Functional Genomics Reveals Diverse Cellular Processes that Modulate Tumor Cell Response to Oxaliplatin

Kelly A. Harradine¹, Michelle Kassner², Donald Chow², Meraj Aziz², Daniel D. Von Hoff², Joffre B. Baker¹, Hongwei Yin², and Robert J. Pelham¹,*

¹Genomic Health, Inc., Redwood City, CA 94002
²Clinical Translational Research Division, Translational Genomics Research Institute, Scottsdale, AZ 85259

*Corresponding Author: Robert J. Pelham, Genomic Health, Inc., 301 Penobscot Drive, Redwood City, CA 94002. Phone: 650-569-2884; Fax: 650-556-1132; Email: rpelham@genomichealth.com

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Running title: Cellular processes regulating response to oxaliplatin
Abstract

Oxaliplatin is widely used to treat colorectal cancer, as both adjuvant therapy for resected disease and palliative treatment of metastatic disease. However, a significant number of patients experience serious side effects from oxaliplatin treatment, including prolonged neurotoxicity, creating an urgent need for biomarkers of oxaliplatin response or resistance to direct therapy to those most likely to benefit. As a first step to improve selection of patients for oxaliplatin-based chemotherapy, we have conducted an in vitro cell-based small interfering RNA (siRNA) screen of 500 genes aimed at identifying genes whose loss of expression alters tumor cell response to oxaliplatin. The siRNA screen identified twenty-seven genes, that when silenced, significantly altered colon tumor cell line sensitivity to oxaliplatin. Silencing of a group of putative resistance genes increased the extent of oxaliplatin-mediated DNA damage and inhibited cell cycle progression in oxaliplatin-treated cells. The activity of several signaling nodes, including AKT1 and MEK1, was also altered. We used cDNA transfection to over-express two genes (LTBR and TMEM30A) that were identified in the siRNA screen as mediators of oxaliplatin sensitivity. In both instances, over-expression conferred resistance to oxaliplatin. In summary, this study identifies numerous putative predictive biomarkers of response to oxaliplatin which should be studied further in patient specimens for potential clinical application. Diverse gene networks appear to influence tumor survival in response to DNA damage by oxaliplatin. Finally, those genes whose loss of expression (or function) is related to oxaliplatin sensitivity may be promising therapeutic targets to increase patient response to oxaliplatin.
Introduction

Oxaliplatin is a third-generation platinum-based chemotherapeutic agent that has significant activity in colorectal carcinoma (CRC). Adjuvant therapy with oxaliplatin, combined with fluoropyrimidine-based (5-FU) chemotherapy, results in significant increases in disease-free survival rates in patients with stage II/III colon cancer [1]. In the metastatic setting, combination therapy with 5-FU and oxaliplatin is the most commonly used front-line regimen, with superior response rates and longer survival than 5-FU alone [2-3]. However, it is apparent that not all patients benefit from oxaliplatin treatment, and in the face of significant side-effects associated with oxaliplatin, most notably prolonged neurotoxicity, there is a great need for clinical tools to guide use of oxaliplatin in those patients who are most likely to derive benefit.

Oxaliplatin induces cytotoxicity through the formation of platinum–DNA adducts, which in turn, activate multiple signaling pathways[4]. Alterations in drug efflux and uptake, DNA repair and inactivation of the apoptosis pathways have been hypothesized to promote resistance to platinum agents such as carboplatin and cisplatin[5-6]. None of these putative markers of oxaliplatin sensitivity and resistance have been clinically validated, and at present, there are no markers established in clinical use for selecting CRC patients for oxaliplatin therapy.

The current clinical practice used for making CRC treatment decisions is determined by clinical and pathological staging. However, these prognostic tools do not predict drug response in an individual patient. Recent insights into the genomics of
cancers have enabled development of diagnostic tests that inform clinical decisions for cancer patients [7-10]. To further advance the personalization of CRC treatment, there is a need for a greater understanding of the genetic alterations in CRC tumors which are associated with patient sensitivity or resistance to oxaliplatin. One approach to identifying genes responsible for drug sensitivity or resistance is the use of in vitro loss-of-function (LOF) genetic screens via RNA-mediated interference (RNAi). Genes that modulate oxaliplatin sensitivity in vitro can be taken forward for testing in clinical studies, and ultimately may serve to be clinically useful predictive biomarkers to guide patient selection for therapy with oxaliplatin.

