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ORIGINAL PAPER

Functional genomics screen identifies proteostasis targets that modulate prion protein (PrP) stability

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Abstract

Prion protein (PrP) adopts either a helical conformation (PrP^{C}) or an alternative, beta sheet-rich, misfolded conformation (PrP^{Sc}). The PrP^{Sc} form has the ability to "infect" PrP^{C} and force it into the misfolded state. Accumulation of PrP^{Sc} is associated with a number of lethal neurodegenerative disorders, including Creutzfeldt-Jacob disease (CJD). Knockout of PrP^{C} protects cells and animals from PrP^{Sc} infection; thus, there is interest in identifying factors that regulate PrP^{C} stability, with the therapeutic goal of reducing PrP^{C} levels and limiting infection by PrP^{Sc}. Here, we assembled a short-hairpin RNA (shRNA) library composed of 25+ shRNA sequences for each of 133 protein homeostasis (aka proteostasis) factors, such as molecular chaperones and co-chaperones. This Proteostasis shRNA Library was used to identify regulators of PrP^{C} stability in HEK293 Hu129M cells. Strikingly, the screen identified a number of Hsp70 family members and their co-chaperones as putative targets. Indeed, a chemical pan-inhibitor of Hsp70s reduced PrP^{C} levels and limited conversion to PrP^{Sc} in N2a cells. These results implicate specific proteostasis sub-networks, especially the Hsp70 system, as potential new targets for the treatment of CJD. More broadly, the Proteostasis shRNA Library might be a useful tool for asking which proteostasis factors are important for a given protein.

Keywords shRNA · Neurodegeneration · Heat shock protein 70 · Drug targets · Chaperone networks · Prion protein (PrP)

Introduction

Creutzfeldt-Jacob disease (CJD) is a deadly neurodegenerative disease that is rapidly progressive and incurable (Colby and Prusiner 2011). CJD and other prion disorders are caused by conversion of prion protein (PrP^{C}) into a misfolded conformer (PrP^{Sc}). The misfolded PrP^{Sc} accumulates as oligomeric aggregates and amyloid fibrils that have been linked to proteotoxicity and neuron loss (Diaz-Espinoza and Soto 2012; Nunziante et al. 2003). The different "strains" of PrP^{Sc}, such as Rocky Mountain laboratory (RML), may represent partially distinct mis-folding pathways (Moda et al. 2015). Recent studies have suggested that other neurodegenerative disorders, such as tauopathies and synucleinopathies, might share features in common with CJD; specifically, the misfolded proteins "spread" to healthy cells and seed misfolding of native proteins (Clavaguera et al. 2009; Iba et al. 2013; Prusiner 2012). Such observations have made the search for anti-prion strategies even more urgent, because ways of blocking prion-like propagation might be more widely applicable than previously imagined.

PrP^{C} is a helical, GPI-linked protein that is composed of a hydrophobic core and disordered octapeptide repeats (Acevedo-Morantes and Wille 2014). PrP^{C} is generated at ER-localized ribosomes and inserted into the ER lumen, where it is matured and subject to modification by *N*-glycosylation (Chakrabarti et al. 2009). Addition of a GPI anchor and further maturation in the Golgi occurs prior to display of PrP^{C} at the plasma membrane (Aguzzi and Falsig 2012). In cell culture, most PrP^{C} resides in lipid rafts, while a small percentage (~ 2%) is also secreted into the cytoplasm and another minor population is abnormally inserted as a transmembrane protein (Mironov et al.

Jennifer Abrams and Taylor Arhar contributed equally to this work.

