Toward stem cell-based phenotypic screens for neurodegenerative diseases

Vikram Khurana, Daniel F. Tardiff, Chee Yeun Chung and Susan Lindquist

Abstract | In the absence of a single preventive or disease-modifying strategy, neurodegenerative diseases are becoming increasingly prevalent in our ageing population. The mechanisms underlying neurodegeneration are poorly understood, making the target-based drug screening strategies that are employed by the pharmaceutical industry fraught with difficulty. However, phenotypic screening in neurons and glia derived from patients is now conceivable through unprecedented developments in reprogramming, transdifferentiation, and genome editing. We outline progress in this nascent field, but also consider the formidable hurdles to identifying robust, disease-relevant and screenable cellular phenotypes in patient-derived cells. We illustrate how analysis in the simple baker's yeast cell *Saccharaomyces cerevisiae* is driving discovery in patient-derived neurons, and how approaches in this model organism can establish a paradigm to guide the development of stem cell-based phenotypic screens.

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Introduction

Neurodegenerative diseases such as Alzheimer disease (AD) and Parkinson disease (PD) are characterized by progressive neuronal loss, leading to disability and deleterious effects on daily living and social function. No preventive or disease-modifying strategy has been successfully developed for any neurodegenerative disease, and there are more than 47.5 million people worldwide with dementia alone. Without a disease-modifying therapy, this number is predicted to almost triple by 2050.[1] Thus, the toll that these diseases will exact on individual patients and caregivers, as well as on the health-care delivery system, portends a public health crisis.

Neuropathologically, the most common neurodegenerative diseases result in the loss of distinct neuronal populations, accompanied by abnormal intraneuronal and/or extraneuronal protein accumulation. For instance, AD is associated with extracellular amyloid-β (Aβ) and intracellular tau accumulation; PD and other synucleinopathies are associated with abnormal intraneuronal accumulation of the protein α-synuclein; and amyotrophic lateral sclerosis (ALS) and some forms of frontotemporal dementia (FTD) are associated with altered localization of TAR DNA-binding protein 43 (TDP-43) in neurons.[2]

A revolution in genetics over the past 15 years has led to the identification of genetic risk factors for neurodegenerative diseases. A number of these risk variants map to genes encoding the misfolding proteins (or molecules directly involved in their production), thereby causally tying these aggregating proteins to the neurodegenerative process. Patients with such mutations generally present with early-onset and aggressive forms of the disease that mimic the pathology of the more common late-onset sporadic diseases. These early-onset disorders tend to display an autosomal dominant pattern of inheritance, suggesting an abnormal 'toxic gain-of-function' disease mechanism. For example, point mutations at the α-synuclein (*SNCA*) locus, or inheritance of one or two extra copies of the wild-type *SNCA* gene, lead to aggressive familial forms of parkinsonism and dementia.[3] Genetic data have enabled the development of animal and cellular pathology models based on overexpression of the individual toxic proteins. While undoubtedly an important advance, these models often do not recapitulate key aspects of disease pathology, and the disease mechanisms have, therefore, remained enigmatic. In addition, it remains unclear whether a simple reduction in the levels of the putative proteotoxic species will be neuroprotective in these diseases.

Against this disheartening background, extraordinary technological advances offer new promise. For example, methods to reprogramme adult somatic cells now allow us to create induced pluripotent stem cells (iPSCs) from patients.[4] These cells can be differentiated to any adult somatic cell, offering an unprecedented opportunity to generate patient-specific neurons and glial cells. Other methods exist to directly 'transdifferentiate' somatic cells to neurons or glial cells without traversing the pluripotent state.[5,6] A biological and drug discovery pathway can now be envisaged in which unbiased screening in patient-derived cells enables the identification of genes and small molecules that influence the neurodegenerative process, potentially even tailored to the specific genetic susceptibilities of individual patients.

Department of Neurology, Massachusetts General Hospital and Harvard Medical School, WACC-835, 15 Parkman Street, Boston, MA 02114, USA (**V.K.**). Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, MA 02142, USA (**D.F.T.**, **C.Y.C.**, **S.L.**).

Correspondence to:
V.K.
vkhurana@post.harvard.edu
S.L.
lindquist_admin@wi.mit.edu

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V.K., D.F.T., C.Y.C. and S.L. are scientific co-founders of Yumanity Therapeutics, a company involved in neurodegenerative disease drug discovery.

Key points

- Cellular 'phenotypic' screening is a powerful unbiased method for elucidating and developing drugs for poorly understood processes such as neurodegeneration
- Neurons and glial cells can now be generated from patient-derived induced pluripotent stem cells (iPSCs)
- Commensurate progress in our ability to manipulate ('edit') human genomes paves the way for iPSC technology to be used for unbiased genetic and small-molecule screening of patient-derived cells
- The main roadblock for the neurodegeneration field is the identification of robust and disease-relevant cellular phenotypes
- A path toward screening of patient-derived cells and target identification can be suggested by investigations in model organisms, including the most screenable eukaryotic cell, baker's yeast

In this Review, we explore the challenges of cellular screening, contrast target-based and phenotypic screens, and place these concepts in the context of novel stem cell-based technologies. We discuss the many hurdles that will need to be overcome for the discovery process to move this field forward. Moreover, we illustrate how it is possible to take advantage of the conservation of fundamental processes in cell biology across the spectrum of eukaryotic evolution. High-throughput screens in simple yeast models can identify genes and compounds that can reverse pathological phenotypes in patient-derived neurons. Perhaps more importantly, the investigational paradigm established in yeast provides a blueprint for the unbiased discovery process in patient-derived cells.

Target-based screens

Principles

In the post-genomic era, the conventional approach employed by the pharmaceutical industry for drug discovery has become the target-based screen (Figure 1, bottom).[7] In this approach, one begins with a 'druggable' target (a cellular process or component that is considered to be disease-modifying). Validation of druggable targets takes many forms, including definitive human genetic data or a link between a target and a disease phenotype in a relevant model organism. An *in vitro* assay is then established and optimized for high-throughput compound screening against a purified target protein. Target-based screening ideally identifies high-affinity compounds with a known target and mechanism of action, and is a superb approach when validated targets are known.

Limitations

The limitations and drawbacks of target-based strategies are numerous. Targets must be identified and validated before a screen can be contemplated, and validated targets for neurodegeneration have been elusive; the approach is also unable to identify new targets. Moreover, the druggable target space available for this approach has been highly restricted, with effort particularly focused on kinases and G-protein-coupled receptors at the expense of many other classes of molecules. Fortunately, there are some notable exceptions. For example, Finley, King and colleagues recently discovered that the deubiquitinating enzyme USP14 inhibits the degradation of ubiquitin–protein conjugates both *in vitro* and in cell lines. They designed a small-molecule screen to identify highly selective USP14 inhibitors, and demonstrated that one such compound facilitates ubiquitin-dependent degradation of various proteins implicated in neurodegeneration.[8,9]

Once a target is actually engaged within a living system, the capacity of biological systems for adaptation can thwart even the cleverest *in vitro* approaches. For example, compounds recovered from target-based screens can be challenged by the need to cross cellular membranes, by diverse defensive cellular metabolic processes, and by multiple mechanisms for excretion. Compounds recovered through *in vitro* assays can have poor chemical liabilities (for example, solubility or reactivity), and may not be appropriately tuned for *in vivo* activity. Off-target effects are particularly difficult to overcome, and might only become apparent within the complexity of a cellular system. For example, the human genome encodes more than 500 kinases, and the ATP-binding pocket—the most common target for drug discovery—is highly conserved among these enzymes, creating formidable potential for off-target effects. Fortunately, new technologies are emerging that can aid identification of these effects within a cellular context before preclinical or clinical testing. For example, engagement of a target by a small molecule stabilizes that target and reduces its interaction with chaperones, a change that can be measured in living cells with high precision.[10] This particular method identified 48 off-target interactions for ponatinib, a drug designed specifically to inhibit forms of the BCR–ABL tyrosine kinase. Interestingly, this drug was later found to have substantial adverse effects in clinical trials, presumably related to such off-target effects.

