FEMS Yeast Research, 18, 2018, foy024

doi: 10.1093/femsyr/foy024
Advance Access Publication Date: 8 March 2018
Research Article

RESEARCH ARTICLE

Yeast screen for modifiers of *C9orf72* poly(glycine-arginine) dipeptide repeat toxicity

Noori Chai[1,2] and Aaron D. Gitler[1,*]

[1]Department of Genetics, Stanford University School of Medicine, 300 Pasteur Drive, M322 Alway Building, Stanford, CA 94305, USA and [2]Neurosciences Graduate Program, Stanford University School of Medicine, 1215 Welch Road, Modular B, Stanford, CA 94305, USA

*Corresponding author: Department of Genetics, Stanford University School of Medicine, 300 Pasteur Drive, M322 Alway Building, Stanford, CA 94305, USA. Tel: (650) 725-6991; E-mail: agitler@stanford.edu
One sentence summary: A genetic screen in yeast reveals insight into Lou Gehrig's disease mechanisms.
Editor: Brooke Bevis

ABSTRACT

A hexanucleotide repeat expansion in the *C9orf72* gene has been identified as the most common cause of amyotrophic lateral sclerosis and frontotemporal dementia. The expanded hexanucleotide repeat is translated by an unconventional mechanism to produce five species of dipeptide repeat (DPR) proteins, glycine-proline (GP), glycine-alanine (GA), glycine-arginine (GR), proline-alanine (PA) and proline-arginine (PR). Of these, the arginine-rich ones, PR and GR, are highly toxic in a variety of model systems, ranging from human cells, to *Drosophila*, to even the budding yeast, *Saccharomyces cerevisiae*. We recently performed a genetic screen in yeast for modifiers of PR toxicity and identified suppressors and enhancers, many of which function in nucleocytoplasmic transport. Whether or not GR toxicity involves similar mechanisms to PR is unresolved. Therefore, we performed a genetic screen in yeast to identify modifiers of GR toxicity and compared the results of the GR screen to results from our previous PR screen. Surprisingly, there was only a small degree of overlap between the two screens, suggesting potential for distinct toxicity mechanisms between PR and GR.

Keywords: ALS; *C9orf72*; yeast; dipeptide repeat protein; screen; GR

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) are devastating human neurodegenerative disorders (Swinnen and Robberecht 2014). The most common genetic cause of ALS and FTD is mutations in the *C9orf72* gene (Renton, Chiò and Traynor 2014). *C9orf72* mutations can cause either disease or sometimes both (Taylor, Brown and Cleveland 2016). The disease-causing mutation is a massive GGGGCC hexanucleotide repeat expansion in the first intron of the *C9orf72* gene (DeJesus-Hernandez *et al.* 2011; Renton *et al.* 2011). Normally, the *C9orf72* gene harbors between 2 and 25 repeats and repeat expansions from hundreds to thousands are considered pathogenic (DeJesus-Hernandez *et al.* 2011; Renton *et al.* 2011). Since *C9orf72* mutations are the common cause of ALS and FTD, there is intense interest in defining the mechanisms by which they cause disease so that insight could be harnessed to develop therapeutic strategies.

Several potential mechanisms could explain how the *C9orf72* repeat expansion causes disease. First, the large GGGGCC repeat in the regulatory regions of *C9orf72* interferes with gene expression, resulting in reduced levels of *C9orf72* transcript and protein—the loss of function could contribute to disease (DeJesus-Hernandez *et al.* 2011; Waite *et al.* 2014). Second, the expanded repeat region is bidirectionally transcribed to form distinct RNA secondary structures that could be toxic by sequestering RNA-binding proteins and splicing factors (DeJesus-Hernandez *et al.* 2011; Gendron *et al.* 2013; Haeusler *et al.* 2014).

Received: 14 December 2017; **Accepted:** 5 March 2018

Third, the sense and anti-sense repeat-containing RNAs are translated in multiple frames, despite the absence of a start codon, by an unconventional form of translation, called RAN (repeat-associated non-AUG) translation (Zu *et al.* 2011), to produce dipeptide repeat (DPR) proteins (Ash *et al.* 2013; Mori *et al.* 2013; Zu *et al.* 2013). The sense transcript produces glycine-alanine (GA), glycine-arginine (GR) and glycine-proline (GP) DPRs, while the anti-sense transcript produces proline-alanine (PA), proline-arginine (PR) and GP DPRs. These DPRs are themselves aggregation prone and accumulate in the brain of *C9orf72* mutation carriers and could thus contribute to disease by a toxic gain-of-function mechanism (Ash *et al.* 2013; Mori *et al.* 2013; Zu *et al.* 2013). These three proposed mechanisms are not mutually exclusive and there is compelling evidence for and against each of them (Gitler and Tsuiji 2016).