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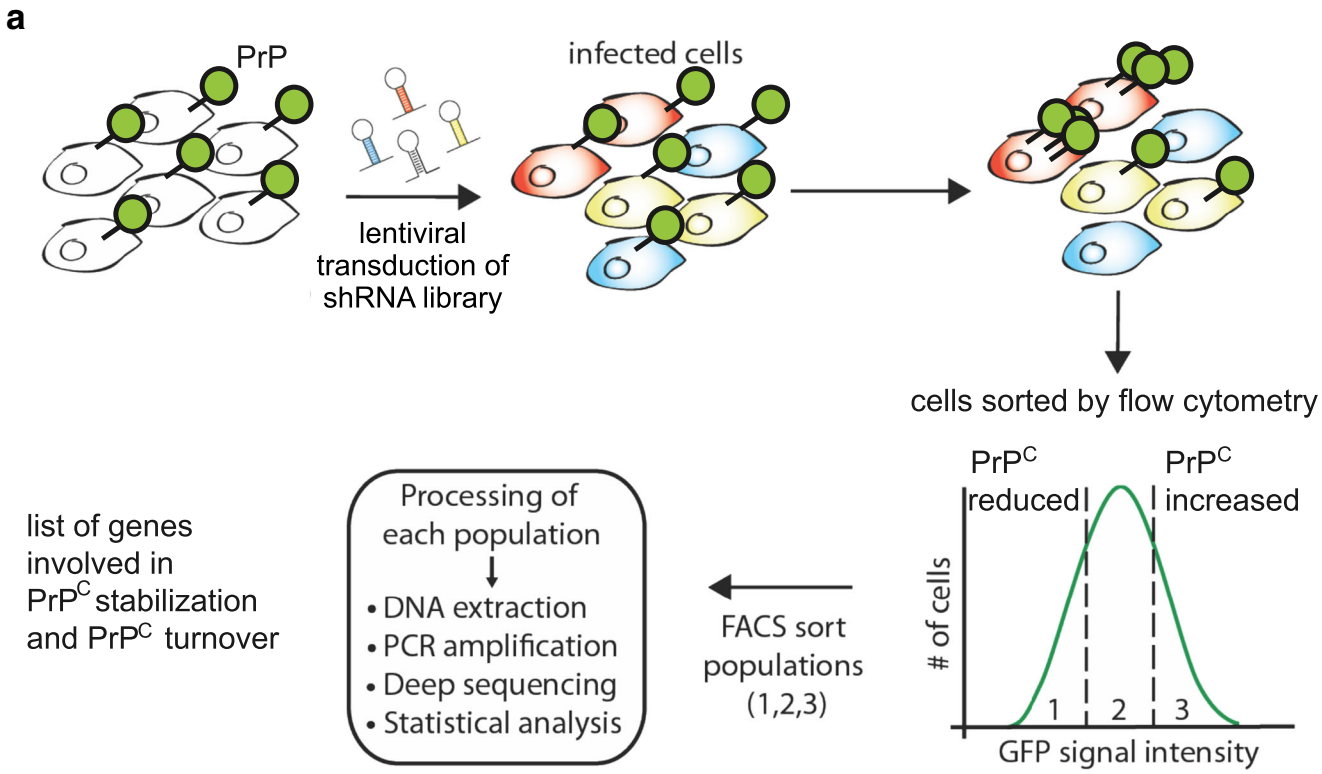

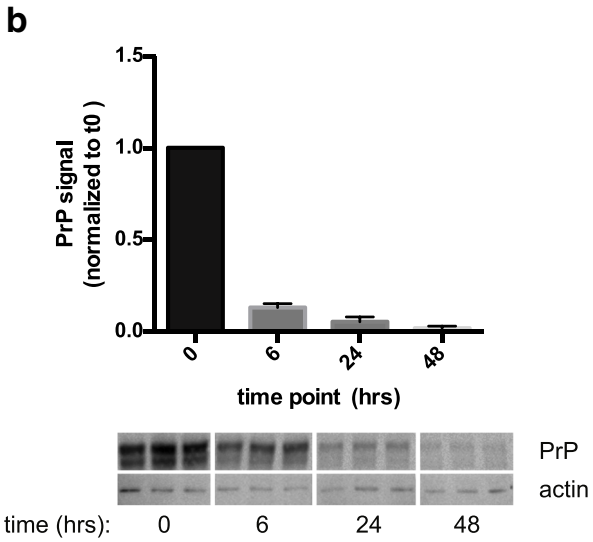

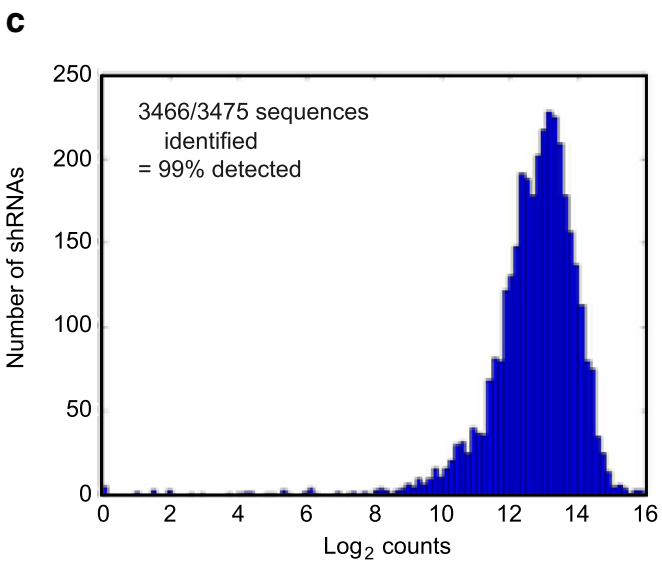

Fig. 1 Design of an shRNA screen to identify proteostasis regulators of PrP^C stability. **a** Schematic for the flow cytometry-based screen. HEK293 cells expressing human 129M PrP are transduced with the Proteostasis shRNA Library, grown for 10 doublings, sorted for PrP^C surface expression (high, med, low), and sequenced. **b** Pulse chase study establishes the lifetime of PrP^C. HEK293 cells expressing mCherry-tagged human 129M PrP were treated with 50uM cycloheximide at t = 0, and cells were lysed at 6, 24, and 48 h post-treatment. Results are the average of experiments performed in triplicate. Error is SEM. **c** Expression tests of the Proteostasis shRNA library suggest high coverage in the lentiviral infection, with ~ 99% of shRNAs expressed