Perhaps the greatest problems of all are inherent to biological systems. Compounds in target-based assays are selected for their ability to inhibit or activate an individual biological target. However, the capacity of cells to reroute signalling pathways and to activate other compensatory mechanisms is great, and remains highly unpredictable.

Phenotypic screens

Pros and cons

In contrast to target-based screens, phenotypic screening involves the unbiased identification of genes or compounds that modify a specific trait (phenotype) within the physiological context of an intact cell or organism (Figure 1, top). Critically, the target is not known *a priori*, and the biology is allowed to 'speak for itself'. Although phenotypic genetic screens are possible in some animal models, including worms (*Caenorrhabditis elegans*), flies (*Drosophila melanogaster*) and zebrafish (*Danio rerio*), genome-wide screens are extremely expensive and time-consuming. Large chemical library screens (in the order of a million compounds) are currently achievable only in cellular models. In the context of neurodegenerative diseases, relevant cellular phenotypes include overt cell

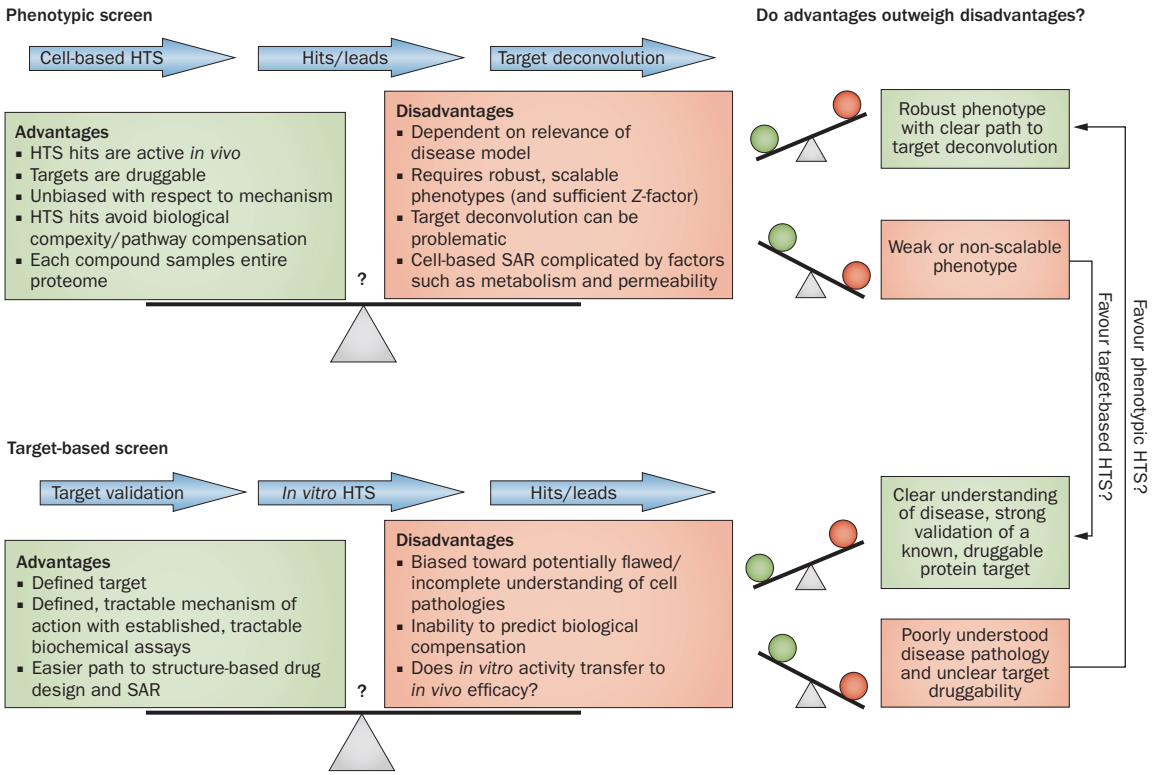

Figure 1 | Tipping the scales: phenotypic versus target-based drug discovery approaches for neurodegenerative diseases. Phenotypic cell-based screens and *in vitro* target-based screens each have advantages and disadvantages. Understanding of the disease, availability of faithful cell-based assays, validation of a given target, and robustness of a screening assay all factor into the choice of technique. In either approach, at least one roadblock must be overcome to validate hits and support their further development into high-value biological probes or early lead compounds. Abbreviations: HTS, high-throughput screen; SAR, structure–activity relationship.

death, altered localization of a disease-relevant protein, and modification of specific cellular stress responses that have been linked to neurodegeneration. The key to any unbiased phenotypic screen is a robust, consistent phenotype that can be modulated and scored for an effect that is directly connected to disease pathology.

The main advantage of the phenotypic screen is that it takes place within a living cell, and accesses the breathtaking complexity of *in vivo* biology. The screen itself filters out compounds that fail due to various chemical liabilities, such as toxicity, permeation and, of course, efficacy. Moreover, various features that influence the function of a protein target within the cell, including binding partners, subcellular localization, molecular crowding and post-translational modification, factor into the actual efficacy of a compound or genetic modifier. Presumably, these advantages contribute to the greater success of first-in-class FDA approvals for targets identified in phenotypic versus target-based screens, despite the latter capturing the lion's share of dollar investment.[7]

Despite these advantages, phenotypic screens also have limitations in the context of neurodegenerative disease. Mammalian cellular models of neurodegeneration are limited by weak and variable phenotypes. For example, elevated levels of α-synuclein expression are sufficient to cause PD in humans, but recreating the toxicity of α-synuclein overexpression is very difficult in the human cell lines that are currently amenable to screening. This phenomenon probably derives from the process of establishing human cell lines that proliferate in culture, which inevitably leads to higher resistance to apoptosis and cell death, as well as to increased rates of genetic and epigenetic change in culture. Primary rodent neuronal cultures offer a more physiological alternative, and toxicity is seen when α-synuclein is overexpressed or when the cells are exposed to exogenous Aβ[11] or α-synuclein.[12,13] However, high-throughput screening is not yet feasible with these neurons. Factors such as cellular heterogeneity, the inability to easily expand or freeze down lines, requirements for viral transduction, and complex protocols all introduce prohibitive time, cost and labour constraints. The absence of robust phenotypes in existing cellular systems has led to the widespread use of toxins—for example, oxidative stressors or inhibitors of the ubiquitin–proteasome system—to mimic disease, but the relevance of these exogenous insults to disease pathogenesis remains far from clear.