We have performed a small interfering RNA screen (siRNA) screen in human CRC cell lines to identify genes whose LOF modulates in vitro tumor cell response to oxaliplatin. Our primary screen targeted 500 genes involved in DNA repair, drug transport, metabolism, apoptosis and regulation of the cell cycle, and utilized four unique siRNA duplexes over seven different oxaliplatin concentrations per gene. Here we report twenty-seven genes whose loss of expression can significantly alter the response (through either increased sensitivity or increased resistance) of colon tumor cell lines to oxaliplatin. These results provide a foundation for future work in clinical samples from CRC patients treated with oxaliplatin and also reveal insights into the heterogeneity of cellular processes regulating tumor cell response to DNA damage.
Materials and Methods

Cell Lines

Colon cancer cell lines HCT 116 (ATCC# CCL-247) and SW480 (ATCC# CCL-228) were obtained from the American Type Culture Collection, and were maintained in McCoy’s 5A medium supplemented with 10% Fetal Bovine Serum, 1.5mM L-glutamine, and 1% Antibiotic-Antimycotic (Invitrogen).

siRNA Screening and Drug Treatments

Four siRNA sequences for each targeted gene were picked from the Whole Human Genome siRNA Library (Qiagen) to create six (6) custom 384-well assay plates. All assay plates included negative control siRNAs (Non-Silencing, All-Star Non-Silencing, and GFP), and two positive control siRNAs for transfection (UBBs1 and All-Star Cell Death Control), all of which were purchased from Qiagen. Selected siRNAs were printed individually into white solid 384-well plates (1μl of 0.667μM siRNA per well for a total of 9ng siRNA) using a Biomek FX (Beckman Coulter). Lipofectamine 2000 (Invitrogen) was diluted in serum-free McCoy’s 5A media and 20μl was transferred into each well of the 384-well plate containing siRNAs (final ratio of 7.4nl lipid per ng siRNA). After an incubation period of 30 minutes at room temperature to allow the siRNA and lipid to form complexes, 20μl of HCT 116 cells (2.5x10^4 cells/ml) in antibiotic-free
McCoy’s 5A media were added into each well. Transfected cells were incubated for 24 hours prior to the addition of 10μl per well of different concentrations of oxaliplatin (35.0, 3.75, 3.0, 2.0, and 1.5 μM) and vehicle control (DMSO) for a total assay volume for a total assay volume of 50μl. Oxaliplatin was obtained from Sigma. Cell viability was measured 72 h post drug treatment using the CellTiter-Glo assay (Promega), measured on an Analyst GT Multimode reader (Molecular Devices). A replicate of the screen was also performed, resulting in total of 56 data points per gene. Cell viability data was normalized to the median value of negative control siRNAs, and IC50 values were calculated using Prism 5.0 (GraphPad).

**Hit Identification and Statistical Analysis**

The effect of siRNA treatment on the IC50 of oxaliplatin was expressed as the log2 fold-shift of the median IC50 of siRNA-treated cells relative to the median IC50 of non-silencing siRNA control-treated cells. Hits were identified as those with a median IC50 shift ≥ median IC50 ±3 median absolute deviations (median±3MAD) [11-12]. To then assign statistical significance to siRNA hits identified from the siRNA screen, we modeled the collective activities of the 4 individual siRNAs used for each gene using redundant siRNA activity (RSA) analysis[13]. Briefly, the normalized, log2 transformed IC50 shifts of all siRNAs were rank ordered, and the rank distribution of all siRNAs targeting the same gene was examined and a P value was calculated based on an iterative hypergeometric distribution formula[13]. siRNAs with P-values <0.05 were
considered as significant. Subsequently, only genes with a median IC$_{50}$ shift $\geq$ median IC$_{50} \pm 3$ MAD and an RSA $P$ value <0.05 were considered robust hits and analyzed further. All other tests of significance were two-sided, and $P$ values <0.05 were considered significant.