2003). In some heritable prion diseases, the percentage of these abnormally processed PrP^C variants is increased, suggesting that mis-trafficking may be involved in pathology (Ben-Gedalya et al. 2011). Cytoplasmic PrP^C is degraded via the ubiquitin proteasome system (UPS), while PrP^C at the plasma membrane is cycled through endosomes in a caveolae-mediated process (P. J. Peters et al. 2003; Ren et al. 2013) and PrP^C in endosomes is thought to be degraded by fusion with lysosomes (Mays et al. 2014; P. J. Peters et al. 2003). Further, some PrP^C is subject to retrograde transport and degradation through the proteasome via

ER-associated degradation (ERAD) (Ma and Lindquist 2001). When any of these pathways are disrupted (e.g., by heritable mutations or aging), PrP^C homeostasis can become imbalanced.

Active production of PrP^C is required for prion disease in mice (Friberg et al. 2012). Moreover, the survival of mice infected with prions is strongly correlated with the expression level of PrP^C, such that mice with little PrP^C are relatively immune to infection and mice over-expressing PrP^C are highly sensitive (Safar et al. 2005). These results strongly suggest that one way to treat prion disease may be to reduce levels of PrP^C (Astolfi et al. 2020; Baldeschi et al. 2020). Indeed, antisense oligonucleotides targeted against PrP mRNA have shown promise in extending the survival of prion-infected mice (Friberg et al. 2012; Raymond et al. 2019). As an alternative to decreasing expression of PrP^C, another attractive strategy is to accelerate PrP^C degradation. When PrP^C folding is slowed, it is recognized by ER-resident chaperones and its degradation is accelerated by ERAD (Andre and Tabrizi 2012; Shao et al. 2014; Watts et al. 2009). Similarly, failure of any of the ER-Golgi checkpoints, such as the glycosylation machinery, promotes PrP^C turnover (Cancellotti et al. 2013; Shao et al. 2014). Thus, there are robust protein quality control (PQC) pathways for degrading PrP^C. While these findings are mechanistically informative, it is not yet clear what specific targets in the PQC pathways might serve as putative drug targets to promote turnover.

Molecular chaperones are primarily responsible for the recognition of misfolded or damaged proteins (Kim et al. 2013). The mammalian chaperone network is composed of ~ 180 chaperones and co-chaperones that work together in dynamic, multi-protein complexes to guide "client" proteins, such as PrP^C, through quality control and triage (Balchin et al. 2016). These chaperones include a large class of heat shock proteins, such as heat shock protein 70 (Hsp70), Hsp90, Hsp60, Hsp110, and Hsp27 (Dahiya and Buchner 2019), which are supported by co-chaperones, adapters, and other related factors. The network of chaperones and co-chaperones is highly interconnected through protein-protein interactions (Freilich et al. 2018), and it is characterized by both "general" factors (e.g., those that play housekeeping functions) and highly specialized components (e.g., co-chaperones that act as scaffolding factors to recruit chaperones into specific cellular tasks) (Kampinga and Craig 2010; Rosenzweig et al. 2019). For example, the Hsp70 sub-network is composed, in part, of up to 13 Hsp70 genes, >45 J-domain proteins (JDPs), and ~ 10 nucleotide exchange factors (NEFs), which enable this system the versatility to perform diverse functions. Indeed, JDPs have been well-studied in yeast for their specialized roles in regulating prion propagation and curing, where different prions and prion variants have distinct JDP requirements (Astor et al. 2018; Oliver et al. 2017; Reidy et al. 2014; Stein and True 2014).

Previous studies have linked specific chaperones to PrP proteostasis. For example, multiple groups have shown that the ER-resident Hsp70 paralog BiP/HSPA5 is involved in regulation of PrP^C retrotranslocation and degradation (Park et al. 2017; S. L. Peters et al. 2016; Zavodszky and Hegde 2019). A few studies have also suggested that PrP^C may be degraded through a mechanism dependent on cytoplasmic Hsp70 (Winklhofer et al. 2003; Zhang et al. 2012) and Hsp60/HSPD1 (Edenhofer et al. 1996). This link is also suggested by observations from animal models; for example, over-expression of the cytoplasmic paralog of Hsp70 protects against prion-related neurodegeneration in a fly model (Fernandez-Funez et al. 2009) and knockdown of Hsp70 has the opposite effect in mice (Mays et al. 2019). Similarly, knockdown of HSPA13, a relatively under-studied Hsp70 paralog, is known to reduce RML PrP^{Sc} prion infection by 40% (Brown et al. 2014), while over-expression of this chaperone reduces incubation time in mice (Grizenkova et al. 2012). Beyond Hsp70, more recent studies have shown a potential interaction of PrP^C with other components of the proteostasis network, including TRiC/CCT (Kudryavtseva et al. 2020), Sti1 (Landemberger et al. 2018), and the Hsp70 co-chaperones, DnaJA2 and DnaJB1 (Beck et al. 2006; Rambold et al. 2007).

