CRISPR–Cas9 screens in human cells and primary neurons identify modifiers of C9ORF72 dipeptide-repeat-protein toxicity

Nicholas J. Kramer1,2,6, Michael S. Haney1,6, David W. Morgens1, Ana Jovičić15, Julien Couthouis1, Amy Li1, James Ousey1, Rosanna Ma1, Gregor Bieri1,2, C. Kimberly Tsui1, Yingxiao Shi3, Nicholas T. Hertz4, Marc Tessier-Lavigne4, Justin K. Ichida3, Michael C. Bassik1* and Aaron D. Gitler1*

Hexanucleotide-repeat expansions in the C9ORF72 gene are the most common cause of amyotrophic lateral sclerosis and frontotemporal dementia (ALS/FTD). The nucleotide-repeat expansions are translated into dipeptide-repeat (DPR) proteins, which are aggregation prone and may contribute to neurodegeneration. We used the CRISPR–Cas9 system to perform genome-wide gene-knockout screens for suppressors and enhancers of C9ORF72 DPR toxicity in human cells. We validated hits by performing secondary CRISPR–Cas9 screens in primary mouse neurons. We uncovered potent modifiers of DPR toxicity whose gene products function in nucleocytoplasmic transport, the endoplasmic reticulum (ER), proteasome, RNA-processing pathways, and chromatin modification. One modifier, TMX2, modulated the ER-stress signature elicited by C9ORF72 DPRs in neurons and improved survival of human induced motor neurons from patients with C9ORF72 ALS. Together, our results demonstrate the promise of CRISPR–Cas9 screens in defining mechanisms of neurodegenerative diseases.

myotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) are devastating human neurodegenerative disorders. ALS is associated with progressive loss of motor neurons from the brain and spinal cord, thus leading to muscle weakness, paralysis, and ultimately death, usually 2–5 years after symptom onset. FTD, the second most common cause of dementia, is associated with a range of cognitive and behavioral symptoms, including changes in personality. There is an emerging appreciation for clinical overlap between ALS and FTD, along with evidence of FTD symptoms in patients with ALS and of motor neuron signs in patients with FTD. The two disorders are also connected by pathology and genetics. Aggregates of the RNA-binding protein TDP-43 in neurons and improved survival of human induced motor neurons from patients with C9ORF72 ALS. Together, our results demonstrate the promise of CRISPR–Cas9 screens in defining mechanisms of neurodegenerative diseases.

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Results

CRISPR–Cas9 screens for modifiers of C9ORF72 DPR toxicity.

We engineered the human immortalized myelogenous leukemia cell line K562 to stably express Cas9 (ref. 43). We chose this cell line for the initial screen for several reasons. The cells grow in suspension and double rapidly, thus allowing us to expand the cultures to the large numbers required to perform genome-wide screens with complex sgRNA libraries. Furthermore, this cell line has been used for several other genome-wide screens, thus allowing us to compare and contrast hits to test for specificity. Similarly to findings in previous reports in other human cell lines 14, synthetic polymers of PR and GR (PR20 and GR20, respectively) added to the cell culture medium were rapidly taken up by K562 cells, and subsequently trafficked to the nucleus and killed cells in a dose-dependent manner (Fig. 1a–d and Supplementary Fig. 1).

To identify genetic modifiers of both PR20- and GR20-mediated toxicity, we conducted genome-wide CRISPR KO screens. We used a lentiviral sgRNA library comprising ten sgRNAs per gene and targeting ~20,500 human genes, along with ~10,000 negative-control sgRNAs 44 (Fig. 1e). We used deep sequencing to track the effect (protective, sensitizing, or neutral) of each sgRNA in a pooled population of KO cells. That is, sgRNAs that protected cells from DPR toxicity were enriched, and those that sensitized cells were depleted from the pooled population of cells. To identify both protective and sensitizing gene KOs in the same screen, we treated the pooled population of cells with a DPR dose sufficient to kill 50% of cells and repeated this treatment four times over the course of the screen to amplify the selection. We performed separate screens for modifiers of PR20 and GR20 toxicity, and repeated each screen two independent times.

To detect statistically significant suppressors and enhancers of DPR toxicity, we used the Cas9 high-throughput maximum-likelihood estimator (casTLE) algorithm as previously described 45. With a false discovery rate (FDR) cutoff of 10%, the screens identified 215 genetic modifiers of PR20 toxicity and 387 genetic modifiers of GR20 toxicity (Fig. 1f, Supplementary Fig. 2a–c and Supplementary Table 1). Results from the PR20 and the GR20 screen were well correlated ($R^2 = 0.61$), thus suggesting similar mechanisms of toxicity (Fig. 1g). We validated individual KO K562 lines by performing competitive-growth assays with PR20 treatment. We tested five
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nonspecific genes involved in general peptide-mediated toxicity indicating that we uncovered specific genetic modifiers rather than nonspecific genes involved in general peptide-mediated toxicity (Supplementary Fig. 2d).