Baker's yeast—lessons from a simple eukaryote

As outlined above, there are numerous challenges to developing high-throughput screening technologies for sophisticated neuronal cultures, but a simple model organism shows promise to identify interesting lead compounds in a more straightforward assay format. Over the past

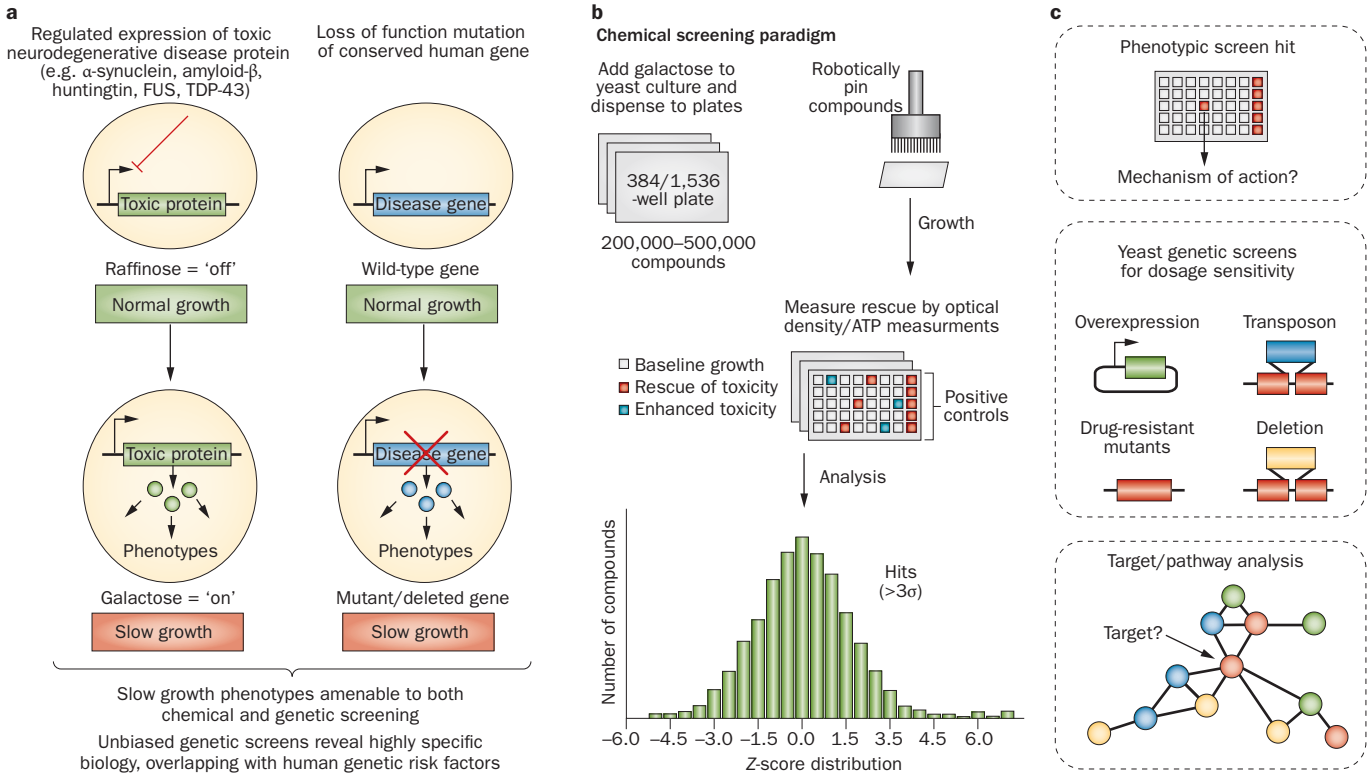

Figure 2 | Phenotypic screening and target identification in baker's yeast. **a** | Phenotypic small-molecule screens discover compounds that reverse slow-growth phenotypes. Reduced growth is conferred by toxic overexpression of a causal disease gene product or a loss-of-function mutation in the yeast homologue of a human disease gene. Slow growth results from deleterious cellular phenotypes that may be antagonized by small-molecule intervention. **b** | HTS are performed in 384-well or 1,536-well formats with optical density or viability (for example, ATP measurement) read-outs to indicate reversal of slow-growth phenotypes. HTS are executed by near-simultaneous induction of toxic protein expression with galactose and administration of compounds (via robotic pinning). After a defined period, effects of each compound on growth are assessed. Statistical analysis enables identification of hits according to deviation (typically >3σ) from the average well measurement across the plate or from control compounds. **c** | Unbiased genetic screens (for example, deletion, overexpression, spontaneous drug-resistant mutants) can be used in yeast to identify a compound's mechanism of action by identifying genes that modify its dose–response relationship. Knowledge of genetic and protein–protein interactions in yeast enables hits to be arranged in coherent molecular networks that facilitate target identification.[75,76] Abbreviation: HTS, high-throughput screens.

decade, we and a number of other groups have studied the toxic consequences of misfolding of neurodegenerative disease-related proteins, including α-synuclein, Aβ, TDP-43, FUS and polyglutamine-expanded proteins, in yeast cells (Figure 2). The inducible expression of these proteins in yeast causes robust and entirely distinct cellular toxicities. The rationale behind the development of this system, extensively reviewed recently,[14] is to model cellular proteotoxicities relevant to neurodegeneration in a simpler eukaryotic 'living test tube' with unparalleled genetic tractability. The robust cell growth and viability phenotypes in yeast are supremely suited to phenotypic screening in high-throughput formats.

The yeast platform is attractive for several reasons. Beyond its genetic tractability and short doubling time, core aspects of eukaryotic cell biology, such as organelle and cytoskeletal biology, protein homeostasis pathways (including proteasomal and lysosomal degradation), intracellular protein trafficking, lipid metabolism, RNA metabolism, and signal transduction, are well conserved in yeast. Critically, recent human genetic studies have strongly implicated perturbation of these conserved eukaryotic pathways—as opposed to specialized neuronal processes—in major neurodegenerative diseases.[15–17] As a consequence, yeast can reasonably be expected to recapitulate key aspects of protein-misfolding pathology and downstream cellular consequences that are relevant to disease.

Yeast cells provide a glimpse into how cellular phenotypic screens might one day play out in more-complex cells. Not only can individual genes be overexpressed or deleted in a yeast cell (Figure 2), but 'variomic' libraries now exist that allow screening of a phenotype (or compound) against thousands of distinct mutations per gene.[18] High-throughput small-molecule screening—of more than 5×10^5 compounds at a time[19]—offers a powerful approach to pharmacologically reverse phenotypes in yeast. Moreover, the genetic toolbox[14] can enable the targets of candidate small molecules to be identified, thus overcoming the most important hurdle of phenotypic screens. Beyond the screening capability itself, the extraordinary depth of data from thousands of yeast genetic screens, conducted over several decades of research, can be coupled to thousands of expression

