p53 in maintaining global genomic NER through up-regulation of XPC, DDB2, and GADD45 (269). Additionally, as discussed above, data on p53 expression and function in TGCTs and other cell types do not support a consistent role for p53—pro-survival or pro-cell death—in the response to cisplatin.

The role of p73 in cisplatin-induced cell death

Another protein in the p53 family, p73, has also been implicated in cisplatin resistance. p73-deficient mouse embryo fibroblasts exhibit increased resistance to cisplatin (251) and loss of p73 induction and p73-mediated apoptosis was demonstrated in a cisplatin-resistant bladder cancer cell line (533). Contrary to the latter data and the demonstrated role of p73 in cisplatin-induced apoptosis (see Section 1.3.4), overexpression of p73 in human ovarian cancer cells was shown to confer cisplatin resistance and to lead to higher expression of the DNA repair and damage response genes *DNA-PK*, *ATM*, *XRCC6*, *XPD*, *XPG*, *XPB*, *XRCC1*, *MGMT*, and *hMLH1* as well as increased repair capability of a cisplatin-damaged substrate (716). The conflicting data on the effects of deficient- and over-expression of p73 may in part be due to the mutual and competing influence of p53 and p73 on gene transactivation and apoptosis. The two closely related proteins p53 and p73 share similar transcriptional activation, DNA binding, and oligomerization domains (346,349); indeed, p53 and p73 can both activate transcription from promoters containing p53-response elements (346,349,510,716) (which may explain why overexpression of p73 leads to increased DNA repair) and these proteins have been shown to compete for DNA binding as p73 can reduce the transactivational activity of p53 (702,715). Furthermore, both proteins are negatively regulated by MDM2 (24,270,361,532,721,781). Both p53 and p73 can activate apoptosis in several cell lines and both proteins are induced following cisplatin treatment (211,251). The similarities between these two proteins can lead to important consequences in the clinical responses to cisplatin. For example, mutant p53 can abrogate p73-mediated sensitivity to cisplatin; p53 polymorphisms found in advanced head and neck cancers influence p73 function and the inhibition of p73 by specific p53 mutations correlates with clinical drug resistance (42). In addition, Strano et al. demonstrated that human tumor-derived p53 mutants associate with p73 *in vitro* and *in vivo*, and these the p53 mutants also inhibit the transcriptional activity of p73 (667). Conversely, overexpression of a tumor-derived truncated transcript of p73, which lacks the acidic N-terminus corresponding to the transactivation domain of p53 and which was found in 46% of breast cancer cell lines surveyed, was shown to partially protect lymphoblastoid cells against cisplatin-induced apoptosis and was shown to reduce the ability of wildtype p53 to promote apoptosis by acting as a dominant negative inhibitor to p53 (211). Thus mutant forms of p53 and p73 may result in the dual inhibition of apoptosis leading to increased drug resistance.

The c-Abl and mitogen-activated protein kinase (MAPK) pathways

Despite the similarities between p53 and p73, the mechanism of activation of these two proteins in response to cisplatin damage is different; p73 is a tyrosine phoshoprotein—unlike p53 which is a serine/threonine phosphoprotein—and p73 but not p53 is phosphorylated and stabilized by c-Abl, a non-receptor tyrosine kinase (5,694,770). Furthermore, c-Abl-dependent induction of p73 and p73-mediated apoptosis following cisplatin treatment is dependent on mismatch repair, whereas p53 induction and p53-mediated apoptosis following cisplatin damage is not dependent on MMR (251,637), Although p53 is not a known phosphorylation target of c-abl, c-abl regulates p53 by preventing the nuclear export of p53 by Mdm2 and by inhibiting both Mdm-2- and E6-E6-AP-mediated p53 ubiquitination within the nucleus (249,646,648). Therefore c-Abl, acting upstream of p73 as well as regulating p53 levels (although only the former mechanism was shown to contribute to cisplatin sensitivity (251)), may also contribute to cisplatin resistance. Accordingly cells deficient in c-Abl are more resistant to cisplatin and cells deficient in MMR, which is required to activate c-Abl in response to cisplatin (512,513), are consequently resistant to the drug (251,513).

In addition to c-Abl, a member of the activator protein-1 (AP-1) transcription factors, c-Jun, has also been shown to stabilize p73 in response to cisplatin treatment, and c-*jun* (-/-) mouse fibroblasts are resistant to cisplatin-induced apoptosis while reintroduction of c-Jun restores p73 induction and cisplatin sensitivity (612,686). The effect of c-Jun on cisplatin sensitivity may be linked to c-Abl activation; c-Abl acts upstream of stress-activated protein kinase/c-Jun NH_2-terminal Jun kinase (SAPK/JNK) (373,374), and JNK activation mediates the expression of c-*jun* (140,386,612,616). Cisplatin activates JNK in a dose-dependent manner while transplatin fails to activate the kinase (78,274,275,556,576). In addition, inhibition of JNK activation correlates with cisplatin resistance; cisplatin-induced apoptosis involves JNK activation whereas acquired cisplatin-resistance is associated with reduced apoptosis and attenuation of JNK activation (78,238). Consequently activation of JNK is associated with better survival in the clinic (244), although several studies showed that JNK-mediated activation of c-Jun (576) and ATF2 (274) may facilitate repair of cisplatin damage and subsequently lead to increased cellular viability following cisplatin treatment. Consistent with the latter observation, another study showed that expression of a non-phosphorylatable dominant negative c-Jun in human glioblastoma cells inhibits AP-1-driven transcription and in turn increases apoptosis induced by a variety of DNA-damaging agents including cisplatin (575). Furthermore, it was shown that c-Jun expression is elevated in cisplatin resistant

human ovarian cancer cells, and elevated c-Jun expression may induce increased levels of GSH (760). Conflicting data on whether mitogen-activated protein kinase activation enhances cisplatin-induced apoptosis or affords protection against cell death following cisplatin treatment is also demonstrated by two studies in drug-resistant and sensitive ovarian cancer cell lines; one study showed that exogenous expression of dominant negative c-Jun or treatment with an inhibitor of MAPK (PD98059) caused increased sensitivity to cisplatin (275), whereas the other study showed that expression of a dominant negative c-Jun or use of a different MAPK inhibitor (SB202190, inhibitor of p38 kinase) reduced apoptosis following cisplatin treatment (438).

The MAPK kinases JNK and p38 were shown to be regulated by the serine/threonine kinase AKT2; constitutively active AKT2 renders cisplatin-sensitive A2780S ovarian cancer cells resistant to cisplatin, whereas inhibition of AKT2 sensitizes A2780S and cisplatin-resistant A2780CP cells to cisplatin-induced apoptosis through regulation of the ASK1/JNK/p38 pathway (771). While AKT2 phosphorylates ASK1 resulting in inhibition of its kinase activity, activated AKT2 blocked ASK1-mediated signaling, including activation of JNK and p38 and the conversion of Bax to its active conformation. Moreover, inhibitors of JNK and p38 or dominant negative forms of the proteins inhibited cisplatin-induced Bax conformation change (771). Another member of the MAPK family, extracellular signal-regulated protein kinase (ERK), also plays a role in cisplatin resistance as HeLa cell variants selected for cisplatin resistance showed reduced activation of ERK following cisplatin treatment, and ERK activation contributed to cell death in this cell type (732).

c-Fos, c-Myc

The activation of other proto-oncogenes may also confer drug resistance by activating pro-survival pathways. For example, higher expression of the oncogene c-*fos* is associated with cisplatin resistance both *in vitro* (354,369,619) and in the clinic (618), and a reduction in c-*fos* expression can reverse cisplatin resistance (232,233,496,617). C-Fos, is a nuclear transcription factor that induces transcription of a number of genes involved in the regulation of cell replication, cell cycle progression, and differentiation through its interactions with members of the c-Jun (described above) and ATF/CREB families to form AP-1 transcription factor (108,186). AP-1 has also been linked to chemotherapeutic resistance (760), and an adenovirus expressing a dominant negative inhibitor of AP-1 DNA binding can sensitize cisplatin-resistant ovarian cancer cells (57). *Fos* RNA is induced

following treatment with various DNA-damaging agents including cisplatin (293), and genes whose expression levels are modulated by c-Fos/AP-1 and that may affect sensitivity to cisplatin include genes involved in proliferation and apoptosis, metallothionein, DNA polymerase β, and c-*myc* (186,233). Like AP-1, the expression of c-Myc protein is higher in cisplatin resistant cells (354,518) and resistance can be reversed by c-*myc* down-regulation (51,118,379,406). c-Myc is another transcription factor and it regulates the cell cycle by modulating the expression of genes such as *cyclin D1*, *cyclin A*, and *eIF4E* (150,624); c-Myc, like c-*fos*, is induced following genotoxic insult including cisplatin damage (542,710) and c-Myc may protect against cell death through the regulation of GSH levels (50,51) and ornithine decarboxylase (ODC) induction (542)♦. Protection against cisplatin by c-Myc was shown to involve the pro-survival NF-κB transcription factor discussed in Section 1.3.4; cisplatin activates NF-κB, which in turn induces c-Myc and results in reduced apoptosis (542).

H-Ras

In addition to c-*fos* and c-*myc*, the oncogene *H-ras* has been implicated in playing a role in cisplatin resistance. Ras protein is involved in the activation of the mitogen-activated protein kinases, which have been shown to mediate cisplatin-induced apoptosis (see section 1.3.4 and above). *Ras* alleles are frequently mutated in cisplatin resistant tumors, whereas tumors for which cisplatin exhibits the most effectiveness (i.e., testicular and ovarian tumors) typically have a low frequency of mutated *ras* alleles (710). Similar to c-Fos, H-Ras can increase expression of metallothionein and DNA polymerase β and increased *ras* expression results in cisplatin resistance (323,354,619). While studies have associated cisplatin resistance induced by h-Ras with increased repair (195,408), a recent study showed that the NER protein ERCC1 is markedly up-regulated by activated H-Ras, and H-Ras-mediated induction of ERCC1 is dependent on an increase in AP-1 transcriptional activity; accordingly, ERCC1 small interfering RNA expression was shown to reduce the oncogenic H-Ras-mediated increase in DNA repair activity as well as to suppress the oncogenic H-Ras-mediated resistance of NIH3T3 cells to cisplatin (766). Although

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♦ ODC is a critical enzyme in polyamine biosynthesis and intracellular polyamines are essential for cell proliferation and differentiation; consequently ODC can protect cells against cisplatin-, H_2O_2-, and radiation-induced apoptosis (542).

expression of activated H-Ras results in cisplatin resistance in human breast cancer and mammary epithelial cells (195,408), a study of 16 human ovarian carcinoma cell lines, both untreated cell lines as well as cell lines with acquired cisplatin resistance, showed that *ras* overexpression or mutation did not correlate with cisplatin sensitivity (291).

The Bcl-2 family

The aforementioned signaling proteins and transcription factors mediate cisplatin resistance by acting upstream of resistance mechanisms described previously. These transcription factors can induce expression of intracellular thiols to inactivate the drug, bypass polymerases for translesion synthesis past adducts, and DNA repair proteins to increase removal of the adducts. However the proteins discussed above also regulate downstream effectors of apoptosis, including caspase activation and the Bcl-2 family of pro- and anti-apoptotic factors. For example, p53 activates transcription of the pro-apoptotic protein Bax, whereas it suppresses transcription of Bcl-2, a factor that inhibits apoptosis through its interaction with Bax (218,487,531). Bax is reduced in cisplatin resistant ovarian cancer cells (554) and expression of Bax in MCF-7 breast cancer cells can sensitize the cells to cisplatin (608). Alternatively, higher expression of pro-survival or anti-apoptotic factors can confer drug resistance, and overexpression of Bcl-2 or Bcl-XL leads to cisplatin resistance (170,171,189,235,281,485,486,555). Furthermore, the susceptibility of testicular tumors cells to cisplatin may be due to the relative proportions of pro- versus anti-apoptotic factors (e.g. Bax: Bcl-2 ratio) that favor apoptosis, with high levels of Bax and low levels of Bcl-2 (111,430,439), although some reports indicate no correlation between endogenous levels of Bcl-2 and Bax expression and cisplatin-induced apoptosis in TGCT cell lines (84,85). Increased expression of another factor of apoptosis, X-linked inhibitor of apoptosis protein (XIAP, Section....), a direct inhibitor of caspase-3, -7, and -9, was also associated with cisplatin resistance in an ovarian resistant subline, alongside with reduced expression of Fas-ligand, an inducer of apoptosis (439). Fas-ligand receptor-mediated apoptosis is achieved through the Fas-associated death domain protein (FADD), and basal level of FADD expression was shown to be higher in the cisplatin sensitive ovarian cancer cell line A2780 compared to resistant subclones (281). Furthermore, cisplatin treatment induced expression of FADD in the sensitive cells whereas it suppressed FADD expression in the resistant clones (281).

Based on the numerous studies on cisplatin resistance, it is clear that multiple factors play a role in both intrinsic and acquired drug resistance. Cells may be inherently resistant to cisplatin due to high repair capacity or a deficiency in triggering apoptosis in response to DNA damage. Alternatively, cisplatin treatment may select cells that develop mechanisms of resistance, such as mutations in signaling proteins (e.g., MMR proteins, p53, and p73) resulting in an inability to induce apoptosis. Investigators continue to probe mechanisms of resistance in order to understand how best to treat cancer and hopefully achieve the clinical success observed with testicular germ cell cancer. However cisplatin resistance even occurs, albeit with lower frequency, in TGCTs, and this resistance may be due to differential thresholds of apoptosis and may be overcome by high doses of cisplatin-based chemotherapy (502). To circumvent the limitation of resistance associated with cisplatin therapy in the treatment of other cancers, new platinum derivatives have been developed. A few of these compounds are discussed below.

1.5 Cisplatin analogs and second generation compounds

The major disadvantages of cisplatin-based therapies, namely dose-limiting toxicity and clinical resistance, have inspired the development of new compounds that retain the potent anticancer effects of cisplatin while circumventing such limitations. Over the past three decades, thousands of platinum derivatives have been investigated for their ability to overcome dose-limiting toxicity and drug resistance associated with cisplatin. Two prevalent cisplatin analogues that have proved effective in cancer chemotherapy are carboplatin (cis-diammine(cyclobutane-1,1-dicarboxylato)-platinum(II)) and oxaliplatin (1,2-diaminecyclohexaneoxalato platinum (II)) (Fig. 1.6). Carboplatin exhibits reduced toxicity but tumors resistant to cisplatin may be cross-resistant to carboplatin (402). However compounds containing a 1,2-diaminocyclohexane (DACH) carrier ligand (e.g., oxalipatin) are non-cross-resistant with cisplatin in cell lines with acquired cisplatin resistance and show clinical activity in tumors intrinsically resistant to cisplatin (592). In addition, DACH compounds such as oxaliplatin lack the nephrotoxicity of cisplatin and the myelosuppression of carboplatin (480). The DACH structure is believed to result in the different spectrum of clinical activity and toxicity of oxaliplatin compared to cisplatin and carboplatin, as the DACH ligand forms bulkier and more hydrophobic DNA adducts. Indeed, the solution structure of the oxaliplatin-GG adduct is significantly different from the cisplatin-GG intrastrand adduct

(98). As a result of their different adduct structures, cisplatin and oxaliplatin adducts are differentially processed by DNA metabolic proteins. For example, the major diguanyl intrastrand crosslinks formed by cisplatin and oxaliplatin are differentially bypassed by polymerases β and η, with the oxaliplatin 1,2-d(GpG) adduct undergoing more efficient translesion synthesis (with respect to both kinetics and fidelity) than the cisplatin 1,2-d(GpG) adduct (98). The different adduct structures also result in differential recognition by cellular proteins, including excision repair (536) and mismatch repair proteins (778). Such differential cellular processing of the adducts leads to different mechanisms of resistance for the two platinum drugs, and hence resistance mechanisms such as mismatch repair deficiency and enhanced replication bypass have reproducibly been shown to discriminate between cisplatin and oxaliplatin (99,435,708). In August 2002, the FDA approved oxaliplatin for the treatment of recurring metastatic colon and rectum carcinomas, where the drug has been shown to improve response rates and progression-free survival when given with 5-fluorouracil.

To mimic the potency of the major 1,2-d(GpG) cisplatin adduct, second generation platinum compounds are modeled on cisplatin and possess certain common characteristics. For example, these compounds are "electroneutral" so that they may pass through non-polar substances such as cell membranes. To form crosslinks, the compounds must also have two good leaving groups (preferably in the cis conformation as the trans isomer of cisplatin is clinically inactive). Additionally, inert carrier ligands, usually tertiary amine groups, increase adduct stabilization through hydrogen bonding with nearby bases. Second generation platinum compounds may also include modifications designed to circumvent the resistance mechanisms associated with cisplatin. In addition to carboplatin and oxaliplatin, numerous other platinum compounds have entered clinical trials during recent years. These compounds include JM335 (trans-ammine (cyclohexylaminedichlorodihydroxo) platinum(IV)), an active trans platinum complex, and AMD473 (previously ZD0473, cis-amminedichloro(2-methylpyridine) platinum(II)), a sterically hindered complex shown to be less reactive towards thiol-containing molecules than cisplatin (289,290). JM335 exhibited antitumor activity *in vitro* and reduced cross-resistance in tumor cell lines with acquired cisplatin resistance (363,365). AMD473 also demonstrated low levels of cross-resistance with cisplatin as well as a broad-spectrum of antitumor effects (289,292,365,472,582), and this drug has undergone initial Phase I and Phase II clinical testing for various tumor types

(219,240,241,347). Other platinum analogues that have undergone various levels of preclinical and clinical testing include nedaplatin (cis-diammine-glycolato-0,0′platinum II, 254-S, CDGP), cycloplatam (ammine cyclopentylamine malato platinum (II)), SKI 2053 R (methyl, isopropyl, dimethylamino, dioxelane malonato platinum (II)), satraplatin (bis(acetato)amminedichloro(cyclohexylamine) platinum (IV), JM216, an orally bioavailable platinum analog that shows activity in prostate cancer (664)), BBR3464 (a trinuclear platinum agent exhibiting differential recognition and processing by cellular proteins compared to cisplatin (64,356,357,467,780)), and the carboplatin analogue lobaplatin (diamminomethyl cyclobutane lactate platinum II, D-19466 (reviewed in (402,468)) (Fig. 1.6). These compounds were designed to improve potency and organospecificity while reducing the side effects of platinum-based chemotherapy.

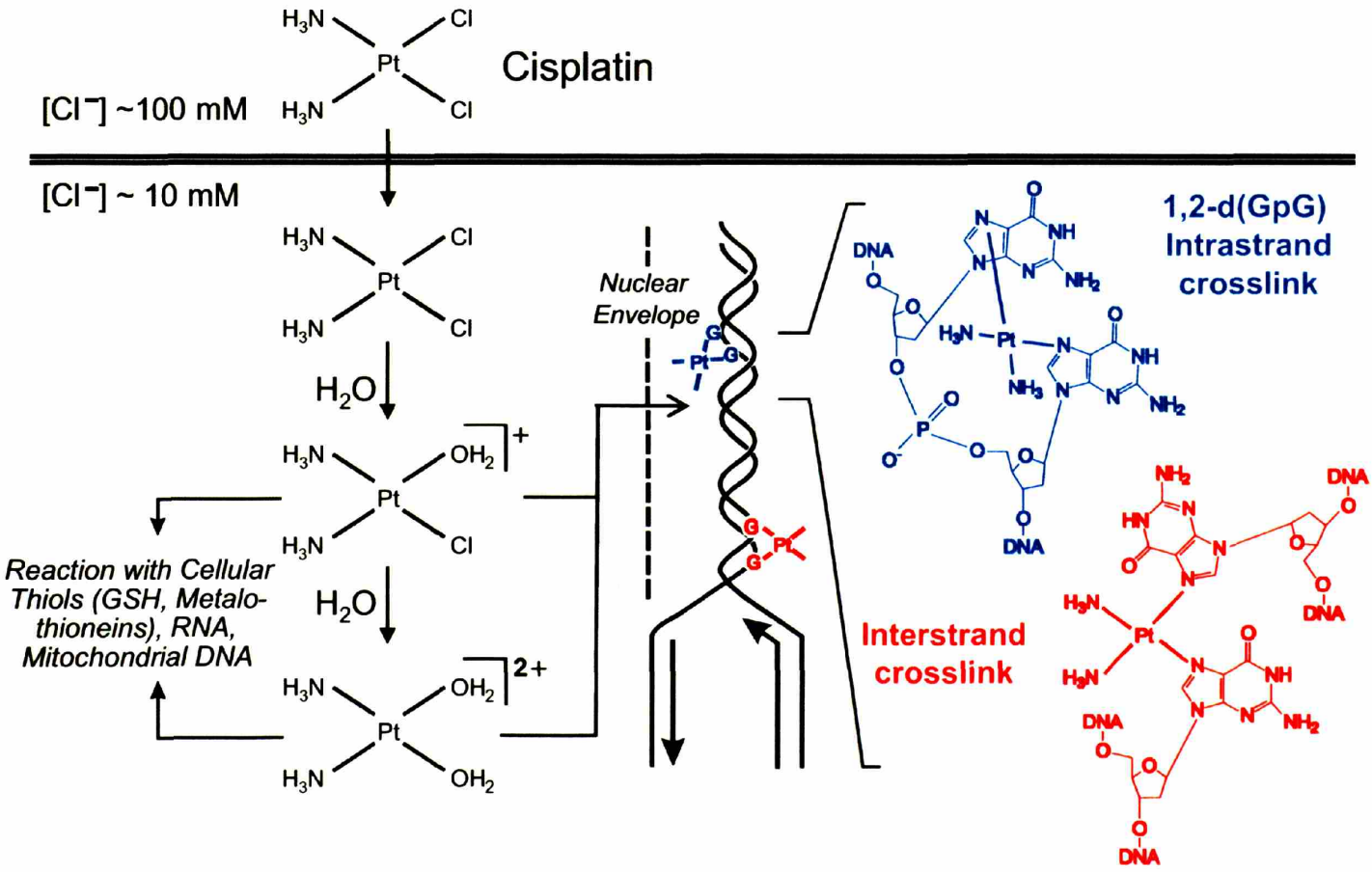
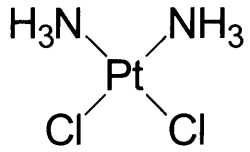
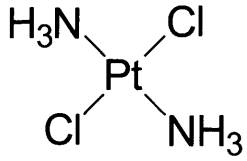


Figure 1.1: Cisplatin undergoes hydrolysis to form $[Pt(NH_3)_2Cl(OH_2)]^+$ and $[Pt(NH_3)_2Cl(OH_2)_2]^{2+}$ once it enters the cell. The aquated compound may then react with various cellular molecules, including thiols, proteins, RNA, and DNA. Cisplatin reacts with DNA to form mainly intrastrand crosslinks. The platinum atom binds covalently to the N7 of purines to form 1,2- or 1,3-intrastrand crosslinks as well as a smaller number of monoadducts and interstrand crosslinks.

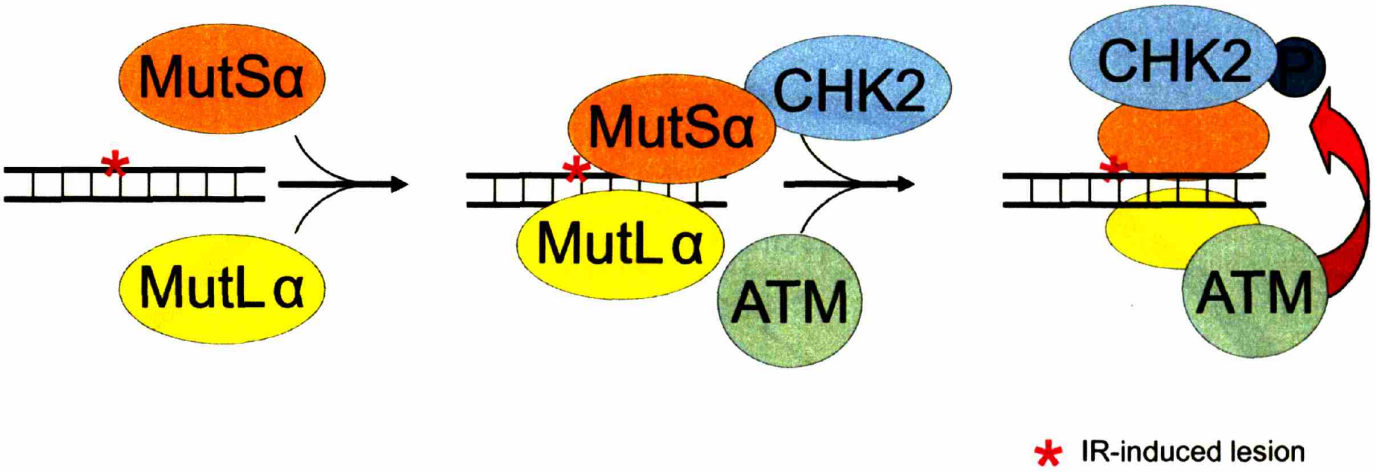


cis-Diamminedichloroplatinum(II)
cis-DDP or cisplatin



trans-Diamminedichloroplatinum(II)
trans-DDP or transplatin

Figure 1.2: Structures of the clinically active drug cisplatin and its inactive isomer, transplatin.



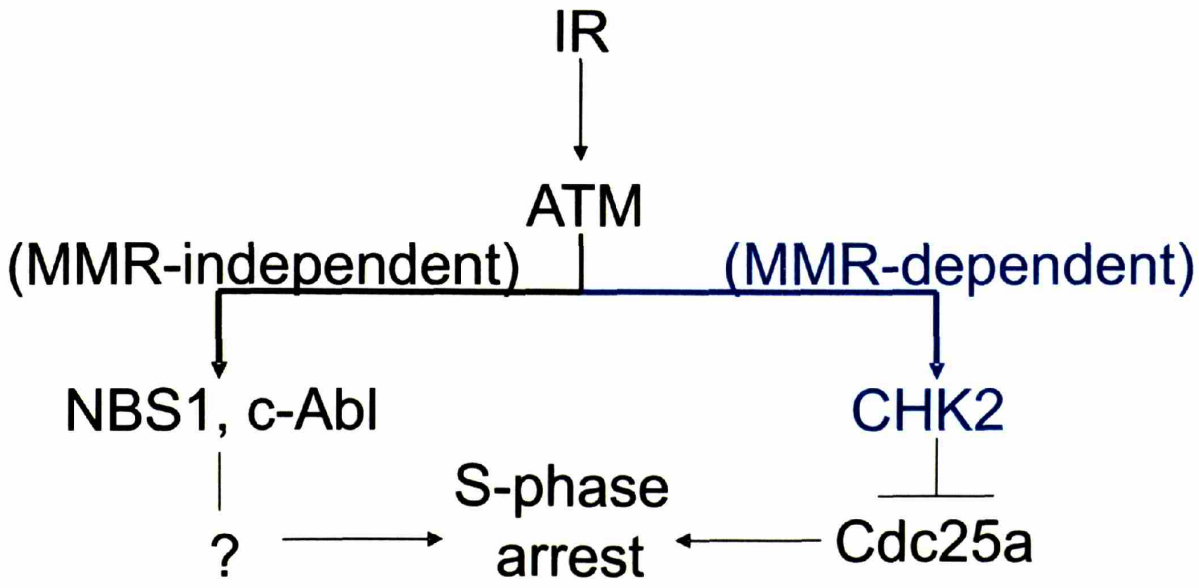


Figure 1.3: Mismatch repair-dependent and -independent signaling events following IR-induced DNA damage. Mismatch repair proteins were shown to be required for the phosphorylation of CHK2 at Thr68 by ATM kinase. Figure based on the results of Brown et al., *Nature Genetics* (33) 2003.

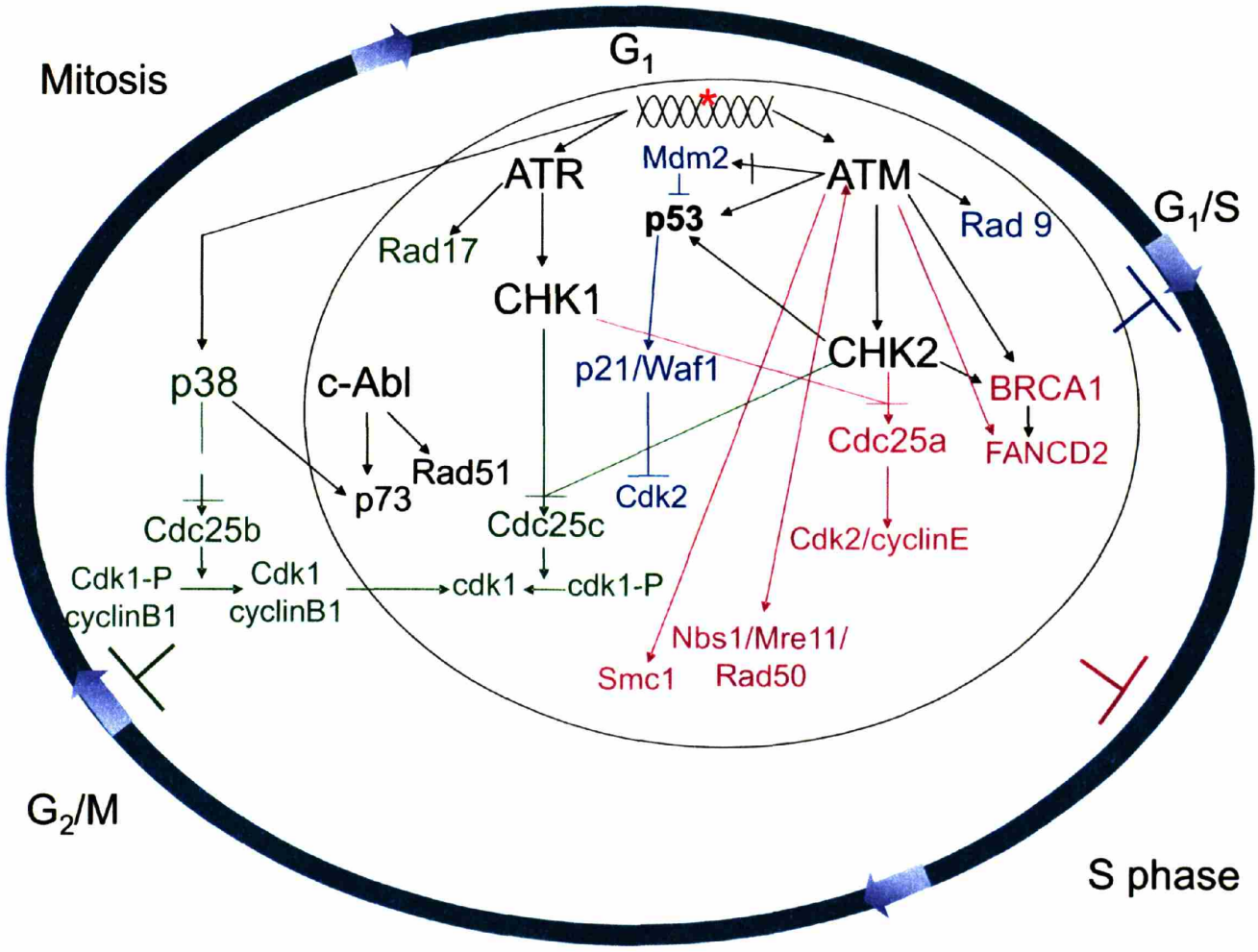


Figure 1.4: A diagram of a select number of signaling events following DNA damage. MMR proteins were shown to interact with ATM and BRCA-1 following DNA damage and c-Abl activation of p73 following cisplatin damage is MMR-depedent. The p38 MAPKpathway also mediates cellular responses to cisplatin damage.

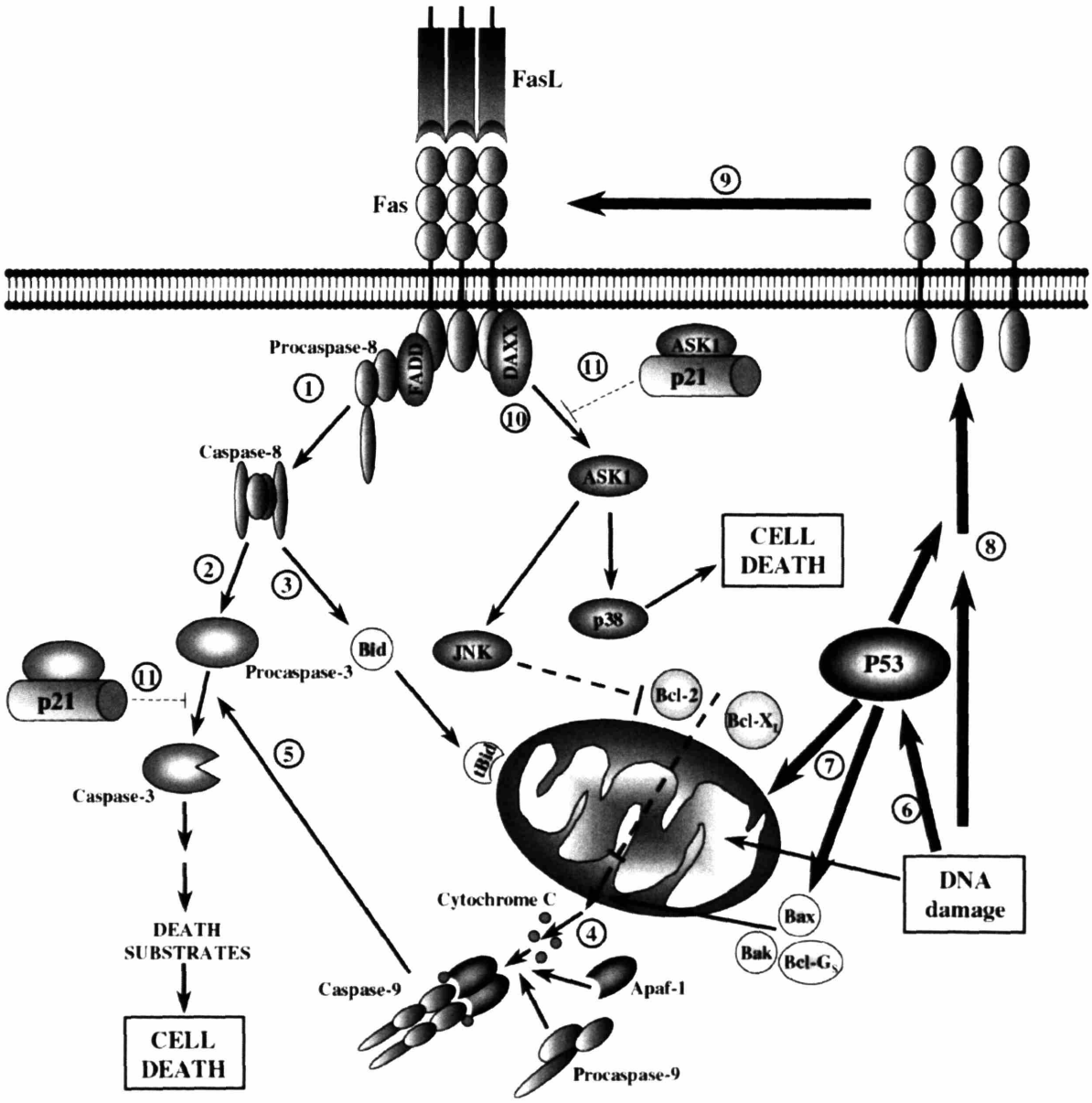
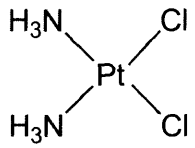
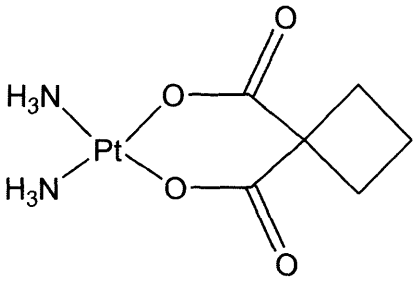


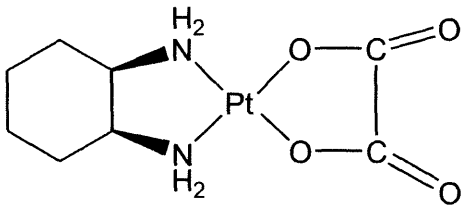
Figure 1.5: Apoptotic pathways incduced by DNA damage. Cisplatin was shown to induce FasL, which triggers apoptosis through trimerisation of the Fas receptor and susequent activation of caspase-8 (1) and caspase 3 (2 and 3). Apoptosis may also be induced via cytochrome C release from the mitochondra (4)i resulting in caspase 9 activiation. The pro and anti-apoptotic factors of the Bcl-2 family are also determinants of apoptosis, and levels of these factors are regulated by p53 and MAPKs as shown. Figure taken from Spierings et al., *J Pathol* 2003 (200).



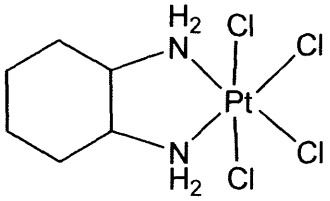
Cisplatin



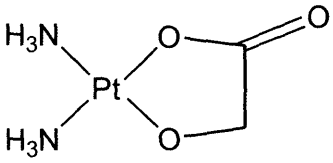
Carboplatin



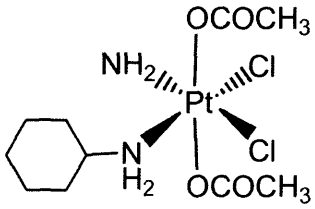
Oxaliplatin



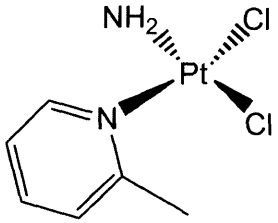
Ormaplatin



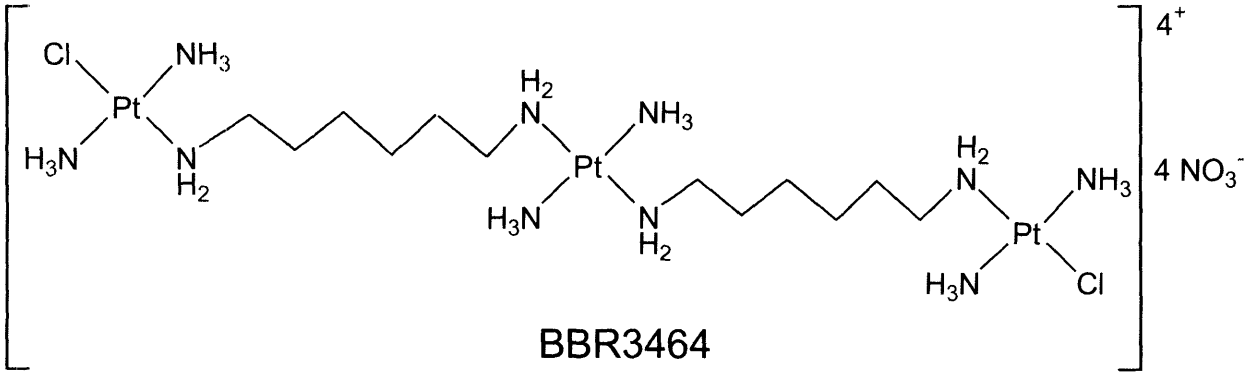
Nedaplatin



Satraplatin



AMD473



BBR3464

Figure 1.6: Cisplatin and platinum analogues. Oxaliplatin and ormaplatin form 1,2-(GpG) adducts carrying the DACH ligand. The only platinum drugs currently approved by the FDA are cisplatin, carboplatin, and oxaliplatin. Satraplatin, however, is under fast-track development for the treatment of hormone-refractory prostate cancer. Previous studies of the mechanism of action of platinum(IV) drugs such as satraplatin suggest that the parent drugs must be reduced before reacting with DNA and exerting chemotherapeutic activity. The trinuclear compound BBR3464 binds to DNA only through noncovalent hydrogen bonding and electrostatic interactions.

Chapter 2: DNA repair pathways that mediate sensitivity to cisplatin: recombination and mismatch repair

2.1 Recombination pathways help cells tolerate cisplatin-induced damage

2.1.1 Recombination pathways in *E. coli* mediate sensitivity to cisplatin

It has long been established that NER is a major pathway for repairing cisplatin-DNA adducts. However as early as 1973, Beck and Brubaker reported that a *recA E. coli* mutant was significantly more sensitive to cisplatin damage compared to wild-type while *recB* and *recC* mutants exhibited intermediate levels of sensitivity (33). Furthermore, their study showed that a *lex1* mutant incapable of inducing recombinational repair was hypersensitive to cisplatin, and the effect of a double mutation in LexA and NER (*lex1 uvrA6*) was cumulative. Husain et al. later also showed that in addition to NER, a RecA-dependent pathway contributed to survival following cisplatin treatment (313). More recent evidence supports an important role for recombination in tolerating cisplatin-induced damage.

The two major pathways of recombination in *E. coli* are depicted in Fig. 2.1 which is based in part on models of S. West and J. Szostak ((680,742) and reviewed in Lusetti and Cox (429)). The first pathway, daughter-strand gap (DSG) repair, involves the RecFOR proteins and is employed when replication fork progression is blocked by DNA lesions. In the DSG pathway (A in Fig. 2.1), persistent cisplatin-DNA adducts (perhaps due to poor nucleotide excision repair of the 1,2 intrastrand crosslink) are encountered by the replication complex (Step 1). Stalled replication results in the formation of a DSG opposite the adduct. The presence of an adduct-binding protein (ABP) may present an even stronger block to replication than the adduct alone. Interactions between the proteins of the RecFOR pathway and the replication fork initiate RecA nucleation and strand exchange (Step 2). The ensuing RecA-catalyzed strand exchange (with the aid of the RecFOR accessory proteins) results in the formation of a Holliday junction (Step 3). Branch migration of the Holliday junction catalyzed by RecA, RuvAB or RecG proteins results in the repair of the DSG and restoration of the replication fork (Step 4). Resolution of the Holliday junction by RuvC restores two double stranded DNA molecules (Step 5). The DSG pathway may serve as a mechanism of damage tolerance because the cisplatin adduct is bypassed by recombinational "repair" and persists in the DNA. The second recombination pathway, double strand break (DSB) repair (B in Fig. 2.1), employs the RecBCD proteins. In the DSB

pathway, the replication complex encounters an unrepaired DSG or a nick opposite the adduct (Step 6). Collapse of the replication fork forms a DSB (bottom) and a DSG (top); the DSG portion of the collapsed replication fork is processed by the DSG pathway (A). Other mechanisms by which the DSB could arise include incisions made by DNA repair proteins (e.g., MutH, MutY); if two adducts are in close proximity on opposite strands, abortive repair activity may result in strand breakage. Alternatively, a single incision made by a DNA repair protein and encountered by the replication complex could lead to replication fork collapse as shown in Fig B Step 6. By the proposed scheme, repair of the DSB requires an intact homologue of the damaged duplex, which would be present in *E. coli* if multiple replication forks are operative (in mammalian cells, the sister chromatid could serve this role). The RecBCD complex (pac-man object) binds the DSB free end (Step 7) and generates ss DNA that is a substrate for RecA nucleation. RecA nucleoprotein filaments catalyze the invasion of the RecBCD generated ss tail into the homologous duplex (Step 8). RecA catalyzed strand exchange and branch migration results in the formation of a Holliday junction and restoration of the replication fork (Step 9). Resolution of the Holliday junction by RuvC yields two intact duplexes (only one molecule is shown; Step 10).

Recent results show that *E. coli* use both DSG and DSB recombination pathways to defend against cisplatin treatment (779). While the formation of DSGs is consistent with previous studies showing that cisplatin adducts inhibit DNA polymerases and cause frequent replication blocks (80,123,266,333,475), the finding that cisplatin induces, in addition, lethal DSBs had not been previously reported. The formation of DSBs has been confirmed biochemically in the recent study described in Chapter 4 using neutral single-cell electrophoresis and by work of Nowosielska and Marinus (522). These recent data thus support the earlier genetic findings by Zdraveski et al. and provide direct evidence that cisplatin-DNA damage leads to the formation of double-strand breaks, and thus as Zdraveski et al. have shown, both DSG and DSB pathways of recombinational repair are required in tolerating cisplatin-induced damage. Work by Courcelle et al. suggests that Rec proteins may not be involved in recombination repair pathways per se but may be critical in avoiding replication fork collapse and in promoting replication fork recovery in the face of DNA damage (132-136). Courcelle et al. propose that RecA, rather than promoting recombination to overcome DNA lesions, is required to maintain replication forks that are blocked by DNA lesions until those lesions are removed by excision repair (135). In support of this view, several studies have shown that Rec function—specifically the function of *recA*,

recF, and *recR* genes—and nucleotide excision repair exhibit a synergistic effect on the recovery of replication following UV irradiation, suggesting that both Rec proteins and excision repair proteins are involved in a common pathway (132,133,136,234,601). However recent work using a drug-induced recombination *lacZ* assay to directly visualize recombination events shows that cisplatin induces recombination in a dose-dependent manner (521,779), which is consistent with the DSB formation analysis showing that cisplatin induces DSBs in a dose-dependent fashion. In addition, while *recBC* and *recD* mutant cells recover replication while *recF* and *recR* mutants do not recover following UV irradiation (132), both *recBCD* and *recFOR* mutants are highly sensitive to cisplatin damage, implying that cisplatin damage requires additional Rec function than the replication recovery function following UV irradiation. Taken together, the data summarized above show that although nucleotide excision repair (NER) has been assigned the central role in modulating the sensitivity of eukaryotic and prokaryotic cells to cisplatin, double-strand break repair by recombination is equally as important as NER in determining cell survival following cisplatin damage.

2.1.2 DSB repair and recombination in eukaryotic cells

Eukaryotic cells also possess several pathways to deal with DNA strand breaks. The primary pathways for the repair of DSBs are homologous recombination (HR), single-strand annealing (SSA), and nonhomologous end-joining (NHEJ), which are reviewed in detail elsewhere (201,262,350,450,520,541,675). Unlike NHEJ, HR and SSA pathways require homology. HR is the most conservative (i.e., the most error-free) pathway and is important for the recovery of collapsed replication forks. In *Saccharomyces cerevisiae*, HR relies on the *RAD52* epistasis group of genes, which includes *RAD50*, *RAD51* (encoding the *E. coli* RecA homologue), *RAD52*, *RAD54*, *RAD57*, *RAD59*, *MRE11*, and *XRS2*. Similarly, numerous mammalian genes have been implicated in HR (based on their sequence similarity to the yeast *RAD52* epistatis group genes) and the mammalian genes include *Rad50*, *Rad51*, *Rad52*, *Rad54*, *Rad54B*, and *Mre11*. Human cells express other Rad51-related proteins including Rad51B, Rad51C, Rad51D, XRCC2 and XRCC3, and numerous interactions among these Rad51 paralogs have been reported (70,419,450,451,620,639,743); for example, both XRCC2 and XRCC3 interact with Rad51 and participate in DSB repair by HR (336,418,561). Like the *E. coli* paradigm, eukaryotic recombination involves strand invasion, branch migration, and Holliday junction formation

and resolution. Rad50, Mre11 (the *E. coli* SbcC and SbcD homologs respectively) and Xrs2 in yeast, and in humans the analogous proteins Rad50, Mre11, and Nbs1 (or nibrin, the protein defected in the ataxia telangiectasia-like disorder, Nijmegen breakage syndrome (200)), otherwise known as the MRN complex, may process the termini of the DSB before Rad51-mediated strand invasion. Rad51-mediated homologous pairing and joint molecule formation is facilitated by Rad54 and RPA (328,559,639,640,711). The products of the genes disrupted in the hereditary breast cancer syndromes, BRCA1 and BRCA2, also interact with the Rad51 protein and are involved in human HR (45,466,498,500,626,630), though the exact function of these proteins is still unclear. Holliday junctions or four-strand intermediates arising from replication fork regression are resolved by the Mus81-Eme1/Mms4 heterodimer (1,117,125,469). In SSA, the ends of a DSB are digested by 5' to 3' exonucleases (perhaps by MRN) until regions of homology on the two sides of the break are exposed. The homologous regions are annealed and the nonhomologous ends are trimmed off and the duplex ends ligated. SSA may create deletions as well as expansions between repetitive sequences and thus entail information loss (588,589). Several proteins involved in DSB repair by HR also participate in SSA; these proteins include Rad52, RPA and the Rad50/Mre11/Xrs2 protein complex in yeast or the MRN complex in human cells. Digestion of the ssDNA tails may be achieved by Rad1/Rad10 in yeast and XPF-ERCC1 endonuclease in human cells. The third DSB repair pathway, NHEJ, is essential for V(D)J (variable [division] joining) recombination and is mediated by DNA-PK. In NHEJ, the Ku heterodimer binds to the ends of a DSB and activates DNA-PKcs and the DNA ligase IV/XRCC4 (X-ray cross-complementing 4) heterodimer, which then ligates the two duplex ends regardless of whether the two ends are from the same chromosome. Although NHEJ was thought to be the major repair pathway for DSBs in mammalian cells, recent studies have demonstrated that homology-directed repair is also a major DSB repair pathway, with up to 50% of observed repair at a specific genomic locus occurring by recombination (131,334,335,412). Furthermore, homologous recombination is stimulated 2-3 fold by a chromosomal DSB (335,499). The relative contributions of HR versus NHEJ may be cell-cycle dependent, as HR was shown to be the predominant recombination pathway in human cells in S phase but not in M or G_1/G_0 phases of the cell cycle (609). Interestingly, NHEJ and homologous repair were shown to both participate in the repair of a single DSB, indicating that the two pathways are not completely separable (590).

