New Therapeutic Strategies for Cancer and Neurodegeneration Emerging from Yeast Cell-based Systems

Clara Pereira[#], Mariana Leão[#], Joana Soares, Cláudia Bessa and Lucília Saraiva*

REQUIMTE, Department of Biological Sciences, Laboratory of Microbiology, Faculty of Pharmacy, University of Porto, Porto, Portugal

Abstract: Despite great advances in understanding the molecular etiology of cancer and neurodegeneration, therapeutic strategies against these diseases are still largely lacking. Hence, acceleration of the discovery of new therapeutic agents against these pathologies is of enormous interest.

This review is focused on the role of multi-faceted and expanding yeast cell-based systems in the search for new drugs and therapeutic targets in cancer and neurodegeneration. Though the obvious limitations of using a microorganism to address human diseases, when used in the early phase and with complementary mammalian systems, it can have a tremendous impact in the discovery of new therapeutic opportunities. In this review, many evidence are provided demonstrating the valuable contribution of yeast in this area. Additionally, several yeast target-based drug screening approaches based on a readily screenable phenotype on genomic technologies increasingly oriented towards genetic and chemical high-throughput analysis are addressed.

Altogether, with this review, we intend not only to recognize previous successes and ongoing work in this area, but also to point out new opportunities that may be of interest for yeast as a model organism and as a powerful system in the discovery of new lead compounds that have the potential to become novel drugs in cancer and neurodegeneration.

Keywords: Yeast, cancer, neurodegeneration, drug discovery, therapeutic targets, target-based screening, genome-wide screening.

1. INTRODUCTION

The high degree of conservation of cellular and molecular processes between the yeast *Saccharomyces cerevisiae* and higher eukaryotes have made this microorganism a valuable model system for numerous studies on devastating human disorders such as cancer and neurodegeneration. Actually, many studies developed in yeast led to novel insights into the function of genes and pathways involved in human pathologies [1,2]. While several factors make yeast an ideal system for biological and functional studies, the extraordinary simplicity of yeast genetics is the main reason for the effectiveness of this microorganism in the development of many types of large-scale genetic and chemical experimental approaches. These approaches have highly contributed to the identification of new therapeutic targets and particularly of new drugs both via target-based and non-target-based drug screenings. With the high number of potential therapeutic targets as well as chemical libraries available to be tested, the development of quick, selective and reliable high-throughput screening (HTS) assays to be applied in the early phase of drug discovery is required. In fact, yeast is an increasingly popular model for pharmacological and/or drug discovery studies (reviewed in [3-5]). Due to several advantages, particularly the possibility to examine a specific cellular process triggered by a defined target (Table **1**), in the last years cell-based assays have become an alternative to cell-free assays for HTS. On the other hand, though cellular screens should be ideally performed with human cells, which represent the most physiological model system, several disadvantages (Table **1**) also discourage the use of these cells in large-scale screenings. In this context, engineered yeast cells have emerged as the model of choice in the first-line drug screening approach, particularly in target-specific screenings where the activity of a defined human protein is measured in a heterologous, yet eukaryotic, cellular environment that provides the natural physiological state of the target and its substrate or interacting partners (Table **1**). The long scientific expertise in yeast genetics and molecular biology has allowed, in the last years, the development of a multitude of genetic tools and cellular selection systems with a high applicability in HTS (reviewed in [3-5]).

In this review, the contribution of yeast model systems for the discovery of drugs and therapeutic targets in cancer and neurodegeneration is discussed. Several target-specific screening approaches based on a readily screenable phenotype and genomic techniques, developed and validated in yeast and with high applicability in genetic and chemical large-scale screenings, are also addressed in this article. In spite of this, it must be noted that in this review these techniques will not be exhaustively described, but instead discussed in what concerns to their contributions for the development of potential therapeutics in cancer and neurodegeneration. We consider that insights emerging from yeast will continue to expand in coming years, contributing in a joint effort with more complex systems to face the challenges of therapeutic discovery in cancer and neurodegeneration.

2. TARGET-BASED DRUG SCREENING APPROACHES

The earliest point at which yeast can contribute to drug discovery is in identifying compounds that cause a desired physiological change, rather than modulate a specific protein. While this non-target-based strategy is being employed in yeast to demonstrate that a drug has activities beyond modulation of the intended target, it is being replaced by the target-based approach. In fact, a difficulty associated with drug discovery approaches that do not begin with a screen against a specific target is that the precise mechanism of action cannot be identified without first determining the molecular target, which is frequently a costly and complex task.

As referred above, the high degree of conservation with mammalian cells in terms of proteins implicated in human diseases, in association with the ease of deleting, mutating or replacing a specific gene, make yeast a very promising system for identifying and testing target-specific drug therapies. Indeed, several yeast models based on the deletion of yeast orthologues of genes involved in cancer and neurodegeneration have been developed in order to

*Address correspondence to this author at the Lucília Saraiva, Laboratory of Microbiology, Faculty of Pharmacy, University of Porto, Rua Jorge Viterbo Ferreira nº 228, 4050-313 Porto, Portugal;
Tel: +351 220428584; E-mail: lucilia.saraiva@ff.up.pt
[#]Authors contributed equally to this work

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Table 1. Advantages and Drawbacks of Yeast Cell-based Assays Over Cell-free and Human Cell-based Assays

Yeast cell-based assays			
Advantages		**Drawbacks**	**Remarks**
Over cell-free assays	**Over human cell-based assays**		
Purification of the target protein is not required; Target proteins are examined in a cellular context (the most natural physiological state); Selection against compounds that are generally cytotoxic, instable in cell culture or that cannot permeate plasma membrane.	A clean read-out in a null background environment for the expression of the human protein; Less expensive to culture; Faster growth in defined media; Easier to propagate in HTS automated systems; Versatile genetic malleability; Multitude of genetic tools and cellular selection systems readily converted to HTS formats: advanced plasmid systems, homologous recombination techniques and selection of easily-scored markers.	Are not the most physiologically relevant model system =>> tests in mammalian cells are ultimately essential to validate the pharmacological relevance of the targets identified.	Cell wall does not limit compound permeability, which is similar to mammalian cells, and the yeast pleiotropic drug resistance exporter proteins are structurally similar to the mammalian multiple drug resistance efflux pumps.