Among the hits from these KO screens were genes encoding some of the same nuclear-import and nuclear-export factors discovered in DPR modifier screens in model organisms1,3,8,9,17, such as XPO6 and IPO11 (Fig. 2), thus suggesting that the same mechanisms are important in mammalian cells. We also identified many novel genetic modifiers of PR and GR toxicity, including genes encoding RNA-binding proteins that have been shown to physically interact with PR and GR17, such as NONO and HNRNPF. We also identified genes involved in RNA splicing, such as SPEN and PQQP1, a process previously observed to be dysregulated in the brains of patients with c9ALS/FTD48 and to be dramatically disrupted in human cell lines treated with DPRs56. In addition to these previously implicated genes, some of the strongest hits in the screen were genes encoding ER-resident proteins (such as TMX2 and CANX) and almost all members of the ER-membrane protein complex (the EMC complex), which is upregulated by ER stress and is thought to be involved in ER-associated degradation54,55. Genes encoding proteasome subunits, as well as chromatin modifiers and transcriptional regulators, such as those involved in histone lysine methylation (KDM6A, KMT2D, KMT2A, and SETD1B), were also significant genetic modifiers of PR and GR toxicity (Fig. 2).

CRISPR–Cas9 screens in primary mouse neurons. To evaluate the roles of the genetic modifiers identified in our human genome-wide screens in a more disease-relevant context, we next designed a strategy to conduct pooled CRISPR–Cas9 screens in mouse primary cortical neurons. In these primary neurons, similarly to human cell lines, synthetic PR20 dipeptides were toxic in a dose-dependent manner (Fig. 3a). However, to better model the endogenous production of DPRs, as occurs in the brain in ALS/FTD, we also used a lentiviral construct for expression of codon-optimized PR50 driven by the neural-specific synapsin promoter52. When expressed in primary neurons, PR50 localized to nuclear puncta and elicited neurodegeneration in the brains of patients with c9ALS/FTD48 and to be dramatically decreased in viability after one round of selection. Given the stringency of this screen, together with the nondividing nature of these neurons, we expected only genetic modifiers with the strongest effects to be enriched. We harvested the surviving neurons and used deep sequencing to assess sgRNA enrichment relative to that in the control populations (Fig. 3c). We identified 13 modifier genes in the synthetic PR20 screen and 17 genes in the lentiviral PR50 expression screen (determined by having a nonzero estimated effect with a 95% credible interval) (Fig. 3d,e, Supplementary Fig. 4d and Supplementary Table 2). The genes that validated in these neuronal cultures showed strong specificity to the method of PR delivery to the neurons. We further validated the two strongest modifier genes from these neuronal screens in an independent primary-neuron culture model of DPR toxicity, using mouse dorsal root ganglion neurons. KO of either Rab7 or Tmx2 protected against PR toxicity in these primary neurons (Fig. 4a–d).

RAB7A and intracellular transport of PR50. A top hit in the screen for modifiers of synthetic PR20-induced toxicity was the endolysosomal trafficking gene RAB7A (Fig. 3d). We hypothesized that the endosomal trafficking pathway is necessary for the intracellular transport of exogenously applied PR50. We tested this hypothesis in wild-type (WT) and RAB7A-knockdown HeLa cells by using immunocytochemistry and found altered subcellular localization of PR50 (Supplementary Fig. 3b) indicating that endolysosomal trafficking is important for the localization and subsequent toxicity of synthetic PR50. We used the CRISPR-interference system to
decrease the levels of RAB7A in HeLa cells. This decrease caused a stereotypical enlargement and mislocalization of LAMP1-positive lysosomal puncta. In control cells, at an early time point after PR20 addition (1 h, 1 µM PR20), PR20 distributed diffusely throughout the cell, including the nucleus, but was not yet enriched in the nucleus (as shown in Fig. 1b, d and Supplementary Fig. 1). However, at
this same early time point in RAB7A-knockdown cells (denoted Rab7-i.1 cells), PR20 accumulated in numerous puncta speckled throughout the cytosol, a result indicative of an early endomembrane compartment (Supplementary Fig. 3b). Given that cell-to-cell spread of DPRs may be relevant to disease45,46, understanding mechanisms of DPR cellular entry and trafficking may inform strategies to block this process.

**C9ORF72 DPRs induce an ER-stress response.** In contrast to the results of the screen with synthetic PR20, none of the trafficking genes present in the sublibrary were hits in the screen in which neuronal toxicity was induced by lentivirus-mediated PR20 expression. In this screen, the strongest genetic modifiers were genes encoding proteins predominantly localized to the nucleus and ER; a top protective KO was the poorly characterized ER-resident transmembrane thioredoxin Tmx2 (Fig. 3c). Because this hit is implicated in ER function, our results suggested that DPR accumulation might induce an ER-stress response. To test this hypothesis, we performed RNA sequencing (RNA-seq) on primary-neuron cultures transduced with a lentivirus for expression of either PR20 or GFP (Fig. 5a). After 4 d of PR20 expression, 126 genes were significantly upregulated, and 133 genes were significantly downregulated (DESeq2, adjusted P < 0.05; Fig. 5b, Supplementary Fig. 5 and Supplementary Table 3). A significantly enriched Gene Ontology (GO) category among upregulated genes was 'apoptotic signaling pathway in response to endoplasmic reticulum stress' (GO 00070059) (Fig. 5c). Genes in this enriched ontology category included Atf4, Bcl2 (also known as Puma), Chac1, Bax, and the ER Ca2+-channel gene Itpr1, for which loss-of-function variants have been shown to cause spinocerebellar ataxia45 (Fig. 5d). Other notable enriched GO categories among these differentially expressed genes included 'neuronal apoptosis', 'ion homeostasis', 'ribonucleoprotein', 'translation', and 'DNA repair'.

