The M1 aminopeptidase NPEPPS is a novel regulator of cisplatin sensitivity

Robert T. Jones[1,15], Andrew Goodspeed[1,3,15], Maryam C. Akbarzadeh[2,4,16], Mathijs Scholtes[2,16], Hedvig Vekony[1], Annie Jean[1], Charlene B. Tilton[1], Michael V. Orman[1], Molishree Joshi[1,5], Teemu D. Laajala[1,6], Mahmood Javaid[7], Eric T. Clambey[8], Ryan Layer[7,9], Sarah Parker[10], Tokameh Mahmoudi[2,11], Tahlita Zuiverloon[2,*], Dan Theodorescu[12,13,14,*], James C. Costello[1,3,*]

[1]Department of Pharmacology, University of Colorado Anschutz Medical Campus, Aurora, CO, USA
[2] Department of Urology, Erasmus MC Cancer Institute, Erasmus University Medical Center Rotterdam, Rotterdam, The Netherlands
[3]University of Colorado Comprehensive Cancer Center, University of Colorado Anschutz Medical Campus, Aurora, CO, USA
[4]Stem Cell and Regenerative Medicine Center of Excellence, Tehran University of Medical Sciences, Tehran, Iran
[5]Functional Genomics Facility, University of Colorado Anschutz Medical Campus, Aurora, CO, USA
[6]Department of Mathematics and Statistics, University of Turku, Turku, Finland.
[7]Computer Science Department, University of Colorado, Boulder
[8]Department of Anesthesiology, University of Colorado Anschutz Medical Campus, Aurora, CO
[9]BioFrontiers Institute, University of Colorado, Boulder
[10]Heart Institute & Advanced Clinical Biosystems Research Institute, Cedars Sinai Medical Center, Los Angeles, California 90048, United States
[11]Erasmus MC, Department of Biochemistry, Rotterdam, The Netherlands
[12]Department of Surgery, Cedars-Sinai Medical Center, Los Angeles, CA, USA
[13]Department of Pathology and Laboratory Medicine, Cedars-Sinai Medical Center, Los Angeles, CA, USA
[14]Cedars-Sinai Samuel Oschin Comprehensive Cancer Institute, Los Angeles, CA, USA
[15]These authors contributed equally
[16]These authors contributed equally
*Corresponding Authors

Corresponding Authors
Tahlita Zuiverloon, MD, PhD
Department of Urology
Erasmus MC Cancer Institute, Erasmus University Medical Center
Dr. Molewaterplein 40
3015GD, Rotterdam
The Netherlands
+31 6 26 41 90 87
t.zuiverloon@erasmusmc.nl

Dan Theodorescu, MD, PhD
Department of Surgery and Pathology
Cedars-Sinai Medical Center
8700 Beverly Blvd.
OCC Mezz C2002
Los Angeles, CA 90048

+1 (310) 423-8431
dan.theodorescu@cshs.org

James C Costello, PhD
Department of Pharmacology
University of Colorado Anschutz Medical Campus
Mail Stop 8303
12801 E. 17th Ave., Rm L18-6114
Aurora, CO 80045
+1 (303) 724-8619
james.costello@cuanschutz.edu

HIGHLIGHTS

- CRISPR screens with multi-omics identify NPEPPS as a driver of cisplatin resistance
- NPEPPS depletion in multiple bladder cancer models enhances cisplatin sensitivity
- LRRC8A and LRRC8D loss increase resistance to cisplatin in CRISPR screens
- Unique resource of functional and multi-omic data is provided to the community

KEY WORDS

Bladder Cancer, CRISPR Screen, Synthetic Lethality, Genomics, Transcriptomics, Proteomics, DNA Repair, Cisplatin, Chemotherapy, NPEPPS, Volume Regulated Anion Channel, VRAC, LRRC8A, LRRC8D, Tosedostat

ABSTRACT

Platinum-based chemotherapeutics are used in many combination regimens in cancer. Despite extensive use across diverse cancer types, there is room for improved efficacy and patient selection for treatment. Here, we use bladder cancer to address both issues. A multi-omic assessment of five human bladder cancer cell lines and their chemotherapy resistant derivatives, coupled with *in vitro* whole-genome CRISPR screens were used to define functional drivers of treatment resistance. We identified 46 genes that sensitized the resistant cell lines to cisplatin plus gemcitabine (GemCis), a standard combination therapy in bladder cancer. Most genes were involved with DNA damage and repair pathways, which have previously been associated with enhanced sensitivity to cisplatin. Evaluating expression of the 46 genes in the whole transcriptome and proteome data in parental and resistant lines identified the puromycin sensitive aminopeptidase, NPEPPS, as a novel hit. Depletion of NPEPPS resulted in sensitizing resistant bladder cancer cells to cisplatin *in vitro* and in xenograft experiments. Pharmacologic inhibition of NPEPPS with tosedostat in cells and in chemoresistant, bladder cancer patient tumor-derived organoids improved response to cisplatin. Prior work found NPEPPS in a protein complex with volume regulated anion channels (VRACs) in several cell line models. Interestingly, depletion of two VRAC subunits, LRRC8A and LRRC8D, known importers of intracellular cisplatin, enhanced resistance to cisplatin. Our findings support NPEPPS as a novel and druggable driver of cisplatin resistance with the potential for rapid translation to clinical investigation.

INTRODUCTION

Platinum-based chemotherapeutics have a long history (Dilruba and Kalayda, 2016) with successful applications in testicular, ovarian, bladder, head and neck, and lung cancers. However, these drugs come with dose-dependent side effects that limit patient eligibility. Additionally, chemoresistance mechanisms can arise, reducing the efficacy of these drugs. While mechanisms of resistance have long been established, including DNA damage repair and drug export (Galluzzi et al., 2012), other mechanisms, such as the import of platinum drugs through volume regulated anion channels (VRACs) are more recently discovered and present new opportunities for therapeutic development (Planells-Cases et al., 2015a; Rottenberg et al., 2021). Despite their limitations, platinum-based drugs remain the standard of care in many cancer types and with a paucity of better treatment options for many patients, these drugs will remain in use for the foreseeable future. Two avenues can be taken to improve patient outcomes, which include discovery of more effective agents or development of strategies that can improve efficacy of platinum-based regimens. The latter would have broad impact across a range of cancer types. Here we focus on the latter approach and use bladder cancer as the model.

Bladder cancer (BCa) accounts for 430,000 new diagnoses and 170,000 deaths worldwide annually (Bray et al., 2018). Cisplatin-based combination chemotherapy, in the form of gemcitabine plus cisplatin (GemCis) or Methotrexate, Vinblastine, Adriamycin, and Cisplatin (MVAC), remains the first-line, standard of care for metastatic BCa, providing a 5-10% cure rate. However, up to 30% of patients are ineligible for cisplatin-based treatment (Galsky et al., 2018) and are offered carboplatin-based combinations. Unfortunately carboplatin combination therapy has been shown to be less effective in BCa (Patel et al., 2020). Alternatively, immune checkpoint therapies (ICT) are being considered as a first-line therapy (Galsky et al., 2020); however, ICT requires a PD-L1 diagnostic test, for which only ~25% patients meet eligibility (Nadal and Bellmunt, 2019). The complete response rates for ICT eligible patients is 20-30% (Balar et al., 2017a, 2017b). Cisplatin-based combination chemotherapy is also standard of care in the neoadjuvant (NAC) setting for the management of localized muscle-invasive bladder cancer (Grossman et al., 2003; Vale, 2005). However, NAC adoption has been relatively slow due to the toxicity of the drugs, the number of patients that are cisplatin ineligible, and the relatively small survival benefit of 5-15% over immediate cystectomy (Witjes et al., 2020). Importantly, in both the metastatic and NAC BCa settings, patient selection and therapeutic efficacy of cisplatin-based chemotherapy are critical unresolved challenges (Patel et al., 2020).

Recently, several large-scale efforts have performed whole genome loss-of-function screening across hundreds of cancer cell lines using CRISPR- and shRNA-based libraries to define pan-cancer and context-specific genetic dependencies (Cowley et al., 2014; McDonald et al., 2017; Tsherniak et al., 2017; Behan et al., 2019). A limitation of these efforts in pharmacogenomics is that cells were grown under basal growth conditions in the absence of treatment. Additionally, those studies were performed in cell lines that had not acquired resistance to the treatment. To better understand the functional drivers of therapeutic resistance, such screens must be done in the presence and absence of the therapy of interest (Goodspeed et al., 2019; Huang et al., 2020; Jost and Weissman, 2018; Olivieri et al., 2020), and in cells that have acquired resistance to the treatment itself. Results from such synthetic lethal screens can be used to prioritize gene candidates that can be targeted to overcome treatment resistance.

In this study, we harnessed the power of CRISPR-based synthetic lethal screening and multi-omic profiling to systematically assess the functional determinants of sensitivity to the treatment regimen of gemcitabine plus cisplatin in a panel of chemoresistant BCa cell lines (**Figure 1A**). In

addition to known mechanisms, we present the finding that upregulation of puromycin-sensitive aminopeptidase, NPEPPS, is a novel mechanism of gemcitabine plus cisplatin resistance, specifically affecting cisplatin sensitivity. We provide validations of these findings *in vitro* and *in vivo*. We next show that pharmacological inhibition of NPEPPS through an orally deliverable, well-tolerated drug, tosedostat, re-sensitizes resistant cells to cisplatin treatment in BCa cell lines and organoids derived from patient tumors that did not response to cisplatin-based chemotherapy .We also provide a unique resource to the community, an R Shiny app for broad comparisons between datasets (CRISPR screens and multi-omic) and cell lines, along with individual gene queries and basic plotting functionality (https://bioinformatics.cuanschutz.edu/GC_Visualization/).

RESULTS

From the Resistant Cancer Cell Line (RCCL) collection (Vallo et al., 2015, 2017), we obtained five human BCa cell lines, KU1919, 5637, T24, TCCSUP, and 253J. For each, we obtained the parental lines (-Par) and their matched derivatives that were made resistant through dose escalation to cisplatin (-Cis), gemcitabine (-Gem), and gemcitabine plus cisplatin (-GemCis) concurrently (**Figure 1A; Table S1**). We confirmed resistance to the associated drugs for all resistant derivatives in comparison to the parental lines and found them to be consistent with those reported by RCCL (**Figure S1**) (Vallo et al., 2015, 2017). These cells represent features and alterations in putative BCa drivers as reported in TCGA (Robertson et al., 2017) and variants reported in ClinVar (Landrum et al., 2018) (**Tables 1**, **S2** and **S3)**.

Genome-wide CRISPR screens identify 46 common synthetic lethal genes

To study the connection between drug resistance and genes, we performed whole-genome loss-of-function screens in each of the five GemCis-resistant cell line derivatives. After transduction of the Brunello CRISPR-Cas9 knockout library (Doench et al., 2016a), we passaged the cells for 10 days to clear essential genes, then split into saline (PBS) or gemcitabine plus cisplatin treatment groups (**Figure 1A**). Each screen was performed at a drug concentration that allowed the GemCis-resistant cells to grow unrestricted, but which significantly inhibited the growth of the associated parental lines (**Table S1**). Screening parameters for each cell line are reported in **Table S4**. We measured sgRNAs 19 and 25 after transduction, which were 9 and 15 days after the start of treatment.

We defined genes as “synthetic lethal” with gemcitabine plus cisplatin treatment as those for which the combined cognate sgRNA counts were significantly lower (moderated t-test, FDR < 0.05) in the gemcitabine plus cisplatin-treated arm compared to the PBS arm when including both days 19 and 25 in the statistical model (**Table S5**). We identified 235 synthetic lethal genes that were significant in KU1919-GemCis, 888 for T24-GemCis, 2099 for TCCSUP-GemCis, 2369 for 253J-GemCis, and 511 for 5637-GemCis. Next, we performed gene set enrichment analysis (Korotkevich et al., 2019) on the full ranked list of genes according to their synthetic lethality. For this analysis, we combined the results to create one ranked gene list by including each of the five cell types in the statistical model directly. As expected, we found that the top ranked pathways were dominated by processes such as DNA repair, Fanconi Anemia, nucleotide excision repair, double-stranded break repair, base-excision repair, and DNA damage bypass mechanisms (**Figure 1B** and **Table S6**). These results are consistent with the known roles of DNA damage detection and repair in cisplatin resistance (Drayton and Catto, 2012; Galluzzi et al., 2012).

Next, we sought to identify the most robust and commonly synthetic lethal candidate genes by identifying only those significant in all 5 cell lines (**Figures 1C** and **S2**). Of the 46 commonly synthetic lethal genes, and illustrated in **Figure 1D**, some increased cell growth in PBS treatment, then reduced growth in gemcitabine plus cisplatin treatment. Other genes had very little impact on cell growth in PBS treatment, but then reduced growth when treated with gemcitabine plus cisplatin. Finally, some genes reduced cell growth in PBS treatment and further reduced growth with gemcitabine plus cisplatin treatment. As expected, nearly all 46 common synthetic lethal candidate genes fell into DNA damage response and repair pathways.

NPEPPS is a novel determinant of response to cisplatin

A recent systematic analysis of hundreds of CRISPR screens in cancer cell lines with comprehensive multi-omic profiling demonstrated that transcript expression markers were the best predictors of gene dependency (Dempster et al., 2020), providing rationale for the use of pre-treatment -omic profiling as a means to study the biological impact of synthetic lethal hits. Hence, to prioritize the 46 common synthetic lethal genes for validation and detailed mechanistic understanding, we performed RNA sequencing and mass spectrometry-based proteomic profiling on cell lysates of all cell lines grown in drug-free media (**Figure 1A**).

We investigated the transcriptome and proteome data by comparing parental to matched drug resistant derivative lines (-Gem, -Cis, and -GemCis) and identified several known mechanisms of chemoresistance. For example, acquired resistance to gemcitabine follows a number of common pathways across multiple tumor types that disrupt intracellular metabolism, such as the loss of deoxycytidine kinase (DCK) or increased expression of ribonucleotide reductase subunit M1 (RRM1) (Bepler et al., 2006; Bergman et al., 2005; Jordheim et al., 2011) (**Figure S3A**). Our data shows that RRM1 is specifically and significantly upregulated in nearly all Gem- and GemCis-resistant derivatives in the T24, TCCSUP, KU1919, and 5637 cell line series by both RNA and protein expression. In addition, and with the TCCSUP-GemCis line being the only exception, we found RRM1 copy number amplified, but not in the parental or the cisplatin resistant cells, providing strong support that a robust and consistently acquired mechanism of gemcitabine resistance in these cells is the copy number amplification and subsequent upregulation of RRM1 (**Figure S3B**). RRM1 is defined as an essential gene in the Dependency Map (Tsherniak et al., 2017), which we also detected in our screen (**Table S7**). Interestingly, in 253J-Gem and 253J-GemCis cell lines, which had minor changes in RRM1 expression, DCK expression was lost at the RNA and protein level with these results being supported by a copy number loss specific to these cells (**Figure S3B**).

Next, we analyzed gene and protein expression together while treating the cell line as a covariate in the statistical model. We found 1557 significantly upregulated genes across the Gem-resistant lines, 1897 in the Cis-resistant lines, and 1530 in the GemCis-resistant lines (moderated t-test, FDR < 0.05; **Table S8**). The proteomics data revealed 9 significantly upregulated proteins across the Gem-resistant cell lines, 1 in the Cis-resistant cell lines, and 10 in the GemCis-resistant cell lines (moderated t-test, FDR < 0.25; **Table S9**). Given the lower number of significant proteins and the relevance of transcript expression in predicting genetic dependency (Dempster et al., 2020), we first investigated the overlap between the CRISPR screen results and the transcriptomes from each of the resistant cell line derivatives compared to the parental cells. Few genes were significantly and consistently upregulated across the resistant derivatives in the list of 46 commonly synthetic lethal genes (**Figure 2A**). The most significantly and consistently upregulated genes were involved in DNA damage response and repair mechanisms, including ERCC6, XPA, REV1, POLH, ERRC8, PRIMPOL, NBN, and members of the Fanconi Anemia pathway. Puromycin sensitive aminopeptidase, NPEPPS, was identified as being the most consistently upregulated gene across the resistant derivatives (**Figure 2A, B**). We similarly found protein levels to be consistently and significantly upregulated (**Figure 2C**). NPEPPS was also a top synthetic lethal hit (**Figure 2D** and **Table S5**). Consistent with the proteomics results, immunoblotting for NPEPPS revealed that it was upregulated in the Cis-resistant and GemCis-resistant lines, with the Gem-resistant lines showing variable upregulation (**Figure 2E**).

