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Chronic cisplatin treatment promotes enhanced damage repair and tumor progression in a mouse model of lung cancer

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Abstract

Chemotherapy resistance is a major obstacle in cancer treatment, yet the mechanisms of response to specific therapies have been largely unexplored *in vivo*. Employing genetic, genomic, and imaging approaches, we have examined the dynamics of response to a mainstay chemotherapeutic, cisplatin, in multiple mouse models of human non-small cell lung cancer (NSCLC). We show that lung tumors initially respond to cisplatin by sensing DNA damage, undergoing cell cycle arrest and inducing apoptosis—leading to a significant reduction in tumor burden. Importantly, we demonstrate that this response does not depend on the tumor suppressor p53 or its transcriptional target p21. Prolonged cisplatin treatment promotes the emergence of resistant tumors with enhanced repair capacity that are cross-resistant to platinum analogs, exhibit advanced histopathology, and possess an increased frequency of genomic alterations. Cisplatin-resistant tumors express elevated levels of multiple DNA damage repair and cell cycle arrest-related genes, including p53-inducible protein with a death domain (*Pidd*). We demonstrate a novel role for PIDD as a regulator of chemotherapy response in human lung tumor cells.

Introduction

Lung cancer is the leading cause of cancer death in the United States with a 5-year survival rate of only ~15% (ACS 2007). The majority of patients with advanced non-small cell lung cancer (NSCLC) are treated with combination therapy that includes a platinum-based compound. However, only ~30% of patients with advanced NSCLC respond to this treatment (Socinski 2004). The remaining ~70% of patients suffer negative side effects associated with drug toxicity without the therapeutic benefits of treatment. Among the ~30% of patients that initially respond, most patients eventually develop resistant disease. Therefore, both inherent and acquired drug resistance are major barriers to successful platinum-based therapy.

Cisplatin (cis-diamminedichloroplatinum(II)) is one of the most widely employed drugs in cancer therapy. Its activity as an anticancer agent was discovered over 40 years ago (Rosenberg et al. 1969), and it became the first FDA-approved platinum compound for cancer treatment in 1978 (Kelland 2007). Cisplatin and platinum-based analogs like carboplatin are currently used to treat many malignancies, including lung, ovarian, head and neck, bladder, and testicular cancer (Socinski 2004). While the major barriers limiting the use and efficacy of platinum-based compounds are toxicity and resistance (Kelland 2007), there are currently no established approaches to identify patients who are likely to respond to cisplatin-based therapy.

Cisplatin and carboplatin bind DNA to form intrastrand and interstrand crosslinks between purine bases. Platinated adducts distort the DNA helix in a manner that is recognized by high-mobility group (HMG) proteins and other proteins involved in the DNA damage response (Wang and Lippard 2005). These adducts impair replication and transcription, which can lead to stalled replication forks and the formation of double strand breaks. A number of DNA repair pathways including mismatch repair (MMR) and nucleotide excision repair (NER) have been implicated in platinum adduct repair and, correspondingly, alterations in these pathways have been implicated in resistance (Wang and Lippard 2005; Helleday et al. 2008). Other signaling pathways such as those involving NF- κ B, c-ABL, JNK, and p73 have also been implicated in cisplatin response *in vitro* (Kharbanda et al. 1995; Gong et al. 1999; Hayakawa et al. 2004; Mabuchi et al. 2004; Leong et al. 2007).

Multiple mechanisms that mediate intrinsic or acquired resistance to cisplatin *in vitro* have been identified (Kelland 2007). Mechanisms that preclude the formation of platinum-DNA adducts include decreased import, increased detoxification, and increased efflux (Hall et al. 2008). For example, impaired uptake of cisplatin due to down-regulation of the copper-transporter 1 (CTR1) protein has been demonstrated in ovarian cancer (Ishida et al. 2002;

Holzer et al. 2006). Increased detoxification by conjugation of cisplatin to glutathione, coupled with increased export, has also been documented in ovarian cancer cell lines derived from the same patient before and after drug resistance (Lewis et al. 1988). However, numerous gene expression studies have failed to identify a single transporter that is universally altered in cisplatin-resistant cell lines. It is therefore likely that multiple genes involved in import, detoxification and efflux can be involved in clinically-relevant resistance. Tissue specificity of transporter expression may also impact the mechanisms of resistance in different tumor types (Bando et al. 1998).

Cisplatin resistance can also occur through enhanced DNA damage repair. NER is thought to be the predominant repair pathway for platinum-DNA adducts. The marked sensitivity of testicular cancer to cisplatin may be due to intrinsically lower levels of the NER pathway proteins, ERCC1 and XPA (Welsh et al. 2004). Additionally, increased expression of ERCC1 in ovarian tumors and cancer cell lines has been associated with cisplatin resistance (Dabholkar et al. 1994; Selvakumaran et al. 2003). Recent clinical trials suggest that patients with tumors with low ERCC1 levels benefit preferentially from cisplatin-based chemotherapy (Olaussen et al. 2006). However, very few DNA repair genes have been functionally validated *in vivo*.

Finally, the role of the tumor suppressor p53 in mediating cisplatin response remains controversial and appears to be cell-type dependent. In some cell lines, p53 mutation is associated with cisplatin resistance (Perego et al. 1996). However, in other cell lines, loss of p53 increases cisplatin sensitivity (Pestell et al. 2000). Since p53 is mutated in approximately 50% of human NSCLC (Ahrendt et al. 2000; Skaug et al. 2000), elucidating its role in chemoresistance has important implications for treatment strategies.

Although much has been learned from studying resistance mechanisms in isolated cell lines, tumors *in vivo* encounter drugs in very different conditions. The tumor microenvironment

may provide signals and physical barriers that alter signaling networks and the context in which cells respond to therapy (Olive et al. 2009). The immune system can also act as a barrier or promoter of tumor behavior. Finally, drug pharmacodynamics differ *in vitro* compared to *in vivo*. Therefore, a systematic attempt to model cisplatin response and resistance *in vivo* may provide insights that cannot be ascertained from *in vitro* studies. Observations in xenograft models first demonstrated that *in vivo* chemotherapy resistance mechanisms were distinct from those *in vitro* (Teicher et al. 1990). Few studies have examined the response of autochthonous tumors to platinum-based therapy *in vivo*. For example, responses to several chemotherapy agents including cisplatin were analyzed in mice bearing *Brca1*^{-/-};*p53*^{-/-} mammary tumors (Rottenberg et al. 2007). Interestingly, these tumors developed resistance to doxorubicin and docetaxel but not to cisplatin, even after repeated doses. Thus, there is still a need for *in vivo* models of inherent and acquired resistance to platinum agents.

We have previously described the development of mouse models for human lung cancer in which expression of oncogenic *Kras* (mutated in approximately 30% of NSCLC) is the initiating event (Jackson et al. 2001; Johnson et al. 2001). In the LSL-*Kras*^{G12D/+} model, Cre-mediated loss of a stop cassette permits expression of the oncogenic *Kras*^{G12D} allele from its endogenous promoter. Mice develop lung adenomas with 100% penetrance that eventually progress to high-grade adenocarcinomas. LSL-*Kras*^{G12D/+} mice that possess conditional mutant or null alleles of *p53* develop lung tumors with a shorter latency and advanced histopathology compared to mice with wild-type *p53* (Jackson et al. 2005). We previously demonstrated a strong similarity between *Kras*^{G12D}-initiated lung tumor models and human NSCLC at the level of gene expression (Sweet-Cordero et al. 2005). Since early-stage and advanced NSCLC are frequently treated with platinum compounds, we investigated the effects of cisplatin treatment on oncogenic *Kras*^{G12D}-initiated lung tumors.