From these RNA-seq results, we confirmed a time-dependent induction of ER-stress-related genes by using PR20 expression and quantitative reverse-transcription PCR (qRT–PCR) (Fig. 5e). The IRE1 stress-sensor of the unfolded-protein response was not activated by PR20 expression in neurons, as determined by the levels of spliced Xbp1 mRNA (Fig. 5f). These data were consistent with activation of the integrated stress response, which converges on eIF2 and the upregulation of the transcription factor Atf4 (ref. 55). To confirm that these ER-stress responses are conserved in other models of DPR toxicity, we performed RNA-seq on primary mouse neurons as well as human K562 cells, each treated with synthetic PR20, ATF4 and related ER-stress-response genes were also induced in each of these models (Fig. 5f, Supplementary Fig. 6 and Supplementary Table 3). Finally, in addition to observing the genetic signatures of ER stress, we also used a pharmacological approach to test the importance of this pathway in response to PR20. The small molecule ISRIB, a potent inhibitor of the cellular response to ER stress48, inhibits ATF4 induction by modulating eIF2α signaling during the integrated stress response49. Pretreatment of K562 cells with 15 nM ISRIB mitigated PR20 toxicity (Fig. 5g), thus providing further evidence of a role of ER stress in DPR-mediated toxicity. Future experiments will be required to validate ISRIB's efficacy in primary neurons and animal models of C9ORF72 DPR toxicity.

**Tmx2 modifies the C9ORF72 DPR-induced ER-stress response.** Several lines of evidence have suggested a potential role for ER stress in C9ORF72 pathogenesis. Transcriptomic analysis of C9ORF72-mutant ALS brain tissue has revealed an upregulation of genes involved in the unfolded-protein response and ER stress60. A specific PERK–ATF4-mediated ER-stress response has been reported in a separate study of brain tissue from patients with c9ALS/FTD compared with ALS61. Additionally, ER stress and disruptions to ER Ca2+ homeostasis have been observed in induced pluripotent stem cell (iPSC)-derived motor neurons from patients with c9ALS/FTD44. Given that a main transcriptional stress response to endogenous and exogenous DPR treatment was the ER stress response, we chose to further investigate Tmx2, a gene encoding a poorly characterized ER-resident protein that was one of the strongest protective hits in both the PR and GR (Fig. 5f, Supplementary Fig. 6). We first confirmed that the sgRNAs targeting Tmx2 effectively knocked out the gene in K562 cells and in the targeted primary-neuron screen expressing PR20. We then validated that the sgRNAs targeting Tmx2 effectively knocked out the gene in K562 cells and primary neurons cultured from Cas9-expressing mice (Fig. 6a and Supplementary Fig. 7a). The decrease in Tmx2 levels was sufficient to markedly suppress C9ORF72 DPR toxicity both in K562 cells (Supplementary Fig. 7b) and in primary mouse cortical neurons (Fig. 6b,c).

Tmx2 is an ER-resident transmembrane thioredoxin protein that may be enriched at the mitochondrial-associated membrane of the
ER62. It is a member of the protein disulfide isomerase family, but whether it is catalytically active remains unclear, given that it contains an SXXC motif instead of the canonical CXXC motif necessary for the oxidoreductase activity used to catalyze the formation or rearrangement of disulfide bonds63. To define the mechanism by which decreased Tmx2 mitigates DPR toxicity, we performed RNA-seq