Following treatment with DNA damaging agents such as ionizing radiation, alkylating agents and cisplatin, Rad51 forms discrete nuclear foci that are believed to be the sites of DNA damage and DSBs (52,247). Homologous recombination requires the recombinase RAD51 and in vertebrate cells, all five RAD51 paralogs. The paralogs form two complexes in solution, a XRCC3/RAD51C heterodimer and a RAD51B/RAD51C/RAD51D/XRCC2 heterotetramer (419,450,451,620,743). Disruption of any one of the five paralog genes prevents subnuclear assembly of the Rad51 recombinase at damaged sites and renders cells 30-100 fold more sensitive to DNA cross-linking agents including cisplatin (52,246,418,682,683). Regulation of Rad51 may be achieved in part by the c-Abl tyrosine kinase, which interacts constitutively with Rad51 and which phosphorylates Rad51 on Tyr-54 *in vitro* (769). Furthermore, treatment of cells with ionizing radiation induces c-Abl-dependent phosphorylation of Rad51, although studies have revealed conflicting roles for this event. One study, for example, showed that c-Abl phosphorylation of Rad51 serves to negatively regulate Rad51 as it inhibits Rad51 DNA binding as well as Rad51–catalyzed strand exchange (769). By contrast, other studies have shown that c-Abl phosphorylation of Rad51 (via formation of a complex with ATM) promotes interaction between Rad51 and Rad52 and is required for IR-induced Rad51 foci formation (103,768).

Like in *E. coli*, recombination in eukaryotic cells protects against cisplatin-induced damage. *S. cerevisiae* deficient in RAD51 and RAD52 are more sensitive to cisplatin compared to wild-type cells, and importantly *rad51Δ* cells are much more sensitive to cisplatin than to UV (259), suggesting that cisplatin repair requires RAD51 function in recombination. In addition, overexpression of the Rad51 paralog Xrcc3 in MCF-7 cells leads to a 2-6-fold increase in cisplatin resistance. This resistance is accompanied by a 2-fold increase in drug-induced Rad51 foci, an increase in cisplatin-induced S-phase arrest, and decreased cisplatin-induced apoptosis (755). Furthermore, Xrcc3 overexpression is associated with increased Rad51C protein levels, which is consistent with the known interaction of these two proteins (450,451). Along the same lines, an inhibitory synthetic peptide specific for Rad51C inhibits assembly of the Rad51 recombinase and sensitizes CHO cells to cisplatin when it is added to growth medium (124). Another protein involved in DSB repair, the Brca1 protein, was shown to promote subnuclear Rad51 foci formation following cisplatin treatment, and *Brca1*(-/-) mutants are 5-fold more sensitive to cisplatin compared with wild-type cells (45). Interestingly, Rad51 and the TFIIH subunit XPD involved in NER may cooperate to tolerate cisplatin damage via recombination repair.

Overexpression of XPD was shown to result in cisplatin resistance, which was associated with increased Rad51-related homologous recombination and increased sister chromatid exchanges whereas no change in NER activity was observed (9). Furthermore, XPD and Rad51 colocalize and coimmunoprecipitate in a human glioma cell line (9). Rad51 is also important for cisplatin resistance in cells expressing the BCR/ABL oncogenic tyrosine kinase, which is a fusion tyrosine kinase (FTK) that arises from reciprocal chromosomal translocations (see Skorski (649,650) for a review on BCR/ABL). FTK-transformed cells display cisplatin resistance as well as increased expression of Rad51, which is dependent on STAT5-dependent transcription and inhibition of caspase-3-dependent cleavage (652,653). In addition, phosphorylation of Rad51 by BCR/ABL is required for enhanced DSB repair and cisplatin resistance. Importantly, cisplatin-induces DSBs in HI-60 cells that are independent of the DNA fragmentation associated with apoptosis (309). Thus recombination mechanisms, in both prokaryotic and mammalian models, help cells tolerate cisplatin-induced damage.

2.2 Mismatch repair mediates cisplatin-induced toxicity

Mismatch repair proteins act on nucleotide mismatches as well as insertion-deletion loops. These proteins are also important for the recognition of homeologous (similar but nonidentical) DNA sequences and can prevent the recombination of two DNA molecules when the sequences exhibit non-homology. Although MMR is critical in maintaining genomic integrity, this pathway paradoxically sensitizes cells to DNA damage by alkylating agents and cisplatin. The possible mechanisms of MMR-mediated sensitivity to cisplatin in both bacterial and mammalian cells are discussed below.

2.2.1 The *E. coli* paradigm of mismatch repair

Methyl-directed mismatch repair in mutation avoidance

During DNA replication, the DNA pol III holoenzyme, its proofreading activity, and the aid of accessory proteins such as ssb result in a cumulative error frequency of ~ 10^{-7} (226). The correction of these misincorporation errors requires post-replicative mismatch repair (Fig. 2.2). Mismatch repair discriminates between template and the newly synthesized daughter strands by the methylation status of d(GATC) sites; in *E. coli*, the DNA adenine methyltransferase (Dam) protein methylates the N6 position of adenine in the sequence d(GATC). While the number of Dam molecules is only ~130 per cell (62), the enzyme

cannot keep pace with the replication fork and consequently the duplex is transiently hemimethylated, thus allowing MMR to distinguish template (methylated) from daughter (unmethylated) strands. MMR is initiated by recognition of a distorted structure arising from mismatched bases; this recognition requires the MutS homodimer, in which the two monomer subunits hold different conformations and form a structural heterodimer (267,397,670). Following MutS binding to the mismatch and ADP to ATP exchange, a MutS/MutL complex is formed and in a process that has still not entirely been elucidated, the complex translocates along DNA (2,8) and forms a tertianary complex with the latent endonuclease MutH. MutL activates MutH in an ATP-dependent reaction (20,257,263) and activated MutH makes an incision 5′ to the dG of an unmethylated d(GATC) site located either 5′ or 3′ to the mismatch (256,490). Incision on the unmethylated strand may occur several kilobases from the mismatch (669,722), and hence MMR is also referred to as long-patch repair. DNA unwinding from the incision site to a point beyond the mismatch error is achieved by helicase II (UvrD) (151,757), and DNA is chewed from the incision site back to the site of the mismatch by one of several exonucleases depending on the directionality of the reaction (e.g., Exo VII or RecJ for 5' to 3', and Exo I, Exo X, or Exo VII for 3' to 5' activity). In the presence of ssDNA-binding protein, DNA polymerase III holoenzyme performs DNA resynthesis and DNA ligase finally seals the nick. The Dam protein then methylates the new strand, completing the MMR reaction.

Mutation avoidance by *E. coli* mismatch repair, as it is described above and reviewed elsewhere in detail (267,394,490,491), relies on hemi-adenine methylation to distinguish between the nascent strand containing the error and the template strand. Indeed, both reduced expression and overexpression of Dam result in hypermutability in *E. coli* (282,447,759). Of relevance to the models of MMR-mediated sensitivity to DNA damaging agents described below, in the case where Dam is absent and the genome is unmethylated at d(GATC) sites, MutH cannot distinguish between the new and template strands. *In vitro* experiments show that in this situation MutH aimlessly makes an incision on either strand, although the endonuclease shows reduced activity on unmethylated compared to hemimethylated substrates (20,740). Furthermore, Au et al. (20) have shown in a reconstituted *in vitro* system that in the absence of d(GATC) methylation MutH can make incisions on both DNA strands and form a double-strand break (DSB). When both strands are methylated, the duplex is resistant to MutH incision. Interestingly, preexisting internal nicks can substitute for MutH function *in vitro* (395), which sheds light on how eukaryotic

MMR, for which there is no known MutH homologue, may discriminate between template and daughter strands.

Anti-recombination function of mismatch repair

In addition to recognizing and correcting post-replicative mismatches, MMR also recognizes mismatches between two DNA molecules undergoing recombination and can abort recombination mid-reaction. In this way, MMR prevents recombination between divergent DNA sequences and performs "anti-recombination", and even low divergence (1% or less) can substantially inhibit homologous recombination in bacteria, yeast, and mammalian cells (152,153,158,720,772,773). Several early studies in *E. coli* showed that mutants deficient in MMR exhibit increased frequencies of intragenic recombination (204,791), and in conjugational crosses between *E. coli* and *Salmonella typhimurium*, the genomes of which exhibit ~16% sequence divergence, elimination of the recipient MMR system was associated with up to a 1000-fold increase in conjugational recombination frequencies (583). Radman (578) suggested that MMR may serve in anti-recombination to ensure the fidelity of recombination events. According to Radman's proposal, mismatches in regions of heteroduplex lead to MMR-dependent excision of the invading strand within a recombination intermediate, resulting in abortion of the strand-exchange reaction. More recent work has demonstrated that the inactivation of MMR leads to a ~6-fold increase in the frequency of intergenic recombination exchanges (398), and biochemical studies using the closely related bacteriophages M13 and fd show that MutS and MutL block RecA-catalyzed strand transfer of M13-fd but not of M13-M13 (homologous) DNA substrates (748,749).

Štambuk and Radman proposed two models of MMR-mediated anti-recombination: a MutH-independent mechanism involving an abortion of the recombination reaction that requires free ends and the UvrD helicase, and a MutH-dependent pathway requiring unmethylated d(GATC) sites and *de novo* DNA synthesis (662) (Fig. 2.3). In their study of interspecies recombination between the linear Hfr DNA from *Salmonella typhimurium* and the *E. coli* circular chromosome, recombination between these two divergent (16% on average) sequences involved DNA synthesis requiring the PriA primosome and RecF functions. This DNA synthesis may be promoted by the inherent instability of the heteroduplex and may provide the unmethylated d(GATC) sites required for the MutH-dependent pathway of anti-recombination; recombination-dependent DNA replication also occurs following DNA damage in *E. coli* (383-385), making the MutH-dependent pathway

relevant for damaged recombination substrates. This MutH-dependent pathway occurs relatively late-stage in the recombination reaction, following DNA synthesis, and also requires MutS and MutL in addition to PriA and RecF. During this late-stage antirecombination, editing occurs on the DNA strand that contains the mismatch in the heteroduplex region derived from the parental sequences, and MutH incision occurs at an unmethylated d(GATC) site in the newly synthesized extension of the mismatch-containing strand (Fig. 2.3B). Unwinding and excision from the unmethylated d(GATC) site to the Holliday junction would interrupt recombination and separate the two parental DNA molecules. The MutH-independent pathway of anti-recombination corresponds to the dissociation of the earliest RecA-catalyzed heteroduplex due to mismatch formation, and hence it is an early-stage editing pathway of the recombination substrates. This early-stage pathway requires MutS, MutL, the UvrD helicase, and RecBCD nuclease for the digestion of the dissociated single-stranded end and may occur prior to the initiation of DNA synthesis (Fig. 9A).

Mismatch repair in cisplatin sensitivity

It was originally established by Fram et al. (224) that mismatch repair proteins potently sensitize cells to specific DNA damaging agents including cisplatin; *E. coli dam* mutant strains, which have decreased adenine methylase activity and are thus unable to distinguish between parental and newly synthesized DNA strands, are hypersensitive to cisplatin. However *dam* mutants with an additional mutation in any of the mismatch repair genes *mutS mutL*, or *mutH* show reduced sensitivity and near wild-type levels of resistance (224,522). Unlike cisplatin, the therapeutically inactive platinum compound, transplatin, does not elicit differential sensitivity in *E. coli dam-* and wild-type strains.

The effect of MMR on cisplatin sensitivity is most likely direct, as MutS recognizes cisplatin-DNA lesions *in vitro* ((778) and Chapter 4). One prevalent model to explain the influence of MMR on cellular sensitivity to DNA damage is the futile repair model. Futile cycling was originally proposed to explain the effect of MMR on cellular sensitivity to methylating agents (351) and has been extended to explain MMR effects on cisplatin sensitivity (a discussion of this model is also presented in Chapter 4). According to this model, MMR initiates repair on the strand opposite the adduct. While the adduct remains following repair synthesis, another cycle of repair is initiated, again on the strand opposite the adduct. Because the adduct is not removed in these repair cycles, the continual and

"futile" repair results in a persistent strand gap, and if encountered by a replication fork this single-strand gap may be converted to a lethal double-strand break (Fig. 5.10). Futile cycling may be more prevalent in cells lacking adenine methylation because of the increased level of MutH substrates (i.e., unmethylated d(GATC) sites). Thus in a Dam-deficient cell, MMR recognition of a cisplatin adduct may result in MutH incision and digestion of either strand, and coupled with replication such repair activity may lead to a double-strand break. Furthermore, in Dam-deficient cells MutH may introduce nicks on both strands, thus forming a double-strand break independent of replication.

An alternative model to futile cycling is MMR-mediated inhibition of recombinational bypass of cisplatin adducts. According to this model, MMR, serving its role in anti-recombination, may recognize cisplatin adducts as heterologies within a recombination intermediate and may abort the recombination reaction. Recent work by Calmann and Marinus show that *in vitro* MutS blocks RecA-catalyzed strand exchange of two recombination substrates when one substrate is modified with cisplatin (90). While the inhibitory effects of MutS were not enhanced by the addition of MutL, the lack of an observed effect of MutL may have been the result of low "divergence" generated by 4-8 adducts versus the high level of divergency (e.g., close to 200 mismatches) in previous studies demonstrating a role of MutL in anti-recombination (749). While MMR-mediated anti-recombination is expected to occur in both Dam-proficient and-deficient cells, the effects of anti-recombination may be more detrimental to cells lacking Dam due to the decreased availability of recombination proteins in these cells; because of the high level of endogenous damage in *dam* mutant cells ((731) and Chapter 3), recombinational proteins in these cells may be operating close to maximal capacity. Thus the cells cannot afford any further damage burden, and inhibition of recombinational repair of cisplatin adducts confers such additional damage burden. This model is discussed in more detail in Chapter 4.

Both models of MMR-mediated sensitivity to cisplatin—futile cycling and inhibition of recombination—are consistent with genetic data showing that in *dam* mutant cells, an additional mutation in *mutS*, *mutL*, or *mutH* abrogates sensitivity to drug. The futile cycling model requires the complete MMR repair reaction involving MutS recognition and MutH incision, and thus mutations in each of the MMR *mut* genes is expected to abrogate sensitivity. Inhibition of recombination, if occurring by the late-stage model proposed by

Štambuk and Radman, also requires MutH function[♦]. Furthermore, both futile repair and anti-recombination models support the hypothesis that MMR leads to strand break formation following cisplatin damage; in futile cycling, MMR may be introducing single nicks after recognition of cisplatin adducts in an attempt to initiate repair. These single nicks may be converted to lethal DSBs if encountered by a replication fork. In anti-recombination, MutS binding to a cisplatin adduct may prevent recombinational bypass of the adduct and consequently lead to strand break formation. Additionally, strand break formation occurring by either mechanism would be increased in a Dam-deficient background, as MutH incisions require unmethylated d(GATC) sites and as the high level of basal damage in *dam* mutants renders these cells more susceptible to the effects of anti-recombination.

2.2.2 Mismatch repair in mammalian cells

Since the initial discovery in *E. coli*, it has been shown in numerous studies that mammalian cells deficient in mismatch repair also exhibit increased survival and tolerance to drug treatment (3,4,177,213,251,497,512,540,567,751). Although the influence of MMR on sensitivity to DNA-damaging agents is more pronounced for alkylating agents than it is for cisplatin damage in *in vitro* systems (up to 100-fold versus ~2-fold) (4,46,47,311,685), the level of cisplatin resistance afforded by MMR deficiency is sufficient to produce a large difference in drug responsiveness *in vivo* in tumor model systems (13,212,216). The available preclinical and clinical data also suggest that tumors with a significant fraction of MMR-deficient cells will exhibit reduced responsiveness to specific drugs including cisplatin (4,212,441,568). The importance of acquired drug resistance by loss of MMR is underscored by studies demonstrating that treatment with cisplatin can lead to an enrichment of MMR-deficient, and thus drug-resistant, cells *in vitro* and in tumor xenograft models (214,215). Furthermore, paired ovarian tumor samples from patients before and after platinum-based chemotherapy showed a reduction in MLH1 staining in 66% of the cases (215), thus demonstrating a model of acquired clinical resistance whereby drug treatment selects for MMR-deficient and consequently drug-resistant cells.

Eukaryotic homologs to E. coli MMR proteins

[♦] This late-stage anti-recombination model also requires PriA function. Interestingly, PriA is required for replication restart following the stalling of the fork at sites of DNA lesions (139,614) and for recombinational bypass of cisplatin adducts (521), and consequently *priA* mutant *E. coli* are hypersensitive to cisplatin treatment (522).

Eukaryotic mismatch repair is functionally homologous to the *E. coli* MMR system, and the yeast and mammalian homologs to the MutS and MutL proteins are shown in Table 2.1. While there are no identified MutH homologs to date, eukaryotic MMR is not thought to be methyl-directed as in *E. coli*. Different mechanisms have been proposed for strand discrimination in eukaryotes. These mechanisms include nick-directed repair (198,296,489,684), where the nicks at the 5' and 3' ends of Okazaki fragments during lagging strand synthesis and the growing 3' end of the leading strand provide the strand discrimination signals. Interestingly, the human MSH2-MSH6 complex translocates away from the mismatch in an ATP-dependent manner *in vitro* (253) and the human pathway of mismatch correction may possess a bidirectional excision capability similar to that of *E. coli* (198). In addition, while human MSH2, MLH1, and PMS2 interact with proliferating cell nuclear antigen (PCNA) and while PCNA is required for the repair initiation step of MMR (260,489,703), it has been proposed that PCNA might confer information on daughter vs. template strands to the MMR machinery based on the position of the replication complex. Alternatively, PCNA might trigger the 3' to 5' exonuclease activities of DNA polymerases in mismatch removal (253). The final stage of MMR, repair synthesis, may be achieved primarily by DNA polymerase δ in human cells (425).

Table 2.1: Eukarytoic homologs to the *E. coli* MutS and MutL proteins*

Organisms			**Eukaryotic protein function**
E. coli	**Yeast**	**Mammals**	
MutS	MSH1	*--nd---*	Mutation avoidance in mitochondria
	MSH2	MSH2	Forms heterodimers with MSH6 (to form MutSα) and MSH3 (to form MutSβ) to repair replication errors, recognize mismatches in recombination intermediates, remove nonhomologous tails (MutSβ only), inhibit recombination between heterologous sequences, and in mammals participates in DNA damage-induced signaling
	MSH3	MSH3	Forms complex with MSH2 to form MutSβ; repairs small insertion/deletion mismatches
	MSH4	MSH4	Forms heterodimer with MSH5 to promote crossing-over in meiosis
	MSH5	MSH5	Forms heterodimer with MSH4 to promote crossing-over in meiosis
	MSH6	MSH6	Forms complex with MSH2 to form MutSα; repairs both base-base and insertion/deletion mismatches
MutL	PSM1	PSM2	Forms heterodimers with MLH1 to repalr replication errors and mismatches in recombination intermediates, inhibits recombination between heterologous sequences, and in mammals participates in DNA damage-induced

			signaling
	MLH1	MLH1	Forms heterodimers with the other MutL homologs
	MLH2	PMS1	Forms heterodimers with MLH1 to repair replication errors and mismatches in recombination intermediates
	MLH3	MLH3	Forms heterodimers with MLH1 to repair replication errors and to promote crossing-over in meiosis

***Adapted from Harfe and Jinks-Robertson (267). *nd*, not determined**

Eukaryotic MMR in mutation avoidance and in anti-recombination

The importance of mammalian MMR in mutation avoidance is underscored by studies demonstrating that mutations in the MutS and MutL homologs may be linked to tumorigenesis. For example, mouse knockouts of *MSH2*, *MSH3*, *MSH6*, *PMS1*, *PMS2*, and *MLH1* have all been constructed, and all, with the exception of *PMS1* and *MSH3* knockouts, show an increased incidence of various types of internal organ tumors (22,158,159,185,577). In addition, MMR is linked to tumorigenesis in humans, as mutations in human MLH1 and MSH2 are associated with 60% and 70% respectively of the cases of hereditary non-polyposis colon cancer (HNPCC) (reviewed in Buermayer (82)), and individuals heterozygous for MLH1 are predisposed to HNPCC as loss of heterozygosity (i.e., loss of function of the wild-type, functional allele) results in highly elevated mutation rates and subsequent tumor development (278). Although MMR deficiency is associated with a reduced ability to trigger apoptosis as well as with hypermutability, Lin et al. recently demonstrated that an $Msh2^{G674A}$ mutation in mice caused DNA repair deficiency that resulted in a strong cancer predisposition while it did not affect the DNA damage response function of Msh2 (413).

Like in *E. coli*, MMR in eukaryotes recognizes mismatches in recombination intermediates. Such mismatch recognition may trigger either mismatch correction or complete abortion of the recombination event (678). The repair of mismatches in recombination intermediates results in gene conversion, whereby information on one chromosome is replaced with information from the homologous chromosome ((191,268,680) and reviewed in 1025, 1026, 1027, 1008, 1031}). Studies in yeast have supported a role of MMR in limiting heteroduplex formation (via blockage or reversal of strand-exchange) in a mismatch-dependent manner (104,105,511,516,679). Furthermore, MMR was also shown to inhibit single-strand annealing between non-homologous sequences in yeast (671,672). Although few studies have examined the role of MMR in anti-recombination in mammalian cells, the data available suggest that recombination between divergent sequences is

increased in MMR-deficient cells (190,718), although according to one study the effects of MMR are only observed for spontaneous recombination as the efficiency of double-strand break-induced recombination was not affected by sequence divergence in mismatch repair proficient or deficient backgrounds (718).

Roles of mammalian MMR in mediating cisplatin sensitivity

As mentioned previously, the presence of an intact MMR system in mammalian cells confers sensitivity to cisplatin, as is observed with *E. coli*. Like the *E. coli* MutS damage recognition protein, the mammalian MutS homologs bind cisplatin-modified DNA *in vitro*. Human MutSα specifically recognizes the major 1,2-instrastrand crosslink but fails to recognize the major 1,3-intrastrand crosslink produced by tansplatin (179,473), and thus MutS recognition of the different adducts correlates with the toxicity profile of the agents as transplatin is clinically inactive (see Chapter 1, Section 1.2). MutSα binding to cisplatin-DNA adducts is enhanced when the complementary strand contains T opposite the 3' and C opposite the 5' guanine in the crosslink while binding to an adduct opposite correct bases is much lower (756), suggesting that replicative bypass may enhance MutSα interactions with cisplatin adducts. Importantly, however, Duckett et al. showed that the major 1,2-d(GpG) adduct effectively competes with a G:T mismatch substrate for human MutSα binding *in vitro*, with the binding constant for the cisplatin crosslink estimated to be about an order of magnitude less than the binding constant for the G:T mismatch (179). Such binding affinity of MutSα for the major cisplatin crosslink is consistent with a significant effect *in vivo* and is of particular significance as human MSH2 is highly expressed in testis and ovary (473); cancers arising in these organs are the cancers best treated by cisplatin therapy. Thus the two models of MMR-mediated sensitivity in *E. coli*—futile cycling and the inhibition of recombinational bypass of cisplatin adducts—are also applicable in mammalian cells. The mammalian MutS homologs may recognize a cisplatin adduct and initiate repair; if repair is initiated on the strand opposite the adduct, the lesion remains leading to additional cycles of repair and a persistent strand gap, which may in turn lead to more lethal DNA damage and cell death. This model, as mentioned above, has been proposed for alkylation damage, where sensitivity to methylating agents such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and *N*-methyl-*N*-nitrosourea (MNU) is abrogated in cells deficient in MMR (47,67,97,250,311,359). Interestingly, cytotoxicity and steady-state chain elongation assays indicate that hMLH1 or hMSH6 defects result in 1.5-4.8-fold increased cisplatin resistance

and 2.5-6-fold increased replicative bypass of cisplatin adducts (708). Thus defects in hMutLα and hMutSα may contribute to increased net replicative bypass of cisplatin adducts and therefore to drug resistance by preventing futile cycles of translesion synthesis and mismatch correction. Supporting this hypothesis is the finding that extracts from cells lacking hMLH1 and hMSH2 showed ~3-fold reduction in cisplatin damage-specific DNA synthesis (207). While MMR status had no effect on NER activity, such MMR-dependent repair synthesis may reflect a reduction in DNA synthesis associated with adverse processes (e.g., futile cycling) rather than productive repair activity. In addition, while mammalian MMR, like *E. coli* MMR, also serves to monitor recombination events and to prevent recombination between divergent DNA sequences, it may also prevent the recombinational bypass of cisplatin adducts. The aforementioned study showing that hMLH1 or hMSH6 defects result in 1.5-4.8-fold increased cisplatin resistance and 2.5-6-fold increased replicative bypass of cisplatin adducts is also consistent with the notion that MMR may prevent the recombinational bypass of cisplatin adducts during replication.

Although the futile repair and anti-recombination hypotheses are operable in mammalian cells, recent data suggests that MMR in mammalian cells may serve as a "sensor" of DNA damage (217,666). In support of this view, the human mismatch repair proteins MSH2, MSH6, and MLH1 were found to be associated with other DNA damage response proteins, including BRCA1, ATM, BLM, and the RAD50-MRE11-NBS1 protein complex, to form a DNA damage surveillance complex (736). Furthermore, cisplatin is reported to activate signaling kinases such as the c-Jun NH2-terminal kinase (JNK/stress-activated protein kinase) (512,513) and c-Abl tyrosine kinase (251,637) in a MMR-dependent manner (refer to Chapter 1, Section 1.3.4 for a detailed discussion of MMR-mediated apoptosis in response to cisplatin). Thus while MMR may serve upstream in DNA damage signaling, conferring signals of damage level to effectors in apoptosis, the effects of MMR deficiency in cisplatin toxicity may also be linked to an inability to trigger apoptosis in mammalian cells.

Taken together, the results on MMR and recombination show that recombination is a critical means for tolerating cisplatin-induced damage and that MMR, acting either by futile cycling or by an abortive repair mechanism, enhances the cytotoxic effects of cisplatin. However further work is required to determine if MMR is acting primarily as a block to

recombinational repair or as an enzymatic pathway leading to the production of additional strand breaks.

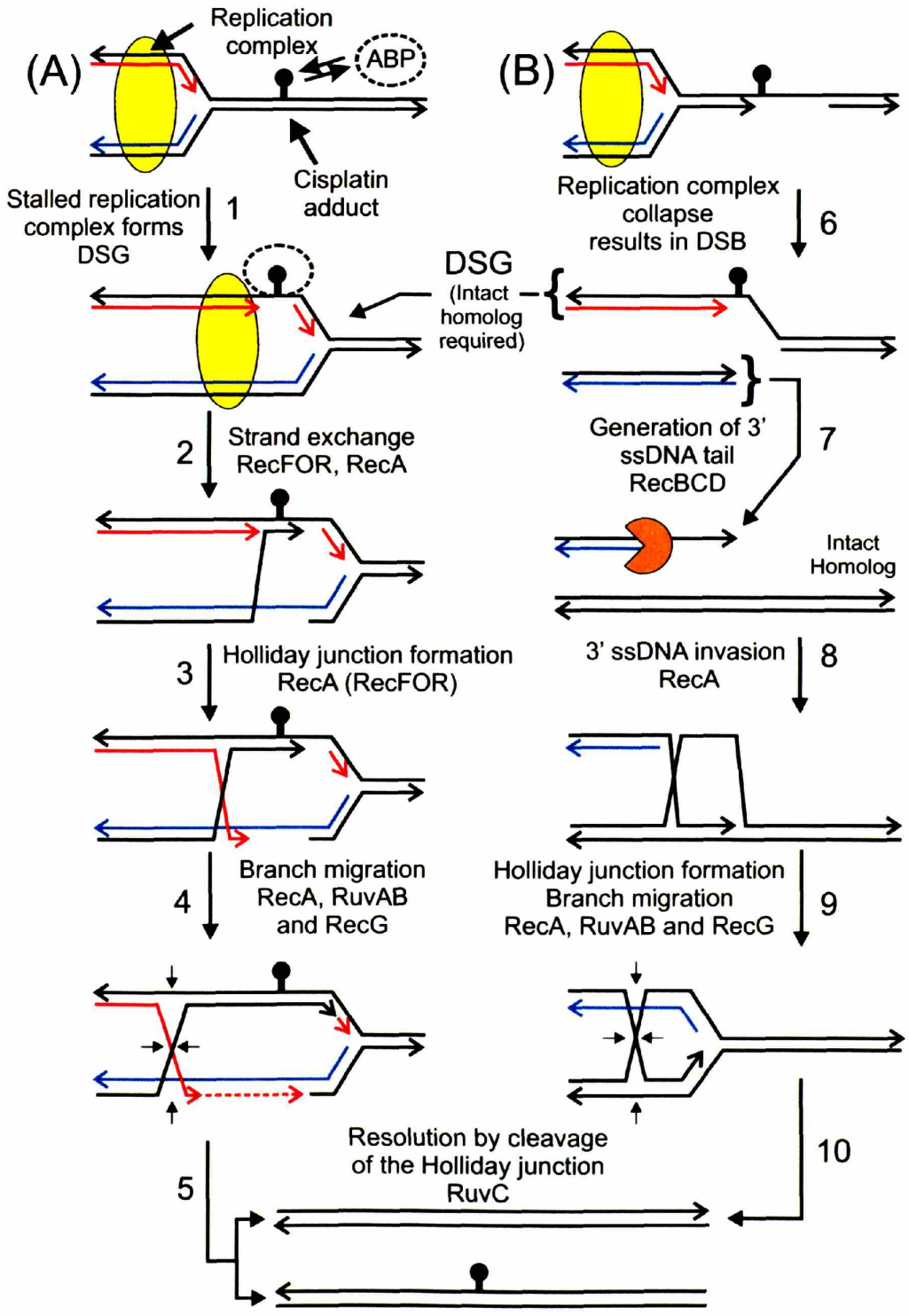


Figure 2.1: Recombination repair pathways in *E. coli*. Both daughter-strand gap repair (A) and double-strand break repair (B) pathways help cells tolerate replication-blocking lesions including cisplatin. Figure taken from Zdraveski et al. *Chem Biol* 2000 (7).

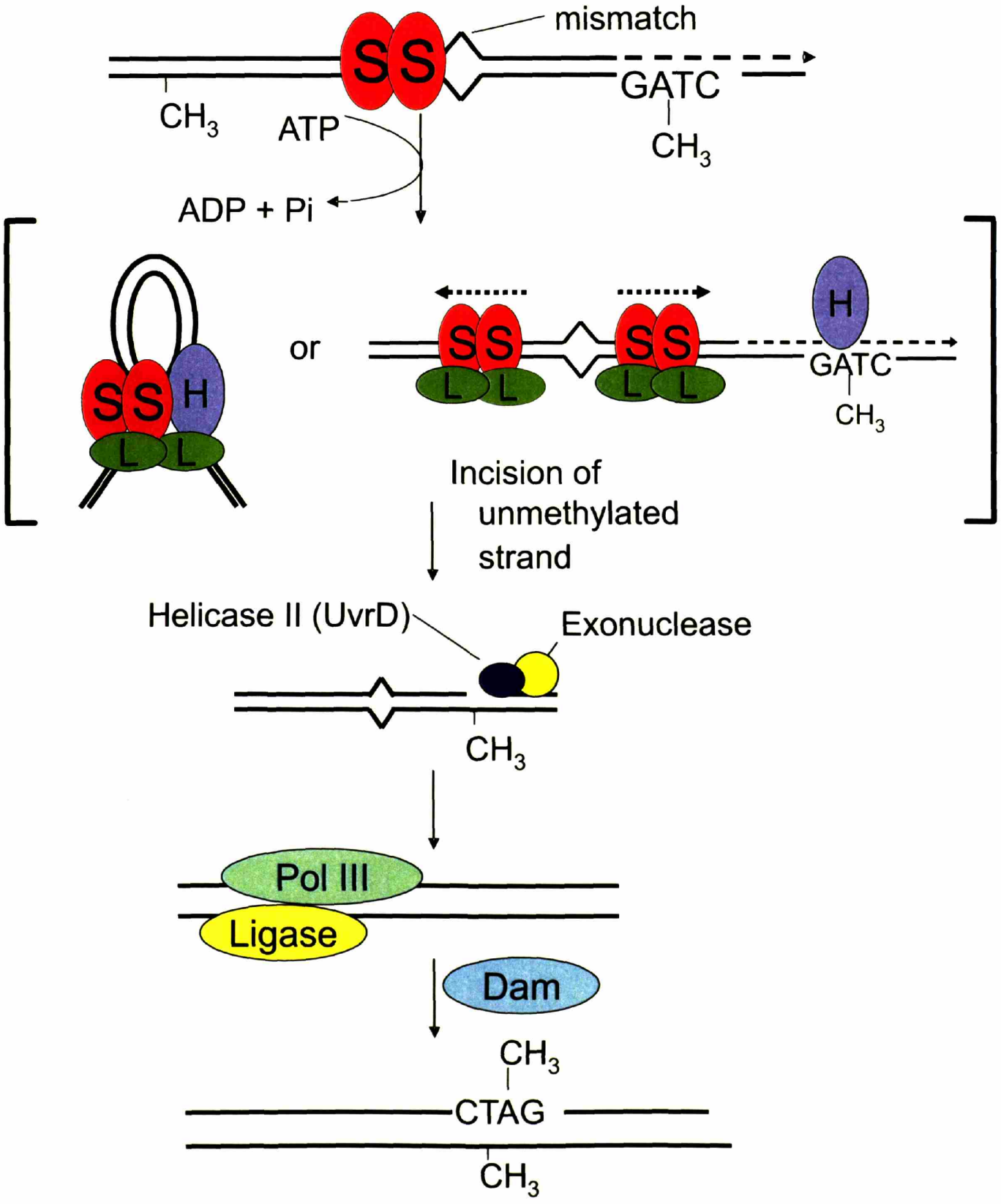


Figure 2.2: Methyl-directed mismatch repair in *E. coli*. MutS homodimer binds the mismatch. A complex is formed with MutL and MutH to form either a loop structure (left), or the MutS/MutL complexes diffuse away from the mismatch and reach MutH (right). MutH makes on incision on the unmethylated strand, followed by exonuclease digestion, DNA resynthesis and ligation.

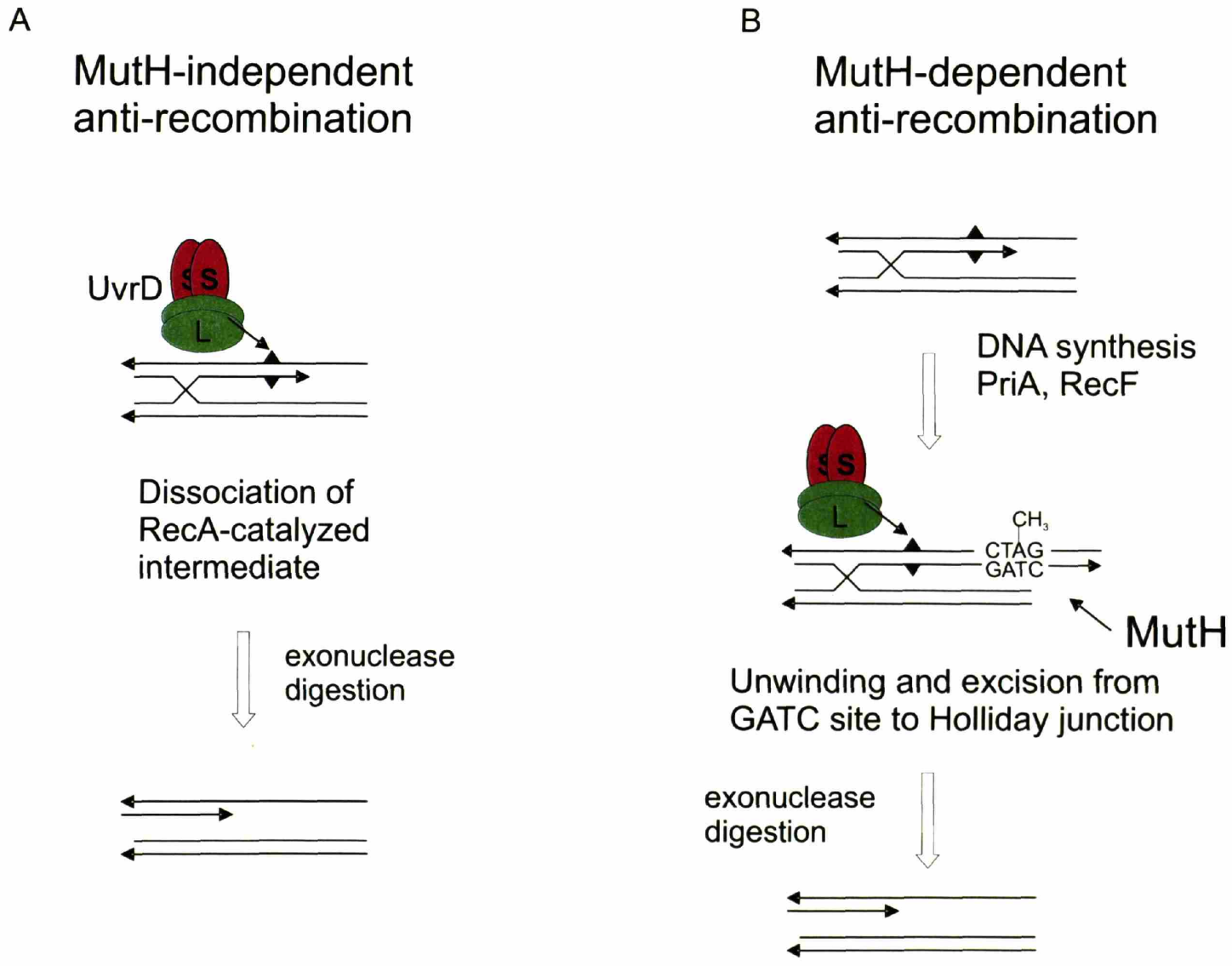


Figure 2.3: Anti-recombination pathways in E. coli. Mismatch repair may reverse the earliest RecA-catalyzed recombination intermediate (A), or, in a MutH-dependent pathway, mismatch repair may degrade the invading strand (B).

Chapter 3: Materials and methods

3.1 Bacterial strains

The strains used in this study are derivatives of AB1157 and the complete genotypes are listed in Table 3.1. We used the *dam-16::Kan* strain (GM3819) which carries a deletion of a large part of the *dam* gene. The auxotrophic phenotype of each mutant used in this work was confirmed by growth on the appropriate supplemented minimal medium. The strains used for the *sulA*::*lacZ* reporter assay are lambda *ind sulA*::*lacZ* lysogens of AB1157 (GM4352), GM3819 (GM4355) and GM5556 (GM5878).

The *dam mutH* mutant strain was constructed by P1 transduction. Briefly, the *Tn10* marker, which encodes TetR_N and TetR_C, was amplified from GM5555 genomic DNA using the primers; 5' - CAC TTG TCG GGC TGG TTA CGC CAG AGA ATT TAA AAC GCG ATA AAG CTC GAC ATC TTG GTT ACC GTG AAG - 3' and 5' - ATG GCT TCG GTA AGC GCT TTC GCA TTC GCT GCT TTC GGT CGT ATC CGC GGA ATA ACA TCA TTT GGT GAC - 3'. These primers contain 24 bases at their 3' ends to anneal to *Tn10* for PCR amplification, and 45 bases at the 5' end to target the resulting PCR fragment for recombination with the *mutH* gene (see below). The 2 kb product, identified by separating a fraction of the reaction products on a 1% agarose gel, was purified using the QIAquick PCR Purification kit. This DNA fragment was used for *mutH* gene replacement in KM22 cells as previously described by Murphy et al. (507) and as described briefly below.

KM22 cells (obtained from M. Marinus, UMass Worcester) were prepared for transformation by diluting 100 µL of an overnight culture in 10 mL LB plus 1 mM IPTG, growing (37°C, 250 rpm) until OD_{600} = 0.6, washing the cells twice with 15 mL cold water, and resuspending the final cell pellet in 75 µL 1 mM MOPS/20% glycerol. Approximately 100 µ L of cells and 500 ng of DNA was combined in a chilled electroporation cuvette and a 2.5 kV pulse at 129 Ohms was applied using a Electro Cell Manipulator 600 electroporation system (Bantex). Immediately after electroporation, cells were transferred to a culture tube containing 3 mL LB and 1 mM IPTG and grown at 37°C as above. After three hours, cells were harvested by centrifugation, resuspended in 100 µL LB, plated on LB containing 4 µg/mL Tetracycline, and incubated over night at 37°C. The next day, successful transductants were picked and grown over night in liquid culture with 12 µg/mL Tetracycline. Cultures were then analyzed individually for the correct genotype by performing PCR using primers to amplify *mutH*. The primers used for *mutH* analysis were 5'- ATG TCC CAA CCT

CGC CCA CT -3' and 5'- ACT GGC CCG TCA TTT TCT GAT CCA GTA G -3'. Because the 45 base sequences used to target the *Tn10* insertion were located 100 bases from each end of the mutH gene (690 bp), insertion of *Tn10* into *mutH* would replace the central 400 bases of *mutH* with ~2 kb. Therefore the mutH insertion deletion product would have an expected size of ~2.2 kb. Gel analysis on a fraction of the PCR products from all transformants analyzed showed a ~2.2 kb fragment, confirming insertion of *Tn10*. The KM22 *mutH::Tn10* allele was then used to construct the *mutH*⁻ derivative of GM3819 by P1 transduction, essentially as described by Miller.

TABLE 3.1 Genotypes of *E. coli* K-12 strains used in this study

Strain	Genotype	Source
AB1157	F⁻ *thr-1 araC14 leuB6*(Am)Δ *(gpt-proA)62 lacY1 tsx-33 supE44*(AS) *galK2*(Oc) *hisG4*(Oc) *rfbD1 mgl-51 rpoS396*(Am) *rpsL31*(Str^R) *kdgK51 xylA5 mtl-1 argE3*(Oc) *thi-1*	DeWitt and Adelberg (164)
GM3819	*dam-16*::Kan *thr-1 leuB6 thi-1 argE3 hisG4 proA2 lacY1 galK2 mtl-1 xyl-5 ara-14 rpsL31 tsx-33 supE44 rfbD1 kdgK51*	Parker and Marinus (543)
GM5555	As AB1157 but *mutS215::*Tn*10*	Lab. stock
GM5556	As GM3819 *dam-16*::Kan but *mutS215::*Tn*10*	Lab. stock
	As GM3819 *dam-16*::Kan but *mutH*::Tn10	This work (P. Rye)

3.2 Chemicals

Cisplatin was obtained from Sigma-Aldrich and dissolved in ddH2O at 37°C. The concentration of drug was determined by UV/Visible scan (165,417,737). The chemicals used as part of the Affymetrix GeneChip protocol were obtained from the suggested vendors.

3.3 Cytotoxicity Analysis

Overnight cultures were diluted 1000-fold and grown in Luria-Bertani medium until the cells reached exponential growth as determined by OD_{600}. The exponentially growing cells were resuspended in M9 minimal medium at a cell density of 2 X 10^8 cells/ml and treated with drug at the indicated doses for 2 hours at 37°C. Following treatment appropriate dilutions in M9 medium were plated on LB plates and incubated at 37°C until visible colonies could be counted. Results from three independent experiments plated in duplicate were averaged and plotted against drug concentration, ±SEM (standard error of the mean).

3.4 Array analysis

3.4.1 RNA preparation

Total RNA isolation and purification

Overnight cultures were diluted 1000-fold with fresh LB medium and cultured further. Log phase cultures were diluted to a cell density of 2 X 10^8 cells/ml in M9 salts and incubated at 37°C for two hours with or without drug, after which they were resuspended in LB broth for 90 minutes. OD_{600} were measured and total RNA was isolated from cells by extraction using the MasterPure RNA Purification Kit (Epicentre Technologies) followed by DNAse digestion according to the manufacturer's protocol. The isolated total RNA was quantitated by absorption at 260 nm (typical yield from a 15 ml culture was 250-500 µg of total RNA), and the purity was determined by the ratio of absorption values at 260/280nm. RNA quality was determined by formaldehyde agarose gel electrophoresis (1.2% agarose in FA Buffer pH 7.0 (20 mM 3-[N-morpholino]propanesulfonic acid, 5 mM sodium acetate, 1 mM ethylenediaminetetraacetic acid (EDTA))) or by analysis on an Agilent 2100 Bioanalyzer. All samples visualized by gel electrophoresis or by the bioanalyzer electropherogram showed clear distinct bands correlating to 16S and 23S ribosomal RNA, indicating that no detectable RNA degradation occurred and that RNA integrity was maintained throughout the RNA isolation procedure (data not shown).

mRNA enrichment, fragmentation, and labeling for arrays

mRNA was enriched from total RNA as described in the Affymetrix GeneChip Expression Analysis Technical Manual for GeneChip *E. coli* Sense Genome Arrays. In brief, reverse transcriptase and primers specific to 16S and 23S rRNA were used to

synthesize complementary cDNAs. Then rRNA was removed by treatment with RNase H (Epicentre Technologies), which specifically digests RNA within an RNA:DNA hybrid. The cDNA molecules were removed by DNase I (Amersham Biosciences) digestion and the enriched mRNA was purified on Qiagen RNeasy columns according to the manufacturer's protocol. Enriched mRNA was then fragmented by heat and ion-mediated hydrolysis. The 5'-end RNA termini were then modified by T4 polynucleotide kinase (New England Biolabs) and γ-S-ATP (Boehringer Mannheim). Next a biotin group (PEO-iodeacetyl-Biotin, Pierce Chemical) was conjugated to 5'-ends of the RNA. The conjugated product was purified using RNA/DNA Mini Column Kit (Qiagen) and the efficiency of the labeling procedure was determined by a gel-shift assay on a 4-20% TBE Gel (Invitrogen, data not shown). Target hybridization and probe array washing, staining, and scanning were performed as described in the Affymetrix Manual.

3.4.2 Data analysis

Basal comparisons between mutant strains and wild-type

Experiments for array analysis were performed in biological replicates with three independent experiments for each strain. Signal intensities of each array were normalized by Robust Multi-array Average (RMA) version 0.1 release (55,319) and normalized signal intensities for each gene were averaged across the three biological replicates for each strain. Changes in gene expression are given as signal log ratios (base 2), and analysis of variance (ANOVA) was used to compare gene expression values in each mutant strain using wildtype *E. coli* as the baseline for comparison. All microarray data analysis (i.e., RMA normalization and ANOVA) was performed using the Array Analyzer module version 1.1 in S-Plus (Insightful) version 6.0. Transcripts were filtered based on a *p*-value threshold of 0.05 as well as a fold change threshold of two (signal log ratio $1 \leq x \leq -1$). Differentially regulated genes were annotated and categorized according to general function by the NCBI Clusters of Orthologous Groups database. We determined the number of d(GATC) sites in the promoter/regulatory regions of genes differentially expressed in our analysis by searching the upstream sequence regions (-400 base pairs from the transcriptional start site) of each gene. Sequences searched were provided on *coli*base (http://colibase.bham.ac.uk/), and the number of expected GATC sites occurring by chance in 400 bp was taken from Oshima et al. (535) and estimated to be one GATC site every 256 base pairs (or between one and two sites in the searched regions). Raw microarray data

and RMA-normalized data is available at the NCBI Gene Expression Omnibus (GEO) repository under the accession number GSE2928.