identify modulators of proteins involved in these pathologies. Actually, a number of familiar drugs used against human targets specifically inhibit the orthologous protein in yeast. One example of this is the sensitivity of mutants defective in recombinational repair of DNA double-strand breaks (DSBs) (e.g. yeast *rad50* mutants) to agents that cause DSBs (e.g. the topoisomerase poison etoposide), which legitimizes the use of yeast biology to identify and study potential anticancer drug targets [6]. By screening for agents that elicit cytotoxicity in cells harboring cancer-related mutations, it has been possible to identify new anticancer agents. For instance, using this strategy several compounds (such as NSC-142496) were identified as selective for *bub3*Δ mutant (bub3p is a component of the mitotic checkpoint; defects in this component have been observed in some human tumors, giving rise to the 'Chromosome Instability' phenotype), inhibiting the growth of a *bub3*Δ mutant but not of wild-type (wt) cells. Afterward, it was shown that human tumor cell lines treated with NSC-142496 accumulated in G2 with the formation of tubulins [6]. Other example involves Sir2, a yeast orthologue of SIRT1 of the sirtuin family of NAD-dependent histone acetyltransferase. For oncology, inhibitors of SIRT1 are indicated as potential therapeutics. Bedalov and colleagues [7] screened 6,000 diverse National Cancer Institute (NCI) compounds and among 11 hits identified, a compound (splitomycin), revealed to be a Sir2 acetyltransferase inhibitor. Other models were developed based on the deletion of yeast orthologues of genes involved in neurodegeneration. For instance, the gene frataxin causing Friedreich ataxia, an autosomal recessive neuro- and cardiodegenerative disorder, is conserved in yeast. Decreased expression of frataxin in humans or the frataxin orthologue in yeast, Yfh1p, is associated with mitochondrial dysfunction. Using yeast depleted on Yfh1p, a HTS assay was developed in which mitochondrial function was monitored by reduction of the tetrazolium dye in a growth medium with a respiratory carbon source [8]. From a large chemical library, several compounds were identified in yeast that rescued mitochondrial defects, being their effectiveness confirmed in secondary screenings in mammalian cell lines. Another model that relies on the presence of conserved disease associated proteins is the prion screening model developed by Bach and colleagues [9]. Though yeast prions are not true orthologues of human prions, they show similarities concerning transmission of phenotype in protein-only mode [10]. The model developed is a two-step assay against the prions responsible for the [PSI+] and [URE3] phenotype and allowed the identification of several promising compounds as confirmed by their efficiency in mammalian prion models [9].

Yeast-based screens that are highly specific can be designed even for targets that do not have yeast orthologues. The possibility of reproducing the function of human proteins in the cellular environment of this heterologous organism has prompted researchers to set up target-specific assays with engineered yeast cells overexpressing human proteins involved in cancer and neurodegeneration. A more generalizable approach is to simply determine whether the human target protein expressed in yeast confers any phenotype that can be used as a starting point for screening, such as growth arrest. In fact, many target proteins involved in cancer and neurodegeneration are cytotoxic when expressed in yeast by interfering with growth-regulatory pathways. The restoration of yeast growth through the inhibition of the activity of the foreign protein by a drug or by co-expression of a second human protein formed the basis of several chemical and genetic screenings, respectively, for inhibitors of human target proteins. Similarly, based on the capacity to increase the growth arrest-induced by the expressed protein, activators of human target proteins can be also screened using this strategy. The design and validation of such screens are particularly straightforward and several works have already demonstrated their efficacy in the discovery of therapeutic agents of the target protein. Despite of this, more versatile targeted-based screening approaches based on the use of reporter systems (for example *LacZ* and Luciferase) have been proposed as complementary or even as alternative to the cellular growth selection systems.

One promising drug target in cancer that has been widely studied in yeast is the p53 tumor supressor protein, which is a sequence-specific transcription factor inactivated in human cancers through the expression of p53 mutants or, in tumors with a wt p53, through endogenous negative regulators as MDM2 and MDM4. As such, an important anticancer strategy has been focused on the reactivation of p53 function by targeting mutant p53 or by impacting on the interaction between p53 and MDM2/4 negative modulators [12]. Recently, due to its key role in cell proliferation and death, inactivation of p53 has been also recognized as a promising therapeutic

strategy in neurodegeneration [13]. At the beginning of the 1990s, evidences were provided corroborating the remarkable similarities between the transcriptional activity of p53 in yeast and mammalian cells [14,15]. Simultaneously, a cytotoxic effect of human wt p53 was also reported in *S. cerevisiae* [16]. This study provided the opportunity to score the effect of cofactors (as MDM2/4) or small molecule modulators of p53 activity through a simple yeast phenotypic assay based on the interference with p53-induced growth arrest. The validation of this cell system to search for p53 inhibitors was recently demonstrated by using the selective p53 transcriptional inhibitor pifithrin (PFT)-α that completely abrogated p53-induced growth arrest [17]. Besides its simplicity, an additional advantage of this phenotypic assay relates to the fact that it allows the identification of factors that act on p53 transcriptional-independent functions recently identified in yeast [17]. More recently, a highly defined dual-luciferase functional assay was developed in *S. cerevisiae* based on a previous system designed to address functions of p53 mutants and target response elements by varying the p53 levels [18,19]. This genetically well-defined and cost-effective assay can be used in parallel to mammalian cell-based assays for investigating the effectiveness of small molecules either on wt and mutant p53 transactivation potential or on the functional interaction between wt p53 and cofactors like MDM2 and 53BP1 (Fig. **1**). In the latter case, the sensitivity and robustness of this assay was validated by testing the inhibitors of p53-MDM2 interaction, RITA and Nutlin 3A. Similarly to that observed in mammalian cells, both RITA and Nutlin 3A could relieve the MDM2-dependent inhibition of wt p53 transactivation function. Interestingly, it was observed that RITA could also impact p53-53BP1 functional interactions [20].

An additional promising therapeutic target in cancer is PARP1. PARP1 activity in human cells is induced by double strand DNA breaks and inhibitors of this enzyme might be useful as modulators of DNA-damage responses. Using a yeast strain expressing human PARP1 novel inhibitors of this enzyme were identified [21], which have been shown to improve the efficacy of DNA-damaging agents as anticancer drugs [22].

Caspase family members are also key therapeutic targets in cancer and neurodegeneration. In fact, it is well-known that deregulations in the expression or activity of these proteases can lead to the development of these human apoptotic diseases [23,24]. Methods for the HTS against these targets are generally limited to cell-free assays with the several limitations mentioned above. Based on this, and on the high complexity of caspase-signalling pathways, several research groups have used yeast as a simpler cell system for an independent analysis of human caspase family members in order to identify specific regulators. It must be noted that though yeast