**Fig. 5 | Transcriptional analysis of PR-treated primary neurons and K562 cells.** a, RNA-seq of primary mouse neurons treated with lentiviruses for expression of either PR50 or GFP. Differentially expressed genes were determined with DESeq2. b, MA plot of differential gene expression of PR50-expressing neurons compared with control GFP-expressing neurons (n = 3 independent cell culture experiments; red, significantly upregulated genes, adjusted P value < 0.05; blue, significantly downregulated genes, adjusted P value < 0.05; calculated by DESeq2). c, Selected GO terms enriched in significantly upregulated genes (red) or significantly downregulated genes (blue), determined with DAVID 6.8. d, Fold changes in expression of differentially expressed genes (n=3, adjusted P value < 0.001; calculated by DESeq2; error bars, s.e.m.) grouped by GO category. e, qRT–PCR validation of Atf4, Atf5, Chac1, Ddit3, and Bbc3 upregulation in neurons expressing PR50 for 3, 4, or 5 d, compared with control neurons (n = 2 independent cell culture experiments; one-way ANOVA; ***P < 0.001, **P < 0.01, *P < 0.05; error bars, s.e.m.). f, RNA-seq in K562 cells treated for 24 h with synthetic PR20, showing upregulation of ER-stress-related genes. Relative expression of significantly differentially expressed genes determined by DESeq2 (n = 3, adjusted P < 0.001; error bars, s.d.) plotted as fragments per kilobase sequence per million mapped reads for each gene relative to untreated cells. g, K562 cells preincubated with 15 nM ISRIB or control (DMSO) were treated with 15 μM PR20 (red) or left untreated (black) for 24 h; then measured by flow cytometry (forward- and side-scatter analysis). Measurements were performed in triplicate, and mean ± s.d. are plotted (**P < 0.005, two-tailed t test). h, Reverse transcriptase PCR analysis to measure spliced Xbp1 mRNA (relative to Gapdh) in cortical neurons transduced with PR50 for 3, 4, or 5 d (+Thaps., 200 nM thapsigargin treatment, 2 h).
Fig. 6 | Decreased Tmx2 protects primary neurons against PR-mediated toxicity. a, Tmx2 measured in primary Cas9⁺ cortical neurons (immunoblot) 10 d after sgRNA transduction (uncropped blots shown in Supplementary Data 1). b, Quantification of lactose dehydrogenase levels (LDH) in primary cortical neurons 5 d after transduction with PR₅₀ or GFP lentiviruses. Tmx2 knockdown by sgRNA lentivirus transduction on DIV1 (Tmx2 sgRNA) protected primary neurons against PR₅₀-induced cytotoxicity relative to control (Safe sgRNA) (one-way ANOVA; n = 3; *P < 0.05; error bars, s.e.m.). c, Quantification of activated caspase 3/7 (DEVDase activity; RLU, relative light units) in primary neurons 4 d after transduction with PR₅₀ or GFP lentiviruses. log₂ fold change (Tmx2 sgRNA/Safe sgRNA in PR₅₀-treated conditions) of differentially expressed genes, determined with DESeq2 (n = 3; adjusted P < 0.001; error bars, s.e.m.) enriched in select GO categories. e, Select GO-term enrichment, determined with DAVID 6.8 (red, upregulated genes; blue, downregulated genes), for differentially expressed genes (adjusted P < 0.001) identified by comparing RNA-seq results between control (Safe sgRNA) and Tmx2-KO (Tmx2 sgRNA) neurons expressing PR₅₀. f, qRT-PCR validation of Atf3 expression changes after PR₅₀ expression in control (Safe sgRNA) and Tmx2-KO (Tmx2 sgRNA) neurons (n = 3; P < 0.001; one-way ANOVA; error bars, s.e.m.). g, V5-tagged ATF3 overexpression by lentiviral transduction of primary cortical neurons confers protection of PR₅₀-induced dendrite degeneration (blue, DAPI; green, anti-MAP2 (dendrite marker); magenta, anti-V5 (ATF3-V5 overexpression); SYN, lentiviral synapsin promoter; scale bars, 20 µm). Ctl., control. h, Quantification of the percentage area of MAP2 immunofluorescence (n = 5, one-way ANOVA, Dunnett’s multiple comparisons test, ***P < 0.001; error bars, s.e.m.). i, Quantification of active caspase 3/7 (DEVDase) in PR₅₀ or GFP-expressing cortical neurons ± ATF3 overexpression (n = 5, one-way ANOVA, Dunnett’s multiple comparisons test ****P < 0.0001; error bars, s.e.m.). n refers to independent cell culture experiments for each experiment.
Lowering Tmx2 and survival of C9-ALS motor neurons. Given the strong protective effect of Tmx2 knockdown on PR-mediated toxicity in rodent primary neurons, we tested whether lowering Tmx2 levels might modify the survival phenotypes in neuronal cells from patients with ALS harboring endogenous C9orf72 GGGGCC expansions. We generated induced motor neurons (iMNs) from iPSCs from patients with C9orf72 ALS and unaffected individuals by using transcription-factor-mediated lineage conversion, as previously described\(^{13,66}\). These C9-ALS–patient–derived iMNs, as compared with control iMNs, showed poorer survival after glutamate addition (Fig. 7, Supplementary Fig. 8 and ref. 13). Using a seven factor (7F) differentiation method to generate iMNs, we tested two independent shRNAs targeting Tmx2 and found that both shRNAs, as compared with control shRNAs, significantly increased the proportion of surviving C9orf72 iMNs in two independent C9-ALS lines (Fig. 7a,b) but did not increase the proportion of surviving control iMNs (Supplementary Fig. 8). We repeated this experiment by using a different differentiation method (Dox-NIL) to generate iMNs and found a similar protective effect of decreased Tmx2 in C9-ALS but not control iMNs, though there was a degree of variability in the extent of rescue among patient lines with this differentiation method (Supplementary Fig. 9). Further studies will be required to assess the effects of Tmx2 on C9orf72–related pathologies in additional patient cell lines and ALS models, but these results suggest that our screening strategy may be useful to identify potent modifiers of ALS–related phenotypes.

Discussion

Here, we used comprehensive CRISPR–Cas9 KO screens in human cells with further validation screens in primary neurons to discover modifiers of C9orf72 DPR toxicity. We identified nucleocyttoplasmic–transport machinery, confirming the results from previous studies in model organisms\(^{15,16}\), and also identified new genes that suggest that ER function and ER stress are important in c9FTD/ALS pathogenesis. The gene KOs that mitigated toxicity, such as Tmx2, may serve as future therapeutic targets. Notably, decreased Tmx2 expression conferred protection from PR toxicity across multiple cellular assays, including in primary rodent neurons.

