# Understanding and evolving prions by yeast multiplexed assays

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**Abstract:**

Yeast genetics made it possible to derive the first fundamental insights into prion composition, conformation, and propagation. Fast-forward 30 years and the same model organism is now proving an extremely powerful tool to comprehensively explore the impact of mutations in prion sequences on their function, toxicity, and physical properties. Here, we provide an overview of novel multiplexed strategies where deep mutagenesis is combined to a range of tailored selection assays in yeast, which are particularly amenable for investigating prions and prion-like sequences. By mimicking evolution in a flask, these multiplexed approaches are revealing mechanistic insights on the consequences of prion self-assembly, while also reporting on the structure prion sequences adopt *in vivo.*

## From classic prions to a wide set of prion-like proteins

Prions are infectious proteins that can self-template and self-propagate, acting as protein-based epigenetic elements [1]. Prion-forming sequences have challenged for decades our understanding of sequence–structure–function relationships. Not only have these proteins proved that the same primary sequence can adopt several stable folds, but also that their ability to self-assemble can provide phenotypic advantage, at least under certain circumstances. If in the last five years we all warmed up to the idea of functional self-assembly, thanks to extensive efforts aiming at deciphering the role of condensation inside the cell [2], the hypothesis that the aggregation of specific proteins could be a common means to heritable phenotypic variability was far from trivial to formulate 30 years ago when the first yeast prions [*URE3*] and [*PSI+*] were characterized [3]. It is only thanks to pioneering work in *S. cerevisiae* that we now have a better idea of how prions arise and of the range of phenotypic outcomes they can result in. [4]. While paving the way for our current understanding of these sequences, the clever genetic manipulation of yeast in these early studies led to landmark mechanistic and structural insights, such as inferring the parallel in- register β-sheet arrangement of [*URE3*] and [*PSI+*] [5,6] (Figure 3a).

There is now growing evidence that a wider set of intrinsically disordered proteins in yeast can give rise to specific phenotypes upon self-assembly and that these can be inherited over several generations [11,12]. Although these sequences do not share all properties of classic prions (e.g. they do not necessarily form amyloids) [11], they are commonly also classified as prions or prion-like proteins. Even a subset of the human proteome is nowadays considered to be prion-like. These sequences are found in 1% of protein-coding genes [13], they are intrinsically disordered, and their low-complexity composition resembles that of classic yeast prions: rich in Q, N, but also S and Y. These sequences encode the information required for self-templated aggregation. Surprisingly, yeast proved to be an excellent model to explore many of these sequences, as they can be easily swapped for the prion domain of yeast proteins and assessed for their ability to support specific inheritable phenotypes [14,15].

Classic alignment approaches are not useful when looking at prions, as conservation is mostly evident at the level of composition rather than down to their exact primary sequence [16]. Composition is conserved, for example, across 21 fungi that diverged over one billion years ago and homologs of prion proteins in different species have retained the ability to aggregate [17]. Moreover, the self-assembly properties of some more recently discovered prions show patterns of conservation all the way to humans [18•,19]. Nonetheless, many single amino acid changes in prion sequences are enough to prevent or enhance prion formation [20]. These polymorphisms are a key element in preventing prion propagation between species, a phenomenon commonly known as species barrier [21]. One single change in the amino acid sequence is sometimes sufficient to affect the ability of a 'mutated' monomer to template the aggregation of a wild-type molecule [22].

## Fitness trade-offs

In yeast, the ability of prion sequences to switch to an aggregated state provides fitness advantages in a wide range of scenarios (bet hedging), with adaptation arising from the possibility of quickly acquiring complex traits that would be less likely to appear upon sequential selection of mutations contributing to them [23]. A typical example is the aggregation and sequestration of the yeast translation-termination factor Sup35p into aggregates, leading to the read-through of premature stop codons and the onset of the [*PSI+*] phenotype [4]. On the other hand, several recently discovered prions result instead in fitness advantage [24] by potentiating the action of their causal protein [18•]. The discovery of prions in at least a third of the wild yeast strains tested supports their role in the adaptation of yeast through a changing environment and it was even suggested that stress may promote prion switching [21,23,25–27].