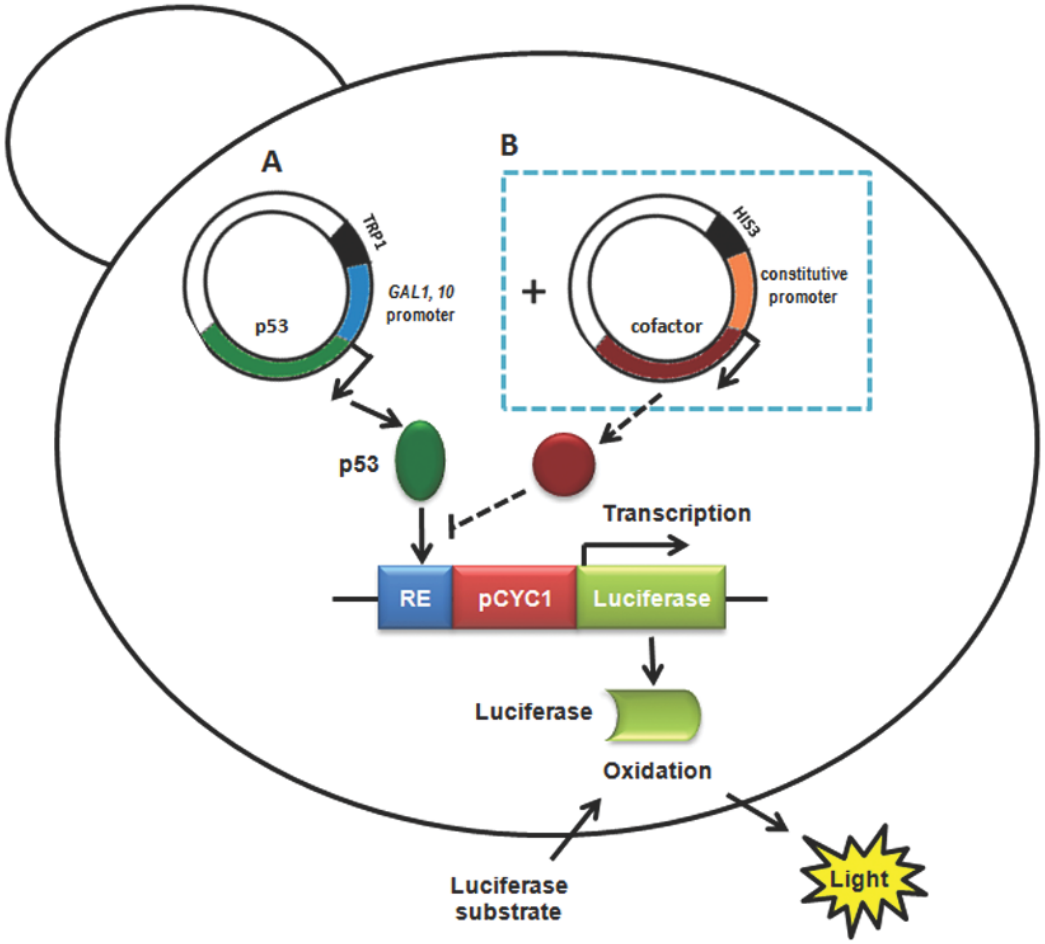

Fig. (1). Yeast-based p53 dual-luciferase transactivation assay using cells (A) expressing human wt/mutant p53 or (B) co-expressing human wt p53 and a cofactor (e.g. MDM2 or 53BP1). The assay exploits the variable expression of p53 proteins and utilizes the *Firefly* and *Renilla* luminescent reporters integrated as single copies at different chromosomal loci in haploid strains or at the same chromosomal location in diploid strains (heteroalleles). While a common minimal promoter controls low-level constitutive expression of both reporters, p53-dependent expression of the *Firefly* reporter is attained through a specific p53 response element (RE) placed upstream of the minimal promoter (pCYC1) [20]. The p53 transcriptional activity is evaluated by quantification of luciferase activity used as reporter gene, which is directly proportional to the light units measured in a plate reader.

encodes a metacaspase (Yca1p), several reports have demonstrated that the activity of human caspases in yeast is independent of Yca1p [25]. A yeast approach to monitor the caspase activity was developed using a reporter system consisting of a transcription factor linked by caspase cleavage sites to the intracellular domain of a transmembrane protein. Caspase activation induces the release of the transcription factor from the membrane, which in turn drives the transcriptional activation of a reporter gene, as bacterial *lacZ*, therefore resulting in a β-galactosidase activity dependent on the caspase activation. This assay led to the identification of *Drosophila* IAP1 as an inhibitor of the *Drosophila* caspase DCP-1 [26]. More recently, the versatility of this assay as a HTS strategy of chemical and genetic libraries was validated for several human caspases [27]. However, the most commonly used strategy to study human caspases in yeast is a phenotypic assay based on the growth arrest induced by these proteins. Though caspases-3, -6, -7 and -9 do not auto-activate when expressed in *S. cerevisiae*, different strategies have been used to circumvent this problem. Usually, engineered auto-activated caspase variants that undergo spontaneous proteolytic processing or folding have been used, namely: *i)* reverse caspases, in which the small subunit precedes its prodomain and large subunit [28,29]; *ii)* caspases without the N-terminal prodomain from the coding sequence [29]; *iii)* by separate co-expression of the large and small subunits of an active caspase [30]; *iv)* by joining in-frame the caspase cDNA to the coding regions for *Escherichia coli* β-galactosidase (*lacZ*) [31]. It was shown that caspase-induced growth arrest can be abolished by co-expression with the pan-caspase inhibitor, baculovirus protein p35 [28] or inhibitors of apoptosis proteins (IAPs) [32]. Recently, the abolishment of reverse caspase-3-induced yeast growth inhibition by the small molecule inhibitor of caspase-3 Ac-DEVD-CMK was also demonstrated [33]. Together, these works opened the possibility of using yeast-based caspase assays to screen for genetic and chemical inhibitors of caspase family members. In fact, using this strategy new inhibitors of caspase-3 were identified among a chemical library of vinyl sulfones [33].

Yeast is also considered a valuable model system for the individual analysis of human Bcl-2 family members, which are also major therapeutic targets in apoptotic diseases. In fact, studies on Bcl-2 family members in yeast have provided important insights into the biology of these key regulators of mitochondrial apoptotic signal transduction. Interestingly, though the amenability of yeast to HTS, only few works (mentioned in the gene overexpression section) have used this cell system to search for regulators of Bcl-2 family members. In spite of this, enough data are provided that corroborate the use of yeast as an efficient phenotypic first-line screening assay to search for small molecules that may specifically modulate each member of this family or the regulatory mechanisms of their functions. Indeed, yeast-based growth interference screens are suitable for proteins for which *in vitro* assay are unavailable, such as the pro-apoptotic proteins Bax and Bak. Both proteins induce apoptosis by triggering cytochrome *c* (cyt *c*) release from mitochondria. Therefore, this assay can be used for screening new modulators of cyt *c* release.