There are five distinct DPRs produced from the C9orf72 GGGGCC-repeat expansion: glycine–alanine (GA), glycine–arginine (GR), glycine–proline (GP), proline–alanine (PA), and proline–arginine (PR) (GP is produced from both the sense and antisense transcript). Our studies here focused on two of these DPRs, GR and PR. The relative contribution of each DPR to neurodegeneration and the potential for synergistic effects among multiple DPRs remains to be defined. Although the evidence from model systems for a role of DPRs in neurodegeneration is compelling, there are some caveats. These studies, including ours, used either exogenous addition of synthetic DPRs to cells or high levels of transgenic expression of specific DPRs, which might not accurately reflect the physiological levels of DPRs. Moreover, studies of C9ALS/FTD neuropathology have also produced results that seem to not support a role of DPRs as the drivers of neurodegeneration. The abundance and localization of DPR pathology does not appear to correlate with neurodegeneration and clinical phenotypes\(^{76–79}\). Finally, GA and GP DPRs seem to be the most abundant but are the least toxic in model systems\(^{11,15,16}\), and the most toxic DPR, PR, is extremely rare\(^{7,10}\). One possible explanation for this apparent disconnect is that the most toxic DPRs (for example, GR and PR) do not accumulate to high enough levels (because they are so toxic) before causing neuron death, whereas the others (for example, GA and GP) can accumulate to higher levels because they are more benign\(^{7,11}\).

Beyond the DPRs, RNA foci from the sense (GGGGCC) and antisense (GGGGCG) repeat transcripts, as well as loss of function of the C9orf72 gene could also contribute to disease. These different mechanisms may also interact with one another. For example, loss of C9orf72 function may make neurons or glia more vulnerable to the RNA foci or DPRs. Additionally, our work here provides evidence that DPRs can elicit an ER-stress response, and recent work shows that ER stress can increase RAN translation\(^{72,74}\), thus suggesting the potential for a feed-forward loop. It will be important to define the relative contributions of each of these facets of C9orf72 pathology (loss of function, RNA foci, and DPRs) to disease so that effective therapeutic strategies can be developed. If the DPRs are indeed key contributors to disease, our work here identifies novel suppressors of DPR toxicity that may be potential therapeutic targets.

Beyond the types of CRISPR–Cas9 screens used here, the CRISPR–Cas9 system has been adapted for use in other types of genetic screens. Through use of Cas9 with a deactivated nuclease (dCas9), which is unable to generate double-strand breaks, specific genomic regions can be targeted for transcriptional control\(^{57,59}\). Fusing a KRAB effector domain to Cas9 results in transcriptional repression (CRISPR interference)\(^{77}\). Alternatively, CRISPR activation uses dCas9 fused with various transcriptional–activation domains, thereby empowering genome–wide transcriptional activation (i.e., overexpression) screens\(^{56,78–80}\). Thus, the same logic applied to yeast, worm, and fly genetic screens for up- or downregulation of genes can now be achieved in human cells on a genome–wide scale. We anticipate that CRISPR screens in human cells and primary
neurons will be a powerful addition to the experimental toolbox to study mechanisms of neurodegenerative disease.

Methods

Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41588-018-0070-7.

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Methods

Cell culture. K562 cells (ATCC) were cultured in RPMI-1640 (Gibco) medium with 10% FBS (HyClone), penicillin (10,000 U/ml), streptomycin (10,000 µg/ml), and l-glutamine (2mM). Cells were grown in log phase by maintaining the population at a concentration of 500,000 cells/ml. K562 cells were maintained in a controlled humidified incubator at 37 °C, with 5% CO2.

Primary mouse cortical neurons were dissociated into single-cell suspensions from E16.5 mouse cortices with a papain dissociation system (Worthington Biochemical Corporation). Neurons were seeded onto poly-l-lysine–coated plates (0.1% (wt/vol) and grown in Neurobasal medium (Gibco) supplemented with B-27 serum-free supplement (Gibco), GlutaMAX, and penicillin-streptomycin (Gibco) in a humidified incubator at 37 °C, with 5% CO2. Half media changes were performed every 4 or 5 d, or as required. For gene-knockout experiments in Cas9(r) cortical neurons, lentiviruses encoding sgRNAs expressed from a U6 promoter were transduced on DIV1. Cultures were incubated for at least 10 d with the sgRNAs to allow adequate knockdown of target protein levels before further experiments were performed. Mouse experiments were approved by the Stanford Administrative Panel on Animal Care (APLAC).

Dorsal root ganglion cultures. 24-well plates were coated overnight with PDL (100 µg/ml) and, after a wash, were coated with laminin (1.33 mg/ml) for 3 h. DRGs were dissected from E12.5 mouse embryos and dissociated in 0.05% trypsin–EDTA. Dissociated neurons were plated in Neurobasal medium supplemented with 2% B27, 0.45% n-glucose, 1% GlutaMAX, 100 U/ml penicillin, and 100 U/ml streptomycin. On DIV1, the cultures were fed with fresh Neurobasal/B-27 medium containing 5µM 5-fluoro-2′-deoxyuridine and 5µM uridine to inhibit the growth of non-neuronal cells. On DIV1, cells were transduced with lentiviruses for expression of sgRNAs targeting a safe genomic region (Safe), TMX2, or Rab7. Cultures were maintained with 10% FBS (Hyclone), penicillin (10,000 IU/ml), streptomycin (10,000 µg/ml), and l-glutamine (2mM) for 24 h at 4 °C, then centrifugation at 1,500 g for 5 min to remove cellular debris, and concentrated tenfold with Lenti-X concentrator (Clontech) before being added to cell cultures. GFP expressing cells were cocultured with WT/GFP– cells and were subsequently treated with 10µM PR20. The percentage of GFP+ cells was quantified by fluorescence-activated cell sorting with a BD Accuri C6 flow cytometer after 48 h.