**Validation siRNA Knockdown**

For validation of siRNA hits, ON-TARGETplus siRNAs (Thermo Scientific), containing pools of 4 siRNAs per gene, were utilized (Supplementary Table S3). Seventy µl of HCT 116 or SW480 cells (1.0x10$^5$ cells/ml) were plated in black, clear-bottomed 96-well plates in antibiotic-free McCoy’s 5A medium and allowed to adhere overnight. Cells were then transfected with 25nM siRNA using DharmaFECT transfection reagent (Thermo Scientific). Following a 4 hr incubation, 10µl per well of an 11-point, 2-fold serial dilution of oxaliplatin (50µM maximum concentration) was then added, for a total assay volume of 100µl. Assays were performed in triplicate, with ON-TARGETplus Non-Targeting siRNA (Thermo Scientific) as a negative control, with biological replicates. Cell viability was measured 72 h later using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega), and IC$_{50}$ values calculated using Prism 5.0 (GraphPad). siRNA knockdown after 72 hr was validated by qRT-PCR using the Roche LightCycler480 (Supplementary Fig. S3A).
Overexpression Experiments

Full-length LTBR or TMEM30A ORFs were cloned into pCMV-XL4 or pCMV-XL5, respectively (Origene) and validated by sequencing. Transfection was performed using Turbofectin 8.0 (Origene) in 96-well format as per manufacturer’s instructions using 100ng of cDNA per well. Following a 4 hr incubation, 10µl per well of an 11-point, 2-fold serial dilution of oxaliplatin (50µM maximum concentration) was then added. Assays were performed in triplicate, using the empty pCMV-XL4 vector or empty pCMV-XL5 vector as negative control, with biological replicates. Cell viability was measured 0, 24, 48, and 72 hours later (Fig. 2C and Supplementary Fig. S3C) using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega), and IC₅₀ values calculated using Prism 5.0 (GraphPad). Transcript levels following cDNA overexpression was measured by qRT-PCR at 0, 24, 48, and 72 hours using the Roche LightCycler480, with transcript levels normalized to the housekeeping gene, β-actin, and results expressed as normalized expression relative to empty pCMV-XL4 or empty pCMV-XL5 vector-transfected cells ($2^{\Delta\Delta C_{p}}$) (Supplementary Fig. S3B).

DNA Damage, Phosphorylation and Caspase 3-Cleavage Assays

DNA damage was assessed by quantification of Apurinic/apyrimidinic (AP) sites (BioVision) following manufacturer’s instruction.

Phosphorylation status of AKT1 (Ser473), MEK1 (Ser217/221), p38 MAPK (Thr180/Tyr182), STAT3 (Tyr705), and NFκB p65 (Ser536), was determined using the
PathScan Signaling Nodes Multi-Target Sandwich ELISA (Cell Signaling Technology) as per manufacturer's instructions. The phosphorylation status of p53 (Ser15), Bad (Ser112), PARP (Asp214), and cleavage status of Caspase-3 was determined using the PathScan Apoptosis Multi-Target Sandwich ELISA (Cell Signaling Technology), following manufacturer’s instructions. Raw signal intensity was normalized to either total Akt or Bad protein levels. Assays were performed in duplicate, and the log$_2$ fold-change (OD$_{450}$ siRNA-treated cells/OD$_{450}$ non-silencing siRNA-treated cells), following median normalization, was converted into a heatmap using Java TreeView.

**Cell Cycle Analysis**

Transfections were performed as described above in the validation experiments, using six-well plates (5x10$^5$ cells/well). Cells were collected by gentle trypsinization, followed by centrifugation at 500 rpm for 5 min, fixed with 70% ethanol at -20°C, washed with PBS, and re-suspended in 0.5 ml of PBS containing propidium iodide (10 µg/ml). After a final incubation at 37°C for 30 min, cells were analyzed by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson). Data were analyzed using FlowJo software (Tree Star).