Given the known roles of chaperones in regulation of PrP^C homeostasis, this class of proteins seems likely to include appealing drug targets. Indeed, treatment with a pharmacological inhibitor of FKBPs is known to cause degradation of PrP^C, and Hsp90 inhibition is hypothesized to affect PrP^C processing and conformation (Ochel et al. 2003; Stocki et al. 2016). However, the chaperone network has not been systematically surveyed to identify which members may be the most valuable drug targets. To address this gap, we assembled an shRNA library targeting 133 chaperones and related factors, termed the Proteostasis shRNA Library. Using this collection and a flow cytometry-based selection approach, we identified chaperones that regulate cell-surface levels of PrP^C in HEK293 cells expressing human 129M PrP. This screen identified benchmark chaperones, such as Hsp90 (HSP90AA1) and Hsp70s (BiP/HSPA5, HSPA13), as well as unexpected factors, including co-chaperones of both Hsp70s and Hsp90s. Importantly, we also found that pharmacological inhibitors of Hsp70 reduced PrP^C levels and limited PrP^{Sc} infectivity. Together, these results suggest that the proteostasis network contains putative drug targets for the treatment of prion diseases, such as CJD. More broadly, the Proteostasis shRNA Library might be a useful tool for asking which chaperones are important for a given "client" protein.

Results

Identification of proteostasis factors that regulate PrP^C stability

To identify proteostasis factors that regulate the stability of PrP^C, we used an optimized design algorithm (Kampmann et al. 2015) to assemble a library of 500 non-targeting, control shRNAs and

25 shRNA sequences for each of the 133 human chaperone ORFs, termed the Proteostasis shRNA Library. Recent work has shown how using large numbers of shRNA sequences can minimize off-target effects (Kampmann et al. 2013). Using this approach, we focused on HEK293 Hu129M cells, which overexpress membrane-anchored PrP^C with the 129M mutation, a risk factor for CJD (Will et al. 2000). Cells were lentivirally transduced with the shRNA library, and the transduced cells were grown for ~10 doublings (Fig. 1a), ideally allowing sufficient time for both knockdown of the shRNA targets and also for PrP^C turnover, based on pulse chase studies (Fig. 1b). Notably, the majority of shRNAs (~ 99%) were detected in the cell population, showing high coverage in the lentiviral infection (Fig. 1c). Using flow cytometry and an anti-PrP antibody, transduced cells were sorted into populations of low, medium, and high PrP^C surface expression (Fig. 1a). Genomic DNA was isolated from the cells in each population, and shRNA-encoding constructs were PCR amplified from the genomic DNA. The frequencies of these shRNA-encoding constructs in each population were determined by deep sequencing (Bassik et al. 2013). Hits were corrected for growth of the cells, to remove non-specific effects on viability.

To identify regulators of PrP^C stability, we then compared the frequencies of shRNAs from the High vs Low PrP^C (Fig. 2a) and High vs Med PrP^C (Fig. 2b) populations. The High vs Low comparison was expected to be more inclusive of factors involved in PrP^C stability, while the High vs. Medium comparison was chosen to potentially highlight the most critical genes. This approach revealed proteostasis factors that, when knocked down, would either increase or decrease surface PrP^C levels. Genes that were statistically significant hits ($-\mathrm{Log}_{10}$ P value > 2) are labeled in Fig. 2a and b. Among the identified genes were those that have previously been implicated in PrP proteostasis, including subunits of TRiC/CCT, Hsp60/HSPD1, and Hsp90/Hsp90AA1 (Edenhofer et al. 1996; Kudryavtseva et al. 2020; Ochel et al. 2003), confirming that benchmark genes could be identified using this approach. Strikingly, many of the other hit genes belong to the Hsp70 sub-network (orange points, Fig 2a/b). Multiple members of the Hsp70 family cluster were identified, including representatives from both the ER (BiP/HSPA5, HYOU1) and cytoplasm (HSPA13). In addition, multiple Hsp70 co-chaperones, including HSPA4, DnaJB1, DnaJC3, and DnaJC6, were also identified, with DnaJC6 emerging as the strongest "hit."

To further emphasize hits that significantly increased or decreased PrP^C, each gene was assigned a gene score (defined in "Methods"). To illustrate which sub-networks might be important for PrP^C stability, we projected the gene scores from the High vs Low (Fig. 2c) and High vs Med comparisons (Supplemental Fig. 1D) onto a schematic map, in which members of specific chaperone families are clustered and lines between clusters indicate known protein-protein interactions. From this analysis, one striking observation is that most proteostasis factors, at least under these conditions, do not seem to be significantly involved in PrP^C stability (white boxes, no phenotype). However, given the possibility of false negatives, some regulators of PrP^C stability may have gone undetected. In both comparisons, significant hits (denoted by bold, colored gene labels in Fig. 2c, Supplemental Fig. 1D) were identified in the Hsp70 sub-network, the Hsp90 sub-network, and the chaperonins. Although a broad role for Hsp70s in PrP^C homeostasis had been suggested, several of the specific factors identified here had not previously been implicated in PrP proteostasis; for example, the hypoxia-associated Hsp70 family member, HYOU1, and the JDP, DnaJC6 (Fig. 2c). Together, these findings implicate the Hsp70 systems, present in both the ER and cytoplasm, as being important for PrP^C stability.