Pretreatment of ISRIB in PR20-exposed K562s. ISRIB (Sigma SML0843) was dissolved in DMSO and added to the K562 cell line at a concentration of 15 nM 3 h before treatment with PR20. An equal amount of DMSO was added to cells that were not pretreated with ISRIB. 15 µM of PR20 was then added to these cells, and a third group of K562s were not treated with ISRIB or PR20 and served as an untreated control. 24 h after PR20 treatment, cells were measured with a BD Accuri C6 flow cytometer for forward- and side-scatter analysis. All experiments were performed in triplicate.

CRISPR–Cas9 screen for DPR toxicity in primary mouse cortical neurons. Cortical tissue from mouse E16.5 embryos (G70(Rosa)26sgFPik;IcSg-Cas9;Ksp95ΔF)1; Jackson Laboratory stock no: 024858) was dissected and dissociated into a single-cell suspension using a papain dissociation system (Worthington Biochemical Corporation). 7 million cells were seeded onto 10-cm dishes in Neurobasal medium supplemented with B-27 serum-free supplement (Gibco), GlutaMAX (Gibco), and penicillin–streptomycin (Gibco). Half the culture medium was replenished every 4 or 5 d. One day after seeding, neurons were infected with a lentiviral library containing ~3,000 sgRNA elements consisting of 178 genes (5% FDR for PR20 genome-wide screen hits), ten sgRNAs per gene, and 1,000 negative-control sgRNAs, synthesized and cloned as previously described. Transductions were performed with a titer resulting in a 50–70% infection rate, and the other half were treated with 1.5µM synthetic PR20 for 24 h, thus resulting in approximately 90% cell death.

Lentiviral-mediated DPR screen. Half the cells were left untreated (control group), and the other half were treated with 1.5µM synthetic PR20 for 24 h. Half the cells were infected with control lentivirus particles for expression of GFP from the synapsin promoter (control group), and the other half were infected with a lentivirus for expression of codon-optimized PR20 from the synapsin promoter. Lentiviral PR20 resulted in approximately 90% cell death between 4 and 6 d. To harvest DNA from the remaining live cells, cultures were washed three times in PBS, treated with DNase I (Worthington Biochemical Corporation) for 10 min at 37 °C (to remove any extracellular gDNA from dead neurons), then washed again three times in PBS to remove residual DNase I. Genomic DNA was harvested with a DNeasy Blood and Tissue Kit (Qiagen) including proteinase K digestion to inactivate residual DNase I. sgRNA

K562 competitive-growth assays. K562 cells stably expressing Cas9 were infected with sgRNAs targeting each gene of interest for validation from the genome-wide screen. sgRNA plasmids also encoded GFP. Cells were selected for stable sgRNA expression with puromycin (1µg/ml) and were confirmed to be GFP+ by fluorescence-activated cell sorting with a BD Accuri C6 flow cytometer after 48 h.

CRISPR–Cas9 screen for DPR toxicity in primary mouse cortical neurons. Cortical tissue from mouse E16.5 embryos (G70(Rosa)26sgFPik;IcSg-Cas9;Ksp95ΔF)1; Jackson Laboratory stock no: 024858) was dissected and dissociated into a single-cell suspension using a papain dissociation system (Worthington Biochemical Corporation). 7 million cells were seeded onto 10-cm dishes in Neurobasal medium supplemented with B-27 serum-free supplement (Gibco), GlutaMAX (Gibco), and penicillin–streptomycin (Gibco). Half the culture medium was replenished every 4 or 5 d. One day after seeding, neurons were infected with a lentiviral library containing ~3,000 sgRNA elements consisting of 178 genes (5% FDR for PR20 genome-wide screen hits), ten sgRNAs per gene, and 1,000 negative-control sgRNAs, synthesized and cloned as previously described. Transductions were performed with a titer resulting in a 50–70% infection rate, and the other half were treated with 1.5µM synthetic PR20 for 24 h, thus resulting in approximately 90% cell death.

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amplification and library preparation for deep sequencing was performed as described above. Gene-level effects and confidence scores were calculated as in the primary screen. The significance of the gene-level effects was determined on the basis of the 95% credible intervals calculated in casTLE45. A gene was considered a hit if its 95% credible interval did not contain zero.

**RNA sequencing.** Total RNA quality control was performed with a Eukaryote Total RNA Nano assay on an Agilent 2100 Bioanalyzer System for all RNA samples before library preparation for RNA sequencing. mRNA libraries were prepared for Illumina paired-end sequencing with an Agilent SureSelect Strand Specific RNA-Seq Library Preparation kit on an Agilent Bravo Automated Liquid Handling Platform. Libraries were sequenced on an Illumina NextSeq sequencer. Alignment of RNA-sequencing reads to the transcriptome was performed with STAR and ENCODE standard options, read counts were generated with rsem, and differential expression analysis was performed in R with the DESeq2 package6. All bioinformatics analyses were performed on Sherlock, a Stanford HPC cluster.