Prion and prion-like domains (PRDs and PRLDs) were also shown to drive adaptive reversible protein condensation through liquid-liquid phase separation. Proteins containing these sequences can sense and rapidly respond to cellular stress — pH, starvation, and temperature — by condensing and temporarily sequestering or releasing proteins and transcripts [28••–30]. Condensed Ded1p promotes translation of stress mRNA upon heat-induced stress, an adaptive mechanism that has been fine-tuned to the growth temperature of each species [28••]. Another example consists of the protein Whi3 whose aggregation controls cell cycle in multinucleate cells, via sequestration of cyclin mRNAs, inducing phenotypic heterogeneity even in the absence of stress [31,32].

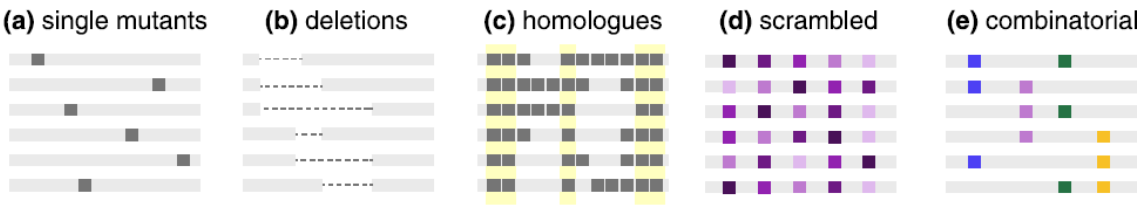
While providing interesting examples of the adaptive role of prions, these observations do not exclude that the states these sequences adopt can be toxic under certain circumstances. Indeed, the loss of function caused by aggregation can be detrimental and even lethal. Even when loss of function is not detrimental, such as for Ure2p, the prion state can still drastically affect fitness, suggesting that the prion itself or intermediate assemblies in its formation are toxic for the cells [33]. On this line, a few versions of [*PSI+*] and [*URE3*] obtained in the lab have even been named 'suicidal' due to their high toxicity [34]. Although prions are found in wild yeast strains, their frequency is lower than certain viruses that are notoriously detrimental [35], suggesting they are actually overall harmful for the cells. This assumption has however been challenged by i) modeling the frequency of other reversible epigenetic elements [36,37] and ii) the finding that specific prions such as [*RNQ+*] and [Het-S] are instead detected in a vast majority of natural isolates [23,38].

In the human proteome, PRLDs are particularly enriched in nucleic acid binding proteins [39,40], suggesting a role for these sequences in promoting functional condensation and temporal sequestration of RNAs also in more complex organisms. Many PRLDs exist in essential genes and aggregation of prion-like proteins plays a role in signal transduction from fungi to humans. This is the case of Het- S polymerization at the basis of heterokaryon incompatibility [41] and of the antiviral signaling cascade activated by MAVS prion switching [42]. However, PRDs and PRLDs can also drive pathological aggregation in devastating diseases, such as Fatal Familial Insomnia and Amyotrophic Lateral Sclerosis, and mutations in these protein regions cause dominant forms of disease [43–45]. The phenotypic consequences of these mutations are diverse and how different cell and tissue specificities depend on the identity and function of the aggregating sequence has not been fully elucidated yet. Altogether, the role and impact of these sequences in the human proteome is just another reminder of how the interplay between beneficial and detrimental effects of self-assembly and their sequence determinants is not trivial to decipher.

## The multiplexed era

Altogether, prion conformation, function, toxicity, and environment all concur to the final phenotypic outcome of each cell. This interplay is particularly challenging to decipher, calling for well-defined assays to report — if possible — on just one of these biological mechanisms at once. We suggest that high-throughput approaches such as multiplexed assays of variant effects (MAVEs) can be a useful tool to decouple the different layers of these complex systems. The basic principle behind MAVEs, also known as deep mutational scanning (DMS), is the construction of a library of thousands of variants that can be selected for a specific phenotype in a cell-based assay [46]. The performance of each variant is quantified by sequencing the population before and after selection [47]. Using mutations to disrupt or enhance a process in order to understand it is the main power of MAVEs where variant libraries can be rationally designed to address a range of questions, from prion polymorphism to explore evolutionary paths or prion sequence space in a hypothesis-free manner [48–50] (Figure 1). Libraries can be selected in parallel assays that report on different phenotypes in variable experimental conditions mimicking fluctuating environments. Overall, the versatility of MAVEs makes them very suitable for studying the different layers of prion biology and the scale required for these approaches makes yeast an excellent system to employ: simple manipulation, large population, and fast generation time.