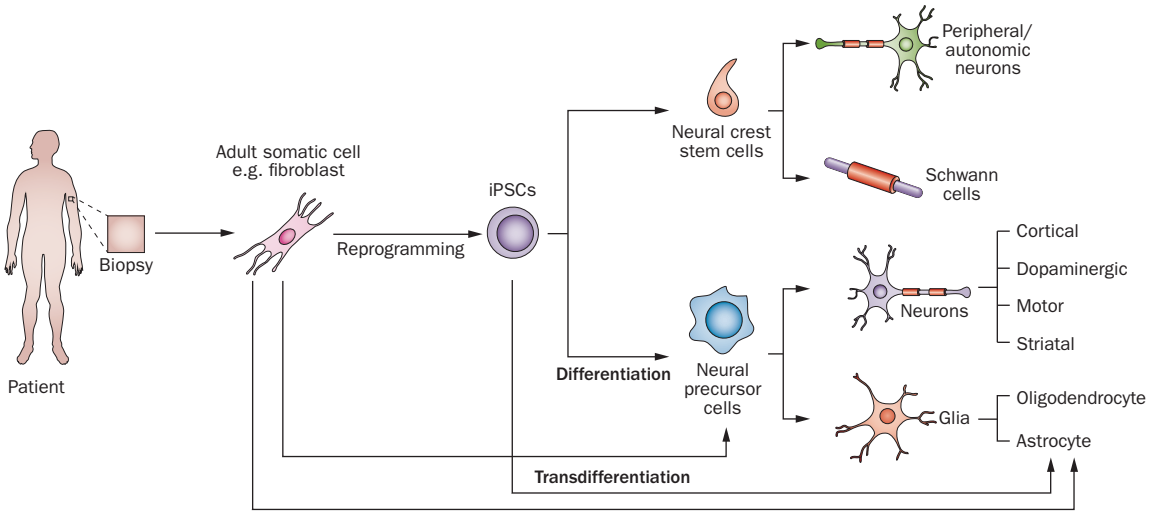

Figure 3 | Reprogramming, differentiation and transdifferentiation schemes relevant to neurodegenerative disease modelling. Fibroblasts are one example of a somatic cell type that can be reprogrammed to iPSCs. Reprogramming is generally achieved through ectopic expression of a combination of pluripotency-associated transcription factors (original 'Yamanaka factors': Oct4, Sox2, Klf4 and c-Myc). Differentiation proceeds by patterning a cell from pluripotent to precursor to mature differentiated states through exposure to patterning factors established from studies of neurodevelopment. Neuroepithelial stem cells include neural crest stem cells and neural precursor cells. Neural crest stem cells give rise to PNS neurons and Schwann cells, among other derivatives. Neural precursor cells generate glia and neuronal subtypes of the CNS, and motor neurons. Transdifferentiation refers to transition between two states—for example, from a somatic cell to a progenitor or mature neural cell, or from an iPSC to a mature neural cell—without passing through an intervening progenitor state. Transdifferentiation is typically achieved through ectopic expression of lineage-specific transcription factors. Notable successes include transdifferentiation of fibroblasts to cortical neurons,[6] motor neurons[85] and oligodendroglial progenitors,[5] and from iPSCs to cortical glutamatergic neurons.[41] These protocols all produce mixed populations of cells, with variable efficiency between iPSC lines and between different rounds of differentiation. Abbreviation: iPSC, induced pluripotent stem cell.

profiles, as well as to protein interaction data. This resource provides unparalleled molecular networks for establishing links among genes, small-molecule targets and cellular pathways.[20]

Fortunately, optimism for the predictive value of the yeast platform for more-relevant model systems has been borne out by discoveries in diverse neuronal models, including worms, flies and rodents,[14,21–23] and in human genetic studies. For example, genetic and cell-based studies in yeast implicated perturbed vesicle trafficking as a critical upstream consequence of α-synuclein toxicity,[21,24,25] a finding that has been validated in neurons[26,27] and confirmed by human genetic studies.[28] A yeast screen against Aβ toxicity directly tied the human AD genetic risk factor *PICALM* to Aβ, and demonstrated its mode of protection through rescue of defective endocytosis.[11,23] Furthermore, unbiased small-molecule screens against Aβ toxicity in yeast have identified agents already known to have neuroprotective effects, which have now entered clinical trials in AD.[29]

Perhaps more importantly, the distinct genetic modifiers uncovered in screens of different proteotoxicities in yeast overlap with known human genetic risk factors. In addition to the *PICALM* findings described above,[11] the PD-related genes *PARK9* (*ATP13A2*), *PARK17* (*VPS35*) and *PARK18* (*EIF4G1*) modify α-synuclein toxicity in yeast,[22,30] and *ATXN2* was identified as a novel ALS risk factor on the basis of a genetic interaction between the yeast *ATXN2* homologue and TDP-43.[31] In the sections that follow, we will establish how these observations have begun to inform our studies on patient-derived neurons, both to explore disease mechanisms and to validate lead compounds.

Patient-based neurodegeneration models

Advantages and limitations

Moving away from yeast, emerging patient-based cellular models of neurodegenerative diseases lie at the other end of the spectrum of complexity. The generation of patient-derived neurons[32] or glial cells[33,34] enables aspects of neurodegeneration to be modelled 'in a dish'. These cell lines have clear advantages. First, they can be differentiated to specific cell types, with the potential to reveal mechanisms if, for example, some cells are vulnerable and others resistant to disease pathology. Second, their patient origin reinforces their capacity to recapitulate disease-relevant phenotypes. Third, they have the potential to recapitulate pathologies even when those pathologies are not fully understood. Last, they provide a replenishable source of cells that can be expanded and frozen down for later use.

Innovation in reprogramming and stem cell technologies has exploded in recent years (Figure 3). Cells of diverse origins, including fibroblasts obtained through skin biopsy,[35] mononuclear peripheral blood cells obtained through blood draw,[36] and even renal epithelial cells present in urine,[37] have been reprogrammed to iPSCs. In parallel, the methods to generate iPSCs have evolved. Whereas the initial protocols utilized retroviruses that integrated into the genome, new, efficient non-integrating