Results

Short-term response to cisplatin

LSL-*Kras*^{G12D/+} mice were treated intraperitoneally (ip) with a single dose of cisplatin (7 mg/kg) 12-16 wks after tumor initiation by intranasal Adeno-Cre (AdCre) infection (higher doses led to death and excessive weight loss in pilot studies (**Supp Fig S1**)). Mice were sacrificed at different time points following cisplatin treatment to analyze the effects on cell cycle and cell survival in tumors. As indicated by BrdU incorporation, cisplatin led to a reduction in the number of cells entering the cell cycle that was maximal 72 hrs after a single dose, with full recovery by 120 hrs (**Fig. 1A**). In contrast, the kinetics of the apoptotic response as measured by cleaved caspase 3 (CC3) staining had two waves of activity that peaked at 24 and 72 hrs, and returned to control levels by 120 hrs after treatment (**Fig. 1B**). A maximal decrease in mitotic index was observed 24 hrs after cisplatin treatment and persisted through 72 hrs (**Supp Fig. S2**).

To investigate whether p53 activation mediates apoptosis and cell cycle arrest in response to cisplatin in this model, we crossed LSL-*Kras*^{G12D/+} mice with conditional *Trp53*^{F2-10/F2-10} mice (Jonkers et al. 2001), hereafter referred to as *p53*^{fl/fl}. Delivery of AdCre to the lungs of LSL-*Kras*^{G12D/+}; *p53*^{fl/fl} mice leads to simultaneous activation of oncogenic *Kras* and loss of *p53* function (Jackson et al, 2005). *Kras*^{G12D/+} lung tumors null for *p53* had significantly higher basal proliferation indices than tumors with wild-type *p53* ($p < 0.003$), while *p53* heterozygous lung tumors had intermediate levels of proliferation (**Supp Fig. S3**). However, in response to cisplatin, both *p53* heterozygous and null lung tumors exhibited cell cycle arrest similar to that seen in *Kras*^{G12D/+} tumors with wild-type *p53* (**Fig. 1C**). The majority of tumors had significant decreases in BrdU incorporation 72 hrs after cisplatin, regardless of *p53* status (**Supp Fig. S4**). While the maximum number of apoptotic cells observed in *Kras*^{G12D/+}; *p53*^{fl/fl} tumors in response to cisplatin was decreased compared to *Kras*^{G12D/+}, we detected a statistically

significant increase in both cases (**Fig. 1B, D**). Thus, cell cycle arrest induced by cisplatin is not dependent on p53 in this model, and apoptosis is at least partially p53 independent as well. We confirmed the lack of dependence on p53 for cell cycle arrest in this model by crossing LSL-*Kras*^{G12D/+} mice to mice lacking a functional allele of *p21* (Brugarolas et al. 1995). *Kras*^{G12D/+}; *p21*^{-/-} lung tumors had similar cell cycle arrest and apoptosis profiles in response to cisplatin compared to controls (**Supp Fig. S5**). Taken together, these data suggest that cisplatin response *in vivo* is not dependent on the p53-p21 pathway.

To investigate the kinetics of cisplatin adduct formation and DNA damage signaling at a cellular level, we analyzed cisplatin-treated tumors for the presence of platinum (Pt)-DNA adducts using a Pt-1,2-d(GpG) intrastrand cross-link-specific monoclonal antibody (Liedert et al, 2006). This antibody recognizes the most frequently occurring adduct formed by cisplatin, which is associated with its cytotoxicity and anticancer activity (Liedert et al. 2006; Dzagnidze et al. 2007). Pt-DNA adducts were detected in the lung as early as three hours after a single dose of cisplatin (data not shown and **Fig. 1E,F**). Platinum adduct formation can cause stalling of replication forks which leads to collapse and the generation of DNA double strand breaks (Henry-Mowatt et al. 2003). This leads to activation of checkpoint kinases, ATM and ATR, and their downstream substrates, Chk2 and Chk1, which recruit other repair proteins to sites of damaged DNA (Pabla et al. 2008). The phosphorylated form of the histone variant H2AX (γ -H2AX) is a critical component of this repair complex and thus, can be used as a marker of DNA damage signaling. In cisplatin-treated *Kras*^{G12D/+} tumors, we detected γ -H2AX four hours (the earliest time point examined) after cisplatin treatment with maximal staining 12-24 hrs following treatment (**Fig. 1G,H, Supp Fig. S6**, and data not shown). Basal phosphorylation of the checkpoint kinase Chk2 (Thr68) was detected in untreated tumors, and increased phosphorylation of both Chk1 (Ser345) and Chk2 (Thr68) was clearly evident after cisplatin treatment (**Fig. 1I-L**). Taken together, these data demonstrate that tumors sense DNA damage

in response to cisplatin within 4 hrs and respond by cell cycle arrest and cell death associated with activation of both Chk1 and Chk2. In *Kras*^{G12D/+};*p53*^{fl/fl} lung tumors analyzed 4-24 hrs after a single dose of cisplatin, we did not detect obvious differences in DNA damage signaling compared to *p53* wild-type tumors (**Supp Fig. S7**). We observed very few tumors with patterns of BrdU or γ -H2AX staining that deviated significantly from the mean at the indicated time points, suggesting that most tumors initially respond to cisplatin-induced DNA damage in this model (**Supp Fig. S4** and data not shown).

Long-term response to cisplatin

To analyze the long-term effects of cisplatin therapy on *Kras*^{G12D}-initiated lung tumors, we treated mice 12 wks following AdCre infection with cisplatin once a week for 2 wks followed by a 2 wk rest period to allow recovery from toxicity and repeated this regimen for a total of 4 doses of cisplatin (**Fig. 2A**, Group 3). Tumor response was measured by determining the ratio of tumor area to total lung area (TA/LA) in histological sections. Treatment with cisplatin significantly reduced tumor burden in the treated G3 group (n=8) compared to the control G1 mice (n=7) ($p < 0.0002$) (**Fig. 2B-D**).

To determine whether this response was dependent on intact *p53*, we treated LSL-*Kras*^{G12D/+};*p53*^{fl/fl} mice with a similar treatment regimen. Upon sacrifice, the basal tumor volume in untreated LSL-*Kras*^{G12D/+};*p53*^{fl/fl} mice was much greater than those with wild-type *p53*. However, despite this increase in volume, LSL-*Kras*^{G12D/+};*p53*^{fl/fl} mice treated with cisplatin (n=11) also had a significant reduction in tumor burden compared to controls (n=10) ($p < 0.0001$), again demonstrating that wild-type *p53* is not required for response to cisplatin (**Fig. 2B, EF**).