256 We examined an independent whole-genome CRISPR screen that tested 27 general genotoxic
257 agents (Olivieri et al., 2020) and here report new findings in support of NPEPPS as a novel
258 mediator of cisplatin resistance. We found that cells with NPEPPS loss were specifically
259 depleted in response to cisplatin, but not gemcitabine (**Figure 3A**). This result strongly supports
260 the robustness of our findings as Olivieri et al. used different CRISPR libraries (TKOv2 and
261 TKOv3) and cell line (retinal pigment epithelium-1, RPE1). Moreover, our screen results for all
262 five cell lines were highly correlated with the three cisplatin screens (**Figure S4A**). Strikingly,
263 nearly all 46 hits were significant hits associated with cisplatin, but not gemcitabine in Olivieri et
264 al. (**Figure S4B**).
265
266 To validate our screen findings that NPEPPS depletion enhances sensitivity to gemcitabine plus
267 cisplatin treatment in GemCis-resistant BCa cells, and parse its role in both cisplatin and
268 gemcitabine resistance, we generated stable NPEPPS shRNA knockdowns in the KU1919-
269 GemCis cell line (**Figure 3B**). The KU1919-GemCis line was selected for further experiments
270 throughout this work because it had the strongest combination of a synthetic lethal result and
271 gene/protein upregulation (**Figure 2**). We found that NPEPPS knockdown preferentially
272 increased cisplatin, but not gemcitabine sensitivity (**Figure 3C, D**). The same result was found
273 using siRNA in the KU1919-GemCis cell line and shRNA and/or siRNA in T24-GemCis and
274 253J-GemCis cells (**Figure S5**). We also found NPEPPS mRNA increased with cisplatin
275 treatment in both KU1919-parental and KU1919-GemCis cells after 24 hours of treatment
276 (**Figure 3E**). These results indicate that NPEPPS mediates sensitivity to gemcitabine plus
277 cisplatin primarily by its effect on resistance to cisplatin.
278
279 Several agents inhibit NPEPPS activity (Drinkwater et al., 2017). Tosedostat, an orally available
280 M1 aminopeptidase prodrug has antileukemic activity with a favorable toxicity profile in patients
281 (CHR-2797, CAS 238750-77-1) (Cortes et al., 2013; van Herpen et al., 2010; Krige et al., 2008;
282 Löwenberg et al., 2010; Mawad et al., 2016). We tested the response of the KU1919-GemCis,
283 T24-GemCis, and TCCSUP-GemCis resistant cells to serial doses of tosedostat at the resistant
284 doses of gemcitabine, cisplatin, and gemcitabine plus cisplatin. Consistent with NPEPPS
285 depletion, tosedostat did not sensitize cells to gemcitabine (**Figure 3F** and **Table S1)**. The
286 strongest combined effect was seen with cisplatin and gemcitabine plus tosedostat treatment
287 (**Figure S6)**. These results show that GemCis-resistant BCa cells can be re-sensitized to
288 cisplatin-based treatments at varying degrees by genetic and pharmacologic inhibition of
289 NPEPPS.
290

291 Volume regulated anion channels and their impact on chemoresistance in bladder 292 cancer cells

293
294 NPEPPS is one of 13 M1 aminopeptidases that cleaves amino acids from the N-terminus of
295 polypeptides. NPEPPS is involved in cell growth, development and antigen presentation
296 (Constam et al., 1995; Menzies et al., 2010; Saric et al., 2004; Towne et al., 2008). A role of
297 NEPPPS in chemotherapeutic response is yet to be described. To begin characterizing the
298 mechanisms NPEPPS uses to drive cisplatin resistance, we investigated NPEPPS protein
299 interaction partners in the BioPlex interactome, a database that has collected affinity-purification
300 mass spectrometry measurements of systematically over-expressed, tagged proteins (Huttlin et
301 al., 2020). Remarkably, among the small number of proteins that were observed to interact with
302 NPEPPS, were all five members of the volume regulated anion channel (VRAC), leucine rich
303 repeat containing 8 VRAC subunit A-E (LRRC8A-E) (**Figure 4A**). Supporting this finding, other
304 affinity-purification mass spectrometry experiments independently reported the interaction
305 between NPEPPS and VRAC members across different cell lines (Kasuya et al., 2018; Syeda et

306 al., 2016). Equally interesting was that none of the other 12 M1 aminopeptidases were found in
307 complex with any VRAC members in the BioPlex interactome.
308
309 VRACs directly respond to osmotic stress by trafficking osmolytes such as chlorine, potassium,
310 and taurine, across the plasma membrane to regulate cell volume (Voss et al., 2014).
311 Importantly, subunits LRRC8A and LRRC8D are mediators of platinum drug resistance in
312 chronic myelogenous leukemia cells. Knockout experiments in kidney and colorectal cell lines
313 showed that 50-70% of intracellular cisplatin is transported through these channels (Planells-
314 Cases et al., 2015a), mediated by LRRC8A and LRRC8D. Similar findings were subsequently
315 found in ovarian cancer and alveolar carcinoma cell lines (Sørensen et al., 2014, 2016a,
316 2016b). Thus, we focus on the LRRC8A and LRRC8D subunits for further analysis.
317
318 We revisited our CRISPR screens and RNAseq data to determine if loss of LRRC8A and/or
319 LRRC8D impacted cisplatin resistance. As predicted, LRRC8A and LRRC8D loss provided a
320 growth advantage to cells treated with gemcitabine plus cisplatin (**Figure 4B, C**). Most strikingly,
321 LRRC8A was the 1st and LRRC8D was the 11th ranked gene that when lost provided a growth
322 advantage in gemcitabine plus cisplatin treatment (**Figure 4D**). LRRC8A and/or LRRC8D
323 mRNA expression was reduced for most of the Cis- or GemCis-resistant cell lines, with the
324 Gem-resistant lines showing variable differential expression (**Figure 4E, F**). Most notable,
325 LRRC8D gene expression in the TCCSUP-Cis and TCCSUP-GemCis cells was completely lost
326 (**Figure 4F**). We found that in these cell lines, there is a deep deletion at the LRRC8D locus
327 (**Figure S7**). NPEPPS loss in the TCCSUP-GemCis lines showed the weakest synthetic lethal
328 result compared to the other four GemCis-resistant lines (**Figure 2D**) and LRRC8D loss had no
329 effect on TCCSUP-GemCis growth (**Figure 4C**), while LRRC8A loss did in fact increase growth
330 (**Figure 4B**). Taken together, these data support a functional dependency between NPEPPS
331 and VRAC subunits LRRC8A and LRRC8D in relation to cisplatin resistance.
332
333 Given that VRACs transport cisplatin and carboplatin (Planells-Cases et al., 2015a) and finding
334 NPEPPS in complex with LRRC8A and LRRC8D (Huttlin et al., 2020; Kasuya et al., 2018;
335 Syeda et al., 2016), we hypothesized that NPEPPS may be a negative regulator of VRAC
336 activity, consequently reducing import of intracellular cisplatin. Thus, we tested the impact of
337 NPEPPS on osmolytes known to be transported through VRACs. NPEPPS knockdown in
338 KU1919-GemCis-shN39 cells resulted in a significantly lower levels of intracellular taurine,
339 hypotaurine, creatine, phosphocreatine, and several other amino acids (**Figure 4G** and **Table**
340 **S10**), which are known to be exported via VRACs. In addition, intracellular levels of taurine were
341 reduced even further when cells with knockdown of NPEPPS were also treated with 10μM
342 cisplatin, which is the resistance dose for the KU1919-GemCis cells (**Figure 4H**). This suggests
343 that cisplatin further stimulates channel activity when NPEPPS is decreased, which allows for
344 increased export of taurine, and as we show next increases cisplatin import.
345
346 To evaluate NPEPPS impact on cisplatin import, we directly measured intracellular cisplatin
347 using the metal ion detection capabilities of mass cytometry (Chang et al., 2015). Intracellular
348 cisplatin was measured after 4 hours of treatment at 10μM for KU1919-parental, KU1919-
349 GemCis, and KU1919-GemCis-shN39 cells. As expected, KU1919-GemCis cells showed
350 decreased uptake of cisplatin compared to KU1919-parental cells. Knockdown of NPEPPS
351 shifted the intracellular levels of cisplatin to be nearly double (median Pt 195 = 77) of the
352 GemCis-resistant cells (median Pt 195 = 44.5) and half of the parental cells (median Pt 195 =
353 158), suggesting that NPEPPS depletion allows increased import of cisplatin (**Figure 4I** and
354 **S8A,B**).
355

356 Furthermore, we measured protein levels of LRRC8A and LRRC8D after 48 hours of PBS or
357 10μM cisplatin treatment in NPEPPS knockdown or nontargeting control KU1919-GemCis cells.
358 Supporting the mass cytometry results (**Figure 4I**) and the result that taurine is exported at a
359 higher rate upon cisplatin stimulation in the KU1919-GemCis-shN39 cells (**Figure 4H**), NPEPPS
360 knockdown increased DNA damage as measured by increased γH2AX foci (**Figure 4J** and
361 **S8C**). However, we did not find major changes in LRRC8A or LRRC8D expression in response
362 to NPEPPS knockdown or cisplatin treatment (**Figure 4J** and **S8C**). Taken together, these data
363 and previous studies, support a role for NPEPPS in regulating cellular osmolyte homeostasis via
364 an interaction with VRACs.
365

366 **Genetic and pharmacologic inhibition of NPEPPS enhances chemotherapy**
367 **sensitivity *in vivo* and in patient tumor-derived organoids**

368

369 To test if NPEPPS depletion would sensitize tumor cells to gemcitabine plus cisplatin treatment
370 *in vivo*, we established subcutaneous xenografts using the KU1919-GemCis cells with either
371 NPEPPS shRNA knockdown or non-targeting shRNA control. When tumors reached roughly
372 200mm^3, mice were randomized into four groups: shCtrl1 with PBS, shCtrl1 with gemcitabine
373 plus cisplatin, shN39 with PBS, and shN39 with gemcitabine plus cisplatin. Treatment was
374 delivered through intraperitoneal injection, with PBS or gemcitabine plus cisplatin administered
375 three times weekly for four weeks. Tumor volumes were monitored until they reached the
376 predetermined endpoint of 2cm^3. NPEPPS knockdown alone and gemcitabine plus cisplatin
377 treatment alone had significant impact on tumor growth compared to vehicle-treated, shRNA
378 controls. The combination of NPEPPS knockdown and gemcitabine plus cisplatin treatment led
379 to an even stronger and more significant impact on tumor growth (**Figure 5A**). We further
380 analyzed tumor growth using linear mixed-effects models aimed at capturing trends in tumor
381 volume change in relation to pre-treatment baseline tumor volume across the four groups.
382 According to this model, tumor growth inhibition by NPEPPS knockdown (p=0.00178), GemCis
383 treatment (p=5.49e-7), or the combination of NPEPPS knockdown and gemcitabine plus
384 cisplatin treatment (p=1.47e-8) were all consistent effects over the treatment period (**Figure**
385 **S9A, B**). We validated NPEPPS knockdown in the pre-xenograft inoculate cells and after
386 tumors were removed from mice upon reaching the 2cm^3 endpoint (**Figure S9C**). Survival
387 analysis using tumor volume as the endpoint showed that mice treated with gemcitabine plus
388 cisplatin had a 14-day survival advantage. Similarly, knockdown of NPEPPS resulted in a 14-
389 day survival advantage. Mice treated with gemcitabine plus cisplatin and with NPEPPS
390 knockdown tumors had a 25-day survival advantage, a statistically significant improvement
391 (Logrank test, $p<0.0001$) (**Figure 5B**).
392

393 Next, we extended our work to evaluate the impact of the drug combination of tosedostat and
394 cisplatin on *ex vivo* patient tumor-derived BCa organoids. We generated three independent
395 organoid models from patient cystectomy samples that did not respond to gemcitabine plus
396 cisplatin NAC (**Figure S10A**). Based on targeted mutations or global copy number alterations,
397 the organoids had similar characteristics of the tumor tissues from which they were derived
398 (**Figure S10B, C**). We treated the organoids with cisplatin and tosedostat for six days and then
399 removed the drugs to allow recovery for 10 days (**Figure S10D)**. Cisplatin with tosedostat
400 resulted in significant decreases in viability across all organoids (**Figures S11-S13**). Viability of
401 vehicle control and tosedostat treated organoids show an initial decrease of roughly 20%, but
402 these cells recover over time to be equal to the vehicle control. We saw no different in 5μM or
403 20μM tosedostat (**Figure S11D**). After 10 days of recovery from treatment, organoids were
404 reseeded at different densities based on overall growth and allowed to re-establish and grow for
405 an additional 6 days, then cell viability was assessed (**Figures 5C-E** and **S11-13**). Treatment

406 with increasing concentrations of Cis alone resulted in a progressively decreased viability of
407 organoids. Strikingly, the addition of tosedostat to cisplatin resulted in further reductions at
408 concentrations in which cisplatin treatment alone had minor effects (**Figure 5C-E**). Notably, the
409 combined treatment with tosedostat lowered the effective concentration to 2μM in patients 1 and
410 2, and to 25μM in the highly treatment resistant patient 3 (**Figure 5C-E**). These findings are
411 translationally relevant since the maximum concentration of cisplatin in patients is 14μM (Liston
412 and Davis, 2017) and provide additional validation using a human-derived model system that
413 tosedostat enhances cisplatin activity.
414
415 The increase in NPEPPS mRNA that has been observed in response to chronic (**Figure 2B, C**)
416 and acute cisplatin treatment *in vitro* and in the *ex vivo* organoids (**Figure 3E**) suggests that
417 high levels of NPEPPS expression are part of an acquired or adaptive rather than intrinsic
418 mechanism of drug resistance in tumors that have been exposed to cisplatin. Hence, pre-
419 treatment tumor NPEPPS levels may not necessarily be a biomarker of chemotherapy response
420 in bladder cancer. However, given the relationship of NPEPPS to VRACs described previously
421 and findings that levels of LRRC8A and LRRC8D are predictive of cisplatin response in ovarian
422 cancer (Planells-Cases et al., 2015a), we reasoned that such relationships would also be true in
423 BCa. Using TCGA data from muscle invasive bladder cancer (Robertson et al., 2017), we
424 compared patients with and without a record of platinum-based treatment (Goodspeed et al.,
425 2019) with respect to amplification, copy number, and expression of either LRRC8A or
426 LRRC8D. Notably, patients with LRRC8A and/or LRRC8D copy number gain or overexpression
427 that received platinum-based treatment showed significantly improved overall survival in
428 contrast to those with no record of this treatment modality (**Figure 5F**, **G**). Together, these
429 findings support VRAC subunits LRRC8A and LRRC8D as pre-treatment biomarkers of
430 response to cisplatin-based chemotherapy (Rottenberg et al., 2021).
431
432

DISCUSSION

NPEPPS has been suggested to play a role in a range of cellular processes including promoting autophagy, regulating cell cycle progression, and antigen processing (Constam et al., 1995; Menzies et al., 2010; Saric et al., 2004; Towne et al., 2008). The majority of what is known about NPEPPS has been from studies in the brain, where it targets the degradation of polyglutamine sequences and misfolded protein aggregates associated with a number of neurodegenerative diseases, including Alzheimer's disease, Huntington's Disease, and Parkinson's disease (Karsten et al., 2006; Kudo et al., 2011; Menzies et al., 2010; Schönlein et al., 1994; Yanagi et al., 2009). As reported in gnomAD, NPEPPS is a highly conserved gene and constrained based on several metrics of intolerance to genetic variation in the population (Karczewski et al., 2020). NPEPPS is also ubiquitously expressed across human tissues (Uhlen et al., 2017). However, despite these features, genetic modification in mice is tolerable (Osada et al., 1999; Towne et al., 2008) and as we have shown from our CRISPR screen results, knockout is not essential (**Figure 2D**). Overall, NPEPPS presents a viable therapeutic target and we have shown that its downregulation genetically or pharmacologically re-sensitizes treatment-resistant cells back to cisplatin.

Broadly, aminopeptidases have been therapeutically targeted as potential cancer treatments (Hitzerd et al., 2014). More specifically, NPEPPS is a zinc containing M1 aminopeptidase. Tosedostat was developed as a target of M1 aminopeptidases and the intracellular metabolized product CHR-79888 is the most potent inhibitor of NPEPPS reported (Krige et al., 2008; Reid et al., 2009). There have been a total of 11 clinical trials with tosedostat as reported in *clinicaltrials.gov* (Cortes et al., 2013; van Herpen et al., 2010; Krige et al., 2008; Löwenberg et al., 2010; Mawad et al., 2016). The focus of its application has been in leukemias and myelomas, with several applications in solid tumors. The few clinical trials completed have reported tosedostat as being well tolerated by patients, but with modest effect as a cancer treatment alone and inferior outcomes in elderly AML patients when combined with cytabine and daunomycin (Janssen et al., 2021). A few examples of tosedostat in combination with cytarabine, azacitidine, capecitabine or paclitaxel have been tried, but there are no reports of tosedostat being tried in combination with platinum-based chemotherapy, supporting the novel application of cisplatin-based chemotherapy plus tosedostat that we propose in this study.

Another exciting potential application of NPEPPS inhibition is to provide alternative treatment options for BCa patients. Many patients are ineligible for cisplatin-based chemotherapies, leaving them with less effective options, such as carboplatin. VRACs also transport carboplatin at similar amounts as cisplatin (Planells-Cases et al., 2015a), thus combining an NPEPPS inhibitor, such as tosedostat, with carboplatin could provide a more effective and less toxic drug combination option for cisplatin ineligible patients. A further area of novel development would be the impact of NPEPPS inhibition on ICT with its known effect on MHC class I antigen presentation on dendritic cells (Towne et al., 2008). ERAP1 and ERAP2, other M1 aminopeptidases in the same family as NPEPPS, have been linked to boosting T cell and NK cell mediated immune response in cancer (Compagnone et al., 2019); however the impact of NPEPPS on antigen presentation in tumor cells is yet to be investigated. Interestingly, low ERAP2 was associated with improved response to anti-PD-L1 in luminal bladder cancer (Lim et al., 2018). The impact of NPEPPS inhibition in immunotherapies requires further study.

