Supporting Information

Genome editing-enabled HTS assays expand drug target pathways for Charcot-Marie-Tooth disease

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Supplementary Materials and Methods

Generation and maintenance of the S16 cell line expressing Gaussia luciferase

TALE arrays listed below were cloned into TALEN expression vector bearing TALE domain truncation points that enable genome editing activity at endogenous loci (*1*) using the following sequences of the repeat variable residues:

HD-NI-NG-HD-NG-NI-HD-NN-NG-NN-NI-NG-HD-HD-NG and

NI-NN-NI-HD-NN-NN-NI-NG-NN-NN-NG-NN-HD-NN-NG-HD-NK, which target the following sequences (target sites in capital letters): CATCTACGTGATCC tgcggaaacgcgaatgaggcgccc GACGCACCATCCGTCT. The primers used to assay TALEN cleavage in the Cel-1 endonuclease assay (*1*) are:

F: 5'-TGG AGA ACC CTC CAG TTA CC-3', R: 5'-AGC AAT CCC CAC TCA ACT GT-3'

To make the secreted nanoluciferase line, a 2nd pair of TALENs was prepared by Sidansai Biotechnology to the following sequence, with individual binding sites in capital letters

TCCCGCTGGCCCTCCTT agtggcatcatctacgtg ATCCTGCGGAAACGCGAatga

Recombination cassettes were generated by cloning the GLuc reporter between two ~800 bp recombination arms flanking the *Pmp22* stop codon. Reporters were cloned such that luciferase was in frame with the *Pmp22* ORF, with an intervening 2a ribosome stuttering sequence. In addition, a furin cleavage site was placed at the 3' end of *Pmp22* to remove the C-terminal 2a peptide. The S16 cells were transfected with the two TALEN pairs and the recombination cassettes using the Amaxa nucleofection instrument as described (*2*). Individual clones were

screened for GLuc reporter activity, and correct insertion was determined by PCR and Southern blot.

For the secNLuc clones, recombination cassettes contained both secNLuc and GFP. After transfection, GFP-expressing clones were sorted using a FACSAria II sorter (BD Biosciences) at the UW Comprehensive Cancer Center Flow Laboratory. After expansion of sorted cells, a second round of flow cytometry showed a robust GFP signal, and cells were sorted individually into 96-well plates containing S16-conditioned medium. After expansion of the colonies, 25uL aliquots of culture supernatant were collected from all wells and transferred to a black 96-well luminometer plate. Clones expressing secNLuc were screened to identify several that yielded a PCR fragment indicating recombination at the *Pmp22* locus. PCR primers used a primer upstream of the 5' recombination arm and a reverse primer in the 2a sequence:

Pmp22 rec. F: AGGTGGTCAAACTTGAACATG, 2a R: CACGTCACCGCATGTTAGAAG

The genetically modified cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine growth serum (Hyclone). S16 cells were transfected with control and Sox10 siRNA as described (*2*) using the Amaxa 4D nucleofection instrument using SE Cell line solution according to manufacturer's directions.

Cell-based Gaussia luciferase (GLuc) and Nanoluciferase (NLuc) assays

The GLuc-expressing S16 cells were dispensed into white solid-bottom 1536 well plates (Cat# 00019130, Aurora, Carlsbad, CA) in fresh assay medium using a multidrop combi dispenser (Thermo Fisher Scientific). Compounds were transferred to the plates in 23nl by a pin tool (Kalypsys, San Diego, CA) in the concentration range of 1.6nM to 57μM along with DMSO and titrations of Bortezomib positive control from top 575 nM (S1013, Selleck Chemicals, Houston, TX) plated in column 1-4 in each plate. The plates were incubated for 24h at 37°C, and one volume of BioLux *Gaussia* Luciferase Flex assay reagent (E3308, New England BioLabs, Ipswich, MA) was added to them using a BioRAPTR FRD (Beckman Coulter, Brea, CA) for a luciferase reporter read-out measured by a ViewLux plate reader (PerkinElmer, Waltham, MA) (See **Supplementary Table 1** for more detail). Small scale experiments employed a previously described protocol for measuring GLuc activity (*3*), using either a Promega GloMax plate reader or a single tube Monolight 3010 luminometer. NLuc-expressing S16 cells were plated and treated similar to the GLuc cells and assays for measuring nanoluciferase activity were purchased from Promega (Madison, WI).