Using another cohort of LSL-*Kras*^{G12D/+} and LSL-*Kras*^{G12D/+};*p53*^{fl/fl} mice, we asked whether the impact of the 4-dose regimen of cisplatin could prolong survival of tumor-bearing

mice. Unexpectedly, despite the significant reduction in tumor burden in LSL-*Kras*^{G12D/+} mice observed after the treatment regimen (**Fig. 2B**), there was no improvement in survival (**Fig. 2G**). In contrast, LSL-*Kras*^{G12D/+}; *p53*^{fl/fl} mice treated with four doses of cisplatin survived significantly longer (n=11) than mice treated with PBS (n=8) ($p < 0.002$) (**Fig. 2H**). Tumors in LSL-*Kras*^{G12D/+} mice develop much more slowly than tumors that lack *p53*, and untreated LSL-*Kras*^{G12D/+} mice do not die from their lung tumor burden until 7-13 wks after treated mice receive the fourth dose of cisplatin—a considerable time frame for residual treated tumors to re-grow. Indeed, tumor burden at the time of death in LSL-*Kras*^{G12D/+} mice treated with four doses of cisplatin was not significantly different from control animals (data not shown). In contrast, *Kras*^{G12D/+}; *p53* null lung tumors develop extremely rapidly, and these tumors typically kill untreated animals near the time when treated mice are receiving their fourth and final dose of cisplatin. When we treated LSL-*Kras*^{G12D/+} mice with continuous dosing of cisplatin beyond four doses, mice experienced a significant survival benefit (**Supp Fig. S8**). The fact that treatment with cisplatin significantly prolongs survival of mice with *p53* null lung tumors further demonstrates that *p53* is not required for drug response and therapeutic benefit. This suggests that loss of *p53*, while a predictor of poor prognosis and more aggressive tumors in mice, still permits therapeutic benefit from cisplatin.

Next, to investigate whether residual *Kras*^{G12D/+} tumors present at the end of the treatment regimen were resistant to cisplatin, we treated a cohort of LSL-*Kras*^{G12D/+} mice as described above with four total doses of cisplatin or PBS, waited 4 wks, and then treated them with a final 72 hr dose of cisplatin before sacrifice (**Fig 2A**, G2 vs. G4). When both sets of mice received a final dose of cisplatin, tumors from mice that had received previous cisplatin treatment no longer demonstrated a significant reduction in BrdU incorporation like the naïve tumors (**Fig. 2I**, and **Fig 1A**), suggesting that the pretreated tumors have acquired resistance to cisplatin treatment.

Dynamics of tumor response to cisplatin

To gain further insight into the dynamics of cisplatin response in this model, we employed *in vivo* micro-computed tomography (microCT) imaging. LSL-*Kras*^{G12D/+} mice were treated with PBS or cisplatin according to the regimen described above and imaged prior to treatment, 5 days after the second dose of cisplatin, and 10 days after the fourth and final dose of cisplatin. We focused on tumors whose boundaries could clearly be defined in multiple scans over time. Untreated *Kras*^{G12D/+} lung tumors grew slowly (average tumor volume doubling time of ~35 days) with highly variable growth rates. Following two doses of cisplatin, most tumors in the LSL-*Kras*^{G12D/+} model showed a reduction in tumor volume (**Fig. 3A-C**). During the dosing break (between doses 2 and 3), cisplatin-treated tumors resumed growth but generally remained sensitive after the third and fourth doses (**Fig. 3A-B**). However, some tumors stopped responding to the third and fourth doses of cisplatin (**Fig. 3A**). Thus, while we cannot rule out that innate resistance occurs in individual clones within tumors, it does not appear to be a characteristic of bulk tumors. Importantly, in mice that received four doses of cisplatin and received a final dose of cisplatin approximately 6 wks later, treated tumors no longer responded, again suggesting that tumors become resistant after 4 doses of cisplatin (**Fig. 3B**).

Kras^{G12D/+}; *p53* null lung tumors grow much faster than those with wild-type *p53* (doubling time ~7 days) and, therefore, it is more straightforward to observe a significant impact on tumor growth. Indeed, a single dose of cisplatin caused a significant reduction in tumor growth in this model as observed by microCT (data not shown). Unlike *Kras*^{G12D/+} tumors with wild-type *p53*, *p53* null tumors did not regress, but progressed despite therapy (**Fig. 3D**). In a smaller study, we quantified total tumor burden by microCT in LSL-*Kras*^{G12D/+}; *p53*^{fl/fl} mice treated with PBS or 4 doses of cisplatin. Cisplatin treatment clearly impeded tumor growth, but tumors continued to progress despite therapy (**Fig. 3E**).

Mechanism of cisplatin resistance *in vivo*

Previous studies suggest that cisplatin resistance in human cancer may be complex as no single factor has been able to explain resistance in full. *In vitro* studies suggest that decreased uptake, increased detoxification and increased efflux of platinum from cells may all be mechanisms of resistance. In addition, platinum adducts may be more rapidly repaired in resistant tumors. Finally, tumor cells may utilize error-prone translesion DNA polymerases in order to tolerate higher levels of adducts. To distinguish among these possibilities, we treated long-term PBS or cisplatin-treated mice with a final dose of cisplatin and stained tumor sections with the antibody to Pt-1,2-intrastrand DNA crosslinks to monitor the kinetics of adduct levels. Strikingly, twenty-four hours after a final dose of cisplatin, long-term treated tumors had significantly decreased levels of Pt-1,2-d(GpG) adducts compared to tumors from mice previously treated with PBS (G2 vs. G4, **Fig. 4A-C**), whereas adduct levels in the normal surrounding lung cells were similar (**Supp Fig. S9**). Tumors that completely lacked adducts at this time point were found only in lungs from long-term cisplatin-treated animals. To support this observation, we quantified the levels of γ -H2AX in PBS and cisplatin-treated tumors that had received a final 24 hr dose of cisplatin. We observed a significant reduction in γ -H2AX staining in resistant tumors (**Fig. 4D-F**), consistent with the lack of adducts at this time point.

These data suggest that the mechanism of cisplatin resistance in this model is not mediated by tolerance of platinum adducts *in vivo*. However, these data do not discriminate between resistance mechanisms in which damage never occurs (ie. import/ detoxification/ export) or in which damage occurs but is rapidly repaired. To discriminate between these possibilities, we used atomic absorption spectroscopy (AAS) to quantify platinum levels in lysates from individual lung tumors taken from animals treated with PBS or cisplatin (4 total doses) plus a final dose of cisplatin given at 0, 2, 4, 12, 24, 48, or 72 hrs before sacrifice. Chronic cisplatin treatment did not cause a significant decrease in platinum levels within

tumors at any time point examined (**Supp Fig. S10**), demonstrating that platinum is able to enter tumors similarly in naïve and pretreated tumors. This result suggests that decreased import and/or rapid efflux are not the driving forces behind cisplatin resistance in this model.

Increased DNA repair has been proposed as a mechanism of platinum resistance (Martin et al. 2008). We reasoned that if rapid repair was occurring, we should detect a difference in the kinetics of Pt-1,2-d(GpG) adduct formation and potentially markers of DNA damage signaling. To explore this possibility, we treated long-term PBS and cisplatin-treated mice with a final dose of cisplatin and examined the kinetics of platinum adducts at early time points (< 24 hrs) following a final dose of cisplatin (**Fig. 4G-I**). Adduct levels were blindly scored as absent, low, or high (-, +, or ++) on at least 20 tumors per treatment group. As expected, adducts were not present in naïve tumors (long-term PBS, LT PBS), but surprisingly, had persisted in non-tumor lung areas of pretreated animals for at least 4 weeks following their last dose of cisplatin (long-term cisplatin, LT Cis) (**Fig. 4G**). At four hours after a final dose of cisplatin, 81% of cisplatin-pretreated tumors (21/26) had similar levels of adducts as naïve tumors treated with cisplatin (**Fig 4H**). Thus, the majority of tumors showed similar levels of Pt-1,2-d(GpG) adducts regardless of whether or not they had previously received cisplatin. By eight hours after a final dose of cisplatin, 59% of cisplatin-pretreated tumors (13/22) had similar levels of adducts as naïve cisplatin-treated tumors (data not shown). By twenty-four hours after a final dose of cisplatin, tumors that completely lacked adducts were found only cisplatin-pretreated tumors (**Fig. 4I**). These data demonstrate that Pt-1,2-d(GpG) adducts are present in sensitive and most resistant tumors at early time points, but are more rapidly cleared in tumors pretreated with cisplatin.