The data we provide supports the role of NPEPPS and VRACs in cisplatin-based response in BCa, thus we have scoped our conclusions to BCa. However, results outside of this study support a molecular mechanism with broader impact. The evidence that supports the interaction between NPEPPS and VRACs were derived from several different cell types and the evidence

484 that implicates VRACs in platinum-based chemotherapy sensitivity is from ovarian cancer
485 (Planells-Cases et al., 2015b; Sørensen et al., 2014, 2016a, 2016b). If the NPEPPS-VRAC
486 mechanism of platinum-based chemotherapy resistance is a general mechanism, then there are
487 clear implications for any cancer type that uses platinum-based treatments. Hence, we can
488 propose a hypothetical model (**Figure 6**) where a cancer cell imports cisplatin, which in turn
489 causes DNA damage and eventually cell death. An inherent mechanism of resistance can
490 simply be the number of VRACs in a tumor cell, where downregulation of VRAC subunits can
491 lead to treatment resistance, such as was previously found in ovarian cancer, or the opposite
492 effect seen with LRRC8A or LRRC8D upregulation in BCa (**Figure 5F, G**). In our model,
493 NPEPPS interacts with LRRC8A and/or LRRC8D to inhibit channel activity, thus providing
494 resistance to cisplatin and overall chemoresistance. If proven to be true, our insight into this
495 mechanism opens up opportunities for novel therapeutic strategies to reverse or prevent the
496 development of cisplatin resistance, such as the development of agents that block NPEPPS
497 interactions with VRACs.
498
499 This work is not without its limitations. There is strong evidence from independent studies with
500 multiple different cell lines and experimental techniques that NPEPPS and VRAC subunit
501 proteins interact (Huttlin et al., 2020; Kasuya et al., 2018; Syeda et al., 2016). It would be very
502 surprising this would not be the case for BCa. We have also shown in multiple settings that
503 inhibiting NPEPPS genetically or pharmacologically results in re-sensitizing resistant BCa cells
504 to cisplatin. However, where and when NPEPPS interacts with VRACs in the cell is yet to be
505 determined. In addition, NPEPPS could have effects on treatment response outside of the
506 VRACs. Our work is also limited by the fact that we have not shown that NPEPPS depletion
507 leads directly to carboplatin sensitization in BCa, but that is likely given the known VRAC
508 relationships (Planells-Cases et al., 2015a). Despite these study limitations, the implications of
509 NPEPPS as a therapeutic target for better treatment response has the potential to be translated
510 into novel treatment regimens for improved patient outcomes.
511
512 In conclusion, our finding that NPEPPS mediates cisplatin-based chemoresistance is both novel
513 and actionable. We provided *in vitro*, *in vivo*, and *ex vivo* evidence that this mechanism is robust
514 and therapeutically targetable. Future directions will include determining the detailed
515 mechanism of NPEPPS-mediated treatment response, particularly though the interaction with
516 VRACs, and generating additional pre-clinical data testing NPEPPS inhibitor efficacy and
517 toxicities. Cisplatin-based chemotherapeutic regimens are mainstays of treatment across many
518 cancer types and these novel findings lay the groundwork for improved treatment of patients
519 harboring these tumors (Rottenberg et al., 2021). Our findings also have implications into other
520 platinum agents, such as carboplatin which would further improve efficacy of this agent in
521 additional cancer types. Finally, for the benefit of the research community, we make the -omic
522 and CRISPR screen data publicly available through an R Shiny app
523 (https://bioinformatics.cuanschutz.edu/GC_Visualization/) which will provide a rich source for
524 novel analysis in the mechanisms of chemotherapy resistance.
525
526

527 **Materials and Methods**
528
529 **Key Resource Table**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
NPEPPS	Invitrogen	PA5-22383
NPEPPS	Invitrogen	PA5-83788
GAPDH (D16H11) XP Rabbit mAb	Cell Signaling Technologies	5174S
Anti-LRRC8A, Rabbit polyclonal	LSBio	LS-B16989
Anti-LRRC8D, Rabbit polyclonal	SinoBiological	104245-T32
Phospho-Histone H2A.X (Ser139) Monoclonal Antibody (3F2)	Invitrogen	MA1-2022
Anti-mouse IgG (whole molecule), peroxidase antibody in rabbit	Sigma-Aldrich	A9044
Anti-rabbit IgG (whole molecule), peroxidase-conjugated (HRP)	MP Biomedicals	855689
Bacterial and Virus Strains		
Library Efficiency™ DH5α competent cells	ThermoFisher	18263012
Endura™ ElectroCompetent cells	Lucigen	60242
Chemicals, Peptides, and Recombinant Proteins		
Gemcitabine hydrochloride	Sigma	Y0000675
Gemcitabine hydrochloride (mouse experiment)	BOC Sciences	122111-03-9
Cisplatin	Sigma	PHR-1624
Cisplatin (mouse experiment)	Sigma	11344357
Tosedostat	Sigma	SML2303
Tosedostat (mouse experiment)	BOC Sciences	BCMV18265-2B
Tosedostat (organoids)	Tocris	3595
Puromycin dihydrochloride	Sigma	P9620
Fetal Bovine Serum (FBS)	VWR Seradigm Life Sciences	89510-186
RIPA Lysis and Extraction Buffer	ThermoFisher	89900
T-PER™ Tissue Protein Extraction Reagent	ThermoFisher	PI78510
Halt™ Protease and Phosphatase Inhibitor Cocktail, EDTA-free (100X)	ThermoFisher	PI78443
Polybrene [10mg/mL]	EMD Millipore	TR-1003-G
Lipofectamine 3000 Reagent	ThermoFisher	L3000075
Polyethylenimine, linear (PEI)	Polysciences, Inc.	23966
Lipofectamine® RNAiMAX Reagent	ThermoFisher	13778075
0.5% Trypsin-EDTA (10x)	Gibco	15400-54
DPBS (1x)	Gibco	14190-144
4% formalin	Sigma	HT501128
2.5% Low-Melting Agarose	Sigma	2070
Herculase II Fusion Enzyme	Agilent	600679
DNAseI	Invitrogen	18068015
Deoxynucleotide (dNTP) Solution Mix	New England Biolabs	N0447L
Critical Commercial Assays		
Puregene Cell and tissue Kit	Qiagen	158388
RNase A Solution	Qiagen	158924

SureSelect Human All Exon v6	Agilent	G9611
Amersham ECL Rainbow Marker -Full Range	Sigma	RPN8000E
Precision Plus Protein™ Kaleidoscope™ Prestained Protein Standards	Biorad	1610375
SuperSignal West Pico Plus	ThermoFisher	34580
SuperSignal West Fempto	ThermoFisher	34094
4–20% Mini-PROTEAN® TGX™ Precast Protein Gels	Biorad	4561096, 4561094
4X Protein Sample Loading Buffer for Western Blots	LI-COR	928-40004
KAPA library Quant Kit ROX Low	Kapa Biosystems	KK4873
Pierce™ BC Protein Assay Kit	ThermoFisher	23225
Universal Plus mRNA –Seq +UDI kit	Nugen Technologies	9144-96
RNAEasy Plus	Qiagen	74106
QIAmp DNA Mini-Kit	Qiagen	51304
Cell-ID 20-plex Pd Barcoding Kit	Fluidigm	201060
MycoAlert	Lonza	LT07-318
SensiFAST Probe No-ROX Mix	Bioline	BIO-86005
SuperScript™ II Reverse Transcriptase	Invitrogen	18064022
NPEPPS TaqMan® Gene Expression Assay	Thermofisher	Hs00196905_m1
HMBS TaqMan® Gene Expression Assay	Thermofisher	Hs00196905_m1
alamarBlue™ Cell Viability Reagent	Invitrogen	DAL 1025
Biorad Protein Assay Dye reagent concentrate	Biorad	5000006
Deposited Data		
Raw RNA sequencing data	This paper	GEO:XX
Raw whole exome sequencing	This paper	GEO:XX
Affymetrix GSA V3 organoid CAN	This paper	GEO:XX
Raw mass spectrometry proteomics	This paper	Peptide Atlas:XX
CRISPR screening results	This paper	**Table S13**
Bladder tumor proteomics	ProteomeXchange	PXD010260
TCGA bladder cancer patient gene expression	cBioPortal	https://cbioportal-datahub.s3.amazonaws.com/blca_tcga_pub_2017.tar.gz
TCGA bladder cancer survival data	(Goodspeed et al., 2019)	Supplementary Table 4
DepMap Common essential and non-essential genes (20Q1)	https://depmap.org/portal/download	common_essentials.csv, nonessentials.csv
Experimental Models: Cell Lines		
T24 parental	Resistant Cancer Cell Line Collection (https://research.kent.ac.uk/industrial-biotechnology-centre/the-resistant-cancer-cell-line-rccl-collection/)	N/A
T24 gemcitabine resistant		N/A
T24 cisplatin resistant		N/A
T24 gemcitabine and cisplatin resistant		N/A
TCCSUP parental		N/A
TCCSUP gemcitabine resistant		N/A
TCCSUP cisplatin resistant		N/A
TCCSUP gemcitabine and cisplatin resistant		N/A
253J parental		N/A
253J gemcitabine resistant		N/A

253J cisplatin resistant		N/A
253J gemcitabine and cisplatin resistant		N/A
KU1919 parental		N/A
KU1919 gemcitabine resistant		N/A
KU1919 cisplatin resistant		N/A
KU1919 gemcitabine and cisplatin resistant		N/A
5637 parental		N/A
5637 gemcitabine resistant		N/A
5637 cisplatin resistant		N/A
5637 gemcitabine and cisplatin resistant		N/A
293FT	ThermoFisher	R70007
Experimental Models: Organisms/Strains		
Mouse: Nu/J (JAX)	The Jackson Laboratory	002019
Oligonucleotides		
CRISPR screening library primers	**Table S11**	N/A
Recombinant DNA		
Human CRISPR Knockout Pooled Library (Brunello) - 1 vector system (lentiCRISPRv2)	Addgene	73179
psPAX2	Addgene	12260
pMD2.G	Addgene	12259
shCtrl1	University of Colorado Functional Genomics Facility	SHC002
shCtrl2	University of Colorado Functional Genomics Facility	SHC016
shN38	University of Colorado Functional Genomics Facility	TRCN0000073838
shN39	University of Colorado Functional Genomics Facility	TRCN0000073839
shN40	University of Colorado Functional Genomics Facility	TRCN0000073840
Software and Algorithms		
BWA-MEM (0.7.17)	(Li, 2013)	http://bio-bwa.sourceforge.net
Samblaster (0.1.24)	(Faust and Hall, 2014)	https://github.com/GregoryFaust/samblaster
Samtools (1.8)/HTSlib (1.9)	(Li et al., 2009)	http://www.htslib.org/
GATK Base Quality Score Recalibration (BQSR)	GATK4 v4.1.8	https://gatk.broadinstitute.org/hc/en-us/articles/360035890531-Base-Quality-Score-Recalibration-BQSR-
Calling Somatic SNVs and Indels with Mutect2	(Benjamin et al., 2019)	https://www.biorxiv.org/content/10.1101/861054v1.full.pdf

Somatic copy number variants	GATK4 v4.1.8	http://genomics.broadinstitute.org/data-sheets/PPT_Somatic_CNV_WKST_ASHG_2016.pdf
The Nextflow (20.04.1) pipeline implementing the workflows for this paper	This paper	https://github.com/javaidm/layer_lab_vc
SavvyCNV: genome-wide CNV calling from off-target reads v0.10	(Laver et al., 2019)	https://www.biorxiv.org/content/10.1101/617605v1
BBTools	BBMap – Bushnell B. – sourceforge.net/projects/bbmap/	https://jgi.doe.gov/data-and-tools/bbtools/
Image Studio	LiCor	https://www.licor.com/bio/image-studio/
STAR (2.6.0a)	(Dobin et al., 2013)	https://github.com/alexdobin/STAR
FLowJo (10.7.1)	(FlowJo, 2019)	https://www.flowjo.com/
Biodiscovery Nexus CN7.5	N/A	https://www.biodiscovery.com/products/Nexus-Copy-Number
GenomeStudio (1.9.4)	Genotyping Module	https://www.illumina.com/techniques/microarrays/array-data-analysis-experimental-design/genomestudio.html
Limma R package (3.44.3)	(Ritchie et al., 2015)	https://bioconductor.org/packages/release/bioc/html/limma.html
edgeR R package (3.30.3)	(Robinson et al., 2010)	https://bioconductor.org/packages/release/bioc/html/edgeR.html
fgsea R package (1.14.0)	(Sergushichev, 2016)	https://bioconductor.org/packages/release/bioc/html/fgsea.html
ggplot2 R package (3.2.2)	(Wickham, 2009)	https://ggplot2.tidyverse.org/
ggpubr R package (0.4.0)	N/A	https://cran.r-project.org/web/packages/ggpubr/index.html
ClusterProfiler	(Yu et al., 2012)	https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html

caRpools R package	(Winter et al., 2016)	https://cran.r-project.org/web/packages/caRpools/index.html
DEseq2 R package	(Love et al., 2014)	https://bioconductor.org/packages/release/bioc/html/DESeq2.html
Maven	(Clasquin et al., 2012)	http://genomics-pubs.princeton.edu/mzroll/index.php
openSWATH	(Röst et al., 2014)	http://openswath.org/en/latest/
vsn (3.12)	(Huber et al., 2002)	https://bioconductor.org/packages/release/bioc/html/vsn.html
lme4 (1.1-26)	(Bates et al., 2015)	https://cran.r-project.org/web/packages/lme4/index.html
lmerTest (3.1-3)	(Kuznetsova et al., 2017)	https://cran.r-project.org/web/packages/lmerTest/index.html
PyProphet	(Teleman et al., 2015)	http://openswath.org/en/latest/docs/pyprophet.html
MSstats R package	(Choi et al., 2014)	https://www.bioconductor.org/packages/release/bioc/html/MSstats.html
TRIC	(Röst et al., 2016)	https://github.com/msproteomicstools/msproteomicstools

Cell Culture

All human BCa cell lines as reported in the Key Resource Table were obtained from the Resistant Cancer Cell Line (RCCL) Collection and were grown in Iscove's Modified Dulbecco's Medium (IMDM) with 10% Fetal Bovine Serum (FBS). Cells were passaged every two to three days. Resistance to gemcitabine and cisplatin were confirmed at the reported resistance doses from the RCCL (**Table S1** and **Figure S1**). Lentivirus production utilized 293FT cells (ThermoFisher), which were maintained in DMEM (high glucose) supplemented with 0.1mM non-essential amino acids (NEAA), 6mM L-glutamine, 1mM sodium pyruvate, and 500μg/mL geneticin (G418) with 10% FBS added. Cells were routinely monitored for mycoplasma and confirmed negative at multiple times during this study using MycoAlert (Lonza). All cells were grown at 37°C with 5% CO_2 in a humidified incubator.

All molecular characterization efforts (RNA sequencing, whole exome sequencing, and mass spectrometric proteomics) were performed on cells from independent passages and in drug-free, complete media to identify stable molecular changes rather than treatment induced transient adaptations. Cells were routinely passaged through drug-containing media at the

resistant doses (**Table S1**) to confirm resistance was maintained and early passage cells were utilized whenever possible.

RNA sequencing

Sample preparation

All cell lines were grown for several passages in the absence of antibiotics, gemcitabine or cisplatin. Cell pellets were snap frozen from sub-confluent dishes from 3 separate passages (replicates) for each of the 20 cell lines sequenced (5 cell lines, each with 4 derivatives: parental, G-resistant, C-resistant, GC-resistant). RNA was extracted using the RNAeasy Plus Kit (Qiagen). Cells were lysed and passed through QIAShredder column (Qiagen) according to the manufacturer's protocol. gDNA elimination columns (Qiagen) were used to remove any residual gDNA from the purified RNA. RNA integrity was assessed on the High Sensitivity ScreenTape Assay on the Tape Station2200 (Agilent Technologies) and only samples with an RIN score of 8 or higher were used for sequencing. RNA library preparation was performed using the Universal Plus mRNA –Seq +UDI kit (Nugen Technologies) according to the manufacturer's specification. Each library was sequenced to a minimum of 40 million clusters or 80 million 150bp paired-end reads on a NovaSeq 6000 instrument (Illumina) at the University of Colorado Cancer Center Genomics Shared Resource.

Data processing

Illumina adapters and the first 12 base pairs of each read were trimmed using BBDuk and reads <50bp post trimming were discarded. Reads were aligned and quantified using STAR (Dobin et al., 2013) against the Ensembl human transcriptome (GRCh38.p12 genome (release 96)). Ensembl genes were mapped to HGNC gene symbols using HGNC and Ensembl BioMart. Gene counts were generated using the sum of counts for transcripts of the same gene. Lowly expressing genes were removed if mean raw count <1 or mean CPM (counts per million) <1 for the entire dataset. Reads were normalized to CPM using the edgeR R package (Robinson et al., 2010). Differential expression was calculated using the voom function in the limma R package (Ritchie et al., 2015). In addition to two-group comparisons, single drug comparisons for all cell lines were generated with cell line as a covariate (**Table S7**).

Alignment and transcript quantification

```
STAR --runThreadN 12 --runMode genomeGenerate --sjdbGTFfile
Homo_sapiens.GRCh38.96.gtf --genomeFastaFiles
Homo_sapiens.GRCh38.dna_sm.primary_assembly.fa

STAR --readFilesIn Read1.fastq.gz Read2.fastq.gz --readFilesCommand zcat --runThreadN 6 --
alignEndsProtrude 13 ConcordantPair --outFilterScoreMinOverLread 0.66 --
outFilterMatchNminOverLread 0.66 --outSAMtype BAM SortedByCoordinate --quantMode
GeneCounts
```

Pathway analysis

Gene set enrichment analysis was performed using the full list of genes ranked by fold change for the indicated comparison and the fgsea R package (Sergushichev, 2016) using gene sets from the Molecular Signatures Database (v7.0) (Liberzon et al., 2011). Over representation analysis was performed using the ClusterProfiler R package with genes less than an adjusted p-value 0.05 (Yu et al., 2012) or Metascape (Zhou et al., 2019). General plots were generated

591 with the ggplot2 and ggpubr R packages (Wickham, 2009). Heatmaps were generated with the
592 ComplexHeatmap R package following z-score transformation (Gu et al., 2016).