Pairwise comparisons between drug-treated and mock-treated cultures

Signal intensities of each array were normalized by a variation of Robust Multi-array Average (RMA) (55,319) called GCRMA (753) and normalized signal intensities for each gene were averaged across the replicates for each strain. Probe sets that were designated as absent for all chips were filtered out of the data set. Pairwise comparisons were made for each gene to determine cisplatin-induced fold changes within each strain, and changes in gene expression are given as signal log ratios (treated/untreated, base 2). To compare gene expression values of treated versus mock treated samples, a value of significance was determined using the Local Pooled Error test (LPE) (324), and *p*-values were adjusted by the Benjamini and Hochberg method (40) to allow a false discovery rate of 0.10. Signal log ratios for each gene having a *p*-value ≤ 0.10 are listed in Chapter 5, Tables 5.3-5.6. To include basal gene expression changes (e.g., gene expression values measured in a *dam* mutant compared to the wild-type strain), signal log ratios and LPE p-values for a mutant (mock-treated) versus wild-type (mock-treated) were also calculated for each gene. All data analysis, including RMA normalization and LPE, was performed using the Array Analyzer module version 2.0.2 in S-Plus version 6.2 (Insightful), and GCRMA-normalized data is available at the Gene Expression Omnibus repository under accession number GSE2999. Differentially expressed genes were grouped according to general function categories provided by NBCI Clusters of Orthologous Groups (COGs).

3.5 Semi-quantitative real-time RT-PCR

Total RNA was isolated from cultures and quality of RNA was determined as described above. DNAse digestion for total RNA was performed using amplification grade Deoxyribonuclease I (Invitrogen). Briefly, total RNA was incubated with 1 U DNase I per microgram RNA in 200 mM Tris-HCl pH 8.4, 20 mM $MgCl_2$, 500 mM KCl for 15 minutes at room temperature. The reaction was terminated by the addition EDTA (2 mM final concentration) and by heating for 10 minutes at 65° C. cDNA synthesis was performed using random hexamer primers (Invitrogen) and the Omniscript Reverse Transcriptase Kit (Qiagen) according to the manufacturer's protocol. In order to ensure that the amplification observed in the PCR reactions was due to cDNA template made from mRNA and not from contaminating genomic DNA, controls were carried out for each sample using the same

conditions except that reverse transcriptase was not added to the reactions. Semiquantitative real-time PCR reactions were performed with QuantiTect SYBR Green (Qiagen) according to the manufacturer's protocol on a DNA Engine Opticon thermal cycler (MJ Research). Primers specific to each target gene were designed using Primer3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi/)) and are listed in Table 3.2.: Optimal melting temperatures for each primer pair were determined by performing real time analysis with a temperature gradient ranging over 10 degrees (± 5°C from the optimal calculated T_m for each primer pair) and negative controls with no template cDNA were performed to ensure that primers alone did not yield an amplification product. Relative gene expression values for the samples were measured by including a standard curve analysis for each gene assay; a separate batch of wildtype cDNA template was used to make a series dilution, and amplified product from this dilution series was used to make a standard curve by which to quantify the relative amount of product in each experimental sample. To account for variation in the efficiency of the reverse transcription reaction between samples, we performed RT-PCR for the constitutively expressed *gapA* (D-glyceraldehyde 3-phosphate dehydrogenase) gene and normalized the gene expression values detected in each sample to the value determined for *gapA*. Each experiment was carried out in triplicate so each relative gene expression value reported for each strain represents the average of three independent biological replicates. The student t-test (two sample, two tailed) was used to determine if the expression values of a given gene were significantly different between strains.

Table 3.2 Primer sequences for real-time RT-PCR

Gene	Left primer	Right primer
recA	5′-TACAGCTACAAAGGTGAGAAGATCG-3′	5′-TTCGCTATCATCTACAGAGAAATCC-3′
lexA	5′-GCATATTGAAGG TCATTATCAGGTC-3′	5′-ACCGTTACGTACATCCTGAGTTTT-3′
recN	5′-GTACAGCTGTTCCTCTGTCA CAAC-3′	5′-GTCATTTCCTGCAG TAGAGAGGTT-3′
sulA	5′-CAACTTCTACTGTTGCCATTGTTAC-3′	5′-AGAGCTGGCTAATCTGCATTA CTT-3′
yebG	5′-CGAAGAGAAAATGTCGTTT ACCAG-3′	5′-CTCAGCACATCTTTTTGTTCTGC-3′
dinI	5′-AGTA TGCGTTTCCTGATAATGAAGG-3′	5′-TATTCGCTGACAAACCAGTCAT-3′
uvrA	5′-ATAAAGTGGTGTTGTACGGTTCTG-3′	5′-CACGGAC GATTACTGATAAACTTG-3′
uvrB	5′- GTTTCCACTATTCCACGTTTTACC -3′	5′-GTAGTTTTCAATCCCCGAACAGTA-3′
cho	5′-GTGGTACGGCGTT TAACTTCTC-3′	5′-GTTAACGCTTTTGCCGATATAGAG-3′
ruvA	5′-CCTGTTTTTATGAACTCCCTGAAG-3′	5′-CTCAACGGCATTAACG AACTG-3′
ruvB	5′-GTTCGTTCACAGATGGAGATTTTC-3′	5′-GA TCTCATCAATAAACAGCACGTC-3′
priA	5′-GTGTGATTTAGCAAGTGAAACACC-3′	5′-TTTCCAGT ACGCTGAGATAAACCT-3′
priB	5′-GAAAGGTCAGTCCATCAGGAAT-3′	5′-CCGACCGTTATACTGTGAGTAATG-3′
gapA	5′-TATGACTGGTCC GTCTAAAGACAA-3′	5′-GGTTTTCTGAGTAGCGGTAGTAGC-3′
rrlG	5′-GAATCAGTGTGTGTGTTAGTGGAAG-3′	5′-CACTATCGGTCAGTCAGGAGTATTT-3′

3.6 Neutral single cell gel electrophoresis

Cultures were grown as described above. Microgel electrophoresis was performed as described in detail by Singh (641,643,644). Briefly, small aliquots (0.25 µl) of cells were mixed with 50 µl of 0.5% agarose (biotechnology grade 3:1, Amresco). Agarose containing cells was then immediately transferred to an MGE microscope slide (Erie Scientific, Portsmouth, NH) pre-coated with 50 µl agarose, and after cooling another layer of agarose (200 µl) containing 5µg/ml RNAse A (Amresco), 0.25% sodium *N*-lauroyl sarcosine, and 0.5 mg/ml lysozyme (Amresco) was added with a coverglass. Slides were incubated at 4°C for 10 minutes, after which they were transferred to a humidified chamber at 37°C and incubated for 30 minutes. Coverglasses were then removed and the slides immersed in lysis buffer (2.5 M NaCl, 100 mM EDTA tetra sodium salt, 10 mM Tris pH 10, 1% sodium *N*-lauroyl sarcosine, 0.1% Triton X-100) and incubated for 8 hours. Slides were then immersed in buffer for protein digestion (2.5 M NaCl, 10 mM EDTA, 10 mM Tris pH 7.4) containing 1 mg/ml Proteinase K for 2 hours at 37°C. After protein digestion, the slides were placed in a modified electrophoresis unit (TECA 2222, Ellard Instrumentation, Monroe, WA) with buffer (300 mM sodium acetate, 100 mM Tris pH 9) and allowed to equilibrate for 20 minutes, after which electrophoresis was performed for 1 hour at 12 V (0.4 V/cm) with buffer recirculation at 100 ml/min. Following electrophoresis slides were incubated in solution

containing 1:1 ethanol: 20 mM Tris pH 7.4 containing 1 mg/ml spermine for 30 minutes, and slides were then transferred to fresh solution for another 30 minutes. Slides were allowed to air dry until analysis. Dried slides were stained with 50 µl of 0.25 µM YOYO-1 iodide (Molecular Probes) in 2.5% dimethyl sulfoxide and 0.5% sucrose and immediately viewed on a Nikon Eclipse E600 fluorescence microscope equipped with a 100X/1.25 oil-immersion Fluor lens and a B-2A filter set (exciter, 490 nm; emitter, 515 nm). Three to four independent experiments were performed for each strain, and each experiment consisted of two slides per strain with at least 50 observations taken per slide, giving a total of at least 400-600 observations per strain. Pictures were analyzed by Komet analysis software version 4.0.2 (Kinetic Imaging Ltd.) and by visually counting individual strand breaks or tails for each cell. The frequency at which no DSBs occurred, one DSB occurred, and so on was determined for each strain. We assume that one tail corresponds to one double-strand break (54). To determine if the frequency at which DSBs occurred between mutant and wildtype was different, we performed the Mann-Whitney U test to compare the frequency of DSBs in each mutant strain to the frequency observed in wildtype. For drug-treated cultures, pictures were analyzed by Komet image analysis software or by visually counting strand breaks where counting was feasible. To determine if the frequency at which DSBs occurred in each group was different, we performed the Mann-Whitney U test to compare the frequency of DSBs upon increasing drug dose within strains as well as between mutant strain and wild-type at each drug dose.

3.7 Electrophoretic mobility shift assay

3.7.1 Preparation of platinum-modified DNA probes.

Platinum-modified DNA probes were prepared as previously described (473,563). Briefly, oligonucleotides were purchased from Integrated DNA Technologies and purified by gel electrophoresis. The platination reaction was carried out in 5 mM Na_3PO_4 buffer, pH 7.4, at 37 °C for 18-21 h, and the platinated DNA was purified by high performance liquid chromatography on a C18 column (reverse-phase) and the presence of the adduct was confirmed by MADLI-TOF (data not shown). The complementary strands (bottom strands in Fig. 5.1) were radiolabeled with [γ-^{32}P] dATP and the DNA strands were hybridized by heating the top and bottom strands for 5 min at 80 °C and then letting the mixture cool for 14-20 h. The sequences of the DNA duplexes are shown in Fig. 5.1. Concentrations were determined by measuring A_{260}.

3.7.2 Binding assay

MutS was purchased from USB and provided in 50% glycerol. Before the binding assay glycercol was removed by dialysis against 20 mM KPO_4, pH 7.4, 0.1 mM EDTA, 1mM PMSF and 10 mM beta-mercaptoethanol. Binding assays contained the radiolabeled DNA duplex probes (1-20 nM), either unmodified or modified with cisplatin, and MutS present at 0-200 nM or 0-20 pmoles. Binding reactions were carried out in 10 µl solutions containing 20 mM Tris pH 7.6, 5 mM MgCl2, 0.1 mM DDT, 0.01 mM EDTA, and 50 ng of nonspecific salmon sperm (Stratagene) or chicken erythrocyte competitor DNA for 30 minutes on ice. Samples were then loaded onto 6% (29:1) acrylamide:bis) native polyacrulamide gels containing TAE buffer (90 mM Tris (pH 8.0), 2.0 mM EDTA, 90 mM boric acid) and 5% sucrose, and separated by electrophoresis at room temperature in TAE buffer at ~ 25 mA (140V) for 2 h. Quantitative analysis was determined by Molecular Dynamics Storm system and ImageQuant software.

3.8 SulA::lacZ reporter assay

Overnight cultures in LB broth were diluted ten-fold in minimal salts and 3 ml portions placed on MacConkey agar plates for 10 min and the residual liquid removed by aspiration. The plates were allowed to dry at room temperature and filter paper disks were placed on the surface. Cisplatin (1.2 mg/ml) was added to the disks in 5, 10, and 20 µl volumes. Water was used on the control disk. The plates were incubated overnight at 37°C.

Chapter 4: Analysis of global gene expression and double-strand break formation in DNA adenine methyltransferase (dam) deficient and mismatch repair deficient *Escherichia coli*

Adapted from:
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Abstract

DNA adenine methylation by DNA adenine methyltransferase (Dam) in *Escherichia coli* plays an important role in processes such as DNA replication initiation, gene expression regulation, and mismatch repair. In addition, *E. coli* deficient in Dam are hypersensitive to DNA-damaging agents. We used genome microarrays to compare the transcriptional profiles of *E. coli* strains deficient in Dam and mismatch repair (*dam*, *dam mutS*, and *mutS* mutants). Our results show that >200 genes are expressed at a higher level in the *dam* strain while an additional mutation in *mutS* suppresses the induction of many of these same genes. We also show by microarray and semiquantitative real-time RT-PCR that both *dam* and *dam mutS* strains show de-repression of LexA-regulated SOS genes as well as the up-regulation of other non-SOS genes involved in DNA repair. To correlate the level of SOS induction and the up-regulation of genes involved in recombinational repair with the level of DNA damage, we used neutral single-cell electrophoresis to determine the number of double-strand breaks per cell in each of the strains. We find that *dam* mutant *E. coli* have a significantly higher level of double-strand breaks compared to the other strains. We also observe a broad range in the number of double-strand breaks in *dam* mutant cells with a minority of cells showing as many as ten or more double-strand breaks. We propose that the up-regulation of recombinational repair in *dam* mutants allows for the efficient repair of double-strand breaks whose formation is dependent on functional mismatch repair.

4.1 Introduction

The DNA adenine methyltransferase (Dam) protein methylates the N6 position of the adenine residue at d(GATC) sites of the *E. coli* genome. Dam methylation is a postreplicative process (444), and consequently the newly synthesized daughter strand is unmethylated for a short time after passage of the replication fork. This transient hemimethylated state following DNA replication plays a crucial role in processes such as the regulation of gene expression (130,442,538), DNA mismatch repair (37,538,622), and the timing of chromosome replication initiation (23,420,526,603). By altering the recognition sequences of transcriptional regulators and RNA polymerases, Dam methylation may affect the ability of proteins to bind the upstream regions of genes and in such a way may serve to regulate gene expression. Because d(GATC) sites are not randomly distributed in the *E. coli* genome (279,535), Dam deficiency may therefore have a direct effect on gene expression patterns.

In methyl-directed mismatch repair, hemimethylated d(GATC) sites serve as the strand discrimination signal so that mismatch repair can differentiate between parent (methylated) and daughter (unmethylated) strands (490). The mismatch repair system relies on three unique proteins: MutS, MutL, and MutH. If there is a misincorporation error following the replication fork, MutS recognizes the mismatch and a protein-DNA complex is formed with MutS, MutL and the latent endonuclease MutH. Activated MutH then makes an incision on the unmethylated, or newly synthesized, strand at a d(GATC) site located either 5' or 3' to the mismatch (20,126,256). Methylation status therefore allows mismatch repair to act on the new strand while preserving the sequence of the template strand, and in this way mismatch repair helps protect the genome against mutations arising from misincorporated deoxynucleotides. In the case where Dam is absent and the genome is unmethylated at d(GATC) sites, MutH cannot distinguish between the new and template strands; *in vitro* experiments show that in this situation MutH aimlessly makes an incision on either strand, although the endonuclease shows reduced activity on unmethylated compared to hemimethylated substrates (20,740). Furthermore, Au et al. (20) have shown in a reconstituted *in vitro* system that in the absence of d(GATC) methylation MutH can make incisions on both DNA strands and form a double-strand break (DSB).

E. coli deficient in Dam exhibit pleiotropic changes that have helped uncover many functions of adenine methylation. Dam-deficient strains display a mutator phenotype (447),

which most likely results from mismatch repair activity on template rather than daughter strands following replication errors. Interestingly, a mutator phenotype is also conferred by the overexpression of Dam (282,759), which may result in fully methylated DNA following the replication fork that is resistant to MutH incision. Other studies have shown that the SOS response is constitutively induced (sub-induced) in the absence of Dam (524,558,607). The induction of SOS response genes in *dam* mutant strains may be due to the presence of single-stranded DNA resulting from errant MutH incisions. However *dam mutS/L/H* strains in which mismatch repair is inactivated also show SOS sub-induction (557). Dam-deficient *E. coli* are also hyper-recombinogenic (445,446) and double mutants deficient in both Dam and recombinational repair (*dam recA*, *-B*, *-C*, and *dam ruvA*, *-B*, *-C*) are inviable (443,447,557,731). Wang and Smith (731) have shown that the requirement for recombination in *dam E. coli* is correlated to the mismatch repair-dependent production of double-strand breaks (DSBs), and accordingly mutations in mismatch repair (*mutL* or *mutS*) allow the recovery of *dam rec* mutants.

Wang and Smith (731) determined the relative levels of DSBs in *dam*, *recB* (temperature sensitive mutant, Ts), *dam recB*(Ts) and *dam recB*(Ts) *mutS/L* cells; they could only detect DSBs in the *dam recB*(Ts) strain and not in the *dam*$^-$ only cells. However we expect *dam* mutant cells to exhibit a higher level of DSBs compared to wildtype because *dam* mutants deficient in DSB repair are inviable (443,447,558). Furthermore, *dam* mutants are hypersensitive to exogenous DNA-damaging agents and this hypersensitivity is abrogated by an additional mutation in mismatch repair (224); this hypersensitivity may be due to basal differences in *dam* mutants that, like DSB formation, are dependent on mismatch repair. In order to help elucidate the global changes resulting from Dam deficiency and the role of mismatch repair in producing these changes, we determined the global gene expression profiles of wildtype, *dam*, *dam mutS*, and *mutS E. coli* strains by microarray analysis of ~4,200 open reading frames. We also measured the number of DSBs in each of the strains and correlate the level of basal DNA damage with the induction of genes involved in recombination and DSB repair. Our findings show that many genes involved in carbohydrate metabolism and transport, energy production and conversion, cell motility, and translation are up-regulated in *dam* mutant *E. coli*, whereas an additional mutation in *mutS* suppresses gene induction. We also see up-regulation of several SOS-response genes in both *dam* and *dam mutS* mutants. However only the *dam* mutant cells

show a higher level of DNA DSBs compared to wildtype, and double-strand break formation in *dam* mutant *E. coli* is dependent on functional mismatch repair.

4.2 Results

4.2.1 Dam-deficient strains show a higher number of basal gene expression changes compared to the *mutS* strain

We measured the global gene expression patterns in four *E. coli* strains using the Affymetrix microarray platform. We isolated mRNA from three independent biological replicate cultures for wildtype, *dam*, *dam mutS*, and *mutS* strains, and the gene expression values from the replicates were averaged. Gene expression changes in mutant strains are given as signal log ratios (mutant/wildtype, log base 2) and analysis of variance (ANOVA) was performed to compare gene expression levels in each of the mutant strains to the gene expression levels measured in wildtype *E. coli*. Figures 4.1A-C show the log *p*-value versus signal log ratio for all transcripts, and the bold lines mark the boundaries by which the data were filtered; the bold horizontal line represents the *p*-value threshold while the two bold vertical lines mark the signal log ratio threshold applied to the data. Genes whose expression show at least a ± 2-fold change compared to wildtype (signal log ratio ± 1) with a *p*-value ≤ 0.05 are represented in Figs. 4.1D and 4.1E and are listed in Tables 4.2-4.4. When we compare the basal gene expression differences between each of the mutant strains and wildtype, the results show that while the *mutS* strain does not display many differences from wildtype at the transcriptional level, both *dam* and *dam mutS* strains show differential expression of 206 and 114 genes, respectively. The majority of genes differentially expressed are expressed at higher levels (induced) in the mutant strains compared to wildtype (Fig. 4.1D). Among the most prominent groups of up-regulated genes are those encoding ribosomal subunit proteins and products involved in carbohydrate transport and metabolism (Fig. 4.1E). In particular, several genes encoding proteins in the sugar phosphotransferase system (PTS) and maltose transport are up-regulated in the Dam-deficient strains. *Dam E. coli* also show up-regulation (≥ 2-fold induction, $p \leq 0.05$) of many genes involved in energy production and conversion (24 genes), cell motility (17 genes) including flagellar biosynthesis, amino acid transport and metabolism (9 genes), transcription (8 genes) as well as genes of unknown function (12 genes). *Dam mutS E. coli* also show up-regulation of genes involved in cell motility (5 genes) and genes of unclassified or unknown function (24 genes).

The higher level of global transcription in the Dam-deficient strains may be due to genes whose regulation is directly controlled by adenine methylation. While some transcriptional activators bind unmethylated DNA (63), the absence of Dam methylation may lead to the up-regulation of genes whose promoters/regulatory regions are normally methylated and therefore whose transcriptional activators are usually bound only following replication when the DNA is transiently unmethylated. Alternatively, genes whose repressors require adenine methylation for DNA binding affinity may also be up-regulated in the absence of Dam. Under such scenarios, Dam deficiency would directly affect the transcription of genes and would lead to increased gene expression. Although we were unable to find a correlation between gene expression level and the presence of d(GATC) sites in upstream sequence regions (-400 base pairs from the transcriptional start sites) in *dam* or *dam mutS* strains (Supplementary Tables 1 and 2), we do see expression changes of certain genes known to be regulated in a Dam-methylation-dependent manner. For example, *dnaA* expression is reduced in both *dam* and *dam mutS* strains (signal log ratio = -.368, p = .035 and -.412, p = .025, respectively) as shown previously in *dam* mutant strains (69,421). Methylation of d(GATC) sites in the dnaAp2 promoter, two of which are in the -35 and -10 sequences, may affect the affinity of DNA binding proteins that regulate the expression of *dnaA* (69,143). In addition, we find expression of the phase variation *flu* gene (*agn43*) to be slightly down-regulated (signal log ratio = -.383, p = .016) in *dam E. coli*. While *agn43* transcription is controlled in a methylation-dependent manner, it is DNA methylation of three d(GATC) sites in the regulatory region that prevents binding of the repressor OxyR and that therefore leads to *agn43* expression and the ON phase (130,725). Løbner-Olesen et al. (421) have shown that a 10-fold overproduction of Dam leads to a 20-fold induction of *flu* expression.

4.2.2 The effects of MutS on gene expression in a *dam* mutant background: differences between *dam* and *dam mutS* strains

Both *dam* and *dam mutS* strains show a high level of gene induction compared to wildtype (Fig. 4.1A and B). The *dam mutS* strain, however, shows higher variability among some of these induced genes (Fig. 4.1B); specifically, 39 genes that show 2-fold higher expression in *dam mutS* compared to wildtype are filtered out of the data set due to their high variability (i.e., they do not meet the p-value threshold), and of these 39 genes 15 encode ribosomal proteins. The remaining genes are mostly involved in protein and carbohydrate metabolism. Data in Fig. 4.1D and 4.1E show the filtered data set that

includes genes showing at least ± 2-fold change and $p \leq 0.05$. Fig. 4.1E shows that many of the expression changes in *dam* and *dam mutS* strains fall under common categories, with carbohydrate transport/metabolism and translation accounting for close to 50 % of the transcriptional changes in these strains. Many of the genes induced in *dam mutS* are also induced in the *dam* strain; that is, the genes induced in *dam* include the majority of the genes induced in *dam mutS*, and it is the case that the *dam* strain appears to show up-regulation of more genes rather than induction of a distinct set of genes within the categories shown in Fig. 4.1E (refer to Tables 4.2-4.4 for a complete list of genes). There is a striking difference between these two strains, however, in the number of induced genes encoding for products in energy production and conversion. The *dam* strain displays a high number of induced genes falling under this category whereas the *dam mutS* strain does not (Fig. 4.1E). Therefore it appears that MutS deficiency in a *dam* mutant background reduces the number of induced genes in this category as well as decreases the overall number of transcriptional changes. This effect of MutS is only observed in the *dam* background, as *mutS E. coli* closely resemble wildtype and show transcriptional changes for only 17 genes. The transcriptional changes observed in the mutant strains are not attributable to different growth stages, as all four strains showed similar growth rates in culture at the time RNA was isolated (data not shown).

4.2.3 *Dam* and *dam mutS* strains display constitutive SOS and up-regulation of genes involved in DNA recombination and repair

The SOS response is induced when RecA is activated in the presence of single-stranded DNA (723). Activated RecA then facilitates the autocleavage of the LexA repressor and the transcriptional activation of those genes whose operons are normally bound and repressed by LexA. Our microarray data show that several LexA-regulated genes are induced in *dam* and to a lesser extent in *dam mutS E. coli*. The analysis shows that the genes *lexA*, *recA*, and *yebG* are all induced at least two-fold in the *dam* strain compared to wildtype ($p \leq .001$, Table 4.1). The LexA-regulated genes *sulA* and *recN* also show induction in the *dam* strain, with signal log ratios (*dam*/WT) of 0.95 ($p = .004$) and 0.762 ($p = .022$) respectively. Many of these genes (*lexA*, *recN*, *sulA*, and *yebG*) are also induced in the *dam mutS* strain (Table 4.1). The SOS genes involved in nucleotide excision repair (*uvrA*, *uvrB*, and the recently characterized gene *cho* (494)) do not show induction in the *dam* and *dam mutS* strains by microarray analysis, although our RT-PCR results show

that the *cho* gene is expressed at a significantly higher level in the *dam* strain compared to wildtype (see below and Table 4.1).

RecN, a protein known to be involved in DSB repair (387,560), is not required for *dam* mutant survival as *dam recN* mutants are viable (557). However we find that *recN* is significantly induced in the *dam* and *dam mutS* strains. We also see a moderate but significant ($p < 0.05$) induction of a non-SOS gene *recG* in the *dam* strain (signal log ratio = .300, $p = .013$). The RecG helicase catalyzes branch migration of three- and four-stranded DNA junctions *in vitro* and is proposed to catalyze fork regression *in vivo* (464,465,594); RecG has been shown to be important for tolerating DSBs (387,471) and may be required for *dam* mutant viability (443).

Several other genes involved in recombinational repair show unique up-regulation in *dam* and *dam mutS E. coli*. The primosomal gene *priB* shows up-regulation in these strains. PriB is a structural protein of the primosome, which allows replication restart at recombination intermediates including sites of template damage where the replication fork collapses (515,613). The genes encoding for the subunits of the *E. coli* histone-like protein (HU), *hupA* and *hupB*, are also induced in Dam-deficient *E. coli*. HU is involved in recombinational repair (174,411), and *hupAB* double mutants are hypersensitive to both γ-irradiation (60) and UV-induced damage (411).

We performed semi-quantitative real time PCR to confirm the gene expression changes we observed by microarray analysis. For the RT-PCR studies, we isolated total RNA from three independent replicates using the same procedure we used for the microarray experiments. After DNAse digestion, cDNA for each sample of isolated total RNA was made using random hexamer primers and reverse transcriptase, and controls with no reverse transcriptase were performed for each sample to ensure that genomic DNA was removed by digestion. To determine the relative expression levels for a particular gene, all cDNA samples and no reverse transcriptase controls were run in the same assay along with samples for a standard curve. We accounted for variation between samples by normalizing the expression level of each gene in a sample to the amount of housekeeping gene message (*gapA*) in that sample. After normalization to *gapA* level, we set the expression level for each gene detected in wildtype to a value of one and adjusted the expression levels in the other strains accordingly (Fig. 4.2). We performed the student t-test to compare the amount of transcript measured in a mutant strain to the amount in wildtype, and

comparisons for which $p \leq 0.05$ are designated in Figure 4.2 by an asterisk. Our RT-PCR results confirm the microarray data and show that the SOS response genes *recA*, *lexA*, *sulA*, *dinI*, and *yebG*, are all expressed at higher levels in *dam* compared to wildtype, while the genes *lexA* and *yebG* are also expressed at a significantly higher level in *dam mutS* compared to wildtype *E. coli* (Fig. 4.2A). We also used real-time RT-PCR to measure the levels of other SOS genes involved in recombinational repair and show that the genes *recN*, *ruvA*, and *ruvB* are highly expressed in *dam* and *dam mutS* strains compared to wildtype (Fig. 4.2B). The *uvrC* homologue, *cho*, which has been shown to be up-regulated as part of the SOS response (206), is also significantly induced in the *dam* strain (Fig. 4.2C). The SOS response genes are not induced in *mutS E. coli*.

The relative expression changes determined by array and RT-PCR correlate as to the direction of change for the LexA-regulated genes discussed. Expression changes measured by these methods however do not always show the same absolute magnitudes of change. In most cases RT-PCR detected slightly lower magnitudes of change. For example, in *dam E. coli*, the SOS gene displaying the highest level of induction by microarray analysis is *recA* (4-fold induction), while RT-PCR detected a two fold induction of *recA* in *dam* compared to wildtype (Table 4.1). In general, however, our levels of SOS response gene induction in *dam* cells compared to wildtype are consistent with those determined by Peterson et al. (558) using the beta-galactosidase reporter assay. The RT-PCR data also confirm the microarray data in showing that the non-SOS genes *priB* and *hupB* are moderately induced in *dam E. coli*.

TABLE 4.1 Differential basal gene expression (mutant/WT) of several SOS response genes and other genes involved in DNA maintenance determined by microarray analysis and RT-PCR

lexA-regulated SOS response gene		Array Signal log ratio* (ANOVA p-value)			RT-PCR Signal log ratio* (t-test p-value)		
Gene name	Blattner Number	*dam*/WT	*dam mutS*/WT	*mutS*/WT	*dam*/WT	*dam mutS*/WT	*mutS*/WT
lexA	b4043	1.56 (.001)	.914 (.051)	.223 (.498)	.970 (.009)	.997 (.023)	.283 (.238)
recA	b2699	2.02 (.000)	1.69 (.000)	.348 (.278)	1.19 (.001)	.986 (.088)	-.009 (.491)
recN	b2616	.752 (.022)	.835 (.008)	.269 (.350)	1.66 (.013)	1.53 (.015)	-.593 (.278)
sulA	b0958	.950 (.004)	.784 (.010)	.232 (.427)	1.47 (.004)	.870 (.068)	-.086 (.424)
yebG	b1848	1.60 (.001)	1.14 (.001)	.049 (.856)	1.72 (.046)	1.59 (.012)	.314 (.305)
ruvA	b1861	.240 (.541)	.182 (.784)	.227 (.553)	1.21 (.002)	1.09 (.073)	.317 (.147)
ruvB	b1860	.094 (.843)	-.159 (.569)	.107 (.802)	1.12 (.003)	1.07 (.005)	.584 (.113)
dinI	b1061	.652 (.091)	.448 (.232)	.360 (.446)	1.32 (.002)	.420 (.256)	-.832 (.135)
uvrA	b4058	-.277 (.240)	-.308 (.195)	-.121 (.584)	.637 (.041)	.669 (.060)	.441 (.166)
uvrB	b0779	-.155 (.468)	-.095 (.671)	.273 (.283)	.966 (.047)	.821 (.064)	.327 (.231)
cho/ *ydjQ*	b1741	-.150 (.520)	-.156 (.331)	-.133 (.414)	1.52 (.014)	.974 (.050)	.275 (.210)
Genes involved in DNA repair/replication restart (non-SOS)		Array Signal log ratio* (ANOVA p-value)			RT-PCR Signal log ratio* (t-test p-value)		
Gene name	Blattner Number	*dam*/WT	*dam mutS*/WT	*mutS*/WT	*dam*/WT	*dam mutS*/WT	*mutS*/WT
priA	b3935	.026 (.872)	-.317 (.162)	-.197 (.332)	.071 (.300)	-.283 (.290)	-.264 (.319)
priB	b4201	2.19 (.001)	1.63 (.018)	.356 (.502)	.874 (.055)	.813 (.073)	.509 (.218)
hupA	b4000	1.04 (.015)	.209 (.555)	0.100 (.859)	-.219 (.355)	.269 (.324)	-.034 (.474)
hupB	b0440	1.89 (.001)	1.60 (.003)	.523 (.176)	.556 (.023)	.283 (.296)	.114 (.314)

Table 4.1: *Signal log ratios represent the log of expression ratios (mutant/WT), base 2. Therefore a signal log ratio of 1 is the equivalent to a 2-fold change in expression level. Genes listed were tested by both array and RT-PCR. ANOVA values were determined using the array analyzer module in SPlus and

represent the significance for expression differences between mutant and WT. The student t-test was performed to compare the expression values of a given gene measured by RT-PCR in mutant versus wild-type strains.

4.2.4 Dam-deficient *E. coli* exhibit a higher level of DSBs

We performed neutral single cell microgel electrophoresis to determine the level of DSBs in the genomes of wildtype, *dam, dam mutS,* and *mutS* mutant cells. Using the single cell electrophoresis method developed by Singh (643,644), we were able to detect a single double strand break in the genome of a cell. We assume that one linear tail corresponds to a single double-strand break (54) as shown in Fig. 4.3A; an individual cell with no DSBs appears as a head with no tail, whereas a cell with DSBs appears as a head followed by linear tails indicative of the number of breaks in the genome. For these experiments, cultures were grown as described above for the gene expression studies and three independent experiments were performed. A total of 600 to 1000 single cells for each strain were analyzed by Komet analysis software (Kinetic Imaging Ltd.) and by the visual counting of the number of DSBs. Our data show that Dam-deficient *E. coli* have a significantly higher level of basal DSBs (Mann-Whitney U test, $P < .02$) compared to the level of DSBs in the wildtype strain, with *dam* cells having on average 1.2 breaks per cell (Fig. 4.3B). The *dam mutS* and *mutS* strains do not show a significantly higher level of double strand breaks compared to wildtype.

Although the average number of DSBs detected in the *dam* strain is 1.2 breaks per cell, we observe a broad range of breaks per cell. A small percentage of the *dam* cells (6 %) show levels of double strand breaks between 5 and 12, while 42% of the cells show no DSBs and the majority of cells display between 1 and 4 DSBs. We expect that this broad range in the number of DSBs is due to differences in the cell population with respect to the number of active DNA replication forks and the stages of recombination substrates in each cell. In other words, DSBs are lethal lesions and most likely do not persist for long in the cells, and therefore we were able to capture relatively few cells with a high level of damage that had not yet completed recombinational repair. The short persistence of DSBs in the cell would explain why previous studies could only detect DSBs in *dam rec* double mutant strains deficient in recombinational repair and were unable to detect DSBs in *dam* only mutant cells (522,544). The other strains did not show a broad range of DSB formation; 80-90% of the cells showed no DSBs while 1-3 DSBs were detected in the remaining cells.

Komet image analysis software analyzes photometric data and quantifies the fluorescence of the head and tail. The software provides measurements such as the mean intensities of the head and tail and the tail length. Percent DNA in the head or tail represents a percentage of the total measured intensity (head and tail), and cells with a higher level of DNA damage will display a higher percentage of tail DNA. Figure 12C shows a box plot of the distribution of cells for each strain according to the percentage of tail DNA. The horizontal line in each box represents the median of the data, while the box represents the inter-quartile range, or the range including 50% of the data. The lines extending from the top and bottom of each box mark the minimum and maximum values within the data set that fall within an acceptable range, and any values outside of this range (outliers) are displayed as individual points. The *dam* cells display the broadest range of data, with many cells showing a very high percentage of DNA in the tail. The distribution of cells by percent tail DNA is similar for wildtype, *dam mutS*, and *mutS* strains, although *dam mutS E. coli* show a few outliers with a high level of damage. The data support the idea that mismatch repair induces DSBs in *dam* cells and causes a high level of damage; while only a subset of *dam* mutant cells exhibit very high levels of damage, we speculate that the lethal damage is repaired efficiently and does not persist for long in the cells, resulting in a majority of the cells showing lower levels of damage.

4.3 Discussion

Dam-deficient *E. coli* exhibit pleiotropic changes including an increased mutation rate, uncoordinated DNA replication initiation, and transcriptional alterations (420,448,538). Many of these phenotypes result from the absence of hemimethylated DNA following passage of the replication fork. Dam-deficient *E. coli* also exhibit a hypersensitivity to DNA damage and a dependence on recombinational repair, and both phenotypes can be suppressed by inactivating mismatch repair (224,466,731). In the present study, we determined the global transcriptional changes in *dam*, *dam mutS* and *mutS E. coli*. Our data show that *dam* and *dam mutS* strains exhibit the greatest number of transcriptional changes compared to wildtype. Although gene expression can be regulated by Dam methylation, most of the gene expression changes observed in these strains appears to be due to secondary effects of Dam deficiency rather than from direct methylation-mediated gene regulation. The majority of genes induced in *dam* and *dam mutS* strains are genes encoding products of carbohydrate transport and metabolism, translation, and in the *dam*

strain, energy production and conversion. The up-regulation of genes involved in metabolism, energy production, and translation in *dam E. coli* may be indicative of the great effort this strain must devote to growth and to coping with asynchronous DNA replication and DNA damage. We also see induction of several *lexA*-regulated genes in *dam* and *dam mutS* strains, which is consistent with the previous findings of others (524,535,558). Furthermore, mismatch repair appears to be contributing to the transcriptional changes observed in the *dam* strain; adding a mutation in *mutS* suppresses many of the gene expression changes in the *dam* strain, as the *dam mutS* strain shows induction of fewer genes in categories such as amino acid and carbohydrate transport and metabolism, energy production and conversion, and translation. Our data suggest that due to the many functions of adenine methylation, a deficiency in Dam increases the overall stress level in the cells, including stress caused by DNA damage, and that introducing an additional mutation in mismatch repair abrogates some of this stress. A deficiency in MutS alone does not result in many changes at the transcriptional level.

Two previous studies have examined the effects of Dam methylation on global gene expression patterns. Løbner-Olesen et al. (421) found few gene expression changes in Dam-deficient strains that were either *dam* null or that expressed 30% of the level of Dam relative to wildtype. However their study showed that Dam overexpression resulted in altered expression of numerous genes, and the effects of Dam overproduction were almost identical to the gene expression effects they observed in *seqA* mutant cells, or cells in which reinitiation of replication occurs at *oriC* repeatedly during a single replication cycle (72,651). Because most of the genes whose expression was altered did not contain d(GATC) sites in their promoter regions, the authors proposed that the gene expression changes observed in Dam overproducing and *seqA* mutant strains are due to the increased amount of fully methylated DNA and the resulting alterations in chromosome structure. Although their study did not find many gene expression changes in the *dam* null strain compared to wildtype, their findings are similar to ours in the respect that Dam methylation appears to have little direct effect on gene regulation. The second study by Oshima et al. (535) tested the gene expression changes in *dam* mutant cells under aerobic and low aerobic conditions. This study, like ours, found many gene expression changes in the Dam-deficient strain. Furthermore, genes involved in amino acid metabolism, energy metabolism, and the environmental stress response were up-regulated in the *dam* mutant under aerobic conditions, which is consistent with our data.

Our data show that several SOS response genes are constitutively induced in the *dam* and *dam mutS* strains. Dam-deficient strains have a high basal level of single-strand breaks compared to wildtype (447) and thus single-strand breaks may be the primary SOS-inducing signal. Previous work has indicated that damage in *dam* strains also consists of DSBs; expression of *recA*, *recB*, *recC*, *ruvA*, *ruvB*, and *ruvC* is essential for *dam* mutant viability although expression of *recN*, *recO*, *recF*, and *recR* is not required (443,558). Furthermore, the presence of DSBs and the requirement for DSB recombinational repair is dependent on functional mismatch repair; Wang and Smith (731) have shown that mutations in mismatch repair (*mutS* or *mutL)* suppress the formation of DSBs and the requirement for recombination in a dam^{-} rec^{-} background. However the basal level of DSBs in *dam* mutants has not been measured in recombination proficient strains. Using a single-cell assay where we can detect a single DSB per cell, we measured the level of DSBs in *dam* and *dam mutS* *E. coli* that are proficient in recombinational repair. Based on our data, *dam E. coli* have on average 1.2 double strand breaks per cell, whereas wildtype and *dam mutS* have an average of 0.17 and 0.37 DSBs per cell, respectively. Therefore, while inactivating MutS abrogates the need for DSB repair in *dam* mutant cells, we propose that mismatch repair contributes to nearly all of DSBs detected in *dam* cells.

It is important to note that one phenotype of *dam* mutants is asynchronous cell division and multiple firings of the origin of replication during the cell cycle (420,603). Such multiple firings may lead to hyper-ploidy and an increase in DNA fragments as multiple DNA duplexes are being synthesized. Thus the number of tails in Dam-deficient strains may be higher due to this phenotype. The contribution of multiple DNA molecules within the cell, however, should be the same for both *dam* and *dam mutS* mutant strains as both strains lack DNA adenine methylation. While the level of DSBs is higher in *dam* mutants than it is in *dam mutS* mutant cells, DSB formation in *dam* mutants is therefore dependent on mismatch repair.

As mentioned in the results, we believe the level of mismatch-repair induced DSBs in the *dam* strain to be well above the average of 1.2 breaks per cell. Because we observed a broad range of values, with the highest level reaching 12 breaks in a *dam* cell, we speculate that the DSBs are repaired efficiently and do not persist for long in the cells, and thus we could only capture a small population of cells with unrepaired DSBs. This scenario would explain why other methods, such as neutral sucrose-gradients (731) and pulse-field gel

electrophoresis (522), could only detect an increased level of DSBs in *dam*$^-$ *rec*$^-$ backgrounds. Our results are also consistent with the findings of McCool et al. (462) demonstrating that SOS expression in *dam* mutant cells follows a two-population model in which some cells show high SOS expression while other cells do not; our data show that at least one form of DNA damage—DSB formation—in *dam* mutant cells is stochastically formed and is not present at uniform levels in the culture at a given moment in time.

Despite the aforementioned correlative data on DSB formation, the dependence on recombination and functional mismatch repair in a *dam* mutant strain, SOS induction is not suppressed by an additional mutation in *mutS*. Therefore other SOS inducing signals must be present in *dam* and *dam mutS* strains. While recombinational repair, and specifically DSB repair, is required for *dam* mutant viability (443,557), induction of SOS is also critical for survival, and *lexA dam* double mutants in which the LexA repressor cannot be inactivated are inviable (558).

The mechanism by which mismatch repair induces DSBs in the absence of Dam methylation is not fully understood. Mismatch repair may be making dual incisions at unmethylated d(GATC) sites, forming DSBs (20,245). Alternatively, MutH-catalyzed single incisions could result in DSBs when these single-strand nicks are encountered by a replication fork, resulting in replication fork collapse and a DSB (see reference (443) for a discussion of this model). To account for the increased level of DSBs observed in *dam* mutant cells, MutH would need to make more single-strand incisions in *dam* mutant cells than in *dam*$^+$ cells. Increased MutH-catalyzed single-strand incisions may occur in *dam* mutant cells because of the higher presence of MutH substrate, or unmethylated d(GATC) sites, and an increased level of single-strand breaks has indeed been observed in *dam* cells (447). In growing *dam* mutant cells, therefore, the coupling of a high presence of single-strand gaps with multiple replication forks may result in replication fork collapse and may account for the increased level of DSBs observed in the *dam* strain. Under this model and based on our results, the frequency at which replication forks encounter single-strand gaps in *dam E. coli* could result in as many as ten or more DSBs. However due to the up-regulation of recombinational repair in this strain, *dam* mutant cells efficiently repair these DSBs, and thus the average level of DSBs observed in the culture is only one to two breaks per cell. Unlike the first model where MutH catalyzes two incisions on complementary strands resulting in a DSB, this second model relies on replication for the formation of DSBs.

The replication-dependence of DSB formation and recombination in *dam* mutant cells is currently being tested.

The present study demonstrates the importance of functional mismatch repair in contributing to DNA damage and gene expression changes in *dam* mutant cells. We have shown that DSB formation in *dam* mutant cells is dependent on functional mismatch repair; *dam* mutant cells have a higher level of DSBs compared to wildtype whereas *dam mutS* and *mutS* mutant cells do not. We have also shown that the SOS response and genes involved in recombinational repair are induced in *dam* mutant *E. coli*. While it has been shown that mismatch repair sensitizes cells to DNA damaging agents in a *dam* mutant background (224), the high level of mismatch repair-induced basal damage in *dam* mutant cells helps explain why this strain is hypersensitive to DNA-damaging agents. The following chapter examines the role of mismatch repair in mediating toxicity to exogenous DNA-damaging agents using the strains characterized in this chapter.

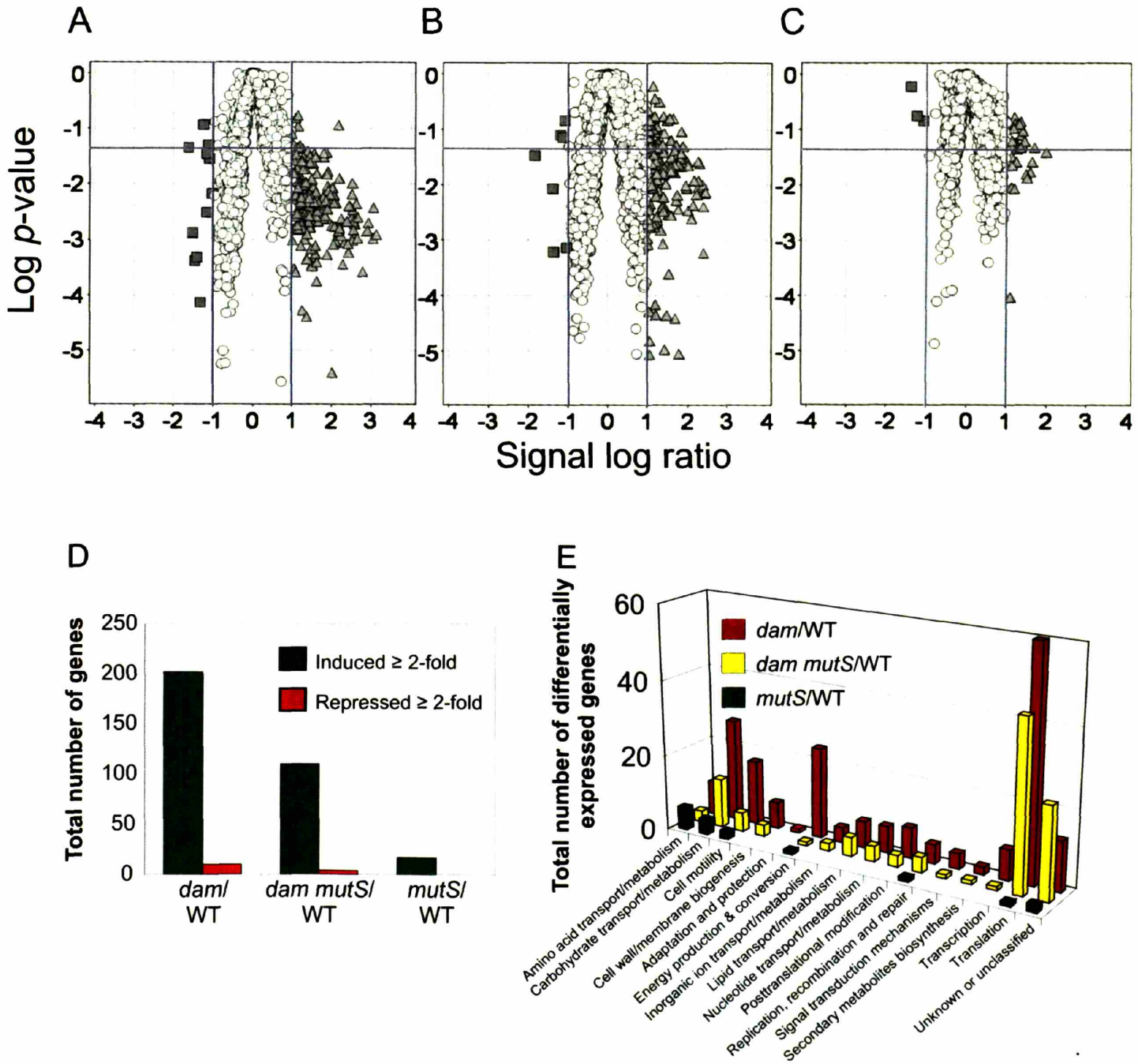


Figure 4.1: Global transcriptional changes in *dam*, *dam mutS*, and *mutS E. coli* compared to wild-type. ANOVA was performed using wild-type expression level as a baseline, and log *p*-values are plotted against signal log ratios for *dam* (A), *dam mutS* (B), and *mutS* (C) mutant cells. Circles represent genes that do not meet the 2-fold cutoff marked by the vertical bold lines while dark grey squares represent genes expressed at a 2-fold lower level and light grey triangles represent genes expressed at a 2-fold higher level compared to wild-type. The horizontal bold line shows the*p*-value threshold applied to filter the data (*p* 0.05) and (D) and (E) show the filtered data. (D) Total number of genesinduced/repressed 2-fold in each of the strains. Only genes for which the magnitude of induction or repression is at least 2-fold (signal log ratio 1 and -1) and for which *p* 0.05 are represented. (E) Genes in (D) categorized according to general function as provided by the NCBI COG database.