Chk1 and Chk2 are checkpoint kinases that are activated by DNA damage signals mediated by ATM and ATR. Therefore, we analyzed Chk1 and Chk2 phosphorylation in long-term cisplatin-treated vs. control mice with or without a final 12 hr dose of cisplatin as another

marker of whether cells were experiencing DNA damage. We observed a clear difference in the dynamics of phosphorylation of these two DNA-damage signaling proteins. Chk1 phosphorylation occurred in response to cisplatin in tumors from both naïve and long-term treated mice (G1 vs. G2, and G3 vs. G4, **Supp Fig S11**) demonstrating that resistant tumors activate the DNA damage response and, thus, that cisplatin is entering these cells. In contrast, Chk2 phosphorylation was induced after an initial dose of cisplatin (G1 vs. G2) and then remained high even in tumors that had not been given cisplatin for several weeks (G3 in **Supp Fig S11**). This finding suggests that these two signaling pathways may be responding to distinct DNA damage signals as result of cisplatin treatment—one that is transient (Chk1) and another that is persistent (Chk2). Furthermore, it suggests there is a fundamental difference in the DNA damage response mechanism in naïve and long-term cisplatin-treated lung tumors. Taken together, our data strongly argue that increased DNA damage repair is the predominant mechanism of cisplatin resistance *in vivo* in this model.

Cross-resistance to platinum analogs

Cisplatin resistant tumors in the clinical setting are often cross-resistant to other platinum analogs. To determine whether cisplatin-resistant tumors were cross-resistant to other platinum agents, we treated long-term PBS or cisplatin-treated tumors with a single dose of carboplatin (50 mg/kg in saline) and analyzed tumors 24 hrs later for the presence of Pt-1,2-d(GpG) adducts and DNA damage signaling (γ -H2AX). Carboplatin induces the same type of Pt-1,2-d(GpG) crosslinks as cisplatin in cells, although at a slightly reduced frequency (Blommaert et al. 1995). Indeed, in our studies with carboplatin, staining for this adduct was less intense compared to a single dose of cisplatin (7 mg/kg) (data not shown). In long-term cisplatin-treated tumors, carboplatin produces fewer adducts (data not shown) and reduced DNA damage signaling evident by γ -H2AX staining compared to naïve tumors (**Supp Fig.**

S12). These data indicate that, just as encountered in clinical resistance (Wang and Lippard 2005), cisplatin-resistant tumors in this model are cross-resistant to other platinum analogs.

Comparative genomic analysis of naive vs. cisplatin-treated tumors

We performed DNA copy number analysis to identify potential genomic deletions or amplifications that might implicate particular genes involved in acquired resistance. LSL-*Kras*^{G12D/+} mice were treated with long-term PBS or cisplatin as described in G1 and G3 (**Fig. 2**) and then sacrificed ~8 wks after their final dose of cisplatin, a total of 24-30 wks following tumor induction by AdCre. DNA was isolated from individually micro-dissected tumors and subjected to representational oligonucleotide microarray analysis (ROMA) (Lakshmi et al. 2006). Of 11 long-term PBS-treated lung tumors analyzed, only two (18%) had detectable whole chromosomal aberrations (**Fig. 5A**). This observation is consistent with the low frequency of DNA copy number changes that we previously reported in this model using BAC arrays (Sweet-Cordero et al. 2006). In contrast, 19 of 23 long-term cisplatin-treated tumors (83%) harbored whole chromosomal aberrations, including gains and losses of whole chromosomes (**Fig. 5B-H**). A subset of tumors was analyzed for copy number changes with independent methodologies including Agilent Array CGH, Affymetrix SNP Arrays (Broad Institute, Cambridge, MA), and Solexa sequencing (Illumina); these techniques consistently validated the whole chromosomal changes identified by ROMA (data not shown).

Strikingly, histological analysis of a subset of these tumors revealed that the majority of cisplatin-treated tumors were higher grade (11/14 as Grade 2+, 79%) compared to untreated tumors (2/10 as Grade 2+, 20%) (**Fig. 5I-L**). The only two PBS-treated tumors with whole chromosomal changes were also blindly scored as Grade 2+ tumors, whereas eight PBS-treated tumors with undetectable copy number changes were scored as low-grade (Grade 2 or less). Therefore, high-grade tumors are consistently associated with chromosomal

abnormalities, whereas low-grade tumors have apparently normal DNA copy numbers in this model. These data suggest that long-term cisplatin treatment either selects for and/or promotes tumor progression accompanied by alterations in chromosome number.

Gene expression analysis of cisplatin response and resistance *in vivo*

The data presented above suggest that long-term cisplatin treatment creates tumors that are fundamentally different from naïve tumors. To characterize these potential differences, we performed gene expression analysis to examine cisplatin response and resistance. First, we examined the dynamics of gene expression changes in response to cisplatin using laser capture microdissection to isolate RNA from individual tumors at 24, 48 and 72 hrs after a single dose of cisplatin. We analyzed expression of *p21*, *Mdm2*, *Bax* and *Bcl2* using real-time PCR. Maximal differential expression of these genes occurred 72 hrs after cisplatin treatment despite the fact that the DNA damage response and cell death occurred earlier (data not shown). We then performed a more global analysis of gene expression at the same time point after cisplatin therapy using microarrays. cDNA from individually-microdissected lung tumors (n=49) was analyzed using Affymetrix 430A Genechips. Samples from mice treated in the 4 groups shown in **Figure 2** were included: G1 (n=13), G2 (n=11), G3 (n=9), G4 (n=7) as well as normal lung (n=9) (**Supp Table S1**).