Another strong hit identified by the screen was the Hsp90 co-chaperone Cdc37. Although Hsp90 has multiple co-chaperones, such as Aha1/AHSA1 and p23/PTGES3, their knockdown had little effect on PrP^C surface expression (Fig. 2c) and only Cdc37 was a significant "hit." Cdc37 is selectively required for processing of kinases by the Hsp90 system (Verba and Agard 2017), so it is possible that this pair regulates a signaling pathway important for PrP^C stability. It is also interesting that knockdown of Hsp90/HSP90AA1 and Cdc37 had opposing effects on PrP^C surface expression, with the Hsp90 increasing PrP^C and Cdc37 decreasing it. The distinct phenotype of Cdc37 knockdown suggests that specifically targeting only the Cdc37-dependent functions of Hsp90, or the co-chaperone itself, could be a viable strategy for promoting PrP^C turnover. Because Cdc37 is not yet validated, future studies will be necessary to validate this co-chaperone as a regulator of PrP^C stability.

Hsp70 inhibition reduces PrP^C and PrP^{Sc} infectivity

To explicitly probe whether this screen might identify putative drug targets, we focused on the Hsp70s. Briefly, the function of Hsp70s is dependent on ATPase activity that is promoted by interactions with NEFs and JDPs (Fig. 3). The small molecule JG-48 and its analogs are known to stall ATP cycling in Hsp70s by binding to an allosteric site that is conserved in both ER- and cytoplasmic paralogs (Li et al. 2013; Rousaki et al. 2011; Shao et al. 2018) and "trapping" the ADP- state (Li et al. 2015) (Fig. 3a). Previous work demonstrated that stabilization of the client-bound state is associated with increased client turnover (Young et al. 2016). Thus, to test whether pan-inhibition of Hsp70s would reduce PrP^C levels, HEK293 cells expressing human PrP tagged with mCherry were treated with JG-48 or a structurally similar, negative control (JG-258) (Shao et al. 2018). Indeed, we found that treatment with JG-48 reduced PrP^C levels by > 80%, with an EC_{50} value of ~ 0.7 μM, while JG-258 was inactive. To assess whether pan-Hsp70 inhibition could decrease PrP^{Sc} propagation, we utilized prion-infected mouse