Of the five distinct DPRs produced from the *C9orf72* repeat (GA, PA, GP, GR and PR), the arginine-rich ones (GR and PR) are particularly toxic. They are potently toxic to human cells and cause neurodegeneration in *Drosophila melanogaster* and human motor neurons derived from induced pluripotent stem cells (iPSCs) (Kwon *et al.* 2014; Mizielinska *et al.* 2014; Wen *et al.* 2014). These phenotypes do not depend on the repeat itself, because synthetic DPRs or use of constructs codon optimized to produce the DPRs without the GGGGCC or GGCCCC repeat sequence still cause cell death (Kwon *et al.* 2014; Mizielinska *et al.* 2014). This permits interrogation of DPR-specific toxicity pathways and their contributions to disease, without confounds of potential RNA-mediated toxicity.

Just like for human cells and *Drosophila*, GR and PR DPRs are also toxic in yeast cells. We recently used this toxicity as the basis for a genetic screen for modifiers of DPR toxicity (Jovičić *et al.* 2015). We focused on PR and identified several yeast genes that suppressed and enhanced toxicity. These studies illuminated genes in the nucleocytoplasmic transport pathway as potent modifiers of PR toxicity in yeast (Jovičić *et al.* 2015). Studies in other systems, including *Drosophila* and iPSC-derived neurons also provided evidence that *C9orf72* mutations disrupt nucleocytoplasmic transport (Zhang *et al.* 2015; Freibaum *et al.* 2015; Boeynaems *et al.* 2016). Since our previous study focused on PR, in this paper, we performed additional screens to identify modifiers of GR toxicity, to define the commonalities and differences between how GR and PR elicit toxicity.

MATERIALS AND METHODS

Yeast strains, media and plasmids

Yeast cells were grown in rich media or in synthetic media lacking uracil and containing 2% glucose (SD/-Ura), raffinose (SRaf/-Ura) or galactose (SGal/-Ura). To generate yeast expressing a GR dipeptide protein containing 100 repeats (GR_{100}), we utilized a codon-optimized GR sequence, as described previously (Jovičić *et al.* 2015). The ATG-DPR construct was synthesized by Genscript (Piscataway, USA) and was flanked by attB sites. Constructs were further subcloned into a pDONR221 plasmid and subsequently used in Gateway LR reactions with pAG416GAL-ccdB (Alberti, Gitler and Lindquist 2007) to produce yeast expression vectors. The resulting pAG416GAL-GR_{100} construct was transformed into Y7092 yeast using the PEG/lithium acetate method. Spotting assays verified GR_{100} toxicity in yeast.

Yeast transformation and spotting assays

Yeast procedures were performed according to the standard protocols. We used the PEG/lithium acetate method to transform yeast with plasmid DNA. For spotting assays, yeast cells were grown overnight at 30°C in liquid media containing SRaf/-Ura until they reached log or mid-log phase. Cultures were then normalized for OD_{600}, serially diluted and spotted with a Frogger (V&P Scientific, San Diego, USA) onto synthetic solid media containing glucose (SD/-Ura) or galactose (SGal/-Ura) lacking uracil and were grown at 30°C for 2–3 days.

Yeast genetic screen

We used synthetic genetic array analysis (Tong and Boone 2006) to identify nonessential yeast deletions that modify *C9orf72* GR_{100} toxicity. We performed this screen as described in Jovičić *et al.* (2015), using a Singer RoToR HAD (Singer Instruments, Emeryville, USA). We mated MATα strain expressing GR_{100} under galactose promoter control to the yeast haploid deletion collection of nonessential genes (MATa, each gene deleted with KanMX cassette conferring resistance to G418). Following diploid selection and sporulation, we selected haploids carrying both deletion and GR_{100} expression cassette. Colony sizes were measured using the ht-colony-measurer software (Collins *et al.* 2006). We performed the entire screen for three independent times. Individual hits were validated by independent transformations and spotting assays.

RESULTS

We screened a library of all 4850 nonessential yeast gene knockouts to identify deletions that could suppress GR_{100} toxicity (Fig. 1A and B). These types of genetic modifiers are an interesting class (gene deletions that suppress a phenotype) because they could represent potential drug targets. We identified 133 yeast deletions that suppressed GR_{100} toxicity (Table 1). We validated several modifiers from a variety of functional categories by individual transformations and spotting assays (Fig. 1C). Gene ontology analysis via YeastMine revealed an enrichment for cytoplasmic translation ($P = 7.292e{-}7$) and ribosomal small subunit biogenesis ($P = 3.323e{-}4$). The majority of the genes found in these categories encode ribosomal proteins and proteins involved in rRNA processing and ribosome synthesis in the nucleolus (Table 1). These ribosome-associated modifiers could act by reducing translation of the toxic GR100 protein. However, we did not identify these modifiers as suppressors of toxicity in deletion screens for other toxic proteins (PR_{50}, FUS, and TDP-43) (Sun *et al.* 2011; Armakola *et al.* 2012; Jovičić *et al.* 2015), suggesting that loss of these ribosomal proteins does not reduce expression of toxic proteins in general, but instead selectively affects GR_{100}. Immunoblots to quantify GR_{100} were inconclusive (data not shown) and so the specific mechanism of action for these ribosomal hits remains to be determined.