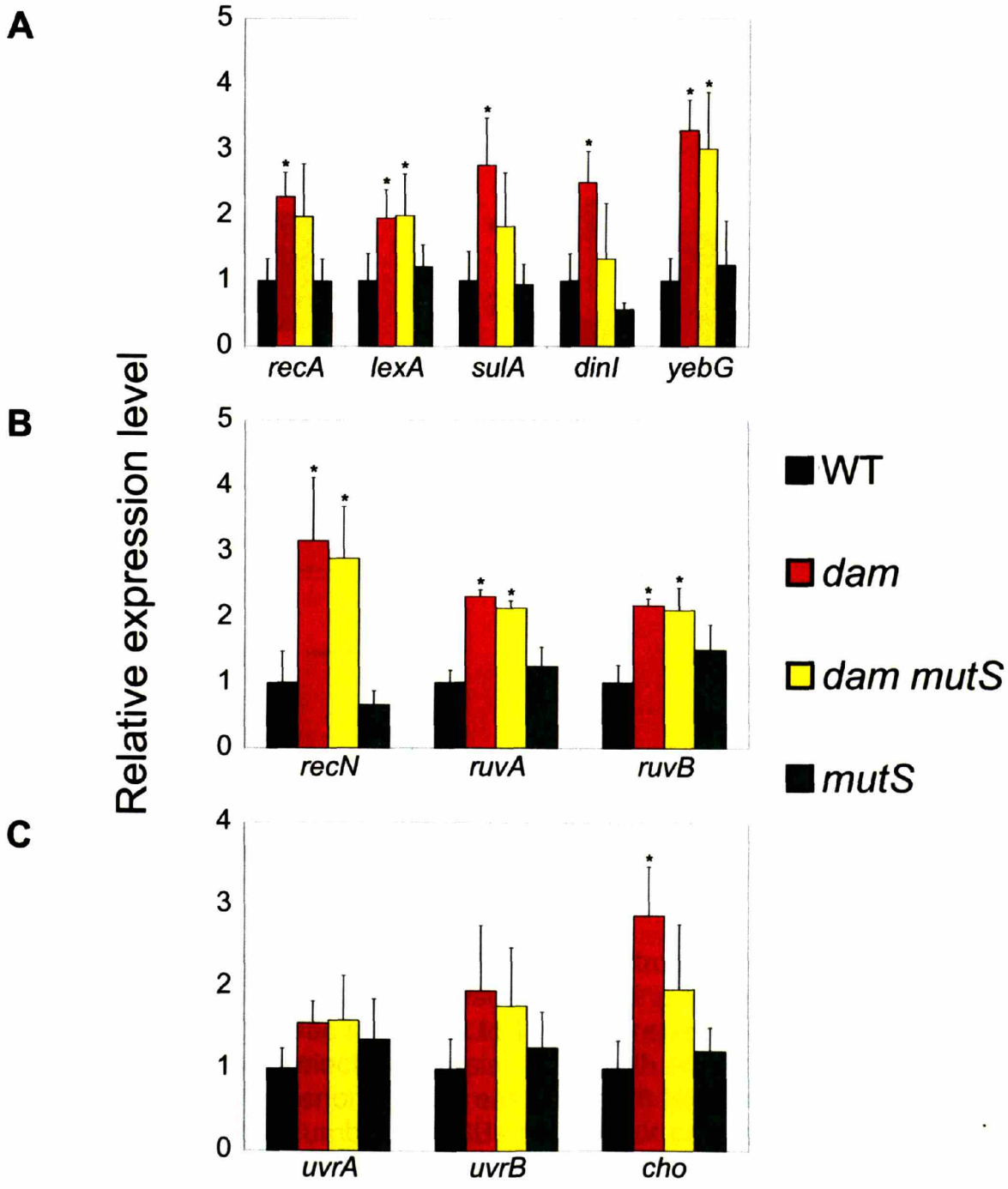


Figure 4.2: Relative expression levels for several SOS genes determined by semi-quantitative real-time PCR. Student t-tests were performed to compare the transcript levels of a gene detected in a mutant strain to that detected in the wild-type strain. Comparisons for which p 0.05 are designated by an asterisk, and error bars represent standard deviation. SOS response genes represented include genes involved in SOSregulation (A), recombination (B), and nucleotide excision repair (C).

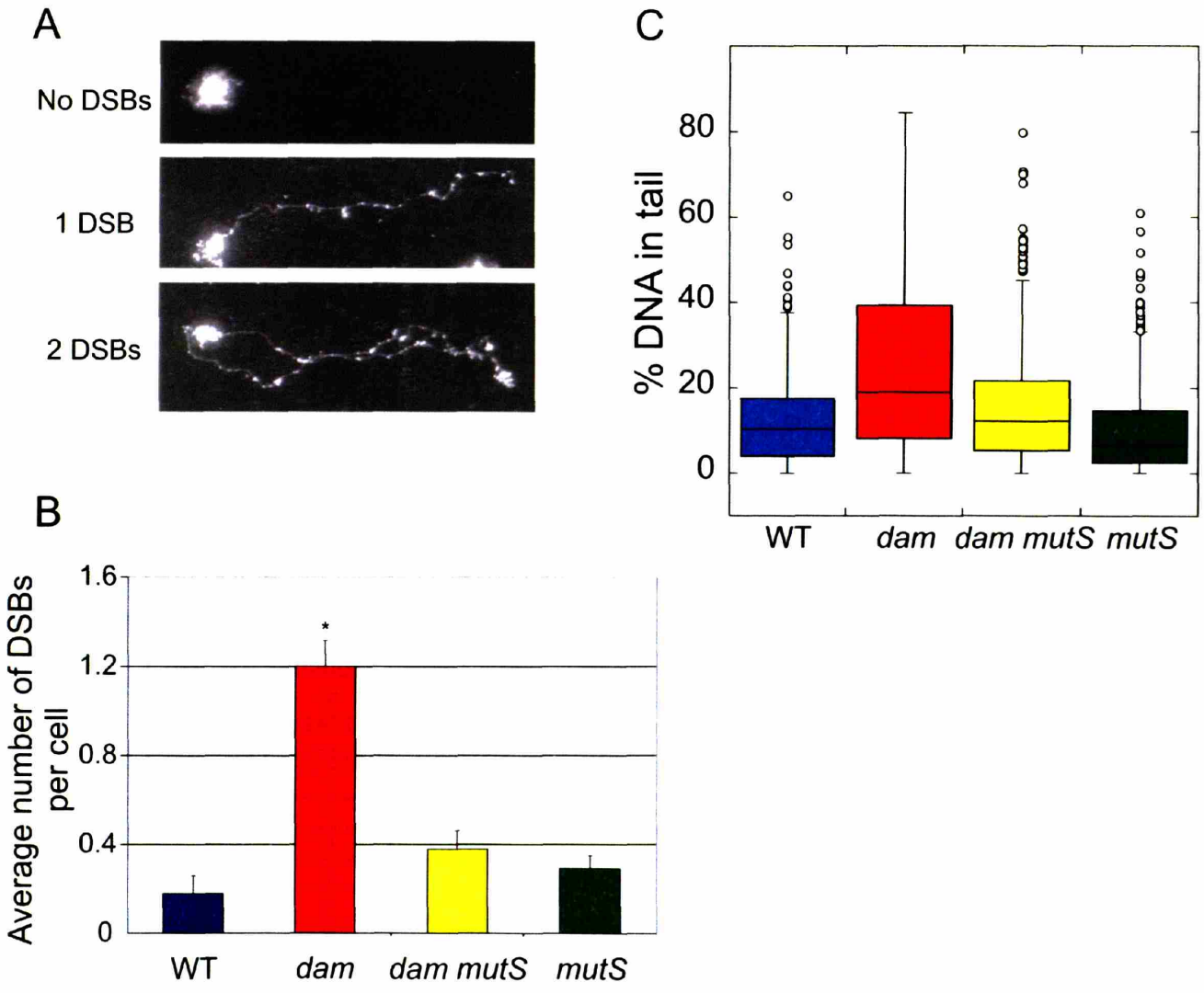


Figure 4.3: *E. coli* strains analyzed by single-cell microgel electrophoresis. Six hundred Individual cells for each strain were analyzed by visually counting the number of breaks and by Komet analysis software (Kinetic Imaging Ltd.) A) Representative pictures of individual cells analyzed by microgel electrophoresis. Cells with no tails indicate no double-strand breaks (DSBs) in the genome, whereas cells with tails indicate the number of strand breaks in the genome. B) Number of DSBs per cell for each strain determined by counting the tails of each cell for each of the strains. Error bars represent SE, *Mann-Whitney U test $P < .02$ C) Results from Komet analysis. Percent of DNA in the tail represents a percentage of the total DNA (total fluorescence) detected in both the head and tail. Box plot shows the distribution of cell data for each strain. Horizontal lines for each box represent the median value, with the box representing 50% of the data (box upper and lower limits represent the upper and lower quartiles, respectively). Vertical lines extending from the box show the full range of data, and outliers are shown as individual points (outliers are defined as values greater than the upper quartile +1.5 X interquartile distance).

Table 4.2: Genes differentially expressed at the basal level (± 2-fold) in *dam* mutant *E. coli* compared to wildtype. Only genes for which ANOVA $p \leq 0.05$ are listed.

Amino acid transport and metabolism					
Gene	**Blattner Number**	**Number of GATC sites***	**Signal Log Ratio**	**ANOVA p-value**	**Gene Product and Function**
argV	b2694	-	1.03	0.020	Arginine tRNA2; tandem quadruplicate genes
aspC	b0928	2	1.26	0.026	aspartate aminotransferase
dapA	b2478	3	1.24	0.005	dihydrodipicolinate synthase
gcvH	b2904	1	1.04	0.018	in glycine cleavage complex, carrier of aminomethyl moiety via covalently bound lipoyl cofactor
glyA	b2551	0	1.32	0.003	serine hydroxymethyltransferase
oppA	b1243	1	1.16	0.012	oligopeptide transport; periplasmic binding protein
tdh	b3616	1	1.46	0.007	threonine dehydrogenase
tnaB	b3709	1	1.48	0.031	low affinity tryptophan permease
ydgR	b1634	1	1.61	0.007	putative transport protein

Carbohydrate transport and metabolism					
Gene	**Blattner Number**	**Number of GATC sites***	**Signal Log Ratio**	**ANOVA p-value**	**Gene Product and Function**
crr	b2417	1	1.07	0.026	PTS system, glucose-specific IIA component
deoB	b4383	1	1.05	0.041	phosphopentomutase
eno	b2779	3	1.13	0.045	enolase
fba	b2925	0	1.48	0.002	fructose-bisphosphate aldolase, class II
galK	b0757	2	1.45	0.003	galactokinase
galM	b0756	2	1.15	0.002	galactose-1-epimerase (mutarotase)
glk	b2388	2	1.07	0.000	glucokinase
glpF	b3927	2	1.58	0.035	facilitated diffusion of glycerol
glpT	b2240	0	1.25	0.010	sn-glycerol-3-phosphate permease
gnd	b2029	3	1.17	0.004	gluconate-6-phosphate dehydrogenase, decarboxylating
gpmA	b0755	0	1.09	0.019	phosphoglyceromutase 1
malE	b4034	2	2.80	0.000	periplasmic maltose-binding protein; substrate recognition for transport and chemotaxis
malF	b4033	2	1.17	0.002	part of maltose permease, periplasmic
malG	b4032	3	1.02	0.001	part of maltose permease, inner membrane
malK	b4035	2	2.44	0.001	ATP-binding component of transport system for maltose
malP	b3417	4	1.34	0.006	maltodextrin phosphorylase
mgsA	b0963	0	1.36	0.011	methylglyoxal synthase
mtlD	b3600	0	1.30	0.030	mannitol-1-phosphate dehydrogenase
pfkA	b3916	4	1.27	0.002	6-phosphofructokinase I
pgk	b2926	3	1.44	0.002	phosphoglycerate kinase
ptsG	b1101	1	1.24	0.009	PTS system, glucose-specific IIBC component
ptsH	b2415	1	1.01	0.030	PTS system protein HPr
ptsI	b2416	2	1.15	0.024	PEP-protein phosphotransferase system enzyme I
rbsB	b3751	3	1.02	0.030	D-ribose periplasmic binding protein

Carbohydrate transport and metabolism					
talB	b0008	1	1.12	0.008	transaldolase B
treB	b4240	5	2.30	0.000	PTS system enzyme II, trehalose specific
treC	b4239	1	1.19	0.015	trehalase 6-P hydrolase

Cell motility					
Gene	**Blattner Number**	**Number of GATC sites***	**Signal Log Ratio**	**ANOVA p-value**	**Gene Product and Function**
	b3836	2	1.28	0.000	orf, hypothetical protein
flgB	b1073	1	1.36	0.009	flagellar biosynthesis, cell-proximal portion of basal-body rod
flgC	b1074	1	1.62	0.006	flagellar biosynthesis, cell-proximal portion of basal-body rod
flgD	b1075	0	1.04	0.018	flagellar biosynthesis, initiation of hook assembly
flgE	b1076	2	1.75	0.004	flagellar biosynthesis, hook protein
flgF	b1077	4	1.51	0.009	flagellar biosynthesis, cell-proximal portion of basal-body rod
flgG	b1078	1	1.59	0.005	flagellar biosynthesis, cell-distal portion of basal-body rod
flgL	b1083	1	1.17	0.023	flagellar biosynthesis; hook-filament junction protein
flgN	b1070	2	1.14	0.014	protein of flagellar biosynthesis
fliC	b1923	0	1.55	0.023	flagellar biosynthesis; flagellin, filament structural protein
fliG	b1939	3	1.12	0.002	flagellar biosynthesis, component of motor switching and energizing, enabling rotation and determining its direction
fliL	b1944	0	1.23	0.001	flagellar biosynthesis
fliN	b1946	3	1.18	0.011	flagellar biosynthesis, component of motor switch and energizing, enabling rotation and determining its direction
prlA	b3300	2	1.67	0.002	putative ATPase subunit of translocase
secB	b3609	3	1.08	0.007	protein export; molecular chaperone; may bind to signel sequence
secG	b3175	0	1.63	0.015	protein export - membrane protein
yajC	b0407	1	1.38	0.000	orf, hypothetical protein

Cell wall/membrane biogenesis					
Gene	**Blattner Number**	**Number of GATC sites***	**Signal Log Ratio**	**ANOVA p-value**	**Gene Product and Function**
ompA	b0957	1	1.82	0.036	outer membrane protein 3a (II*;G;d)
ompC	b2215	1	2.08	0.004	outer membrane protein 1b (Ib;c)
ompF	b0929	0	2.22	0.002	outer membrane protein 1a (Ia;b;F)
ompT	b0565	0	1.04	0.012	outer membrane protein 3b (a), protease VII
ompX	b0814	1	2.10	0.002	outer membrane protein X
pal	b0741	2	1.32	0.007	peptidoglycan-associated lipoprotein
spr	b2175	0	1.48	0.002	putative lipoprotein

tolC	b3035	3	1.13	0.003	outer membrane channel; specific tolerance to colicin E1; segregation of daughter chromosomes

Cell division					
Gene	**Blattner Number**	**Number of GATC sites***	**Signal Log Ratio**	**ANOVA p-value**	**Gene Product and Function**
sulA	b0958	4	0.95	0.004	suppressor of lon; inhibits cell division and ftsZ ring formation

Energy production and conversion					
Gene	**Blattner Number**	**Number of GATC sites***	**Signal Log Ratio**	**ANOVA p-value**	**Gene Product and Function**
ackA	b2296	0	1.18	0.045	acetate kinase
atpA	b3734	0	1.54	0.009	membrane-bound ATP synthase, F1 sector, alpha-subunit
atpB	b3738	1	1.01	0.007	membrane-bound ATP synthase, F0 sector, subunit a
atpC	b3731	2	1.29	0.025	membrane-bound ATP synthase, F1 sector, epsilon-subunit
atpD	b3732	3	1.69	0.004	membrane-bound ATP synthase, F1 sector, beta-subunit
atpE	b3737	3	1.45	0.002	membrane-bound ATP synthase, F0 sector, subunit c
atpF	b3736	4	1.41	0.003	membrane-bound ATP synthase, F0 sector, subunit b
atpG	b3733	1	1.45	0.006	membrane-bound ATP synthase, F1 sector, gamma-subunit
atpH	b3735	1	1.28	0.003	membrane-bound ATP synthase, F1 sector, delta-subunit
cydA	b0733	3	1.11	0.026	cytochrome d terminal oxidase, polypeptide subunit I
fdoI	b3892	1	1.08	0.015	formate dehydrogenase, cytochrome B556 (FDO) subunit
frdA	b4154	2	1.11	0.002	fumarate reductase, anaerobic, flavoprotein subunit
frdB	b4153	0	1.61	0.030	fumarate reductase, anaerobic, iron-sulfur protein subunit
galT	b0758	1	1.52	0.000	galactose-1-phosphate uridylyltransferase
glcB	b2976	0	1.89	0.015	malate synthase G
glpC	b2243	1	1.10	0.000	sn-glycerol-3-phosphate dehydrogenase (anaerobic), K-small subunit
glpK	b3926	1	1.41	0.039	glycerol kinase
lpdA	b0116	0	1.26	0.026	lipoamide dehydrogenase (NADH); component of 2-oxodehydrogenase and pyruvate complexes; L-protein of glycine cleavage complex
mdh	b3236	0	1.90	0.006	malate dehydrogenase
nuoB	b2287	0	1.29	0.009	NADH dehydrogenase I chain B
nuoH	b2282	2	1.14	0.019	NADH dehydrogenase I chain H
nuoK	b2279	1	1.04	0.004	NADH dehydrogenase I chain K
nuoL	b2278	2	1.15	0.004	NADH dehydrogenase I chain L
ppa	b4226	0	1.66	0.003	inorganic pyrophosphatase

Inorganic ion transport and metabolism					
Gene	**Blattner Number**	**Number of GATC sites***	**Signal Log Ratio**	**ANOVA p-value**	**Gene Product and Function**
	b2431	2	1.52	0.000	orf, hypothetical protein
focA	b0904	2	1.17	0.008	probable formate transporter (formate channel 1)
phnA	b4108	1	1.03	0.005	orf, hypothetical protein
sodB	b1656	1	1.29	0.018	superoxide dismutase, iron

Lipid transport and metabolism					
Gene	**Blattner Number**	**Number of GATC sites***	**Signal Log Ratio**	**ANOVA p-value**	**Gene Product and Function**
accB	b3255	1	1.26	0.006	acetylCoA carboxylase, BCCP subunit; carrier of biotin
accC	b3256	1	1.22	0.002	acetyl CoA carboxylase, biotin carboxylase subunit
accD	b2316	2	1.13	0.002	acetylCoA carboxylase, carboxytransferase component, beta subunit
acpP	b1094	0	1.38	0.000	acyl carrier protein
fabB	b2323	2	1.40	0.001	3-oxoacyl-[acyl-carrier-protein] synthase I
fabF	b1095	1	1.06	0.005	3-oxoacyl-[acyl-carrier-protein] synthase II
fabI	b1288	0	1.11	0.006	enoyl-[acyl-carrier-protein] reductase (NADH)

Nucleotide transport and metabolism					
Gene	**Blattner Number**	**Number of GATC sites***	**Signal Log Ratio**	**ANOVA p-value**	**Gene Product and Function**
cdd	b2143	3	1.04	0.016	cytidine/deoxycytidine deaminase
deoD	b4384	2	1.97	0.005	purine-nucleoside phosphorylase
guaB	b2508	2	1.15	0.022	IMP dehydrogenase
nupC	b2393	2	1.58	0.005	permease of transport system for 3 nucleosides
prsA	b1207	2	1.28	0.008	phosphoribosylpyrophosphate synthetase
purA	b4177	2	1.50	0.012	adenylosuccinate synthetase
udp	b3831	0	1.05	0.051	uridine phosphorylase

Posttranslational modification, protein turnover, chaperones					
Gene	**Blattner Number**	**Number of GATC sites***	**Signal Log Ratio**	**ANOVA p-value**	**Gene Product and Function**
ahpC	b0605	1	1.12	0.011	alkyl hydroperoxide reductase, C22 subunit; detoxification of hydroperoxides
grpE	b2614	3	1.83	0.030	phage lambda replication; host DNA synthesis; heat shock protein; protein repair
hflB	b3178	1	1.47	0.024	degrades sigma32, integral membrane peptidase, cell division protein
slyD	b3349	4	1.20	0.010	FKBP-type peptidyl-prolyl cis-trans isomerase (rotamase)
sspA	b3229	0	1.10	0.020	regulator of transcription; stringent starvation protein A

Posttranslational modification, protein turnover, chaperones					
tig	b0436	0	1.42	0.005	trigger factor; a molecular chaperone involved in cell division
tpx	b1324	1	1.14	0.027	thiol peroxidase
yeaA	b1778	1	-1.01	0.007	orf, hypothetical protein

Replication, recombination and repair					
Gene	**Blattner Number**	**Number of GATC sites***	**Signal Log Ratio**	**ANOVA p-value**	**Gene Product and Function**
hupA	b4000	0	1.04	0.015	DNA-binding protein HU-alpha (HU-2)
hupB	b0440	3	1.89	0.001	DNA-binding protein HU-beta, NS1 (HU-1)
priB	b4201	4	2.19	0.001	primosomal replication protein N
recA	b2699	1	2.02	0.000	DNA strand exchange and renaturation, DNA-dependent ATPase, DNA- and ATP-dependent coprotease
smf_1		1	-1.31	0.000	orf, fragment 1

Signal transduction mechanisms					
Gene	**Blattner Number**	**Number of GATC sites***	**Signal Log Ratio**	**ANOVA p-value**	**Gene Product and Function**
crp	b3357	0	1.13	0.034	cyclic AMP receptor protein
dksA	b0145	1	1.18	0.003	dnaK suppressor protein
yebJ	b1831	1	1.16	0.008	orf, hypothetical protein
ygaG	b2687	1	1.42	0.005	orf, hypothetical protein

Secondary metabolites biosynthesis, transport and catabolism					
Gene	**Blattner Number**	**Number of GATC sites***	**Signal Log Ratio**	**ANOVA p-value**	**Gene Product and Function**
fabG	b1093	1	1.18	0.001	3-oxoacyl-[acyl-carrier-protein] reductase
srlD	b2705	1	1.64	0.000	glucitol (sorbitol)-6-phosphate dehydrogenase

Transcription					
Gene	**Blattner Number**	**Number of GATC sites***	**Signal Log Ratio**	**ANOVA p-value**	**Gene Product and Function**
cspA	b3556	0	1.34	0.019	cold shock protein 7.4, transcriptional activator of hns
cspB	b1557	0	1.22	0.050	cold shock protein; may affect transcription
fliA	b1922	3	1.13	0.008	flagellar biosynthesis; alternative sigma factor 28; regulation of flagellar operons
fliM	b1945	1	1.25	0.006	flagellar biosynthesis, component of motor switch and energizing, enabling rotation and determining its direction
lexA	b4043	1	1.60	0.001	regulator for SOS(lexA) regulon

Transcription					
rpoA	b3295		2.54	0.001	RNA polymerase, alpha subunit
rpoZ	b3649	0	1.04	0.014	RNA polymerase, omega subunit
yeeD	b2012	1	1.19	0.009	orf, hypothetical protein

Translation					
Gene	**Blattner Number**	**Number of GATC sites***	**Signal Log Ratio**	**ANOVA p-value**	**Gene Product and Function**
asnS	b0930	0	1.50	0.012	asparagine tRNA synthetase
efp	b4147	3	1.14	0.026	elongation factor P (EF-P)
fusA	b3340	1	1.78	0.004	GTP-binding protein chain elongation factor EF-G
glnS	b0680	4	1.10	0.003	glutamine tRNA synthetase
infB	b3168	4	1.08	0.029	protein chain initiation factor IF-2
rplA	b3984	0	2.02	0.002	50S ribosomal subunit protein L1, regulates synthesis of L1 and L11
rplB	b3317	1	2.49	0.004	50S ribosomal subunit protein L2
rplC	b3320	8	2.58	0.002	50S ribosomal subunit protein L3
rplD	b3319	1	2.68	0.002	50S ribosomal subunit protein L4, regulates expression of S10 operon
rplE	b3308	0	3.06	0.001	50S ribosomal subunit protein L5
rplF	b3305	3	2.08	0.006	50S ribosomal subunit protein L6
rplI	b4203	3	1.81	0.006	50S ribosomal subunit protein L9
rplJ	b3985	0	2.04	0.008	50S ribosomal subunit protein L10
rplK	b3983	1	1.80	0.005	50S ribosomal subunit protein L11
rplL	b3986	2	1.29	0.044	50S ribosomal subunit protein L7/L12
rplM	b3231	2	1.50	0.003	50S ribosomal subunit protein L13
rplN	b3310	1	1.64	0.003	50S ribosomal subunit protein L14
rplO	b3301	1	2.53	0.002	50S ribosomal subunit protein L15
rplP	b3313	2	2.26	0.002	50S ribosomal subunit protein L16
rplQ	b3294	5	2.21	0.013	50S ribosomal subunit protein L17
rplR	b3304	2	2.53	0.006	50S ribosomal subunit protein L18
rplS	b2606	0	1.67	0.005	50S ribosomal subunit protein L19
rplT	b1716	3	1.92	0.008	50S ribosomal subunit protein L20, and regulator
rplU	b3186	1	1.60	0.003	50S ribosomal subunit protein L21
rplV	b3315	1	2.92	0.001	50S ribosomal subunit protein L22
rplW	b3318	4	2.55	0.010	50S ribosomal subunit protein L23
rplX	b3309	2	1.83	0.005	50S ribosomal subunit protein L24
rpmA	b3185	1	1.46	0.004	50S ribosomal subunit protein L27
rpmB	b3637	3	1.91	0.009	50S ribosomal subunit protein L28
rpmC	b3312	4	2.06	0.001	50S ribosomal subunit protein L29
rpmD	b3302	2	3.07	0.004	50S ribosomal subunit protein L30
rpmF	b1089	1	1.40	0.003	50S ribosomal subunit protein L32
rpmG	b3636	0	1.88	0.037	50S ribosomal subunit protein L33
rpmH	b3703	3	1.92	0.017	50S ribosomal subunit protein L34
rpmI	b1717	2	1.85	0.003	50S ribosomal subunit protein A
rpmJ	b3299	1	1.33	0.001	50S ribosomal subunit protein L36
rpsA	b0911	2	1.63	0.000	30S ribosomal subunit protein S1
rpsB	b0169	1	2.26	0.001	30S ribosomal subunit protein S2

Translation					
rpsC	b3314	4	2.56	0.003	30S ribosomal subunit protein S3
rpsD	b3296	2	2.34	0.001	30S ribosomal subunit protein S4
rpsE	b3303	0	2.94	0.002	30S ribosomal subunit protein S5
rpsF	b4200	0	2.58	0.001	30S ribosomal subunit protein S6
rpsG	b3341	2	2.61	0.002	30S ribosomal subunit protein S7, initiates assembly
rpsH	b3306	2	2.64	0.004	30S ribosomal subunit protein S8, and regulator
rpsI	b3230	0	2.01	0.005	30S ribosomal subunit protein S9
rpsJ	b3321	1	2.43	0.001	30S ribosomal subunit protein S10
rpsK	b3297	4	2.66	0.001	30S ribosomal subunit protein S11
rpsL	b3342	1	2.18	0.001	30S ribosomal subunit protein S12
rpsM	b3298	1	2.11	0.001	30S ribosomal subunit protein S13
rpsN	b3307	4	2.13	0.005	30S ribosomal subunit protein S14
rpsO	b3165	3	2.03	0.006	30S ribosomal subunit protein S15
rpsP	b2609	0	2.40	0.002	30S ribosomal subunit protein S16
rpsQ	b3311	1	1.13	0.011	30S ribosomal subunit protein S17
rpsR	b4202	1	1.87	0.005	30S ribosomal subunit protein S18
rpsS	b3316	1	1.99	0.006	30S ribosomal subunit protein S19
rpsT	b0023	0	1.38	0.010	30S ribosomal subunit protein S20
thrS	b1719	2	1.39	0.000	threonine tRNA synthetase
trmD	b2607	6	1.82	0.002	tRNA methyltransferase; tRNA (guanine-7-)-methyltransferase
tsf	b0170	2	1.54	0.023	protein chain elongation factor EF-Ts
tufA	b3339	0	1.93	0.007	protein chain elongation factor EF-Tu (duplicate of tufB)
tufB	b3980	0	2.46	0.004	protein chain elongation factor EF-Tu (duplicate of tufA)
yfjA	b2608	4	1.75	0.004	orf, hypothetical protein
yjgF	b4243	4	1.45	0.025	orf, hypothetical protein

Unclassified, unknown or general function prediction only					
Gene	**Blattner Number**	**Number of GATC sites***	**Signal Log Ratio**	**ANOVA p-value**	**Gene Product and Function**
fliZ	b1921	2	1.22	0.002	orf, hypothetical protein
hns	b1237	0	1.24	0.002	DNA-binding protein HLP-II (HU, BH2, HD, NS); pleiotropic regulator
lamB	b4036	1	3.15	0.001	phage lambda receptor protein; maltose high-affinity receptor
malM	b4037	0	1.29	0.002	periplasmic protein of mal regulon
slyB	b1641	2	1.91	0.003	putative outer membrane protein
smp	b4387	0	1.13	0.018	orf, hypothetical protein
wzzB	b2027	1	1.06	0.006	regulator of length of O-antigen component of lipopolysaccharide chains
ybgF	b0742	2	1.12	0.016	orf, hypothetical protein
yceD	b1088	1	1.49	0.001	orf, hypothetical protein
yeaC	b1777	1	-1.09	0.029	orf, hypothetical protein
yebF	b1847	1	1.11	0.004	orf, hypothetical protein
yebG	b1848	2	1.60	0.001	orf, hypothetical protein

*Number of GATC sites in the upstream sequence regions was determined by searching -400 bp from the start site of each gene.

Table 4.3: Genes differentially expressed at the basal level (±2-fold) in *dam mutS* mutant *E. coli* compared to wildtype. Only genes for which ANOVA $p \leq 0.05$ are listed.

Amino acid transport and metabolism					
Gene	**Blattner Number**	**Number of GATC sites***	**Signal Log Ratio**	**ANOVA p-value**	**Gene Product and Function**
artI	b0863	0	1.21	0.002	arginine 3rd transport system periplasmic binding protein
artQ	b0862	2	1.06	0.000	arginine 3rd transport system permease protein
ydgR	b1634	1	1.27	0.047	putative transport protein

Carbohydrate transport and metabolism					
Gene	**Blattner Number**	**Number of GATC sites***	**Signal Log Ratio**	**ANOVA p-value**	**Gene Product and Function**
	b2097	1	1.07	0.000	orf, hypothetical protein
crr	b2417	1	1.08	0.050	PTS system, glucose-specific IIA component
fba	b2925	0	1.17	0.013	fructose-bisphosphate aldolase, class II
galK	b0757	2	1.12	0.024	galactokinase
galM	b0756	2	1.04	0.004	galactose-1-epimerase (mutarotase)
glk	b2388	2	1.43	0.000	glucokinase
gpmA	b0755	0	1.02	0.023	phosphoglyceromutase 1
malE	b4034	2	2.12	0.002	periplasmic maltose-binding protein; substrate recognition for transport and chemotaxis
malK	b4035	2	1.60	0.018	ATP-binding component of transport system for maltose
pfkA	b3916	4	1.22	0.006	6-phosphofructokinase I
pgk	b2926	3	1.10	0.027	phosphoglycerate kinase
ptsH	b2415	1	1.17	0.035	PTS system protein HPr
ptsI	b2416	2	1.18	0.045	PEP-protein phosphotransferase system enzyme I

Cell motility					
Gene	**Blattner Number**	**Number of GATC sites***	**Signal Log Ratio**	**ANOVA p-value**	**Gene Product and Function**
flgB	b1073	1	1.20	0.023	flagellar biosynthesis, cell-proximal portion of basal-body rod
flgC	b1074	1	1.43	0.020	flagellar biosynthesis, cell-proximal portion of basal-body rod
flgE	b1076	2	1.41	0.016	flagellar biosynthesis, hook protein
fliL	b1944	0	1.02	0.005	flagellar biosynthesis
prlA	b3300	2	1.55	0.003	putative ATPase subunit of translocase

Cell wall/membrane biogenesis					
Gene	**Blattner Number**	**Number of**	**Signal Log Ratio**	**ANOVA p-value**	**Gene Product and Function**

		GATC sites*			
ompA	b0957	1	1.86	0.037	outer membrane protein 3a (II*;G;d)
ompC	b2215	1	1.39	0.050	outer membrane protein 1b (Ib;c)
ompX	b0814	1	2.41	0.001	outer membrane protein X

Energy production and conversion					
Gene	**Blattner Number**	**Number of GATC sites***	**Signal Log Ratio**	**ANOVA p-value**	**Gene Product and Function**
ppa	b4226	0	1.26	0.031	inorganic pyrophosphatase

Inorganic ion transport and metabolism					
Gene	**Blattner Number**	**Number of GATC sites***	**Signal Log Ratio**	**ANOVA p-value**	**Gene Product and Function**
	b2431	2	1.54	0.000	orf, hypothetical protein
focA	b0904	2	1.13	0.010	probable formate transporter (formate channel 1)

Lipid transport and metabolism					
Gene	**Blattner Number**	**Number of GATC sites***	**Signal Log Ratio**	**ANOVA p-value**	**Gene Product and Function**
accB	b3255	1	1.09	0.012	acetylCoA carboxylase, BCCP subunit; carrier of biotin
accC	b3256	1	1.25	0.003	acetyl CoA carboxylase, biotin carboxylase subunit
accD	b2316	2	1.05	0.004	acetylCoA carboxylase, carboxytransferase component, beta subunit
acpP	b1094	0	1.19	0.002	acyl carrier protein
fabB	b2323	2	1.10	0.010	3-oxoacyl-[acyl-carrier-protein] synthase I

Nucleotide transport and metabolism					
Gene	**Blattner Number**	**Number of GATC sites***	**Signal Log Ratio**	**ANOVA p-value**	**Gene Product and Function**
dut	b3640	3	1.07	0.000	deoxyuridinetriphosphatase
guaB	b2508	2	1.26	0.012	IMP dehydrogenase
nupC	b2393	2	1.24	0.016	permease of transport system for 3 nucleosides
prsA	b1207	2	1.01	0.053	phosphoribosylpyrophosphate synthetase

Posttranslational modification, protein turnover, chaperones					
Gene	**Blattner Number**	**Number of GATC sites***	**Signal Log Ratio**	**ANOVA p-value**	**Gene Product and Function**

slyD	b3349	4	1.07	0.028	FKBP-type peptidyl-prolyl cis-trans isomerase (rotamase)
tig	b0436	0	1.29	0.008	trigger factor; a molecular chaperone involved in cell division
yeaA	b1778	1	-1.36	0.001	orf, hypothetical protein

Replication, recombination and repair					
Gene	**Blattner Number**	**Number of GATC sites***	**Signal Log Ratio**	**ANOVA p-value**	**Gene Product and Function**
hupB	b0440	3	1.60	0.003	DNA-binding protein HU-beta, NS1 (HU-1)
priB	b4201	4	1.63	0.018	primosomal replication protein N
recA	b2699	1	1.70	0.000	DNA strand exchange and renaturation, DNA-dependent ATPase, DNA- and ATP-dependent coprot
smf_1		1	-1.04	0.001	orf, fragment 1

Signal transduction mechanisms					
Gene	**Blattner Number**	**Number of GATC sites***	**Signal Log Ratio**	**ANOVA p-value**	**Gene Product and Function**
ygaG	b2687	1	1.47	0.010	putative 2-component transcriptional regulator

Secondary metabolites biosynthesis, transport and catabolism					
Gene	**Blattner Number**	**Number of GATC sites***	**Signal Log Ratio**	**ANOVA p-value**	**Gene Product and Function**
srlD	b2705	1	1.16	0.011	glucitol (sorbitol)-6-phosphate dehydrogenase

Transcription					
Gene	**Blattner Number**	**Number of GATC sites***	**Signal Log Ratio**	**ANOVA p-value**	**Gene Product and Function**
rpoA	b3295	2	2.15	0.004	RNA polymerase, alpha subunit

Translation					
Gene	**Blattner Number**	**Number of GATC sites***	**Signal Log Ratio**	**ANOVA p-value**	**Gene Product and Function**
fusA	b3340	1	1.49	0.022	GTP-binding protein chain elongation factor EF-G
rplA	b3984	0	1.41	0.015	50S ribosomal subunit protein L1, regulates synthesis of L1 and L11
rplB	b3317	1	1.84	0.021	50S ribosomal subunit protein L2
rplC	b3320	8	1.84	0.032	50S ribosomal subunit protein L3
rplD	b3319	1	1.93	0.025	50S ribosomal subunit protein L4, regulates

					expression of S10 operon
rplE	b3308	0	2.27	0.012	50S ribosomal subunit protein L5
rplF	b3305	3	1.70	0.023	50S ribosomal subunit protein L6
rplJ	b3985	0	1.84	0.030	50S ribosomal subunit protein L10
rplM	b3231	2	1.02	0.031	50S ribosomal subunit protein L13
rplO	b3301	1	2.42	0.004	50S ribosomal subunit protein L15
rplP	b3313	2	1.97	0.010	50S ribosomal subunit protein L16
rplR	b3304	2	2.00	0.026	50S ribosomal subunit protein L18
rplU	b3186	1	1.10	0.040	50S ribosomal subunit protein L21
rplV	b3315	1	2.46	0.007	50S ribosomal subunit protein L22
rplW	b3318	4	1.90	0.049	50S ribosomal subunit protein L23
rpmA	b3185	1	1.11	0.046	50S ribosomal subunit protein L27
rpmC	b3312	4	1.74	0.005	50S ribosomal subunit protein L29
rpmD	b3302	2	2.31	0.024	50S ribosomal subunit protein L30
rpmF	b1089	1	1.41	0.004	50S ribosomal subunit protein L32
rpmI	b1717	2	1.54	0.020	50S ribosomal subunit protein A
rpmJ	b3299	1	1.24	0.002	50S ribosomal subunit protein L36
rpsA	b0911	2	1.27	0.002	30S ribosomal subunit protein S1
rpsB	b0169	1	1.76	0.007	30S ribosomal subunit protein S2
rpsC	b3314	4	2.40	0.010	30S ribosomal subunit protein S3
rpsD	b3296	2	2.03	0.003	30S ribosomal subunit protein S4
rpsE	b3303	0	2.44	0.007	30S ribosomal subunit protein S5
rpsF	b4200	0	1.69	0.041	30S ribosomal subunit protein S6
rpsG	b3341	2	2.28	0.009	30S ribosomal subunit protein S7, initiates assembly
rpsH	b3306	2	2.03	0.022	30S ribosomal subunit protein S8, and regulator
rpsI	b3230	0	1.61	0.031	30S ribosomal subunit protein S9
rpsJ	b3321	1	1.98	0.009	30S ribosomal subunit protein S10
rpsK	b3297	4	2.24	0.007	30S ribosomal subunit protein S11
rpsL	b3342	1	1.69	0.016	30S ribosomal subunit protein S12
rpsM	b3298	1	1.78	0.004	30S ribosomal subunit protein S13
rpsN	b3307	4	1.40	0.045	30S ribosomal subunit protein S14
rpsP	b2609	0	1.73	0.052	30S ribosomal subunit protein S16
rpsQ	b3311	1	1.13	0.020	30S ribosomal subunit protein S17
rpsS	b3316	1	1.42	0.038	30S ribosomal subunit protein S19
rpsT	b0023	0	1.05	0.054	30S ribosomal subunit protein S20
thrS	b1719	2	1.16	0.002	threonine tRNA synthetase
trmD	b2607	6	1.34	0.023	tRNA methyltransferase; tRNA (guanine-7-)-methyltransferase
tufA	b3339	0	1.78	0.023	protein chain elongation factor EF-Tu (duplicate of tufB)
tufB	b3980	0	2.27	0.026	protein chain elongation factor EF-Tu (duplicate of tufA)
yfjA	b2608	4	1.32	0.029	orf, hypothetical protein

Unclassified, unknown or general function prediction only					
Gene	**Blattner Number**	**Number of GATC sites***	**Signal Log Ratio**	**ANOVA p-value**	**Gene Product and Function**
b0332	b0332	3	1.03	0.001	orf, hypothetical protein

Unclassified, unknown or general function prediction only					
b2655	b2655	2	1.16	0.009	orf, hypothetical protein
ecs0702	b0667	0	1.41	0.003	putative RNA
glgS	b3049	2	-1.82	0.033	glycogen biosynthesis, rpoS dependent
insA_2		0	1.39	0.004	IS1 protein InsA
lamB	b4036	1	2.18	0.012	phage lambda receptor protein; maltose high-affinity receptor
pheL	b2598	1	1.03	0.002	leader peptide of chorismate mutase-P-prephenate dehydratase
slyB	b1641	2	1.59	0.021	putative outer membrane protein
yadH	b0128	2	1.06	0.002	orf, hypothetical protein
ybjX	b0877	1	1.42	0.006	putative enzyme
ycdV	b1031	0	1.79	0.000	putative ribosomal protein
yceD	b1088	1	1.35	0.002	orf, hypothetical protein
yeaC	b1777	1	-1.38	0.008	orf, hypothetical protein
yebF	b1847	1	1.40	0.001	orf, hypothetical protein
yebG	b1848	2	1.11	0.011	orf, hypothetical protein
yecR	b1904	0	1.09	0.002	orf, hypothetical protein
ydhI	b1643	2	1.00	0.001	orf, hypothetical protein
ydiH	b1685	0	1.21	0.000	orf, hypothetical protein
ygiA	b3036	1	1.48	0.000	orf, hypothetical protein
yhcE	b3217	2	1.21	0.000	orf, hypothetical protein
yi82	1	1	1.23	0.027	IS186 and IS421 hypothetical protein
yihM	b3873	1	1.08	0.004	orf, hypothetical protein
yneK	b1527	1	1.02	0.025	orf, hypothetical protein
ytfI	b4215	0	1.05	0.010	orf, hypothetical protein

*Number of GATC sites in the upstream sequence regions was determined by searching -400 bp from the start site of each gene.

Table 4.4: Genes differentially expressed at the basal level (±2-fold) in *mutS* mutant *E. coli* compared to wildtype. Only genes for which ANOVA $p \leq 0.05$ are listed.

Amino acid transport and metabolism				
Gene	**Blattner Number**	**Signal Log Ratio**	**ANOVA p-value**	**Gene Product and Function**
gadB	b1493	1.10	0.000	glutamate decarboxylase isozyme
trpA	b1260	1.71	0.048	tryptophan synthase, alpha protein
trpB	b1261	1.65	0.027	tryptophan synthase, beta protein
trpC	b1262	1.52	0.013	N-(5-phosphoribosyl)anthranilate isomerase and indole-3-glycerolphosphate synthetase
trpD	b1263	1.49	0.016	anthranilate synthase component II, glutamine amidotransferase and phosphoribosylanthrani
trpE	b1264	1.13	0.023	anthranilate synthase component I

Carbohydrate transport and metabolism				
Gene	**Blattner Number**	**Signal Log Ratio**	**ANOVA p-value**	**Gene Product and Function**
eno	b2779	1.12	0.052	enolase
fba	b2925	1.12	0.009	fructose-bisphosphate aldolase, class II
malE	b4034	1.60	0.017	periplasmic maltose-binding protein; substrate recognition for transport and chemotaxis
malK	b4035	1.35	0.048	ATP-binding component of transport system for maltose

Cell motility				
Gene	**Blattner Number**	**Signal Log Ratio**	**ANOVA p-value**	**Gene Product and Function**
flgE	b1076	1.25	0.027	flagellar biosynthesis, hook protein
fliC	b1923	1.26	0.045	flagellar biosynthesis; flagellin, filament structural protein

Energy production and conversion				
Gene	**Blattner Number**	**Signal Log Ratio**	**ANOVA p-value**	**Gene Product and Function**
ppa	b4226	1.13	0.033	inorganic pyrophosphatase

Replication, recombination and repair				
Gene	**Blattner Number**	**Signal Log Ratio**	**ANOVA p-value**	**Gene Product and Function**
dps	b0812	1.02	0.029	global regulator, starvation conditions

Translation				
Gene	**Blattner Number**	**Signal Log Ratio**	**ANOVA p-value**	**Gene Product and Function**
rplO	b3301	1.42	0.052	50S ribosomal subunit protein L15

Unclassified, unknown or general function prediction only				
Gene	**Blattner Number**	**Signal Log Ratio**	**ANOVA p-value**	**Gene Product and Function**
lamB	b4036	2.00	0.039	phage lambda receptor protein; maltose high-affinity receptor
yfiD	b2579	1.20	0.009	putative formate acetyltransferase

Chapter 5: Cisplatin-induced gene expression and double-strand break formation in DNA adenine methyltransferase (dam) deficient and mismatch repair deficient *Escherichia coli*

Adapted from:

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Abstract

Loss of DNA mismatch repair (MMR) confers resistance to DNA-damaging agents including the anticancer drug cisplatin. Cisplatin tolerance in MMR-deficient cells was initially discovered in *E. coli*, where methylation deficient *dam* mutants exhibit hypersensitivity to cisplatin and *dam* mutants with an additional mutation in MMR show near wild-type levels of resistance. A prevalent explanation for this observation is the futile repair model, which proposes in *dam* mutants MMR attempts futile cycles of repair opposite cisplatin-DNA adducts. However it has recently been shown that MutS may contribute to toxicity by preventing the recombinational bypass of a cisplatin-modified substrate, and recombination is an essential mechanism for tolerating cisplatin damage. To help elucidate the role of MMR in mediating cisplatin-induced toxicity, we examine the global transcriptional responses of wild-type, *dam*, *dam mutS*, and *mutS* mutant *E. coli* following cisplatin treatment and determine a dose-response of several SOS and DNA repair genes. While the hypersensitivity of recombination-deficient strains suggests that cisplatin treatment may induce secondary DNA damage in the form of strand breaks, we also measure the level of double-strand breaks (DSBs) in each strain by single-cell electrophoresis. Our results show that Dam-deficient *E. coli* exhibit the most robust transcriptional response following cisplatin treatment. In addition, cisplatin treatment induces DSB formation and the SOS response in a dose-dependent manner, and DSBs are greatest in the hypersensitive *dam* mutant strain. The higher level of cisplatin-induced DSBs in *dam* mutants is dependent on MutS and may explain why this strain is hypersensitive to cisplatin.

5.1 Introduction

The anticancer drug cisplatin exhibits clinical efficacy in the treatment of testicular cancer, where cisplatin-based therapies can afford cure rates exceeding 90% (58). However the effectiveness of the drug in treating other human cancers is limited by harmful side effects (422), and human cancers can acquire resistance to the drug which further reduces its clinical utility. While it is generally accepted that the toxic effects of the drug arise from its reaction with DNA to form mainly cisplatin-DNA intrastrand crosslinks (80,209,775), the mechanisms by which cisplatin-DNA adducts induce cell death are not well understood. Previous work has implicated DNA mismatch repair (MMR) in mediating cisplatin-induced toxicity. *E. coli* (224) and human cells (177,213,497,540,567) deficient in MMR exhibit increased survival and tolerance to drug treatment, and similarly loss of MMR in mouse xenografts confers resistance to cisplatin treatment (216). Moreover, treatment of human tumor cells with cisplatin leads to an enrichment of MMR-deficient cell populations that are resistant to the drug (214,215). Our lab and others have also shown that the bacterial mismatch recognition protein MutS (778) and its human homologue MutSα (179,473,756) bind cisplatin-modified DNA *in vitro*. Despite the aforementioned correlative data, the exact mechanisms by which MMR contributes to the toxic effects of cisplatin are not known, and the biochemical events downstream of MutS recognition of cisplatin lesions have not been fully elucidated.