593

594 **Proteomics**

595 ***Sample preparation***

596 All cell lines were grown for several passages in the absence of antibiotics, gemcitabine or
597 cisplatin, then seeded at 100,000 – 200,000 cells per well and grown for 48 hours in IMDM +
598 10% FBS. Approximately 48 hours after seeding cells the supernatant was aspirated and cells
599 were washed 3 times with cold phosphate buffered saline (PBS). Cells were lysed in 100μl of
600 8M Urea, 50mM Tris-HCl, pH 8.0. Lysates were transferred to pre-chilled 1.5mL microcentrifuge
601 tubes and centrifuged at 15000 RCF for 10 minutes to pellet. The supernatant was then
602 transferred to a clean, pre-chilled tube and frozen. Lysate replicates were collected in triplicate
603 from different passages. Cell pellets were lysed in 8M Urea supplemented with 0.1% Rapigest
604 MS compatible detergent. DNA was sheared using probe sonication, and protein concentration
605 was estimated by BCA (Pierce, Thermo Scientific). A total of 30μg protein per sample was
606 aliquoted, and samples were diluted to <2M Urea concentration using 200mM ammonium
607 bicarbonate while also undergoing reduction with DTT (10mM) and then alkylation with IAA
608 (100mM). The pH of diluted protein lysates was verified as between 7-8, and samples were
609 digested with sequencing grade Trypsin/Lys-C enzyme (Promega) in the presence of 10%
610 Acetonitrile for 16 hours at 37°C. Samples were acidified adding formic acid to 1%, and speed
611 vac dehydration was used to evaporate acetonitrile. Peptides were desalted on C18 tips (Nest
612 group) and dried to completion. Prior to MS, peptides were resuspended in 0.1% Formic Acid
613 solution at 0.5μg/μL concentration with 1:40 synthetic iRT reference peptides (Biognosys).

614 ***Data acquisition***

615 Peptides were analyzed by liquid chromatography coupled with mass spectrometry in data
616 independent acquisition (DIA) mode essentially as described previously (Robinson et al., 2020).
617 Briefly, 4μL of digested sample were injected directly unto a 200 cm micro pillar array column
618 (uPAC, Pharmafluidics) and separated over 120 minutes reversed phase gradient at 1200
619 nL/min and 60°C. The gradient of aqueous 0.1% formic acid (A) and 0.1% formic acid in
620 acetonitrile (B) was implemented as follows: 2% B from 0 to 5 min, ramp to 4% B at 5.2 minutes,
621 linear ramp to 28% B at 95 minutes, and ramp to 46% B at 120 minutes. After each analytical
622 run, the column was flushed at 1200 nL/min and 60°C by injection of 50% Methanol at 95% B
623 for 25 minutes followed by a 10 minutes ramp down to 2% B and a 5 minute equilibration to 2%
624 B. The eluting peptides were electro sprayed through a 30 um bore stainless steel emitter
625 (EvoSep) and analyzed on an Orbitrap Lumos using data independent acquisition (DIA)
626 spanning the 400-1000 m/z range. Each DIA scan isolated a 4 m/z window with no overlap
627 between windows, accumulated the ion current for a maximum of 54 seconds to a maximum
628 AGC of 5E5, activated the selected ions by HCD set at 30% normalized collision energy, and
629 analyzed the fragments in the 200-2000m/z range using 30,000 resolution (m/z = 200). After
630 analysis of the full m/z range (150 DIA scans) a precursor scan was acquired over the 400-1000
631 m/z range at 60,000 resolution.

632 ***Peptide library generation***

633 To construct a comprehensive peptide ion library for the analysis of human BCa we combined
634 several datasets, both internally generated and external publicly available data resources were
635 utilized. First, we utilized a previously published (Berle et al., 2018) human bladder tumor
636 proteomics experiment by downloading raw files from the online data repository

637 (ProteomeXchange, PXD010260) and searching them through our internal pipeline for data
638 dependent acquisition MS analysis (Parker et al., 2016) against the uniprot human reviewed
639 canonical sequence database, downloaded July 2019, using internal peptides to perform
640 retention time alignment (Parker et al., 2015). To this library, we appended a sample specific
641 library generated from DIA-Umpire extraction of pseudo-spectra from one full set of replicates
642 from the experimental bladder tumor cell lines. A final, combined consensus spectrast library
643 containing all peptide identifications made between the internal and external dataset was
644 compiled and decoy sequences were appended.

645 ***Data analysis***

646 Peptide identification was performed as previously described in (Parker et al., 2015, 2016).
647 Briefly, we extracted chromatograms and assigned peak groups using openSWATH (Röst et al.,
648 2014) against the custom BCa peptide assay library described above. False discovery rate for
649 peptide identification was assigned using PyProphet (Teleman et al., 2015) and the TRIC (Röst
650 et al., 2016) algorithm was used to perform feature-alignment across multiple runs of different
651 samples to maximize data completeness and reduce peak identification errors. Target peptides
652 with a false discovery rate (FDR) of identification <1% in at least one dataset file, and up to 5%
653 across all dataset files were included in the final results. We used SWATH2stats to convert our
654 data into the correct format for use with downstream software MSstats27. Each individual data
655 file was intensity normalized by dividing the raw fragment intensities to that files total MS2
656 signal. MSstats (Choi et al., 2014) was used to convert fragment-level data into protein-level
657 intensity estimates via the 'quantData' function, utilizing default parameters with the exception of
658 data normalization, which was set to 'FALSE'. For plotting purposes, protein intensities were
659 VSN normalized, log-transformed, and replicate batch effects were removed using the
660 removeBatchEffect function in the limma R package. The limma package was also used to
661 calculate differential protein expression. Multiple hypothesis correction was performed using the
662 Benjamin Hochberg method.

663

664 **Whole exome sequencing**

665 ***Sample preparation***

666 All cell lines were grown for several passages in the absence of antibiotics, gemcitabine or
667 cisplatin. Cell pellets were snap frozen from sub-confluent dishes for each of the 20 cell lines
668 sequenced (5 cell lines, each with 4 derivatives: parental, Gem-resistant, Cis-resistant, GemCis-
669 resistant). gDNA isolation was performed using the Puregene cell and tissue kit (Qiagen) with
670 the addition of RNase A Solution (Qiagen) according to manufacturer's instructions. gDNA was
671 quantified using a Qubit 4.0, then sheared using a Covaris S220 Sonicator to 200bp. Libraries
672 were constructed using the Sure Select All Exon v6 library kit (Agilent) following the XT library
673 preparation workflow. Completed libraries were run on the 4200 Tape Station (Agilent) using
674 D1000 screen tape. Libraries were quantitated using the Qubit, diluted to 4nM prior to
675 verification of cluster efficiency using qPCR, then sequenced on the NovaSeq 6000 instrument
676 (Illumina) (150bp, paired-end) at the University of Colorado Cancer Center Genomics Shared
677 Resource. Mean insert size across all cell lines was 177.8 bp and mean coverage was 193.7X
678 with > 96.8% at >30X. Individual call line quality control metrics are reported in **Table S12**.

679 ***Data processing***

680 The analysis pipeline was developed using Nextflow. For the raw fastq files, Fastqc was used to
681 assess overall quality. For computational efficiency, raw sequence reads were partitioned using
682 BBMap (partition.sh) into 40 partitions. They then were aligned to the GRCh38 reference
683 genome (including decoy sequences from the GATK resource bundle) using the BWA-MEM

684 short read aligner (Li, 2013), and merged back into single BAM files using Samtools. The
685 resulting BAM files were de-duplicated using Samblaster (Faust and Hall, 2014), and sorted
686 using Samtools. These duplicate-marked bams were further passed through the GATK Base
687 Quality Score Recalibration in order to detect systematic errors made by the sequencing
688 machine when it estimates the accuracy of base calls. The dbSNP (version 146) (Sherry et al.,
689 2001), the 1000 Genome Project Phase 1 (1000 Genomes Project Consortium et al., 2015), and
690 the Mills and 1000G gold standard sets (Mills et al., 2011) were used as databases of known
691 polymorphic sites to exclude regions around known polymorphisms from analysis. After
692 alignment, Samtools (Li et al., 2009), Qualimap (Okonechnikov et al., 2016), and Picard tools
693 (2018) were run to acquire various metrics to ensure there were no major anomalies in the
694 aligned data.

695 ***Alignment***

696 bwa mem -K 100000000 -R "read_group" -t 64 -M ref_fasta read_1 read_2

697 ***Marking duplicates***

698 samtools sort -n -O SAM sample_bam | samblaster -M --ignoreUnmated

699 ***Base Quality Score Recalibration***

700 gatk BaseRecalibrator -I sample_bam -O sample.recal.table -R ref_fasta --known-sites
701 known_sites

702 ***Whole exome sequencing variant calling***

703 We used Mutect2 from the GATK toolkit for SNVs and short indels (Benjamin et al., 2019).
704 Mutect2 is designed to call somatic variants and makes no assumptions about the ploidy of
705 samples. It was run in *tumor-only* mode to maximize the sensitivity albeit at the risk of high false
706 positives. We used tumor-only mode to call variants for each cell line separately. Mutect2
707 workflow is a two steps process. In the first step, it operates in high sensitivity mode to generate
708 intermediate callsets that are further subjected to filtering to generate the final variant calls.
709 Annotation of variants was performed using Annovar (Wang et al., 2010) with the following
710 databases: refGene, cytoBand, exac03, avsnp150, clinvar_20190305, gnomad211_exome,
711 dbnsfp35c, cosmic90. Intergenic variants were removed along with variants that were identified
712 at greater than 0.001% of the population according to ExAC or gnomAD, or had a depth < 20.

713 ***Mutect2 raw callset:***

714 gatk Mutect2 -R ref_fasta -I bam_tumor -tumor Id_tumor --germline-resource germline_resource
715 -O raw_vcf

716 ***Mutect2 filtering:***

717 gatk FilterMutectCalls -V raw_vcf --stats raw_vcf_stats -R ref_fasta -O filtered_mutect2_vcf

718 ***Copy number calling using GATK***

719 Base quality score recalibrated bams were used as the input. The covered regions for the
720 exome kit were converted into bins (defining the resolution of the analysis) for coverage
721 collection. Read-counts, that form the basis of copy number variant detection, were collected for
722 each bin. The read-counts then go through denoising, modelling segments, and calling the final
723 copy ratios.

724 ***Preprocess intervals***

725 gatk PreprocessIntervals --intervals intervals_bed_file --padding 0 --bin-length 0 -R ref_fasta --
726 interval-merging-rule OVERLAPPING_ONLY -O preprocessed_intervals_list

727 ***Collect read counts***

728 gatk CollectReadCounts -I sample_bam -L preprocessed_intervals} --interval-merging-rule
729 OVERLAPPING_ONLY -O sample.counts.hdf5

730 ***Denoise read counts***

731 gatk DenoiseReadCounts -I sample.counts.hdf5 --standardized-copy-ratios
732 sample_std_copy_ratio --denoised-copy-ratios sample_denoised_copy_ratio

733 ***Model Segments***

734 gatk ModelSegments --denoised-copy-ratios denoised_copy_ratio --output-prefix id_sample -O
735 output_dir

736 ***Call copy ratio segments***

737 gatk CallCopyRatioSegments -I sample.modelled_segments -O sampled.called.segments

738 ***Cell line authentication***

739 Variant calls from the Mutect2 pipeline were filtered for each cell line to identify high confidence
740 variants according to the filtering criteria above. These high confidence variants were then
741 compared to the variants reported for all cell lines in the DepMap (https://depmap.org/portal/) for
742 the Cancer Cell Line Encyclopedia (CCLE_mutations_hg38.csv, sample_info.csv) and COSMIC
743 (CosmicCLP_MutantExport.tsv) as measured by the jaccard distance, the intersection of
744 variants divided by the union of variants. Cells listed in CCLE or COSMIC were the rank ordered
745 for each BCa cell line in this study according to the jaccard distance. Results are reported in
746 **Table S13**.

747

748 **Metabolomics**

749 ***Sample preparation***

750 Cell lines were cultured for several passages in IMDM + 10% FBS (IMDM10). Prior to
751 experiment, cells were cultured in IMDM10 to ~80% confluence and then dissociated. For
752 dissociation, cells were washed once with room temperature PBS and then incubated with PBS
753 + 0.05% Trypsin-EDTA for 10-15 minutes. Cells were neutralized with IMDM10 and then fully
754 dissociated by gentle pipetting. After dissociation, cells were counted by Trypan blue staining
755 and then replated at 1e6 cells. 24 hours after plating, cells were treated with either IMDM10 or
756 IMDM10 + 10μM cisplatin. Day 0 cell cultures were immediately processed for metabolomics
757 analysis. To prepare cell pellets for metabolomics analysis, day 0 cells were dissociated and
758 then centrifuged at 300RCF for 10 minutes at 4°C. Cells were suspended in PBS, centrifuged a
759 second time, and then resuspended in PBS and counted. Day 0 cells were centrifuged a third
760 time, the supernatants were aspirated, and the dry cell pellets were snap frozen in liquid
761 nitrogen and stored at -80°C until metabolite extraction. 72 hours after plating, cells were
762 processed for metabolomics analysis as described for the day 0 cell cultures.

763 ***Data generation and analysis***

764 Metabolites from frozen cell pellets were extracted at 2e6 cells/mL in ice cold 5:3:2
765 MeOH:acetonitrile:water. Extractions were carried out using vigorous vortexing for 30 min at
766 4°C. Supernatants were clarified by centrifugation (10 min, 18,000 g, 4°C) and 10 μL analyzed
767 using a Thermo Vanquish UHPLC coupled to a Thermo Q Exactive mass spectrometer. Global
768 metabolomics analyses were performed using a 5 min C18 gradient in positive and negative ion
769 modes (separate runs) with electrospray ionization as described in (Gehrke et al., 2019;

770 Nemkov et al., 2019). For all analyses, the MS scanned in MS^1 mode across the m/z range of
771 65 to 950. Peaks were annotated in conjunction with the KEGG database, integrated, and
772 quality control performed using Maven as described in (Nemkov et al., 2015). Data was
773 variance stabilization normalized (Huber et al., 2002), log2-transformed, and differential
774 abundance calculations were done using limma (Ritchie et al., 2015) with time and/or treatment
775 as covariates in the linear model.

776

777 **Cell Line Drug Treatments**

778 Gemcitabine (Sigma) and cisplatin (Sigma) stocks were resuspended in 0.9% saline solution
779 and tosedostat (Sigma and BOC Sciences) was resuspended in DMSO. All stocks solutions
780 were stored protected from light and kept frozen until use. For cell culture dose-response, cells
781 were seeded in 96-well tissue culture plates with 500-2000 cells per well depending on growth
782 rate and duration of experiment. Cells were seeded and allowed to attach overnight followed by
783 replacing the media with fresh, pre-warmed media just prior to treatment. Drug dilutions were
784 performed serially and using complete media (IMDM + 10% FBS) and the associated drug
785 treatments. Growth inhibition was measured using confluence estimates over time on the
786 IncuCyte ZOOM (Essen Bioscience, Ann Arbor, MI) over varying amounts of time depending on
787 each experiment. Details for timing and replicates for each experiment are included in their
788 respective figure legends.

789

790 **Antibodies and Western Blotting**

791 Whole cell lysates were prepared from cultured cells using RIPA lysis and extraction buffer
792 (ThermoScientific). Lysates from xenograft tissues were prepared using tissue protein extraction
793 reagent (T-PER) and glass tissue homogenizer. All lysates were prepared on ice and with the
794 addition of Halt protease and phosphatase inhibitor cocktail and EDTA (ThermoFisher). Protein
795 concentration of lysates were quatified with BCA protein assay (Pierce™, ThermoFisher). All
796 lysates were prepared with 4X Licor Loading buffer with DTT added boiled for 10 minutes prior
797 to gel loading. All western blots were run using PROTEAN TGX precast 4-15% or 4-20%
798 gradient gels (Biorad) and transferred to either 0.2μm or 0.44μm nitrocellulose membranes.
799 Transfer was done for 1.5-2hrs in cold TrisGlycine buffer (Biorad) with 20% methanol prior
800 blocking for 1hr at room temperature in 5% BSA in TBS-T. Primary antibodies were diluted and
801 incubated overnight at 4°C on a rocker. Membranes were washed 3 or 4 times in fresh TBS-T
802 prior a 1 hour room temperature incubation in an appropriate secondary antibody. Membranes
803 were washed 3-4 times in TBS-T, developed with enhanced SuperSignal West Pico Plus or
804 SuperSignal West Fempto (ThermoFisher) and imaged using Li-Cor Odyssey® Fc instrument.
805 Densitometry was performed using LiCor Image Studio™ software. Statistical comparisons
806 using densitometry measurements were done using a one-way ANOVA with Tukey post hoc to
807 control for the experimentwise error rate.

808

809 **Cisplatin induced NPEPPS mRNA expression**

810 Total RNA was isolated from cells and organoids using Trizol (ThermoFisher) and standard
811 phenol-chloroform based extraction methods. Residual DNA was digested with DNAse I (Life
812 technologies). cDNA synthesis was performed using Superscript II Reverse Transcriptase kit
813 (Life technologies) using random primers. RT-qPCR reactions were performed on a CFX
814 Connect Real-Time PCR Detection System thermocycler (Biorad) using TaqMan™ gene
815 expression assays for NPEPPS and HMBS as a housekeeping gene (ThermoFisher) in
816 combination with SensiFAST™ Probe No-ROX Kit (Bioline, Toronto, Canada). Expression data
817 was calculated using $2^{-\Delta\Delta Ct}$. All cell line experiments were performed in triplicate from

818 independently grown cells. Comparisons were made between control treatment (0μM cisplatin)
819 using a t-test.