Though ribosomal genes were statistically enriched in the screen, additional functional clusters emerged (Table 1). One such cluster consisted of six *ADE* genes ($P = 3.488e{-}5$) and *BAS1*, all of which are involved in purine nucleotide biosynthesis (Cherry *et al.* 2012). Similarly, DNA damage repair genes including *RAD50*, *RAD51* and *RAD52* were identified in the screen, and this specific pathway has been implicated in GR toxicity in iPSC-derived neurons (Cherry *et al.* 2012; Lopez-Gonzalez *et al.* 2016). We also identified numerous genes involved with various forms of RNA-interacting processes including nucleocytoplasmic transport, tRNA synthesis and the mRNA life cycle. Similar genes, or in the case of *NUP107*, identical genes, involved in nucleocytoplasmic transport and RNA export and degradation were also been identified in GGGGCC repeat and PR toxicity

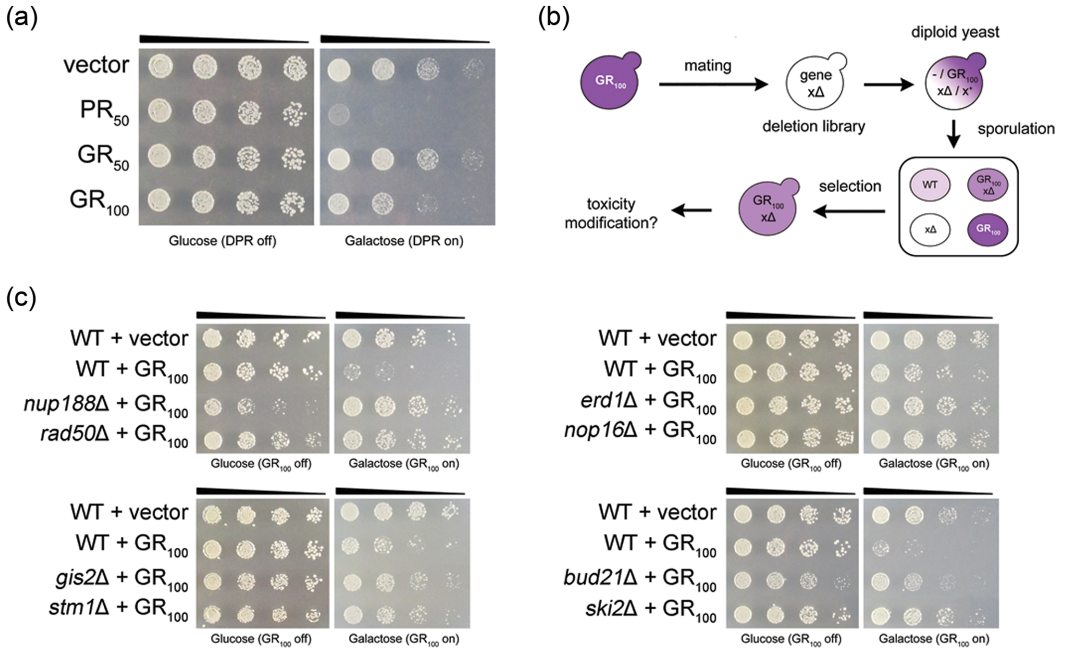

Figure 1. A yeast deletion screen reveals genetic suppressors of GR_{100} toxicity. **(A)** GR toxicity is length-dependent and less severe than PR toxicity in yeast. Five-fold serial dilutions of yeast cells were spotted onto glucose- or galactose-containing plates. Galactose induced expression of GR or PR in yeast, while glucose repressed DPR expression. **(B)** Schematic of the yeast deletion screen. **(C)** Example spotting assays validating specific hits from the deletion screen. Expression of GR is no longer toxic in strains lacking Nup188 (nuclear pore protein), Rad50 (double stranded break repair protein), Erd1 (ER protein), Nop16 (nucleolar protein), Gis2 (translational activator of specific mRNAs), Stm1 (ribosome preservation factor), Bud21 (ribosomal biogenesis protein) or Ski2 (RNA helicase).

screens in *Drosophila* (Freibaum *et al.* 2015; Zhang *et al.* 2015; Boeynaems *et al.* 2016).

We next compared the hits from the GR_{100} to hits from other screens we have performed on ALS-related proteins, including PR_{50}, FUS and TDP-43 (Table 1). Six of the hits from the GR screen were also hits in the PR screen. This number is small, in part because there were only 13 hits from the PR deletion screen (Jovičić *et al.* 2015) and because some hits from the PR screen were identified in only two out of three rounds of the GR screen. Nevertheless, the overlapping hits are informative, pointing to a role for the shared arginine content in the way these genes interact with and modify these arginine-rich DPRs. Also, while the individual genes between the PR and GR yeast screens diverged, the classes of genetic modifiers that have emerged from this GR_{100} screen have been implicated in GR and PR biology in *Drosophila* and mammalian cell systems (Kwon *et al.* 2014; Boeynaems *et al.* 2016, 2017; Lee *et al.* 2016). There was no overlap with the TDP-43 screen (Armakola *et al.* 2012). Surprisingly, the biggest overlap of hits came from the GR_{100} and FUS screens, with 22 shared suppressors of toxicity (Table 1) (Sun *et al.* 2011). This result could be due to the fact that the FUS protein contains several domains containing arginine/glycine/glycine (RGG) repetitive sequences (Boeynaems *et al.* 2017; Ozdilek *et al.* 2017) that may behave similarly to the repetitive GR_{100} sequence when overexpressed in yeast.