Biochemical GLuc and NLuc assays

Medium was collected from the culture flasks of either GLuc-expressing or NLuc-expressing S16 cells prior to cell plating and filtered through a 0.22μm filter. Four μl of GLuc enzyme-containing medium were plated in white solid-bottom 1536 well plates as described above. Compounds were transferred to the plates and DMSO and titrations of U-74389G (U5882, Sigma-Aldrich, St. Louis, MO) positive control from top concentration of 115μM were added to the media as in the cell-based assays. Secreted GLuc medium was incubated for 24h at 37°C, and assayed for enzyme luminescence as described above.

Two μl of NLuc assay substrate (Nano-Glo luminescence assay, Promega, Madison, WI) was plated with a BioRAPTR FRD and treated with either compounds or DMSO and titrations of Cilnidipine (C1493, Sigma-Aldrich) positive control from top concentration of 115 μM in each plate as in the cell-based assays. NLuc substrate reagent and compounds were incubated for 5m at room temperature and one volume secreted NLuc medium was added with a BioRAPTR FRD. NLuc enzyme luminescence was measured by a ViewLux plate reader as above.

CellTiter-Glo assay

The GLuc-expressing and NLuc-expressing S16 cells were dispensed into white solid-bottom 1536 well plates, treated and incubated with compounds, DMSO and titrations of Digitonin (D141, Sigma-Aldrich) positive control from top concentration of 115 μM controls as in the respective reporter cell-based assays. The plates were incubated for 24h at 37°C, and one volume of CellTiter-Glo luminescent cell viability assay reagent (Promega) was added to them using a BioRAPTR FRD. Luminescence was measured as described above.

Quantitative reverse transcription-PCR analysis

qRT-PCR was carried out essentially as previously described (*4*). In six well plates cells (2×10^5 cells/well) were plated and treated with either DMSO or bryostatin (final concentration of 1 or 10 nM). After 24h, the cells were harvested using an RNeasy Plus Micro kit (Qiagen) for purification of RNA, 1μg of which was converted into cDNA using superscript III first-strand synthesis supermix (Invitrogen) according to a manufacturer's protocol. Quantitative PCR was performed in either SYBR green-based reactions or Taqman-based customized 384-well micro fluidic arrays using a ViiA7 system (Applied biosystems, Foster City, CA). See **Supplementary Table 3** for primer sequences. Abundance of gene expression was first normalized to beta-actin (ActB, NM_031144.2) serving as an endogenous loading control across samples and then determined relative to the untreated sample for each gene using the comparative Ct method (*5*).

Western blot analysis

S16 cells were plated in 6 well tissue culture plates and treated with each siRNA as described [21]. After 24h, lysates were harvested and analyzed by immunoblotting for PMP22 (1:1000, Cat# ab61220, Abcam) as described [21].

Data analysis for qHTS

Data from each assay was normalized plate-wise to corresponding intra-plate controls as described previously (*6*). The same control data sets were also used for the calculation of Z' factors from each assay (*7*). In all assays, wells containing 0.6% DMSO, the solvent for the test compounds, were used as no-response controls. For the GLuc assay, the luminescence signal, presumed to be proportional to the change in expression level of the gene construct, from each drug-containing well on the plate was normalized to the median readings of n=16 wells of 0.57 μM bortezomib and n=32 wells of DMSO-treated wells from the same plate. Similarly, for NLuc: n=16 0.57 μM bortezomib treated wells, n=16 DMSO wells; for CellTiter-Glo on the S16 GLuc and secNluc cell lines: n=16 wells 57.5 μM digitonin and n=16 (GLuc) or n=32 (NLuc) DMSO wells. For the enzyme supernatant assays, GLuc sample data was normalized to 115 μM