820

821 **siRNA-mediated knockdown experiments**

822 NPEPPS and non-targeting siRNA smartpools were purchased from Horizon Discovery and
823 resuspended in Dharmacon 5X siRNA Buffer. Transfections were performed using
824 Lipofectamine RNAiMax (ThermoFisher) transfection reagent according to the manufacturer's
825 specifications. Briefly, cells were grown to ~60% confluence in 6-well plates prior to being
826 transfected and allowed to incubate overnight. The following day cells were trypsinized and
827 replated into 96-well plates at 1000-2000 cells per well and allowed to attach overnight. Cells
828 from the initial transfection were also replated into 6-well plates to collect protein and RNA to
829 confirm knockdown. The following day, cells were treated using their previously established
830 resistance doses of gemcitabine, cisplatin, or both (**Table S1**), and their relative growth rates
831 were measured on the IncuCyte ZOOM (Essen Bioscience, Ann Arbor, MI) over time.

832

833 **shRNA-mediated knockdown experiments**

834 Lentiviral production and transduction were carried out by the University of Colorado Cancer
835 Center Functional Genomics Shared Resources. Plasmids from The RNAi Consortium (TRC)
836 collection (TRC construct numbers TRCN0000073838, TRCN0000073839 and
837 TRCN0000073840) were used for targeting NPEPPS were selected based on predicted
838 knockdown efficiency; non-targeting controls used were SHC002 and SHC016. 2μg of target
839 shRNA construct and 2μg of 3:1 ratio of psPAX2 (Addgene) and pMD2.G (Addgene) were
840 transfected into HEK293FT cells using 2 μg of Polyethylenimine (Polysciences, Inc.). Lentiviral
841 particle containing media was filtered using 0.45μm cellulose acetate syringe filter and used for
842 transduction. Puromycin selection was performed at doses used for CRISPR library screening
843 or in some cases, cells were re-selected with higher doses of puromycin (10μg/mL), in order to
844 ensure complete elimination of non-transduced cells. Selected cells were frozen at early
845 passage and early passage cells were used for all experiments.

846

847 **Intracellular cisplatin measurements using CyTOF**

848 Cell lines were cultured for several passages in IMDM + 10% FBS. Prior to experiment, cells
849 were cultured in IMDM10 to be 50-80% confluence overnight and then treated the next day with
850 varying concentrations of cisplatin or PBS as indicated and then dissociated after 4 hours of
851 treatment. For dissociation, cells were washed twice with room temperature PBS and then
852 incubated with PBS + 0.05% Trypsin-EDTA for 10-15 minutes. Cells were neutralized with
853 IMDM10 and then fully dissociated into single-cell suspension by gentle pipetting. After
854 dissociation, cells were counted by Trypan blue staining and then placed in separate tubes at 3
855 x 10^5 cells. Individual samples were then fixed, permeabilized, and labeled using unique
856 barcodes using the Cell-ID 20-plex Pd Barcoding kit (Fluidigm) according to the manufacturer
857 protocol. Barcoded samples were pooled across cell line condition and cisplatin concentration,
858 incubated with Cell-ID Intercalator-Ir, mixed with equilibration beads and acquired on a Helios
859 mass cytometer (Fluidigm). Post-acquisition data were normalized to equilibration beads and
860 debarcoded, using the bead-normalization and single-cell-debarcoder packages from the Nolan
861 Laboratory GitHub page (https://github.com/nolanlab). Relative cisplatin intensity (defined by
862 ^{195}Platinum isotopic mass intensity) was analyzed among nucleated ^{191}Iridium+ ^{193}Iridium+
863 events (defined by Boolean gating within FlowJo 10.7.1).

Whole Genome CRISPR Screening

Plasmid library expansion and quality control

Whole genome CRISPR Screening was performed using the Human CRISPR Knockout Pooled Library (Brunello) - 1 vector system (Addgene and a gift from John Doench to the Functional Genomics Facility at the University of Colorado Anschutz Medical Campus) (Doench et al., 2016b). Two distinct plasmid expansions were performed. And the library distribution was assessed using next generation sequencing to determine the impact on overall library was modest following re-expansion. Library width was calculated as previously described (Imkeller et al., 2020; Joung et al., 2017) by dividing the 10th percentile of the library distribution by the 90th percentile using the log2 average expression of all sgRNAs in the library and found to be 6.7 and 7.13 for batch 1 and 2 respectively. All quality control metrics for each sample are reported in **Table S14**. Different screening parameters were used based on the cell line screened these are summarized in **Table S4.**

Lentivirus Production and Titration

For the two plasmid batches, two distinct protocols for lentivirus production were utilized. The first batch was generated by using Polyethylenimine, linear (PEI; Polysciences Inc.) and the second using lipofectamine 3000. For the first batch, 293FT cells were seeded at a density of 36,800 cells/cm^2 into a 4-layer CELLdisc (Greiner) using DMEM + 10% FBS along with Antibiotic and antimycotic solution. Transfection mix consisting 47.6μg pMD2G (Addgene), 95.2μg of psPAX2 (Addgene), and 190.5μg of Brunello Whole genome knockout library (Addgene) was mixed with 448μl PEI (1 mg/mL) and 3mL OptiMEM, vortexed for 30 seconds and allowed to incubate at room temperature for 20 minutes. Fresh media containing transfection mix were added to the CELLdisc using up to 270mL of media. The next day media was changed for 280mL fresh media followed by a 48 hour incubation. After this 48 hour incubation the viral supernatant was harvested and filtered through a cellulose acetate filter system (ThermoScientific) and frozen at -80°C.

The first method had low functional virus titer, so we attempted a different virus production method for subsequent screens. In the second batch of virus production we utilized lipofectamine 3000 instead of PEI, eliminated use of multilayer flasks and centrifuged to remove debris as opposed to filtering. Briefly, 293FT cells were plated in T225 flasks to be 80% confluent after 24hrs. 2hrs before transfection, media was changed and 40mL of fresh media was used per T225 flask. The lipofectamine 3000 protocol was followed according to manufacturer's instructions and scaled based on the volume of virus being prepared. For each T225 flask 2mLOptiMEM was mixed with 40μg Brunello whole genome library plasmid, 30μg of psPAX2 and 20μg of pMD2.G and 180μl of P3000. This mix was added to a tube containing 2mL OptiMEM and 128μl Lipofectamine 3000, which was scaled according to the number of T225 flasks being prepared. Transfection mix was mixed thoroughly by pipetting up and down slowly, and allowed to incubate at room temperature for 15 minutes. Transfection mix was then added dropwise to the plates of 293FT cells with gentle swirling and incubated overnight (~16hr). The following morning, the media was changed and 60mL of fresh media was added to each T225 flask. This was allowed to incubate overnight and replaced the following morning. This first lentiviral supernatant was stored at 4°C to be pooled with a subsequent 48 hour collection. Upon collection, viral supernatants had 1M HEPES added at 1%. Following the second virus collection, supernatants were pooled and centrifuged at 1250rpm for 5 minutes to pellet debris. Lentivirus was stored in polypropylene tubes as polystyrene is known to bind lentivirus, and all tubes were flash frozen in liquid nitrogen and stored at -80°C. Despite the

911 changes to the lentiviral production protocols, functional lentiviral titers were not improved using
912 these changes to the methodology, but feel it is worth noting these changes in protocol to
913 account for any possible variability associated with this change.

914 Lentivirus was titered functionally based on protocols adapted from the Broad Institute's Genetic
915 Pertubation Platform's public web portal (https://portals.broadinstitute.org/gpp/public/).

916 ***Screening Parameter Optimization***

917 All screening parameters for each cell lines including cell polybrene and puromycin sensitivity,
918 screening coverage, technical and biological replicates performed, and gemcitabine and
919 cisplatin treatment concentrations are reported in **Table S4.**

920 ***DNA Isolation***

921 Cell pellets of $2.0X10^7$ were snap frozen in liquid nitrogen in 1.5mL tubes and stored at -80 prior
922 to extraction. When possible at least $8.0X10^7$ cell were used for 4 separate genomic DNA
923 isolation which were pooled to account for any variation with pellet size. DNA isolation was
924 performed using the Puregene cell and tissue kit (Qiagen) with the addition of RNase A Solution
925 (Qiagen) according to manufacturer's instructions. DNA concentration was measured in
926 quadruplicate using either a nanodrop spectrophotometer (Thermo), Qubit® dsDNA assay (Life
927 Technologies) and the average DNA content per cell was determined.

928 ***Library preparation***

929 The minimum number of cell equivalents of gDNA to maintain equal coverage was used for
930 library preparation. In all screens, the minimum coverage based on cell number was multiplied
931 by the average gDNA content per cell for each individual cell line to determine the minimum
932 number for 10μg PCR reactions needed to maintain coverage. A minimum coverage of 500-fold
933 per sgRNA in the library was targeted for each independent sample or replicate but this was
934 increased in some cases where screening was carried out with greater depth (see **Table S4** for
935 coverage and replicate information).

936 Library preparation was performed using primers sequences designed by the Broad Institute's
937 Genetic Perturbation Platform (https://portals.broadinstitute.org/gpp/public/) and utilized a pool
938 of eight P5 primers with to introduce a stagger in reads associated with each library and sample
939 specific P7 primer that contained a unique sample index sequence for each timepoint, replicate,
940 or treatment condition to be sequenced in the same pool (**Table S11**). All library preparation
941 primers were resuspended at 100μM.

942 Each library preparation PCR reaction contained the following components: 1μl Herculase II
943 Fusion Enzyme (Agilent), 2.5μl Deoxynucleotide (dNTP) Solution Mix (New England Biolabs),
944 0.5μl P5 primer pool, 0.5μl P7 index primer, 20μl 5X Reaction Buffer (Agilent), 10μg of gDNA
945 and nuclease-free water to bring the total reaction volume to 100μl. Samples underwent 23
946 cycles of thermal cycling followed by a quality assessment by electrophoresis on 2% agarose
947 gel to ensure consistent library amplification across multiple wells and samples for each plate.

948 Each unique library had 10μl pooled from all PCR reactions performed on that unique sample
949 and mixed thoroughly. 50-100μl of the pooled library preparation reactions was used to perform
950 magnetic bead-based purification and elimination of any residual free primer using a 0.8X ratio
951 SPRIselect beads (Beckman Coulter) according to the manufacturer's instructions. Libraries
952 were then assessed for appropriate amplicon size and complete elimination of free primer peaks
953 using the High Sensitivity ScreenTape Assay on the Tape Station2200 (Agilent) and quantified
954 using the qPCR-based quantification in order to ensure only NGS-compatible amplicon was
955 quantified using the Library Quant ROX Low Kit (Kapa Biosystems) on a QuantStudio™ 6

956 Realtime PCR System (ThermoFisher). Following qPCR quantification, all libraries were
957 normalized to a standard concentration (typically 20-40nM) depending on the lowest
958 concentration library to be pooled, and then requantified by qPCR to ensure all samples were
959 within ~10-20% of the pool mean target concentration. After confirming accurate library
960 quantification and normalization, samples were pooled at an equimolar ratio and submitted for
961 sequencing. Libraries were sequenced on the NovaSeq 6000 instrument (Illumina) (150bp,
962 paired-end) at the University of Colorado Cancer Center Genomics Shared Resource.

963 ***CRISPR screening bioinformatic pipeline and analysis***

964 sgRNA counts were extracted directly from R1 raw sequence reads using a custom perl script
965 that uses regular expression string matching to exactly match sgRNA sequence flanked by 10
966 bases of vector sequence. The vector sequence was allowed to have one error before and after
967 the sgRNA sequence. sgRNAs were tabulated for each sample based on the sgRNA sequence
968 (**Table S15**). The sgRNA IDs of the Brunello library were updated to current HGNC gene names
969 using the Total Approved Symbols download from HGNC, accessed on 9/1/2020
970 (https://www.genenames.org/download/statistics-and-files/). Transcript IDs were matched when
971 possible and when matches were not found, past symbols and aliases were updated to current
972 names. Finally, 5 sgRNAs with missing updated gene names were manually curated using
973 literature searches. Library distribution was calculated using the caRpools R package (Winter et
974 al., 2016) (**Table S11)**. The DESeq2 R package (Love et al., 2014) was used to calculate
975 differential abundance of genes (**Table S5**). Gene counts were generated using the sum of
976 counts for sgRNAs of the same gene. Synthetic Lethality compared GemCis day 19 and
977 GemCis day 25 vs. Saline day 19 and Saline day 25 with the day as a covariate. In the
978 comparison integrating all cell lines, cell line was additionally modeled as a covariate. Gene
979 essentiality was calculated by comparing Saline day 25 to Saline day 0 and in the integrated all
980 cell lines comparison, cell line was modeled as a covariate. Common synthetic lethal genes
981 were defined as being statistically significantly differentially lost (FDR < 0.05 and Log2 FC < 0)
982 in each of the 5 cell lines. Gene set enrichment analysis (GSEA) was performed using the fgsea
983 R package run with 10000 permutations (Sergushichev, 2016) with the KEGG and Reactome
984 gene sets from MSigDB (Liberzon et al., 2011). Heatmaps were generated with the
985 ComplexHeatmap R package following z-score transformation (Gu et al., 2016). Other plots
986 were generated using the ggplot2 R package.

987 **Xenograft experiments**

988 Six-week-old, female Nu/J mice (Jackson Labs) were allowed to acclimate for at least one week
989 prior to initiating any experiments. Mice had free access to food and water in pathogen-free
990 housing and cared for in accordance NIH guidelines and all experiments were performed under
991 protocols approved by the University of Colorado Denver Institutional Animal Care and Use
992 Committee (IACUC).

993 For KU1919-GC xenografts, cells that had been stably transduced with non-targeting control
994 (shCtrl1, SHC002) and NPEPPS (shN39, TRCN0000073839) shRNA constructs. Mice were
995 divided into groups of 22 and 23 for the non-targeting control and NPEPPS shRNA constructs
996 respectively. Mice were injected with $4X10^6$ cells in phenol red- and serum-free RPMI mixed
997 with equal volume Matrigel Matrix (Corning) to total 100μl volume. Tumors were allowed to
998 engraft for 9 days following injection and mice were randomized based on tumor volume within
999 each shRNA condition into groups of 11 or 12 to be treated with combination gemcitabine plus
1000 cisplatin or DPBS. Treatment was initiated 13 days post-inoculation with dosing adjusted based
1001 on individual mouse weight.

1002 Cisplatin (Sigma) and gemcitabine hydrochloride (BOC Sciences) were both resuspended in
1003 0.9% saline and stored protected from light at -80°C as individual aliquots. Prior to treatment

fresh aliquots of gemcitabine and cisplatin were thawed and diluted to their final concentration with 1X DPBS (Gibco). Mice were treated three times weekly on a Monday, Wednesday and Friday schedule for four weeks total. All mice in the gemcitabine plus cisplatin treated groups were given 50mg/kg gemcitabine and 2mg/kg cisplatin that were mixed and administered as a single intraperitoneal injection, while control mice were administered an equivalent volume of DPBS.

Mouse health was monitored daily and all tumor volume measurements and weights were measured 3x weekly schedule. Tumor volume was calculated using the formula (L x W^2)/2, for which *L is* the length of the long axis and *W* is the width of the axis perpendicular to the long axis measurement. All measurements were performed using digital calipers. Animal were humanely euthanized with CO_2 followed by cervical dislocation when tumors reached a predetermined endpoint of $2cm^3$ or when weight loss exceeded 15% body weight. Mice that were removed from study due to weight loss were censored in the survival analyses.

Linear mixed-effects model of tumor growth

Linear mixed-effects models were used to model longitudinal observations of xenograft tumor growth volumes normalized by their corresponding baseline volume. Mixed-effects models from the R-package *lme4* (Bates et al., 2015) and Satterthwaite's approximation for degrees of freedom for the fixed effects from *lmerTest* (Kuznetsova et al., 2017) were used for model fitting and inspection in the R statistical software (4.0.3). Volume changes compared to baseline were log_2-transformed. The final model was structured as:

$$log_2\left(\frac{y_{i,t}}{y_{i,:}}\right) = \beta_0 + \beta_1 x_{i,t} + \beta_2 x_{i,t}^2 + \beta_3 x_{i,t} KD_i + \beta_4 x_{i,t} GC_i + \beta_5 x_{i,t} KD_i\, GC_i + \gamma_{0,i} + \gamma_{1,i} x_{i,t} + \varepsilon_{i,t}$$

where β is the fixed effects capturing population-level trends, γ is the normally distributed random effects capturing individual-level variation, ε is the i.i.d. normally distributed residual term, i is the unique individual identifier, t notes the time points, $x_{i,t} \in \{2, 4, 5, 7, 9, 11, 14, 16, 18, 21, 23, 25, 28\}$ depicted days since initiating interventions, $y_{i,:}$ is tumour volume at baseline prior to treatments upon randomization, and $y_{i,t}$ were the observed tumour volumes over the treatment period measured in mm^3. The model was fit using Restricted Maximum Likelihood and built iteratively until the underlying model assumptions and model convergence criteria were met. To this end, a quadratic growth term (β_2) was added on top of the linear growth term (β_1) and intercept (β_0), allowing slightly non-linear relative growth patterns to be captured by the otherwise linear model. Binary indicators $KD_i \in \{0,1\}$ and $GC_i \in \{0,1\}$ were used to model knockdown of NPEPPS (KD), GemCis therapy (GC), or both combined. The corresponding model terms were captured in β_3, β_4 and β_5, respectively. Finally, the model allows for individual-specific random effects for intercept ($\gamma_{0,i}$) and linear growth slope ($\gamma_{1,i}$). Shapiro-Wilk test was used to examine the underlying normality assumption for $\gamma_{0,i}$ and $\gamma_{1,i}$ with *p*=0.1373 and *p*=8901, respectively, indicating that these random effects followed underlying assumptions of normality. After inspection of the residual plots (**Figure S9B**), this final model was deemed suitable for population-level statistical inference via the fixed effects. This population-level model fits are visualized in **Figure S9A**. These population-level estimates are as follows:

Fixed effect	**Estimate**	**Std. error**	**df**	**t**	***p*-val**
β_0 (intercept)	0.05054	0.08422	54.28	0.600	0.55091
β_1 (linear slope)	0.1236	0.01493	65.52	8.276	8.92e-12 ***
β_2 (quadratic slope)	0.00308	0.0002242	389	13.740	< 2e-16 ***
β_3 (knockdown)	-0.0605	0.01821	44.97	-3.322	0.00178 **
β_4 (GC)	-0.1063	0.01821	44.97	-5.837	5.49e-07 ***

β_5 (knockdown + GC)	-0.1233	0.01791	45.28	-6.884	1.47e-08 ***

1045

1046 **Survival analyses from TCGA**

1047 Copy number and gene expression data for patients with muscle-invasive bladder cancer in the
1048 TCGA cohort (PanCancer Atlas) were downloaded from cBioPortal (Cerami et al., 2012; Gao et
1049 al., 2013). Patient survival and platinum-based treatment annotation was from our previous work
1050 (Goodspeed et al., 2019). Patients were separated into treatment groups, platinum-based
1051 treatment (n = 100) or unrecorded treatment (n = 204), and then stratified based on copy
1052 number gain or amplification, or mRNA upregulation (z-score > 1) of LRRC8A or LRRC8D. The
1053 Logrank test was used to test the difference in overall survival between the stratified patient
1054 groups.