DISCUSSION

Here, we have used a yeast genetic screen to identify suppressors of *C9orf72* GR_{100} toxicity, which provide clues into the potential mechanisms of GR toxicity. While recent studies have focused on the highly toxic PR species or grouped GR and PR together due to their shared arginine content, there has been little done to parse apart potential differences in GR and PR biology, even though such differences exist. From our screen, we have discovered that there is divergence in the genes that suppress GR and PR toxicity when deleted in yeast.

Several factors could contribute to this divergence. First, PR is more toxic than GR (Fig. 1A). This increased toxicity might contribute to the low number of genetic modifiers identified in the PR deletion screen (13) compared to the GR screen (133) (Jovičić *et al.* 2015), since the threshold for suppressing PR toxicity is greater than for GR toxicity. In that case, we could be missing real commonalities between PR and GR, which may be detectable with a less-toxic PR species. And indeed, in other experimental systems, nucleolar and ribosomal proteins, which were modifiers of GR toxicity in yeast, can interact physically with PR (Lee *et al.* 2016; Lin *et al.* 2016; Boeynaems *et al.* 2017). Both PR and GR have also been shown to disrupt the nucleolus and ribosome biogenesis (Kwon *et al.* 2014). The positively charged arginines in both species most likely contribute to these interactions.

However, when we consider the biochemistry of these species, it is important to consider the glycines and prolines in addition to the arginines. Glycine, with a single hydrogen for a sidechain, is dramatically different from proline, which contains a large cyclic side chain that imparts a high degree of structural rigidity to proline-containing peptides. Understanding why the proline content appears to confer increased toxicity at shorter lengths will be an important next step in the field. Furthermore, the specific glycine content is also biologically relevant, as repetitive glycine/arginine rich (GAR) domains occur in numerous proteins and is in fact the second-most common RNA binding domain in the human genome (Ozdilek *et al.* 2017).

The existence of GAR domains, as opposed to proline/arginine rich domains, provides an opportunity for the GR dipeptide species to have a unique impact on the cell. The GR repeats could mimic a protein's GAR domain, thereby wreaking havoc when inserted within specific GAR domain-mediated

Table 1. List of yeast deletion strains that suppress GR_{100} toxicity.

GR100 suppressors	Systematic name	PR_{50} suppressor	FUS suppressor	TDP-43 suppressor	Function	Human ortholog(s)
Ribosomal small subunit biogenesis (16/133, $P = 3.323e{-}4$)						
rps0aΔ	YGR214W				ribosomal 40S subunit protein; rRNA maturation	RPSA
rps1bΔ	YML063W				ribosomal 40S subunit protein	RPS3A
rps6aΔ	YPL090C		*rps6bΔ*		ribosomal 40S subunit protein	RPS6
rps8aΔ	YBL072C		yes		ribosomal 40S subunit protein	RPS8
rps11aΔ	YDR025W				ribosomal 40S subunit protein	RPS11
rps11bΔ	YBR048W				ribosomal 40S subunit protein	RPS11
rps16bΔ	YDL083C				ribosomal 40S subunit protein	RPS16
rps18aΔ	YDR450W				ribosomal 40S subunit protein	RPS18
rps24aΔ	YER074W				ribosomal 40S subunit protein	RPS24
sac3Δ	YDR159W				ribosome biogenesis; mRNA export	SAC3D1/MCM3AP
nsr1Δ	YGR159C	yes	yes		pre-rRNA processing; ribosome biogenesis	
ltv1Δ	YKL143W		yes		Ribosomal small subunit export	LTV1
hcr1Δ	YLR192C				pre-rRNA processing; translation initiation	EIF3J
tsr2Δ	YLR435W		yes		potential role in pre-rRNA processing	TSR2
bud21Δ	YOR078W				part of the ribosomal small subunit processosome	
bud22Δ	YMR014W				rRNA maturation; ribosome biogenesis	SRFBP1
Additional ribosomal proteins and ribosome-associated processes (23/133)						
rpl12aΔ	YEL054C				ribosomal 60S subunit protein	RPL12
rpl19bΔ	YBL027W		yes		ribosomal 60S subunit protein	RPL19
rpl21bΔ	YPL079W				ribosomal 60S subunit protein	RPL21
rpl34aΔ	YER056C-A				ribosomal 60S subunit protein	RPL34
rpl37aΔ	YLR185W				ribosomal 60S subunit protein; pre-rRNA processing	RPL37
rpl38Δ	YLR325C				ribosomal 60S subunit protein	RPL38
rps29aΔ	YLR388W				ribosomal 40S subunit protein	RPS29
rpp1bΔ	YDL130W				component of the ribosomal stalk	RPLP1
rpp2bΔ	YDR382W		yes		component of the ribosomal stalk	RPLP2
cgr1Δ	YGL029W				pre-rRNA processing; nucleolar integrity	CCDC86
hpm1Δ	YIL110W				methyltransferase; modification of ribosomal protein	METTL18
jjj1Δ	YNL227C				ribosome biogenesis	
kap120Δ	YPL125W				karyopherin; nuclear import of ribosomal maturation factor Rpf1p	IPO11
kns1Δ	YLL019C				serine/threonine kinase; ribosome and tRNA biogenesis; rRNA transcription	CLK1-4
nop12Δ	YOL041C				pre-rRNA processing; ribosome biogenesis	HNRNPD/DL/A0/AB
nop16Δ	YER002W		yes		ribosome biogenesis	NOP16
rrp8Δ	YDR083W				methyltransferase; modification of ribosomal protein; pre-rRNA processing	RRP8
stm1Δ	YLR150W				translation and ribosome preservation during nutrient stress; binds G-quadruplexes	SERBP1, HABP4
tif4631Δ	YGR162W				ribosome biogenesis; translation initiation	EIF4G
syh1Δ	YPL105C				unknown function, but associates with nuclear pore and ribosomes	GIGYF1/2
tma19Δ	YKL056C				associates with ribosomes	TPT1, 1P8
ygl088wΔ	YGL088W		yes		unknown function, but partially overlaps with a snoRNA	
yor309cΔ	YOR309C		yes		dubious open reading frame (ORF), but partially overlaps with NOP58	