Yeast has also been successfully used as drug screening tool in the search for potent and selective small molecule modulators of individual mammalian protein kinase C (PKC) isoforms. Due to the regulation of major cellular processes, such as proliferation and death, PKC isoforms are also key therapeutic targets in cancer and neurodegeneration (reviewed in [34]). As for caspases and Bcl-2 family proteins, yeast arose as a promising cell system for an individual analysis of mammalian PKC isoforms expressed in a simpler eukaryotic cellular environment without the interference of other isoforms or endogenous regulators. The observation that PKC activators (e.g. PMA) induced growth inhibition in yeast cells expressing a mammalian PKC isoform that was proportional to the degree of PKC activation, while PKC inhibitors (e.g. chelerythrine) reverted the effects of a PKC activator, led to the establishment of the yeast PKC phenotypic assay [35-39]. This assay was used to quantitatively characterize the potency and isoform-selectivity of commonly used PKC activators and inhibitors [35-44]. In fact, many of these compounds have been used as prototypes of potent and non-selective PKC modulators. However, the studies in yeast revealed a differential regulation of PKC isoforms by these compounds (Tables **2** and **3**), being some of them selective to a particular PKC isoform, namely PA (Table **2**) and NPC 15437 (Table **3**). These data concerning the isoform-selective profile of commercially available PKC modulators are extremely relevant and should be taken account when they are used as analytical probes in experimental studies involving these kinases. These studies also argued against the selectivity of compounds such as dPPA (Table **2**), commonly used as a selective activator of PKCβI, and rottlerin (Table **3**), commonly used as a selective inhibitor of PKCδ. In fact, *in vivo*, these compounds were also effective on PKCη. Finally, an unexpected action of MPMA and 4αPMA as PKC inhibitors (Table **3**) led to question the frequent use of these phorbol esters as negative controls. Additionally, the use of yeast-based PKC assay in the screening of several chemical libraries led to the identification of new potent PKC activators and inhibitors, some of them selective to a particular isoform, such as PKCδ, PKCη and PKCζ (Tables **2** and **3**). The new isoform-selective PKC modulators identified in yeast may have promising pharmacological applications as analytical probes, therapeutic agents and/or compounds that can be chemically modified in order to further improve the potency and selectivity to a PKC isoform. Using the yeast PKC assay, the molecular targets involved in the biological activities of several compounds were uncovered. For instance, although mezerein and its analogue daphnetoxin share similarities on their toxicity, the antitumor properties of mezerein were not shared by daphnetoxin. This could be explained based on their differential efficacy to activate PKC isoforms. Indeed, when compared with mezerein, daphnetoxin is less potent on the pro-apoptotic PKCδ and much more potent on the pro-survival PKCα (Table **2**). It was also shown that euxanthone-induced neuroblastoma cells differentiation was associated with a remarkable selectivity to activate PKCζ (Table **2**), a PKC isoform involved in the differentiation of neuroblastoma cells. More recently, it was revealed that coleon U, a diterpene compound with anti-proliferative effects on several human tumor cell lines, was a potent and selective activator of nPKCδ and ε (Table **2**).

Many yeast-based models for screening toxicity suppressors of human proteins involved in neurodegenetation, namely Amyloid β (Aβ), have been developed. Aβ disorders include several progressive neurodegenerative disorders, such as Alzheimer's disease (AD), characterized by the oligomerization and deposition into plaques of an amyloid peptide, the Aβ [45]. Aβ is a cleavage product derived from the amyloid precursor protein (APP) by the action of specific proteases known as secretases. Because secretases are crucial in the formation of this toxic peptide and are not fully identified, several systems were developed in yeast in order to search for new proteases. One of these systems used the yeast *GAL* reporter system to screen for proteases that cleave at the C-terminal of APP. When APP is processed, the *Gal4* transcription factor is released activating *GAL*-reporter genes. This methodology was applied to the screening of a library of human cDNAs, and among other hits, authors found caspase-3 and -8 [46], which validated this screening approach since these caspases are known to cleave APP generating a fragment also implicated in AD [47]. Another novel approach to identify APP processing secretases used an engineered APP (fused to invertase) that only allows the growth of invertase-null yeast upon processing of APP [48]. Restoration of yeast growth upon expression of human BACE-1, responsible for β-secretase activity in humans, indicates processing of APP. This system, initially developed for the identification of novel secretases, was also adapted for the screening of small molecule inhibitors of BACE-1 [49]. Taking advantage that, like in humans, Aβ aggregates in

Table 2. Characterization of the Potency and Selectivity of Known PKC Activators and Identification of New Isoform-selective PKC Activators Using the Yeast PKC Assay

	cPKCs		nPKCs			aPKC	References
	PKCα	**PKCβI**	**PKCδ**	**PKCε**	**PKCη**	**PKCζ**	
Known PKC activators							
Phorbol-12-myristate-13-acetate (PMA)	++	++	++	--	+++++	*NE*	[40]
Phorbol-12,13-didecanoate (PDD)	+++	+++	+++	--	+++	*NE*	[40]
Phorbol-12,13-dibutyrate (PDBu)	+	+	+	--	++++	*NE*	[40]
Phorbol-13-monoacetate (PA)	*NE*	*NE*	*NE*	--	**x**	*NE*	[40]
Phorbol-12,13-diacetate (PDA)	*NE*	*NE*	+	--	+	*NE*	[40]
Phorbol-12-monomyristate (PMM)	*NE*	*NE*	+	--	+	*NE*	[40]
12-deoxyphorbol-13-phenylacetate-20-acetate (dPPA)	*NE*	+	*NE*	*NE*	++	*NE*	[40]
Mezerein	+	+	+++	--	--	*NE*	[43]
New PKC activators							
Daphnetoxin	++	++	+	--	*NE*	*NE*	[43]
Coleon U	*NE*	*NE*	**x**	**x**	--	*NE*	[45]
Xanthone	+	*NE*	*NE*	--	**x**	*NE*	[42]
2-Hydroxyxanthone	*NE*	*NE*	*NE*	--	*NE*	**x**	[42]
3-Hydroxyxanthone	*NE*	*NE*	*NE*	--	*NE*	**x**	[42]
2-Methoxyxanthone	*NE*	*NE*	*NE*	--	*NE*	**x**	[42]
4-Methoxyxanthone	*NE*	*NE*	+	--	+	**x**	[42]
1,2-Dihydroxyxanthone	*NE*	*NE*	*NE*	--	*NE*	**x**	[42]
1,7- Dihydroxyxanthone (Euxanthone)	+	+	++	--	++	+++	[44]
1,2-Dimethoxyxanthone	*NE*	*NE*	*NE*	--	**x**	*NE*	[42]
3,4-Dimethoxyxanthone	*NE*	*NE*	**x**	--	+	+	[42]

(+): Degree of potency; (--): Not determined; (NE): No effect; (X): Selective.

yeast, this cell system was also used to develop models that allow screening for small molecules that reduce the aggregation/oligomerization of Aβ peptide [50,51].

Besides Aβ, other proteins show aberrant protein aggregation and misfolding as key pathological features in neurodegeneration, namely α-synuclein in Parkinson's disease (PD) and polyQ in Huntington's disease (HD). For these proteins, yeast has also been used as screening tool for drug discovery [52,53]. Like in mammalian systems, expression of human α-synuclein in yeast results in a dose-dependent toxicity [52]. Several groups used this α-synuclein-induced growth arrest as a readily screenable phenotype model system to test large chemical libraries. In these screenings, two cyclic peptides [54] and four 1,2,3,4-tetrahydroquinolinones [55] with promising therapeutic potential were found. The efficiency of the hit compounds identified in these studies were tested and confirmed in neuronal models of PD. Additionally, in another study, through the screening of a library of about 10,000 flavonoids promising inhibitors of α-synuclein-induced toxicity, such as quercetin and (-)-epigallocatechin-3-gallate, were identified [56]. Interestingly, the (-)-epigallocatechin-3-gallate was also found to be active against aggregation of a Huntingtin polyQ tract in yeast [57]. About polyQ, most of the studies performed in yeast used the N-terminal of Huntingtin (which includes the polyQ tract) as a surrogate of proteins with expanded polyQ tracts. PolyQ in yeast aggregates for long tracts (Q72 and Q103, associated with human disease), but not for the normal small size tract Q25 [53]. Using this model, inhibitors of aggregation of polyQ tract where searched among a library of natural compounds. The (-)-epigallocatechin-3-gallate was effective in reducing the aggregation in yeast and also the toxicity in neuronal models. This may indicate some overlap between polyQ- and α-synuclein-mediated cellular dysfunction. In fact, using a library of natural compounds, hit compounds effective in reducing aggregation for both proteins were found [58]. Once again, the identified compounds were cathechins, demonstrating the potential of this group of compounds as therapeutic agents in pathological protein aggregation. Distinct classes of compounds, including a sulfobenzoic acid derivative identified in yeast as potently active against polyQ aggregation, showed to be effective in protecting against neurodegeneration in a *Drosophila* model [59]. Additionally, promoters of inclusion formation [60] and autophagic inducers [61] found in yeast, showed to be effective in secondary screens in mammals cells. Though overlapping effects of polyQ and α-