1055 **Tumor-derived Organoids**

1056 ***Culture of the organoids***

1057 Human bladder tissue was obtained from the Erasmus MC Bladder Cancer Center, Rotterdam,
1058 the Netherlands. Bladder tumor-derived organoids from biopsies obtained through TURBT or
1059 cystectomy were isolated and cultured using a method based on (Mullenders et al., 2019) with
1060 modifications (Akbarzadeh/Scholtes et al. in prep). Briefly, Bladder tissues were washed with
1061 Advanced DMEM/F12 (Gibco) supplemented with 10mM HEPES (Gibco), 1% GlutaMax (Gibco)
1062 and 100 µg/ml primocin (InvivoGen), henceforth Ad+++. Tissue was minced and incubated at
1063 37°C with the digestion solution (collagenase 2.5mg/ml in EBSS) and isolated cells were passed
1064 through 70µM strainer (Falcon), washed with Ad+++ and seeded in 50 µl drops of BME (R&D
1065 system) containing 10000-15000 cells in 24 well suspension plates (Greiner). Bladder tumor
1066 organoids were cultured in a culture medium containing Ad+++ supplemented with 1 × B-27
1067 (Gibco), 1.25 mM N-acetylcysteine (Sigma), 10 mM nicotinamide, 20µM TGFβ receptor inhibitor
1068 A83-01, 100ng/ml recombinant human FGF10 (Peprotech), 25 ng/ml recombinant human FGF7
1069 (Peprotech), 12.5 ng/ml recombinant human FGF2 (Peprotech), 10µM Y27632 Rho Kinase
1070 (ROCK) Inhibitor (Sigma) and conditioned media for recombinant Rspondin (2.5% v/v), and
1071 Wnt3A (2.5% v/v). The medium was changed every three days. Organoids were passaged at a
1072 1:3 to 1:6 ratio every 7 days using cell dissociation solution- non enzymatic (Sigma) and plated
1073 in fresh BME matrix droplets.

1074 ***Drug screening***

1075 Organoids were collected 7 days after passaging, passed through a 100µM strainer and 1000
1076 organoids were seeded per well of a 48-well plate in BME matrix droplets. After 24h, cisplatin
1077 (Sigma) resuspended in PBS was added at different concentrations (2, 10, 25, and 50 µM) with
1078 or without tosedostat (20 µM) (Tocris) resuspended in DMSO. Organoids were cultured for the
1079 first 6 days in the presence of drugs followed by drug withdrawal, where organoids were grown
1080 in organoid culture media for 10 days. The entire content of the wells in different treatment
1081 groups was collected, washed and reseeded after disaggregation in fresh BME, and cultured for
1082 6 days. Cell viability was assayed using alamarBlue (Invitrogen) according to the manufacturer's
1083 instructions after 6 days of drug incubation, 10 days of drug withdrawal, and 6 days post
1084 reseeding. Viability data was normalized using organoid wells treated with vehicle control.

1085 ***SNaPshot mutation and microarray analysis***

1086 Tumor, organoid, and matched normal DNA was isolated using with the QIAmp DNA Mini-Kit
1087 (Qiagen) according to the manufacturer's protocol. Presence hotspot mutations in the *TERT*
1088 promoter sequence chr5:1,295,228C>T, chr5:1,295,248G>A and chr5:1,295,250C>T
1089 [GRCh37/hg19]), *FGFR3* (R248Q/E, S249C, G372C, Y375C, A393E, K652E/M) and *PIK3CA*

(E542K, E545G/K and H1047R) were assessed on tumor, normal and organoid DNA by SNaPshot mutation analysis with the same methods as previously described (Allory et al., 2014; Hurst et al., 2009; Junker et al., 2008). Copy number aberration analysis was performed using single-nucleotide polymorphism (SNP) microarrays (Affymetrix GSA V3, Affymetrix) on primary tumor DNA, matched DNA collected from normal urothelium plus stromal tissue from the same sample but from a distant location from the tumor, and organoid DNA using standard protocols. SNP data (log-R ratio, B-allele frequency) were visualized to identify potential CNVs via Biodiscovery Nexus CN7.5. (Biodiscovery) and the GenomeStudio genotyping module (Illumina).

Organoid phenotyping and tumor histology

Tissue processing and H&E staining was performed using standard procedures. For hematoxylin-eosin (H&E) staining of organoids, wells of BME-embedded organoids were fixated with 4% formalin (Sigma) and 0.15% glutaraldehyde (produced in-house) at room temperature for 2 hours. Fixated BME and organoids were washed with PBS and engulfed in 2.5% Low-Melting Agarose (Sigma) prior to paraffin embedding. H&E staining was performed on 4μM paraffin sections of both tumor and organoid tissue. Stained whole-slides, as well as prior 3D organoid cultures were imaged by bright-field microscopy (Olympus IX70).

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Author Contributions

Conceptualization: R.T.J., T.M., T.Z., D.T., J.C.C.
Methodology: R.T.J., A.G., H.V., A.J., T.D.L., M.A., E.C., S.P., T.M., T.Z., D.T., J.C.C.
Software: A.G., T.D.L., M.J., R.L., J.C.C.
Validation: R.T.J., A.G., M.S., A.J., C.T., M.V.O., T.Z.
Formal Analysis: R.T.J., A.G., M.S., H.V., T.D.L., M.V.O., M.J., E.C., S.P., T.Z., D.T., J.C.C.
Investigation: R.T.J., M.A., M.S., H.V., A.J., C.T., M.V.O., E.C., S.P., T.Z., J.C.C.
Resources: A.J., C.T., E.C., R.L., T.Z.
Data Curation: R.T.J., A.G., A.J., T.D.L., M.J., R.L., J.C.C.
Writing – Orginal Draft: R.T.J., A.G., D.T., J.C.C.
Writing – Review & Editing: R.T.J., A.G., M.A., M.S., H.V., M.V.O., M.J., T.D.L., E.C., S.P., T.M., T.Z., D.T., J.C.C.
Visualization: R.T.J., A.G., M.A., M.S., M.V.O., T.D.L., E.C., T.Z., D.T., J.C.C.
Supervision: R.L., T.M., D.T., J.C.C.
Project Administration: R.T.J., A.J., T.M., T.Z., D.T., J.C.C.
Funding Acquisition: T.M., T.Z., D.T, J.C.C.

Declaration of Interests

J.C.C. is co-founder of PrecisionProfile.

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1140

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Figure Legends

Figure 1. Project overview and synthetic lethal screen results. (**A**) Human bladder cancer cell lines were made resistant to cisplatin, gemcitabine, or gemcitabine plus cisplatin through dose escalation. All cell lines were profiled using -omic technologies. The gemcitabine plus cisplatin resistant cells were subjected to a pooled CRISPR screen to identify synthetic lethal gene-to-drug relationships. (**B**) Aggregate gene set enrichment results for the synthetic lethal screen results across all cell lines reveal DNA damage response and repair pathways. Each tick mark represents a gene in the associated pathway. The bars are normalized enrichment scores (NES) with the FDR corrected p-values reported in the bars. (**C**) When results from all cell lines were evaluated individually, a total of 46 commonly synthetic lethal genes were identified; all counts are reported in **Figure S2**. (**D**) The percentage change in the aggregate of the sgRNAs targeting the 46 commonly synthetic lethal genes are reported across saline (PBS) or gemcitabine plus cisplatin treatment arms of the CRISPR screen.

Figure 2. NPEPPS is identified as a commonly upregulated and synthetic lethal hit. (**A**) Differential gene expression of the 46 common synthetic lethal genes as measured by RNAseq across all cell lines, comparing the treatment resistant derivative (Gem-, Cis-, GemCis-resistant) to the associated parental cell line. Asterisks indicate a statistically significant result (moderated t-test, *FDR < 0.05). The top bar plot is the aggregate count of significant results across all 15 comparisons. Genes are ranked by the count of statistically significant upregulated hits. (**B**) RNAseq (compared to parentals; *FDR < 0.05), (**C**) mass spectrometry proteomics (compared to parentals, *FDR < 0.25), and (**D**) CRISPR screen results (*FDR < 0.05) for NPEPPS. (**E**) Representative immunoblots and densitometry quantification for independent triplicates (mean ± SEM) for NPEPPS in all cell lines (*FDR < 0.05).

Figure 3. Genetic and pharmacological inhibition of NPEPPS resensitizes GemCis-resistant cells. (**A**) NPEPPS was found to be synthetic lethal with cisplatin in a CRISPR screen for 27 genotoxic agents in RPE1 cells by (Olivieri et al., 2020). (**B**) Immunoblot for NPEPPS across several control and shRNAs targeting NPEPPS. (**C, D**)KU1919-GemCis cells with knockdown of NPEPPS treated with increasing doses of cisplatin or gemcitabine. A total of 3 technical replicates per dose (mean ± SEM). Independent experiments are reported in **Figure S5**. (**E**) NPEPPS mRNA is upregulated in response to cisplatin treatment in a dose dependent manner if both KU1919 parental and GemCis-resistant cells. Independent triplicate experiments are shown (mean ± SEM) (t-test compared to 0μM; *p < 0.05, **p < 0.05). (**F**) Pharmacologic targeting of NPEPPS with tosedostat in GemCis-resistant cells treated with cisplatin, gemcitabine, and gemcitabine plus cisplatin treatment. A total of 3 technical replicates per dose are shown (mean ± SEM). Independent experiments are reported in **Figure S6**.

Figure 4. NPEPPS interacts with volume regulated anion channel (VRAC) subunits LRRC8A and LRRC8D to mediate cisplatin response. (**A**) NPEPPS is found to interact with all VRAC subunits, LRRC8A-E, using the BioPlex interactome (Huttlin et al., 2020). (**B, C**) Knockout of LRRC8A and LRRC8D through the CRISPR screen resulted in increased cell growth upon gemcitabine plus cisplatin treatment in GemCis-resistant cell lines (moderated t-test; *FDR < 0.05). (**D**) Genes ranked based on $\log_2$ fold change from the synthetic lethal CRISPR screens across all cell lines. LRRC8A-E and the 46 common synthetic lethal genes are labeled. (**E, F**) LRRC8A and LRRC8D gene expression measured by RNAseq (compared to parentals; *FDR < 0.05). (**G**) Volcano plot of metabolites measured from KU1919-GemCis cells with or without NPEPPS knockdown (shN39). Time and treatment (cisplatin 10μM) were covariates in the linear model to calculate differential expression using a moderated t-test;

horizontal grey line is $-\log_{10}$(FDR = 0.05). (**H**)Taurine abundance measured in KU1919-GemCis cells with non-targeting shRNA controls or shRNA targeting NPEPPS. Cells were also measured at 48 hours with cisplatin treatment or PBS, vehicle control. (**I**) Intracelullar cisplatin levels were measured after 4 hours of treatment at 10μM cisplatin using CyTOF, with the number of cells analyzed as indicated. The median Pt 195 measurement for the KU1919-parental line = 158, for the KU1919-GemCis line = 44.5, and for the KU1919-GemCis- shN39 line = 77. (**J**) Immunoblot of LRRC8A, LRRC8D, and γH2AX in KU1919-GemCis-shCtrl1 and KU1919-GemCis-shN39 cells treated with PBS or 10μM cisplatin for 38 hours.

Figure 5. Genetic and pharmacological inhibition of NPEPPS resensitizes *in vivo* and *ex vivo* models of bladder cancer to cisplatin-based chemotherapy. (**A**) Tumor volume of KU1919-GemCis xenografts measured over time and across 4 treatment groups considering non-targeting shRNA controls (shCtrl1), shRNA targeting NPEPPS (shN39), PBS vehicle control (PBS), or gemcitabine plus cisplatin treatment (GemCis). (**B**) Survival analysis of xenograft models with a defined endpoint of a tumor volume > $2cm^3$. Logrank test was applied to test significance. (**C, D, E**) Patient tumor-derived organoids were derived from patient tumors that did not respond to gemcitabine plus cisplatin chemotherapy. Organoids were treated for 6 days, allowed to recover for 10 days, then reseeded at different ratios depending on overall growth, then allowed to grow for an additional 6 days. Cell viability was quantified in triplicate after reseeding (t-test; * $p<0.05$, ** $p<e^{-3}$, *** $p<e^{-5}$). (**F**) Survival analysis of muscle-invasive bladder cancer in the TCGA stratified based on copy number amplification, gain or overexpression of LRRC8A or LRRC8D. Patients all had a record of cisplatin-based chemotherapy treatment. (**G**) Survival analysis for patients stratified by LRRC8A or LRRC8D as in (**F**), but did not have any record of cisplatin-based treatments.

Figure 6. Proposed model of NPEPPS-mediated cisplatin resistance. Normal functioning cells will import cisplatin through the volume regulated anion channels (VRAC), with LRRC8A and LRRC8D being the primary subunits. A mechanism of cisplatin resistance is to have inherently down-regulated VRACs. We propose that NPEPPS interacts with LRRC8A or LRRC8D directly to decrease VRAC activity, which prevents export of taurine and import of cisplatin, hence driving cisplatin resistance.

Figure S1. Dose response for all cell line derivatives. Parental, cisplatin-resistant, gemcitabine-resistant, and gemcitabine plus cisplatin (GemCis)-resistant cells for each of the five bladder cancer cell lines were treated with increasing doses of cisplatin or gemcitabine. Dose response curves were calculated. The resistant derivative lines were more resistant to the associated drug. Data represent a single experiment with each condition measured in technical triplicate wells (mean ± SEM).

Figure S2. Venn diagram of synthetic lethal genes. The number of genes that were statistically significantly synthetic lethal (FDR < 0.05) are reported.

Figure S3. Resistance mechanisms of gemcitabine. (**A**) Gemcitabine is processed through several mechanisms to have its downstream treatment impact, leading to chain termination, impaired DNA repair, and finally apoptosis. Deoxycitadine kinase (DCK) and ribonucleotide reductase subunit M1 (RRM1) are known mechanisms of gemcitabine resistance. (**B**) Transcript and protein expression are reported for both genes (ND = not detected; *FDR < 0.01). In addition, copy number results are shown for all cell lines.

1570 **Figure S4. Synthetic lethal screen comparison.** (**A**) The results from the CRISPR screen
1571 reported in this study were correlated with the synthetic lethal screen results for 27 genotoxic
1572 agents from (Olivieri et al., 2020). The screen results from the five cell lines reported here are
1573 highlighted by the black box and were highly correlated. The drug that was most correlated with
1574 our screen results was cisplatin. (**B**) The synthetic lethal results for the top 46 hits reported here
1575 are shown including results from the Olivieri et al dataset. 42 genes are reported as four genes
1576 did not map to the Olivieri et al dataset. The synthetic lethal scores were z-score normalized for
1577 display purposes and show that the 42 genes were also synthetic lethal in the three cisplatin
1578 screens, but not in the gemcitabine screen.
1579
1580 **Figure S5. shRNA and siRNA targeting NPEPPS resensitizes to cisplatin and gemcitabine**
1581 **plus cisplatin.** (**A**) Dose response for shRNA targeting NPEPPS (shN38 = TRCN0000073838;
1582 shN39 = TRCN0000073839; shN40 = TRCN0000073840) or non-targeting controls (shCtrl1 =
1583 MISSION pLKO.1-puro Non-Mammalian shRNA Control; shCtrl2 = MISSION pLKO.1-puro Non-
1584 Target shRNA Control). Cells were treated with cisplatin or gemcitabine separately. Data for
1585 KU1919 shown from two independent experiments with 3 technical replicates per dose (mean ±
1586 SEM). Data from 253J represent a single experiment with 3 technical replicate wells per dose
1587 (mean ± SEM). (**B**) Immunoblot analysis of NPEPPS protein across the different shRNAs and
1588 cell lines. (**C**) Cell confluency was measured using Incucyte Zoom across untransfected cells,
1589 siRNA controls (siCtrl), and siRNA transfected cells targeting NPEPPS. Cells were treated with
1590 PBS or gemcitabine plus cisplatin (GemCis) at the resistant doses for each cell line (**Table S1**).
1591 Data shown represent a single experiment with 3 technical replicates (mean ± SEM) per
1592 timepoint represented.
1593
1594 **Figure S6. Response to tosedostat in combination with gemcitabine, cisplatin, or**
1595 **gemcitabine plus cisplatin.** Dose response curves for serial increased doses of tosedostat at
1596 120 hours at the associated resistant doses of gemcitabine or cisplatin for each cell line (**Table**
1597 **S1**). Data represent triplicate wells per dose, per experiment (mean ± SEM).
1598
1599
1600 **Figure S7. LRRC8D gene expression and copy number alterations.** Gene expression shows
1601 that LRRC8D is not detected in the TCCSUP-Cis and TCCSUP-GemCis cell lines. This result is
1602 supported by a focal copy number deletion detected using WES data that is specific to the
1603 TCCSUP-Cis and TCCSUP-GemCis cell lines (*FDR < 0.01).
1604
1605 **Figure S8. Intracellular cisplatin measured using CyTOF.** KU1919-Parental, KU1919-
1606 GemCis and KU1919-GemCis-shN39 cells were treated with PBS vehicle (0μM) or 10μM
1607 cisplatin for 4 hours and then intracellular cisplatin was measured using cytometry by time of
1608 flight (CyTOF). Results from (**A**) replicate 1 and (**B**) replicate 2 are shown. (**C**) Immunoblotting
1609 for γH2AX, LRRC8A, and LRRC8D were performed in KU1919-GemCis-shCtrl1 or KU1919-
1610 GemCis-shN39 cells after 48 hours of treatment with 10μM cisplatin or PBS vehicle control. This
1611 is an independent replicate from the immunoblot reported in **Figure 4J**.
1612
1613 **Figure S9. Xenograft tumor growth modeling and validation.** (**A**) Fixed effects population-
1614 level model fit (thick lines) overlaid on top of the observations (grey lines). Longitudinal tumor
1615 volumes were divided by the baseline tumor volume and then log_2-transformed before
1616 modelling. (**B**) Residuals for the final mixed-effects model (y-axis) coupled with the original
1617 observations (x-axis). No systematic trends were detected in model diagnostics, suggesting that
1618 the single fitted model successfully captured variation over the treatment arms and individuals.
1619 (**C**) Immunoblot on the left is from KU1919-GemCis cells that were injected into mice to

establish tumors. Immunoblot on the right are from tumor samples after mice met the endpoint of the experiment of > 2cm^3.