**Functional annotation of siRNA Hits**
Functional annotation of gene hits and networks of interacting proteins was determined through the use of Ingenuity Pathways Analysis (Ingenuity Systems, www.ingenuity.com).
Results and Discussion

A siRNA screen identifies genes that control tumor cell sensitivity to oxaliplatin.

We screened a custom siRNA library targeting 500 genes with putative roles in DNA damage repair, apoptosis, regulation of the cell cycle, drug metabolism and transport, using the colorectal cancer tumor cell line, HCT 116 (Supplementary Table S1). The siRNA library contained four siRNAs targeting each gene, with each siRNA transfected individually. The screen was performed in duplicate, with a non-silencing siRNA negative control. siRNAs were used at 17 nM to reduce off-target effects. Twenty-four hours after transfection, 5 different concentrations of oxaliplatin (35.0, 3.75, 3.0, 2.0, and 1.5 μM) and vehicle control (DMSO) were added and cell viability was measured seventy-two hours after addition of drug. The mean Z’ factor for the screen was 0.78, suggesting that our assay had a robust signal-to-noise ratio (Supplementary Fig. S1B).

Two stringent criteria were used to limit the discovery of false positive genes. First, we identified all genes whose silencing shifted the IC₅₀ of oxaliplatin ≥±3 median absolute deviations from the median IC₅₀ of oxaliplatin in control cells, an approach (median ± k MAD) that has been shown to be robust to outliers and effective in controlling the false positive rate in siRNA screens[11-12]. Second, we modeled the collective activities of the four individual siRNAs used for each gene using the redundant siRNA activity (RSA) analysis[13]. siRNAs with P-values <0.05 were
considered significant (Supplementary Table S2). Twenty-seven high confidence hits that satisfied both of these criteria were identified (Fig. 1; Table 1) and analyzed further.

**Validation of selected hits from siRNA screen.**

Based on functional categorization, we selected twelve hits from our primary screen and tested their validity with additional siRNAs. Using independently targeted pools of four siRNAs (ON-TARGETplus siRNAs) to reduce off-target effects and increase specificity of silencing, we categorized re-tested genes as validated if the resulting IC$_{50}$ of oxaliplatin was shifted >50% from the IC$_{50}$ of oxaliplatin in cells treated with non-silencing siRNAs. All twelve of the hits selected for validation exceeded this 50% threshold (Fig. 2A).

We then examined the impact of nine of these genes in the oxaliplatin-resistant SW480 colorectal tumor cell line[14]. Silencing of each of these nine genes (CUL4B, LTBR, MBD4, MCM3, NHEJ1, PRDX4, SFHM1, and TMEM30A), all of which conferred increased sensitivity to the HCT 116 tumor cell line, also increased sensitivity of the SW480 tumor cell line to oxaliplatin (Fig. 2B).

To independently test whether the expression of the identified genes relates to tumor cell sensitivity to oxaliplatin, we assayed the effects of over-expression of two genes, LTBR or TMEM30A, on tumor cell response to oxaliplatin. Transient over-expression of full-length LTBR or TMEM30A (validated by RT-qPCR; Supplementary Fig. S3B) increased the IC$_{50}$ of oxaliplatin >2-fold (Fig. 2C), significantly increasing the
resistance of the HCT 116 cell line to oxaliplatin, as predicted by the results with siRNA silencing.

Alterations in DNA damage and activity of signaling nodes in tumor cells with increased or decreased sensitivity to oxaliplatin.

To begin to address the cellular mechanisms responsible for modulated cell sensitivity to oxaliplatin, we asked if siRNA silencing of the identified screening hits altered the amount of DNA damage in tumor cells treated with oxaliplatin. Platinium-DNA adducts formed upon exposure to platinium-based chemotherapies are thought to be primarily removed through the nucleotide excision repair pathway (NER). Using an \textit{in vitro} assay that measures the number of apurinic/apyrimidinic sites on the DNA of oxaliplatin-treated cells, we found that siRNA-silencing of CUL4B and NHEJ1, both with known roles in the repair of DNA damage via the NER\[15-16\], significantly increased the amount of DNA damage relative to oxaliplatin-treated control cells (Fig. 3A). siRNA silencing of two other hits with known roles in DNA replication and repair, MBD4 and MCM3\[17-18\], also increased the amount of DNA damage accumulated upon treatment with oxaliplatin (Fig. 3A), although the increase did not reach statistical significance ($P < 0.05$).