Table 3. Characterization of the Potency and Selectivity of Known PKC Inhibitors and Identification of New Isoform-selective PKC Inhibitors Using the Yeast PKC Assay

	cPKCs		nPKC		aPKC	
	PKCα	**PKCβI**	**PKCδ**	**PKCη**	**PKCζ**	**References**
Known PKC inhibitors						
Chelerythrine	+	+	+	++	+	[39]
NPC 15437	+	+	+	**X**	+	[39]
Ro 32-0432	+	++	++	+++	++	[39]
Tamoxifen	*NE*	+	++	+++	+	[39]
Rottlerin	*NE*	+	++	++	+	[39]
D-Sphingosine	+	+	+	+	+	[39]
New PKC inhibitors						
MPMA	++	++	++	++	--	[40]
4αPMA	++	++	++	++	--	[40]
3,4-Dihydroxyxanthone	+	+	+	+++	++	[42]
1-Formyl-4-hydroxy-3-methoxyxanthone	++	++	++	++	++	[42]
Trans-(±)-Kielcorin C	*NE*	+	+	+	**X**	[46]
Cis-(±)-Kielcorin C	*NE*	+	+	+	++	[46]
Trans-(±)-Kielcorin D	*NE*	*NE*	++	++	++	[46]
Trans-(±)-Isokielcorin D	*NE*	+	+	*NE*	++	[46]
Trans-(±)-Kielcorin E	*NE*	+	+	*NE*	+	[46]

(+): Degree of potency; (--): Not determined; (*NE*): No effect; (X): Selective.

synuclein is suggested by the chemical screening, specific pathways of toxicity for each protein may also exist since the cyclic peptides active against α-synuclein referred above were ineffective against polyQ [54].

Moreover, a yeast system expressing TDP-43, involved in Amyotrophic Lateral Sclerosis (ALS), led to the discovery of several 8-hydroxyquinolines effective not only against TDP-43, but also against α-synuclein and polyQ proteins, with some compounds showing a differential selectivity for the tested proteins [62]. An 8-hydroxyquinoline is already in phase II clinical trials for AD. This study indicates that these compounds can be promising against other neurological diseases as well.

3. YEAST GENOME-WIDE SCREENING APPROACHES

3.1. Yeast Two-hybrid System

The yeast two-hybrid (Y2H) system is the best known genetic assay in yeast. It was developed more than 20 years ago as a tool to identify and characterize protein-protein interactions [63]. This assay was developed in *S. cerevisiae* taking advantage of the properties of the transcriptional factor *GAL4*, which is composed by two domains, a DNA-binding domain (DBD) and a transactivation domain (AD) that can be separated and individually fused to the interest proteins. Upon co-expression, protein-protein interaction is detected because reconstitution of the transcription factor leads to the expression of a reporter gene (Fig. **2A**). The reporter can be a nutrient, such as histidine (*HIS3*), a colorimetric reporter such as *lacZ* (reviewed in [64]) or the recently described Green Fluorescence Protein (GFP) reporter that allows to couple Y2H system with high throughput flow cytometry [65]. While the original Y2H was only applicable for soluble proteins, an adaptation for membrane-bound proteins (split-ubiquitin based) was afterward developed [66]. Protein interactions can be tested in a one to one fashion (both proteins are known) or one protein can be used as a bait for a mixture of proteins expressed from cDNA libraries from which the interacting preys are identified by sequencing (screening approach). Y2H can be used in a large scale format and has been applied to build whole genome protein–protein interaction networks [67,68].

The Y2H system has been extensively used in the research of protein-protein interactions involved in human diseases, thus only few recent applications will be addressed in this review. For example, this technique was important to uncover not only the interaction between the anti-apoptotic protein translationally controlled tumor protein (TCTP) and p53, but also the critical binding sites between these two proteins. Binding is important for TCTP to destabilize p53, preventing it to induce apoptosis which may lead to malignant transformation [69]. Likewise, using the Y2H system another p53 interactor, the ubiquitination factor E4B, whose overexpression correlates to p53 inactivation in brain tumors, was identified [70]. As referred in previous section, APP processing plays a crucial role in AD. With this system, the engulfment adaptor protein 1 (GULP1) was identified as a novel APP-interacting protein with a role in APP processing [71]. Another protein involved in AD is the microtubule-binding protein tau. It was found that the pathological