U74389G (n=16 wells) and NLuc sample data to 57.5 μM Cilnidipine, with DMSO as neutral controls (n=16 and 32 wells, respectively). MSR values (*8*) were calculated using the cumulative potency differences of the de-interlaced duplicate control titrations from each plate as previously described (*9*), tabulated in Microsoft Excel. The normalized data from each assay plate set was corrected using DMSO-only containing assay plates read at the start and end of each assay run, and the resulting inter-plate titration data fit, using in-house software, to the standard hill equation and dose-response curves automatically classified as described previously (*10*). Dose-response plots were created with Prism (GraphPad) for 2-axis plots, and Origin (OriginLab) for 3-axis plots.

To discern if the response of the assay system for each re-acquired compound was best fit by a 3- or 4- (Hill Equation) or 5-parameter (bell-shaped curve) model, the aggregated data sets for each of 4 or 8 runs of the compounds for each assay type were fit using GraphPad Prism. The equation with the lower number of parameters was used as the null hypotheses, and the equation with more parameters used as the alternate hypotheses and fits compared using the extra sum-of-squares F test. The simpler model was selected unless the P value was greater than 0.05. For 3- or 4- parameter fits, the absolute value of the activity span normalized to bortezomib was constrained to fall below 150, and for 5-parameter fits, the absolute value of the overall span was constrained below 300. The equation with the highest number of parameters was selected from the pairwise F tests. Reported EC_{50} values for bell curves were selected from the lower-concentration side if the span (top - bottom) values for the fits exceeded 0.5 times the median data range for the 4 runs of the compound, such that the ascending-slope was the dominant feature of the curve fit. Otherwise, the higher concentration descending-slope value was reported as the EC_{50}.

Supplementary Tables

Supplementary Table 1. *qHTS protocol table*

Supplementary Table 2. *Reporter cell line assay performance summary statistics*

Supplementary Table 3. *Primer assay IDs for Applied Biosystems qPCR micro fluidic card*

Supplementary Figure Legends

Supplementary Figure 1. *S16 secNLuc reporter cell line isolation and characterization.* (**A**) Plot shows GFP positive cells obtained by flow cytometry following transfection with TALEN pair and secNluc/GFP recombination cassette to enrich for GFP positive cells. Sorted cells were first gated using side scatter and forward scatter to obtain single cells, and assayed for GFP fluorescence at 525 nm on the y-axis. The x-axis is a far red channel (710 nm) as a negative control to ensure that fluorescence is from GFP. The upper box contains cells that were sorted into individual wells for further analysis. (**B**) Schematic illustrating a section of the genomic structure of the *Pmp22* secNLuc-P2A-GFP locus and primers used to characterize correct

integration of the reporters. Multiple independent clones of the S16 reporter line were tested for insertion at the *Pmp22* locus using PCR. A reverse primer situated in the 2a sequence and a forward primer in the *Pmp22* intron (upstream of the recombination arm, see arrows in diagram were used to amplify a 900 bp fragment in properly recombined clones. Standards shown are 750 and 1000 bp. (**C**) Characterization of genomic clones for secNLuc activity (see *Methods* for assay details).

Supplementary Figure 2. *PDF file containing primary qHTS CRCs selected for potency and low cytotoxicity.* Data based on selection of compounds with a ≥ 10-fold ΔIC_{50} between S16 reporter assay(s) and the cytotoxicity of the compound as measured by Cell TiterGlo. Empty fields for S16NLuc data for bryostatin and romidepsin reflect changes in library composition between when the assays were conducted.

Supplementary Figure 3. *PDF file containing the CRCs from the follow-up assays.* Across the panel, chemical structure, S16 NLuc, S16 GLuc, S16 element-driven FLuc, and S16 cytotoxicity.