The phenomenon of cisplatin tolerance in mismatch repair deficient cells was initially discovered in *E. coli*; methylation deficient *dam* mutants exhibit high sensitivity to cisplatin whereas *dam* mutants with an additional mutation in either of the mismatch repair genes *mutS* or *mutL* show near wild-type levels of resistance (224). *Dam* encodes DNA adenine methyltransferase which acts at GATC sites of the *E. coli* genome. Because Dam methylation is a postreplicative process, a replicating chromosome is transiently hemimethylated following the replication fork. This hemimethylated state allows MMR to distinguish between the parent and the newly synthesized, or unmethylated, daughter strand when initiating repair of a misincorporation error. Mismatch recognition requires MutS, which specifically binds mismatches and small insertion/deletion loops (670). MutS, along with MutL and ATP, are required to activate the latent endonuclease MutH (2,257), which preferentially binds hemimethylated DNA and makes an incision 5' to the G at a GATC site in the unmethylated strand (20,740). While fully methylated DNA is resistant to MutH incision, in adenine unmethylated DNA such as the genomic DNA found in *dam* mutants, both strands can be substrates for MutH incision (20).

A prevalent explanation for MMR-mediated sensitivity to DNA damage is the futile-cycling model originally proposed to explain MMR-mediated sensitivity of cells to alkyation damage (351). According to this model, in the case where error-prone polymerases bypass a cisplatin adduct and insert an incorrect base opposite the adduct or in the case of *dam* mutants where the strand discrimination signal is lost, mismatch repair attempts repair at the sites opposite cisplatin-DNA adducts. The cisplatin lesion remains, however, and thus another repair cycle is initiated leading to futile repair cycling and a persistent gap in the DNA; futile cycling coupled with replication leads to replication fork collapse and a lethal double-strand break (DSB) at the site of the gap. Previous findings have supported this model to the extent that MutS specifically recognizes DNA modified with cisplatin (778). However it has recently been shown *in vitro* that MutS can inhibit RecA-mediated strand exchange of two recombination substrates when one substrate is modified with cisplatin (90); this observation indicates that MMR may recognize two DNA molecules as divergent sequences when one molecule contains cisplatin and may abort the strand exchange reaction (in this way MMR is functioning not in post-replicative MMR but in antirecombination (398,662,748,749)). Therefore MutS binding to a cisplatin adduct may prevent recombinational bypass of the adduct, and we have previously shown that recombination is an essential mechanism for tolerating cisplatin damage (779).

It is important to note that *E. coli* deficient in Dam are hyper-recombinogenic (445,446) and require recombination for viability (443,557). The requirement for recombination in *dam* mutants is dependent on functional MMR and is most likely due to the higher level of DNA double-strand breaks (DSBs) found in *dam* mutants, which is likewise dependent on functional MMR (593,731). Thus in a *dam* mutant cell treated with cisplatin, where both MMR and cisplatin are independently generating recombination substrates, the recombinational repair capacity of the cell may simply be overloaded, thus causing cell death; inactivating mismatch repair alleviates some of the burden on the recombination machinery and thus increases tolerance to exogenous or cisplatin-induced damage. It is also possible, however, that the hypersensitivity of *dam* mutants to cisplatin treatment is not due to an additive level of MMR-induced and cisplatin-induced recombination substrates; rather MMR may mediate toxicity to cisplatin by mechanisms such as futile cycling or antirecombination as described above. While futile cycling and antirecombination mechanisms of MMR-mediated toxicity to cisplatin have not been directly tested *in vivo*, more work is necessary to describe the role of MMR in mediating cisplatin-induced toxicity.

In the present study we examine the global transcriptional responses of wild-type, *dam*, *dam mutS*, and *mutS* mutant *E. coli* after treatment with a toxic dose of cisplatin in order to help

elucidate the role of mismatch repair in mediating cisplatin-induced toxicity. We also determine the dose-response at the transcriptional level of several SOS response genes and other genes involved in DNA repair by real-time reverse transcription-polymerase chain reaction (RT-PCR). Furthermore, in order to examine the role of MMR in the production of DSBs following cisplatin treatment, we determine by neutral single-cell microgel electrophoresis the number of DSBs in each strain following treatment with increasing doses of cisplatin. Our results show that the hypersensitive *dam* mutant strain responds to cisplatin treatment with the most robust transcriptional response with over 180 genes differentially expressed following drug treatment. Our results also show that cisplatin induces DSBs in a dose-dependent manner and that *dam* mutants exhibit the greatest level of cisplatin-induced DSBs as well as the greatest up-regulation of SOS genes. The level of DSBs in *dam* mutants surpasses the level that would be expected if both MMR and cisplatin were inducing strand breaks only by independent mechanisms (i.e., the level of strand breaks is not additive). Thus these results support a model whereby mismatch repair proteins increase the level of secondary lesions resulting from cisplatin damage in a Dam-deficient background.

5.2 Results

5.2.1 MutS binds to the major cisplatin adduct

The observation that MutS binds to DNA globally modified with a low number of cisplatin adducts—on the average of three adducts per 162-bp oligonucleotide (778)—suggests that MutS may recognize an oligonucleotide modified with a single cisplatin adduct as well. We constructed a 24-bp probe containing a single, centrally located 1,2-d(GpG) cisplatin crosslink, the major and presumably most cytotoxic cisplatin-DNA adduct (Fig. 5.1B), and we examined the binding by purified *E. coli* MutS to the modified probe by a DNA-retardation band shift assay (Fig. 5.1C). The binding of MutS to the radiolabeled probe modified to contain a 1,2-d(GpG) cisplatin crosslink was readily observed by a discrete, retarded band in the polyacrylamide gel specific to the lanes that included MutS and the platinated probe. The fraction of the retarded band shifted for the platinated probe increased with the increase of MutS concentration. For example, the percentage of the shifted probe doubled (from 0.59% to 1.24%, relative to the amount of total probe in each lane) when the MutS concentration was increased from 50 nM to 100 nM. Under identical conditions, MutS did not cause a shift of the unmodified control homoduplex 24-bp probe. This result is consistent with previous reports showing that the MutS homologue hMSH2 binds to a 100-bp probe site specifically modified with one 1,2-d(GpG) intrastrand cisplatin crosslink (473) and that the hMutSα complex can recognize a 32-bp probe

modified with a single 1,2-d(GpG) intrastrand crosslink (179). We also show that MutS retains its ability to bind a G/T mismatch substrate when this substrate is modified with cisplatin (Fig. 5.1D)

5.2.2 *Dam* mutant *E. coli* are hypersensitive to cisplatin

We treated log-phase cultures of wild-type, *dam*, *mutS*, and *dam mutS E. coli* strains with varying doses of cisplatin and show that the *dam* mutant is hypersensitive to cisplatin treatment compared to the isogenic derivatives *dam mutS* and *mutS* (Fig. 5.2). Our data thus confirm previous results that *E. coli* lacking DNA adenine methylation are more sensitive to DNA damage and that a mutation in the mismatch repair protein MutS abrogates this sensitivity (224). We also examined the sensitivity of a *mutH* derivative of the *dam* mutant (*dam mutH*) and show that a mutation in the endonuclease also abrogates sensitivity.

5.2.3 Cisplatin-induced global gene expression

We used Affymetrix *E. coli* genome microarrays to determine the gene expression changes in each strain after treatment with 150 μM cisplatin. Exponentially growing cultures were treated for two hours in minimal salts medium containing drug, after which the cultures were allowed to recover in LB broth for 90 minutes before RNA isolation. Conditions such as dose and recovery time were optimized to yield the greatest transcriptional response overall (data not shown) as well as the greatest differential toxicity between the strains (Fig. 5.2). Although our survival data show that 150 μM is a highly toxic dose, at the time we isolated total RNA 90 minutes after drug treatment, the wild-type, *dam mutS*, and *mutS* treated cultures grew by approximately 1 doubling while the *dam* treated culture had maintained a cell density of 2.1-2.3 x 10^8 cells/ml by OD_{600}, or a cell density slightly higher than the original starting cell density when treatment began (data not shown). Mock treated cultures at this time had grown to a cell density of ~6 x 10^8 cells/ml and had underwent ~1.5 doublings. Therefore treatment with a high dose of drug did not cause massive cell death 90 minutes after exposure, even in the hypersensitive *dam* mutant strain, and we were thus able to determine changes in gene expression following cisplatin treatment.

We used the local-pooled-error (LPE) test (324) to compare gene expression levels of each strain treated with drug to the gene expression levels measured in each mock-treated strain. Strain-specific pairwise comparisons for each gene were made by taking the ratio of the transcript level measured in the drug-treated strain to the transcript level in the mock-treated strain, and changes in transcript levels between drug-treated and untreated cultures are

expressed as signal log ratios (treated/untreated, log base 2). Fig. 5.3 shows the signal log ratios for all ~4,200 open reading frames plotted against the $\log_{10} p$-value for all four strains. Differentially expressed genes were filtered based on signal log ratio and *p*-value thresholds; only genes with signal log ratios $1 \leq X \leq -1$, which is at least a $\pm$ 2-fold change, and LPE *p*-values adjusted by the Benjamini and Hochberg method (40) to give a false discovery rate of 10% are included in Table 5.1. The most genes that exhibited at least a two fold-change and that were removed based on high *p*-values (i.e., genes that exhibited high fold-change but also high variability—open circles in Fig. 5.3) were found for *dam* and *dam mutS* strains. All genes with an adjusted *p*-value ≤ 0.10 and signal log ratios meeting the 2-fold change threshold are listed in Tables 5.3-5.6. We also listed genes that meet the *p*-value threshold but that only show moderate (less than 2-fold) differential gene expression to include genes with small changes in transcript levels that may result in significant biological effects.

The data show that the mutant strains respond to cisplatin treatment at the transcriptional level with many changes, both induction and repression, while wild-type *E. coli* do not show many gene expression changes (Table 5.1). Both *dam* and *mutS* strains exhibit down-regulation of a large number genes involved in carbohydrate transport and metabolism following cisplatin treatment, including genes encoding products of the sugar phosphotransferase system (PTS). We have previously shown that many of these genes exhibit higher basal transcript levels in *dam* mutant *E. coli* compared to wild-type (593). We also see down-regulation of many genes encoding products in energy production and conversion in *dam* mutant *E. coli*; interestingly several of these same genes are also up-regulated at the basal level in *dam E. coli* compared to wild-type (593). Of these down-regulated genes, *dam E. coli* show strong down-regulation of genes encoding subunits of fumarate reductase, an enzyme that reduces fumarate to succinate in anaerobic respiration (Table 5.4). In all four strains genes encoding products in flagellar biosynthesis are down-regulated following cisplatin treatment (Tables 5.3-5.6). In addition to the large number of down-regulated genes following cisplatin treatment, *dam* mutants also exhibit up-regulation of many transcripts including genes involved in inorganic ion transport and metabolism as well as in DNA metabolism (Table 5.4). Overall the data show that the hypersensitive *dam* mutant strain responds to cisplatin treatment with the most robust transcriptional response with both up- and down-regulation of many genes following drug exposure.

Table 5.1: Total number of differentially expressed genes following cisplatin treatment (at least 2-fold change, adjusted $p \leq 0.10$)

Gene Category	Wild-type		*dam*		*dam mutS*		*mutS*	
	Repressed	Induced	Repressed	Induced	Repressed	Induced	Repressed	Induced
Amino acid transport and metabolism	-	-	6	2	2	1	10	2
Carbohydrate transport and metabolism	3	-	18	2	2	-	12	1
Cell division	-	1	2	2	-	-		1
Cell motility	2	-	19	3	4	1	10	1
Cell processes (adaptation/ protection)	-	1	-	1	-	-	-	1
Cell wall/membrane biogenesis	1	-	4	2	-	-	2	1
Coenzyme transport and metabolism	-	-	-	1	-	-	-	-
Energy production and conversion	2	1	19	3	1	-	3	-
Inorganic ion transport and metabolism	-	-	1	8	-	3	1	-
Intracellular trafficking, secretion, and vesicular transport	-	-	-	1	-	-	-	-
Lipid transport and metabolism	-	1	1	1	-	-	-	1
Nucleotide transport and metabolism	-	-	5	4	-	-	-	-
Phage, transposon, or plasmid	-	-	-	1	-	-	-	-
Posttranslational modification, protein turnover, chaperones	-	-	-	1	-	-	-	1
Replication, recombination and repair	1	2	3	12	-	1	-	4
Secondary metabolites biosynthesis, transport and catabolism	-	-	1	1	-	-	-	2
Signal transduction mechanisms	-	-	1	1	-	-	2	-
Transcription	1	1	6	4	1	-	1	4
Translation	1	-	3	8	-	4	2	5
Unknown or unclassified	4	9	15	24	1	6	9	13
Total	**15**	**16**	**104**	**82**	**11**	**16**	**52**	**37**

5.2.4 Cisplatin induces the SOS response

The SOS response is characterized by the coordinated de-repression of over 30 genes regulated by the LexA repressor. The SOS response is induced when RecA is activated in the presence of single-strand DNA; activated RecA then facilitates the proteolytic cleavage of the LexA repressor, which binds a ~20 base pair consensus sequence in the promoter/operon regions of certain genes and thus prevents transcription in the absence of an SOS inducing signal (723). Previous studies have shown that cisplatin induces the SOS response in *E. coli* (44,366). A comparison of the expression of LexA-regulated genes in drug-treated versus untreated strains shows that several SOS genes are induced following drug treatment. In wild-type *E. coli*, seven of the 16 induced genes ($p < 0.10$) following cisplatin treatment are the SOS response genes *dinI, recA*, *oraA*, *recN*, *lexA*, *sulA*, and *yebG*, and in *mutS E. coli* some of the genes with the highest level of induction are SOS genes (e.g., *dinI, recA*, *recN*, *yebG*, *sbmC*, and *sulA* all show a greater than 8-fold change in expression level, or signal log ratios above 3). The gene *yebG* also shows induction in *dam mutS E. coli* following cisplatin treatment. The genes *dinI*, *recA*, *oraA*, *ruvB*, *uvrA*, *uvrB*, and *yebG* are also significantly induced in *dam* treated *E. coli*; however several of these genes, such as *lexA* and *recA*, exhibit a lower level of induction in *dam* mutants compared to wild-type and *mutS* treated strains (Table 5.2). The lower level of SOS induction following cisplatin treatment in both *dam* and *dam mutS* strains may be due to the high basal level of SOS gene expression in these strains; because *dam* and *dam mutS E. coli* show constitutive up-regulation of several SOS response genes compared to wild-type (SOS sub-induction) (524,558,593,607), the level of induction following drug treatment may be lower in these strains than the level observed for wild-type and *mutS* strains. Indeed, examining the relative probe set signal intensities for these genes shows that the expression level following cisplatin treatment is similar for the mutant strains; however the basal expression levels for several SOS response genes (and *lexA* in particular) is higher in the *dam* mutant strain (Fig. 5.4).

We performed real-time RT-PCR to confirm our array results showing that the SOS response and DNA repair are induced upon drug treatment. The same drug treatment conditions and RNA isolation procedure used in the array analysis were also used for the RT-PCR experiments. However in addition to the high dose of 150 μM cisplatin used in the array experiments, we used intermediate doses to determine any dose response at the transcriptional level. Three independent experiments were performed, and the transcript levels for each gene measured from each experiment were averaged. Real-time RT-PCR results confirm the array

data in showing that SOS is highly induced in all strains following cisplatin treatment. With the exception of *sbmC*, we see the highest expression level in the *dam* mutant strain for all of the SOS genes tested at each drug dose (Fig. 5.5). *Dam mutS E. coli* also show a high level of induction of the SOS response genes, with the exception of *cho* endonuclease, which shows induction only in *dam* mutant *E. coli* following cisplatin treatment. *Cho* encodes for an endonuclease homologue to *uvrC* and has been shown to be part of the SOS regulon (494). The RT-PCR results also show that, with the exception of *lexA* and *sbmC*, SOS gene expression following cisplatin treatment is dose-dependent. However, whereas microarray analysis showed similar relative expression values for SOS genes among the mutant strains, real-time RT-PCR shows that SOS gene induction is greatest in the *dam* mutant following drug treatment.

The SOS gene with the greatest fold induction, by RT-PCR, among all the strains is *sulA*, which shows at least a 20-fold induction in all strains at the highest dose. *OraA* also shows high induction by RT-PCR, especially in the *dam* and *dam mutS* strains (40- and 30-fold induction, respectively). This gene shows over an 11-fold change in *dam E. coli* by array analysis. *OraA* is a readthrough product of the *recA* transcript (537); it encodes for a repressor of RecA called RecX, and previous work has shown that RecX can inhibit both recombinase and coprotease functions of RecA (665). Like the *recA* gene, *oraA* is inducible and was shown to have a 10-fold induction after UV exposure (137).

A cluster of genes located approximately at 1,200,000 bp on the *E. coli* chromosome show up-regulation following cisplatin treatment in all strains by array analysis. These genes are *ymfG, J, L, M* (Tables 5.3-5.6). Although these genes have no known function, they have previously been shown to be induced in a LexA-dependent manner following UV irradiation (137). We confirmed by real-time RT-PCR that the gene *ymfG* (b1141) is induced after treatment with cisplatin (Table 5.2 and Fig. 5.6).

In addition to real-time RT-PCR, we used a *lacZ* gene reporter assay to determine the dose-dependency of SOS response in each of the strains. For this assay wild-type, *dam*, and *dam mutS* mutant strains that carry a *sulA-lacZ* fusion gene were used to visualize SOS induction; increased expression of *sulA*, and consequently of *lacZ*, is visualized by β-galactosidase activity. The dose-dependent induction of *sulA-lacZ* is shown in Fig. 5.7. This assay also clearly shows the high level of SOS sub-induction in *dam* mutant cells as the number of red colonies, or colonies expressing LacZ, is higher throughout the area of the plate. As mentioned above, constitutive SOS induction has previously been reported for *E. coli* lacking DNA adenine methyltransferase (524,558,593).

5.2.5 Expression of genes involved in DNA maintenance and metabolism

Because the cytotoxic effects of cisplatin are believed to arise from the drug's reaction with DNA, we examined changes in expression of non-SOS genes known to be involved in DNA repair and maintenance. Array analysis shows that the genes *hupA* and *hupB* are down-regulated only in the *dam* mutant strain following cisplatin treatment (Table 5.4). These similar yet distinct genes encode the two subunits HUα and HUβ, respectively, of the *E. coli* nucleoid-associated protein HU. The dimeric protein HU acts as a histone-like protein and is capable of introducing negative supercoiling in the presence of toposiomerase I (41). HU also shows a binding preference for particular DNA structures such as cruciform DNA (56) as well as ssDNA or gaps (96) and plays a critical role in recombination and SOS induction (411,484). In addition to *hupA* and *hupB*, the genes encoding the primosomal proteins, PriA and PriB, also show unique regulation following drug treatment. PriA and PriB are involved in the assembly of the replication fork at recombination intermediates (254,325,613); PriA initiates reloading of the replicative helicase DnaB at branched DNA structures, and PriA along with PriB, PriC, Rep, and DnaT, reassembles the replication fork at recombination intermediates (613,614). Previous work has shown that a *priA dam* double mutant is inviable (522) and that *priA* mutant *E. coli* are very sensitive to cisplatin (521). In addition, we have previously shown that *priB* is expressed at a higher basal level in *dam* (4-fold) and *dam mutS* (3-fold) mutant strains when basal gene expression levels in the mutants are compared to the basal transcript levels in wild-type (593). RT-PCR analysis shows that *priA* and *priB* are induced following high doses of cisplatin in *dam* and to a lesser extent in *dam mutS E. coli*. We also examined gene expression changes for the two genes encoding DNA gyrase, *gyrA* and *gyrB*. The array data show that these genes are induced following cisplatin treatment in the *dam* mutant strain and real-time RT-PCR confirms this observation (Table 5.2 and Fig. 5.6). DNA gyrase, a type IIA topoisomerase, has the ability to actively introduce negative supercoils in DNA and is essential for viability (239,476). In addition, DNA gryase, along with HU (629), plays a role in suppressing illegitimate recombination (316,317,509,635).

Table 5.2: Differential gene expression of SOS response genes and genes involved in DNA repair and maintenance determined by microarray analysis and RT-PCR

LexA-regulated SOS response genes		Array Signal log ratio*				RT-PCR Signal log ratio*			
Gene name	Blattner Number	WT	*dam*	*dam mutS*	*mutS*	WT	*dam*	*dam mutS*	*mutS*
lexA	b4043	**2.43**	**.58**[c]	1.69[c]	**2.79**	**2.06**	**2.47**[c]	**1.00**[c]	**1.82**
recA	b2699	**5.45**	**2.06**[c]	2.55[c]	**4.62**	**3.87**	**4.67**[c]	**3.67**	**3.23**
oraA	b2698	**1.02**	**3.47**	1.73	**1.63**	**3.99**	**5.45**	**4.84**	**3.79**
recN	b2616	**2.17**	2.04[c]	1.52[c]	**3.24**	**3.25**	**3.69**[c]	**2.57**[c]	**3.42**
sulA	b0958	**3.66**	1.89[c]	2.76[c]	**3.64**	**4.73**	**4.81**[c]	**4.49**[c]	**4.49**
yebG	b1848	**4.62**	**2.42**[c]	**3.48**[c]	**5.60**	**3.49**	**3.94**[c]	**2.82**[c]	**2.63**
ruvA	b1861	.34	1.37	1.04	1.35	**1.57**	**2.63**[c]	**1.49**	**1.33**
ruvB	b1860	.38	**1.75**	**1.48**	.67	**1.89**	**2.63**[c]	**2.17**[c]	**1.18**
dinI	b1061	**4.46**	**2.96**	3.70	**4.22**	**3.43**	**4.15**[c]	**4.24**	**4.13**
uvrA	b4058	.19	**2.57**	.69	.27	**1.90**	**3.99**	**2.73**	**1.04**
uvrB	b0779	.52	**2.88**	1.81	1.33	**2.23**	**3.33**[c]	**2.91**[c]	**2.11**
cho/ ydjQ	b1741	-.02	.89	.06	.04	**0.80**	**1.68**[c]	0.13[c]	0.16
umuC	b1184	.07	1.68	.97	**.97**	**3.60**	**3.72**	**3.49**	**4.06**
umuD	b1183	1.40	1.23	1.87	**2.22**	**5.07**	**4.80**	**4.07**	**4.81**
sbmC	b2009	2.09	-.22	.53	**3.23**	**1.36**	0.95	-.72	**1.35**
*ymfG***	b1141	**2.99**	1.38	**3.40**	**3.61**	**5.68**	**4.47**	**4.50**	**6.03**
Genes involved in DNA repair/replication restart (non-SOS)		Array Signal log ratio*				RT-PCR Signal log ratio*			
Gene name	Blattner Number	WT	*dam*	*dam mutS*	*mutS*	WT	*dam*	*dam mutS*	*mutS*
gyrA	b2231	.88	**2.73**	1.26	.17	1.15	**3.32**	**3.09**	0.54
gyrB	b3699	.10	**2.11**	1.31	.19	0.82	**3.62**	**2.17**	0.51

Table 2: *Signal log ratios represent the log 2 of expression ratios (drug treated 150μM /untreated) for each strain (WT, wild-type); therefore a signal log ratio of 1 is the equivalent to a 2-fold change in expression level. Genes listed were tested by both array and RT-PCR. Signal log ratios in bold indicate that the *p*-value is below the threshold for significance ($p \leq 0.10$ for microarray and 0.05 for RT-PCR). [c]Genes that show sub-induction (at least 2-fold) in mutant versus wild-type when tested by this method (i.e., microarray or RT-PCR) (593). **Open reading frames in the *intE* region, including *ymfG* (b1141), were shown to be induced following UV exposure in a LexA-dependent manner (137).

5.2.6 Cisplatin induces double-strand break formation in a dose-dependent manner and *dam* mutant *E. coli* exhibit the highest level of DSBs following cisplatin treatment

It has previously been shown that cisplatin induces recombination and that DSB recombinational repair is an important mechanism for tolerating cisplatin treatment (779). These observations suggest that cisplatin damage leads to the formation of DNA strand breaks

that require recombination for repair, and possible mechanisms for cisplatin-induced strand breaks include replication fork collapse at sites of cisplatin-DNA adducts. It has also been shown that *dam* mutant *E. coli* have an increased level of DSBs whose formation is dependent on functional mismatch repair (593,731). In *dam* mutants, the combination of mismatch repair-dependent and cisplatin-induced recombination substrates could explain why this strain is hypersensitive to cisplatin treatment. However it has also been shown previously (778) as well as in the present study that the mismatch damage recognition protein MutS recognizes cisplatin-modified DNA *in vitro*, and Calmann and Marinus (90) have recently shown *in vitro* that adding MutS protein can inhibit RecA-mediated strand exchange of two recombination substrates when one substrate is modified with cisplatin. Thus *dam* mutants may be hypersensitive to cisplatin due to an additive effect of mismatch repair-induced damage and cisplatin-induced damage; alternatively, in addition to inducing DSBs in a Dam-deficient background, mismatch repair may also increase the level of cisplatin-induced damage by either blocking the recombinational repair of this damage or by acting at sites of cisplatin adducts. Under both scenarios, a deficiency in Dam leads to an increased level of DSB formation in a *mutS*$^{+}$ background. However under the latter scenario where MutS binding to cisplatin adducts leads to an increased level of DNA damage, we would expect the level of DSBs in *dam* mutants treated with drug to be higher than the sum of DSBs that occurs at the basal level in *dam* mutants and that occurs following drug treatment in wild-type cells. Furthermore, if there is a synergistic effect between mismatch repair and cisplatin in generating strand breaks, we expect this effect to be dependent on Dam deficiency, since survival data show that *mutS* mutant and wild-type strains are equally sensitive to treatment and thus a mutation in *mutS* alone does not lead to increased resistance. Accordingly, if a mechanism involving the interplay between MutS and cisplatin under the latter scenario described above requires a deficiency in Dam, then the level of damage in wild-type and *mutS* strains should be relatively the same following cisplatin treatment.

We performed neutral single-cell electrophoresis (54,641,644) to determine the level of DNA double-strand breaks in each of the strains following cisplatin treatment. This method allows the detection of a single DSB per cell (54) (Fig. 5.8A). Our data show that cisplatin induces DSBs in each of the strains and that the number of breaks increases with increasing dose of drug (Fig. 5.8B). We performed the Mann-Whitney U test to compare the frequency of DSBs per cell with increasing drug dose for each strain. Comparisons for which $P < .05$ are designated by an asterisk in Fig. 5.8B. The data show that 25 μM cisplatin causes a significant ($P < .05$) increase in the frequency of DSBs per cell in all strains except in the *dam* mutant

strain. Although there is an increase in the average number of DSBs per cell in *dam* mutant cells following 25 μM cisplatin, the lack of significance by the Mann-Whitney test may be attributed to the high basal frequency of DSBs in *dam* untreated cells (593). Along the same lines, the relatively small increase in DSBs in *dam* mutants observed at 100 and 150 μM doses is most likely due to the high percent of highly damaged cells at these doses (Fig. 5.8C), and therefore the data for *dam* mutant cells at these drug doses in Fig. 5.8B likely represent an underestimation of the level of DSBs.

The results in Fig. 5.8B show that the level of cisplatin-induced DSBs is higher in the *dam* mutant strain compared to the other strains. In addition to determining the dose-dependence of DSB formation within each strain, we performed the Mann-Whitney U test to compare the frequency of DSBs per cell between mutant and wild-type strains at each dose to determine the effect of genotype on cisplatin-induced DSB formation. By the Mann-Whitney U test, the frequency of DSBs per cell in *dam* mutants differs significantly ($P < .05$) from the frequency observed in the wild-type strain at doses 25 and 100 μM. The other strains did not show significant differences compared to wild-type in the frequency of DSBs per cell at each dose.

The data in Figure 5.8B include cells with as many as fifteen double-strand breaks because fifteen approaches the maximum number of DSBs we could accurately count visually. Any cell with more than fifteen DSBs was considered highly damaged and the percent of cells for each strain with a highly damaged chromosome are represented in Fig 20C. The *dam* mutant strain shows the greatest percent of cells with highly damaged chromosomes following cisplatin treatment. Taken together the data show that the level of DSBs following cisplatin treatment is greatest in *dam* mutant cells and that an additional mutation in *mutS* reduces the level of DSBs to the level observed in wild-type and *mutS* strains, and thus the DSB data correlates with drug sensitivity for these strains. The dependence on a *mutS*+ background for the high level of cisplatin-induced DSBs in *dam* mutant cells supports a mechanism by which mismatch repair contributes to the level of cisplatin-induced damage. Furthermore, because *mutS* and wild-type strains show similar levels of damage following treatment, this mechanism requires a deficiency in Dam.

In wild-type, *dam mutS*, and *mutS* strains, the number of cisplatin-induced DSBs increases from 1-2 breaks to 4-5 breaks at the highest drug dose. The average number of DSBs per cell in *dam* mutants is ~1 DSB at the basal level. If we assume that DSBs are additive and take the level of DSBs induced by cisplatin in wild-type cells and add the number of basal DSBs occurring in *dam* untreated cells, then the level of DSBs in *dam* mutants upon increasing drug dose should range from 2-3 breaks to 5-6 breaks per cell. While the level of DSBs in *dam* mutants surpasses these estimations upon increasing drug dose, MMR processing of cisplatin adducts, based on our data, is contributing at least 4-5 DSBs per *dam* mutant cell at the two highest drug doses tested. However because of the percent of highly damaged cells at these doses, this calculation is most likely an underestimation of the number of MMR-mediated DSBs following drug treatment in *dam* mutant cells.

In addition to visually counting the number of tails, we used image analysis software to determine the level of DNA damage in each strain following drug treatment. Komet (Kinetic Imaging, UK) image analysis software quantifies the fluorescence of the head and tail regions for each cell as well as the tail length. Cells with a high level of DSBs exhibit higher percentage of fluorescence in the tail region (Fig. 5.9A) as well as longer DNA migration, and one measurement to represent the data is the Olive tail moment, or the product of the fluorescence of migrated DNA and the distance between the head and center of gravity of DNA in the tail. A high Olive tail moment thus corresponds to a high level of DNA damage. Fig. 5.9B shows a box plot representation of the Olive tail moments and shows that damage is dose-dependent and reaches the highest level in the *dam* mutant strain. As with the visual quantification of DSBs, cells with very high damage that showed little or no head could not be analyzed by the software and were not included in this analysis; therefore the apparent decrease in damage at 150 μM cisplatin in *dam* mutants can be accounted for by those cells that exhibited such high levels of damage.

5.3 Discussion

A significant shortcoming of cisplatin-based chemotherapy is acquired resistance or tolerance to the drug. Drug tolerance has been associated with MMR status, where cells deficient in MMR exhibit a higher tolerance to drug treatment compared to their MMR-proficient counterparts (177,213,216,497,540,567). To help elucidate the role of mismatch repair in mediating cisplatin toxicity, we performed gene expression analysis and measured DSB formation following cisplatin treatment in *E. coli* strains differing in MMR status. It has

previously been shown that in a *dam* mutant background, where the signal distinguishing between parent and newly synthesized daughter strands is lost, cells are hypersensitive to cisplatin; however introducing an additional mutation in a mismatch repair gene (i.e., a mutation in either *mutS* or *mutL*) abrogates this sensitivity and essentially restores wild-type levels of resistance. Importantly, purified MutS interacts with the major cisplatin-DNA adduct, supporting the possibility that a direct interaction between MMR and cisplatin lesions influences cisplatin-induced toxicity. While we sought to uncover possible mechanisms of drug-induced toxicity that are dependent on MMR by comparing the global gene expression profiles of sensitive (*dam* mutant) and resistant (*dam mutS* mutant) strains, our results show that genome-wide expression patterns following cisplatin treatment show a general toxicity response. Cisplatin treatment caused the down-regulation of many genes involved in amino acid and carbohydrate metabolism as well as in energy production and conversion. Down-regulation of transcripts in amino acid metabolism and energy production gene groups has also been shown for *E. coli* exposed to cadmium (726). The lower expression of these genes in drug-treated cells may be the result of decreased cell growth; cells experiencing high levels of DNA damage may not be initiating cell division and may therefore be shutting down processes required for cell growth.

While global transcriptional responses reflect general toxicity, the expression changes of certain genes involved in DNA repair and maintenance follow patterns of drug sensitivity. The real-time RT-PCR results show that the SOS response is induced in a dose-dependent manner and to the greatest extent in the hypersensitive *dam* mutant strain. The high level of SOS induction indicates the presence of an SOS-inducing signal, which is most likely single-stranded DNA. The level of SOS induction also correlates with the level of DSBs detected in each strain, with *dam* mutants showing the highest level of DSB formation following cisplatin treatment. While it has been suggested that recombinational repair and the SOS response may be functioning near maximum capacity in *dam* mutants in order to deal with endogenous MMR-induced DSBs (443), our data show that the SOS response is largely induced in the *dam* mutant strain following cisplatin treatment and thus this mutant has the capacity to further induce SOS following exogenous stress. Further SOS induction beyond the basal level may be attributable to the fact that our data represents the average of ~10^8 cells in a cell culture, and McCool et al. have shown that constitutive SOS in *dam* mutants can best be represented by a two-population model where SOS expression is highly induced only in a small population of cells (462). We have also previously shown that DSB formation in *dam* mutants is a stochastic process and that at a certain point in time a

dam mutant cell may have as many as ten or more DSBs produced endogenously by mismatch repair, though the average level of DSBs in the culture may only be between one and two (593). Thus only at certain times in the life of a *dam* mutant cell may recombinational repair be functioning near maximal capacity, and thus upon cisplatin treatment SOS is further induced.

Although the SOS response is induced in all strains following cisplatin treatment, certain genes show differential regulation between the strains. For example, *gyrA* and *gyrB*, which encode DNA gyrase, are only induced in the Dam-deficient strains. DNA gyrase is an essential protein for DNA replication and is a unique topoisomerase due to its ability to introduce negative supercoils in the presence of ATP (239). In addition, DNA gyrase may interact with the histone-like protein HU to suppress short-homology-independent illegitimate recombination (629). Interestingly, *sbmC*, which is an SOS-inducible gene encoding an endogenous gyrase inhibitor (GyrI), is only up-regulated in wild-type and *mutS* strains and not in the Dam-deficient strains following cisplatin treatment. SbmC (GyrI) was shown to reduce the level of DSBs mediated by other non-chromosomal gyrase inhibitors and induction of *sbmC* as part of the SOS response may be important in reducing exogenous DNA-damage that is amplified by gyrase activity (100,101). In support of this role for GyrI, overexpression of *sbmC* was shown to confer resistance to mitomycin C (MMC) treatment (738) whereas a null mutation in *sbmC* leads to 2-fold more sensitivity to MMC (26). While DNA gyrase mediates short-homology-independent illegitimate recombination, this protective effect of *sbmC* expression may also be due to increased recombination in *sbmC* overexpressing cells; although such increased recombination upon *sbmC* induction has not been tested, overexpression of the DNA gyrase A subunit suppresses the illegitimate recombination observed in HU null cells (629). In addition to DNA gyrase and SbmC (GyrI) differential expression, *oraA*, a gene located down-stream of *recA* encoding an inhibitor of RecA called RecX, is induced to a greater degree in *dam* mutants. While *recA* and *oraA* genes are separated by a hairpin terminator that limits the level of the read-through *recA-oraA* transcript to approximately 5-10% of the level of the *recA* transcript (537), SOS induction of *recA* may lead to higher read-through and a proportionately higher amount of RecX protein. Thus although SOS induction and induced expression of *recA* serve to protect cells against strand breaks, perhaps higher read-through of *recA-oraA* in *dam* mutants is contributing to the hypersensitivity of these cells.

The present study shows that cisplatin induces DSBs in a dose-dependent manner and thus supports previous observations that cisplatin is highly recombinogenic and that

tolerance to cisplatin requires both RecBCD and RecFOR pathways of recombinational repair (521,779). In addition, cisplatin-DNA adducts are replication-blocking lesions (116,123,266,277,333) and *E. coli priA* mutants are hypersensitive to cisplatin treatment (521), indicating that replication restart in double-strand break repair (385) is important in tolerating cisplatin damage. Thus one mechanism for DSB formation following cisplatin treatment involves replication fork collapse at sites of cisplatin lesions. In our experiments the 90 minute recovery time following treatment allowed the cultures to undergo DNA replication, and thus DSBs may have occurred by replication fork collapse. Indeed, preliminary data indicate that the majority (~75%) of DSBs induced by cisplatin occur during DNA replication while the remaining ~25% of DSBs occur in non-replicating DNA (Nowosielska and Marinus, unpublished results).

In addition to the dose-dependence of DSB formation following cisplatin treatment, the data show that the level of DSBs is greatest in *dam* mutant cells. Because *dam mutS* cells show similar levels of cisplatin-induced DSBs as wild-type and *mutS* mutant cells, MMR contributes to the level of cisplatin-induced strand breaks in a *dam* mutant background. To address the role of MMR in the production of cisplatin-induced strand breaks, it is worthwhile to first consider the basal level of DSBs in *dam* mutants. We and others have previously shown that endogenous strand breaks in *dam* mutant cells depend on functional mismatch repair (593,731). While MutS may be introducing nicks on both strands at a GATC site (20), the inviability of *dam priA* double mutants (522) suggests DSBs arise when the replication fork encounters a single nick, which in *dam* mutants lacking adenine methylation may be more prevalent due to the abundance of MutH substrates, or unmethylated GATC sites, as well as the presence of multiple replication forks (420). The MMR-dependent production of endogenous DSBs in *dam E. coli* renders these mutants dependent on recombination for survival, and accordingly this requirement for recombination is eliminated by an additional mutation in mismatch repair (443). In the present study, because the level of DSBs in *dam* mutants surpasses the level expected if both MMR and cisplatin were inducing strand breaks only by independent mechanisms, the data support the idea that mismatch repair is contributing to the level of cisplatin-induced DSBs in *dam* mutants; in other words, mismatch repair and cisplatin are interacting to produce a higher level of damage than would otherwise occur if the two mechanisms—replication fork collapse at cisplatin adducts and fork collapse at MutH-catalyzed nicks—were operating independently.

One mechanism to describe how MMR and cisplatin produce strand breaks is futile cycling (Fig. 5.10A) (351). MMR may be introducing single nicks after recognition of cisplatin adducts in an attempt to initiate repair. In the absence of the strand discrimination signal, repair may be initiated on the strand opposite the lesion, and thus the lesion remains leading to additional repair cycles and a persistent single-strand gap. If this single nick is encountered by a replication fork, a lethal DSB will occur. Thus the presence of cisplatin adducts and recognition of these adducts by MutS may trigger downstream mismatch repair processes and increase the level of single MutH incisions. Such an increase in MutH incisions would require that the DNA be unmethylated, as fully methylated DNA is resistant to MutH cleavage. This mechanism may explain why mismatch repair-mediated sensitization of cells to cisplatin requires a *dam* mutant background, and genetic studies support this model to the extent that a *mutH* mutation was recently shown to abrogate the sensitivity of *dam* mutants (522). It is also worth mentioning that due to multiple firings of the origin of replication during the cell cycle (420), the presence of multiple replication forks in *dam* mutants provides these mutants with more opportunities to encounter both cisplatin lesions as well as single-strand gaps produced by MMR.

An alternative model to the mechanism described above involves the blocking of recombinational repair (Fig. 5.10B). It has recently been shown that MutS, performing its role in antirecombination (91,398,662), can inhibit RecA-mediated strand exchange of two recombination substrates when one substrate is modified to contain cisplatin adducts (90). While recombination is a critical mechanism for tolerating cisplatin damage (521,779), MMR may be contributing to cisplatin toxicity by blocking the recombinational bypass of cisplatin adducts, which would lead to an increased level of strand breaks in the cell. Importantly, it was recently demonstrated that a *mutSDelta800* mutation, which retains function in mutation avoidance but not in anti-recombination, rescues *dam* mutants from cisplatin hypersensitivity, thus supporting the hypothesis that anti-recombination may play a predominant role in MMR-mediated cisplatin toxicity (90). However for this mechanism to account for the hypersensitivity of *dam* mutants to cisplatin, which is dependent on MMR, MutS inhibition of recombinational repair would need to occur with greater efficiency in *dam* mutants compared to the other strains. Calmann and Marinus (90) propose that MutS inhibition of strand exchange is reversible but may require RuvAB proteins. While the basal level of damage in *dam* mutants requires recombination and specifically the RecA and RuvAB proteins for repair (443), this reversal may not occur in *dam* mutants in which RuvAB proteins are occupied (perhaps near full capacity) in dealing with the high level of basal

damage (i.e., the proteins are less available to enable the reversal of strand exchange inhibition). Dam-deficient cells also deficient in mismatch repair, however, have better recombinational repair abilities as they lack endogenous damage. Wild-type and *mutS* mutants have a similar basal state of damage as *dam mutS* cells (593), and thus these strains are better equipped to deal with cisplatin damage as their recombinational repair machinery is more available.

The present study shows that cisplatin induces double-strand breaks in a dose-dependent manner and that *dam* mutants exhibit the greatest level of cisplatin-induced strand breaks as well as the greatest up-regulation of SOS genes. Furthermore, MutS is required to enhance the level of cisplatin-induced strand breaks in *dam* mutant cells. While it is not clear if DSBs are arising by MMR-catalyzed incisions or by MMR-mediated antirecombination, the level of DSBs can account for the differential sensitivity and SOS induction observed in the strains. Thus these results support a model whereby mismatch repair proteins increase the level of secondary lesions resulting from cisplatin damage in a Dam-deficient background. Furthermore, these findings, in conjunction with previous results showing that recombination is important in tolerating cisplatin damage, suggest that DSBs may be the primary lethal lesions following cisplatin treatment. Unfolding the exact mechanisms by which mismatch repair contributes to the level of cisplatin-induced double-strand breaks requires further study.

Table 5.3: Wild-type *E. coli* treated with cisplatin compared to wildtype mock-treated *E. coli*.

A. Differentially expressed genes (± 2-fold) in wildtype *E. coli* treated with cisplatin compared to wildtype mock-treated *E. coli* (LPE $p \leq 0.10$).

Carbohydrate transport and metabolism					
Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
manX	b1817	-2.06	0.000	0.056	PTS enzyme IIAB, mannose-specific
mglB	b2150	-2.06	0.000	0.024	galactose-binding transport protein; receptor for galactose taxis
deoB	b4383	-1.58	0.000	0.005	phosphopentomutase

Cell division					
Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
sulA	b0958	3.66	0.000	0.000	suppressor of lon; inhibits cell division and ftsZ ring formation

Cell motility					
Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
fliC	b1923	-2.98	0.000	0.027	flagellar biosynthesis; flagellin, filament structural protein
flgF	b1077	-2.21	0.000	0.019	flagellar biosynthesis, cell-proximal portion of basal-body rod

Cell processes (adaptation/protection)					
Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
dinI	b1061	4.46	0.000	0.000	damage-inducible protein I

Cell wall/membrane biogenesis					
Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
murE	b0085	-2.62	0.001	0.088	meso-diaminopimelate-adding enzyme

Energy production and conversion					
Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
dctA	b3528	-2.10	0.000	0.059	uptake of C4-dicarboxylic acids
cyoA	b0432	-1.48	0.000	0.023	cytochrome o ubiquinol oxidase subunit II
yfaE	b2236	1.13	0.001	0.085	orf, hypothetical protein

Lipid transport and metabolism					
Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
fabD	b1092	1.93	0.000	0.027	malonyl-CoA-[acyl-carrier-protein] transacylase

Replication, recombination, DNA repair and modification					
Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
yi21_6	b4272	-1.96	0.000	0.000	IS2 hypothetical protein
recN	b2616	2.17	0.000	0.043	protein used in recombination and DNA repair
recA	b2699	5.45	0.000	0.043	DNA strand exchange and renaturation, DNA-dependent ATPase, DNA- and ATP-dependent coprotease

Transcription					
Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
flgM	b1071	-1.21	0.000	0.062	anti-FliA (anti-sigma) factor; also known as RflB protein
lexA	b4043	2.43	0.001	0.078	regulator for SOS(lexA) regulon

Translation					
Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
rmf	b0953	-3.18	0.000	0.006	ribosome modulation factor

General function prediction or unknown function					
Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
yodD	b1953	-2.21	0.000	0.059	orf, hypothetical protein
yccJ	b1003	-2.17	0.001	0.093	orf, hypothetical protein
	b2080	-1.75	0.001	0.083	orf, hypothetical protein
ydjK	b3100	-1.34	0.000	0.013	orf, hypothetical protein
oraA	b2698	1.02	0.000	0.001	regulator, OraA protein
yhdG	b3260	1.59	0.000	0.039	putative dehydrogenase
ydfZ	b1541	1.89	0.000	0.002	orf, hypothetical protein
ymfM	b1148	2.76	0.000	0.000	orf, hypothetical protein
ymfG	b1141	2.99	0.000	0.006	Excisionase-like protein from lambdoid prophage 14
yebF	b1847	3.02	0.000	0.006	orf, hypothetical protein
ymfL	b1147	3.42	0.000	0.000	orf, hypothetical protein
yebG	b1848	4.62	0.000	0.000	orf, hypothetical protein
ymfJ	b1144	4.92	0.000	0.000	orf, hypothetical protein

B. Genes with signal log ratios $-1 \leq x \leq 1$ but for which LPE $p \leq 0.10$.

Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
	b2335	-0.67	0.000	0.035	putative fimbrial protein
	b2641	-0.14	0.000	0.001	orf, hypothetical protein
gmd	b2053	-0.09	0.000	0.000	GDP-D-mannose dehydratase
	b1579	0.30	0.000	0.059	putative transposase
ybaP	b0482	0.46	0.000	0.000	putative ligase
nrdA	b2234	0.78	0.001	0.078	ribonucleoside diphosphate reductase 1, alpha subunit, B1

Table 5.4: *Dam* mutant *E. coli* treated with cisplatin compared to *dam* mock-treated *E. coli.*

5.4A Differentially expressed genes (± 2-fold) in *dam E. coli* treated with cisplatin compared to *dam* mock-treated *E. coli* (LPE $p \leq 0.10$).