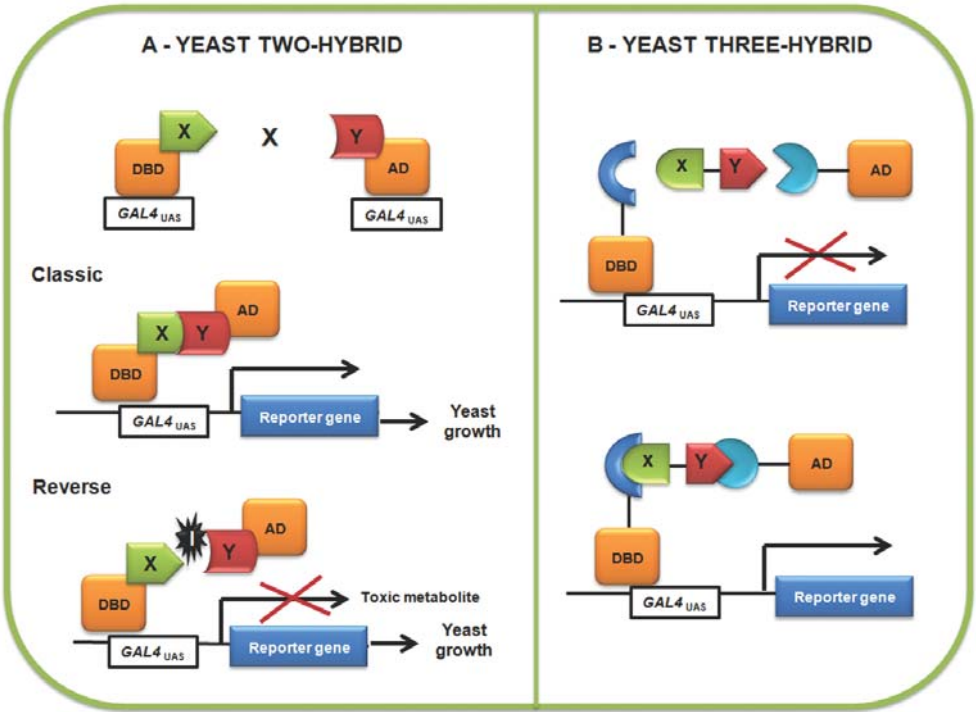

Fig. (2). Principle of the yeast two-hybrid (A) and yeast three-hybrid (B) systems. (A) The coding sequence of protein X (bait) is fused to the *GAL4*-DBD and the coding sequence of protein Y is fused to the *GAL4*-AD. Recombinant vectors are co-inserted, by mating or co-transformation, into an adapted yeast strain. Only if the two fusion proteins X and Y interact, the functionality of the transcription factor is reconstituted, *GAL4* binds to its specific UAS (Upstream Activation Sequence) leading to the transcription of the reporter gene. While in the classic yeast two-hybrid (Y2H) activation of the reporter gene leads to cell growth, in the reverse Y2H, activation of the reporter gene leads to cell death by production of a toxic compound. When an inhibitor (I) disrupts the protein-protein interaction, the reconstitution of the transcription factor is compromised and the reporter gene is not activated allowing yeast to grow. **(B)** The yeast three-hybrid (Y3H) is a variant of the classic Y2H that uses a third molecule composed of a covalently linked heterodimer of two small ligands (X and Y). The *GAL4*-DBD is fused to the receptor for the ligand X and the *GAL4*-AD is fused to the receptor for the ligand Y. The heterodimer allows the proximity between DBD and AD, forming a trimeric complex that leads to the activation of the reporter gene.

effect of a tau mutant is due to a poor membrane insertion due to the loss of interaction with annexin A2 [72].

In 1996, Vidal and colleagues developed a reverse Y2H system applicable for drug screening [73]. This Y2H was named reverse because in this case cells grow when the interaction does not occur, for instance due to drugs that disrupt protein-protein interactions. Because several relevant proteins exhibit undesirable interactions (e.g. p53-MDM2), the inhibition of these interactions may be a relevant therapeutic strategy [74]. Almost simultaneously, Licitra and Liu [75] developed a yeast three-hybrid (Y3H) assay for small molecule target identification. They extended the Y2H approach to small molecule-protein interactions by dimerization of two receptor proteins via a bridging heterodimeric ligand. One ligand-receptor pair serves as an anchor, while the other ligand-receptor pair is the small molecule-protein interaction of interest (Fig. **2B**). As a proof of principle, a dexamethasone-FK506 heterodimer was used to isolate FK506-binding protein 12, the known target of FK506, from a Jurkat cDNA library. Though the obvious applications of this Y3H-based compound/protein display system, few works have taken advantage of this approach in target discovery. In spite of this, it was successfully used to scan the proteome for targets of small molecule kinase inhibitors [76]. More recently, a new approach combining Y3H screening with affinity chromatography was used for the profiling of clinically approved drugs (such as erlotinib, atorvastatin and the anti-inflammatory drug sulfasalazine), providing new insights about their mechanisms of action [77].

Together, though some drawbacks of these genetic systems, namely the high number of false-positives, the continuous improvement of these techniques, namely the adaptation to flow cytometry that highly accelerate the analysis, justifies their widely employment in molecular biology.

3.2. Gene Overexpression

In the last years, several genome-wide yeast gene overexpression libraries have been generated [78-80] and applied in diverse studies. Overexpression screens are typically performed using a high-copy library, in which a cDNA is expressed under an artificial or native promoter. This technique became quite popular in yeast because this organism is easily transformed by extrachromosomal plasmids, the selection of transformed cells is quite straightforward, and there are several regulatable promoters available allowing a considerable control over gene expression. In the last years, overexpression libraries have been applied to studies concerning human disease-associated proteins that induce a screenable phenotype, like growth arrest. In this way, suppressors and enhancers of the human protein can be scored as described in the previous section. With this strategy, proteins and pathways involved in the protein toxicity that may be potential therapeutic targets can be uncovered. For example, through a screen of yeast genes for toxicity suppressors of PD-associated protein α-synuclein, genes involved in the endoplasmic reticulum-to-Golgi vesicular trafficking were identified [81]. The mammalian homologue of one of these genes, Rab1, rescued the loss of dopaminergic neurons in animal models of PD [81]. Moreover, using the same strategy, components of vesicular trafficking pathways were also identified among modifiers of Lrrk2 toxicity [82]. Like α-synuclein, Lrrk2 is associated to PD, suggesting a major role of vesicular trafficking in this disease. The applicability of the overexpression library screenings was extended to many

other neurodegenerative disorders, including ALS by expressing the FUS/TLS protein [83], and AD through the study of the Aβ amyloigenic peptide [84]. In both screenings, new suppressors of toxicity were discovered, and for the Aβ peptide a previously unknown connection between this peptide and the phosphatidylinositol binding clathrin assembly protein (PICALM), also involved in AD, was established. Similarly, an unknown surprising connection was also discovered between the ALS-associated protein TDP-43 and ataxin 2 (an orthologue of yeast Pbp1), a protein associated with the neurodegenerative disorder spinocerebellar ataxia type 2. The potential modulation of TDP-43 by ataxin 2, revealed by yeast-based assays was confirmed in a *Drosophila* model and supported by data from ALS patients. These studies opened new perspectives for therapeutic intervention in these disorders [85].

Taking advantage of the amenability of yeast to large-scale screenings, other authors performed gene overexpression screenings not with a yeast library, but instead with a library of human cDNAs inserted in yeast expression vectors. The success of this strategy was demonstrated by the identification of new human apoptotic regulators, such as Bax inhibitor 1 (BI-1), a suppressor of Bax toxicity that interacts with Bcl-2 but not with Bax [86], the bifunctional apoptosis regulator (BAR) [87], and HMGB1 (a chromosomal high mobility group box 1 protein that is highly expressed in human primary breast carcinoma) [11].

In parallel to the development of screens for genetic modifiers, overexpression libraries have been applied in drug target discovery. The approach named multicopy-suppression profiling is based on the assumption that if a protein is the target of a given drug its overexpression should lead to drug resistance [88,89]. For example, using this technique in combination with haploinsuficiency and homozygous profiling, Hoon and colleagues [90] correctly identified Dfr1, Erg11 and Tor1 as the targets of methotrexate, fluconazole and rapamycin, respectively. Multicopy-suppression profiling was also used to identify secondary drug targets that may be responsible for cisplatin resistance, one of the most common anticancer drugs [91].

Together, the studies herein reported uncover the potentialities of overexpression libraries either in the discovery of new therapeutic targets by performing large-scale screenings for genetic modifiers of disease-associated proteins or in drug discovery/development by multicopy suppression profiling.

3.3. Gene Deletion

3.3.1. Homozigous Deletion

The homozigous deletion is characterized by the complete deletion of non-essential genes in either haploid or diploid strains. Nowadays, there are more than 20,000 strains available from several sources that have been used in multiple studies [92,93]. For example, homozigous deletion has been used to find drug targets by detecting gene deletions that result in drug resistance. Additionally, it has been used to identify individual genes or pathways that buffer the drug target pathway and are therefore required for growth in the presence of the drug (confer drug sensitivity). The observation of these phenotypic alterations, resistance and sensitivity to a drug, allows to infer the global mechanism of action of the drug with the establishment of an index of potential genetic and pharmacologic interactions [88,89,94].