Figure S10. Patient tumor-derived organoids generated from muscle-invasive bladder cancer patients treated with platinum-based chemotherapy. (**A**) Clinical time course of muscle-invasive bladder cancer patients from whom patient tumor-derived organoid lines were initiated after radical cystectomy. Patients are not lost to follow-up but censored, at the time of publication. TURBT = transurethral resection of bladder tumor. (**B**) Bright-field images of organoids together with H&E staining of patient tumors and organoids. (**C**) SNaPshot mutation analysis of patient tumors and organoids on hotspot mutations in fibroblast growth factor receptor 3 (*FGFR3*) or telomerase reverse transcriptase (*TERT*). Copy number plot of the entire genome for the primary tumor and organoids from patient 65 demonstrate the genomic similarity of the tumor-derived organoids. Intensity values of each bin are plotted as colored dots, with each chromosome represented by a different color. (**D**) Overall experimental design for treating the organoids with cisplatin, tosedostat, or the combination cisplatin plus tosedostat. Organoids were withdrawn from treatment after 6 days of treatment and reseeded after 16 days of treatment. Cell viability was measured using alamarBlue.

Figure S11. Patient tumor-derived organoids derived from patient 1 and treated with tosedostat and/or cisplatin. (**A**) Organoids were treated for 6 days at the indicated concentrations of drug. Cell viability was quantified using alamarBlue. (**B**) Organoids were then withdrawn from treatment for 10 days and assayed again for cell viability using alamarBlue. (**C**) Organoids were reseeded and allowed to regrow for 6 days and cell viability was tested using alamarBlue. (**D**) Comparison of tosedostat doses over the course of treatment and withdrawal. All comparisons were done in triplicate (t-test; * $p<0.05$, ** $p<e^{-3}$, *** $p<e^{-5}$).

Figure S12. Patient tumor-derived organoids derived from patient 2 and treated with tosedostat and/or cisplatin. (**A**) Organoids were treated for 6 days at the indicated concentrations of drug. Cell viability was quantified using alamarBlue. (**B**) Organoids were then withdrawn from treatment for 10 days and assayed again for cell viability using alamarBlue. (**C**) Organoids were reseeded and allowed to regrow for 6 days and cell viability was tested using alamarBlue. All comparisons were done in triplicate (t-test; * $p<0.05$, ** $p<e^{-3}$, *** $p<e^{-5}$).

Figure S13. Patient tumor-derived organoids derived from patient 3 and treated with tosedostat and/or cisplatin. (**A**) Organoids were treated for 6 days at the indicated concentrations of drug. Cell viability was quantified using alamarBlue. (**B**) Organoids were then withdrawn from treatment for 10 days and assayed again for cell viability using alamarBlue. (**C**) Organoids were reseeded and allowed to regrow for 6 days and cell viability was tested using alamarBlue. All comparisons were done in triplicate (t-test; * $p<0.05$, ** $p<e^{-3}$, *** $p<e^{-5}$).

Tables

Feature	KU1919	T24	TCCSUP	5637	253J
Sex	Male	Female	Female	Male	Female
Stage	T3	Ta	N/A	N/A	T4
Grade	G3	G3	G4	G2	G4
Base47 Subtype	N/A	Basal	Basal	Luminal	Basal
TP53		Y126X	E349X		
HRAS		G12V			

NRAS	Q61R				
PIK3CA			E545K		E545G
TERT					
ARID1A	Y1052X				
KMT2D	T2441Pfs*44			Q2813X	
KDM6A	Q915X				
FAT1		S2682X	D1536N		
KMT2C		R4225X; A3559T			
ERBB2				S310F	
ERBB3		E1219K			
EP300		C1201Y			
FBXW7			S66X		
ASXL2			E330Q		
ATM				H1876Q	
AKT1	E17K				
RYR2		R2401H			
NFE2L2					G81S
RB1			LOSS	Y325X	
E2F3			AMP	AMP	
PPARG				AMP	
CCND1	AMP				
CDKN2A	LOSS				LOSS

1662 **Table 1.** Clinicopathologic characteristics and genetic drivers for five cell lines.

Figure 1

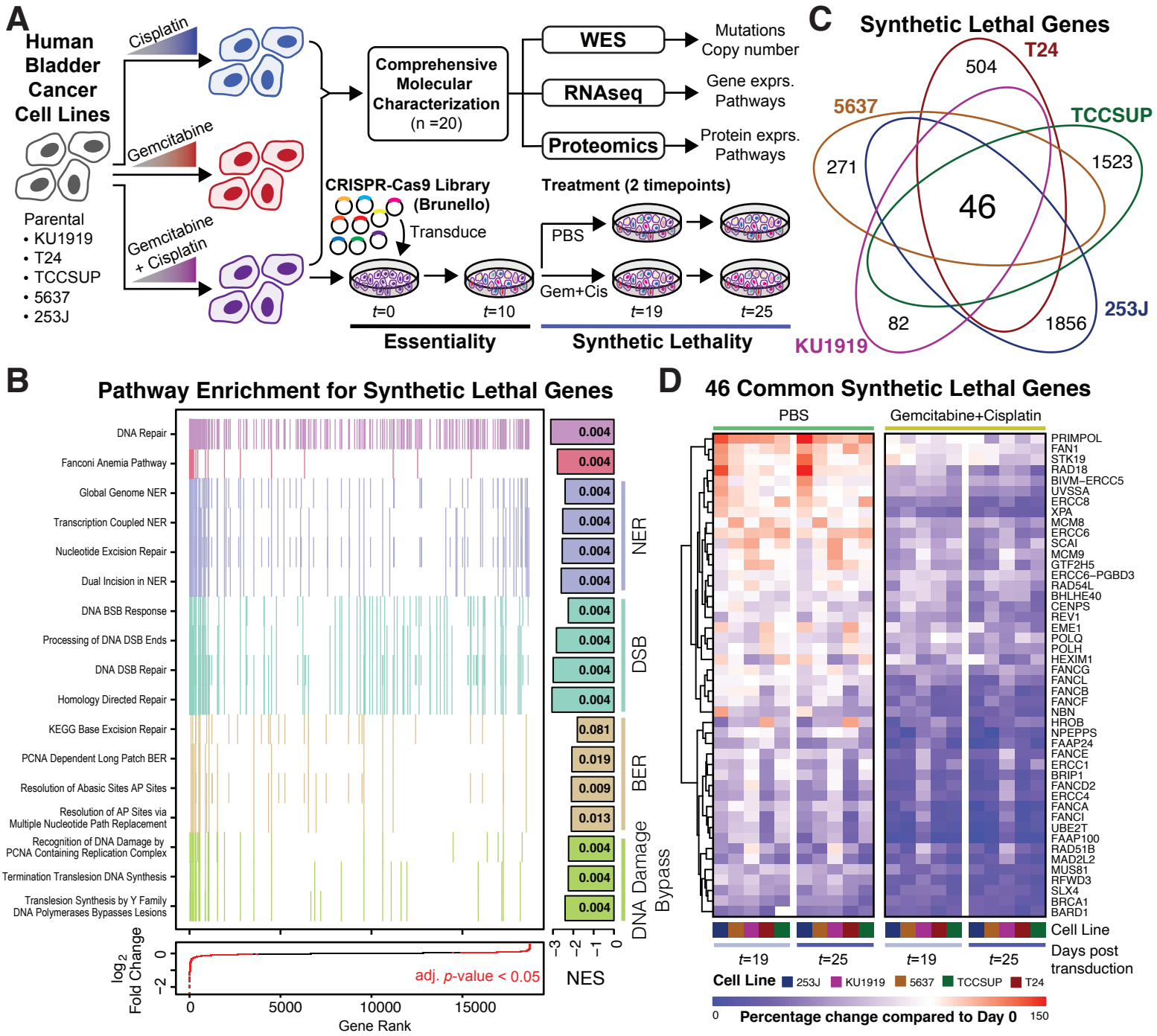

A
Human Bladder Cancer Cell Lines
Parental
• KU1919
• T24
• TCCSUP
• 5637
• 253J
Cisplatin
Gemcitabine
Gemcitabine + Cisplatin
Comprehensive Molecular Characterization (n =20)
WES
RNAseq
Proteomics
Mutations Copy number
Gene exprs. Pathways
Protein exprs. Pathways
CRISPR-Cas9 Library (Brunello)
Transduce
Treatment (2 timepoints)
PBS
Gem+Cis
t=0
t=10
t=19
t=25
Essentiality
Synthetic Lethality
C
Synthetic Lethal Genes
T24
5637
TCCSUP
253J
KU1919
504
271
1523
46
82
1856
B
Pathway Enrichment for Synthetic Lethal Genes
DNA Repair
Fanconi Anemia Pathway
Global Genome NER
Transcription Coupled NER
Nucleotide Excision Repair
Dual Incision in NER
DNA BSB Response
Processing of DNA DSB Ends
DNA DSB Repair
Homology Directed Repair
KEGG Base Excision Repair
PCNA Dependent Long Patch BER
Resolution of Abasic Sites AP Sites
Resolution of AP Sites via Multiple Nucleotide Path Replacement
Recognition of DNA Damage by PCNA Containing Replication Complex
Termination Translesion DNA Synthesis
Translesion Synthesis by Y Family DNA Polymerases Bypasses Lesions
0.004
0.081
0.019
0.009
0.013
NER
DSB
BER
DNA Damage Bypass
NES
log2 Fold Change
adj. p-value < 0.05
Gene Rank
D
46 Common Synthetic Lethal Genes
PBS
Gemcitabine+Cisplatin
PRIMPOL
FAN1
STK19
RAD18
BIVM−ERCC5
UVSSA
ERCC8
XPA
MCM8
ERCC6
SCAI
MCM9
GTF2H5
ERCC6−PGBD3
RAD54L
BHLHE40
CENPS
REV1
EME1
POLQ
POLH
HEXIM1
FANCG
FANCL
FANCB
FANCF
NBN
HROB
NPEPPS
FAAP24
FANCE
ERCC1
BRIP1
FANCD2
ERCC4
FANCA
FANCI
UBE2T
FAAP100
RAD51B
MAD2L2
MUS81
RFWD3
SLX4
BRCA1
BARD1
Cell Line
Days post transduction
Cell Line 253J KU1919 5637 TCCSUP T24
Percentage change compared to Day 0

Figure 2

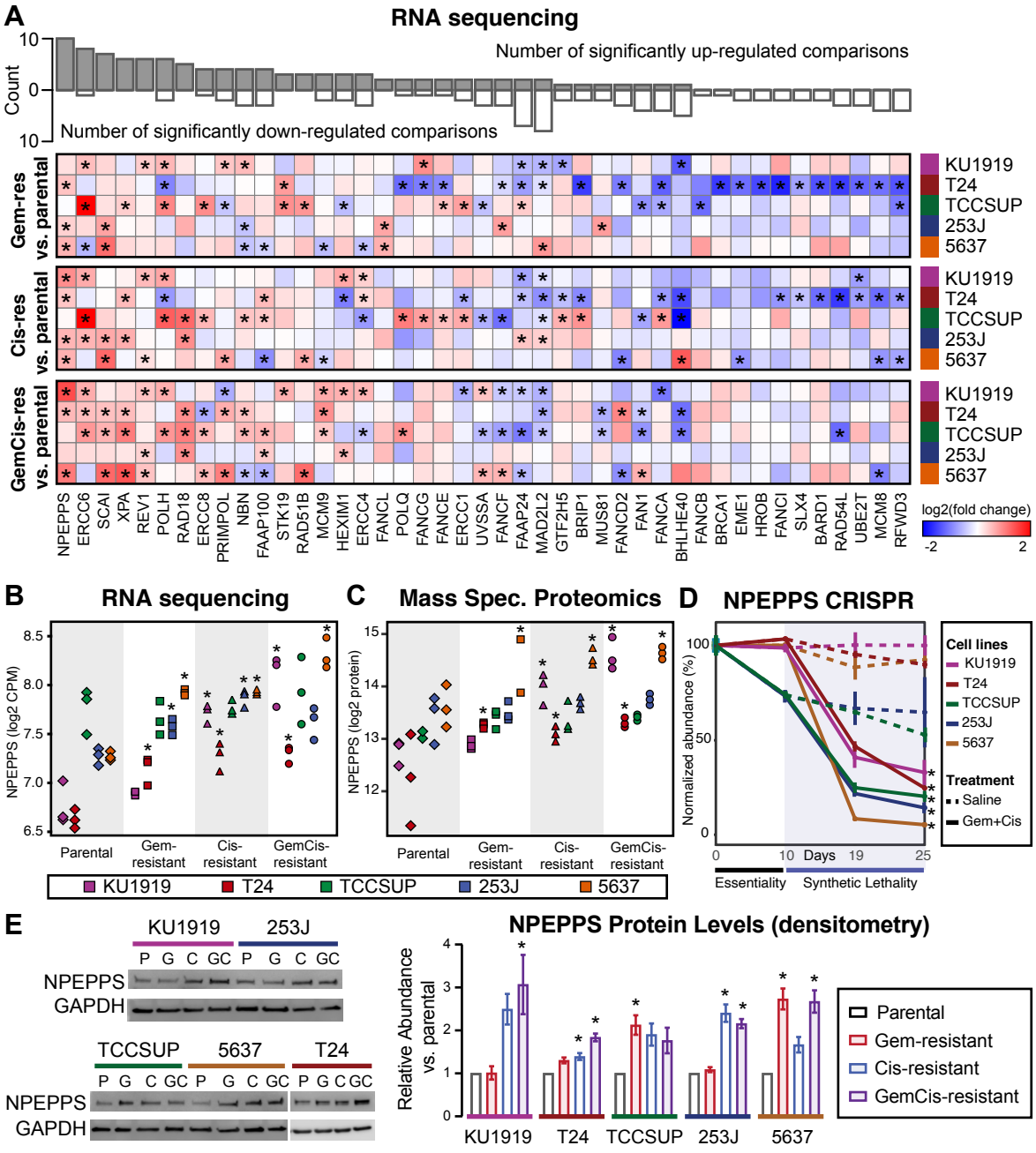

A
RNA sequencing
Number of significantly up-regulated comparisons
Count
Number of significantly down-regulated comparisons
Gem-res vs. parental
Cis-res vs. parental
GemCis-res vs. parental
KU1919
T24
TCCSUP
253J
5637
NPEPPS ERCC6 SCAI XPA REV1 POLH RAD18 ERCC8 PRIMPOL NBN FAAP100 STK19 RAD51B MCM9 HEXIM1 ERCC4 FANCL POLQ FANCG FANCE ERCC1 UVSSA FANCF FAAP24 MAD2L2 GTF2H5 BRIP1 MUS81 FANCD2 FAN1 FANCA BHLHE40 FANCB BRCA1 EME1 HROB FANCI SLX4 BARD1 RAD54L UBE2T MCM8 RFWD3
log2(fold change)
-2
2
B
RNA sequencing
NPEPPS (log2 CPM)
Parental
Gem-resistant
Cis-resistant
GemCis-resistant
C
Mass Spec. Proteomics
NPEPPS (log2 protein)
D
NPEPPS CRISPR
Normalized abundance (%)
Days
Cell lines
Treatment
Saline
Gem+Cis
Essentiality
Synthetic Lethality
E
KU1919
253J
TCCSUP
5637
T24
P G C GC
NPEPPS
GAPDH
NPEPPS Protein Levels (densitometry)
Relative Abundance vs. parental
Parental
Gem-resistant
Cis-resistant
GemCis-resistant