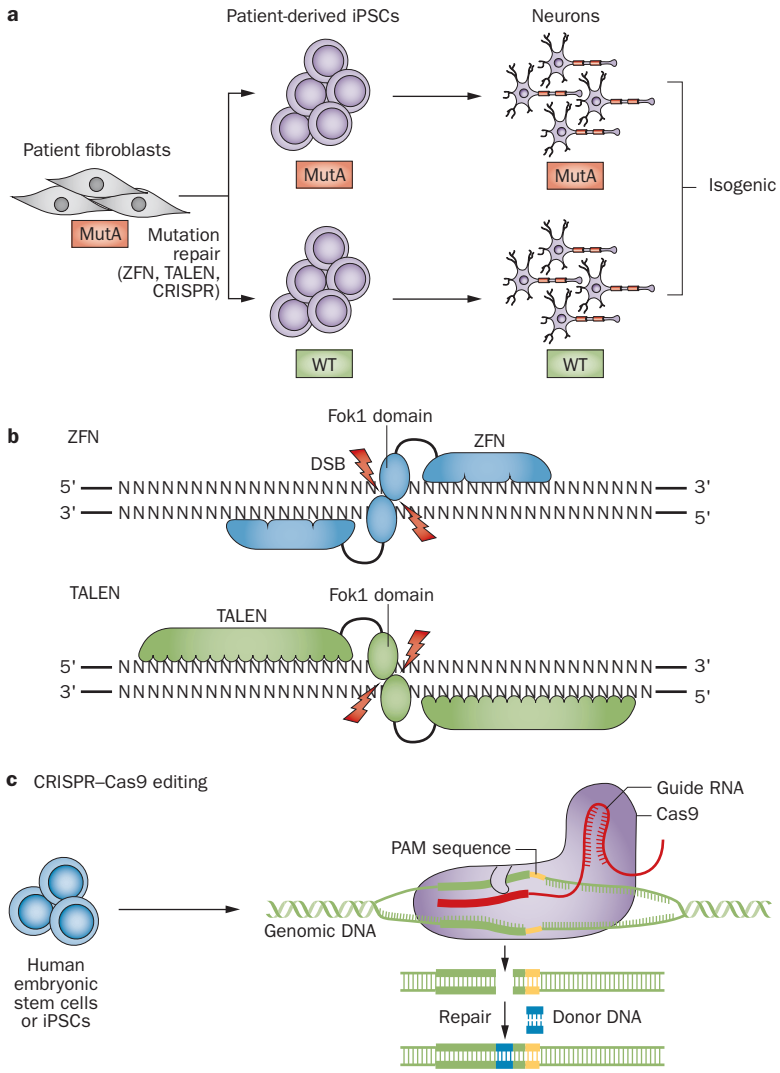

Figure 4 | Genome-editing techniques to create isogenic mutation-corrected controls. **a** | Fibroblasts from mutation-harbouring patients are reprogrammed to iPSCs. Mutations can be corrected via three widely used genome-editing techniques: ZFN, TALEN and CRISPR–Cas9. Each involves precise introduction of DSBs by a nuclease at a defined genomic locus.[86] The break can be repaired by homologous recombination or non-homologous end joining. The former leads either to complete repair, or to repair or introduction of a mutation if an exogenous oligonucleotide is present. Through this mechanism, mutations can be corrected or inserted in pluripotent cells. The latter leads to error-prone repair, frequently with indel or frameshift mutations. Genome-edited iPSCs can be differentiated into neurons to allow isogenic comparisons between diseased and corrected lines. **b** | Schematic showing paired DNA-binding domains of ZFN and TALEN proteins that flank the site at which the Fok1 nuclease cuts the DNA. **c** | CRISPR has revolutionized genome editing because of its relative simplicity. The technique only requires a nuclease (Cas9) and a guide RNA directed toward a specific target sequence. Binding specificity is established by the guide RNA and a specific three-nucleotide 'PAM' sequence. Abbreviations: DSB, double-strand break; indel, insertion or deletion of DNA bases; TALEN, transcription activator-like effector nuclease; WT, wild-type; ZFN, zinc finger nuclease.

methods, including mRNA-based[4,38] or episomal plasmid-based[32,39,40] reprogramming, have become well established and reproducible. In addition, transdifferentiation techniques, in which neurons or glial cells are directly induced from either fibroblasts[5,6,33,34] or iPSCs[41] without requiring a neural progenitor stage, can speed up the process.

These remarkable advances should empower the generation of cellular models that are highly relevant to neurodegenerative diseases. One could imagine these cells circumventing the aforementioned problems of immortalized cell lines, overexpression or toxins, instead relying on innate genetic differences to cause cellular pathologies. They could also provide a more plentiful source of cells than is possible with primary neuronal cultures. Advances in the ease of reprogramming are likely to enable this technology to be applied on a population-wide scale in the near future.

Nevertheless, substantial challenges remain for patient-based models of neurodegeneration. The first hurdle arises from multiple levels of heterogeneity: first, from genetic background differences between cell lines from different individuals; second, between iPSC clones from the same individual; third, within the same cell lines over time in cell culture; and last, from the impure and often unpredictable mixed-cell populations arising during the differentiation process.[42] Practically speaking, stem cell-based experiments can also be prohibitively expensive and time-consuming.

Genome editing can offset these difficulties to a certain extent. If a patient has a defined causative mutation, this technique can correct the mutation to create an 'isogenic' control iPSC line (Figure 4),[42–45] thereby substantially reducing heterogeneity between disease and control cell lines. However, most cases of neurodegenerative disease are late-onset and 'sporadic', with unclear contributions of genetic and environmental factors. Without knowing the environmental contributions, only genetic factors will be recapitulated in the *in vitro* cell model system. Also, owing to the lack of a known causative mutation, it is not possible to make isogenic controls from patients with sporadic disease. In such cases, the standard solution is to generate many patient and healthy control lines, with the required number of lines being directly related to the robustness of the phenotype. In time, as common and rare gene variants underlying complex disease phenotypes become better characterized, stratification of patients (and their derivative cell lines) will become increasingly important to reduce the phenotypic noise in sporadic disease models.

The relationship of ageing to neurodegeneration is an important consideration. Most neurodegenerative diseases, even when caused by highly penetrant monogenic mutations, are adult-onset in nature. It is not clear *a priori* whether cultured cells differentiated over weeks or months (and, thus, developmentally immature) can recapitulate relevant neurodegenerative processes. Early phenotypes are likely to be subtle, if present at all. That said, the onset of pathology may be far earlier than previously recognized. We now know that the clinical manifestations of neurodegenerative disease lag behind the initial onset of the cellular pathology by as much as several decades. This phenomenon has been studied most comprehensively for AD, in which a plethora of biomarker studies have identified Aβ and tau alterations that precede clinical manifestations of even mild cognitive impairment by many years.[46] In PD, 50–80% of dopaminergic

neurons have died by the time that patients manifest motor symptoms, and some nonmotor manifestations of this disease (for example, loss of smell, or constipation) precede motor symptoms by many years.[47,48] Thus, phenotypes could emerge in patient-derived neurons within the short time available in culture conditions. However, the relative importance of these early phenotypes compared with the late-onset degenerative phenotypes observed in postmortem brain tissue from patients with end-stage disease remains to be seen. Another open question is whether correction of these early pathologies in the dish is directly applicable to the clinical demand of arresting a process of active neurodegeneration.

Two general strategies have emerged to accentuate the relatively subtle phenotypes expected in neurons derived from patients with chronic neurodegenerative diseases. The typical approach has been to administer an additional toxic insult thought to be relevant to the disease. In PD, for example, oxidative stressors and mitochondrial toxins are often applied to enhance or induce phenotypes in neurons.[49–51] A more recently developed approach accelerates the ageing of patient-derived neurons by expressing a mutated form of lamin A (also known as progerin). This mutant protein accelerates a broad spectrum of ageing phenotypes in cells from human patients. For instance, in an iPSC model of PD, it accelerates neurodegenerative and intracytoplasmic aggregation phenotypes.[52] These approaches introduce their own biases when used in the modelling of disease, but may nevertheless prove pivotal for achieving successful high-throughput screens.

Screening efforts to date

A desirable goal is to establish higher eukaryotic model systems that are amenable to genome-wide forward genetic screening and interrogation of well over 100,000 compounds. The recent explosion in genome-editing technology brings the types of whole-genome and chemical genomic techniques that are well established in yeast within the realm of possibility for patient-based cellular models. The main bottleneck—namely, identifying readily screenable and disease-relevant cellular phenotypes—is, thus, a biological one.