Amino acid transport and metabolism					
Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
tnaA	b3708	-5.61	0.000	0.000	tryptophanase
gcvH	b2904	-3.85	0.000	0.001	in glycine cleavage complex, carrier of aminomethyl moiety via covalently bound lipoyl cofactor
ansB	b2957	-3.82	0.000	0.002	periplasmic L-asparaginase II
gcvP	b2903	-2.33	0.003	0.069	glycine decarboxylase, P protein of glycine cleavage system
aspC	b0928	-2.22	0.001	0.042	aspartate aminotransferase
asd	b3433	-1.50	0.005	0.103	aspartate-semialdehyde dehydrogenase
gltL	b0652	1.51	0.002	0.051	ATP-binding protein of glutamate/aspartate transport system
cysH	b2762	1.58	0.000	0.000	3'-phosphoadenosine 5'-phosphosulfate reductase

Carbohydrate transport and metabolism					
Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
malE	b4034	-6.34	0.000	0.000	periplasmic maltose-binding protein; substrate recognition for transport and chemotaxis
lamB	b4036	-5.62	0.000	0.000	phage lambda receptor protein; maltose high-affinity receptor; Maltoporin precursor
malK	b4035	-4.61	0.000	0.000	ATP-binding component of transport system for maltose
rbsB	b3751	-3.95	0.000	0.000	D-ribose periplasmic binding protein
treB	b4240	-3.87	0.000	0.001	PTS system enzyme II, trehalose specific
glpF	b3927	-3.68	0.000	0.001	facilitated diffusion of glycerol
ptsG	b1101	-3.60	0.000	0.004	PTS system, glucose-specific IIBC component
manX	b1817	-3.53	0.000	0.002	PTS enzyme IIAB, mannose-specific
galK	b0757	-3.42	0.000	0.011	galactokinase
manY	b1818	-3.23	0.001	0.027	PTS enzyme IIC, mannose-specific

mgsA	b0963	-3.11	0.004	0.087	methylglyoxal synthase
mglB	b2150	-2.96	0.000	0.009	galactose-binding transport protein; receptor for galactose taxis
malP	b3417	-2.58	0.000	0.006	maltodextrin phosphorylase
ptsH	b2415	-2.44	0.004	0.089	PTS system protein HPr
malM	b4037	-2.44	0.003	0.073	periplasmic protein of mal regulon
gatZ	b2095	-2.40	0.001	0.037	putative tagatose 6-phosphate kinase 1
ptsI	b2416	-1.82	0.003	0.074	PEP-protein phosphotransferase system enzyme I
glgB	b3432	-1.52	0.001	0.044	1,4-alpha-glucan branching enzyme
	b2386	1.07	0.001	0.022	putative transport protein
yidY	b3710	1.08	0.001	0.027	putative transport protein

Cell division

Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
minD	b1175	-1.70	0.004	0.092	cell division inhibitor, a membrane ATPase, activates minC
ftsN	b3933	-1.61	0.002	0.051	essential cell division protein
gidA	b3741	1.70	0.000	0.009	glucose-inhibited division; chromosome replication?
mreB	b3251	1.95	0.002	0.053	regulator of ftsI, penicillin binding protein 3, septation function

Cell motility

Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
fliC	b1923	-4.83	0.000	0.000	flagellar biosynthesis; flagellin, filament structural protein
flgF	b1077	-4.60	0.000	0.000	flagellar biosynthesis, cell-proximal portion of basal-body rod
flgC	b1074	-4.43	0.000	0.000	flagellar biosynthesis, cell-proximal portion of basal-body rod
flgN	b1070	-4.27	0.000	0.000	protein of flagellar biosynthesis
flgG	b1078	-4.21	0.000	0.000	flagellar biosynthesis, cell-distal portion of basal-body rod
flgB	b1073	-4.03	0.000	0.000	flagellar biosynthesis, cell-proximal portion of basal-body rod
flgE	b1076	-3.72	0.000	0.000	flagellar biosynthesis, hook protein
flgD	b1075	-3.67	0.000	0.005	flagellar biosynthesis, initiation of hook assembly
fliL	b1944	-3.60	0.001	0.030	flagellar biosynthesis
fliG	b1939	-3.52	0.000	0.009	flagellar biosynthesis, component of motor switching and energizing, enabling rotation and determining its direction
fliN	b1946	-3.13	0.002	0.055	flagellar biosynthesis, component of motor switch and energizing, enabling rotation and determining its direction
fliM	b1945	-3.06	0.000	0.004	flagellar biosynthesis, component of motor switch and energizing, enabling rotation and determining its direction
fliZ	b1921	-2.93	0.001	0.040	orf, hypothetical protein; Flagellar biogenesis protein
flgL	b1083	-2.72	0.001	0.027	flagellar biosynthesis; hook-filament junction protein
fliS	b1925	-2.48	0.001	0.025	flagellar biosynthesis; repressor of class 3a and 3b

					operons (RflA activity)
fimA	b4314	-2.44	0.000	0.019	major type 1 subunit fimbrin (pilin)
flgI	b1080	-2.28	0.000	0.015	homolog of Salmonella P-ring of flagella basal body
fliD	b1924	-2.21	0.000	0.010	flagellar biosynthesis; filament capping protein; enables filament assembly
fliP	b1948	-1.17	0.003	0.068	flagellar biosynthesis
ycaH	b0915	0.98	0.002	0.058	putative EC 1.2 enzyme
yghE	b2969	1.13	0.001	0.036	putative general secretion pathway for protein export (GSP)
exbD	b3005	2.60	0.000	0.002	uptake of enterochelin; tonB-dependent uptake of B colicins

Cell processes (adaptation/protection)					
Gene	**Blattner Number**	**Signal Log Ratio**	**Raw p-value**	**Adjusted LPE p-value**	**Gene Product and Function**
dinI	b1061	2.96	0.004	0.088	damage-inducible protein I

Cell wall/membrane biogenesis					
Gene	**Blattner Number**	**Signal Log Ratio**	**Raw p-value**	**Adjusted LPE p-value**	**Gene Product and Function**
ompC	b2215	-3.66	0.000	0.000	outer membrane protein 1b (Ib;c)
ompF	b0929	-3.24	0.000	0.002	outer membrane protein 1a (Ia;b;F)
galE	b0759	-2.92	0.001	0.025	UDP-galactose-4-epimerase
glgS	b3049	-2.29	0.005	0.106	glycogen biosynthesis, rpoS dependent
rfaG	b3631	1.20	0.000	0.011	glucosyltransferase I; lipopolysaccharide core biosynthesis
gidB	b3740	2.46	0.000	0.000	glucose-inhibited division; chromosome replication?

Coenzyme transport and metabolism					
Gene	**Blattner Number**	**Signal Log Ratio**	**Raw p-value**	**Adjusted LPE p-value**	**Gene Product and Function**
lipA	b0628	1.86	0.000	0.016	lipoate synthesis, sulfur insertion

Energy production and conversion					
Gene	**Blattner Number**	**Signal Log Ratio**	**Raw p-value**	**Adjusted LPE p-value**	**Gene Product and Function**
glpK	b3926	-5.65	0.000	0.000	glycerol kinase
mdh	b3236	-4.58	0.000	0.000	malate dehydrogenase
mtlA	b3599	-4.40	0.000	0.000	PTS system, mannitol-specific enzyme IIABC components
frdB	b4153	-3.92	0.000	0.014	fumarate reductase, anaerobic, iron-sulfur protein subunit
frdD	b4151	-3.51	0.002	0.055	fumarate reductase, anaerobic, membrane anchor polypeptide
glcB	b2976	-3.47	0.000	0.000	malate synthase G
pflB	b0903	-3.38	0.000	0.000	formate acetyltransferase 1
galT	b0758	-3.16	0.000	0.009	galactose-1-phosphate uridylyltransferase

sucD	b0729	-3.06	0.005	0.100	succinyl-CoA synthetase, alpha subunit
frdA	b4154	-2.91	0.000	0.016	fumarate reductase, anaerobic, flavoprotein subunit
glpC	b2243	-2.61	0.003	0.077	sn-glycerol-3-phosphate dehydrogenase (anaerobic), K-small subunit
nuoI	b2281	-2.07	0.001	0.029	NADH dehydrogenase I chain I
yhdH	b3253	-1.51	0.002	0.055	putative dehydrogenase
nuoE	b2285	-1.42	0.002	0.058	NADH dehydrogenase I chain E
glcF	b2978	-1.21	0.001	0.030	glycolate oxidase iron-sulfur subunit
fdhF	b4079	-1.20	0.003	0.073	selenopolypeptide subunit of formate dehydrogenase H
	b1588	-1.19	0.001	0.046	putative oxidoreductase, major subunit
fdoH	b3893	-1.19	0.000	0.005	formate dehydrogenase-O, iron-sulfur subunit
dld	b2133	-1.13	0.001	0.035	D-lactate dehydrogenase, FAD protein, NADH independent
	b1628	1.28	0.000	0.012	orf, hypothetical protein
yqhD	b3011	2.02	0.000	0.004	putative oxidoreductase
ndh	b1109	2.84	0.000	0.000	respiratory NADH dehydrogenase

Inorganic ion transport and metabolism

Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
bfr	b3336	-3.24	0.000	0.000	bacterioferrin, an iron storage homoprotein
cysP	b2425	1.22	0.000	0.002	thiosulfate binding protein
pstS	b3728	1.36	0.000	0.007	high-affinity phosphate-specific transport system; periplasmic phosphate-binding protein
phoU	b3724	1.49	0.000	0.017	negative regulator for pho regulon and putative enzyme in phosphate metabolism
cysJ	b2764	1.69	0.001	0.038	sulfite reductase (NADPH), flavoprotein beta subunit
pstB	b3725	1.76	0.001	0.030	ATP-binding component of high-affinity phosphate-specific transport system
cysI	b2763	1.95	0.000	0.000	sulfite reductase, alpha subunit
mgtA	b4242	2.65	0.000	0.000	Mg2+ transport ATPase, P-type 1
sodA	b3908	2.73	0.000	0.000	superoxide dismutase, manganese

Intracellular trafficking, secretion, and vesicular transport

Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
spy	b1743	1.79	0.000	0.012	periplasmic protein related to spheroblast formation; P pilus assembly/Cpx signaling pathway, periplasmic inhibitor/zinc-resistance associated protein

Lipid transport and metabolism

Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
yhaE	b3125	-2.81	0.001	0.044	putative dehydrogenase
fadD	b1805	1.36	0.002	0.055	acyl-CoA synthetase, long-chain-fatty-acid--CoA ligase

Nucleotide transport and metabolism

Gene	Blattner Number	Signal Log	Raw p-value	Adjusted LPE p-	Gene Product and Function

		Ratio		value	
udp	b3831	-5.16	0.000	0.000	uridine phosphorylase
deoD	b4384	-2.97	0.001	0.023	purine-nucleoside phosphorylase
deoC	b4381	-2.61	0.000	0.018	2-deoxyribose-5-phosphate aldolase
nupC	b2393	-2.21	0.004	0.089	permease of transport system for 3 nucleosides
ybeK	b0651	-1.99	0.002	0.063	putative tRNA synthetase
	b2084	1.12	0.000	0.006	orf, hypothetical protein; Xanthine dehydrogenase, molybdopterin-binding subunit B
apt	b0469	1.33	0.004	0.089	adenine phosphoribosyltransferase
pyrF	b1281	1.41	0.001	0.029	orotidine-5'-phosphate decarboxylase
nrdA	b2234	2.16	0.000	0.017	ribonucleoside diphosphate reductase 1, alpha subunit, B1

Phage, transposon, or plasmid

Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
pspB	b1305	2.30	0.000	0.006	phage shock protein

Posttranslational modification, protein turnover, chaperones

Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
hslJ	b1379	1.18	0.001	0.033	heat shock protein hslJ

Replication, recombination, DNA repair and modification

Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
hupB	b0440	-3.16	0.000	0.002	DNA-binding protein HU-beta, NS1 (HU-1)
dps	b0812	-3.10	0.001	0.035	global regulator, starvation conditions
hupA	b4000	-2.57	0.003	0.068	DNA-binding protein HU-alpha (HU-2)
mutM	b3635	1.18	0.003	0.074	formamidopyrimidine DNA glycosylase
dnaA	b3702	1.49	0.003	0.067	DNA biosynthesis; initiation of chromosome replication; can be transcription regulator
ruvB	b1860	1.75	0.000	0.005	Holliday junction helicase subunit A; branch migration; repair
dnaG	b3066	1.80	0.000	0.006	DNA biosynthesis; DNA primase
recA	b2699	2.06	0.003	0.072	DNA strand exchange and renaturation, DNA-dependent ATPase, DNA- and ATP-dependent coprotease
gyrB	b3699	2.11	0.003	0.072	DNA gyrase subunit B, type II topoisomerase, ATPase activity
ntpA	b1865	2.17	0.003	0.077	dATP pyrophosphohydrolase
uvrA	b4058	2.57	0.000	0.002	excision nuclease subunit A
gyrA	b2231	2.73	0.005	0.100	DNA gyrase, subunit A, type II topoisomerase
uvrB	b0779	2.88	0.001	0.028	DNA repair; excision nuclease subunit B
tpr	b1229	3.26	0.000	0.000	a protaminelike protein
deaD	b3162	3.27	0.001	0.025	inducible ATP-independent RNA helicase

Secondary metabolites biosynthesis, transport and catabolism

Gene	Blattner	Signal	Raw p-	Adjusted	Gene Product and Function

	Number	Log Ratio	value	LPE p-value	
srlD	b2705	-2.61	0.003	0.068	glucitol (sorbitol)-6-phosphate dehydrogenase
	b1519	1.19	0.002	0.057	putative enzyme

Signal transduction mechanisms

Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
rseA	b2572	-1.60	0.005	0.102	sigma-E factor, negative regulatory protein
uhpA	b3669	1.15	0.000	0.003	response regulator, positive activator of uhpT transcription (sensor, uhpB)

Transcription

Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
fliA	b1922	-4.13	0.000	0.000	flagellar biosynthesis; alternative sigma factor 28; regulation of flagellar operons
flgM	b1071	-3.12	0.000	0.000	anti-FliA (anti-sigma) factor; also known as RflB protein
cspG	b0990	-2.79	0.000	0.002	homolog of Salmonella cold shock protein
cspB	b1557	-2.65	0.000	0.017	cold shock protein; may affect transcription
cspI	b1552	-2.32	0.004	0.082	cold shock-like protein
trpR	b4393	-1.17	0.000	0.000	regulator for trp operon and aroH; trp aporepressor
ybdS	b0612	1.02	0.005	0.103	putative a membrane protein; citrate carrier; response regulator of citrate/malate metabolism
yqfE	b2915	1.05	0.001	0.032	orf, hypothetical protein
arsR	b3501	1.29	0.004	0.087	transcriptional repressor of chromosomal ars operon
fis	b3261	3.34	0.000	0.000	site-specific DNA inversion stimulation factor; DNA-binding protein; a trans activator for transcription

Translation

Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
yjgF	b4243	-3.80	0.000	0.001	orf, hypothetical protein
rpsV	b1480	-3.42	0.002	0.053	30S ribosomal subunit protein S22
rmf	b0953	-2.24	0.003	0.067	ribosome modulation factor
fmt	b3288	1.13	0.004	0.087	10-formyltetrahydrofolate:L-methionyl-tRNA(fMet) N-formyltransferase
rbfA	b3167	1.50	0.001	0.029	ribosome-binding factor A
serU	b1975	1.68	0.003	0.077	Serine tRNA2
yciL	b1269	2.12	0.001	0.031	orf, hypothetical protein
yabO	b0058	2.13	0.000	0.006	orf, hypothetical protein
ileX	b3069	2.24	0.001	0.038	Isoleucine tRNA2
argS	b1876	2.31	0.000	0.001	arginine tRNA synthetase
argW	b2348	2.35	0.003	0.072	Arginine tRNA5

General function prediction or unknown

Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function

yfiD	b2579	-4.47	0.000	0.000	putative formate acetyltransferase
hdeA	b3510	-4.09	0.000	0.009	orf, hypothetical protein
yahO	b0329	-3.28	0.002	0.049	orf, hypothetical protein
elaB	b2266	-3.25	0.002	0.059	orf, hypothetical protein; Uncharacterized conserved protein
tnaL	b3707	-3.24	0.001	0.024	tryptophanase leader peptide
yliH	b0836	-3.09	0.000	0.002	putative receptor
glcG	b2977	-2.73	0.000	0.001	orf, hypothetical protein
yccJ	b1003	-2.71	0.001	0.025	orf, hypothetical protein
osmE	b1739	-2.70	0.001	0.033	activator of ntrL gene; Osmotically inducible lipoprotein E precursor
hns	b1237	-2.59	0.000	0.016	DNA-binding protein HLP-II (HU, BH2, HD, NS); pleiotropic regulator
	b1044	-2.33	0.000	0.013	orf, hypothetical protein; Predicted HD superfamily hydrolase
ygiW	b3024	-2.32	0.004	0.087	orf, hypothetical protein
ygdI	b2809	-2.28	0.005	0.102	orf, hypothetical protein
yecR	b1904	-2.21	0.002	0.057	orf, hypothetical protein
ybgO	b0716	-1.53	0.002	0.058	orf, hypothetical protein
	b2641	1.00	0.000	0.002	orf, hypothetical protein
yijF	b3944	1.00	0.001	0.045	orf, hypothetical protein; Uncharacterized protein conserved in bacteria
yjjX	b4394	1.07	0.001	0.025	orf, hypothetical protein
	b1152	1.18	0.005	0.096	orf, hypothetical protein
glpG	b3424	1.19	0.002	0.048	protein of glp regulon
	b2511	1.26	0.000	0.017	putative GTP-binding factor
ycfJ	b1110	1.65	0.004	0.085	orf, hypothetical protein
thdF	b3706	1.72	0.001	0.034	GTP-binding protein in thiophene and furan oxidation
yeeE	b2013	1.78	0.000	0.001	putative transport system permease protein
yfgB	b2517	1.83	0.000	0.003	orf, hypothetical protein
ymfM	b1148	2.02	0.001	0.031	orf, hypothetical protein
ybeB	b0637	2.04	0.000	0.005	orf, hypothetical protein
ymfL	b1147	2.18	0.000	0.004	orf, hypothetical protein
yhdG	b3260	2.24	0.001	0.040	putative dehydrogenase
	b1970	2.40	0.000	0.000	orf, hypothetical protein
yebG	b1848	2.42	0.005	0.103	orf, hypothetical protein
	b0100	2.50	0.000	0.001	orf, hypothetical protein
ycfR	b1112	2.62	0.000	0.011	orf, hypothetical protein
yebF	b1847	2.64	0.002	0.055	orf, hypothetical protein
yjcB	b4060	2.81	0.000	0.000	orf, hypothetical protein
yebE	b1846	3.29	0.000	0.000	orf, hypothetical protein
ymfJ	b1144	3.39	0.000	0.000	orf, hypothetical protein
oraA	b2698	3.47	0.000	0.000	regulator, OraA protein
yhcN	b3238	3.48	0.000	0.000	orf, hypothetical protein

5.4B Genes with signal log ratios $-1.0 \leq x \leq 1.0$ but for which LPE $p \leq 0.10$.

Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function

pgi	b4025	-0.95	0.004	0.085	glucosephosphate isomerase
narH	b1225	-0.94	0.000	0.010	nitrate reductase 1, beta subunit
ygfJ	b2877	-0.86	0.001	0.035	orf, hypothetical protein
ynfE	b1587	-0.70	0.001	0.039	putative oxidoreductase, major subunit
yhjW	b3546	-0.62	0.000	0.010	predicted membrane-associated, metal-dependent hydrolase
rzpR	b1362	-0.59	0.001	0.029	putative Rac prophage endopeptidase
gltV	b4008	-0.54	0.000	0.009	Glutamate tRNA2
tsr	b4355	-0.54	0.000	0.001	methyl-accepting chemotaxis protein I, serine sensor receptor
glyY	b4165	-0.53	0.002	0.055	Glycine tRNA3
ucpA	b2426	-0.53	0.000	0.002	putative oxidoreductase
sbcB	b2011	-0.53	0.005	0.103	exonuclease I, 3' --> 5' specific; deoxyribophosphodiesterase
gltW	b2590	-0.51	0.000	0.009	Glutamate tRNA2
gltT	b3969	-0.50	0.000	0.002	Glutamate tRNA2
gltU	b3757	-0.48	0.000	0.002	Glutamate tRNA2
menB	b2262	-0.48	0.002	0.055	dihydroxynaphtoic acid synthetase
mdoG	b1048	-0.47	0.002	0.062	periplasmic glucans biosynthesis protein
mdoH	b1049	-0.40	0.003	0.068	membrane glycosyltransferase; synthesis of membrane-derived oligosaccharide (MDO)
ybjT	b0869	-0.36	0.000	0.000	putative dTDP-glucose enzyme
hcaA2	b2539	-0.35	0.000	0.000	small terminal subunit of phenylpropionate dioxygenase
ppc	b3956	-0.34	0.004	0.085	phosphoenolpyruvate carboxylase
t150	b3558	-0.33	0.003	0.070	IS150 putative transposase
dcd	b2065	-0.24	0.002	0.046	2'-deoxycytidine 5'-triphosphate deaminase
thiF	b3992	-0.20	0.000	0.002	thiamin biosynthesis, thiazole moiety
glnA	b3870	-0.15	0.004	0.087	glutamine synthetase
basS	b4112	-0.12	0.000	0.003	sensor protein for basR
acs	b4069	-0.07	0.000	0.000	acetyl-CoA synthetase
cyoA	b0432	0.14	0.005	0.104	cytochrome o ubiquinol oxidase subunit II
hsdM	b4349	0.23	0.000	0.000	host modification; DNA methylase M
yhiP	b3496	0.28	0.000	0.012	putative transport protein
exbB	b3006	0.28	0.000	0.007	uptake of enterochelin; tonB-dependent uptake of B colicins
cysA	b2422	0.30	0.001	0.026	ATP-binding component of sulfate permease A protein; chromate resistance
hyfB	b2482	0.37	0.005	0.094	hydrogenase 4 membrane subunit
betB	b0312	0.39	0.003	0.067	NAD+-dependent betaine aldehyde dehydrogenase
uxuB	b4323	0.41	0.004	0.081	D-mannonate oxidoreductase
recG	b3652	0.44	0.004	0.082	DNA helicase, resolution of Holliday junctions, branch migration
ytfH	b4212	0.49	0.001	0.031	orf, hypothetical protein
purD	b4005	0.50	0.001	0.038	phosphoribosylglycinamide synthetase
ydiE	b1705	0.55	0.005	0.103	orf, hypothetical protein
argB	b3959	0.56	0.000	0.001	acetylglutamate kinase
yhiQ	b3497	0.56	0.003	0.068	orf, hypothetical protein
yjbF	b4027	0.58	0.001	0.029	orf, hypothetical protein
pdhR	b0113	0.60	0.001	0.033	transcriptional regulator for pyruvate dehydrogenase complex
ybjF	b0859	0.60	0.002	0.054	putative enzyme
mreC	b3250	0.63	0.005	0.101	rod shape-determining protein
	b1297	0.63	0.004	0.092	putative glutamine synthetase (EC 6.3.1.2)

entF	b0586	0.64	0.004	0.087	ATP-dependent serine activating enzyme (may be part of enterobactin synthase as component F)
livJ	b3460	0.65	0.002	0.057	high-affinity amino acid transport system; periplasmic binding protein
gntU_2	b3435	0.67	0.002	0.059	low-affinity gluconate transport permease protein, fragment 2
suhB	b2533	0.67	0.005	0.105	enhances synthesis of sigma32 in mutant; extragenic suppressor, may modulate RNAse III lethal action
hisG	b2019	0.67	0.002	0.051	ATP phosphoribosyltransferase
yhcP	b3240	0.68	0.004	0.087	orf, hypothetical protein
alr	b4053	0.68	0.001	0.028	alanine racemase 1
agaA	b3135	0.70	0.003	0.068	putative N-acetylgalactosamine-6-phosphate deacetylase
yhjY	b3548	0.72	0.002	0.053	putative lipase
recD	b2819	0.73	0.001	0.028	DNA helicase, ATP-dependent dsDNA/ssDNA exonuclease V subunit, ssDNA endonuclease
yheG	b3326	0.73	0.004	0.089	putative general secretion pathway for protein export (GSP) (TYPE II TRAFFIC WARDEN ATPASE)
yjaI	b4002	0.77	0.000	0.000	orf, hypothetical protein
	b3122	0.77	0.003	0.076	orf, hypothetical protein
gltD	b3213	0.78	0.004	0.089	glutamate synthase, small subunit
yhiN	b3492	0.78	0.000	0.015	orf, hypothetical protein
yjcD	b4064	0.78	0.004	0.089	orf, hypothetical protein
yjdL	b4130	0.80	0.002	0.058	putative peptide transporter
bisZ	b1872	0.81	0.004	0.090	biotin sulfoxide reductase 2
yi82_1	b0017	0.82	0.002	0.052	IS186 and IS421 hypothetical protein
exuT	b3093	0.83	0.004	0.085	transport of hexuronates
dnaC	b4361	0.84	0.004	0.087	chromosome replication; initiation and chain elongation
agaW	b3134	0.85	0.002	0.046	PTS system N-acetylgalactosameine-specific IIC component 2
uvrD	b3813	0.88	0.002	0.048	DNA-dependent ATPase I and helicase II
rnd	b1804	0.90	0.002	0.047	RNase D, processes tRNA precursor
yjaB	b4012	0.91	0.002	0.056	orf, hypothetical protein
cirA	b2155	0.91	0.001	0.032	outer membrane receptor for iron-regulated colicin I receptor; porin; requires tonB gene product
yjeN	b4157	0.92	0.000	0.014	orf, hypothetical protein
rem	b1561	0.93	0.003	0.072	SWIB-domain-containing proteins implicated in chromatin remodeling
phnN	b4094	0.94	0.000	0.011	ATP-binding component of phosphonate transport
smf_1	b3286	0.95	0.004	0.083	orf, fragment 1

Table 5.5: *Dam mutS* mutant *E. coli* treated with cisplatin compared to *dam mutS* mock-treated *E. coli*.

5.5A Differentially expressed genes (± 2-fold) in *dam mutS E. coli* treated with cisplatin compared to *dam mutS* mock-treated *E. coli* (LPE $p \leq 0.10$).

Amino acid transport and metabolism					
Gene	**Blattner Number**	**Signal Log Ratio**	**Raw p-value**	**Adjusted LPE p-value**	**Gene Product and Function**

tnaA	b3708	-5.01	0.000	0.000	tryptophanase
appA	b0980	-1.01	0.000	0.042	phosphoanhydride phosphorylase; pH 2.5 acid phosphatase; periplasmic
trpL	b1265	2.10	0.000	0.002	trp operon leader peptide

Carbohydrate transport and metabolism					
Gene	**Blattner Number**	**Signal Log Ratio**	**Raw p-value**	**Adjusted LPE p-value**	**Gene Product and Function**
malE	b4034	-4.94	0.000	0.000	periplasmic maltose-binding protein; substrate recognition for transport and chemotaxis
lamB	b4036	-4.71	0.000	0.001	phage lambda receptor protein; maltose high-affinity receptor; Maltoporin precursor

Cell motility					
Gene	**Blattner Number**	**Signal Log Ratio**	**Raw p-value**	**Adjusted LPE p-value**	**Gene Product and Function**
fliC	b1923	-3.90	0.000	0.003	flagellar biosynthesis; flagellin, filament structural protein
flgB	b1073	-3.68	0.000	0.012	flagellar biosynthesis, cell-proximal portion of basal-body rod
flgC	b1074	-3.55	0.000	0.004	flagellar biosynthesis, cell-proximal portion of basal-body rod
flgF	b1077	-3.31	0.000	0.000	flagellar biosynthesis, cell-proximal portion of basal-body rod
exbB	b3006	1.00	0.000	0.012	uptake of enterochelin; tonB-dependent uptake of B colicins

Energy production and conversion					
Gene	**Blattner Number**	**Signal Log Ratio**	**Raw p-value**	**Adjusted LPE p-value**	**Gene Product and Function**
mtlA	b3599	-2.92	0.001	0.085	PTS system, mannitol-specific enzyme IIABC components

Inorganic ion transport and metabolism					
Gene	**Blattner Number**	**Signal Log Ratio**	**Raw p-value**	**Adjusted LPE p-value**	**Gene Product and Function**
cysA	b2422	1.07	0.000	0.047	ATP-binding component of sulfate permease A protein; chromate resistance
cysI	b2763	2.18	0.000	0.051	sulfite reductase, alpha subunit
sodA	b3908	3.46	0.000	0.003	superoxide dismutase, manganese

Replication, recombination, DNA repair and modification					
Gene	**Blattner Number**	**Signal Log Ratio**	**Raw p-value**	**Adjusted LPE p-value**	**Gene Product and Function**
tpr	b1229	2.03	0.000	0.000	a protaminelike protein

Transcription					
Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
fliA	b1922	-3.53	0.000	0.043	flagellar biosynthesis; alternative sigma factor 28; regulation of flagellar operons

Translation					
Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
leuX	b4270	3.13	0.000	0.012	Leucine tRNA5 (amber [UAG] suppressor)
ileY	b2652	3.72	0.000	0.000	Isoleucine tRNA2 variant
ileX	b3069	4.00	0.000	0.000	Isoleucine tRNA2
argW	b2348	4.07	0.000	0.052	Arginine tRNA5

General function prediction or function unknown					
Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
tnaL	b3707	-3.18	0.000	0.009	tryptophanase leader peptide
	b0100	1.64	0.000	0.052	orf, hypothetical protein
ymfM	b1148	2.69	0.000	0.039	orf, hypothetical protein
ymfL	b1147	3.37	0.000	0.035	orf, hypothetical protein
ymfG	b1141	3.40	0.000	0.001	Excisionase-like protein from lambdoid prophage 14
yebG	b1848	3.48	0.000	0.031	orf, hypothetical protein
ymfJ	b1144	4.76	0.000	0.007	orf, hypothetical protein

5.5B Genes with signal log ratios $-1 \leq x \leq 1$ but for which LPE $p \leq 0.10$.

Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
ybbA	b0495	-0.93	0.000	0.052	putative ATP-binding component of a transport system
yafW	b0246	-0.91	0.000	0.047	orf, hypothetical protein
yafV	b0219	-0.84	0.000	0.008	putative EC 3.5. amidase-type enzyme
ybaP	b0482	-0.83	0.000	0.059	putative ligase
yjfJ	b4182	-0.64	0.000	0.008	putative alpha helical protein
yi91a	b0255	-0.64	0.000	0.000	IS911 hypothetical protein, variant (IS911A)
yrfH	b3400	-0.01	0.000	0.000	orf, hypothetical protein
	b1706	0.23	0.000	0.012	orf, hypothetical protein
ilvA	b3772	0.26	0.000	0.000	threonine deaminase (dehydratase)
yigB	b3812	0.60	0.000	0.016	putative phosphatase
fdnH	b1475	0.63	0.001	0.064	formate dehydrogenase-N, nitrate-inducible, iron-sulfur beta subunit
parC	b3019	0.64	0.001	0.068	DNA topoisomerase IV subunit A
proW	b2678	0.68	0.001	0.073	high-affinity transport system for glycine betaine and proline
livK	b3458	0.72	0.000	0.062	high-affinity leucine-specific transport system;

					periplasmic binding protein
	b2084	0.91	0.001	0.101	orf, hypothetical protein

Table 5.6: *MutS* mutant *E. coli* treated with cisplatin compared to *mutS* mock-treated *E. coli.*

5.6A Differentially expressed genes (± 2-fold) in *mutS E. coli* treated with cisplatin compared to *mutS* mock-treated *E. coli* (LPE $p \leq 0.10$).

Amino acid transport and metabolism					
Gene	**Blattner Number**	**Signal Log Ratio**	**Raw p-value**	**Adjusted LPE p-value**	**Gene Product and Function**
mtr	b3161	-5.00	0.000	0.000	tryptophan-specific transport protein
trpA	b1260	-4.23	0.000	0.000	tryptophan synthase, alpha protein
trpB	b1261	-2.80	0.000	0.000	tryptophan synthase, beta protein
trpC	b1262	-2.73	0.000	0.000	N-(5-phosphoribosyl)anthranilate isomerase and indole-3-glycerolphosphate synthetase
trpD	b1263	-2.58	0.000	0.000	anthranilate synthase component II, glutamine amidotransferase and phosphoribosylanthranilate transferase
yqjC	b3097	-2.53	0.001	0.035	orf, hypothetical protein; Lactoylglutathione lyase and related lyases
trpE	b1264	-2.49	0.000	0.000	anthranilate synthase component I
fliY	b1920	-1.53	0.000	0.011	putative periplasmic binding transport protein
ybjU	b0870	-1.14	0.002	0.058	putative arylsulfatase
ycjI	b1326	-1.13	0.001	0.030	putative carboxypeptidase
hisL	b2018	1.19	0.001	0.034	his operon leader peptide
pepE	b4021	1.77	0.000	0.000	peptidase E, a dipeptidase where amino-terminal residue is aspartate

Carbohydrate transport and metabolism					
Gene	**Blattner Number**	**Signal Log Ratio**	**Raw p-value**	**Adjusted LPE p-value**	**Gene Product and Function**
gatB	b2093	-3.25	0.000	0.000	galactitol-specific enzyme IIB of phosphotransferase system
manY	b1818	-2.98	0.000	0.000	PTS enzyme IIC, mannose-specific
manX	b1817	-2.85	0.000	0.002	PTS enzyme IIAB, mannose-specific
lamB	b4036	-2.72	0.000	0.000	phage lambda receptor protein; maltose high-affinity receptor; Maltoporin precursor
malE	b4034	-2.32	0.000	0.000	periplasmic maltose-binding protein; substrate recognition for transport and chemotaxis
malK	b4035	-2.15	0.000	0.000	ATP-binding component of transport system for maltose
manZ	b1819	-2.14	0.001	0.029	PTS enzyme IID, mannose-specific
gatA	b2094	-1.79	0.000	0.006	galactitol-specific enzyme IIA of phosphotransferase system
malP	b3417	-1.69	0.000	0.000	maltodextrin phosphorylase

gatZ	b2095	-1.22	0.005	0.101	putative tagatose 6-phosphate kinase 1
malQ	b3416	-1.07	0.001	0.021	4-alpha-glucanotransferase (amylomaltase)
gapC_1	b1417	-1.07	0.002	0.055	glyceraldehyde 3-phosphate dehydrogenase C, interrupted/erythrose-4-phosphate dehydrogenase
rpiA	b2914	1.01	0.000	0.008	ribosephosphate isomerase, constitutive

Cell division

Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
sulA	b0958	3.64	0.000	0.000	suppressor of lon; inhibits cell division and ftsZ ring formation

Cell motility

Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
fliS	b1925	-2.34	0.000	0.000	flagellar biosynthesis; repressor of class 3a and 3b operons (RflA activity)
flgH	b1079	-2.04	0.000	0.001	flagellar biosynthesis, basal-body outer-membrane L (lipopolysaccharide layer) ring protein
flgE	b1076	-2.04	0.000	0.016	flagellar biosynthesis, hook protein
flgF	b1077	-1.97	0.001	0.038	flagellar biosynthesis, cell-proximal portion of basal-body rod
fliZ	b1921	-1.91	0.000	0.013	flagellar biogenesis protein
fliN	b1946	-1.91	0.000	0.004	flagellar biosynthesis, component of motor switch and energizing, enabling rotation and determining its direction
fliM	b1945	-1.79	0.000	0.005	flagellar biosynthesis, component of motor switch and energizing, enabling rotation and determining its direction
cheA	b1888	-1.70	0.000	0.003	sensory transducer kinase between chemo- signal receptors and CheB and CheY
flgG	b1078	-1.57	0.005	0.101	flagellar biosynthesis, cell-distal portion of basal-body rod
fliG	b1939	-1.17	0.003	0.074	flagellar biosynthesis, component of motor switching and energizing, enabling rotation and determining its direction
yhcD	b3216	1.11	0.004	0.081	putative outer membrane protein

Cell processes (adaptation/protection/stress response)

Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
dinI	b1061	4.22	0.000	0.000	damage-inducible protein I

Cell wall/membrane biogenesis and cell structure

Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
yaeT	b0177	-1.21	0.000	0.018	orf, hypothetical protein

csgF	b1038	-1.12	0.004	0.078	curli production assembly/transport component, 2nd curli operon
rhsC	b0700	1.80	0.000	0.008	rhsC protein in rhs element

Energy production and conversion

Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
cydA	b0733	-1.68	0.001	0.041	cytochrome d terminal oxidase, polypeptide subunit I
hybD	b2993	-1.24	0.000	0.002	probable processing element for hydrogenase-2
dmsA	b0894	-1.22	0.002	0.051	anaerobic dimethyl sulfoxide reductase subunit A

Inorganic ion transport and metabolism

Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
trkG	b1363	-1.16	0.000	0.005	trk system potassium uptake; part of Rac prophage

Lipid transport and metabolism

Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
	b1394	1.05	0.000	0.013	putative enzyme

Posttranslational modification, protein turnover, chaperones

Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
ppiC	b3775	1.87	0.000	0.004	peptidyl-prolyl cis-trans isomerase C (rotamase C)

Replication, recombination, DNA repair and modification

Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
dinD	b3645	2.18	0.000	0.000	DNA-damage-inducible protein
sbmC	b2009	3.23	0.000	0.000	SbmC protein
recN	b2616	3.24	0.000	0.000	protein used in recombination and DNA repair
recA	b2699	4.62	0.000	0.000	DNA strand exchange and renaturation, DNA-dependent ATPase, DNA- and ATP-dependent coprotease

Secondary metabolites biosynthesis, transport and catabolism

Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
ynbF/ paaB	b1389	1.17	0.000	0.007	orf, hypothetical protein; Phenylacetic acid degradation protein paab
	b1011	1.56	0.000	0.012	putative synthetase

Signal transduction mechanisms

Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
yeaG	b1783	-1.64	0.003	0.071	orf, hypothetical protein
cheY	b1882	-1.31	0.004	0.089	chemotaxis regulator transmits chemoreceptor signals to flagelllar motor components

Transcription

Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
cysB	b1275	-1.61	0.002	0.045	positive transcriptional regulator for cysteine regulon
adiY	b4116	1.04	0.000	0.018	putative ARAC-type regulatory protein
yhiE/ gadE	b3512	1.35	0.000	0.018	transcriptional regulator gade; regulates the expression of several genes involved in acid resistance
umuD	b1183	2.22	0.000	0.000	SOS mutagenesis; error-prone repair; processed to UmuD'; forms complex with UmuC
lexA	b4043	2.79	0.000	0.001	regulator for SOS(lexA) regulon

Translation

Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
yadB	b0144	-1.90	0.001	0.024	putative tRNA synthetase
pheM	b1715	-1.04	0.002	0.052	phenylalanyl-tRNA synthetase (pheST) operon leader peptide
iadA	b4328	1.16	0.000	0.003	isoaspartyl dipeptidase
tyrT	b1231	1.33	0.005	0.102	tyrosine tRNA1; tandemly duplicated
	b1809	1.97	0.000	0.000	orf, hypothetical protein
argW	b2348	2.01	0.001	0.021	arginine tRNA5
ileY	b2652	2.67	0.000	0.000	isoleucine tRNA2 variant

General function prediction or unknown

Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
trpL	b1265	-3.69	0.000	0.000	trp operon leader peptide
ygaM	b2672	-2.61	0.000	0.014	orf, hypothetical protein
yebV	b1836	-1.48	0.001	0.041	orf, hypothetical protein
	b1028	-1.44	0.000	0.000	orf, hypothetical protein
yahL	b0326	-1.21	0.000	0.010	orf, hypothetical protein
ynhD	b1682	-1.21	0.002	0.058	putative ATP-binding component of a transport system
	b1044	-1.20	0.002	0.058	predicted HD superfamily hydrolase
yccE	b1001	-1.14	0.000	0.017	orf, hypothetical protein
ykgH	b0310	-1.01	0.000	0.007	orf, hypothetical protein
ygiN	b3029	1.40	0.004	0.079	orf, hypothetical protein
oraA	b2698	1.63	0.000	0.000	regulator, OraA protein
ydcH	b1426	1.64	0.000	0.005	orf, hypothetical protein
ymfM	b1148	2.08	0.000	0.000	orf, hypothetical protein
ydfZ	b1541	2.19	0.000	0.000	orf, hypothetical protein
ymfN	b1149	2.31	0.000	0.001	orf, hypothetical protein; Phage terminase-like protein, large subunit

yebF	b1847	2.83	0.000	0.006	orf, hypothetical protein
ymfH	b1142	2.95	0.000	0.000	orf, hypothetical protein
ymfG	b1141	3.61	0.000	0.000	excisionase-like protein from lambdoid prophage 14
ymfL	b1147	3.86	0.000	0.000	orf, hypothetical protein
tnaL	b3707	4.46	0.000	0.000	tryptophanase leader peptide
yebG	b1848	5.60	0.000	0.000	orf, hypothetical protein
ymfJ	b1144	6.18	0.000	0.000	orf, hypothetical protein

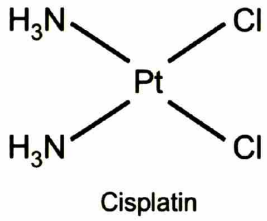
5.6B Genes with signal log ratios $-1 \leq x \leq 1$ but for which LPE $p \leq 0.10$. Genes listed by ascending signal log ratios.

Gene	Blattner Number	Signal Log Ratio	Raw p-value	Adjusted LPE p-value	Gene Product and Function
yqaD	b2658	-0.94	0.002	0.060	Glycosidases
yciG	b1259	-0.94	0.002	0.054	orf, hypothetical protein
ydjO	b1730	-0.93	0.002	0.051	orf, hypothetical protein
ybcV	b0558	-0.92	0.001	0.035	putative an envelop protein
fliD	b1924	-0.85	0.000	0.018	flagellar biosynthesis; filament capping protein; enables filament assembly
fimZ	b0535	-0.81	0.001	0.037	fimbrial Z protein; probable signal transducer
mviM	b1068	-0.79	0.003	0.062	putative virulence factor
fliP	b1948	-0.78	0.004	0.077	flagellar biosynthesis
ymgC	b1167	-0.78	0.004	0.077	orf, hypothetical protein
yddK	b1471	-0.75	0.001	0.024	putative glycoportein
perR	b0254	-0.73	0.003	0.074	putative transcriptional regulator LYSR-type
flhA	b1879	-0.68	0.003	0.063	flagellar biosynthesis; possible export of flagellar proteins
gatD	b2091	-0.66	0.002	0.053	galactitol-1-phosphate dehydrogenase
phoR	b0400	-0.65	0.002	0.056	positive and negative sensor protein for pho regulon
cspG	b0990	-0.60	0.000	0.008	homolog of Salmonella cold shock protein
	b1976	-0.58	0.005	0.094	orf, hypothetical protein
aroH	b1704	-0.56	0.005	0.102	3-deoxy-D-arabinoheptulosonate-7-phosphate synthase (DAHP synthetase, tryptophan repressible)
aspU	b0206	-0.53	0.002	0.059	Aspartate tRNA1 triplicated gene, in rrnH operon
cutF	b0192	-0.46	0.004	0.087	copper homeostasis protein (lipoprotein)
yafV	b0219	-0.44	0.000	0.000	putative EC 3.5. amidase-type enzyme
modC	b0765	-0.43	0.005	0.102	ATP-binding component of molybdate transport
pdhR	b0113	-0.41	0.001	0.027	transcriptional regulator for pyruvate dehydrogenase complex
ybbA	b0495	-0.34	0.000	0.015	putative ATP-binding component of a transport system
rrlG	b2589	-0.34	0.002	0.048	23S rRNA of rrnG operon
ppdA	b2826	-0.33	0.000	0.010	prepilin peptidase dependent protein A
inaA	b2237	-0.31	0.005	0.099	pH-inducible protein involved in stress response
ydhT	b1669	-0.28	0.003	0.069	orf, hypothetical protein
fdnG	b1474	-0.25	0.000	0.004	formate dehydrogenase-N, nitrate-inducible, alpha subunit
eutK	b2438	-0.24	0.002	0.058	Ethanolamine utilization protein eutk precursor
	b2611	-0.14	0.002	0.044	orf, hypothetical protein
yhfZ	b3383	-0.13	0.004	0.077	orf, hypothetical protein

yehQ	b2122	-0.11	0.002	0.046	orf, hypothetical protein
cysP	b2425	-0.11	0.000	0.002	thiosulfate binding protein
yphC	b2545	-0.08	0.000	0.000	putative oxidoreductase
yibH	b3597	-0.07	0.000	0.000	putative membrane protein
eutA	b2451	-0.07	0.000	0.017	Ethanolamine utilization protein, possible chaperon in protecting eutbc lyase from inhibition
ynjA	b1753	-0.06	0.000	0.000	orf, hypothetical protein
rhaA	b3903	-0.05	0.000	0.000	L-rhamnose isomerase
yicP	b3665	-0.02	0.000	0.000	probable adenine deaminase (synthesis xanthine)
ybjT	b0869	-0.02	0.000	0.017	putative dTDP-glucose enzyme
rrfA	b3855	-0.01	0.000	0.000	5S rRNA of rrnA operon
uxuA	b4322	-0.01	0.002	0.053	mannonate hydrolase
basS	b4112	0.05	0.000	0.000	sensor protein for basR
yhiV	b3514	0.08	0.001	0.042	putative transport system permease protein
nrfA	b4070	0.08	0.003	0.063	periplasmic cytochrome c(552): plays a role in nitrite reduction
yhaL	b3107	0.09	0.004	0.084	orf, hypothetical protein
yjaG	b3999	0.14	0.001	0.037	orf, hypothetical protein
yjbR	b4057	0.15	0.001	0.037	orf, hypothetical protein
gmd	b2053	0.17	0.005	0.103	GDP-D-mannose dehydratase
thiF	b3992	0.23	0.000	0.000	thiamin biosynthesis, thiazole moiety
asnA	b3744	0.29	0.000	0.008	asparagine synthetase A
yhhW	b3439	0.29	0.004	0.084	orf, hypothetical protein
yidW	b3695	0.30	0.000	0.000	regulator protein for dgo operon
yhcS	b3243	0.31	0.005	0.101	putative transcriptional regulator LYSR-type
mrcA	b3396	0.31	0.000	0.000	peptidoglycan synthetase; penicillin-binding protein 1A
	b1297	0.32	0.000	0.013	putative glutamine synthetase (EC 6.3.1.2)
yegI	b2070	0.32	0.000	0.013	putative chaperonin
	b2710	0.33	0.001	0.031	putative flavodoxin
pyrE	b3642	0.35	0.001	0.036	orotate phosphoribosyltransferase
yihI	b3866	0.37	0.000	0.000	orf, hypothetical protein
ytfJ	b4216	0.38	0.005	0.098	orf, hypothetical protein
yhiN	b3492	0.42	0.005	0.090	orf, hypothetical protein
	b2868	0.42	0.002	0.049	putative dehydrogenase
ytfL	b4218	0.45	0.001	0.037	putative transport protein
ygjH	b3074	0.47	0.000	0.000	putative tRNA synthetase
mlc	b1594	0.47	0.003	0.064	putative NAGC-like transcriptional regulator
pflD	b3951	0.49	0.002	0.052	formate acetyltransferase 2
yagP	b0282	0.51	0.000	0.008	putative transcriptional regulator LYSR-type
yi82_1	b0017	0.51	0.000	0.000	IS186 and IS421 hypothetical protein
ybiH	b0796	0.51	0.001	0.023	putative transcriptional regulator
	b3042	0.52	0.002	0.054	orf, hypothetical protein
alaX	b2396	0.52	0.004	0.077	Alanine tRNA 2; tandemly duplicated alaW
tra5	1	0.55	0.001	0.035	IS3 putative transposase
bglB	b3721	0.55	0.005	0.097	phospho-beta-glucosidase B; cryptic
	b3122	0.56	0.000	0.000	orf, hypothetical protein
gadA	b3517	0.58	0.000	0.008	glutamate decarboxylase isozyme
yiaI	b3573	0.59	0.005	0.101	orf, hypothetical protein
yjbK	b4046	0.59	0.001	0.026	putative regulator
ycdB	b1019	0.61	0.004	0.081	orf, hypothetical protein
ydcY	b1446	0.63	0.001	0.026	orf, hypothetical protein
clpB	b2592	0.64	0.000	0.002	heat shock protein

acpS	b2563	0.66	0.000	0.011	CoA:apo-[acyl-carrier-protein] pantetheinephosphotransferase
molR_3	b2117	0.67	0.000	0.015	molybdate metabolism regulator, third fragment
cybC	b4236	0.67	0.000	0.000	cytochrome b(562)
xerD	b2894	0.68	0.001	0.039	site-specific recombinase
phnC	b4106	0.68	0.000	0.002	ATP-binding component of phosphonate transport
slyX	b3348	0.69	0.000	0.008	host factor for lysis of phiX174 infection
moaA	b0781	0.70	0.004	0.088	molybdopterin biosynthesis, protein A
yhjK	b3529	0.70	0.000	0.006	orf, hypothetical protein
phnE	b4104	0.71	0.002	0.057	membrane channel protein component of Pn transporter
yfgI	b2506	0.71	0.001	0.039	orf, hypothetical protein
	b2373	0.72	0.000	0.011	putative enzyme
fbp	b4232	0.73	0.002	0.052	fructose-bisphosphatase
ybdF	b0579	0.73	0.003	0.061	orf, hypothetical protein
valS	b4258	0.77	0.000	0.014	valine tRNA synthetase
	b1776	0.78	0.001	0.038	putative oxidoreductase
pdxH	b1638	0.81	0.002	0.049	pyridoxinephosphate oxidase
yqfB	b2900	0.82	0.000	0.000	orf, hypothetical protein
yeeA	b2008	0.82	0.000	0.017	orf, hypothetical protein
fucU	b2804	0.85	0.001	0.034	protein of fucose operon
yjhP	b4306	0.86	0.000	0.000	putative methyltransferase
phnI	b4099	0.89	0.001	0.035	phosphonate metabolism
tyrV	b1230	0.90	0.005	0.093	Tyrosine tRNA1; tandemly duplicated
yghB	b3009	0.90	0.000	0.011	orf, hypothetical protein
ivbL	b3672	0.90	0.000	0.005	ilvB operon leader peptide
yggU	b2953	0.92	0.003	0.073	orf, hypothetical protein
hslU	b3931	0.93	0.003	0.067	heat shock protein hslVU, ATPase subunit, homologous to chaperones
umuC	b1184	0.97	0.000	0.008	SOS mutagenesis and repair
fhuE	b1102	0.99	0.000	0.002	outer membrane receptor for ferric iron uptake
proK	b3545	0.99	0.001	0.022	Proline tRNA1
ileT	b3852	1.00	0.005	0.094	Isoleucine tRNA1, triplicate
mopB	b4142	1.00	0.002	0.058	GroES, 10 Kd chaperone binds to Hsp60 in pres. Mg-ATP, suppressing its ATPase activity

A



Cisplatin

B

5′-CCTCTCCTT**GG**TCTTCTCCTCTCC-3′
3′-GGAGAGGAACCAGAAGAGGAGAGG-5′

5′-CCTCTCCTT**GG**TCTTCTCCTCTCC-3′
3′-GGAGAGGAACTAGAAGAGGAGAGG-5′

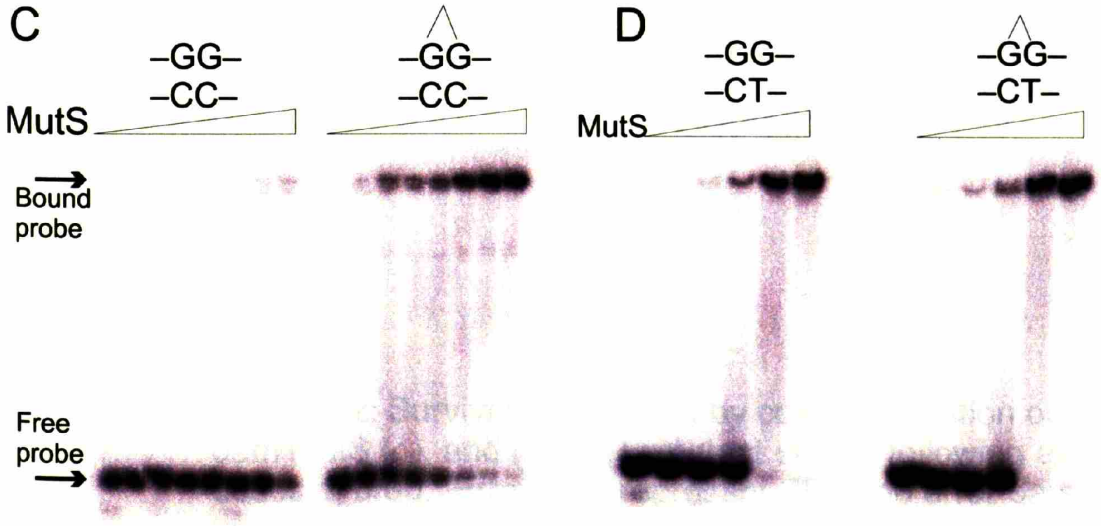


Fig. 5.1 MutS binds to cisplatin-modified DNA. The substrates used are shown in B). The two bold letters indicate the site of the adduct.