**Briefly, thresholds were applied to image stacks to detect NeuN neuronal nuclei, neurons were fixed and stained as described above and quantified with ImageJ.**

**qRT–PCR.** cDNA was reverse transcribed from total RNA samples with a High Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific). TaqMan assays (Applied Biosystems) for ATF3, ATF4, ATF5, CHAC1, DDT1, BCD3, and BAX were used in qPCR reactions with inventoried TaqMan Universal PCR Master Mix (ThermoFisher Scientific) and a StepOne or QuantStudio 3 real-time PCR machine. To measure XBP1 splicing, cDNA was generated as described above, and equal amounts of cDNA (2 μl) were used in a PCR with KOD polymerase (EMD Millipore 71086). Primers used in these experiments are listed in Supplementary Table 5. The cycling conditions were as follows: 95 °C for 5 min; 10 cycles of 94 °C for 20 s, 65–55 °C (−1 °C/loop) for 20 s, and 72 °C for 30 s; and 35 cycles of 94 °C for 20 s, 58 °C for 20 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min. Products were analyzed via 2% agarose gel electrophoresis.

**Western blotting.** Protein lysates were prepared with RIPA buffer supplemented with 1× Halt Protease Inhibitor Cocktail (Pierce). Crude lysates were centrifuged at 12,500 × g for 10 min at 4 °C to remove cellular debris. Clarified lysates were quantified with a Pierce BCA protein assay. Equal amounts of protein were subjected to SDS–PAGE, transferred to nitrocellulose membranes, and immunoblotted according to standard protocols. The following antibodies were used: mouse anti-GAPDH (1:5,000, clone GAPDH-71.1, Sigma G8795), rabbit polyclonal anti-TMX2 (1:1,000, Novus NBP1-87305), and rabbit polyclonal anti-XPO1 (1:2,000, Bethyl Laboratories A303-991A).

**Cytotoxicity assay.** K562 cell viability after DPR treatment was measured by flow cytometry with a BD Accuri C6 flow cytometer by measuring the number of events in forward-scatter and side-scatter gates. Cytotoxicity in primary-neuron cultures was measured by LDH release assays (Promega, CytoTox 96 Non-Radioactive Cytotoxicity Assay) and by NeuN+ quantification of surviving neurons with immunocytochemistry. To quantify the numbers of NeuN+ neurons, cells were fixed and stained as described above and quantified with ImageJ. Briefly, thresholds were applied to image stacks to detect NeuN+ neuronal nuclei, which were then automatically counted with built-in ImageJ plugins. Additional cytotoxicity assays were performed with the Caspase-Glo 3/7 Assay (Promega G8790), a luminescence-based readout of caspase 3/7 activity (DEVase activity) in neurons, according to the manufacturer’s instructions.

**Viruses production for induced motor neuron experiments.** cDNAs for iMN factors (Ngn2, Lin28, Isl1, NeuroD1, Ascl1, Myt1l, and Bnx2) were purchased from Addgene. Each cDNA was cloned into the pMXs retroviral expression vector with Gateway cloning technology (Invitrogen). The H9:RFP lentiviral vector was also purchased from Addgene (ID 37081). Viruses were produced as follows: HEK293 cells were transfected at ~80–90% confluency with viral vectors containing genes of interest and viral packaging plasmids (PIK-MLV-gp and PHDM for retrovirus; pPAX2 and VSVG for lentivirus) with PEI (Sigma-Aldrich). The medium was changed 24 h after transfection. Viruses were harvested at 48 h and 72 h after transfection. Viral supernatants were filtered with 0.45-μm filters, incubated with Lenti-X concentrator (Clontech) for 24 h at 4 °C, and centrifuged at 1,500 g at 4 °C for 45 min. The pellets were resuspended in 300 μl DMEM with 10% FBS and stored at −80 °C.

**Generation of Dox-NIL induced motor neurons.** To generate Dox-NIL iMNs, the Dox-NIL construct was integrated into the AAVS1 safe-harbor locus of the control and C9-ALS patient iPSC lines with CRISPR–Cas9 editing (gRNA sequence in Supplementary Table 4). The conversion of NIL-iMNs was performed in 96-well plates (2 × 104 cells/well), which were sequentially coated with Matrigel (1 h) and laminin (2–4 h) at room temperature. iPSCs were cultured in mTeSr and induced with 1 μg/ml doxycycline on day 1 after plating. On day 3 after plating, the culture was transduced with control shRNA1, control shRNA2, TMX2 shRNA1, and TMX2 shRNA2 with 5 μg/ml polybrene in N3 medium containing DMEM/F12 (Life Technologies), 2% FBS, 1% penicillin/streptomycin, N2 and B27 supplements (Life Technologies), and 10 ng/ml each of GDNF, BDNF, and CNTF (R&D). For the detection of the motor neurons, the culture was transduced with H9:RFP reporter 48 h after shRNA transduction. On day 5, primary mouse cortical glial cells from P1 ICR pups (male and female) were added to the transduced cultures in N3 medium.