Silencing of the identified genes that modulate tumor cell sensitivity to oxaliplatin resulted in altered phosphorylation of several pathway signaling nodes, including mitogen-activated protein kinase cascade, JAK/STAT, and NFκB pathways.
whose activity may be contributing significantly to changes in cell proliferation[19-20].

We performed quantitative analyses to determine the activity of p-Akt1, p-MEK1, p-p38 MAPK, p-Stat3, and p-NFkB p65 (Fig. 3B). Hierarchical clustering of phosphorylation levels (relative to control cells) revealed diverse and non-overlapping clusters of pathway signaling following siRNA silencing of our hits, with the noticeable exception of pNFkB p65, suggesting that multiple distinct cellular mechanisms for each hit are likely responsible for altered cell survival. Similarly, when we probed the activities of several gene regulators of apoptosis, including of p-p53, p-Bad, cleaved caspase 3 and cleaved PARP, distinct clusters of pathway activity were observed, suggesting that, upon siRNA silencing of our hits, both caspase-dependent and caspase-independent pathways regulating changes in apoptosis and/or cell death are modulated in response to DNA damage upon treatment with oxaliplatin (Fig 3C). These results again suggest that a diversity of alterations in cell signaling are responsible for increased tumor cell sensitivity to oxaliplatin, including the modulation of apoptosis and necrosis[5].

**Cell cycle alterations in tumor cells with altered sensitivities to oxaliplatin.**

We also evaluated the effects that siRNA silencing of genes modulating oxaliplatin sensitivity would have on the cell cycle. Cell cycle analysis indicates that upon treatment with oxaliplatin, all siRNA-treated cells, including those with increased siRNA-mediated resistance to oxaliplatin (CDKN1A and p53) exhibited a significant decrease in the percentage of cells in G1 with a concomitant increase in the percentage
of cells in G2/M as compared to control cells (Fig 4). This is consistent with previous observations that G2/M arrest facilitates platinium-mediated cell death [21-22], although it is of note that there were no gross differences between oxaliplatin-sensitive and – resistant cells.

**Network of biological processes identified as potential modulators of tumor cell sensitivity to oxaliplatin.**

To further understand the functional relationships between those genes whose loss of expression altered the sensitivity of tumor cells to oxaliplatin, we performed an extensive bioinformatic analysis using the genes identified the initial screen (Table 1) to identify relevant networks of interacting proteins (Fig. 5).

The largest interaction network is heavily populated with genes that have roles in DNA replication, recombination, repair and cell cycle progression (Fig. 5). It is, however, of interest that this interaction network contains nodes previously not associated with response to oxaliplatin, which link it to proteins from the canonical (BCL10, TRAF6) and non-canonical NFκB pathways (LTBR, TRAF3, PRDX4)[23], as well as the estrogen signaling (ESR1, MPG, MDB2), apoptosis (BCL2, BCL2L10, BCL10, DFFA, CASP3, and BIRC2), and BRCA1/2-signaling pathways (BRCA1, BRCA2, SHFM1, and BRIP1). The diversity of interacting proteins in this network suggests that the response of tumor cells to DNA damage by oxaliplatin, as well as
intrinsic and/or acquired resistance to this drug may be influenced by a larger network of biological processes than previously appreciated[24].

**Perspective**

This study is an attempt to identify putative biomarkers for response to oxaliplatin, as well as potential therapeutic targets for enhancing tumor cell response to oxaliplatin. We have identified twenty-seven genes, as well as associated cellular networks, which may serve to modulate tumor cell response to oxaliplatin.