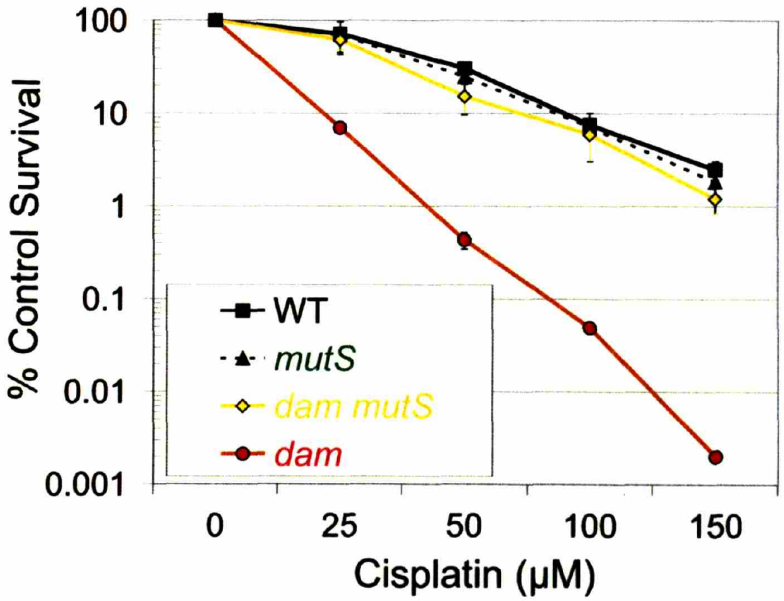


Figure 5.2: Survival determined by colony formation of wildtype (■), dam (●), dam mutS (◆), and mutS (▲) mutant strains following exposure to increasing doses of drug. Error bars represent SEM; no error bars indicates SEM is too small to be visualized on the scale of the plot.

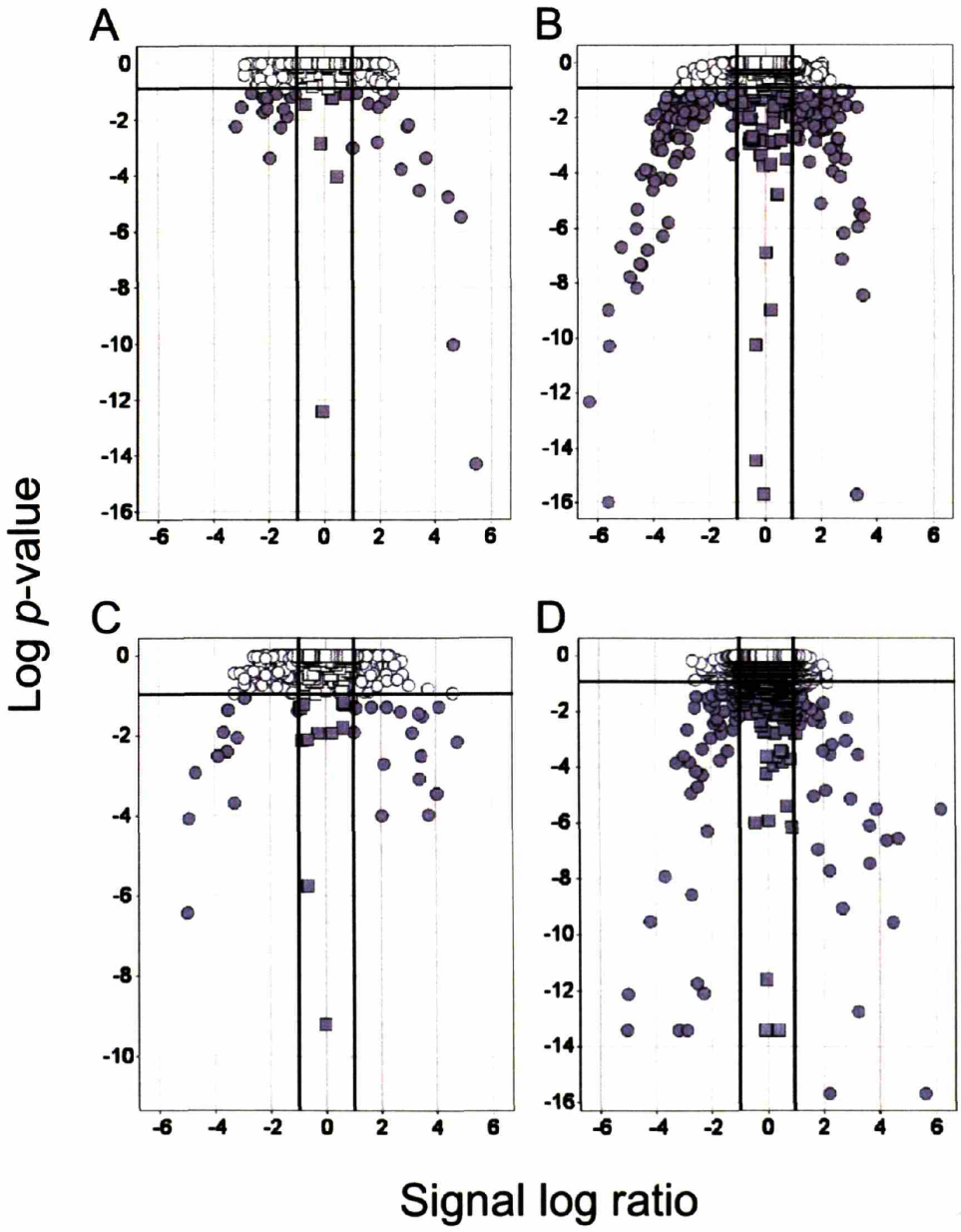


Figure 5.3: Volcano plots of gene expression changes for each strain: wildtype (A), *dam* (B), *dam mutS* (C), *mutS* (D). Each shape represents a gene. Signal log ratio represents the $\log_2$ fold change and is plotted against the $\log_{10}$ p-value. Horizontal bold lines show the p-value threshold and vertical bold lines show the fold change threshold applied to filter the data to include genes for which $p \leq 0.10$ (false discovery rate 10%, grey filled shapes) and that exhibit at least a 2-fold change in expression level (circles).

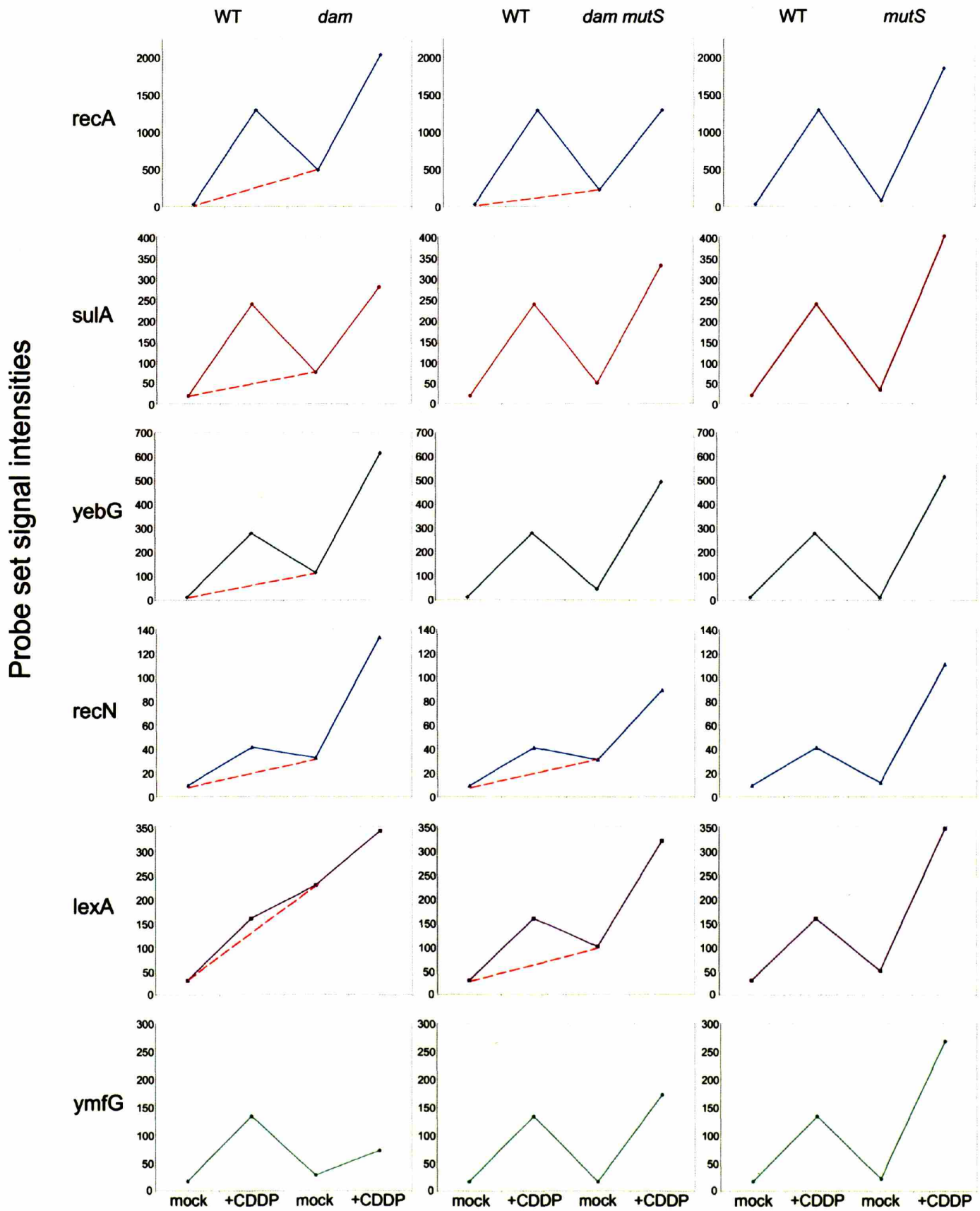


Figure 5.4: Relative probe set intensities for several SOS response genes. Dotted red lines indicate the basal level of induction between mutant and wild-type strains. CDDP = cisplatin

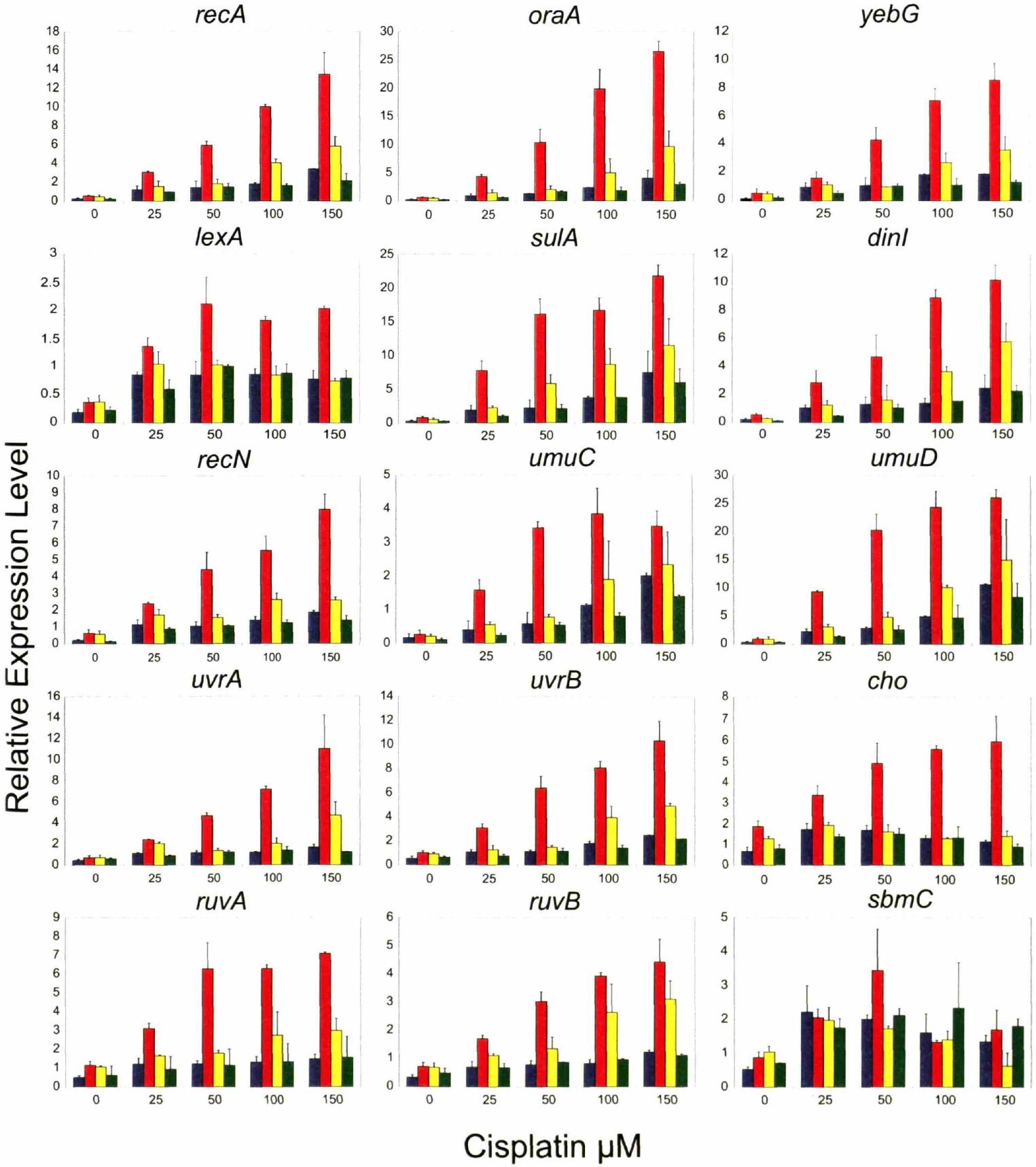


Figure 5.5: Relative expression levels for several SOS genes determined by semi-quantitative real-time RT-PCR. Expression levels represent the average of three independent experiments; error bars represent SD. Wild-type (blue bars), *dam* (red bars), *dam mutS* (yellow bars), and *mutS* (green bars).

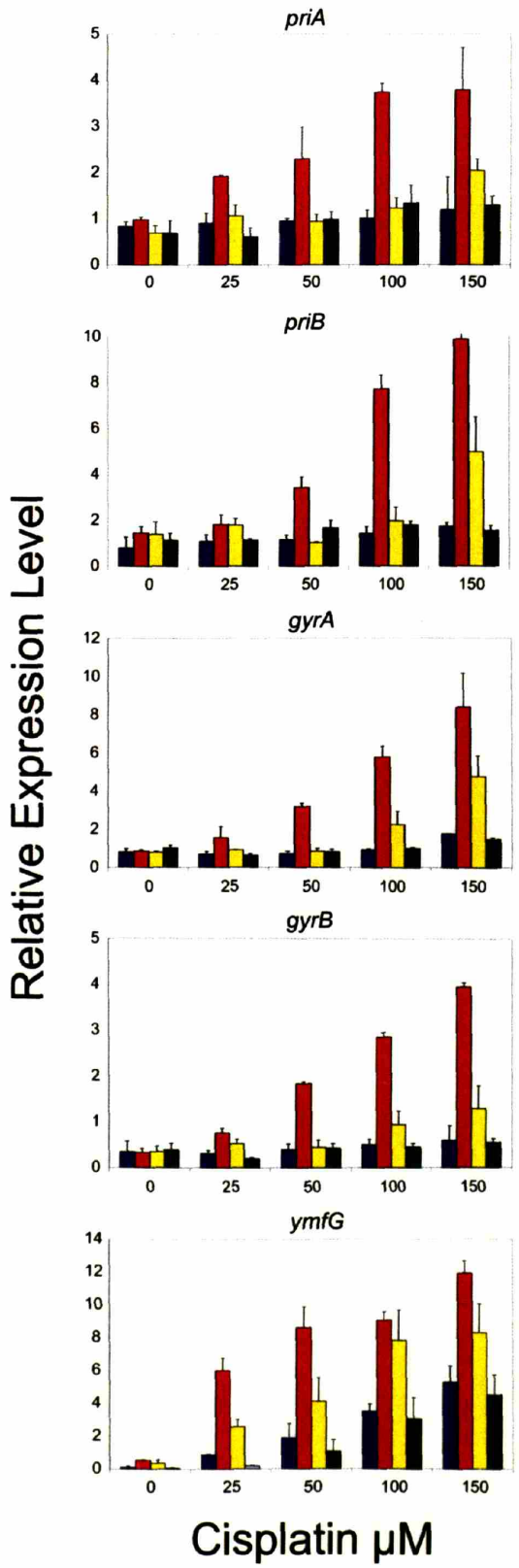


Fig. 5.6: Real-time PCR analysis of genes with known and potential roles in DNA metabolism. *PriA* and *priB* encode for proteins involved in replication restart, *gyrA* and *gyrB* encode the subunits of DNA gyrase, and *ymfG* is a potential LexA-regulated gene.

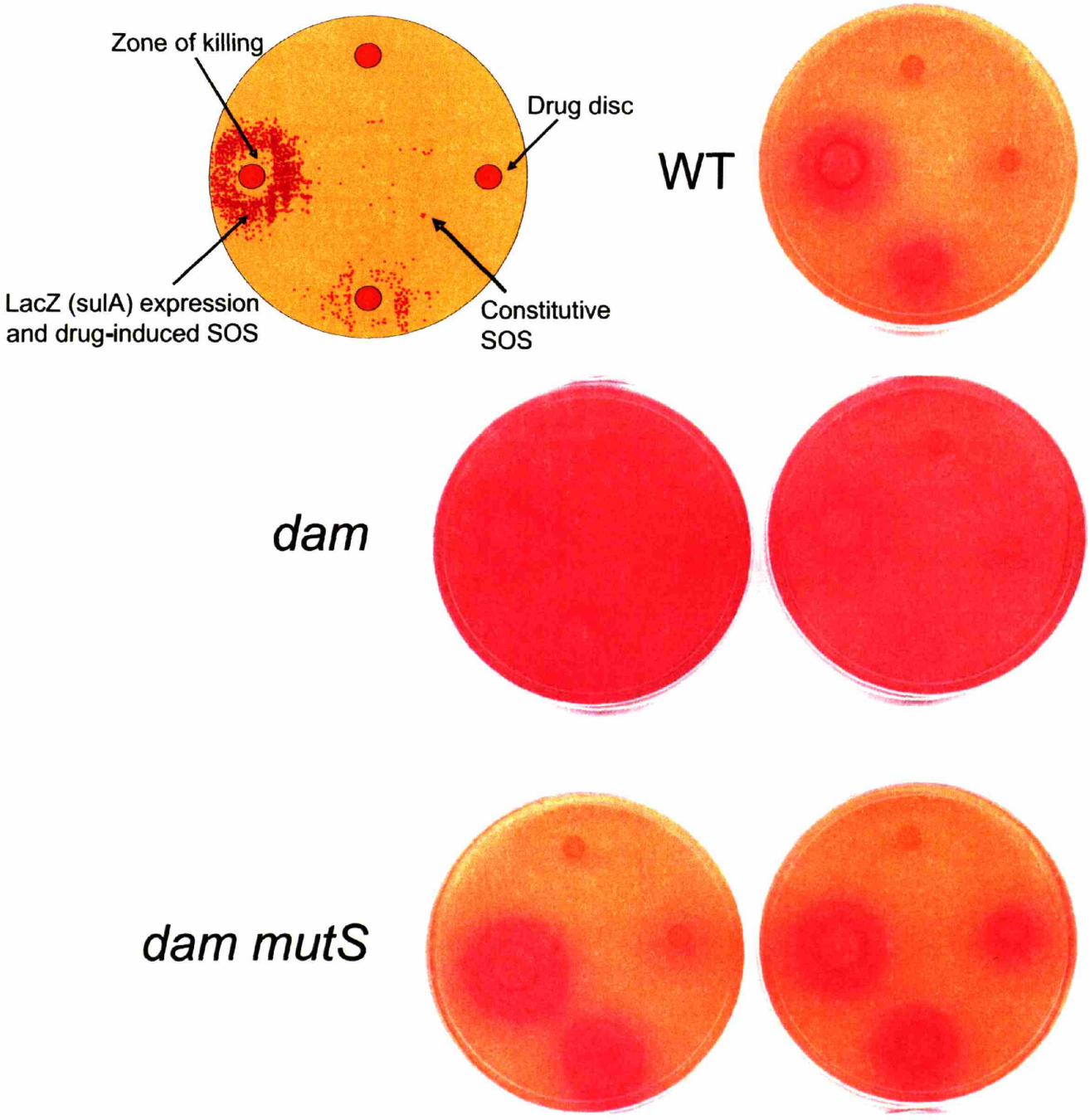


Figure 5.7: *SulA::lacZ* gene reporter assay. The disk at the top of each plate served as the control and 5, 10 and 20 ul cisplatin was added to the disks in a clockwis manner. The strains used were GM4352 (wildtype), GM4355 (*dam*-16) and GM5878 (*dam*-16 *mutS*215). Expression of *sulA*, visualized by LacZ activity, is induced upon increasing dose of cisplatin. The *dam* mutant strain also shows a high level of constitutive *sulA* expression.

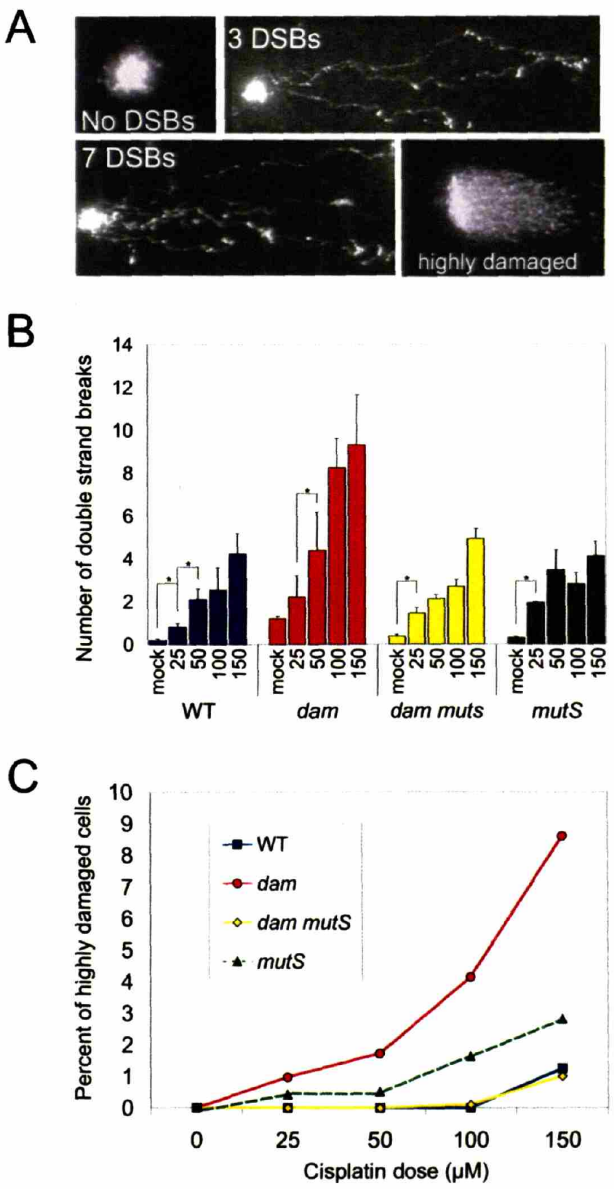


Figure 5.8: *E. coli* strains analyzed by single-cell microgel electrophoresis. 600 individual cells for each strain were analyzed by visually counting the number of breaks A) Representative pictures of individual cells analyzed by microgel electrophoresis. Cells with no tails indicate no breaks in the genome, whereas cells with tails indicate the number of strand breaks in the genome. B) Number of double-strand breaks per cell for each strain determined by counting the tails for each cell. Error bars represent SE, *Mann-Whitney U test $P < .05$ C) The percentage of highly damaged cells for each strain and dose. Cells that showed more than 15 tails were considered highly damaged.

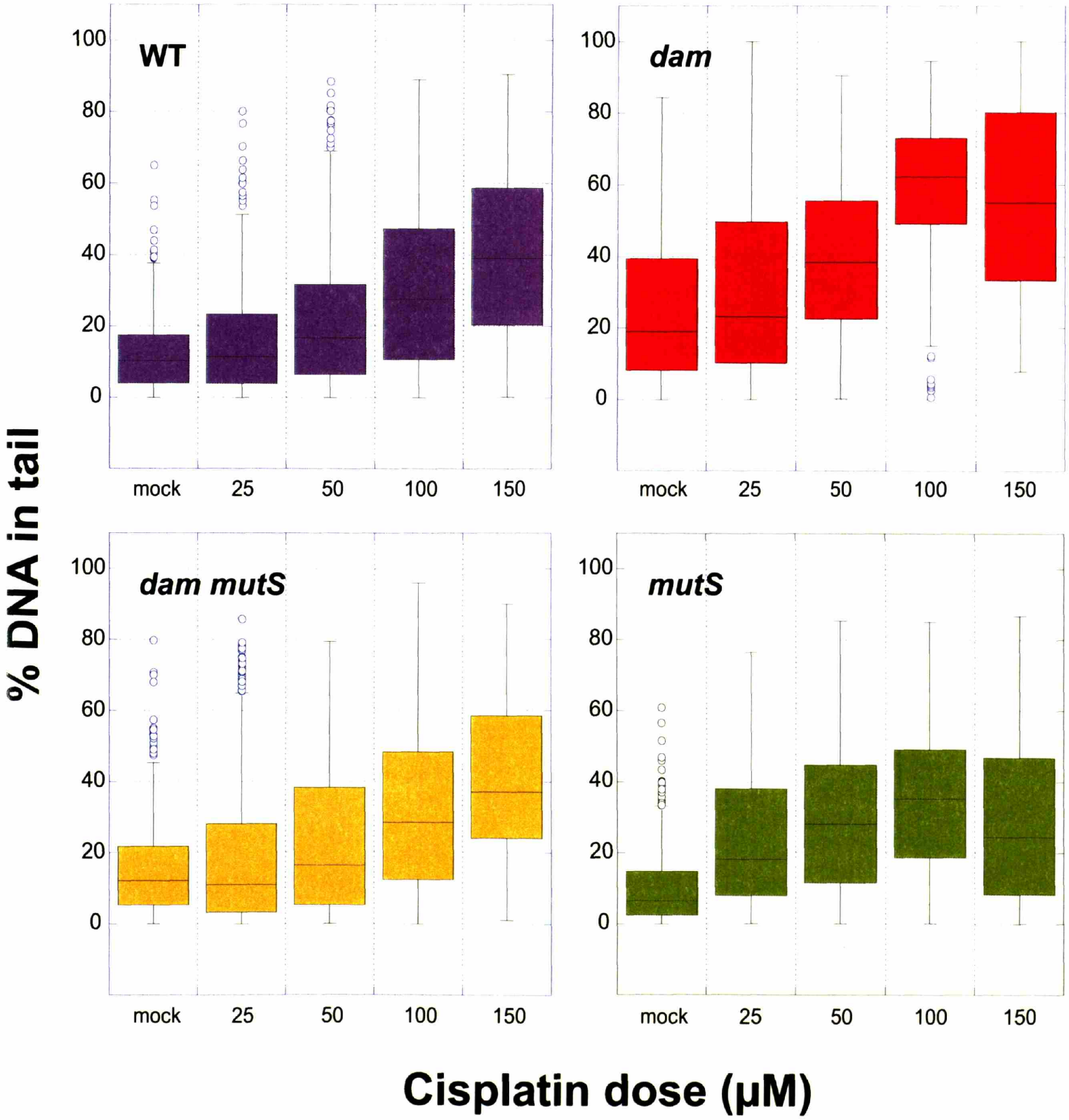


Figure 5.9A: Percent of DNA in the tail region. Percent DNA in tail reflects the flourescence in the tail as a percentage of total flourescence in tail and head region.. Horizontal lines for each box represent the median value, with the box respresenting 50% of the data (box upper and lower limits represent the upper and lower quartiles, respectively). Vertical lines extending from the box show the full range of data, and outliers are shown as individual points (outliers have values that are greater than the upper quartile +1.5 X interquartile distance).

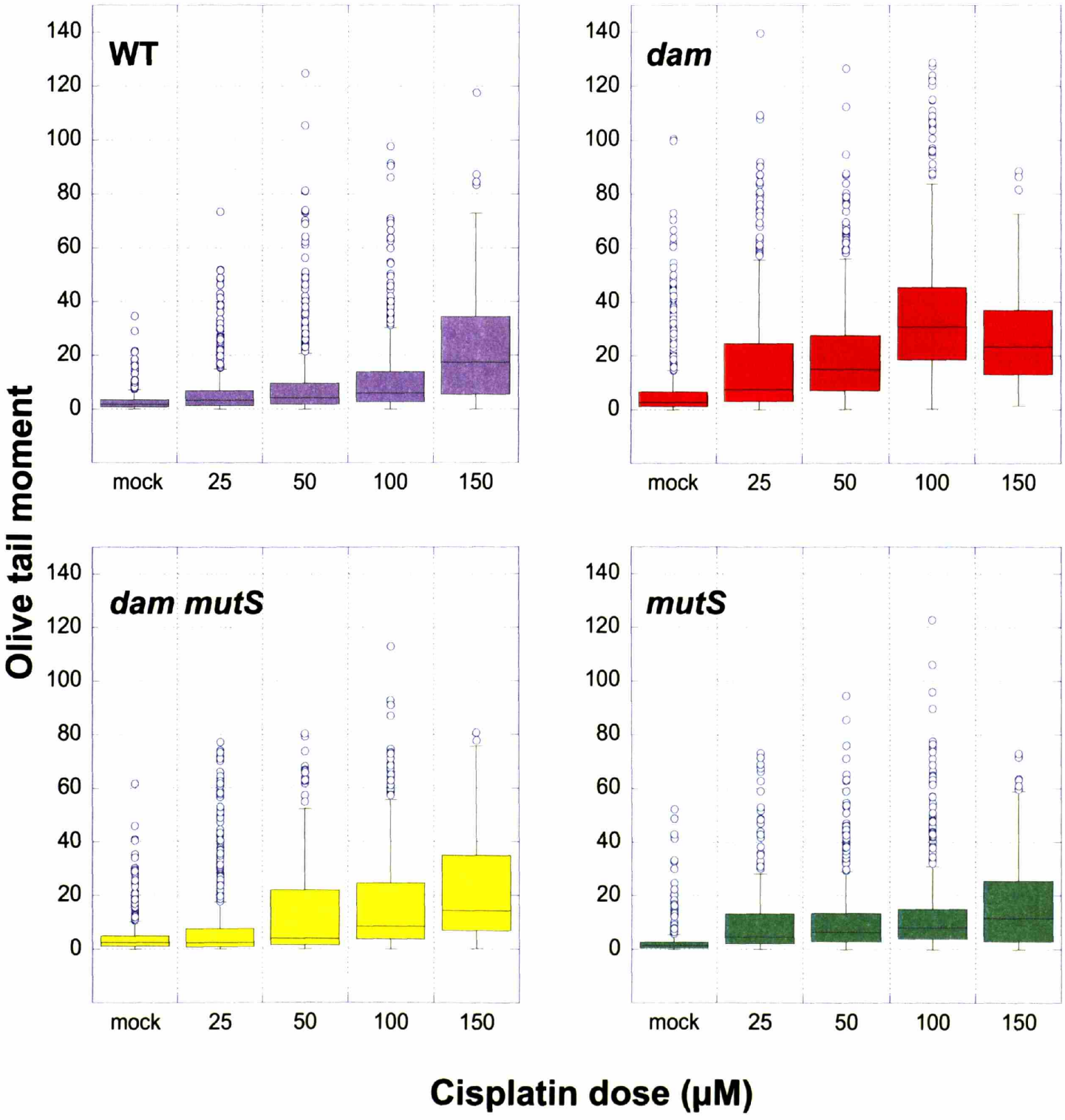


Figure 5.9B: Olive tail moments determined using Komet image analysis software. Horizontal lines for each box represent the median value, with the box respresenting 50% of the data (box upper and lower limits represent the upper and lower quartiles, respectively). Vertical lines extending from the box show the full range of data, and outliers are shown as individual points (outliers have values that are greater than the upper quartile +1.5 X interquartile distance). Olive tail moment: migrated DNA X distance between the head and center of gravity of DNA in the tail.

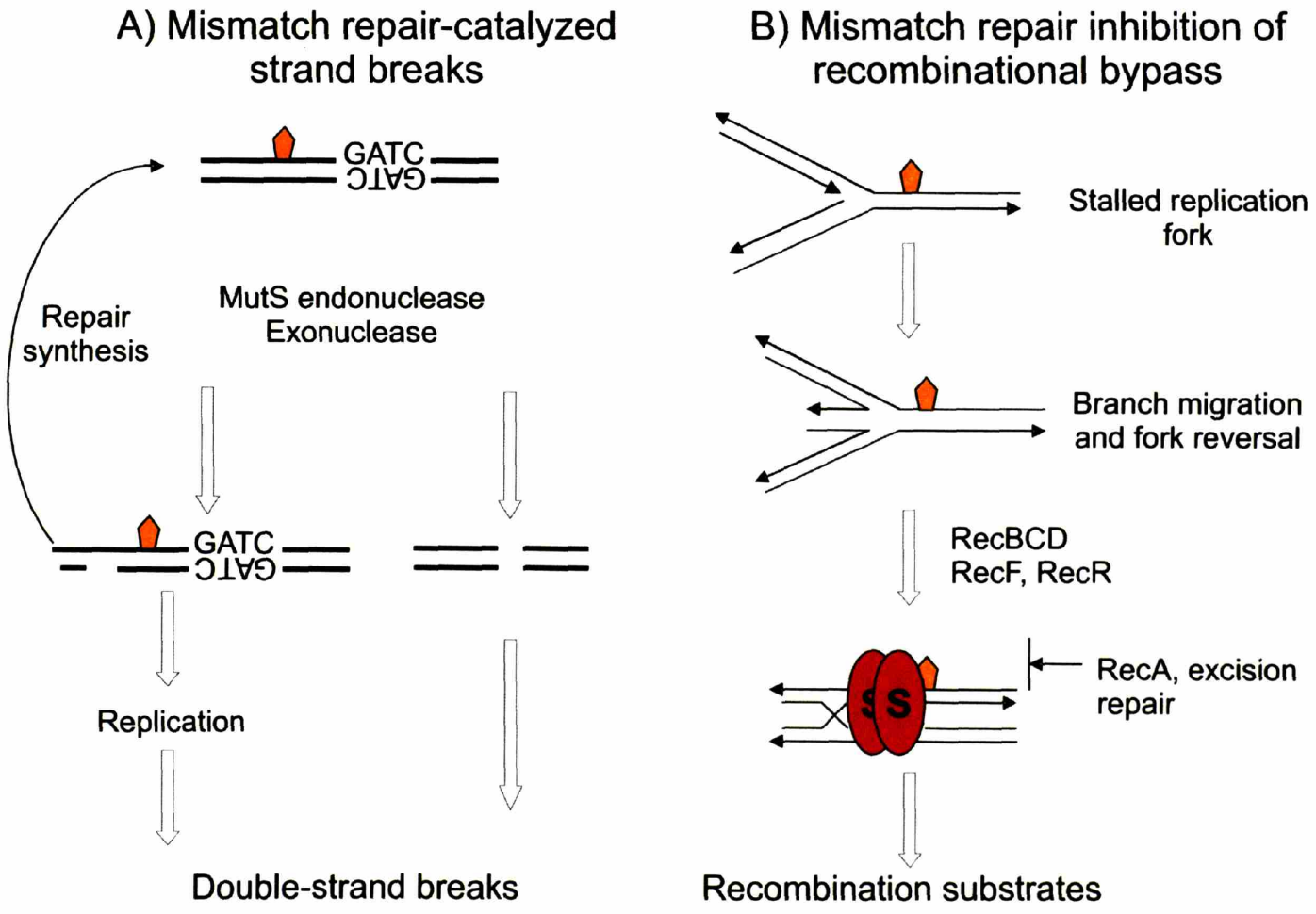


Figure 5.10: Models of the contribution of mismatch repair to cisplatin-induced double-strand break formation. A) Mismatch-repair recognizes a cisplatin-DNA adduct (pentagonal shape) and attempts repair. An incision made on the strand opposite the adduct leads to futile repair cycling and a persistent single-strand gap. Futile cycling coupled with DNA replication would lead to a double-strand break. Alternatively, Mut may catalyze dual incisions on unmethylated DNA, where the strand discrimination signal is absent, thus forming a double-strand break independent of DNA replication. B) MutS may contribute to cisplatin-induced double-strand break formation by preventing the recombinational bypass of cisplatin adducts. The replication fork stalls at the site of the adduct and undergoes fork regression to remove the replication machinery from the cisplatin blocking lesion, and flush ends of the newly synthesized complementary strands may be substrates for RecBCD digestion. MutS binding to the cisplatin adduct may reverse or prevent RecA-catalyzed strand exchange and inhibit recombinational bypass of the adduct. Alternatively, Rec proteins may process and maintain the strands of he replication fork until excision repair has taken place, and MutS binding to the adduct may similarly block excision repair of the adduct.

Chapter 6: Future directions

6.1 DSB formation in dam mutants: MutH-catalyzed DSBs versus replication fork collapse at MutH-catalyzed single-strand breaks

Work in Chapter 4 showed that mismatch repair produces double-strand breaks in *dam* mutant cells. Mismatch repair may introduce dual incisions on complementary strands in unmethylated DNA. Alternatively, MutH may catalyze single incisions (which may occur at greater efficiency *dam* mutants where the genome is unmethylated) and these single incisions may be converted to DSBs following replication fork collapse. The replication dependence of DSB formation therefore may shed light as to which of these mechanisms of DSB formation occurs in cells. For example, if MutH is introducing single nicks, then replication would be required to convert these single nicks into DSBs. However, DSB formation by MutH-catalyzed dual incisions would be independent of DNA replication. Thus a determination of the level of DSBs in replicating and non-replicating *E. coli* may reveal the role of replication in DSB formation. In addition to measuring DSBs in replicating and non-replicating cells, the level of single-strand breaks (SSBs) in each of the strains may provide support for one over the other mechanism of DSB formation. The single-cell electrophoresis method performed under alkaline conditions may be used to determine the level of SSBs in *E. coli* (642,645). If *dam* mutants exhibit a higher level of SSBs than the other strains, and if the level of SSBs is greater than the level of DSBs, then we would suspect that SSBs precede DSB formation and that MutH catalyzes single incisions in *dam* mutants that are only converted to DSBs after collapse of the replication complex. Therefore, determining the level of SSBs and DSBs in replicating and non-replicating *E. coli dam*, *dam mutS*, and wild-type strains may reveal the mechanism of MMR-dependent DSB formation.

6.2 Cisplatin-induced DSBs: MMR futile cycling versus abortive recombinational bypass

As shown in Chapter 5, cisplatin induces double-strand breaks in *E. coli*. Interestingly, the level of cisplatin-induced DSBs is enhanced by MMR in a *dam* mutant background. The DSB data therefore parallel the phenotypes of the four strains; *dam* mutants exhibit the highest degree of sensitivity as well as the greatest level of cisplatin-induced DSBs, while an additional mutation in *mutS* abrogates sensitivity and reduces the number of drug-induced DSBs to the number observed in wild-type and *mutS* strains. Recent *in vitro* data suggest that the influence of MMR on the level of cisplatin-induced DSBs may be due to MMR-mediated inhibition of recombination involving a cisplatin-

modified substrate (90), and this inhibition may be more detrimental to *dam* mutants where the recombination machinery is pre-occupied with repairing endogenous MMR-dependent strand breaks (see Chapter 4). However, similar to the proposed mechanisms of endogenous DSB formation in *dam* mutants, MMR, after recognizing a cisplatin adduct as heterologous DNA, may initiate repair and catalyze dual incisions in a *dam* mutant (directly forming a DSB) or introduce a single incision that is converted to a DSB during replication. The latter mechanism has been termed futile cycling: MMR initiates repair on the strand opposite the adduct, and repair synthesis (which may involve error-prone bypass of the adduct resulting in a mismatch compound lesion) reproduces the substrate for MMR, and repair is thus initiated again. Repeated repair cycles (i.e., futile cycling) results in a persistent single-strand gap, which when coupled with replication would result in a double-strand break. One way to approach this problem is to determine if MMR initiates repair of a cisplatin modified substrate *in vitro.* Such a study would be the complement to the *in vitro* strand-exchange assay testing the abortive recombinational bypass *in vitro.* Reconstituted MMR would require a site specific cisplatin adduct in duplex DNA containing a d(GATC) site located up- or down-stream of the adduct. Such a substrate could be constructed by inserting an oligo containing an adduct into M13 single-strand DNA (using a scaffold oligo and ligase), converting the DNA into duplex DNA in NER-deficient cells (which may result in mismatches opposite the platinated guanine residues), and recovering the duplex plasmid by extraction and isolation. After incubation with the components required for *in vitro* MMR (based on studies by Modrich and colleagues (20,151,394,395,757)), running the reaction product on a gel and determining the nature of the product (i.e., a circular duplex (no incision was made), a linear and circular species (MutH made a single incision), or two linear strands (MutH made dual incisions)) may answer the question if MMR can initiate repair of a cisplatin adduct.

In addition to determining if a cisplatin adduct can stimulate MutH activity on plasmid DNA, we can examine other events downstream of MutH incision to determine the extent of a MMR reaction on platinated DNA. For example, if MutH makes an incision to initiate repair, can the exonucleases digest DNA past a cisplatin adduct? Simple *in vitro* assays can determine the extent of exonuclease digestion of an oligo containing a site specific adduct.

The two functions of MMR—mutation avoidance and anti-recombination—may be separated by examining MMR mutants that are impaired in one function but proficient in the other. Recently, Calmann et al. (91) demonstrated that a *mutSDelta800* mutation (which

removes the C-terminal 53 amino acids of MutS) retains function in mutation avoidance but not in anti-recombination. Interestingly, this mutant form of *mutS* rescues *dam* mutants from cisplatin hypersensitivity (but not from MNNG-induced toxicity), thus supporting the hypothesis that anti-recombination rather than futile-cycling may play a predominant role in MMR-mediated cisplatin toxicity. Different *mutS* and *mutL* mutants, perhaps proficient in adduct binding but not in other activities required for repair initiation (such as ATPase activity or interactions with MutH), would be useful in further examining the differential role of mutation avoidance and anti-recombination in cisplatin toxicity.

6.3 MMR effects in eukaryotes

Studies analogous to the above experiments investigating MMR mutants deficient in either mutation avoidance or anti-recombination (but not both) would also be helpful to examine the role of MMR in cisplatin toxicity in eukaryotic cells. Such MMR mutants in yeast have already been identified (741); two alleles affecting only the anti-recombination function of Pms1p have been identified, and one of these mutations changes an amino acid within the highly conserved ATPase domain. The *in vitro* experiments using the *E. coli* recombination and MMR proteins would be more difficult to transfer to eukaryotic or mammalian cells. Although *in vitro* MMR by human nuclear extracts has been demonstrated (729,730), such studies on a cisplatin-modified substrate may prove problematic due to the relatively high number of nuclear proteins that recognize and bind cisplatin adducts, including other repair proteins (such as NER proteins) that might initiate repair. However studies examining the damage signaling role of MMR in response to cisplatin treatment may shed light on the mechanism of reduced apoptosis in MMR-deficient cells and how such MMR loss contributes to cisplatin resistance. For example, is MMR required for ATM activation of Chk2 kinase in response to cisplatin (as was shown for IR-induced S phase arrest (76))? Similar to experiments using separation-of-function mutants to sparse out the contribution of anti-recombination and mutation avoidance pathways in MMR-mediated cisplatin toxicity, experiments using different eukaryotic mutants proficient in MMR-mediated apoptosis but deficient in the repair of mismatches may provide insight as to which mechanism is critical for cisplatin-induced toxicity. One such MSH2 mutant—a point mutation in the ATPase domain of MSH2 (G674A)—results in increased tumorigenesis in mice; yet this mutant protein retains its ability to bind to a G:T mismatch *in vitro* and tumors carrying the mutant allele remain responsive to cisplatin (413). In addition to studying the MMR-dependent pathways of cisplatin-induced toxicity, the MMR-independent (and p53-

dependent) pathways for cisplatin-induced apoptosis also require further study. Furthermore, is not known whether these pathways are differentially activated for the different cisplatin analogues.

6.4 Lessons learned from cisplatin: the development of novel anticancer agents

Based on the data presented and summarized in this work, cells that are deficient in Dam are essentially "repair deficient" as their recombinational repair machinery is pre-occupied with endogenous mismatch repair-induced strand breaks. Such repair deficiency renders these cells more susceptible to the effects of additional DNA damage. Thus one mechanism for the development of new cancer treatment strategies is to make cancer cells similar to *dam* mutant cells in regard to their DNA repair status. As mentioned in Chapter 1, cisplatin-based therapies are very effective in treating TGCTs, which naturally express lower levels of certain DNA repair proteins. Furthermore, breast and ovarian tumors deficient in BRCA1 or BRCA2, and consequently deficient in recombinational DNA repair mechanisms, are exceptionally sensitive to PARP inhibitors as PARP may be able to compensate for deficient BRCA1/2 pathways (199). Thus, cells whose DNA repair capacity is constitutively under a state of stress present good targets for treatment with DNA damaging agents. While not all tumor cells are inherently repair deficient, other tumor cell characteristics may be exploited to induce selective adduct persistence in tumor versus non-tumor cells (see below).