Important progress has been made for several diseases, particularly those involving loss of a known protein. Work on familial dysautonomia has established a precedent. This disease is characterized by impaired development and progressive degeneration of autonomic and sensory neurons due to aberrant RNA splicing, which reduces the expression of IKAP (IκB kinase complex-associated protein). Consistent with the PNS phenotype in familial dysautonomia, IKAP levels were reduced in neural crest cells derived from patients with this disease.[53] Methodological advances enabled the development of a robust screening platform to identify molecules that could boost these levels. These advances included the ability to generate and freeze down large numbers of iPSC-derived neural crest cells from patients with familial dysautonomia, plating in a 384-well format, and automated RNA extraction with quantitative RT-PCR. A moderate-throughput screen of approximately 7,000 small molecules identified some promising candidates, and provided a deeper mechanistic understanding of IKAP level regulation.[54]

Hopefully, many other neurodegenerative diseases will prove amenable to this screening strategy. These diseases might include Friedreich ataxia, an autosomal recessive condition in which reduced expression of the frataxin protein results most commonly from expanded GAA repeats in the first intron of the *FRDA* gene;[55,56] rare autosomal dominant forms of FTD caused by progranulin haploinsufficiency;[57] and genetic forms of PD related to glucocerebrosidase deficiency[27] or increased activity of leucine-rich repeat serine/threonine-protein kinase 2.[50,58] An interesting example is spinal muscular atrophy, in which enhanced production of *SMN2* (survival motor neuron protein 2) full-length mRNA and SMN protein could, theoretically, compensate for both copies of the *SMN1* gene being deleted or mutated.[59,60] Indeed, Naryshkin and colleagues recently screened around 200,000 compounds for their ability to increase full-length *SMN2* expression in human embryonic kidney cells (Table 1).[61] In this elegant study, compound hits were validated not only in patient-derived motor neurons but also in mouse models of the disease.

Knockdown of polyglutamine-expanded proteins in transgenic mice ameliorates disease progression, and a reduction in the levels of such proteins might offer a readily screenable phenotype in cellular models. However, a caveat is worth noting: such mouse models have generally been constructed through transgenic overexpression of the toxic protein, and may not fully represent the disease process in patients.[62,63] In one particular case, spinocerebellar ataxia type 1, both overexpression and knockdown of the ataxin-1 protein similarly ameliorated mouse phenotypes.[64] In a different type of repeat disorder, iPSCs were generated from patients with intronic expansions of a GGGGCC hexanucleotide repeat in the *C9orf72* gene—a commonly reported mutation in both familial and sporadic cases of ALS and FTD.[65] The expansion did not induce downregulation of the gene; rather, knockdown of the gene reversed the formation of RNA foci, and corrected perturbed gene expression and neurophysiology. This work lays the foundation for a cellular screen targeting this gene.

Despite these tremendous efforts, definitive cases of a disease being caused by a gain or loss of protein function are the exception rather than the rule for chronic neurodegenerative diseases. Most neurodegenerative diseases are sporadic, without a known causative mutation. As noted above, however, autosomal dominant mutations in genes encoding aggregating proteins implicated in the common neurodegenerative diseases—such as AD, PD, FTD and ALS—do provide support for a 'toxic gain-of-function' proteinopathy mechanism in these diseases. As such, screening platforms are being generated to reduce the levels of the aggregating proteins (Table 1). For example, iPSC-based platforms are being developed to test compounds against exogenous Aβ toxicity[66] and endogenous Aβ production.[67] Initial studies have reported toxicity phenotypes in iPSC-derived neurons

Table 1 | Patient iPSC-based screens, and screens that validated hits in patient iPSC-derived cells

Reference(s)	Disease	Cell type screened	Toxins introduced	Screen format	Phenotypes	Small molecules (approximate number)	Active compound hits (% of total)
Lee *et al.* (2012)[54]	Familial dysautonomia	Neural crest stem cells from a patient with familial dysautonomia	None	384-well	Automated RNA extraction with quantitative RT-PCR for IKAP protein	7,000 compounds at 10 μM concentration	43 (0.6)
Höing *et al.* (2012)[74]	Motor neuron disease and, potentially, other neurodegenerative diseases	Mouse ESC-derived motor neurons (HB9: GFP-labelled) co-cultured with astrocytes and microglial cell line (BV2); secondary validation in human neural precursor cells derived from iPSCs	Microglia activated by IFN-γ and lipopolysaccharide	384-well	Neurite length measurements via high content imaging	10,000 compounds at 10 μM concentration	37 (0.3)
Xu *et al.* (2013)[66]	Alzheimer disease	iCell Neurons (mixed population of GABAergic and glutamatergic neurons)	Exogenous amyloid-β_{1-42} peptide	384-well	CellTiter-Glo luminescent cell viability assay	8,000 compounds at 10 μM concentration	19 (0.2)
Yang *et al.* (2013)[73]	ALS	Mouse ESC-derived motor neurons (HB9: GFP-labelled) from wild-type and *SOD*^{G93A} mutant mice; secondary validation in human motor neurons derived from patient iPSCs	Trophic support withdrawal	384-well	GFP-positive cell counting	5,000 compounds at three concentrations (0.1, 1 and 10 μM)	22 (0.4)
Ryan *et al.* (2013)[51] and R. Ambasudhan, personal communication	Parkinson disease	Neural precursor cells from human ESCs with MEF2 luciferase reporter construct	None	384-well	Luciferase activity	2,000 compounds at 10 μM	6 (0.3)
Burkhardt *et al.* (2013)[69]	ALS	Human iPSC-derived motor neurons from patients with ALS	None	384-well	High content screen TDP-43 aggregation	1,800 compounds at 20 μM	38 (2.1)
Barmada *et al.* (2014)[70]	ALS	*In silico* screen with validation in iPSC-derived motor neurons and astrocytes harbouring TDP-43 mutations	NA	NA	*In silico* prediction of autophagy enhancers	>1 million compounds	NA
Naryshkin *et al.* (2014)[61]	Spinal muscular atrophy	HEK 293 cells with validation in patient	NA	?	Firefly luciferase activity (to screen for full-length SMN2 expression)	~200,000 compounds	~2,000 (1.0)

Abbreviations: ALS, amyotrophic lateral sclerosis; ESC, embryonic stem cell; GFP, green fluorescent protein; iPSC, induced pluripotent stem cell; NA, not applicable.

exposed to exogenous human Aβ[66] or harbouring mutations that affect amyloid precursor protein processing.[68] In addition, a recent iPSC-based screen for compounds that ameliorate TDP-43 pathology was performed in a 384-well format, and surveyed the ability of 1,757 compounds to modify an intranuclear TDP-43 aggregation phenotype in patients with sporadic ALS.[69] This particular screen highlights elegant high content imaging-based tools that can be applied to iPSC-derived neurons, although the contribution of large aggregates to neurodegenerative diseases in general awaits clarification. Spectacular advances in high content imaging techniques, including tracking of individual neurons over time, promise to yield novel and screenable phenotypes in patient cell-based models.[70,71]