Table 1. – continued

GR100 suppressors	Systematic name	PR50 suppressor	FUS suppressor	TDP-43 suppressor	Function	Human ortholog(s)
RNA-related processes (15/133)						
*caf120*Δ	YNL278W				part of a transcriptional regulatory complex; mRNA initiation, elongation, degradation	PAK2
*cgi121*Δ	YML036W		yes		part of a tRNA modification complex	TPRKB
*ebs1*Δ	YDR206W				nonsense mediated decay; translation inhibition	SMG5/6/7
*gim3*Δ	YNL153C				part of a prefoldin complex; transcriptional elongation	PFDN4
*gis2*Δ	YNL255C		yes		activation of translation of IRES-containing mRNAs	
*lrp1*Δ	YHR081W				RNA processing, degradation, export	C1D
*nup188*Δ	YML103C				part of nuclear pore complex, nucleocytoplasmic transport	NUP188
*nup84*Δ	YDL116W		yes		part of nuclear pore complex, nucleocytoplasmic transport	NUP107
*she4*Δ	YOR035C				regulation of myosin function; asymmetric mRNA localization	STIP1
*ski2*Δ	YLR398C				RNA helicase; RNA degradation	
*ski8*Δ	YGL213C	yes			RNA helicase; RNA degradation	
*sky1*Δ	YMR216C				serine/arginine kinase; regulation of proteins involved in mRNA metabolism	SRPK1/2/3
*stp1*Δ	YDR463W	yes			transcription factor; potential role in tRNA processing	
*tex1*Δ	YNL253W				mRNA export	THOC3
*tif1*Δ	YKR059W				translation initiation; RNA helicase	EIF4A2
Mitochondrial and NADPH-related metabolic pathways (12/133)						
*aco2*Δ	YJL200C				mitochondrial aconitase isozyme	
*flx1*Δ	YIL134W				mitochondrial flavin adenine dinucleotide transporter	SLC25A32
*idh2*Δ	YOR136W				mitochondrial NAD(+)-dependent isocitrate dehydrogenase	IDH3A
*oxa1*Δ	YER154W				mitochondrial inner membrane insertase	OXA1L
*rcf2*Δ	YNR018W				cytochrome c oxidase subunit	
*zwf1*Δ	YNL241C				glucose-6-phosphate dehydrogenase	H6PD, G6PD
*gor1*Δ	YNL274C				mitochondrial glyoxylate reductase	GRHPR
*gpd2*Δ	YOL059W				NAD-dependent glycerol 3-phosphate dehydrogenase	GPD1, 1L
*gph1*Δ	YPR160W				glycogen phosphorylase; mobilization of glycogen	PYGL/B/M
*stb5*Δ	YHR178W				transcription factor; oxidative stress, stress response	
*nnr2*Δ	YKL151C	yes			NADHX dehydratase	CARKD
*ald6*Δ	YPL061W				aldehyde dehydrogenase	ALDH1A1/A2/A3, ALDH2
Nucleotide biosynthetic pathway (7/133, $P = 3.488e{-}5$)						
*ade1*Δ	YAR015W				purine nucleotide biosynthesis	PAICS
*ade2*Δ	YOR128C				purine nucleotide biosynthesis	
*ade4*Δ	YMR300C				purine nucleotide biosynthesis	PPAT
*ade5, 7*Δ	YGL234W				purine nucleotide biosynthesis	
*ade6*Δ	S000003293				purine nucleotide biosynthesis	PFAS
*ade8*Δ	YDR408C				purine nucleotide biosynthesis	
*bas1*Δ	YKR099W				purine nucleotide biosynthesis; transcription factor	