The need for new chemotherapeutics is based on the fact that although cisplatin is effective in the treatment of TGCTs, for other cancers, including ovarian cancer, platinum-based chemotherapies extend life but unfortunately rarely cure disease. For example, ovarian cancer remains the leading cause of death due to gynecologic malignancies. Recent estimates for 2005 predict that there will be 22,220 new cases of ovarian cancer diagnosed in the United States; these new cases are predicted to be accompanied by 16,210 deaths (330), which would make ovarian cancer the fourth deadliest cancer among women. Current front-line therapies for advanced ovarian cancer include cisplatin/paclitaxel combination therapy, with carboplatin/paclitaxel presenting an alternative treatment with less severe side-effects (Table 1.1). However with an 80% relapse rate, additional and improved therapies are needed. Because of the dose-limiting toxicity and acquired drug resistance, new drugs selectively toxic to cancer cells may be successful in killing cancer cells while proving less toxic to patients.

As discussed in Chapter 1, the trans isomer of cisplatin is clinically ineffective, most likely due to the better capacity of DNA repair to remove adducts formed by transplatin. Furthermore, many studies have shown that ovarian cancer resistant cell lines display increased repair capacity. Based on the proposed mechanisms of cisplatin toxicity, namely repair shielding and transcription factor hijacking discussed in Chapter 1, one proposed mechanism for targeting toxicity to ovarian cancer cells is to treat patients with a DNA damaging drug that would be blocked from repair only in the cancer cells and not in normal healthy cells and that would disrupt normal cellular function by hijacking proteins essential for cell growth. To mimic the repair-shielding mechanism of cisplatin toxicity, the drug's design would need to exploit the differential expression of cellular proteins in cancer cells versus normal cells, and in so doing make adduct repair shielding only operative in the cancer cells. One such protein expressed in many ovarian cancers is the hormone steroid receptor, estrogen receptor alpha. Thus a drug designed to selectively target estrogen receptor positive (ER+) ovarian tumors would bind to the ER and would therefore carry the ER ligand. However to elicit toxicity by a mechanism similar to the platinum compounds (i.e., repair shielding), this drug will also need to damage DNA. In addition to repair shielding, by binding to ERalpha, this new drug will also hijack the receptor away from its natural DNA binding sites (estrogen response elements) and therefore will disrupt the activation of genes important for cell growth. Therefore, this new drug will mimic the property of cisplatin to form DNA adducts that attract nuclear proteins; the hijacking of those proteins is anticipated to block DNA repair and to disrupt the normal functions of those proteins in gene regulation. However unlike cisplatin, this drug will be selective to cancer cells—specifically ER+ cancer cells.

In keeping with the principles outlined above, a novel drug designed by the Essigmann lab contains a non-platinum warhead that reacts with DNA; tethered to the warhead is a ligand for the estrogen receptor (Fig. 6.1). This bifunctional compound will display, in principle, both shielding- and hijacking-based mechanisms of toxicity. As with cisplatin, the compound will react with the N7 atom of purines to form mono and bis-DNA adducts. Also, as with cisplatin, this compound will theoretically persist in DNA by attracting cellular proteins, specifically the ER, to the adduct site. Binding of the ER to the estradiol moiety will prevent repair of the adduct, while ER binding will also prevent the ER from serving its role in promoting cell growth (i.e., the ER will be hijacked away from estrogen response elements in the promoter regions of survival and pro-growth genes, resulting in inhibition of tumor cell growth). Because the ER is expressed in sixty percent of epithelial ovarian cancers (142)

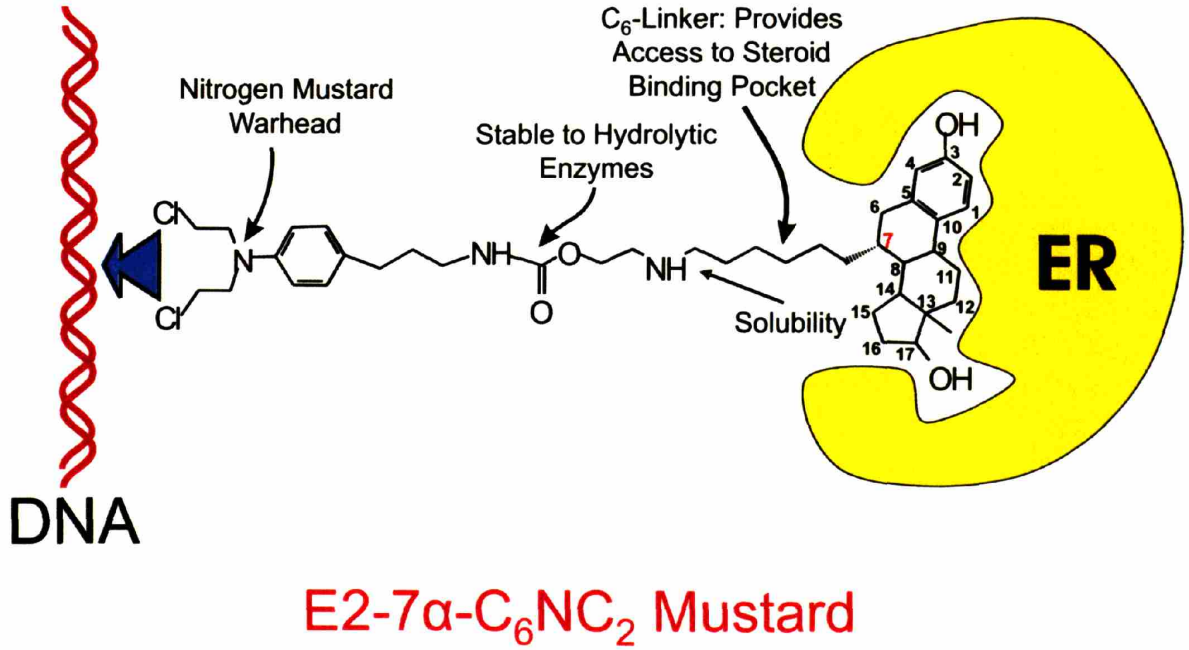
and in nearly seventy percent of breast tumors (318), this agent will in principle selectively target tumor cells by exploiting the differential expression of the ER in tumor and non-tumor cells. Indeed, work in the ER+ breast cancer cell line MCF-7 and a ER- cell line shows that this agent is specifically toxic to ER+ cells and that adducts formed by this agent are differentially repaired in ER+ and ER- cells ((631) and Hillier and Marquis, unpublished results).

Previous work using xenograft tumors in mice have shown that E27a reaches pharmacologic concentration in animals (with acceptable attendant animal toxicity) as well as antitumor activity. However this compound has yet to be tested in an ovarian cancer model. To test the potential of E27a in the treatment of ovarian cancer, a mouse model developed in the Jacks lab may provide an appropriate system. This model is the first mouse model of well-differentiated endometrioid adenocarcinoma of the ovary, and moreover the tumors of these mice express the ER (169). Preliminary work with cisplatin and E27a show nearly identical toxicity profiles on mouse ovarian cancer cells derived from this mouse model (Figs. 6.2 and 6.3). The two compounds show superior characteristics, so far, to carboplatin, the other drug used in front line ovarian cancer therapy. Furthermore, these cell lines are comparably sensitive to cisplatin as two human cell lines—HeLa and the ovarian Caov-3 cell lines (Fig. 6.4). Additional *in vitro* work characterizing the cytotoxic effects of these drugs (such as colony formation assays, cell cycle analysis, and mechanistic studies using different forms of E27a with different abilities to bind the ER and/or DNA) are needed to determine the relative cytotoxic potential of E27a compared to cisplatin. Furthermore, *in vivo* work in this mouse model will hopefully demonstrate that E27a is better tolerated than cisplatin while eliciting similar effects in blocking tumor progression.

Mechanistic studies of E27a may include a determination of ER binding to E27a adducts in cells. ER bound to E27a may be quantitated in cells by chromatin immunoprecipitation experiments in which ER+ cells are treated with ^{14}C-labeled compound, and any cellular proteins bound to DNA are then crosslinked by formaldehyde treatment. After DNA fragmentation and chromatin immunoprecipitation, a determination of ^{14}C will reveal if the compound is bound to DNA, and anti-ER staining will reveal if ER is bound to the compound. Similarly, a pull-down of ERα with any bound substrates using immobilized ERα antibodies may reveal an interaction of compound with the receptor in cells.

In order to determine if E27a treatment limits ER-mediated transcriptional activation, the transcript levels of endogenous estrogen-responsive genes (e.g., c-myc, fos, pS2 and

PR (272,301,389-391)) can be measured following treatment with E27a. First the level of transcriptional activation achieved by estradiol stimulation in ER+ cells can be determined to ensure that ER-mediated transcription is responsive to an agonist. Then semiquantitative real-time RT-PCR and Northern analysis can be used to measure the transcript levels of estrogen-responsive genes in ER+ cells +/-estradiol stimulation. Next the reduction in transcriptional activation achieved by first treating ER+ cells with E27a (and vehicle for the control) followed by estradiol stimulation may show that the estrogen receptor is hijacked in cells; the transcript levels of estrogen-responsive genes such as PR and c-myc may exhibit reduced transcriptional activation in E27a pre-treated cells. In addition to examining the expression of endogenous estrogen-responsive genes, a gene reporter assay may be used to examine the effect of E27a treatment on the transcription of a reporter gene under the control of estrogen response elements. The consensus ERE is a 13-base pair palindromic sequence consisting of inverted repeats 5-GGTCA-3 separated by a 3-bp spacer (e.g., GGTCANNNTGACC) and is found in the 5-flanking region of the *Xenopus* and chicken vitellogenin A2 genes (377,378,724). Cells transfected with the firefly luciferase gene under control of an estrogen-responsive promoter and treated with E27a, control compounds, or vehicle, followed by estradiol stimulation can be used to determine if E27a reduces luceriferase activity and hence the transcriptional activation of genes under the control of EREs.



E2-7α-C_6NC_2 Mustard

Fig 6.1: The basic features of E27a are (a) a nitrogen mustard DNA-damaging warhead, (b) a linker that enhances solubility and is stable to hydrolytic cleavage, and (c) a covalently tethered estradiol moiety, which serves as an estrogen receptor ligand. It was recently reported that the E27a compound binds DNA, and it also binds the ER with the excellent relative binding affinity (RBA) of 46 (the RBA of estradiol is 100).

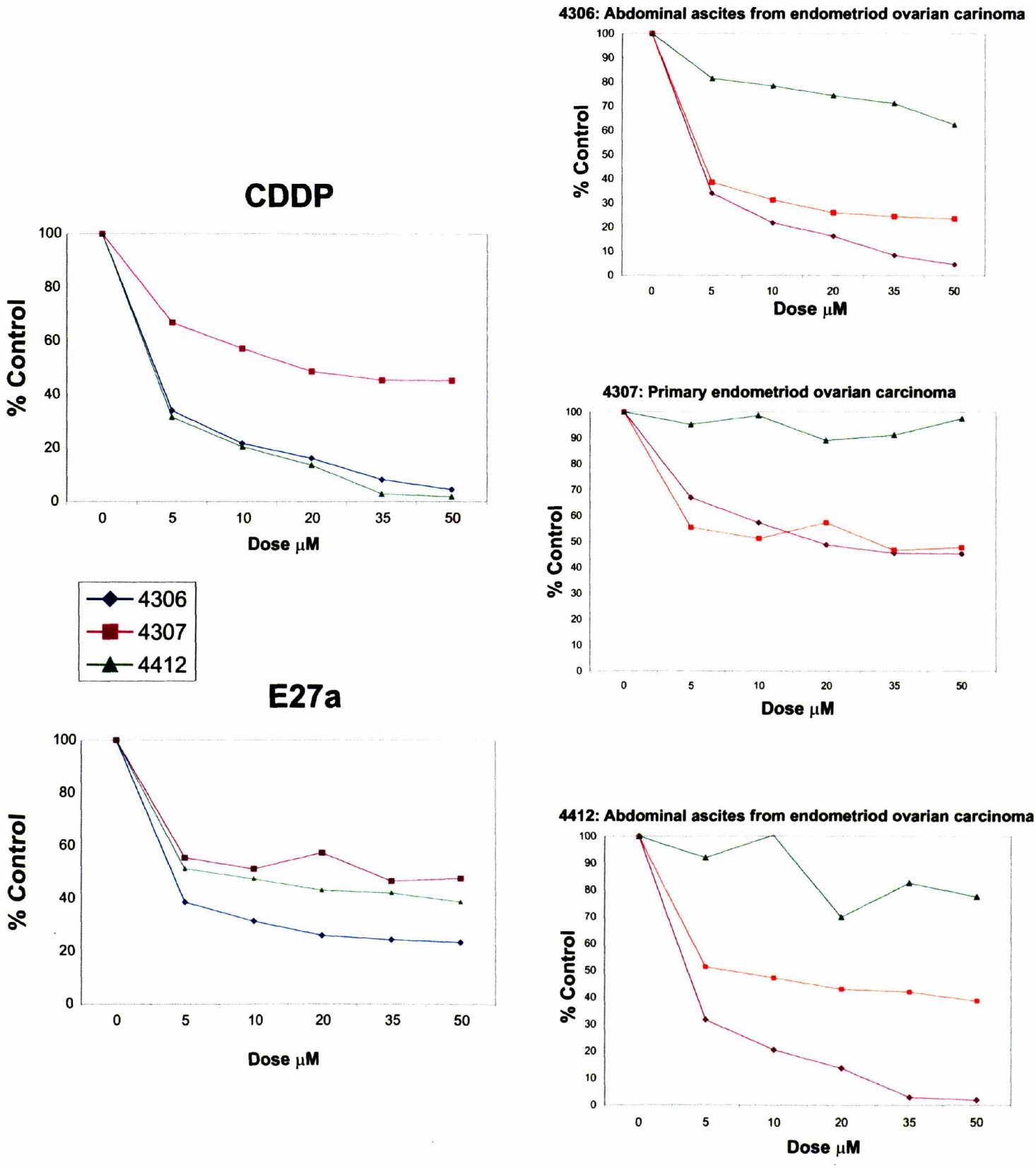


Fig 6.2: Growth inhibition of mouse ovarian cancer cell lines treated with cisplatin, carboplatin, or a novel anticancer drug. Cells were treated for 3 hours and counted 48 hours after treatment.

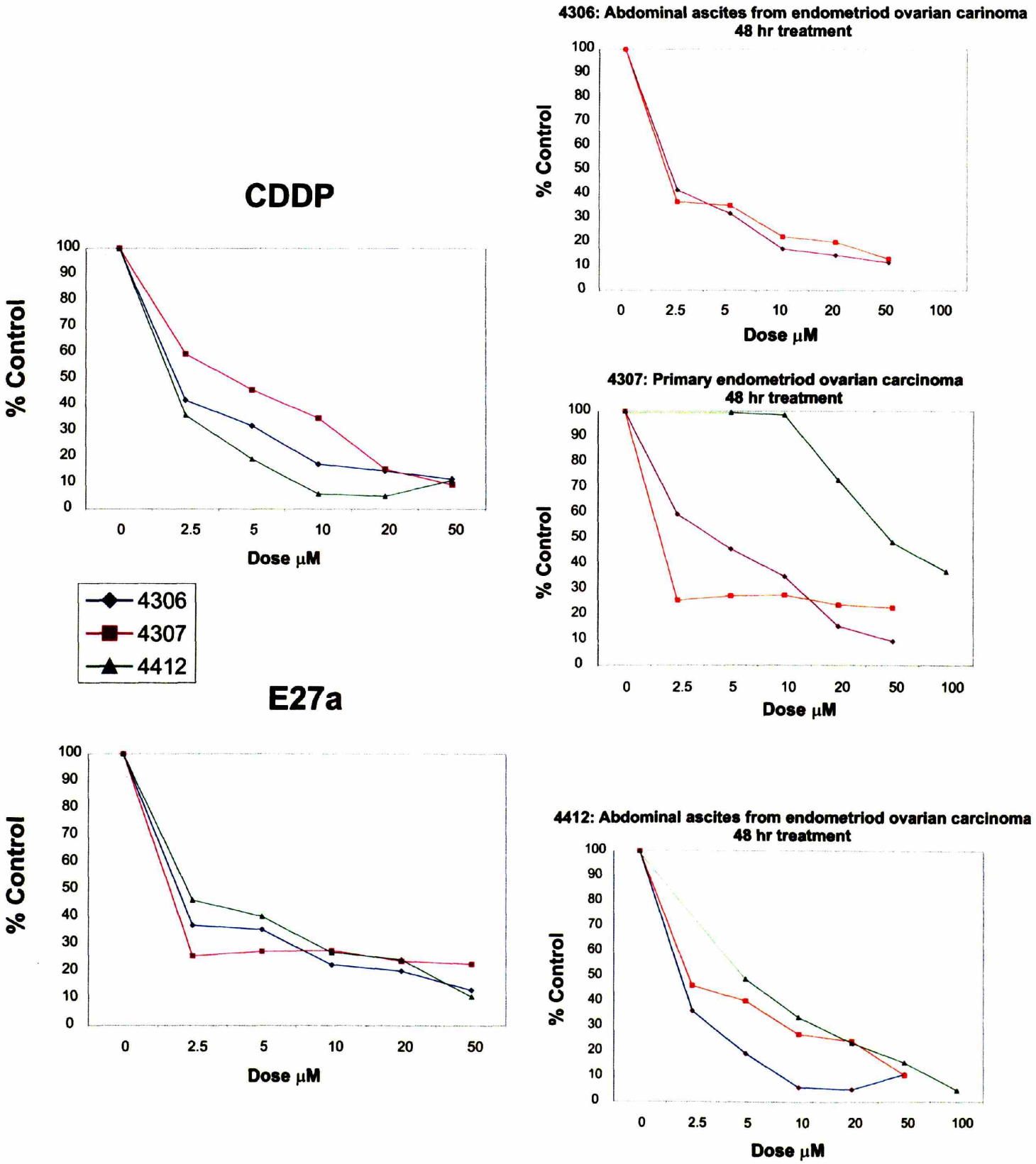


Fig. 6.3: Cells treated for 48 hr, after which cells were trypsinized and counted. CDDP = cisplatin

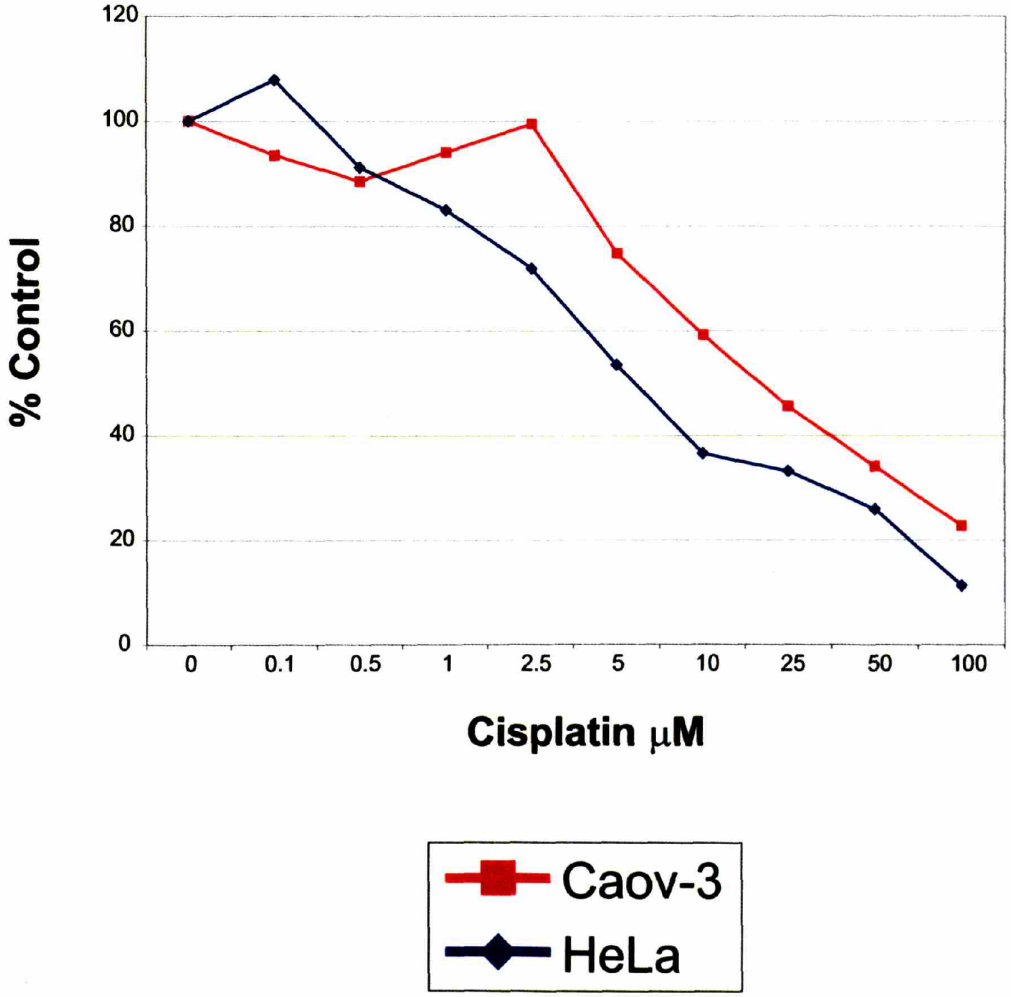


Fig. 6.4: Growth inhibition of human cell lines following cisplatin treatment. Cells treated for 1 hour and counted 48 hours after treatment.

Appendix: Base excision repair and cisplatin damage

A.1 Base excision repair: Cisplatin may hijack base excision repair proteins

A.1.1 AAG binds to cisplatin-DNA adducts, leading to increased mutagenesis

Work by P. Hopkins (307) revealed that some cisplatin crosslinks have a base that is extruded from the helix. This unusual architecture is reminiscent of the structure of damaged DNA bases in the active sites of glycosylases (294,400). Surprisingly, the human 3-methyladenine DNA glycosylase (AAG) can bind each of the cisplatin intrastrand crosslinks *in vitro* (353). AAG is a DNA glycosylase involved in initiation of the first step in base excision repair of a variety of DNA adducts, including 1,N^6-ethenoadenine (εA). Kartalou et al. (353) demonstrated that AAG protein binds cisplatin intrastrand crosslinks with high affinity *in vitro*. Although AAG was unable to release platinum adducts from the DNA, despite the high binding affinity, the presence of cisplatin adducts very effectively inhibited the excision of εA adducts by AAG, suggesting that binding to cisplatin adducts made AAG less available for binding to its natural substrates. Accordingly, if cisplatin could lure away AAG from its natural substrates *in vivo*, the presence of cisplatin adducts could result in decreased repair of other damage in cells, leading to enhanced toxicity and mutagenicity of cisplatin due to the persistence of other DNA lesions normally repaired by AAG (εA in DNA is a natural product derived from lipid oxidation). Such enhanced mutagenicity was observed, and as a parallel Saparbaev et al. (258) recently showed that etheno cytosine in DNA can also hijack AAG away from εA lesions, leading to εA persistence. Thus the mechanism of cisplatin toxicity as it relates to BER is consistent with the hijacking model proposed earlier.

A.2.2 MutY binds to cisplatin-DNA adducts, leading to increased mutagenesis

In addition to AAG, the *E. coli* glycosylase MutY and the human MutY homologue (hMYH) may affect cisplatin mutagenesis *in vivo*. MutY is thought a bifunctional DNA glycosylase (i.e., exhibits both glycosylase and AP lyase activity (611), though the AP lyase activity of MutY is controversial) that is responsible for initiating repair of adenine misincorporated opposite the oxidized lesion 8-oxoG. MutY also shows activity on adenine mispair substrates such as A:G and A:C, albeit with much lower activity (261,654). *Escherichia coli* MutY is a 39 kDa sulfur-iron protein member of the helix–hairpin–helix

and a 13 kDa (p13) domain important in the enzyme's substrate specificity and catalytic efficiency (440). Both the full length protein and p26 bind cisplatin-modified oligonucleotides *in vitro* (Kartalou et al., unpublished results). The role of MutY glycosylase in protecting against oxidized-induced damage and mutagenesis is shown in Fig. A.1. Following MutY activity, the AP site is repaired by the sequential action of an AP endonuclease and gap-filling polymerase that preferentially incorporates dCMP opposite to 8-oxoG and DNA ligase. The resulting C/8-oxoG base pair is then a substrate for the 8-oxoguanine DNA glycosylase (Fpg or MutM). Removal of the 8-oxoG opposite C by Fpg, followed by a gap repair of the abasic site, results in the final restoration of the original C/G base pair.

The bacterial MutY and hMYH exhibit significant sequence homology, and both proteins can bind and cleave at the site of a cisplatin lesion *in vitro* (Kartalou et al., paper in progress). Specifically, the bacterial protein shows enzymatic activity on adenine in a A:G mispair when either the A or G is platinated; hence this protein can initiate a repair event at the site of a cisplatin adduct directly. Mutagenesis analysis also shows that MutY enhances cisplatin-induced mutations in *E. coli*. Such mutations may arise from fixation of the bypass polymerase errors made during translesion synthesis. This mutation fixation would depend on MutY-mediated repair activity on the AG crosslink. For example, if G is misincorporated opposite a platinated A in an AG crosslink, MutY may trigger repair of the crosslink, leaving the G in the opposite strand. The opposite strand containing the bypass error then serves as the template in repair synthesis, leading to an A:T to C:G transversion mutation. The human protein hMYH also binds cisplatin adducts; this protein however was only shown to exhibit enzymatic activity on adenine opposite a platinated G and not a platinated A (opposite G). Thus, based on the model of MutY-dependent mutagenesis just described, the influence of hMYH on cisplatin-induced mutagenesis in human cells may be different than the effects of MutY in bacterial cells. Although MutY does not alter cellular sensitivity to cisplatin in *E. coli*, the effects of hMYH enzymatic activity on sensitivity to cisplatin in human cells have not been studied.

A.2 Construction of a human cell line with deficient expression of hMYH protein

To address the potential roles of hMYH in cisplatin-induced toxicity and/or mutagenesis, RNA interference was used to construct human cells with deficient expression of the hMYH protein. At the time this project was started (in 2001), investigators were testing different duplex siRNAs for knock-down effect by trial-and-error. However today,

many commercial suppliers of siRNAs (including Qiagen, Ambion, among others), sell validated siRNAs for many different proteins. To determine a siRNA sequence that would knock-down hMYH expression, siRNAs designed for five different target sequences of the hMYH cDNA were tested (Fig. A.2). Because the human transcript occurs as several different splice variants (315,529,681), target sites were chosen based on the conserved transcript regions (i.e., only sequences common to all splice variants were considered). A functional assay as well as immunohistochemistry with a polyclonal anti-hMYH antibody were used to assess the extent of knock-down (Figs. A.3 and A.4) Due to factors such as the half-life of the protein and a dilution effect of the siRNAs over time, the extent of knock-down is very transient. Therefore construction of a stable knock-down was attempted. Because targeting the untranslated regions (UTR) would permit functional complementation studies using hMYH cDNA under different UTRs, target #55 was chosen as a target sequence with which to construct a stable-knockdown cell line. Target #11 was also used for stable knock-down expression in order to use two different target sequences and confirm that any knock-down phenotype was not do to off-target effects. In addition to the two hMYH specific targets, a non-specific (control) sequence was used in the stable knock-down clone development. To construct stable human clones with reduced expression of hMYH , a vector containing cDNA encoding for hairpin RNA corresponding to the #55 and #11 target sites as well as a non-specific siRNA sequence were cloned into the p*silencer* plasmid (Ambion). HeLa cells were transfected with the plasmid DNA and selected by G418. G418 resistant cells were seeded so that individual clones could be harvested and characterized. Because the genomic sites containing the hairpin-encoding vector could include genes that affect growth rate and other cellular processes, several clones for each target were selected. Thirty-two clones containing #11, forty-four containing #55, and six negative control clones containing the vector encoding non-specific siRNA were characterized by PCR (Fig. A.5) to ensure that their genomic DNA contained the hairpin encoding construct. Further characterization of these clones is required to determine the level of hMYH protein expression.

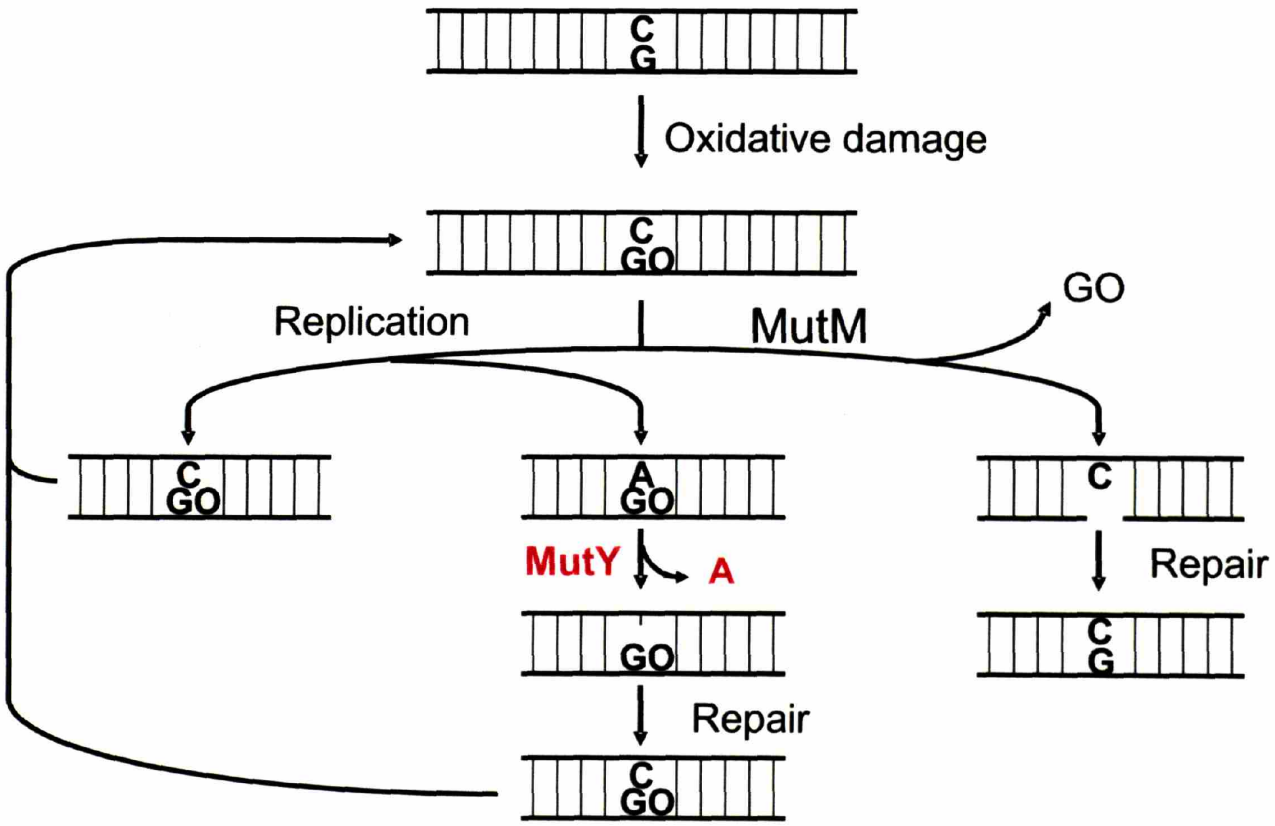


Fig. A.1: MutM and MutY DNA glycosylases participate in the GO system to protect the genome against oxidative damage. MutM removes 8oxoG while MutY removes A opposite 8oxoG.

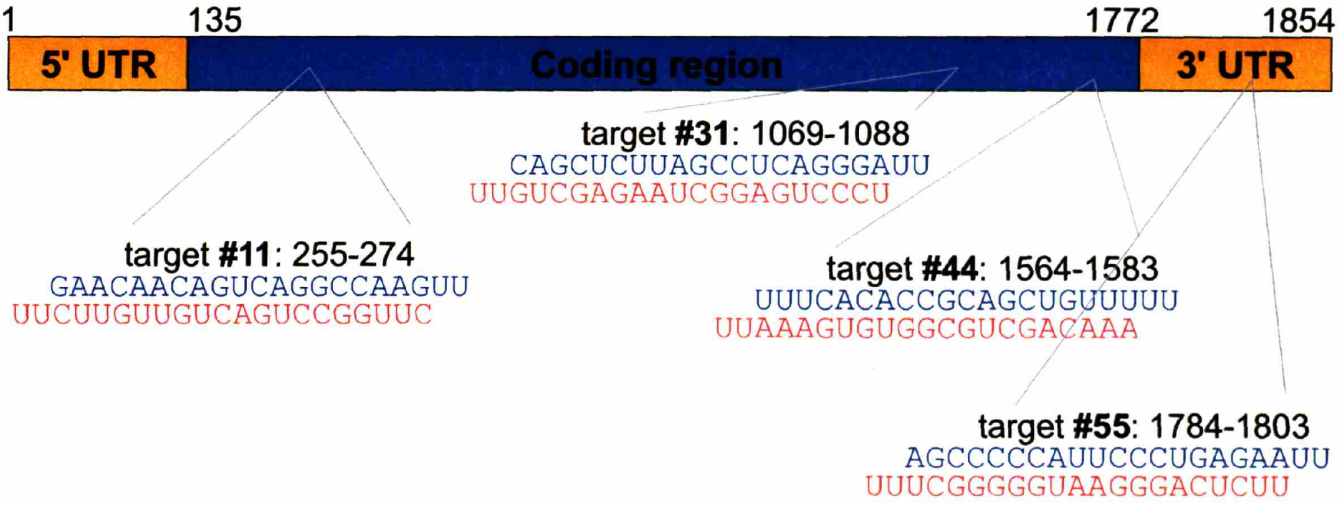
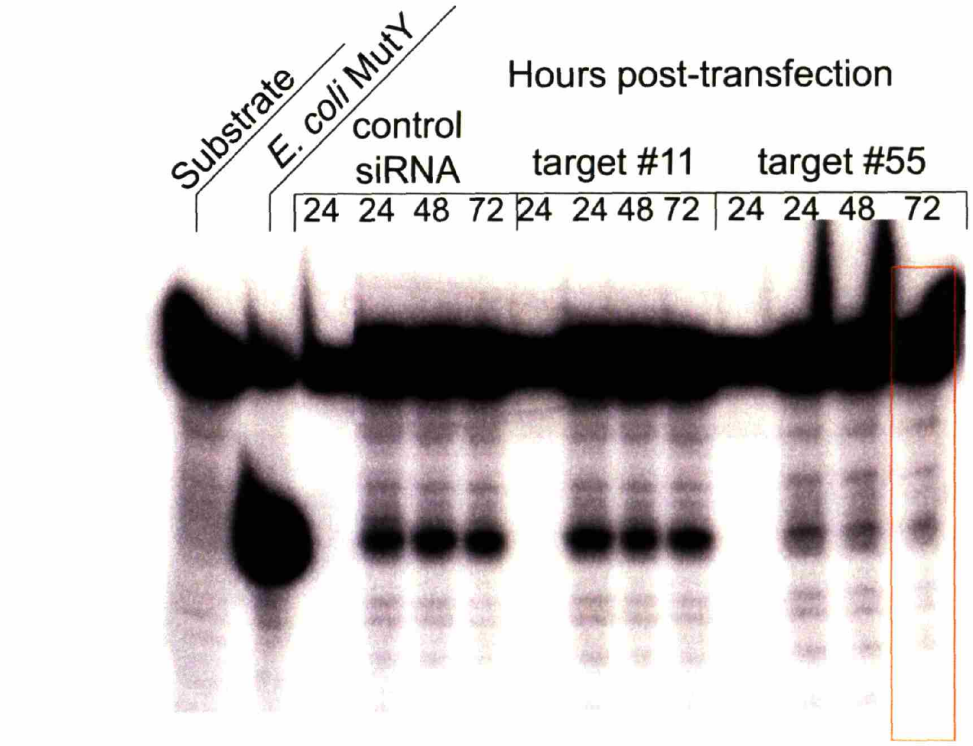


Figure A.2: Target sites of the human MutY homologue for which siRNA duplexes were designed.



Substrate = 5′-CCTCTCCTTGOTCTTCTCCTCTCC-3′
3′-GGAGAGGAACAAGAAGAGGAGAGG-5′

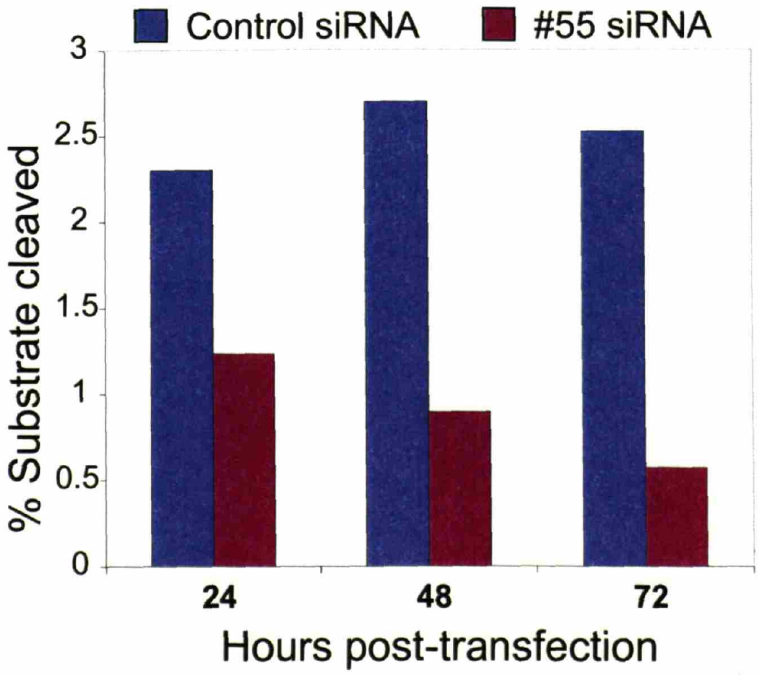


Figure A.3: Transient transfection of HeLa cells with duplex siRNA specific for the human MutY homologue. Post-transfection, cell lysates were prepared and incubated with an oligo susbrate containing an 8oxoG:A mispair. Abasic sites were cleaved with sodium hydroxide and products determined by denaturing gel electrphoresis. Target #55 shows the greatest knock-down effect 72 hours post-transfection.

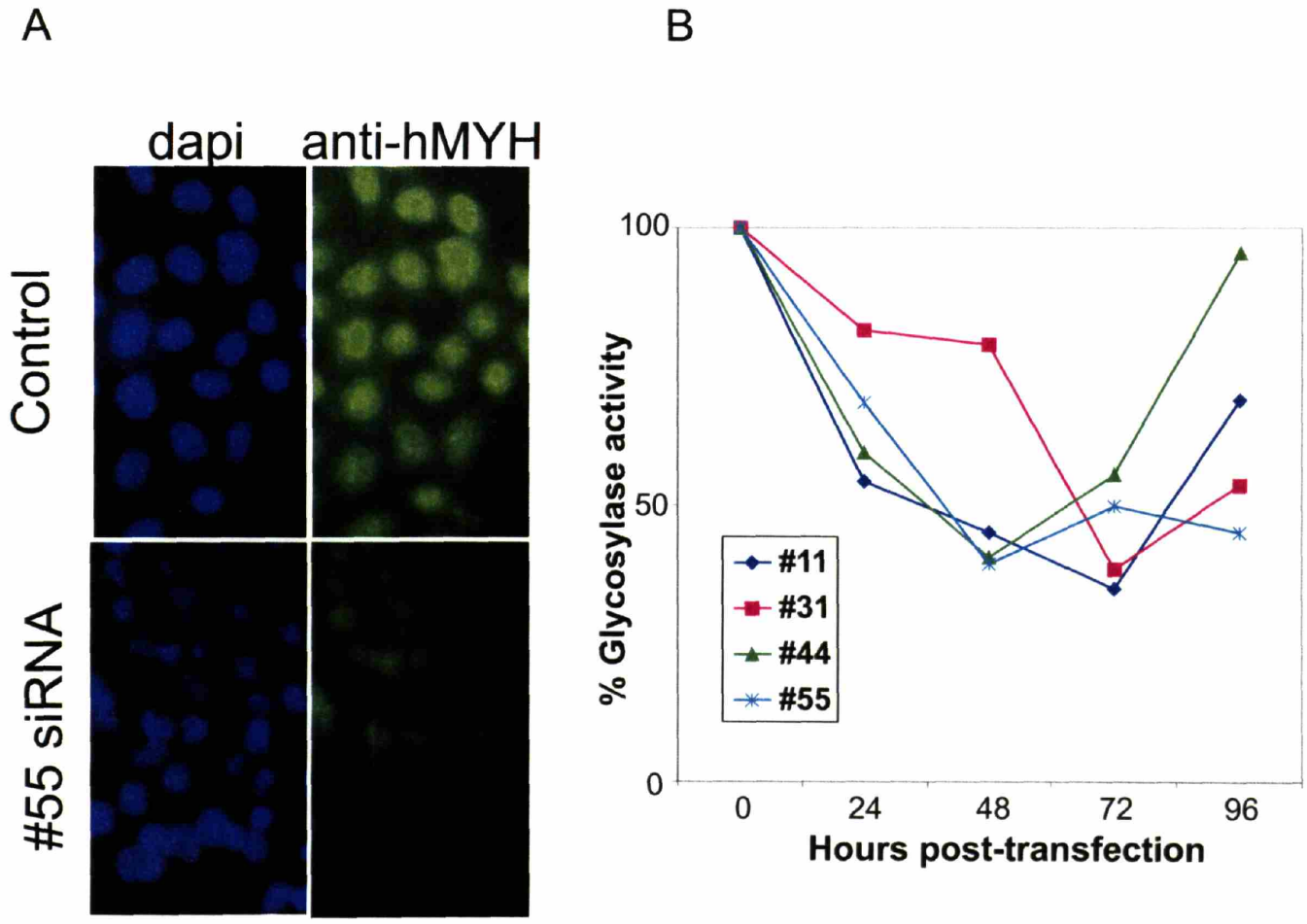
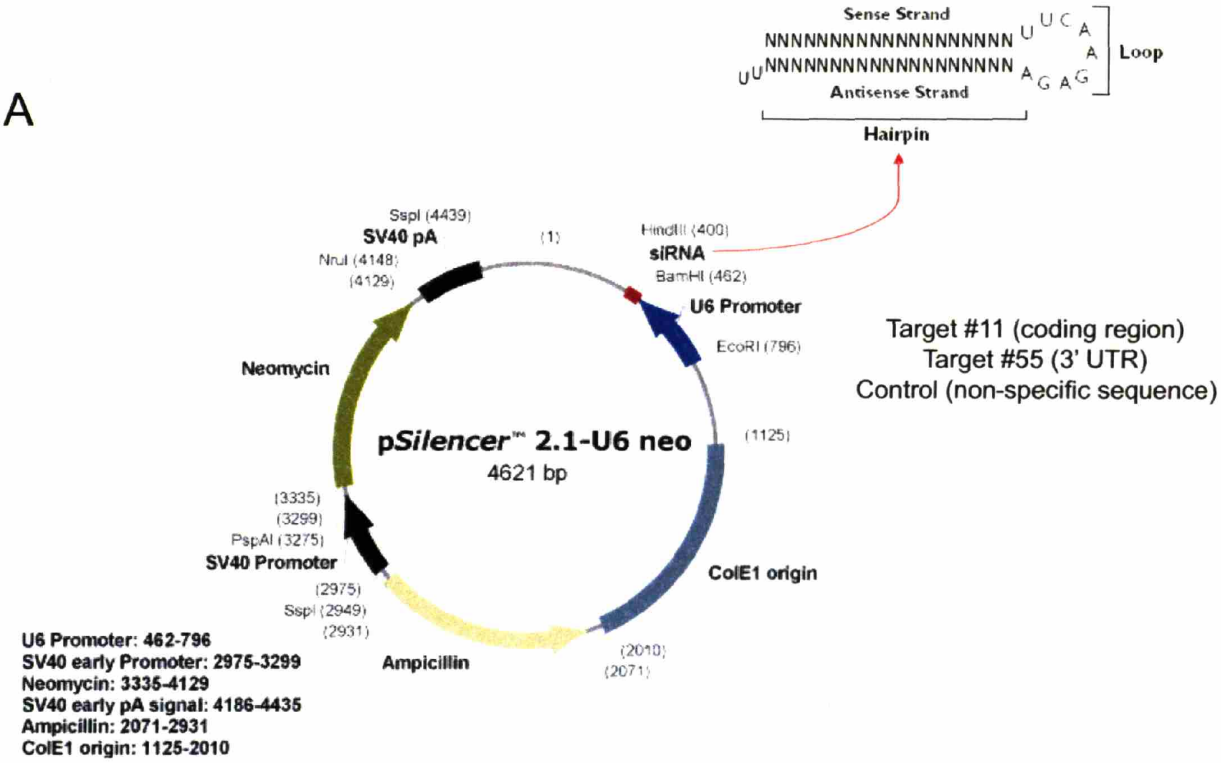


Figure A.4: A) Immunohistochemistry showing reduced expression of hMYH following transcrection with target #55 siRNA. B) Results from four different siRNA duplexes as determined by hMYH activity (Fig. 26).

A



B

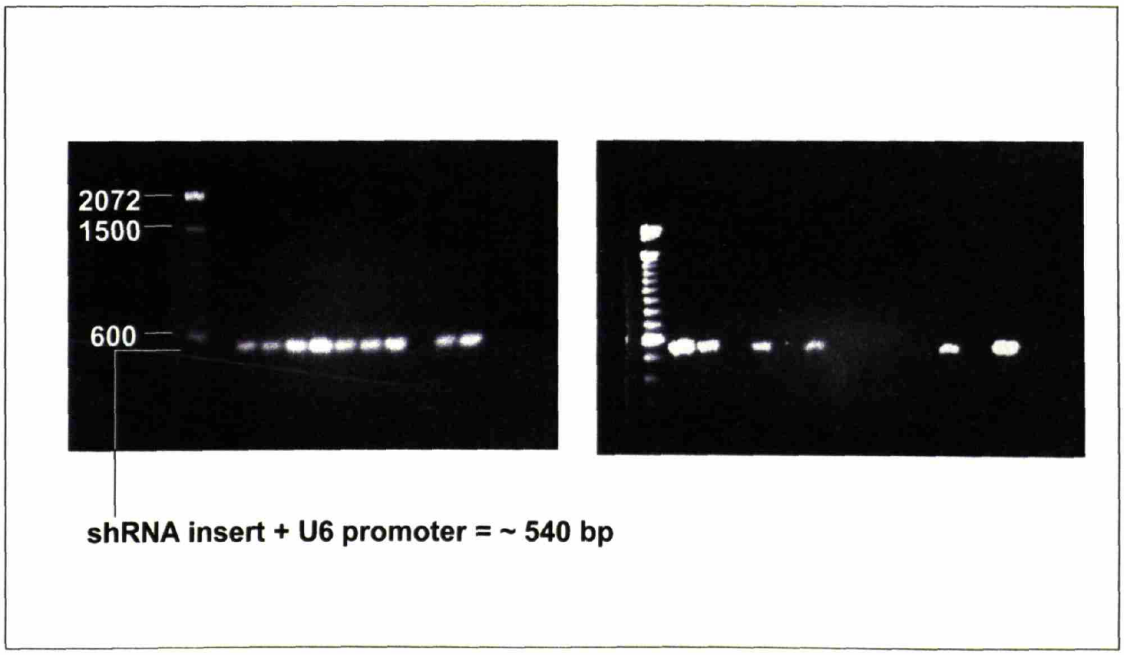


Figure A.5: Vectors encoding short hairpin RNA (shRNA) corresponding to #11 and #55 target sites and a non-specific sequence were cloned into the pSilencer plasmid (A). HeLa cells were transfected with the plasmid and selected by G418 (neomycin) resistance.(B) Individual stable clones were characterized by PCR to confirm that the portion of the plasmid containing the shRNA encoding vector integrated within genomic DNA.

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