As noted above, additional toxic insults often reveal an underlying sensitivity that may provide more-robust phenotypes for screening. For example, Inoue and colleagues identified subtle changes in expression of RNA metabolism genes and increased levels of detergent-insoluble TDP-43 in cells derived from ALS patients harbouring mutations in the *TARDBP* gene, which encodes TDP-43.[72] Survival of motor neurons was decreased in response to the oxidative stressor arsenite, and could be partially rescued with a small molecule. In a separate study, trophic factor withdrawal exaggerated survival differences in mouse embryonic stem cell-derived neurons overexpressing the ALS-associated superoxide dismutase 1 (*SOD1*) mutation in the form of a transgene.[73] From a bioactive library of 5,000 small molecules, one hit was identified that reversed this phenotype. A separate ALS-motivated study used high content imaging to identify compounds that could rescue microglia-induced motor neuron toxicity from a 10,000-compound screen.[74]

Recently, Ryan and Dolabatadi *et al.* showed that PD iPSC-derived neurons with α-synuclein mutations were susceptible to PD-relevant pesticides.[51] By comparing the transcriptional profiles of mutant dopaminergic

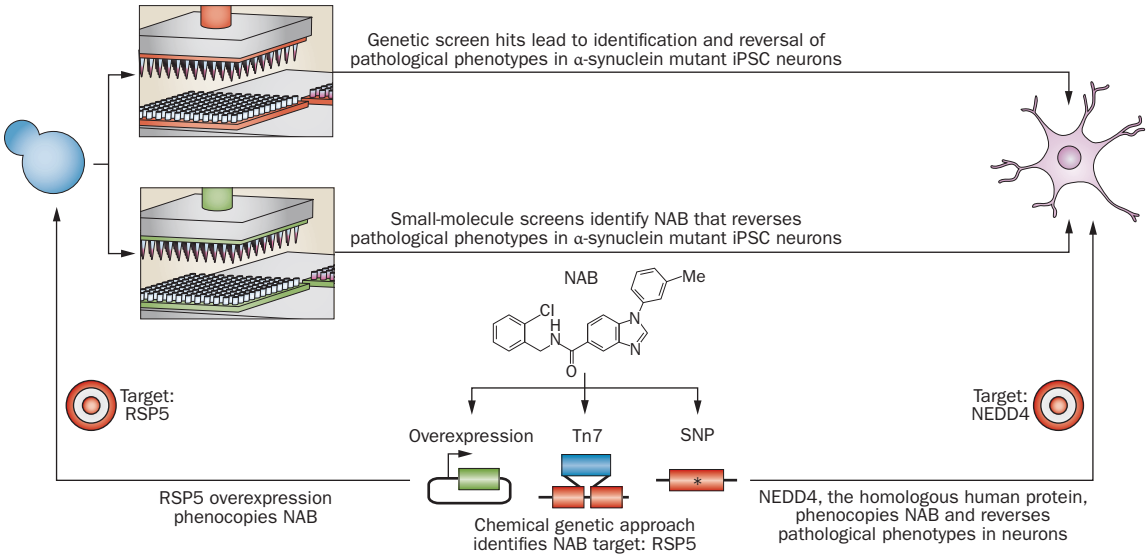

Figure 5 | A yeast and human stem cell dual discovery platform. Proof of principle was established for the toxicity of α-synuclein, a protein that aggregates in Parkinson disease and related disorders, and is mutated in rare families with dementia and parkinsonism. High-throughput genetic screens in yeast (top) yielded biological insights into key processes perturbed by α-synuclein. This in turn enabled the identification of early pathological phenotypes in neurons derived from patients with α-synuclein mutations (compare with isogenic mutation-corrected control cells). Large (>200,000-compound) screens in yeast led to the identification of small molecules that reversed analogous defects in yeast and patient neurons. Chemical genetic approaches in yeast (Figure 2c) were used to discover the mechanism of action of one such molecule, NAB, which was found to activate E3 ubiquitin–protein ligase RSP5. RSP5 overexpression phenocopied NAB in yeast, as did overexpression of the homologous human protein, NEDD4, in patient-derived neurons. Abbreviations: NAB, *N*-aryl benzimidazole; Tn7, transposon (for mutagenesis screen); SNP, single nucleotide polymorphism.

neurons and their isogenic mutation-control counterparts, the researchers revealed that myocyte-specific enhancer factor 2C (MEF2C)-regulated transcription was reduced in the mutant lines. Exposure of neurons to pesticides exacerbated this change in gene expression. The authors thus established a reporter-based immortalized cell line that could be screened for compounds that increase MEF2C-regulated transcription. A screen of 2,000 compounds identified one small molecule that also rescued mutant α-synuclein neurons from pesticide-induced toxicity, supporting the importance of MEF2C in mediating α-synuclein toxicity.[51]

These studies make critical inroads for a nascent field. However, considerable challenges remain. Even if one were to meet the critical need for relevant and screenable phenotypes, the technical and practical difficulties inherent to the system make high-throughput screening daunting. The small-molecule screens reported to date fall far short of the million-molecule libraries used in pharmaceutical industry-scale screens, and even if small molecules can be identified in this way, the problem of finding the target remains.

A yeast-to-patient-neuron platform

The issue of finding the target of a compound recovered in a phenotypic screen brings us back full circle to yeast. The genetic toolbox that is available in this organism (Figure 2c) is capable of finding such targets. In a recent proof-of-principle study, we described a dual yeast and human stem cell-based platform for synucleinopathies that highlights the capacity of yeast proteotoxicity models to reveal and reverse early pathologies in a more disease-relevant patient-derived neuron model (Figure 5).[13,75,76]

Synucleinopathies are defined by α-synuclein aggregation, and include PD, dementia with Lewy bodies and multiple system atrophy.[77] Each of these diseases is characterized by neurodegeneration in multiple neuronal populations; however, they are neuropathologically defined by α-synuclein aggregation within midbrain dopaminergic neurons, cortical neurons and oligodendrocytes, respectively. We first established that phenotypes identified through unbiased genome-wide screening in yeast were conserved in patient-derived neurons.[13] In this discovery platform, the yeast synucleinopathy model comprised yeast cells overexpressing α-synuclein. These cells were subjected to genetic and small-molecule screening. Next, we generated cultures enriched in cortical glutamatergic neurons from iPSCs derived from individuals with *SNCA* mutations, who exhibit prominent cortical α-synuclein pathology and dementia. Importantly, for some of these lines, we used zinc fingers to correct the mutations, thereby providing an isogenic control that was critical in attributing phenotypes directly to the patient's *SNCA* mutation rather than to background genetic variation. A handful of genetic modifiers from yeast were used to inform our investigation of early pathogenic phenotypes in patient neurons, which included nitrosative stress, accumulation of endoplasmic reticulum-associated degradation (ERAD) substrates, and endoplasmic reticulum stress. Furthermore, the human homologues of genes that modified pathology in yeast corrected the same defects

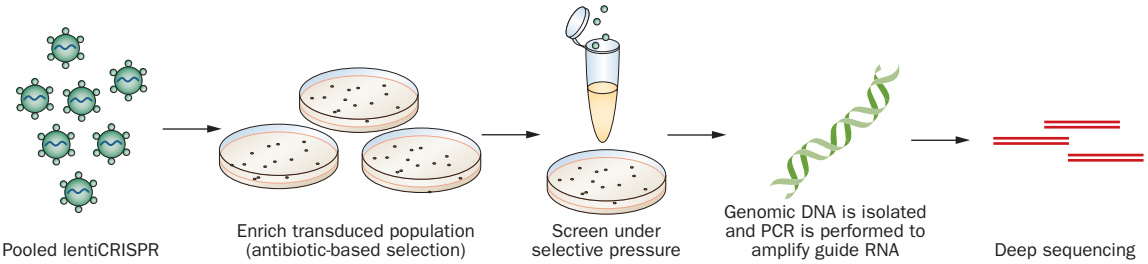

Figure 6 | Pooled CRISPR-based screening. Recent advances in CRISPR technology (see also Figure 4) will enable high-throughput screening of pluripotent stem cells and derivatives, including neurons. In this scheme, a lentiviral library consisting of thousands of pooled guide RNAs is expressed in cells along with the Cas9 endonuclease and the cells are screened under selective pressure. Cells that survive undergo sequencing to determine which genes were responsible for increased survival under pressure. A key challenge for neurodegenerative disease screens is to determine what kinds of selective pressure can be used to identify disease-relevant genetic modifiers. An adaptation of this approach might also be useful for identifying the mechanism of action of small molecules recovered from phenotypic screens.

in human neurons. Thus, the basic underlying pathology caused by α-synuclein was conserved from yeast to human.