Of the genes identified in our screen whose loss of expression results in altered tumor cell sensitivity to oxaliplatin, only three have been previously shown to be associated with tumor cell sensitivity to other platinum-containing chemotherapies, including cisplatin. In our study, the HCT 116 TP53+/+ colon cancer cell line becomes resistant to oxaliplatin upon siRNA-mediated loss of TP53 expression (Fig. 1 and 2A), while previous work has shown that a HCT 116 TP53−/− clone is resistant to oxaliplatin, with a resulting G2/M arrest[25]. That our data provides similar conclusions, using an orthogonal approach (e.g. siRNA knockdown), regarding the role of TP53 in increased tumor cell resistance to oxaliplatin and the resulting cell cycle arrest, only serves to validate our experimental design and screening endpoint, as well as the biological role of TP53 in modulating response to oxaliplatin.

Bartz et al also previously demonstrated, using a genome-wide siRNA screen in a TP53− HeLa cell line, that the loss of SHFM1 or BRIP1 expression enhanced cisplatin
cytotoxicity[26]. Our study demonstrated that the loss of either SHFM1 or BRIP1 expression increased oxaliplatin cytotoxicity in a siRNA screen using the TP53⁺ HCT 116 cell line. It is interesting to note that despite the similar mechanism of action for both cisplatin and oxaliplatin[4], only these two genes (SHFM1 and BRIP1) overlapped between our study and Bartz et al (Supplementary Fig. S2). Likely explanations for these differences include both the underlying mutational status of TP53 in the cell lines used, as well as the more fundamental genomic variation between HeLa and HCT 116 tumor cell lines (derived from a human cervical adenocarcinoma and colorectal carcinoma, respectively) resulting in a differential sensitization upon the loss of additional genes in the presence of DNA-damaging agents such as cisplatin and oxaliplatin[26]. Indeed, eleven genes that were previously identified as enhancers of cisplatin cytotoxicity in Bartz et al [26] were among the 500 genes assayed in our primary siRNA screen (Supplementary Table S1) yet were not found to alter the cytotoxicity of oxaliplatin (Supplementary Table S2). Furthermore, Bartz et al demonstrated that the loss of BRCA1 and BRCA2 selectively enhanced the cytotoxicity of cisplatin in TP53-deficient cells as compared to isogenic wild-type TP53 cells[26]. These observed differences in the modulation of cytotoxicity to oxaliplatin and cisplatin underscore difficulties in identifying generalized predictive biomarkers for drug response, as well as the need to utilize tumors and cell lines of diverse genetic lineages for the identification of robust biomarkers for predicting drug response.

Many genes that regulate diverse aspects of DNA repair, recombination, and replication were represented among our hits, including components of the NER,
nonhomologous end-joining pathway, and mismatch repair (MMR) pathways. The NER portion of this module includes CUL4B, an E3 ubiquitin ligase that binds to damaged DNA and ubiquitinates Histone H2A, modifying the structure of chromatin, and facilitating NER [15, 27]. NHEJ1 encodes a DNA repair protein that is essential for the nonhomologous end-joining pathway, which preferentially mediates repair of double-stranded breaks in DNA[16, 28].

The defects in the mismatch repair pathway (MMR) has long been appreciated as a possible mechanism of resistance to chemotherapy by directly impairing the ability of the tumor cell to detect DNA damage, faithfully replicate DNA, and ultimately the activation of apoptosis[29]. Our data identifies the MMR pathway genes MSH4, MBD2, and MBD4 as drivers of oxaliplatin resistance [30].

Alterations in DNA replication due to defects in the recruitment of minichromosome maintenance proteins (MCMs) onto chromatin during the assembly of the pre-replication complex at origins of replication [18, 31-32], while not previously implicated in drug resistance, may cause tumor cells to develop resistance to chemotherapies. Indeed, MCM3, MCM4, and MCM6, all components of the MCM complex, were identified as modulators of tumor cell sensitivity to oxaliplatin.