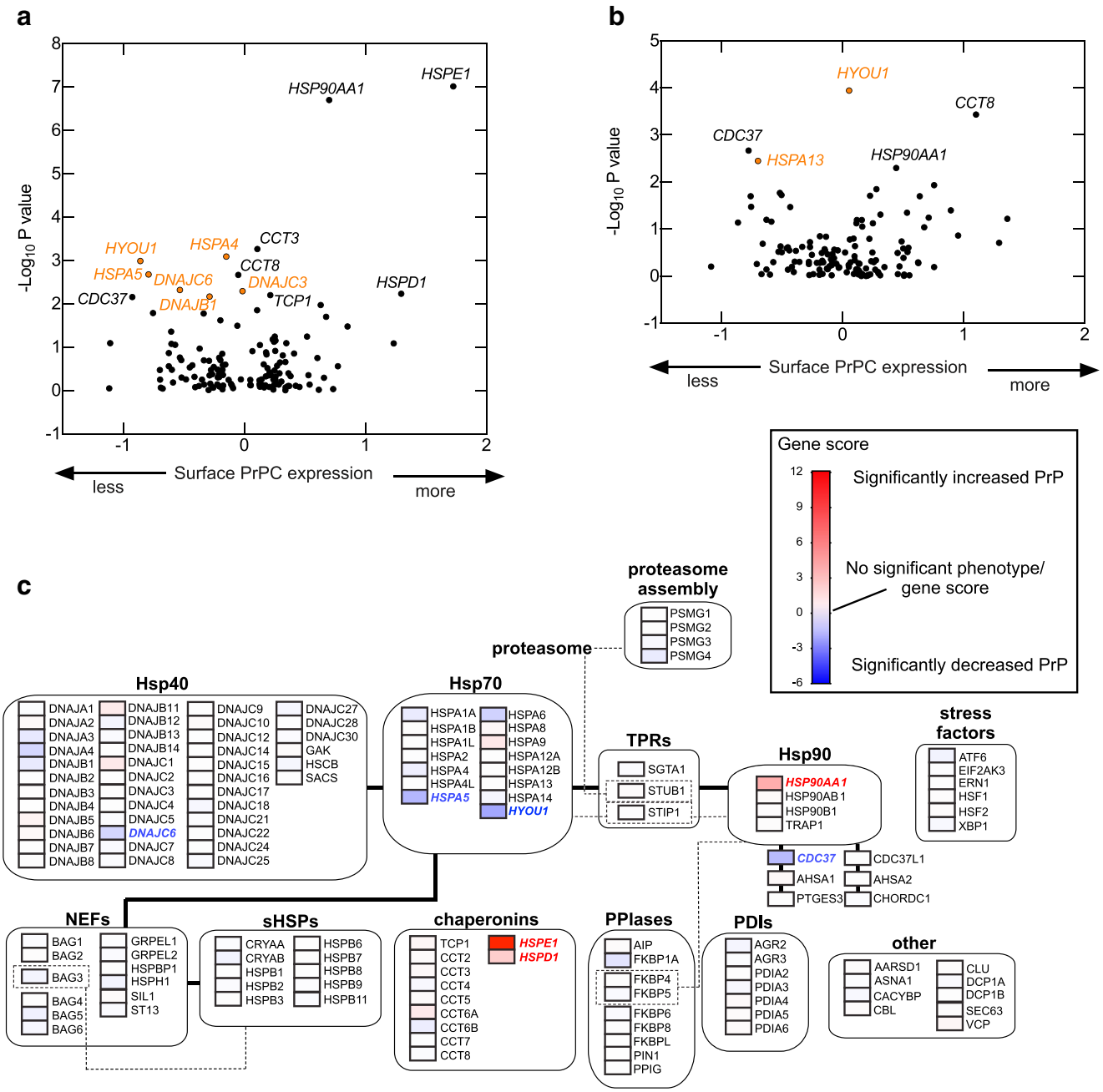

Fig. 2 shRNA screen reveals proteostasis regulators of PrP^C. Genes that affect cell surface levels of PrP^C were compared between High and Low expression groups, and High and Medium expression groups. **a**, **b** Volcano plots showing knockdown effects (increase or decrease in surface PrP levels) and statistical significance of genes in the shRNA library. Genes that are significant hits (−Log10 *P* value > 2.0) are labeled, with orange points representing significant Hsp70s and their co-chaperones. The remainder of the genes targeted by the shRNA library are unlabeled and colored black. **c** Genes targeted by the Proteostasis shRNA library are grouped by their chaperone class. Physical protein-protein interactions (PPIs) between these classes, or between individual members of these classes, are denoted by black lines and dotted lines, respectively. By comparing High and Low populations, genes are scored according to the product of their phenotype and -Log10 *P* value. Statistically significant genes that also have a high gene score (|gene score| > 1) are labeled in bold/italics and colored by their phenotype

neuroblastoma cells (ScN2a-cl3), which stably propagate PrP^{Sc} (Butler et al. 1988; Ghaemmaghami et al. 2010). ScN2a-cl3 cells were incubated with the compounds for 3 days, and PrP^{Sc} levels were subsequently assessed by dot blot. Treatment with JG-48, but not JG-258, suppressed PrP^{Sc} levels in ScN2a-cl3 cells (Fig. 3b). Encouragingly, JG-48 treatment resulted in limited cellular toxicity (Supplemental Fig. 2A). These results suggest a key role for Hsp70s in regulation of PrP^C stability and, moreover, suggest that inhibiting Hsp70 could be a viable therapeutic strategy.

