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Multidrug Sensitive Yeast Strains, Useful Tools for Chemical Genetics

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Takeo Usui

Additional information is available at the end of the chapter

Abstract

The budding yeast *Saccharomyces cerevisiae* is a useful eukaryote model organism for application to chemical biology studies, for example, drug screening, drug evaluation, and target identification. To use yeast for chemical biology research, however, it has been necessary to construct yeast strains suitable for various compounds because of their high drug resistance. Hence, the deletion of all multidrug resistance genes except for those that are important for viability and for genetic experiments/manipulation could increase the drug sensitivity without influencing the transformation, mating, or sporulation efficiency. There are two major factors conferring multidrug resistance in *S. cerevisiae*: one is the drug efflux system and the other is the permeability barrier. We therefore constructed a strain which shows high sensitivity to multiple drugs by disrupting the drug efflux system using ATP-binding cassette transporters and suppressing the membrane barrier system by introducing an ERG6-inducible system. In this review, we discuss the construction of our multidrug-sensitive yeast strains and their application in chemical biology.

Keywords: multidrug-sensitive yeast, drug efflux system, permeability barrier system, drug target identification, drug screening

1. Introduction

1.1. Screening and target identification of bioactive small molecules: important processes in chemical genetics

The screening of bioactive small molecule compounds is the most important process in drug development. Natural products which have structural diversity isolated from microorganisms,

plants, and animals are useful sources in the field of drug development [1]. Structurally, new natural products might show novel activities such as antimicrobial, antiviral, and antitumor activities. These natural products also provide useful information for medicinal chemistry, and allow the development of new synthetic compounds as novel medicines. For example, eribulin, a semi-synthetic derivative of halichondrin B, has been approved as an anti-cancer drug [2–4]. Therefore, the screening and identification of new small molecules open new avenues for drug development. There are two major ways to identify bioactive small molecules: phenotypic screening and target-based screening. Phenotypic screening is based on cytotoxicity [5–7], cell cycle arrest [8], immune-suppression [9], and morphological changes [10] of drug-treated cells, fungi, and bacteria. Target-based screening is performed based on measurable readouts such as enzymatic activity inhibition [11] or drug-protein interaction [12]. These approaches have identified useful small molecules and medicines.

Target identification (Target ID) of small molecules is also quite important in order to develop safe and useful drugs [13]. Thalidomide, a cautionary example, was used as a sedative a half-century ago before it was found to be teratogenic and to cause multiple birth defects [14]. However, thalidomide is also used in the treatment of Hansen's disease, myeloma [14], and so on. In addition, immunomodulatory drugs derived from thalidomide have been developed as a new class of anti-cancer drugs and novel medicines for treating ribosomopathies such as 5q-syndrome [15]. Recently, cereblon, a substrate receptor of the CRL4 E3 ubiquitin ligase, has been identified as a primary target of thalidomide teratogenic [16] and anti-cancer [15] activity. These lines of research provide useful information that cereblon may pose a risk of teratogenic activity and simultaneously serve as an attractive molecular target for immuno-modulatory drug development. To identify the relevant target molecules and target pathways, indirect and direct approaches have been used [13]. The indirect approaches include phenotypic analysis and large-scale analysis such as proteomic and genome-wide analyses. Some specific changes in cell morphology, cell cycle arrest, and other phenotypes provide us useful information for predicting targets of the drugs. Based on this property, Morphobase, an encyclopedic database of the morphological changes that occur in drug-treated cells, has been constructed and applied to drug target discovery [17]. Large-scale analyses such as proteomics, metabolomics, and transcriptome analysis of drug-treated cells have been performed to predict the target pathways of bioactive small molecules [18]. Genome-wide genetic studies are also frequently used for drug target ID. For example, synthetic lethal/sick genetic interaction analyses [19, 20], genome-wide overexpression screening [21], and haploinsufficiency-chemical sensitive assays [22] have been used to analyze the mode of action of various drugs. On the other hand, direct approaches, such as affinity probe approaches and genetic analyses, are quite useful to identify the direct target molecules of drugs. By using affinity probe approaches, the targets of thalidomide [16] and FK506 [23] have been identified. Genetic analysis is another powerful method of identifying not only drug targets [24–29] but also the signaling pathway affected by a drug. Genetic studies using model organisms such as yeast have contributed to identification of the target molecules of bioactive compounds.

The identification of new bioactive small molecules and elucidation of their target molecules/signaling pathways are important not only for developing medicines but also for basic science. Such compounds are a useful tool for understanding the fundamental protein

functions in cells. Well-known examples are famous immunosuppressants such as FK506, cyclosporine, and rapamycin. These compounds inhibit immunophilin and T-cell activation through different mechanisms [30]. Studies of these compounds have revealed their detailed immunoreaction mechanisms [30]. Mitotic inhibitors are another example. Mitotic spindle formation and chromosome segregation are fast processes that are completed within approximately 1 hour. Therefore, by taking advantage of rapid pharmacological intervention, studies using microtubule inhibitors ($\alpha\beta$-tubulin inhibitors [31–33] or γ-tubulin inhibitor [12]), mitotic kinesins (Eg5 [34, 35]), and mitotic kinase inhibitors (aurora kinases [36, 37], Cdk1 [38], Plk1 [39, 40], Mps1 [41, 42]) highlighted useful information regarding the temporal regulation of mitotic spindle architecture and faithful chromosome segregation. These findings could in turn contribute to further drug development. Therefore, target ID of newly found useful bioactive compounds is quite an important process in both basic science and medicine development.