To identify cellular pathways altered in cisplatin-treated tumors, we used gene set enrichment analysis (GSEA) to identify gene sets representing molecular pathways with significant enrichment in control vs. resistant tumors (Subramanian et al. 2005). GSEA provides an enrichment score (ES) that measures the degree of enrichment of a gene set at the top (highly correlated with class 1) or bottom (highly correlated with class 2) of a rank ordered gene-list derived from the data set. A nominal p-value is used to assess the significance of the individual ES score. We also used the pathway analysis tool MetaCore from

GeneGO Inc. to identify cellular processes significantly enriched between treatment groups. Pathways are defined in MetaCore as a set of curated consecutive signals or transformations that have been confirmed by experimental evidence or inferred relationships. We focused our analysis on the top-scoring 200 genes in each transition (G2 vs. G1: genes up-regulated in G2 compared to G1; G1 vs. G2: genes up-regulated in G1 compared to G2, etc.). Consistent with our data in Fig. 2I, cell cycle and proliferation pathways were significantly enriched in naïve tumors compared to tumors treated with a single dose of cisplatin (G1 vs. G2; 6 of the top 8 enriched pathways were associated with cell cycle and cell proliferation; FDR < 0.05, $p < 0.003$). However, cell cycle pathways were not similarly represented in cisplatin-pretreated tumors before and after treatment with the same dose (G3 vs. G4 and G4 vs. G3) (**Supp Table S2**). In addition to changes in cell cycle, pathways enriched in naïve tumors treated with a single dose of cisplatin (G2 vs. G1) included those related to adhesion, transport and immune response (FDR < 0.25, $p < 0.004$). In tumors pretreated with 4 doses of cisplatin and treated with a final challenge of cisplatin, pathways enriched in pretreated tumors (G4 vs. G3) included those related to cell adhesion, G-protein coupled receptor (GPCR) signaling, glutathione metabolism, and p53 signaling, whereas those depleted included pathways related to immune response and apoptosis/survival (FDR < 0.25, $p < 0.05$). Pathways enriched in cisplatin-pretreated tumors compared to naïve tumors (G3 vs. G1) were largely related to immune response (FDR < 0.25, $p < 0.05$). When comparing treatment of naïve tumors to cisplatin-pretreated tumors with a final dose of cisplatin (G4 vs. G2), pathways enriched in cisplatin-pretreated tumors included those related to cell cycle & DNA damage, glutathione and methionine metabolism, cell adhesion and cytoskeletal remodeling, among others (FDR < 0.25, $p < 0.05$) (**Supp Table S2**).

Glutathione-mediated detoxification of cisplatin has been previously implicated in resistance, and we validated that a subset of glutathione-related genes (ie. *Mgst2* and *GstT2*)

were significantly upregulated in pretreated tumors (data not shown). Because our data suggest that the majority of cisplatin-resistant tumors repair adducts more quickly than naïve tumors, we decided to further pursue the cell cycle/DNA damage class of genes. In addition to the enrichment of cell cycle/DNA damage pathways using GeneGO, GSEA identified a DNA damage response gene set enriched in G2 vs. G4 (**Fig 6A**). We validated the expression levels of a subset of these genes by real time RT-PCR on an independent set of tumors. Cisplatin-resistant tumors expressed higher basal levels of some genes (**Fig. 6B**, *Apex1*, *Chek2*, *Rad51*, *Rad52*). Other genes were induced to a higher degree in cisplatin-resistant tumors compared to controls (**Fig. 6C**, *Lrdd*, *Cdkn1a* (*p21*), *Ercc2*, *Rad9*). Together, these data support our observation that cisplatin-resistant tumors have an enhanced ability to repair platinum-DNA adducts, and additionally, they have the capacity to induce expression of genes known to play a role in multiple DNA repair pathways.

PIDD expression induces cisplatin resistance in human cancer cell lines

Of these genes, *Lrdd* (also known as p53-induced protein with a death domain (*Pidd*)) was notable because it had not been previously implicated in cisplatin resistance *in vivo*. PIDD was originally identified as a target gene of p53, whose expression promoted apoptosis in *p53* null cell lines (Lin et al, 2000). Subsequently, it was shown that PIDD is a ~90 kDa protein that is constitutively processed into two smaller C-terminal fragments, PIDD-C and PIDD-CC, by autocatalytic cleavage (Tinel et al., 2007). These fragments participate in different signaling complexes called PIDDosomes, which can act as pro-survival or pro-death signals in response to DNA damage depending on the context (Tinel and Tschopp 2004; Janssens et al. 2005; Tinel et al. 2007; Shulga et al. 2009). More recently, PIDD has been implicated in cell cycle regulation in the context of DNA damage, particularly in NHEJ and the G2/M checkpoint (Shi et al, 2009).

We reasoned that if PIDD is playing a role in cell cycle arrest or repair *in vivo*, it should be induced early after a final dose of cisplatin in resistant tumors. We isolated an independent set of tumors from LSL-*Kras*^{G12D/+} mice (G1 through G4) that were treated with or without a final 8 hr dose of cisplatin and performed real time RT-PCR for *Pidd* expression. Indeed, *Pidd* expression was significantly higher only in tumors pretreated with cisplatin (**Fig. 7A**). To examine the potential role of PIDD in cisplatin response *in vitro*, we treated three human NSCLC cell lines that have *KRAS* mutations and wild-type *P53* with various doses of cisplatin and examined expression of *PIDD* 24 hrs following treatment. In all cell lines examined, cisplatin treatment led to increased levels of *PIDD* mRNA (**Fig. 7B**).

To further investigate the role of PIDD in cisplatin response, we overexpressed PIDD by infecting cells with retroviruses carrying C-terminal Flag-tagged-*PIDD* with a puromycin resistance cassette and selected cells with puromycin (Tinel et al. 2007). Overexpression was confirmed by Western blotting of nuclear and cytoplasmic cell lysates with antibodies directed against Flag and PIDD (**Fig. 7C**, and data not shown). These data demonstrate the presence of the autocatalytically-cleaved forms of PIDD (~51 and ~37 kDa), which were both present in the cytoplasm and also in the nucleus, although at lower levels (**Fig. 7C** and data not shown). Expression of PIDD led to reduced growth rate in each cell line (data not shown), with a corresponding increase in the percentage of cells in G1 of the cell cycle (**Supp Fig S13**). Importantly, in the presence of cisplatin, PIDD expression led to significantly enhanced cell viability (**Fig. 7D, E**). Strikingly, in H460 cells, overexpression of PIDD increased the IC₅₀ by 13-20 fold (**Fig. 7D, E**). In addition, overexpression of PIDD contributed to increased resistance to other DNA-damaging agents, including gemcitabine and etoposide (**Supp Fig. S14**). Because PIDD has been implicated in NF-κB mediated pro-survival signaling, we analyzed expression of the NF-κB subunit, p65, by Western blot of nuclear and cytoplasmic cell fractions (**Supp Fig S15**), but did not detect basal differences as a result of PIDD

overexpression. This does not, however, rule out a role for PIDD in regulating NF- κ B signaling specifically in response to damage. Taken together, the mouse *in vivo* data and the human *in vitro* data support an important role for PIDD in cisplatin resistance in lung cancer.

Discussion

While molecularly targeted therapies hold promise for the future of cancer treatment, most patients are currently treated with cytotoxic agents. Cisplatin is an example of a widely employed anticancer drug about which we have very little understanding of whether a given patient will be responsive or resistant to treatment. An improved understanding of the molecular and genetic basis of cisplatin response and resistance could significantly impact clinical strategies. Previously, mouse models of hematopoietic malignancies were successfully used to study the genetics of chemotherapy response (Schmitt et al. 2000; Schmitt et al. 2002). However, few attempts have been made to model chemotherapy resistance in mouse models of epithelial cancers. Here we have used genetically-engineered mouse models of lung cancer to dissect the molecular and genetic mechanisms of response and resistance to cisplatin therapy *in vivo*.