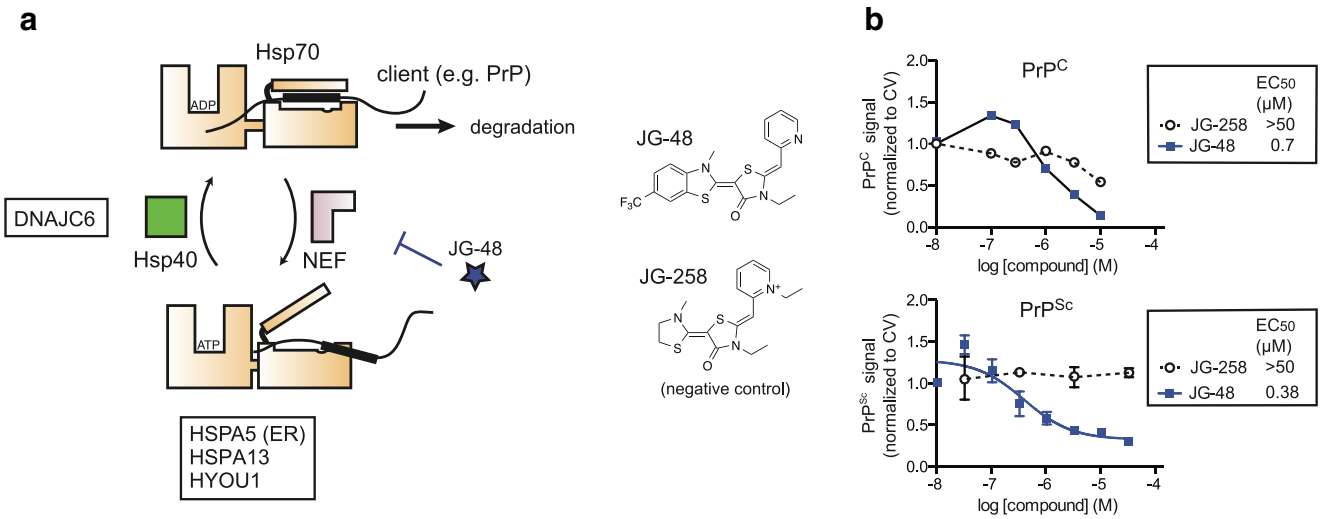

Fig. 3 Hsp70 inhibition reduces PrP^C and PrP^{Sc} infectivity. **a** Schematic of Hsp70 ATP cycling, which regulates binding to clients, like PrP. Hsp70 co-chaperones, such as Hsp40s/JDPs and NEFs, impart selectivity and increase Hsp70 activity. Genes from these classes (Hsp70, Hsp40) were identified as regulators of PrP stability, and are listed by class. **b** JG-48 decreased PrP^C levels in HEK 293 cells expressing mCherry-tagged Hu129M PrP, and decreased PrPSc propagation in ScN2a-cl3 cells. PrP^C and PrP^{Sc} levels were assessed by dot blot, and results are the average of three independent experiments, each performed in quadruplicate. Error is SEM. The chemical structures of tested Hsp70 inhibitors are shown. CV, cell viability

Discussion

PrP^C is a required factor for the pathogenesis of prion diseases, such as CJD. While pharmacological efforts to develop treatments have focused on extending the lifespan of mice infected with PrP^{Sc}, fewer strategies have emerged to regulate PrP^C itself (Giles et al. 2017). Here, we specifically asked which proteostasis factors might be particularly promising targets for reducing PrP^C stability; for example, by increasing its turnover, or blocking its trafficking. We expected to identify numerous possibilities, given that PrP^C has been shown to engage with multiple individual molecular chaperones (Beck et al. 2006; Edenhofer et al. 1996; Jin et al. 2000; Kudryavtseva et al. 2020; Rambold et al. 2007; Zavodszky and Hegde 2019). However, we wanted to take advantage of advances in next-generation, shRNA-based screening to survey a broad set of factors in the network (133 genes).

The screen identified both known and novel regulators of PrP^C, thus highlighting a subset of chaperones as potential therapeutic targets for prion disease. This list included both benchmark factors, such as Hsp70s and Hsp90, and unexpected ones, such as multiple JDPs and Cdc37. Since numerous genes in the Hsp70 system were identified, an important next step was to validate this subnetwork using chemical inhibitors. We found that a pan-inhibitor of Hsp70s reduced PrP^C stability and limited PrP^{Sc} infection in N2a cells. Thus, Hsp70s may be a viable drug target class for prion diseases. Importantly, we envision that the interactions of Hsp70s with PrP^C might be both direct (e.g., binding to PrP^C) and indirect (e.g., broadly remodeling proteostasis). Future efforts should focus on pharmacological inhibition of other identified targets, such as TRiC/CCT and Hsp60/HSPD1. The co-chaperones, such as Cdc37 and DnaJC6, might also serve as selectivity adapters and could be particularly interesting drug targets.

Finally, we propose that shRNA or CRISPRa/i screens, focused on proteostasis factors, will continue to be useful in mapping the reliance of individual "client" proteins on specific chaperones and co-chaperones in mammalian cells. It seems likely that each client may be regulated by a partially distinct sub-set of proteostasis factors. In the future, the flow cytometry approach outlined here could be used to study other soluble and membrane proteins, such as CFTR, and, from those results, sub-networks that are relatively client-specific could emerge. However, as mentioned above, it is likely that these shRNA screens include false positives and negatives, so validation studies and supplemental CRISPRa/i screening will be needed to more comprehensively map the reliance of a client on individual proteostasis factors. Despite these caveats, the Proteostasis shRNA Library has the advantage that dCas9 does not need to be stably expressed in the cell population, making it more versatile for use across multiple cell lines.

Methods

Reagents and antibodies

PrP^{Sc} was detected with the D13 antibody (Williamson et al. 1998), and PrP^C was detected using Rabbit anti-PrP (Abcam ab703). Secondary antibodies included Goat anti-Human IgG F(ab')2 conjugated to HRP (Thermo Scientific 31482) and Goat anti-Rabbit IgG (H+L) conjugated to HRP (Anaspec

AS-28177). The chaperone shRNA library was cloned into the lentiviral backbone plasmid, pMK1275, which is a next-generation shRNA expression vector (Kampmann et al. 2015) with an EF1a promoter and a tagBFP marker. The mCherry-PrP plasmid was provided by Tagan Griffin (UCSF).

Cell culture

ScN2a-cl3 cells, prion-infected mouse neuroblastoma cells expressing high levels of PrP (Ghaemmaghami et al. 2010), were cultured in DMEM supplemented with 1% GlutaMAX, 100 units pen-strep and 10% FBS. ScN2a-cl3 cells were generated by infecting N2a-cl3 cells with the Rocky Mountain Laboratory (RML) strain of mouse scrapie prions as previously described (Butler et al. 1988). HEK293 Hu129M cells were cultured in Eagle's Minimal Essential Medium supplemented with 10% FBS.