Figure 3

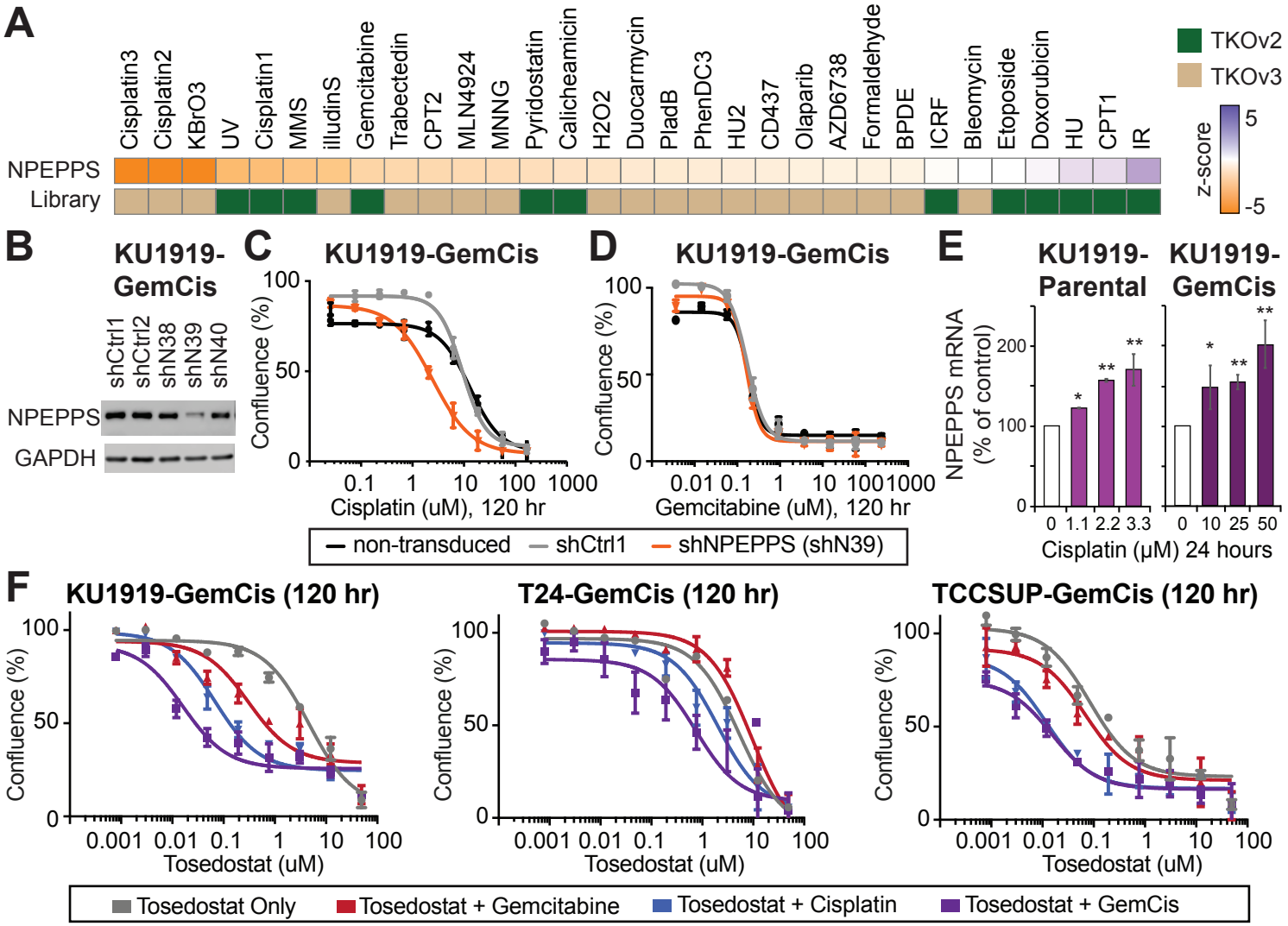

A
Cisplatin3
Cisplatin2
KBrO3
UV
Cisplatin1
MMS
illudinS
Gemcitabine
Trabectedin
CPT2
MLN4924
MNNG
Pyridostatin
Calicheamicin
H2O2
Duocarmycin
PladB
PhenDC3
HU2
CD437
Olaparib
AZD6738
Formaldehyde
BPDE
ICRF
Bleomycin
Etoposide
Doxorubicin
HU
CPT1
IR
NPEPPS
Library
TKOv2
TKOv3
z-score
5
-5
B
KU1919-GemCis
shCtrl1
shCtrl2
shN38
shN39
shN40
NPEPPS
GAPDH
C
KU1919-GemCis
Confluence (%)
Cisplatin (uM), 120 hr
D
KU1919-GemCis
Gemcitabine (uM), 120 hr
non-transduced
shCtrl1
shNPEPPS (shN39)
E
KU1919-Parental
KU1919-GemCis
NPEPPS mRNA (% of control)
Cisplatin (μM) 24 hours
F
KU1919-GemCis (120 hr)
T24-GemCis (120 hr)
TCCSUP-GemCis (120 hr)
Tosedostat (uM)
Tosedostat Only
Tosedostat + Gemcitabine
Tosedostat + Cisplatin
Tosedostat + GemCis

Figure 4

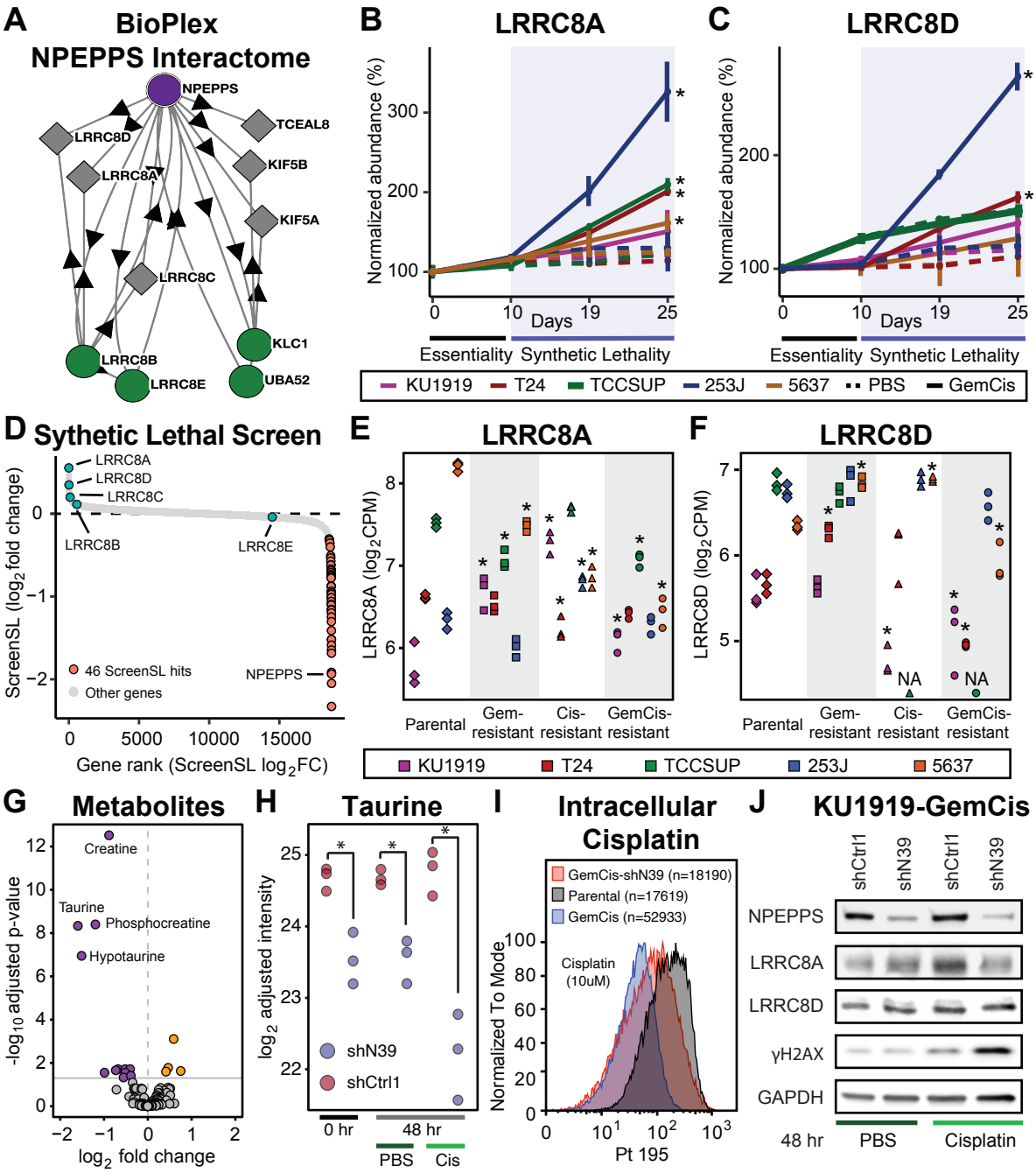

A
BioPlex
NPEPPS Interactome
NPEPPS
TCEAL8
LRRC8D
KIF5B
LRRC8A
KIF5A
LRRC8C
KLC1
LRRC8B
UBA52
LRRC8E
B
LRRC8A
Normalized abundance (%)
Days
Essentiality
Synthetic Lethality
C
LRRC8D
Normalized abundance (%)
Days
Essentiality
Synthetic Lethality
KU1919
T24
TCCSUP
253J
5637
PBS
GemCis
D
Sythetic Lethal Screen
LRRC8A
LRRC8D
LRRC8C
LRRC8B
LRRC8E
NPEPPS
46 ScreenSL hits
Other genes
ScreenSL (log2 fold change)
Gene rank (ScreenSL log2FC)
E
LRRC8A
LRRC8A (log2CPM)
Parental
Gem-resistant
Cis-resistant
GemCis-resistant
F
LRRC8D
LRRC8D (log2CPM)
NA
Parental
Gem-resistant
Cis-resistant
GemCis-resistant
KU1919
T24
TCCSUP
253J
5637
G
Metabolites
Creatine
Taurine
Phosphocreatine
Hypotaurine
-log10 adjusted p-value
log2 fold change
H
Taurine
log2 adjusted intensity
shN39
shCtrl1
0 hr
48 hr
PBS
Cis
I
Intracellular Cisplatin
GemCis-shN39 (n=18190)
Parental (n=17619)
GemCis (n=52933)
Cisplatin (10uM)
Normalized To Mode
Pt 195
J
KU1919-GemCis
shCtrl1
shN39
shCtrl1
shN39
NPEPPS
LRRC8A
LRRC8D
γH2AX
GAPDH
48 hr
PBS
Cisplatin

Figure 5

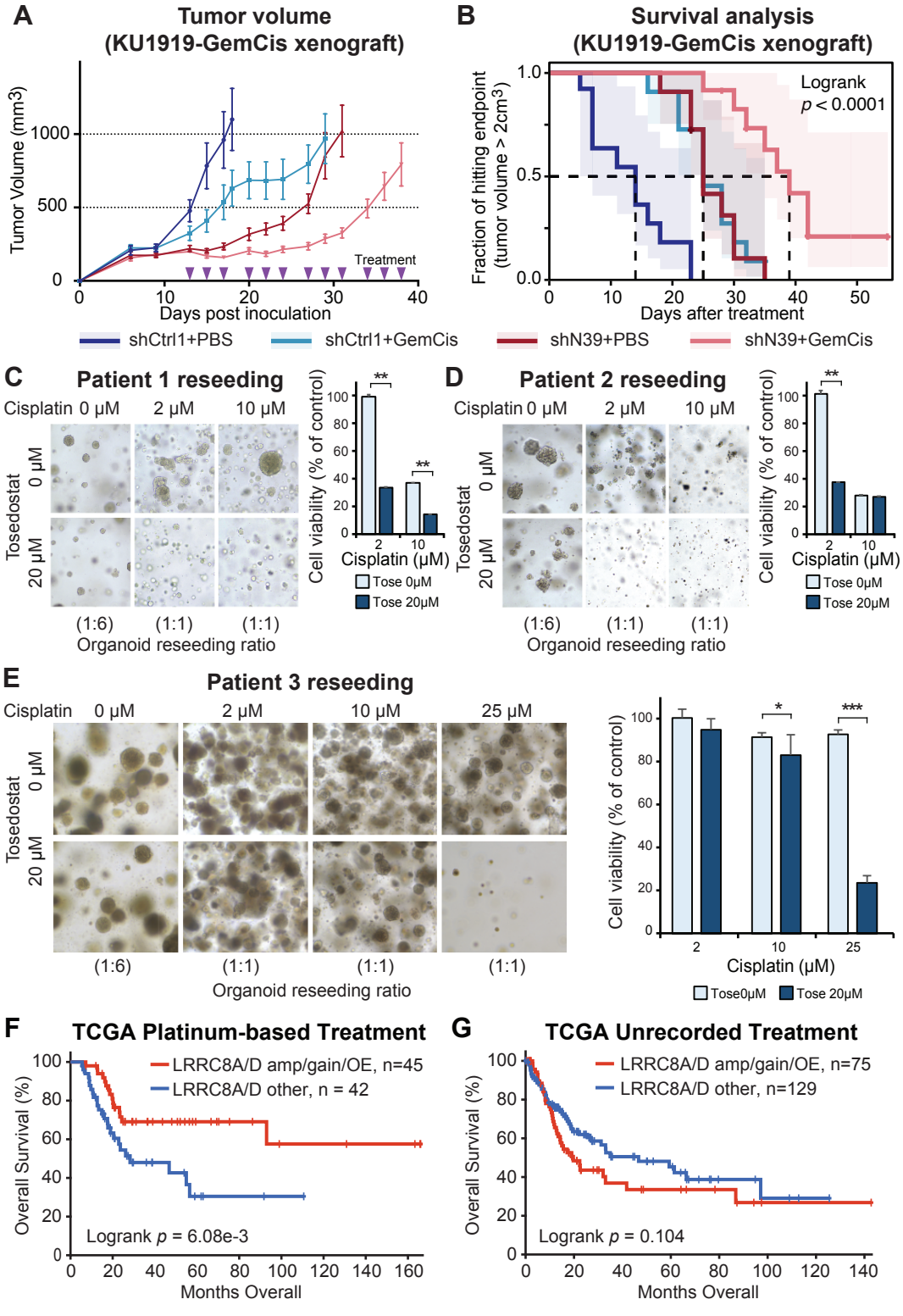
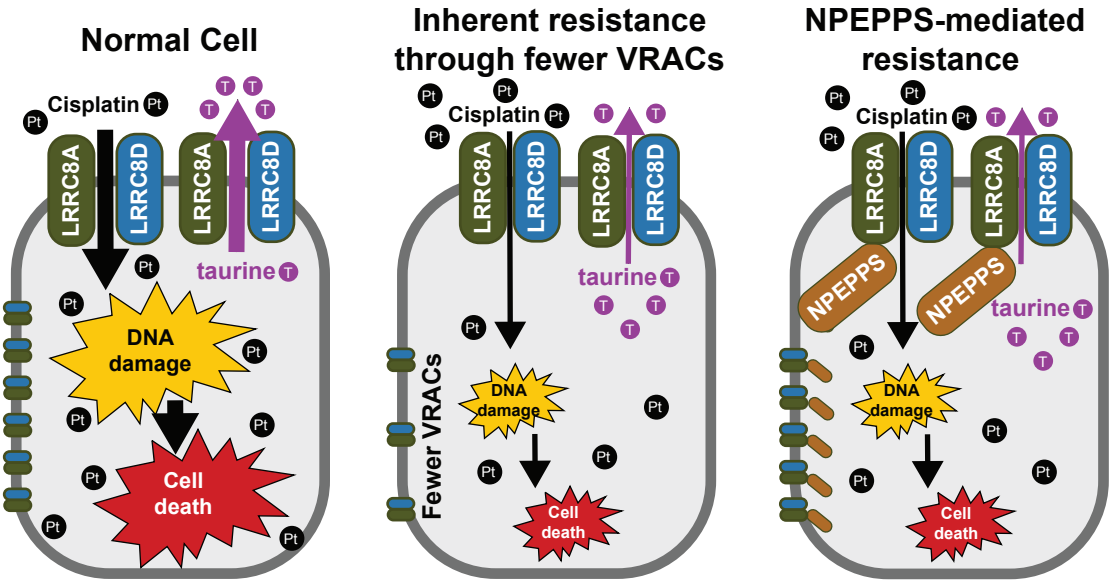

A
Tumor volume
(KU1919-GemCis xenograft)
Tumor Volume (mm3)
Treatment
Days post inoculation
B
Survival analysis
(KU1919-GemCis xenograft)
Fraction of hitting endpoint
(tumor volume > 2cm3)
Logrank
p < 0.0001
Days after treatment
shCtrl1+PBS
shCtrl1+GemCis
shN39+PBS
shN39+GemCis
C
Patient 1 reseeding
Cisplatin 0 μM
2 μM
10 μM
Tosedostat
0 μM
20 μM
(1:6)
(1:1)
(1:1)
Organoid reseeding ratio
Cell viability (% of control)
Cisplatin (μM)
Tose 0μM
Tose 20μM
D
Patient 2 reseeding
Cisplatin 0 μM
2 μM
10 μM
Tosedostat
0 μM
20 μM
(1:6)
(1:1)
(1:1)
Organoid reseeding ratio
Cell viability (% of control)
Cisplatin (μM)
Tose 0μM
Tose 20μM
E
Patient 3 reseeding
Cisplatin
0 μM
2 μM
10 μM
25 μM
Tosedostat
0 μM
20 μM
(1:6)
(1:1)
(1:1)
(1:1)
Organoid reseeding ratio
Cell viability (% of control)
Cisplatin (μM)
Tose0μM
Tose 20μM
F
TCGA Platinum-based Treatment
LRRC8A/D amp/gain/OE, n=45
LRRC8A/D other, n = 42
Overall Survival (%)
Logrank p = 6.08e-3
Months Overall
G
TCGA Unrecorded Treatment
LRRC8A/D amp/gain/OE, n=75
LRRC8A/D other, n=129
Overall Survival (%)
Logrank p = 0.104
Months Overall

Figure 6


Normal Cell
Cisplatin
Pt
LRRC8A
LRRC8D
LRRC8A
LRRC8D
T
taurine
DNA damage
Cell death
Inherent resistance through fewer VRACs
Fewer VRACs
NPEPPS-mediated resistance
NPEPPS