*Figure 1*




Mutational library design. Examples of library design include (a) all possible single mutants in a given sequence to systematically explore the consequences of polymorphism [57•], (b) a range of deleted variants to identify key regions driving protein aggregation [58], (c) sequences mapping entire evolutionary trajectories [28••], (d) scrambled versions of the same sequence to gather insights about structural arrangements [5], and (e) different combinations of double mutants to infer specific residue–residue contacts in prion proteins [50].

## Engineering selection

Next, we report on a series of selection assays that have already been employed or have great potential to be transferred to MAVEs to quantify prion properties at scale (Figure 2).

### Toxicity

Prion toxicity can be assessed in a MAVE simply on the basis of cell viability (Figure 2a): over generations, cells carrying a toxic variant of the prion sequence will be depleted in the population, while those carrying a non- toxic variant will be enriched. This is quantified by deep sequencing before and after expression of the protein, an approach that was employed to quantitatively map toxicity for thousands of variants of the PRLD of the human protein TDP-43 [50].

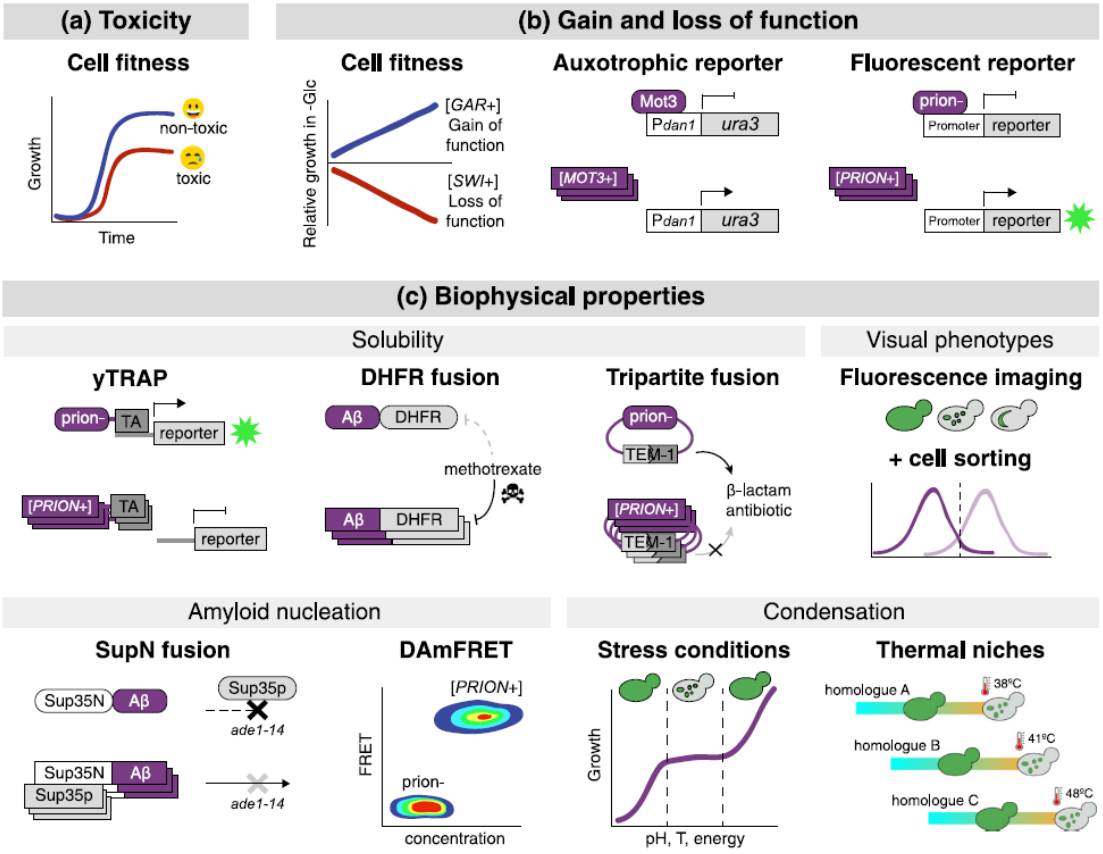
### Gain and loss of function

Cell viability can also be used to select for gain or loss of function (Figure 2b). For example, switching to [*SMAUG+*] or [*GAR+*] represents an adaptive advantage upon glucose depletion: [*SMAUG+*] hyperactivates the function of its causal protein Vts1 [18•], and [*GAR+*] supports yeast growth on mixed carbon sources [27]. In contrast, [*SWI+*] causes a loss of function of its causal protein and slows growth in nonfermentable carbon sources [51]. Thousands of variants of these proteins can be scored for their impact on prion formation by sequencing before and after selection in a medium lacking glucose. Mutational libraries can also be coupled to downstream auxotrophic reporters — such as *ura3* — or fluorescence reporters [52].