We have shown that *Kras*^{G12D}-initiated lung tumors are responsive to cisplatin treatment regardless of loss of *p53*. Tumors initially respond to cisplatin by sensing damage and undergoing cell cycle arrest and death, leading to a significant decrease in tumor burden. We provide genetic evidence that cisplatin efficacy is independent of *p53* loss - and does not require the cdk inhibitor, *p21*. Indeed, an intact *p53*-*p21* pathway was not required for cell cycle arrest, apoptosis, inhibition of tumor growth, or survival benefit in this model. Thus, the *Kras*^{G12D/+};*p53* null lung tumor model resembles human lung cancer in that *P53* loss confers a poor prognosis, but it does not necessarily mean that therapy will not be beneficial (Tsao et al. 2007). However, even though *p53* null tumors respond to cisplatin, our data suggest that there

are fundamental differences in that response compared to tumors with wild-type *p53*.

Specifically, *p53* null tumors exhibit reduced apoptosis, and, instead of regressing in response to cisplatin like *p53* wild-type tumors, their growth was simply impaired. Since most patients with *p53* alterations have point mutations in *p53*, it will be important to compare the effects of cisplatin in these mouse models, which we are currently investigating.

Our studies differ from a recent report that investigated the response of *Brca1*^{-/-};*p53*^{-/-} mouse mammary tumors to treatment with doxorubicin, docetaxel and cisplatin. Tumors in this model acquired resistance to doxorubicin and docetaxel, which was in part mediated by overexpression of P-glycoprotein. Notably, cisplatin is not implicated as a substrate of P-glycoprotein, and *Brca1*^{-/-};*p53*^{-/-} mammary tumors remained sensitive to cisplatin after multiple rounds of treatment (Rottenberg et al. 2007). We hypothesize that the discrepancy in these results could be a consequence of the genetic context of *BRCA* and *P53* deficiency since this combination of genetic alterations has been associated with cisplatin sensitivity (Bartz et al. 2006). Given that these mammary tumors are defective in homologous recombination (HR) and that tumors defective in HR are often sensitive to platinum-based compounds, these studies suggest that HR may be an important repair pathway contributing to cisplatin resistance. Indeed, restoration of wild-type *BRCA2* in *BRCA2*-mutated tumors has been shown to be an important mechanism of therapeutic resistance to cisplatin (Edwards et al. 2008; Sakai et al. 2008). Other genes involved in HR are also up-regulated in resistant tumors in our model (*ie. Rad51, Rad52, Rad9a*). Thus, further studies to test the involvement of HR in resistance in this model may be warranted.

Importantly, we found that cisplatin treatment of LSL-*Kras*^{G12D/+} mice selected for tumors with increased genomic instability that were histologically more advanced. Two possibilities could explain these results. First, tumor cells with abnormal karyotypes could be present prior to chemotherapy and are selected for by repeated doses of cisplatin. Alternatively, cisplatin

treatment itself may induce DNA damage that is not accurately repaired, leading to chromosomal aberrations. Untreated *Kras*^{G12D/+} mice can develop higher grade tumors with whole chromosomal changes at low frequency (Results and Sweet-Cordero, 2006); thus, it is possible that cisplatin enhances the survival of these cells which can eventually develop into more advanced tumors. In either case, our data suggest that in some instances treating with chemotherapy can have no survival benefit and can actually lead to more advanced tumors—in this case, with increased chromosomal changes, more advanced histology, and increased drug resistance. Given that many human cancers have pre-malignant stages of tumor progression, it will be important to investigate whether treating low-grade tumors with DNA-damaging agents can facilitate tumor progression. This knowledge will become more important as the technology to detect earlier stage disease advances. Whether treating high-grade genomically unstable tumors with DNA damaging agents can promote further progression, such as metastasis, is not well understood. This model could be used to investigate this possibility. Notably, the observation that cisplatin treatment can promote genomic instability may not have been uncovered using tumor cell line models that have already acquired high levels of genomic instability.

We demonstrate that prolonged cisplatin treatment leads to resistance in *Kras*^{G12D/-} initiated lung tumors. Acquired cisplatin resistance appears to be mediated by mechanisms that inhibit the ability of cisplatin to sustain adducts on DNA. This result is in agreement with early work pointing to a critical role of 1,2-intrastrand d(GpG) cross-links in mediating the anticancer activity of cisplatin (Lippard 1982). Our data strongly suggest that the most predominant mechanism of resistance in this model is rapid repair of platinum-DNA adducts based on the following observations: 1) Using AAS, both naïve and long-term cisplatin treated tumors had similar levels of platinum following cisplatin treatment, ruling out resistance mechanisms based on platinum entry/export; 2) Analysis of adduct kinetics by

immunofluorescence demonstrated that naïve and long-term cisplatin treated tumors had similar levels of adducts early but that long-term treated tumors exhibited an enhanced ability to remove adducts within 24 hours after a final dose of cisplatin; 3) Chk1 phosphorylation was induced in both naïve and cisplatin pretreated tumors, suggesting that tumors were encountering DNA damage. Notably, Chk2 phosphorylation was persistent in lung tumors that had been treated multiple times with cisplatin, but had not received cisplatin for several weeks. This suggests that high basal phosphorylation of Chk2 is associated with, and could be causally involved in, cisplatin resistance, and that damage signaling between naïve and long-term treated tumors is fundamentally different; 4) Cisplatin pretreated tumors induced expression of genes that have been shown to facilitate DNA repair and resistance (including *Apex1*, *Chek2*, *Rad51*, and *Rad52*, which were basally higher; and *Pidd*, *Cdkn1a (p21)*, *Ercc2*, and *Rad9*, which were induced to higher levels following treatment) (Furuta et al. 2002; Bartz et al. 2006; Wagner and Karnitz 2009; Wang et al. 2009). Together these data suggest that the predominant mechanism of acquired resistance in this model is enhanced damage repair.

While our data suggest that import/export and trans-lesional bypass are not frequent mechanisms of resistance, our data do not exclude the possibility that factors in addition to enhanced DNA damage repair may also contribute to resistance. For example, our gene expression analysis suggests changes in glutathione metabolism and immune response may alter drug response. Furthermore, we observe heterogeneity in adduct formation in resistant tumors in response to a final challenge of cisplatin. In particular, a subset of resistant tumors (~20%) have reduced adduct levels even at early time points (4 hrs) post-cisplatin (**Fig. 4H**). We hypothesize that detoxification of cisplatin by increased glutathione expression may be involved in reducing adduct formation in these tumors. This model will be useful for testing the role of other drug resistance mechanisms *in vivo*.

Our gene expression data suggested that *Pidd* induction correlated with and may play a role in chemotherapy resistance *in vivo*. We demonstrate for the first time that overexpression of PIDD in human lung tumor cells can facilitate cisplatin resistance. In the context of DNA damage, PIDD has previously been implicated in apoptosis, survival, NHEJ and the G2/M checkpoint. Further studies will be necessary to determine whether PIDD-induced chemoresistance is related to its effects on pro-survival NF- κ B signaling, the cell cycle, and/or DNA damage arrest and repair. Functional studies will be necessary to elucidate whether PIDD expression is sufficient to induce chemotherapy resistance *in vivo*, and whether inhibition of PIDD function could potentially have therapeutic applications by synergizing with chemotherapy treatment.

In summary, we have established and characterized a model system for studying response and acquired resistance to cisplatin in lung cancer. *In vivo* treatment with cisplatin in this model recapitulates important features that are seen in the treatment of human lung cancer. Specifically, tumors acquire resistance to cisplatin after prolonged treatment, and this is associated with cross-resistance to other platinum analogs. This model will be useful for comparing the efficacy of novel platinum compounds and combination therapies, as well as their impact on the emergence of drug resistance.