Table 1. – continued

GR100 suppressors	Systematic name	PR50 suppressor	FUS suppressor	TDP-43 suppressor	Function	Human ortholog(s)
Amino acid and other molecular biosynthetic pathways (10/133)						
*alt1*Δ	YLR089C				alanine transaminase; alanine amino acid synthesis and catabolism	CCBL1/2, GPT1/2
*aro1*Δ	YDR127W				synthesis of chorismate, an amino acid precursor	
*cho2*Δ	YGR157W				methyltransferase; phosphatidylcholine biosynthesis	
*dph6*Δ	YLR143W				diphthamide biosynthesis	DPH6
*elo3*Δ	YLR372W				fatty acid and sphingolipid biosynthesis	
*ilv1*Δ	YER086W				threonine deaminase; isoleucine biosynthesis	
*ino1*Δ	YJL153C				inositol, inositol-containing phospholipid biosynthesis	ISYNA1
*ipk1*Δ	YDR315C		yes		synthesis of phytate	IPPK
*met2*Δ	YNL277W				methionine biosynthesis	
*met22*Δ	YOL064C				methionine biosynthesis	
ER-related processes (4/133)						
*erd1*Δ	YDR414C				lumenal ER protein retention	
*get1*Δ	YGL020C				insertion of proteins into the ER membrane	WRB
*lhs1*Δ	YKL073W				chaperone of the ER lumen; protein translocation and folding	
*sse1*Δ	YPL106C		Yes		HSP90 chaperone complex; binds unfolded proteins	HSPA4/A4L/H1
GTPase-related proteins (7/133)						
*aim44*Δ	YPL158C				cytokinesis; regulates Rho1p	
*tus1*Δ	YLR425W				GEF for Rho1p activity	
*lte1*Δ	YAL024C				similar to GDP/GTP exchange factors	RASGEF1A-C
*msb3*Δ	YNL293W				Rab GTPase activation; endocytosis	TBC1D, SGSM3
*gtr1*Δ	YML121W	yes			part of TORC1-stimulating GTPase complex	RRAGA/B
*tco89*Δ	YPL180W				TORC1 subunit	
*tor1*Δ	YJR066W				TORC1 subunit	MTOR
DNA repair (7/133)						
*asf1*Δ	YJL115W				nucleosome assembly; recovery after double-stranded DNA break repair	ASF1A/B
*rad50*Δ	YNL250W		yes		processing double-stranded DNA breaks	RAD50
*rad51*Δ	YER095W				double-stranded DNA break repair	RAD51
*rad52*Δ	YML032C				double-stranded DNA break repair	RAD52
*vps75*Δ	YNL246W				histone chaperone; double-stranded DNA break repair	SET/SIP, TSPYs, FAM197Y1
*mms22*Δ	YLR320W				E3 ubiquitin ligase complex involved in replication repair	
*slx5*Δ	YDL013W				SUMO-targed ubiquitin ligase complex; DNA repair	
Serine/threonine and serine modifiers (8/133)						
*fus3*Δ	YBL016W				mitogen-activated serine/threonine protein kinase	MAPK1,3,4,5,6 or NLK
*ptk2*Δ	YJR059W				serine/threonine protein kinase; regulation of ion transport	TSSKs
*yck3*Δ	YER123W				vacuolar membrane serine/threonine kinase; vacuole fusion	
*pph21*Δ	YDL134C				catalytic subunit of protein phosphatase 2a (serine/threonine phosphatase); mitosis	

Table 1. – continued

GR100 suppressors	Systematic name	PR50 suppressor	FUS suppressor	TDP-43 suppressor	Function	Human ortholog(s)
ppm1Δ	YDR435C		Yes		methyltransferase; methylates the C terminus of Pph21p	LCMT1
rts1Δ	YOR014W				regulatory subunit of protein phosphatase 2A	PPP2R5C/D
kex2Δ	YNL238W				calcium-dependent serine protease	
prb1Δ	YEL060C				vacuolar serine protease	
Acetyltransferases (3/133)						
eaf6Δ	YJR082C				part of acetyltransferase complex; histone acetylation	MEAF6
hpa3Δ	YEL066W				D-Amino acid N-acetyltransferase; histone acetylation	
mak10Δ	YEL053C				NatC N-terminal acetyltransferase	NAA35
Other (8/133)						
alf1Δ	YNL148C		yes		alpha-tubulin folding; microtubule maintenance	TBCB, CLIP3/4
atx1Δ	YNL259C				cytosolic copper metallochaperone	ATOX1
cdc50Δ	YCR094W				endosomal protein; involved with Golgi membrane trafficking	TMEM30A/B/C
clb2Δ	YPR119W		yes		cell cycle progression	CNTD2
fcy22Δ	YER060W-A				purine-cytosine permease	
fen2Δ	YCR028C				H^+-pantothenate symporterH	
sho1Δ	YER118C				transmembrane osmosensor for filamentous growth	
vps64Δ	YDR200C		yes		cytoplasm to vacuole targeting of proteins	TRAF3IP3, SLMAP, CEP170/B, CCDC136
Uncharacterized proteins (13/133)						
brp1Δ	YGL007W				protein of unknown function	
fyv1Δ	YDR024W				dubious ORF	
fyv6Δ	YNL133C				protein of unknown function	
gds1Δ	YOR355W				protein of unknown function	
hhy1Δ	YEL059W				dubious ORF	
irc14Δ	YOR135C				dubious ORF	
mtc7Δ	YEL033W				protein of unknown function	
rtc4Δ	YNL254C				protein of unknown function	
sdd1Δ	YEL057C				protein of unknown function	
ydr417cΔ	YDR417C		yes		dubious ORF	
ygl165cΔ	YGL165C		yes		dubious ORF	
ynl198cΔ	YNL198C	yes			dubious ORF	
ynr005cΔ	YNR005C		yes		dubious ORF	

RNA/protein or protein/protein interactions within the cell. The results from our screen suggest that this is possible, given the large number of shared hits between screens for modifiers of FUS toxicity and GR_{100} toxicity (Table 1). It would be interesting to see whether ectopic expression of other proteins containing GAR domains would be toxic, and if so, whether they would share significant overlap in toxicity modifiers.