The mechanisms through which tumor cells may acquire altered sensitivity to oxaliplatin have been thought to be linked to the DNA-damaging activity of oxaliplatin (e.g. NER, MMR)[4]. However, in addition to DNA replication, several gene hits from our siRNA screen have functional annotations not previously implicated in the
modulation of tumor cell sensitivity to oxaliplatin—several of which may have heretofore unappreciated roles in DNA damage response. LTBR, lymphotoxin-beta receptor, is a member of the tumor necrosis factor receptor (TNFR) superfamily, which stimulates the non-canonical nuclear factor kappaB (NF-kappaB) signaling and cell death via the recruitment of TNFR-associated factor 3 (TRAF3) to the LTBR cytoplasmic domain[19, 33], and plays an important role in the pathobiology of hepatocellular carcinoma[34]. PRDX4, an additional hit from our screen, is thought to activate NF-kappaB by modulating IkB-alpha phosphorylation [35].

It is noteworthy that siRNA knockdown of the gene ERCC1 did not alter tumor cell sensitivity to oxaliplatin. Multiple prior studies have demonstrated that in vitro and in vivo, mRNA levels of ERCC1 are modest predictors of tumor cell sensitivity and patient response to cisplatin[36-39]. However, the utility of ERCC1 in predicting patient response to oxaliplatin is limited to combination 5-FU/oxaliplatin chemotherapies in the clinical setting[40-41]. Our data, while not specifically identifying ERCC1 as a candidate hit, did identify numerous genes involved in the repair of DNA damage, including members of the NER (of which ERCC1 is a key molecule) and DNA mismatch repair pathways (Table 1). It is possible that ERCC1 mRNA levels may predict resistance to oxaliplatin only in the clinical setting of combination therapy with 5-FU[42]. In our screen, we treated cells with oxaliplatin alone in order to reduce the identification of false positive hits and to simplify the screen design. It is also possible that our assay endpoint, by design, resulted in a particularly stringent cutoff for hit selection that yielded only very robust modulators of tumor cell sensitivity to oxaliplatin.
Our results indicate that diverse, and possibly, redundant processes may influence cell proliferation and survival upon treatment with oxaliplatin. That multiple processes may function redundantly to drive cell resistance to DNA damaging agents might make it difficult to find robust biomarkers to predict patient response to oxaliplatin. Finally, protein products of the identified genes suggest therapeutic strategies to sensitize tumor cells to oxaliplatin and other DNA-damaging chemotherapeutic agents.
Figure Legends

Figure 1. Identification and functional classification of genes modulating HCT 116 tumor cell sensitivity to oxaliplatin. Results of 500-gene siRNA screen for genes that modulate sensitivity to oxaliplatin. The median log$_2$ fold shift in the IC$_{50}$ of oxaliplatin following siRNA-treatment is plotted for each gene in the screen. Genes with a median IC$_{50}$ shift $\geq$ median IC$_{50}$ +3 MAD and an RSA $P$ value $<$0.05 are indicated in red (increased resistance to oxaliplatin) or blue (increased sensitivity to oxaliplatin).

Figure 2. Validation of genes identified in a siRNA screen for genes regulating sensitivity or resistance to oxaliplatin. A, siRNA-silencing of gene hits identified in the primary screen was repeated in the HCT 116 tumor cell line with ON-TARGETplus siRNAs, each containing pools of 4 siRNAs per target gene. Cell viability was assayed and IC$_{50}$ of oxaliplatin was calculated 72 h after siRNA transfection and addition of an 11-point, 2-fold serial dilution of oxaliplatin (50μM maximal). B, siRNA-silencing of selected hits was performed using the SW480 tumor cell line as described in 2A. C, Effect of cDNA overexpression of full length LTBR or TMEM30A on the IC$_{50}$ of oxaliplatin. The effect of siRNA-silencing or cDNA overexpression on the IC$_{50}$ of oxaliplatin was expressed as the log$_2$ fold-shift of the mean IC$_{50}$ of siRNA-treated (or cDNA-overexpressing) cells relative to the mean IC$_{50}$ of non-silencing siRNA control-treated (or vector-alone) cells. Cell viability was assayed and IC$_{50}$ of oxaliplatin was
calculated 72 h after cDNA transfection and addition of an 11-point, 2-fold serial dilution of oxaliplatin (50μM maximal). Data represent mean ± SEM (n=3).