When the combined deletion of two non-essential genes results in lethality (synthetically lethal mutants), this indicates that the genes operate in the same or in redundant pathways. By systematic genome-wide synthetic lethality screens, new relationships between genes and pathways were found [95] (Fig. **3A**). Likewise, when a drug is specific for a given target, deletion of a second gene (that would be synthetically lethal with the drug target gene) results in hypersensitivity to the drug (Fig. **3A**) [93]. For example, by sensitivity analysis to the small molecules "clinostatins" that specifically inhibit the G1 cell cycle proteins Cln1 and Cln2, new genes with a role in G1 cell cycle progression were uncovered [96]. Using a similar chemical synthetic lethality strategy, many studies aimed to uncover secondary targets for anticancer agents, with high relevance for tumor resistance or side-effect toxicity [97-100].

Deletion libraries have also been used to unravel pathways that may be relevant for neurodegenerative diseases. For example, by expressing the human TPD-43 in yeast and screening a deletion library, genes that enhance and suppress TDP-43 toxicity were identified [101]. Deletion toxicity suppressors are particularly important because they are potential candidates for drug targets. Expression of α-synuclein in yeast also allowed screening deletion libraries for suppressors. Genes involved in endocytosis and lipid metabolism were identified as important for inclusion formation (believed to be toxic) [102] and toxicity [103]. Using a similar strategy, several loss-of-function suppressors of mutant Huntingtin toxicity were identified, including proteins associated with stress, protein folding, vesicular transport, vacuolar degradation and transcription [103,104]. A relevant identified suppressor was the mitochondrial protein kynurenine 3-monooxygenase involved in the kynurenine pathway [104], a pathway activated in HD patients and in animal models of this disorder [105] and as such, a promising therapeutic target.

Overall, it becomes apparent that gene deletion profiling is an advantageous method that provides a large amount of information in a simple and rapid way. Data provided by this technique can be useful for pharmacology, pharmacogenetics or personalized drug therapy.

3.3.2. Heterozigous Deletion

Heterozigous deletions in diploid strains have mainly been applied for drug-induced haploinsufficiency profiling (HIP). HIP refers to the increased sensitivity observed to a given drug when the dosage of the gene codifying for the drug target is reduced by half in a diploid strain by heterozygous deletion [106]. Particularly valuable for these studies is a deletion collection that includes a 20 base-pair sequence (barcode) linked to the selectable marker (used to replace the gene) [92].

HIP was one of the first assays using the screening of pools of mutants competitively grown under selective conditions (e.g. drug treatment). The selection is based on the fact that, in the presence of the drug, strains carrying deletions in genes essential for growth will be depleted from the pool over time [89]. The proportion of each strain is given by the abundance of the barcodes assessed by hybridizing to high-density arrays [107] or by direct measurement using next generation sequencing [108] (Fig. **3B**).

Giaever and colleagues [106] demonstrated that the activity of the drugs *in vivo* can be accessed by parallel analyses of yeast strains carrying heterozygous deletions of drug target genes. The identification of the well-known target of tunicamycin and two hypersensitive loci validated this technique. On a following study, authors tested 10 different compounds, including anticancer agents in several genome-wide screens [109]. For several compounds, the results corroborated the known interactions of well characterized drugs with their targets. In another work, about 4000 heterozygotes were used to test the activities of 78 chemicals [110]. More recently, a collection of heterozygous was used to identify targets for tenovins, reported as promising p53 activating molecules. Authors found that tenovins act through inhibition of the protein-deacetylating activities of SirT1 and SirT2, two important members of the sirtuin family [111].

HIP reveals to be a feasible and robust technique that alone or in a complementary approach with techniques like homozygous deletion profiling and multicopy-supression profiling can give a valuable contribution for target identification and drug discovery.

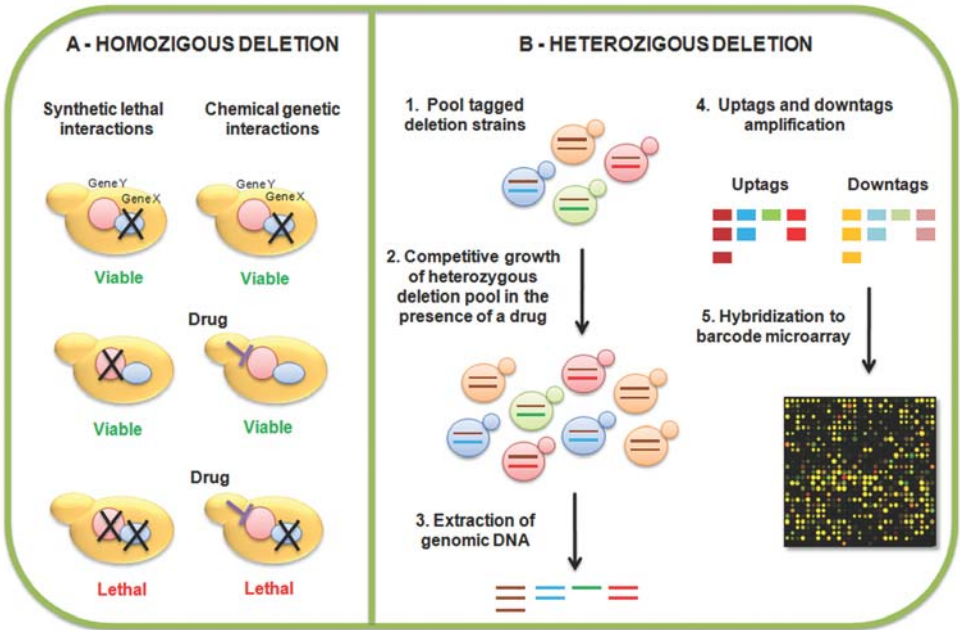

Fig. (3). Techniques based on homozygous (A) or heterozygous (B) gene deletion. (**A**) Synthetic lethal interactions occur when two deletions that individually lead to viable mutants are lethal in a double-mutant combination. Chemical-genetic interactions occur when a deletion mutant is hypersensitive to a sublethal concentration of a growth-inhibitory compound because is synthetically lethal to the gene coding to the drug target. (**B**) Principle of drug-induced haploinsufficiency assays. The yeast deletion collection is pooled with equal abundance of each strain and competitively grown in the presence of a specific drug. Genomic DNA is extracted and the barcodes are amplified by PCR. The PCR products are hybridized to a tag microarray and the relative abundance of each strain is evaluated by hybridization intensity. Reduced intensities identify strains sensitive to the drug tested.

3.4. DNA Microarrays

DNA microarray technologies enable genome-scale expression measurements. Since its development, almost two-decades ago [112,113], DNA microarray quickly became a standard tool in molecular biology due to the ability to analyze thousands of DNA samples simultaneously by hybridization-based assay (Fig. **4**).