Furthermore, in yeast, the majority of GAR domain proteins are nucleolar proteins involved in ribosomal biogenesis (e.g. Gar1, Nsr1, Nop1, Nop3 and Ssb1) or proteins involved in mRNA handling (e.g. Scd6, Npl3, Gbp2, Nab2, Sbp1, etc.), two major groups identified in our screen for modifiers of GR toxicity (Girard *et al.* 1992; Inoue *et al.* 2000; McBride *et al.* 2009; Rajyaguru and Parker 2012). Nsr1, which contains GAR domains, was identified in both the PR and GR screens and GR-specific hits such as Rrp8 and EIF4G have been shown to directly interact with many of the GAR domain-containing proteins listed above (Bousquet-Antonelli *et al.* 2000; Rajyaguru, She and Parker 2012), lending credence to the possibility that GR_{100} toxicity occurs by interfering with the activity of GAR domain-containing proteins.

Additional experiments to investigate exactly how GR_{100} impacts these pathways are required, but overall, this screen has given us a look into the ways through which GR DPRs produced by the *C9orf72* repeat expansion might contribute to disease, and provide potential druggable targets to ameliorate DPR toxicity. The surprising lack of overlap between hits from our GR screen here and our previous PR screen (Jovičić *et al.* 2015) underscores the importance of considering GR and PR toxic mechanisms as distinct and in pursuing approaches to deal with them separately.

FUNDING

This work was supported by NIH grant R35NS097263 (A.D.G.), the Robert Packard Center for ALS Research at Johns Hopkins (A.D.G.), Target ALS (A.D.G.) and the Stanford Brain Rejuvenation Project of the Stanford Neurosciences Institute (A.D.G.).

Conflict of interest. None declared.

REFERENCES

Alberti S, Gitler AD, Lindquist S. A suite of Gateway cloning vectors for high-throughput genetic analysis in *Saccharomyces cerevisiae*. *Yeast* 2007;**24**:913–9.

Armakola M, Higgins MJ, Figley MD *et al*. Inhibition of RNA lariat debranching enzyme suppresses TDP-43 toxicity in ALS disease models. *Nat Genet* 2012;**44**:1302–9.

Ash PE, Bieniek KF, Gendron TF *et al*. Unconventional translation of C9ORF72 GGGGCC expansion generates insoluble polypeptides specific to c9FTD/ALS. *Neuron* 2013;**77**:639–46.

Boeynaems S, Bogaert E, Kovacs D *et al*. Phase separation of C9orf72 dipeptide repeats perturbs stress granule dynamics. *Mol Cell* 2017;**65**:1044–55. e5.

Boeynaems S, Bogaert E, Michiels E *et al*. Drosophila screen connects nuclear transport genes to DPR pathology in c9ALS/FTD. *Sci Rep* 2016;**6**:20877.

Bousquet-Antonelli C, Vanrobays E, Gélugne J-P *et al*. Rrp8p is a yeast nucleolar protein functionally linked to Gar1p and involved in pre-rRNA cleavage at site A2. *RNA* 2000;**6**:826–43.

Cherry JM, Hong EL, Amundsen C *et al*. Saccharomyces genome database: the genomics resource of budding yeast. *Nucleic Acids Res* 2012;**40**:D700–5.

Collins SR, Schuldiner M, Krogan NJ *et al*. A strategy for extracting and analyzing large-scale quantitative epistatic interaction data. *Genome Biol* 2006;**7**:R63.

DeJesus-Hernandez M, Mackenzie IR, Boeve BF *et al*. Expanded GGGGCC hexanucleotide repeat in noncoding region of C9ORF72 causes chromosome 9p-linked FTD and ALS. *Neuron* 2011;**72**:245–56.

Freibaum BD, Lu Y, Lopez-Gonzalez R *et al*. GGGGCC repeat expansion in C9orf72 compromises nucleocytoplasmic transport. *Nature* 2015;**525**:129–33.

Gendron TF, Bieniek KF, Zhang YJ *et al*. Antisense transcripts of the expanded C9ORF72 hexanucleotide repeat form nuclear RNA foci and undergo repeat-associated non-ATG translation in c9FTD/ALS. *Acta Neuropathol* 2013;**126**:829–44.

Girard JP, Lehtonen H, Caizergues-Ferrer M *et al*. GAR1 is an essential small nucleolar RNP protein required for pre-rRNA processing in yeast. *EMBO J* 1992;**11**:673–82.