### Biophysical state

Other genetically engineered yeast systems are suitable to select for specific protein physical states (Figure 2c). The yTRAP system couples the aggregation state of a protein of interest to the activity of a transcription activator acting on a fluorescence reporter, allowing variant discrimination using fluorescence-activated cell sorting [53]. A drug-resistance selection assay has been used in a MAVE to test aggregation of the amyloid beta (Aβ) peptide fused to dihydrofolate reductase (DHFR) [54]. Only when the DHFR is fused to a soluble Aβ variant, but not to an aggregating one, cells will be able to grow in the presence of methotrexate. Similarly, fusion to TEM-1 β-lactamase has been used to identify aggregation-prone sequences both in yeast and in the periplasmic space of *E. coli* [55]. The correct folding of the protein fusions brings the two halves of the enzyme in proximity, restoring function and allowing selection against β-lactam antibiotics. These assays share one common limitation: poorly expressed or degraded sequences would lead to the same phenotypic readout as those that aggregate. There are two systems that instead track nucleation of protein assemblies, that is, the very first step in the formation of self-templating aggregates. One consists in fusing a sequence to the nucleation domain of Sup35p [56,57•]. Nucleation of Sup35p and induction of the [*PSI+*] phenotype is a readout of the ability of the fused sequence to nucleate amyloids. This approach was used to map > 17 000 Aβ variants [57•,58]. The other system, DAmFRET, is also particularly suited to run MAVEs. In this case, nucleation barriers and prion switching are observed by means of amphifluoric FRET and the frequency of nucleation is measured as a function of protein concentration in yeast cells [59•]. The ability of prions to form condensates is also select able, at least for those sequences that were shown to promote cell viability in stress conditions [29,30]. These selection experiments can also be performed at different temperatures to report on the condensation of protein homologs from different species, which have adapted to their thermal niche [28••].