With its relatively small genome and highly tractable genetics, *S. cerevisiae* was the first organism for which cDNA microarrays were used for genome-wide transcriptional profiling [114,115]. These studies have been extensively used to measure whole gene expression upon gene deletion, overexpression, exposure of cells to different conditions and stresses and other manipulations forming a valuable compendium. It can be used, for example, to assign a function to a gene by matching its deletion expression profile with the profile of other gene of known function. If genes have similar functions, their deletion is expected to generate similar expression profiles. A similar assumption can be made for protein inhibitors. That is, if a pharmacological inhibitor of a target protein is specific, it will cause approximately the same genome-wide alterations in gene expression as deletion or mutation of the target gene. This approach was first used and validated by Marton and colleagues [116] by using the immunosuppressive drug FK506. The signature of this drug was observed in yeast cells carrying a null mutation in the FK506 target, establishing that genetic and pharmacological ablation of a gene function result in similar changes in gene expression. This approach was also used to validate the target of potent kinase inhibitors [117].

The comparison of known expression profiles can also be used to uncover genetic modifiers of heterologously expressed foreign genes. An example of this type of analysis was performed for the human Huntingtin protein, upon expression of its polyQ tract in yeast. Using DNA microarray analysis two groups reported defects in translation, comparable with the ones seen in yeast carrying mutations in the conserved histone acetylase complex Spt/Ada/Gcn5 acetyltransferase (SAGA) [118,119]. Moreover, treatment with a histone deacetylase inhibitor ameliorated the dysfunction caused by the expression of polyQ [118,119]. This reinforced the current idea that transcriptional dysregulation associated with histone acetylation may be a primary pathogenic process in polyQ-associated diseases and that histone deacetylase inhibitors may be useful therapeutic molecules.

Microarrays have also provided important information concerning α-synuclein effects in yeast cells. Consistently with several reports in higher organisms, it was observed that α-synuclein-induced dose-dependent toxicity in yeast (referred in previous section) was linked to a downregulation of genes associated with mitochondria and an upregulation of genes associated with oxidative stress [55]. Transcriptional profiling was also used in this study to validate hit compounds found in a screening for suppressors of α-synuclein.

One of the most attractive applications of DNA microarrays is in the study of differential gene expression in disease. Taking advantage of the fact that some genes causing disease in humans are conserved in yeast, Kokko and colleagues [120] studied the fumarase gene that in heterozygous mutations causes the tumor predisposition syndrome hereditary leiomyomatosis and renal cell cancer. Authors evaluated the transcriptional changes associated with two conserved fumarase missense mutations in yeast, one associated with malignant phenotype and another associated only with benign forms. Similarly for the two distinct fumarase mutations, the transcription of the Krebs cycle enzymes was downregulated. These findings, along with other biochemical data also pointing to similar cellular effects of both mutations, indicate that genetic factors separated from the fumarase locus may affect the patient's risk of developing renal cancers. Additionally, they explain the heterogeneity in symptoms observed in families segregating different fumarase mutations.

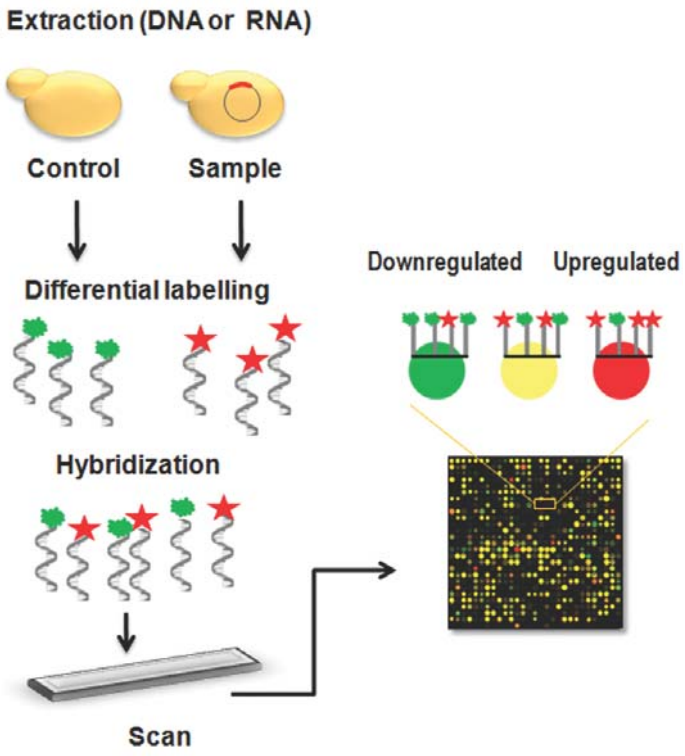

Fig. (4). Principle of DNA microarray assays. Typically, by this methodology DNA, cDNA or mRNA from yeast controls and samples (e.g. expression of a foreign gene, mutation, drug treatment) are isolated and differentially labeled with two fluorophores. Both labeled populations are mixed and hybridized to the DNA microarray. DNA microarrays are arrays of thousands of DNA sequences (e.g. PCR products or oligonucleotides), representing in the case of yeast all 6,200 genes, printed at high density onto a glass slide. Hybridization to spotted cDNA is detected by laser scanning, and the ratio of the dual-color fluorescent signal at any cDNA spot reveals for each target gene the difference in expression between control and sample. The large amount of results obtained is analyzed by clustering algorithms and data visualization tools [122,123].

Altogether, DNA microarrays can generate clues to gene function, which has helped in identifying targets for therapeutic intervention and in assessing drug selectivity. As such, these major tools in molecular biology have greatly accelerated the biomedical research.

4. FINAL REMARKS

Cancer and neurodegeneration are important medical concerns of current aging population. Though enormous advances in understanding the molecular etiology of these disorders, therapeutic options for many patients with these diseases have changed little and outcomes remain disappointing [121]. Based on this, improvement and acceleration of the process of discovery of new therapeutic agents against these pathologies at virtually any step is of great interest from economic and medical viewpoints.

This review is focused on the valuable and expanding role of yeast in the search for new drugs and therapeutic targets in cancer and neurodegeneration. Though the obvious limitations of using a microorganism to identify therapies against human diseases, when used in the first step of the discovery process, coupled with other complementary approaches in mammalian systems, it can have a tremendous impact in the discovery of new therapeutic opportunities. The fact that many human proteins function in this organism made engineered yeast cells the model of choice in several chemical and genetic target-based screenings. Additionally, due to its extraordinary genetic malleability, yeast has proven to be a valuable experimental tool in the development of genomic technologies increasingly oriented towards the high-throughput analysis. Yeast gene disruption/deletion mutants, gene overexpression studies, microarray-based approaches and modified two-hybrid methods continuous to advance and expand. Most importantly, many of these techniques can be performed in a complementary way. For example, combination of the data obtained from genetic screens and transcriptional profiling can be accomplished using a newly developed algorithm providing additional information concerning the phenotype analysed. Also, drug induced haploinsufficiency, homozygous deletion profiling and multicopy suppression profiling can be combined since they provide distinct sets of information, and integration of the results may provide a broader picture of drug action.

Therefore, for the near future, the use of yeast holds great promise in high-throughput analysis to identify novel molecular mechanisms and their participating proteins and to discover lead compounds with potential to become novel drugs in cancer and neurodegeneration. Actually, a typical delay between the early steps in drug discovery, in which yeast is most widely used, and clinical use of any resulting successful compound lead us to believe that most of the benefits of yeast in the discovery and development of therapeutic agents have yet to be realized.

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