Gitler AD, Tsuiji H. There has been an awakening: emerging mechanisms of C9orf72 mutations in FTD/ALS. *Brain Res* 2016;**1647**:19–29.

Haeusler AR, Donnelly CJ, Periz G *et al*. C9orf72 nucleotide repeat structures initiate molecular cascades of disease. *Nature* 2014;**507**:195–200.

Inoue K, Mizuno T, Wada K *et al*. Novel RING finger proteins, Air1p and Air2p, interact with Hmt1p and inhibit the arginine methylation of Npl3p. *J Biol Chem* 2000;**275**:32793–9.

Jovičić A, Mertens J, Boeynaems S *et al*. Modifiers of C9orf72 dipeptide repeat toxicity connect nucleocytoplasmic transport defects to FTD/ALS. *Nat Neurosci* 2015;**18**:1226–9.

Kwon I, Xiang S, Kato M *et al*. Poly-dipeptides encoded by the C9orf72 repeats bind nucleoli, impede RNA biogenesis, and kill cells. *Science* 2014;**345**:1139–45.

Lee KH, Zhang P, Kim HJ *et al*. C9orf72 dipeptide repeats impair the assembly, dynamics, and function of membrane-less organelles. *Cell* 2016;**167**:774–88. e17.

Lin Y, Mori E, Kato M *et al*. Toxic PR poly-dipeptides encoded by the C9orf72 repeat expansion target LC domain polymers. *Cell* 2016;**167**:789–802. e12.

Lopez-Gonzalez R, Lu Y, Gendron TF *et al*. Poly(GR) in C9ORF72-related ALS/FTD compromises mitochondrial function and increases oxidative stress and DNA damage in iPSC-derived motor neurons. *Neuron* 2016;**92**:383–91.

McBride AE, Conboy AK, Brown SP *et al*. Specific sequences within arginine-glycine-rich domains affect mRNA-binding protein function. *Nucleic Acids Res* 2009;**37**:4322–30.

Mizielinska S, Gronke S, Niccoli T *et al*. C9orf72 repeat expansions cause neurodegeneration in Drosophila through arginine-rich proteins. *Science* 2014;**345**:1192–4.

Mori K, Arzberger T, Grasser FA *et al*. Bidirectional transcripts of the expanded C9orf72 hexanucleotide repeat are translated into aggregating dipeptide repeat proteins. *Acta Neuropathol* 2013;**126**:881–93.

Ozdilek BA, Thompson VF, Ahmed NS *et al*. Intrinsically disordered RGG/RG domains mediate degenerate specificity in RNA binding. *Nucleic Acids Res* 2017;**45**:7984–96.

Rajyaguru P, Parker R. RGG motif proteins: modulators of mRNA functional states. *Cell Cycle* 2012;**11**:2594–9.

Rajyaguru P, She M, Parker R. Scd6 targets eIF4G to repress translation: RGG motif proteins as a class of eIF4G-binding proteins. *Mol Cell* 2012;**45**:244–54.

Renton AE, Chio A, Traynor BJ. State of play in amyotrophic lateral sclerosis genetics. *Nat Neurosci* 2014;**17**:17–23.

Renton AE, Majounie E, Waite A *et al*. A hexanucleotide repeat expansion in C9ORF72 is the cause of chromosome 9p21-linked ALS-FTD. *Neuron* 2011;**72**:257–68.

Sun Z, Diaz Z, Fang X *et al*. Molecular determinants and genetic modifiers of aggregation and toxicity for the ALS disease protein FUS/TLS. *PLoS Biol* 2011;**9**:e1000614.

Swinnen B, Robberecht W. The phenotypic variability of amyotrophic lateral sclerosis. *Nat Rev Neurol* 2014;**10**:661–70.

Taylor JP, Brown RH, Cleveland DW. Decoding ALS: from genes to mechanism. *Nature* 2016;**539**:197–206.

Tong AH, Boone C. Synthetic genetic array analysis in *Saccharomyces cerevisiae*. *Methods Mol Biol* 2006;**313**:171–92.

Waite AJ, Baumer D, East S *et al*. Reduced C9orf72 protein levels in frontal cortex of amyotrophic lateral sclerosis and frontotemporal degeneration brain with the C9ORF72 hexanucleotide repeat expansion. *Neurobiol Aging* 2014;**35**:1779.e5–13.

Wen X, Tan W, Westergard T *et al*. Antisense Proline-Arginine RAN dipeptides linked to C9ORF72-ALS/FTD form toxic nuclear aggregates that initiate in vitro and in vivo neuronal death. *Neuron* 2014;**84**:1213–25.

Zhang K, Donnelly CJ, Haeusler AR *et al*. The C9orf72 repeat expansion disrupts nucleocytoplasmic transport. *Nature* 2015;**525**:56–61.

Zu T, Gibbens B, Doty NS *et al*. Non-ATG-initiated translation directed by microsatellite expansions. *Proc Natl Acad Sci* 2011;**108**:260–5.

Zu T, Liu Y, Banez-Coronel M *et al*. RAN proteins and RNA foci from antisense transcripts in C9ORF72 ALS and frontotemporal dementia. *Proc Natl Acad Sci* 2013;**110**:E4968–77.