Figure 2



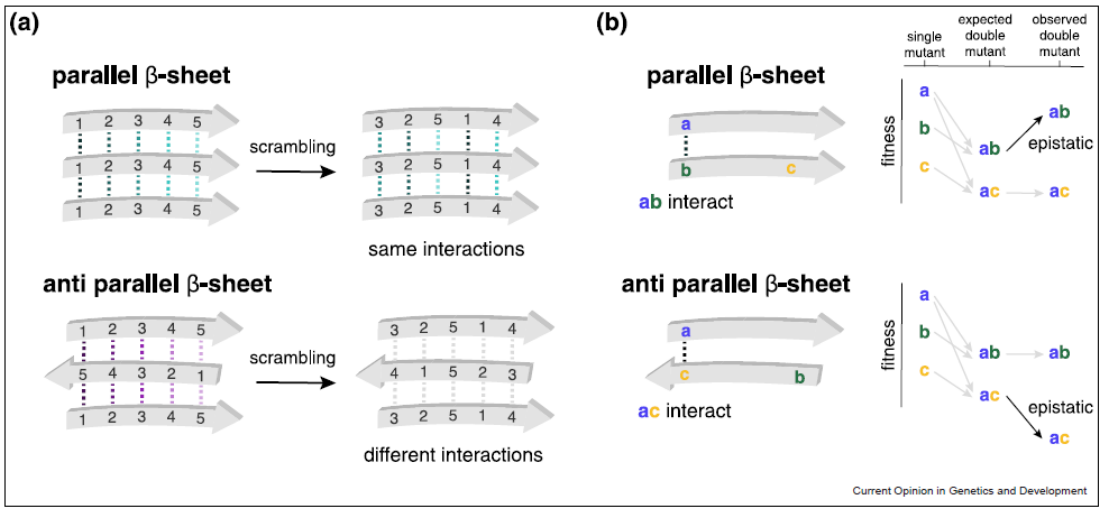

Experimental selection assays to quantify prion properties at scale. (a) Tracking cell growth over time reports on toxic and nontoxic prion variants [50]. (b) Gain and loss of function can be assessed with cell fitness, or an auxotrophic or a fluorescent reporter. Replacing dan1 — transcriptionally repressed by Mot3p in normal conditions — with ura3 and sequencing of variants growing in the absence of uracil can report on the loss of function induced by Mot3p prion switching to [*MOT3+*] [12]. Similarly, replacing flo family genes with ura3 has been used to report on switching to [*SWI+*] and identify antiprion chemical compounds in a high-throughput screening [52]. (c) Solubility can be quantified by the yTRAP system, where the prion is fused to a synthetic transcription-activation domain that recognizes a binding site upstream of a fluorescent reporter gene [53] by means of a DHFR fusion, where soluble protein variants allow the enzyme to remain soluble and functional in the presence of its competitive inhibitor methotrexate, and so to reduce DHF to THF [54,65], thanks to a tripartite fusion, where two domains of TEM-1 β-lactamase are fused to a prion protein. The enzyme can only be reconstituted and hence functional if the prion remains soluble, providing antibiotic resistance in bacteria or yeast cells [55]. Amyloid nucleation can be tracked with a supN fusion, in a yeast strain with a premature stop codon in the adenine gene. Endogenous full-length Sup35p, a translation-termination factor, recognizes the stop codon when soluble. SupN nucleation, induced by nucleation of the protein of interest, recruits Sup35p, causing a read-through of the stop codon and allowing growth in a medium lacking adenine [56,57•]. The DAmFRET system tracks prion nucleation by fusing the protein of interest to a photoconvertible fluorescent protein. Emission of FRET signal quantifies concentration-dependent protein self-assembly in thousands of single cells in one single experiment [59•,66]. Protein condensation can be assessed by cell growth in changing environments. For example, changes in pH, temperature, or nutrient availability can induce protein-phase separation, which ensures cellular fitness during recovery, a mechanism that has been shown to be adaptive and fine-tuned to a specific range of growth temperatures in different species [11,28••]. Finally, prion phenotypes can also be screened and selected with a fluorescent tag and by means of imaging coupled to cell sorting [61••].

Finally, visualizing prions with traditional microscopy showed that they can adopt multiple shapes and have different subcellular localizations [12,50]. Recently, fluorescence imaging has been coupled to cell-sorting, enabling the selection of variants of a library by a multiple set of morphological and spatial traits [60,61••].

**Inferring *in vivo* protein conformation**

Beyond illuminating genotype-to-phenotype relationships, MAVEs of combinatorial libraries can be used to infer structural elements since the genetic interactions between mutations in structurally proximal residues are likely to have nonindependent effects (i.e. be epistatic, Figure 3b) [62,63]. This approach provides a great opportunity to explore *in vivo* conformations of disordered proteins and is particularly appealing to study prions, which, due to their aggregation propensity, are otherwise very difficult to approach by traditional biophysical techniques. In this line, Wickner's vision was absolutely right and ahead of his time: yeast genetics can be extremely informative on protein structure (Figure 3).

*Figure 3*




Protein structure from yeast genetics. (a) In 2005, swapping of sup35 with five scrambled versions of its sequence resulted in a [*PSI+*] phenotype, reporting on the specific arrangement of interactions required for prion switching to [*PSI+*] [5]. (b) In 2019, the interactions between thousands of double mutants in MAVEs were used to predict protein structure on the basis of the principle for which residues in close structural proximity are more epistatic [62,63], a method that proved powerful also to infer the in vivo structural signatures of a human PRLD [50].

**Disclaimer: not just DNA**

There is one element of prion biology that cannot be mimicked well by carefully tailored multiplexed assays. The very same DNA sequence often gives rise to different prion strains that differ in their amyloid structure, stability, and propagation [56,64]. Although this one-to- many relationship between genotype and phenotype cannot be captured by assays relying on DNA variation, we believe that the power of MAVEs to massively assess phenotypes by scanning thousands of genotypes will provide the mechanistic insights required to guide also our understanding of those prion phenotypes not written in the coding sequence.

**Conflict of interest statement**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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