**Figure 3.** Functional analyses of genes modulating sensitivity to oxaliplatin. 

A, Increased levels of DNA damage, as determined by quantification of apurinic/apyrimidinic sites (as % of non-silencing siRNA-treated cells), in CUL4B- and NHEJ1-silenced HCT 116 tumor cells. Cells were transfected, treated with 1.56μM oxaliplatin, and DNA damage was measured after 72 hr. Dashed line indicates 100% of control. Data represent mean ± SEM (n=3);*, P<0.05. 

B, Hierarchical clustering of relative activities of pathway signaling nodes in cells with altered sensitivity to oxaliplatin. The heat map indicates the normalized log2 ratio of the phosphorylation levels of AKT1 (Ser473), MEK1 (Ser217/221), p38 MAPK (Thr180/Tyr182), STAT3 (Tyr705), and NFκB p65 (Ser536) in test siRNA-treated cells (+1.56μM oxaliplatin) relative to non-silencing siRNA-treated cells (+1.56μM oxaliplatin), as assessed by quantitative analysis using a sandwich ELISA with epitope-specific antibodies 72 hr post transfection and addition of oxaliplatin. 

C, Hierarchical clustering of relative activities of key apoptotic regulators, in cells with altered sensitivity to oxaliplatin. The heat map indicates the normalized log2 ratio of the phosphorylation levels of p53 (Ser15), and Bad (Ser112), as well as the cleavage status of PARP and Caspase-3 in test siRNA-treated cells (+1.56μM oxaliplatin) relative to non-silencing siRNA-treated cells (+1.56μM oxaliplatin), as assessed by quantitative analysis using a sandwich ELISA with epitope-
specific antibodies 72 hr post transfection and addition of oxaliplatin. Color bar indicates log₂ of relative activity (phosphorylation or cleavage).

**Figure 4.** Alterations in cell cycle distribution in cells with altered sensitivity to oxaliplatin. X-axis indicates DNA content (as determined by propidium iodide staining), and Y-axis indicates cell count. Color coding indicates G1 (green), S (yellow), or G2/M (blues) phases of the cell cycle. Percentages of each stage are indicated. Cells were transfected, treated with 1.56µM oxaliplatin, and processed for FACS after 72 hr.

**Figure 5.** Network modeling of screening hits identifies networks of multiple cellular processes linked to oxaliplatin sensitivity. Networks of interacting proteins identified using Ingenuity Pathway Analysis. Color indicates genes identified in the siRNA screen whose loss of expression increased tumor cell resistance to oxaliplatin (red) or increased tumor cell sensitivity to oxaliplatin (blue).
References


Figure 1

[Graph showing gene expression levels after treatment with oxaliplatin, with Log₂ Fold Shift IC₅₀ values on the y-axis and Gene names on the x-axis. Notable genes include CDKN1A, KPNA2, SUMO1, and TP53.]
Figure 3

A

Apurinic/pyrimidinic sites (% of NS siRNA-treated cells)

B

CDKN1A
MBD4
NEJ1
LTBR
MCM3
CUL4B
TMEM30A
SHFM1
BRIP1
PRDX4
PTTG1
TP53

p-Akt1 (Ser473)
p-MEK1 (Ser217/221)
p-p38 MAPK (Thr180/Tyr182)
p-Stat3 (Tyr705)
p-NFkB p65 (Ser536)

C

MCM3
MBD4
NEJ1
CDKN1A
PTTG1
BRIP1
CUL4B
SHFM1
TMEM30A
LTBR
PRDX4
TP53

p-p53 (Ser15)
Cleaved Caspase 3 (Asp175)
Cleaved PARP (Asp214)
p-Bad (Ser112)
Figure 5
Table 1

Genes conferring sensitivity to oxaliplatin

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<th>Entrez ID</th>
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<td>B-cell CLL/lymphoma 10</td>
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<tr>
<td>BCL2L10</td>
<td>10017</td>
<td>BCL2-like 10 (apoptosis facilitator)</td>
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Genes conferring resistance to oxaliplatin

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Kelly A Harradine, Michelle Kassner, Donald Chow, et al.

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