Supplementary Figure 4. *GLuc—secNLuc correlation of steroid class.* (**A**) The steroid group arranged according to steroid type, 31 glucocorticoids (open squares), 9 corticosteroids (back solid circles), 7 androgens (open red triangles), 2 progesterones (open blue triangles), and estorgen (open blue circle) and the steroid glycoside Digitonin (solid red circle). Note that compounds that fall at 0 for one cell line may have activity but it fell below a defined cut off, see SI for concentration response curves. Data are the mean of n=4 determinations, error is the SD. (**B**) Example CRCs from apparent non-correlative responses in most cases result from some experimental values exceeding error thresholds. (**C**) Example CRCs from several instances of Prednisolone tested in the follow-up. Two of the four samples (2, 3) pass the error threshold for both the Gluc and Nluc reporter assays. Two other examples (1, 4) either fail in the NLuc or GLuc reporter, though visual inspection suggest the steroids are active with similar EC_{50}s. (**D**) Example of the different pharmacological profile observed from a progesterone agonist, Flugestone (left) and progesterone antagonist, Mifepristone (right).

Supplementary Figure 5. *Extended analysis of dihydropyridines.* (**A**) Chemical structures of representative dihydropyridines (DHPs) found active in the S16 secNLuc reporter assay. (**B**) Activity of DHPs on NLuc activity as determined from S16 secNLuc cell growth medium for varying percentages of media after pre-incubation for either 10 minutes (circles) or 24 h (squares)

References

1. Miller, J. C., Tan, S., Qiao, G., Barlow, K. A., Wang, J., Xia, D. F., Meng, X., Paschon, D. E., Leung, E., Hinkley, S. J., Dulay, G. P., Hua, K. L., Ankoudinova, I., Cost, G. J., Urnov, F. D., Zhang, H. S., Holmes, M. C., Zhang, L., Gregory, P. D., and Rebar, E. J. (2011) A TALE nuclease architecture for efficient genome editing, *Nat Biotechnol 29,* 143-148.

2. Gokey, N. G., Srinivasan, R., Lopez-Anido, C., Krueger, C., and Svaren, J. (2012) Developmental regulation of microRNA expression in Schwann cells., *Mol Cell Biol 32*, 558-568.
3. Tannous, B. A. (2009) Gaussia luciferase reporter assay for monitoring biological processes in culture and in vivo, *Nat Protoc 4*, 582-591.
4. Jang, S. W., Lopez-Anido, C., MacArthur, R., Svaren, J., and Inglese, J. (2012) Identification of drug modulators targeting gene-dosage disease CMT1A, *ACS Chem Biol 7*, 1205-1213.
5. Livak, K. J., and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, *Methods 25*, 402-408.
6. Inglese, J., Auld, D. S., Jadhav, A., Johnson, R. L., Simeonov, A., Yasgar, A., Zheng, W., and Austin, C. P. (2006) Quantitative high-throughput screening: a titration-based approach that efficiently identifies biological activities in large chemical libraries, *Proc Natl Acad Sci U S A 103*, 11473-11478.
7. Zhang, J. H., Chung, T. D., and Oldenburg, K. R. (1999) A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays, *J Biomol Screen 4*, 67-73.
8. Eastwood, B. J., Farmen, M. W., Iversen, P. W., Craft, T. J., Smallwood, J. K., Garbison, K. E., Delapp, N. W., and Smith, G. F. (2006) The minimum significant ratio: a statistical parameter to characterize the reproducibility of potency estimates from concentration-response assays and estimation by replicate-experiment studies, *J Biomol Screen 11*, 253-261.
9. MacArthur, R., Leister, W., Veith, H., Shinn, P., Southall, N., Austin, C. P., Inglese, J., and Auld, D. S. (2009) Monitoring compound integrity with cytochrome P450 assays and qHTS, *J Biomol Screen 14*, 538-546.
10. Southall, N. T. (2009) *Enabling the Large-Scale Analysis of Quantitative High-Throughput Screening Data*, in *Handbook of Drug Screening* (Seethala, R., and Zhang, L., Eds.) 2nd ed., pp 442-464, CRC Press.