Materials and Methods

Mouse breeding and drug treatment

Mice were housed in an environmentally-controlled room according to the Committee of Animal Care. All mice were bred onto a 129svJae background. Mice were infected with 3×10^7 PFU of AdCre (University of Iowa) by nasal instillation as previously described (Jackson et al. 2001) and allowed to develop tumors for 12-16 wks prior to cisplatin treatment. Mice were given freshly prepared cisplatin in PBS at 7 mg/kg body weight intraperitoneally (ip) as indicated (Sigma, and prepared from K_2PtCl_4 supplied as a gift from Engelhard) or carboplatin (50 mg/kg body weight in saline, Sigma). For BrdU labeling experiments, BrdU (5-bromo-2'-deoxyuridine; Sigma) was injected ip (30 mg/kg) 24 hrs prior to sacrifice.

Immunohistochemistry

Antibodies and experimental conditions for immunohistochemistry are described in the Supplementary Material.

MicroCT

At indicated time points, mice were scanned for 15 min under isoflurane anesthesia using a small animal eXplore Locus microCT (GE Healthcare) at 45 μ m resolution, 80kV with 450 μ A current. Images were acquired and processed using GE eXplore software.

DNA copy number analysis

LSL-*Kras*^{G12D/+} mice were treated with long-term PBS or cisplatin (4 total doses over 2 months). After the fourth dose of cisplatin, mice were aged for approximately 4-8 wks in order to allow residual tumors to increase in volume. Mice were sacrificed and individual lung tumors were micro-dissected from the lung surface and snap frozen. DNA was isolated from individual

lung tumors and tail samples from the same animal using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN). Genomic DNA was phenol-chloroform extracted three times and submitted to Cold Spring Harbor Laboratories for ROMA. Briefly, DNA was digested using BglII enzyme, PCR-amplified using universal adaptors and primers, and labeled with fluorophores, Cy3 or Cy5 (Lakshmi et al. 2006). Tumor and tail samples were hybridized onto NimbleGen chips containing 85,000 mouse probes. Lung tumor DNA was compared to tail DNA from the same animal. Raw array data were processed and normalized according to Lakshmi et al, 2006. A moving-median algorithm based on a window of five data points was used to smoothen the normalized data to visualize copy number gains and losses (Kendall et al. 2007).

Gene expression analysis

Mice were sacrificed by cervical dislocation. Lungs were inflated with *RNAlater* (Ambion, Austin, TX, USA), removed, and placed in the same solution. Visible tumors were micro-dissected and immediately frozen on dry ice. Frozen tumor samples were thawed in Trizol solution (Invitrogen, Carlsbad, CA, USA) and then homogenized using first a Kontes pestle (Vineland, NJ, USA) and then a polytron homogenizer. RNA and DNA were isolated from Trizol using the manufacturer's instructions. RNA was further purified using a Qiagen (Valencia, CA, USA) column. RNA was reverse transcribed, linearly amplified and labeled with biotin prior to hybridization to oligonucleotide using an Ovation amplification kit (Nugen, San Carlos, CA). All samples were hybridized to Affymetrix 430A arrays (Santa Clara, CA, USA). For the gene expression trial time course by laser capture, tumors were isolated from *KrasLA2* mice (Johnson et al. 2001).

Microarray expression data were validated on at least 6 independent tumors per treatment group by real time RT-PCR. RNA was isolated by Trizol as described and 1 µg of

total RNA was converted to cDNA using iScript cDNA synthesis kit (Bio-Rad). Real time RT-PCR was performed using gene specific primers and Sybr Green Supermix (Bio-rad) in triplicate on an iCycler real time machine (Bio-rad). Analysis was performed using iCycler software and expression values were based on 10-fold serial dilutions of standards and normalized to *Actin* levels. Human and mouse primers are included in Supplemental methods.

Statistical analysis

All statistical analyses were performed using Graphpad Prism 5.0 (San Diego, CA). For column statistics to determine p values, unpaired two-tailed Student's t tests were performed. For survival curves, log-rank (Mantel-Cox) test was performed. For IC50 analysis, nonlinear fit -- log(agonist) vs. normalized response (variable slope) was performed.

Cell Culture

Human NSCLC lines (H460, SW1573, A549) were cultured according to ATCC. Cells were infected with retroviruses, MSCV-Puro or MSCV-Puro-PIDD (Tinel and Tschopp 2004) and selected with puromycin. For viability assays, cells were seeded in triplicate (6×10^3 /well) in opaque 96 wp and treated the next day with increasing doses of cisplatin, 0-200 μ M. After 48 hrs of treatment, cell viability was measured using CellTiter-Glo (Promega) on a luminometer. PIDD overexpression was validated by Western blotting using antibodies to Flag (M2 clone, Sigma), PIDD (Anto-1 clone, Alexis), and PARP1 (Cell Signaling Technologies, 46D11). For nuclear and cytoplasmic fractionations, lysates were prepared as described in Supplemental material.

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Figure Legends

Figure 1. Cisplatin induces cell cycle arrest and cell death in *Kras*^{G12D}-initiated lung tumors, independent of p53 activity. **A.** Number of BrdU positive cells per lung tumor area from LSL-*Kras*^{G12D/+} mice treated with a single dose (7 mg/kg body weight) of cisplatin and analyzed 0-120 hrs later. **B.** Number of CC3 positive cells per lung tumor area as in A. **C.** Number of BrdU positive cells per lung tumor area from LSL-*Kras*^{G12D/+} mice either heterozygous or homozygous for the *p53*^{fl/fl} allele treated as in A. **D.** Number of CC3 positive cells per lung tumor area from LSL-*Kras*^{G12D/+} mice either heterozygous or homozygous for the *p53*^{fl/fl} allele treated as in A. In A-D, number of tumors analyzed is shown for each bar. Error bars represent standard error of the mean (SEM). Significant changes compared to control are indicated by p * <0.04, ** < 0.006, or *** < 0.0001. **E-L.** PBS-treated lung tumors (E, G, I, K) or cisplatin-treated lung tumors (F, H, J, L) stained with (E,F) Pt-1,2-d(GpG) antibody (8 hr); (G,H) γ -H2AX antibody (24 hr); (I, J) anti-phospho Chk1 (Ser345) antibody (12 hr); or (K, L) anti-phospho Chk2 (Thr68) antibody (12 hr).

Figure 2. Cisplatin treatment significantly reduces lung tumor burden in *Kras*^{G12D}-initiated lung tumors regardless of p53 activity. **A.** Treatment regimens for groups 1-4 (G1-G4). Mice were infected with AdCre to permit expression of *Kras*^{G12D} at time 0 (grey arrow). Cisplatin was given at indicated time points in wks (black arrows) for each group. **B.** Tumor area/total lung area in control (G1) vs. treated (G3) LSL-*Kras*^{G12D/+} mice (white bars, *** p < 0.002) and in LSL-*Kras*^{G12D/+}; *p53*^{fl/fl} mice (black bars, *** p < 0.0001). **C-F.** Representative H&E stains at 2X magnification of (C, E) PBS-treated or (D, F) cisplatin-treated lungs from LSL-*Kras*^{G12D/+} mice (C, D) or LSL-*Kras*^{G12D/+}; *p53*^{fl/fl} mice (E, F). **G-H.** Kaplan-Meier survival curves of (G) LSL-*Kras*^{G12D/+} mice and (H) LSL-*Kras*^{G12D/+}; *p53*^{fl/fl} mice treated with four doses of cisplatin (red) or PBS (black). Black arrows indicate cisplatin treatments at X number of days post AdCre

infection. For H, cisplatin significantly prolongs survival ($p < 0.002$). **I.** Number of BrdU positive cells per lung tumor area in LSL-*Kras*^{G12D/+} mice with or without a final 72 hr dose of cisplatin (** $p < 0.009$). Error bars represent SEM.