Lentiviral production and infection

Lentiviruses were transfected into HEK293T cells using Lipofectamine 2000 (Invitrogen 11668019) and packaging plasmids pMol, pRSV, and pVSV-g. Viral particles were formed for 48 h post transfection, and then the viral supernatant was collected, passed through a 0.45-μm filter, and stored at 4 °C for no longer than 1 week prior to use. Viral supernatant was added to suspended cells immediately following trypsinization, along with 8 μg/mL polybrene (Santa Cruz sc-134220). After 6–8 h, the medium was replaced with regular growth medium. Two days after viral transfection, 1 μg/mL of puromycin (Gibco A11138-03) was supplemented for 48 h to select for cells that were infected with the lentiviral plasmids. Flow cytometry was used to determine infection and selection efficiency via the expression of fluorescent markers encoded by the lentiviral vectors.

Pooled shRNA screen

Lentivirus was prepared as described above and used to infect the HEK293 Hu129M cells. Cells were initially infected at ~ 50–60% efficiency, monitored by BFP intensity, and then were further selected with puromycin to ~ 100%. Under these conditions, there should be good coverage of the library (see Fig. 1c) but also a fraction of the cells will be transduced with multiple viruses, which may contribute to the phenotype. After growth for ~ 10 doublings, cells were sorted via flow cytometry into high, medium, and low PrP^C expression populations using a labeled PrP^C antibody. Each cell population was collected and stored at − 80 °C until genomic DNA was isolated for sequencing. Gene scores were defined as the product between the phenotype and $-\log_{10}(P \text{ value})$.

Genomic DNA isolation, indexing, and PCR purification

Genomic DNA was extracted using MN NucleoSpin® Blood Kit (Macherey-Nagel 740951) for ~ 4–6 million cells per sample. Whole genomic DNA samples were carried forward into indexing PCRs using Q5® High-Fidelity polymerase (New England BioLabs M0492S). PCR-amplified, and indexed, fragments of approximately 280 bp were purified by a two-step SPRI bead purification (Fisher, S. et al. Genome Biology 2011), and concentrations were determined on a Qubit Fluorometer before pooling for deep sequencing on a HiSeq 4000.

Pulse chase

HEK293 cells expressing human PrP tagged with mCherry (between the N-terminal domain and the core domain) were treated with 50 μM cycloheximide at t = 0. Cells were lysed at 6, 24, and 28 h post cycloheximide treatment. PrP^C levels were determined by Western blot. Results are the average of experiments performed in triplicate. Error is SEM.

Pharmacological inhibition of Hsp70

To test for effects on PrP^C levels, HEK293 cells expressing human PrP tagged with mCherry (between the N-terminal domain and the core domain) were treated with a concentration series of each compound for 24 h. PrP^C levels were assessed by dot blot. To assess the effect on PrP^{Sc}, RML-infected mouse neuroblastoma cells (ScN2a-cl3) were treated with a concentration gradient of each compound for 3 days. Cells were lysed and treated to isolate prion only. PrP^{Sc} levels were assessed by dot blot.

Dot blot

Cells were lysed in lysis buffer (10 mM Tris HCl, pH-8, 150 mM NaCl, FW 58.44, 0.5% sodium deoxycholate, 0.5% NP-40) supplemented with 7.5 U/mL Benzonase (Novagen, EMD BioSciences Cat# 7074), and were incubated in a shaker 37 °C incubator for 60 min. For infected ScN2a-cl3 cells, PrP^{Sc} was isolated from lysate by digesting with Proteinase K (Invitrogen Cat# 25530-015) at a final concentration of 25 μg/mL for 60 min in a shaker 37 °C incubator. Proteinase K digestion was stopped by adding PMSF to a final concentration of 3.3 mM. Isolated PrP^{Sc} was denatured by adding guanidine Isothiocyanate (Sigma-Aldrich G6639-250G) to a final concentration of 1.25 M, and samples were allowed to incubate at 37 °C. Samples were spotted onto nitrocellulose membranes, which were then blocked in 5% milk in TBST for 2 h at RT. Membranes were incubated with primary D13 antibody (1:5000) overnight at 4 °C, washed, and

then incubated with goat anti-human secondary antibody (1:5000) for 30–60 min. Membranes were developed using Fempto ECL kit.

Cell viability assays

Cell viability post-treatment with Hsp70 inhibitors was determined by the CellTiter-Glo Luminescent Cell Viability Assay, as previously described. Briefly, treated cells were equilibrated to room temperature for 30 min, and then CellTiter-Glo Reagent was added at a volume equal to the culture volume. After mixing for 2 min and incubating the plate at room temperature for 10 min, luminescence was measured on a Molecular Devices SpectraMax M5 plate reader.

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