**Generation of 7F induced motor neurons.** Reprogramming was performed in 96-well plates (8 × 104 cells/well) that were sequentially coated with gelatin (0.1%, 1 h) and laminin (2–4 h) at room temperature. To enable efficient expression of the transgenic reprogramming factors, iPSCs were cultured in fibroblast medium (DMEM with 10% FBS) for at least 48 h and were either used directly for retroviral transduction or passaged before transduction for each experiment. Seven iMN factors were added in 100–200 μl fibroblast medium per well in 96-well plates with 5 μg/ml polybrene. For iMNs, cultures were transduced with lentivirus encoding the H9:RFP reporter 48 h after transduction with transcription-factor-encoding retroviruses. On day 5, primary mouse cortical glial cells from P1 ICR pups (male and female) were added to the transduced cultures in glia medium containing MEM (Life Technologies), 10% donor equine serum (HyClone), 20% glucose (Sigma-Aldrich), and 1% penicillin–streptomycin. On day 6, cultures were switched to N3 medium containing DMEM/F12 (Life Technologies), 2% FBS, 1% penicillin/streptomycin, N2 and B27 supplements (Life Technologies), 7.5 μM RepSox (Selleck), and 10 ng/ml each of GDNF, BDNF, and CNTF (R&D). The iMN neuron cultures were maintained in N3 medium, which was changed every other day, unless otherwise noted.

**Induced motor neuron survival assay.** H9:RFP+ neurons appeared between days 10 and 13 after initial seeding. Doxycycline was withdrawn at day 10, and the survival assay was initiated at day 20. For the glutamate treatment, 10 μM glutamate was added to the culture medium on day 20 and removed after 24 h. Longitudinal tracking was performed by imaging neuronal cultures in a Nikon Biostation CT instrument once every 24 h starting at day 20. Tracking of neuronal survival was performed with SView 3.0 (DRVision Technologies). Neurons were scored as dead when their soma was no longer detectable on the basis of RFP and GFP fluorescence. All neuron survival assays were performed at least twice, and one of the trials was used for the quantification shown. All trials quantified were representative of other trials of the same experiment. For iMNs from multiple independent donors that were combined into one survival trace in the Kaplan–Meier plots for clarity, the number of iMNs tracked from each line can be found in Supplementary Table 4.

**Statistics.** Statistical tests were performed with GraphPad Prism 7. Two-tailed t tests, one-way ANOVAs, or log-rank tests for survival curves were used as indicated.

**Life Sciences Reporting Summary.** Further information on experimental design is available in the Life Sciences Reporting Summary.

**Data availability.** RNA sequencing data from this study have been deposited in the NCBI GEO database under accession number GSE109177 The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Life Sciences Reporting Summary

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### Experimental design

1. **Sample size**
   - Describe how sample size was determined.
   - No sample size calculation was performed.

2. **Data exclusions**
   - Describe any data exclusions.
   - No data exclusions.

3. **Replication**
   - Describe whether the experimental findings were reliably reproduced.
   - Yes, reliably reproduced.

4. **Randomization**
   - Describe how samples/organisms/participants were allocated into experimental groups.
   - No randomization.

5. **Blinding**
   - Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   - No blinding.

   Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. **Statistical parameters**
   - For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or the Methods section if additional space is needed).

   - n/a Confirmed
     - The exact sample size \( (n) \) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
     - A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly.
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     - A description of any assumptions or corrections, such as an adjustment for multiple comparisons
     - The test results (e.g. \( p \) values) given as exact values whenever possible and with confidence intervals noted
     - A summary of the descriptive statistics, including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
     - Clearly defined error bars

   See the web collection on statistics for biologists for further resources and guidance.

### Software

#### Policy information about availability of computer code

7. **Software**
   - Describe the software used to analyze the data in this study.
   - Source code for analyzing CRISPR screen data is publicly deposited on Bitbucket. See URLs section.
For all studies, we encourage code deposition in a community repository (e.g. GitHub). Authors must make computer code available to editors and reviewers upon request. The *Nature Methods* guidance for providing algorithms and software for publication may be useful for any submission.

### Materials and reagents

#### Policy information about availability of materials

8. **Materials availability**

   Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

   No unique materials used.

9. **Antibodies**

   Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

   - mouse monoclonal anti-FLAG (1:1000, M2 Sigma cat.# F1804), mouse monoclonal anti-MAP2A (1:1000, Millipore cat.# MAB378), mouse monoclonal anti-NeuN (1:1000, Millipore cat.# MAB377), mouse anti-GAPDH (1:5000, clone GAPDH-71.1, Sigma G8795), rabbit polyclonal anti-TMX2 (1:1000, Novus NBP1-87305), rabbit polyclonal anti-XPO5 (1:2000, Bethyl Laboratories A303-991A), mouse monoclonal anti-V5 tag (ThermoFisher R960-25), rabbit monoclonal anti-LAMP1 (Cell Signaling Technologies R960-25)

10. **Eukaryotic cell lines**

   a. State the source of each eukaryotic cell line used.

   b. Describe the method of cell line authentication used.

   c. Report whether the cell lines were tested for mycoplasma contamination.

   d. If any of the cell lines used in the paper are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

   See Methods: Cell Culture. Cell lines were obtained from ATCC.

   No authentication performed

   Yes, all cell lines tested negative for mycoplasma.

   None used.

### Animals and human research participants

#### Policy information about studies involving animals

11. **Description of research animals**

    Provide details on animals and/or animal-derived materials used in the study.

    No animals used.

#### Policy information about studies involving human research participants

12. **Description of human research participants**

    Describe the covariate-relevant population characteristics of the human research participants.

    No human research participants used.