Figure 3. *In vivo* microCT imaging reveals lung tumor regression and stasis in response to cisplatin in LSL-*Kras*^{G12D/+} mice, and decelerated growth in LSL-*Kras*^{G12D/+};p53^{fl/fl} mice. **A.** Tumor volume dynamics of individual cisplatin-treated tumors in LSL-*Kras*^{G12D/+} mice. Black arrows on X-axis indicate cisplatin treatments. Red lines indicate tumors that stopped responding to treatment after 3-4 doses. X-axis indicates days following the first pre-treatment microCT scan which occurred 14 wks post-AdCre infection. **B.** Log2-normalized fold change in tumor volume of individual tumors in PBS (white bars) and cisplatin-treated (black bars) mice. Tumor volumes were quantified before and after dose 1 and 2 (Dose 1-2), before and after dose 3 and 4 (Dose 3-4), and before and after 1 final dose (Final). **C.** Representative microCT lung reconstructions before and after 2 doses of PBS (I & II) or cisplatin (III & IV) with individual lung tumors pseudo-colored. **D.** Tumor volume dynamics of individual cisplatin-treated tumors in response to cisplatin in LSL-*Kras*^{G12D/+};p53^{fl/fl} mice. Black arrows on X-axis indicate cisplatin treatments. **E.** Total lung tumor volume in LSL-*Kras*^{G12D/+};p53^{fl/fl} mice (n= 2 mice per group) treated with four doses of PBS (solid lines with circles) or cisplatin (dashed lines). Arrows on X-axis (days following AdCre infection) indicate one dose of PBS or cisplatin.

Figure 4. Long-term cisplatin-treated lung tumors in LSL-*Kras*^{G12D/+} mice exhibit enhanced adduct repair in response to a final dose of cisplatin. **A.** Representative sensitive lung tumor section (G2) stained with Pt-1,2-d(GpG) from long-term PBS-treated mice given a final 24 hr dose of cisplatin. **B.** Representative resistant tumor section (G4) from long-term cisplatin-treated mice treated as in A. **C.** Number of Pt-1,2-d(GpG)-positive cells per lung tumor area in

long-term PBS (white bar) or cisplatin-treated mice (black bar) given a final dose of cisplatin and sacrificed 24 hrs later ($***p < 0.0001$). **D.** Representative sensitive tumor section (G2) stained with γ -H2AX from long-term PBS-treated mice given a final 24 hr dose of cisplatin. **E.** Representative resistant tumor (G4) from long-term cisplatin-treated mice treated as in **D.** **F.** Number of γ -H2AX positive cells per lung tumor area in long-term PBS (white bar) or cisplatin-treated mice (black bar) given a final dose of cisplatin and sacrificed 24 hrs later ($***p < 0.0001$). Error bars represent SEM. **G-I.** Representative immunofluorescent images of lung tumor sections stained for nuclei (DAPI) or Pt-1,2-d(GpG) (Cy3), and an overlay of these images (Overlay) in mice treated with long-term PBS (LT PBS) or four doses of cisplatin (LT Cis) and given a final dose of cisplatin and analyzed after G) 0, H) 4, or I) 24 hrs. Top panels are 10X magnification and bottom panels are higher magnification zooms. Note that adducts persist in normal parts of the lung even after multiple weeks in LT Cis, 0 hr (G). In panel H (LT Cis, 4 hrs, DAPI), two tumors are separated by a dotted white line. Approximately 20% of tumors in LT Cis mice had reduced adduct levels as early as 4 hrs after a final dose of cisplatin (left tumor) whereas the majority of tumors had similar levels of adducts (right tumor) at this time point.

Figure 5. DNA copy number profiling by ROMA reveals cisplatin treatment enhances the percentage of lung tumors from LSL-*Kras*^{G12D/+} mice with whole chromosomal gains and losses. **A.** Representative genomic profile of lung tumors from PBS-treated mice. Nine of 11 PBS-treated tumors did not exhibit genomic changes. **B-H.** Representative genomic profiles of cisplatin-treated tumors with significant whole chromosomal DNA copy number changes. 19 of 23 cisplatin-treated tumors harbored whole chromosomal changes. X-axis indicates chromosomal position from chr. 1 to 19, and XY chromosomes. Y-axis indicates copy number. **I-J.** Representative H&E stained tumor section from PBS-treated mice with low-grade tumor

histology (20X, I) and a higher magnification panel from the same tumor (40X, J). **K-L.**

Representative H&E stained tumor section from cisplatin-treated mice with high-grade tumor histology (20X, K) and a higher magnification panel from the same tumor (40X, L). Note the larger nuclei, more diffuse nuclear staining, and higher nuclear to cytoplasmic ratio in K-L compared to I-J.

Figure 6. Genes associated with DNA damage and repair are upregulated in cisplatin-resistant lung tumors *in vivo*. **A.** Enrichment plot of the DNA damage gene set identified by GSEA and corresponding heat map for G2 vs. G4. Expression level is represented as a gradient from high (red) to low (blue). **B.** Expression of indicated genes in long-term PBS (LT PBS) versus long-term cisplatin-treated tumors (4 doses, LT Cis). **C.** Expression of indicated genes in LT PBS or LT Cis tumors treated with a final 72 hr dose of cisplatin (LT PBS + 72hr Cis or LT Cis + 72hr Cis). All genes were analyzed in triplicate by real time RT-PCR on 6 independent tumors per treatment group. Expression levels are normalized to *Beta-actin*. ** $p < 0.009$ and * $p < 0.05$. Error bars represent SEM.

Figure 7. Overexpression of PIDD confers resistance to cisplatin in human NSCLC cell lines. **A.** Expression levels of *Pidd* mRNA in LSL-*Kras*^{G12D/+} lung tumors treated with PBS or 4 total doses of cisplatin, with or without a final 8 hr dose of cisplatin (n = 6 tumors per group). $p < 0.01$. Error bars represent SEM. **B.** Expression levels of *PIDD* mRNA in human NSCLC lines treated with increasing doses of cisplatin and harvested 24 hrs following treatment. Y-axis is fold change relative to PBS-treated cells. Expression levels are normalized to *ACTIN*. **C.** PIDD overexpression in human NSCLC lines by Western blot (IB) for Flag, and for Parp to confirm purity of nuclear/cytoplasmic fractions. Upon longer exposure, full length PIDD is apparent in the cytoplasm, and both PIDD cleavage products are also present in the nucleus (data not

shown). **D.** IC₅₀ values for cisplatin treatment in each cell line with MSCV Vector or MSCV-PIDD expression from three independent experiments performed in triplicate. **E.**

Representative survival plots for indicated cell lines expressing MSCV Vector or MSCV-Pidd treated with 0-200 μ M cisplatin (X-axis) and analyzed 48 hrs later using CellTiter-Glo cell viability assay. Y-axis represents percent of viable cells normalized to PBS-treated control. Error bars represent standard deviation.