The robust cellular toxicity caused by α-synuclein in yeast is highly amenable to screening of hundreds of thousands of compounds. In a screen of almost 200,000 small molecules, we identified an *N*-aryl benzimidazole (NAB) compound that reduced α-synuclein toxicity and rescued diverse α-synuclein phenotypes in yeast.[76] The combined results of three different types of genome-wide genetic screen allowed us to identify the mechanism of action of this compound. These independent screens revealed that NAB promoted the activity of the E3 ubiquitin–protein ligase RSP5/NEDD4. Specifically, NAB facilitated protein trafficking steps that were dependent on this ligase and were perturbed by α-synuclein. Importantly, NAB rescued two phenotypes—accumulation of ERAD substrates and generation of nitrosative stress—in PD patient-derived cortical neurons. Moreover, NEDD4 overexpression in human neurons phenocopied the effects of NAB. Thus, phenotypic screening in yeast identified a druggable target in the biology of α-synuclein that could correct multiple aspects of its underlying pathology from yeast to human.

More broadly, this work provides a blueprint for moving forward with phenotypic screening and target identification in patient-based cellular models. As noted above, the primary obstacle to high-throughput screening in patient-derived cells is a dearth of robust screenable phenotypes, owing to both technical limitations of the patient cell-based model systems and our shortcomings in understanding key aspects of the disease mechanisms. Once this obstacle is overcome—and we strongly believe that this is a case of 'when' rather than 'if'—the ongoing revolution of genome-modifying technologies will undoubtedly facilitate the types of large-scale genetic and chemical genomic approaches that have hitherto largely been restricted to yeast.

Systematic and genome-wide gene knockdown in non-dividing cells is now achievable with pooled lentiviral short hairpin RNA (shRNA) libraries.[78] Transduction is followed by massively parallel sequencing to determine the relative abundance of the various shRNAs. The shRNAs that are enriched over time in the experimental versus control condition correspond to genes whose knockdown putatively suppresses toxicity; the converse is true for those that are depleted over time. One interesting recent study utilized high content imaging to screen small interfering RNA (siRNA) libraries encompassing nearly 22,000 genes for their capacity to prevent or facilitate translocation of Parkin—depletion of which causes juvenile parkinsonism—to depolarized mitochondria in HeLa cells.[79] Given that the inability of Parkin to translocate to the mitochondrial membrane is also seen in iPSC-derived neurons with a *PINK1* mutation,[80] it is conceivable that an analogous screen could be performed in patient-derived neurons.

Genome-editing methods, in particular, CRISPR–Cas9-based gene editing (Figure 4), have facilitated targeted mutagenesis in cell lines, with the possibility of introducing multiple mutations or reporter constructs simultaneously. These technologies now make experiments routinely performed in yeast—such as the ability to precisely edit, tag or modulate expression of a particular protein—a possibility in patient cells. Such techniques will greatly facilitate phenotypic screening by enabling organelles and cellular stress responses to be monitored in specific cell types, and will also allow tightly controlled expression of proteotoxic proteins in the same manner as employed in our yeast screens (Figure 2).

Genome-editing approaches can also now create true recessive 'knockout' human cell lines, thereby overcoming the frequent problem of incomplete shRNA-mediated knockdown and deleterious off-target effects. Two recent studies have demonstrated lentiviral delivery of genome-scale CRISPR–Cas9 knockout libraries targeting 7,114 and 18,080 genes, respectively, with tens of thousands of unique guide sequences that enable both negative and positive selection screening in human cells (Figure 6).[81,82] It will be important, however, to complement these studies with overexpression screens. Many types of genetic interaction will not be captured by knockout screens alone, and many deletions will cause intrinsic phenotypes that confound identification of genetic interactions. Indeed, in recent genome-wide deletion and overexpression screens of yeast neurodegenerative disease models (V. Khurana *et al.*, unpublished work),

we found very little overlap in the actual genes recovered, despite strongly overlapping functional categories of genetic hits.

Finally, though not yet reported for primate or human cells, haploid mouse embryonic stem cells, which can differentiate into neurons, have been generated from parthenogenetic embryos. These cells become diploid during the differentiation process, but mutagenesis at the haploid stage creates defined knockout lines.[83,84] Systematic mutagenesis with retroviruses containing reversible gene traps has provided a powerful mammalian platform for forward and reverse mutagenesis of haploid embryonic stem cells.

Conclusions

Unbiased approaches for biological and therapeutics discovery are warranted for disease processes such as neurodegeneration that have poorly defined mechanisms and few, if any, validated drug targets. Until recently, truly unbiased, genome-wide approaches were only conceivable for genetically tractable model organisms. Of these, baker's yeast cells have the most extensive genetic and chemical genetic toolbox. Yeast has proven to be a convenient model system for unravelling key mechanisms underlying neurodegenerative proteinopathies, and their connection to human genetic risk factors. As noted above, these insights have led to the identification of perturbed cellular processes in neuronal models, including those derived from patient iPSCs. Of course, like any model system, yeast cells have limitations. For example, they will not recapitulate many non-cell-autonomous processes involved in neurodegeneration. Also, many human genes do not have yeast homologues. Furthermore, although yeast may shed light on mechanisms underlying the exquisite vulnerability of specific cell types to neurodegenerative proteinopathies, they will not capture the complexity of specialized neuronal and glial populations, and the interplay between them.

Fortunately, the confluence of two game-changing technologies—somatic cell reprogramming and genome editing—promises to usher in a new era for mammalian and human cellular genetics, to achieve in mammalian and human cells what was previously only attainable in yeast. With the appropriate identification of disease-relevant phenotypes in these cells, unbiased high-throughput genetic and small-molecule screening approaches will identify 'druggable' targets for neurodegenerative disease. We can now readily envisage a scenario in which therapeutic strategies are tailored precisely to fit both individual patients and degenerative processes occurring in specific cell types. The hope is that these cellular studies will forge a clear and logical path toward preclinical animal studies, and lead to carefully designed clinical trials in genetically stratified patient populations.

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Author contributions

V.K., D.F.T. and C.Y.C. researched data for the article, and reviewed and edited the manuscript before submission. V.K., D.F.T. and S.L. made substantial contributions to discussions of the content. All authors contributed equally to writing the article.