1.2. *Saccharomyces cerevisiae*, a useful model organism for chemical genetics

Saccharomyces cerevisiae is one of the most frequently used model organisms in chemical genetics. The properties of *S. cerevisiae* along with easy-to-use genetic analyses, mutational analyses, gene disruption, and genome modification have facilitated both chemical screening and target ID (**Table 1**). For example, the target of rapamycin (TOR) has been found by genetics using *S. cerevisiae* [29]. In addition, *S. cerevisiae* is useful for chemical screening [43, 44]. However, *S. cerevisiae* generally shows higher resistance against various compounds compared with mammalian cells, except in the case of a few compounds such as rapamycin (**Table 2**). This disadvantage limits the application of *S. cerevisiae* in chemical screening. Therefore, *S. cerevisiae* showing sensitivities against drug of interest has been quite useful. For example, *S. cerevisiae* quadruple deletion mutant lacking *yrr1*, *yrs1*, *pdr1*, and *pdr3* was constructed for the analyses of target molecule of reveromycin A. However, construction of sensitive yeast suitable for each compound is a time-consuming process. To overcome this drawback, we developed two multidrug-sensitive strains which have proven quite useful for

Compound	Approach	Finding	Ref.
Benomyl	Pathway analysis	Identification of Mad1, Mad2, Mad3 as mitotic spindle checkpoint proteins by using benomyl sensitive mutants	[31]
Benomyl	Pathway analysis	Identification of Bub1, Bub2, Bub3 as mitotic spindle checkpoint proteins by using benomyl sensitive mutants	[32]
Reveromycin A	Target ID	Identification of *ILS1* as a target of reveromycin A	[27]
Curvularol	Target ID	Identification of *RPL3* as a target of curvularol	[28]
Rapamycin	Target ID	Identification of *TOR* as a target of rapamycin	[29]
Eudistomin C	Target ID	Identification of *RPS14* as a target of eudistomin C	[50]
Splitomicin	Screening	Identification of splitomicin as a NAD⁺-dependent histone deacetylase inhibitor	[51]

Table 1. The examples of chemical genetics studies using *S. cerevisiae*.

	Mammalian cell line (HeLa)	Budding yeast (BY4741)
Cycloheximide (μM)	0.2	270
Digitonin (μM)	0.4	1.9
Fluphenazine (μM)	13	51
Latrunculin A (nM)	0.2	>240
4-Nitroquinoline 1-oxide (μM)	0.1	7.1
Rapamycin (nM)	>300	7.1
Staurosporine (μM)	0.1	15.1
Tunicamycin (μM)	1.8	>120

HeLa cells (3×10^3 cells/well in 96 well plate) and BY4741 cells (3.8×10^5 cells/well in 96 well plate) were treated with various concentrations of compounds for 48 and 8 h, respectively. Cell viabilities were determined by WST-8 (Dojindo, Kumamoto, Japan) and IC50 values were calculated.

Table 2. The IC50 values of compounds against HeLa cells and *S. cerevisiae*.

research in chemical biology. There are two major systems conferring multidrug resistance in *S. cerevisiae*: one is the drug efflux system, which exports drugs into vacuoles or outside of cells, and the other is the permeability barrier, which blocks the penetration of drugs into the cells (**Figure 1**). The drug efflux system consists of ATP-binding cassette (ABC) transporters that export xenotoxic compounds outside of cells or inside of vacuoles, and their transcriptional factors [45–47]. *S. cerevisiae* has at least 16 ABC transporters, of which Pdr5p, Snq2p, and Yor1p confer multidrug resistance by exporting bioactive small molecules out of cells. Four transcriptional factors (Pdr1p, Pdr3p, Pdr8p, and Yrr1p) up-regulate the transcription of most of the ABC transporters [45–47]. A permeability barrier is conferred by ergosterol in the yeast plasma membrane. Therefore, ABC transporter-related genes and ergosterol synthesis genes were frequently disrupted to construct drug-sensitive strains. For instance, a strain in which *pdr1*, *pdr3* (genes encoding transcriptional factors for ABC transporters), and *erg6* (a gene involved in ergosterol synthesis) were disrupted was used for drug screening [43]. However, the *erg6* deletion mutant shows decreased transformation and sporulation efficiencies that are essential for yeast genetic analysis. In addition, some of the transporters located in the vacuole membrane are involved in the detoxination of metabolites as well as xenotoxins, and their disruption results in growth defects. Therefore, to make a yeast strain sensitive to a wide range of drugs, it is necessary to suppress both efflux and barrier systems without affecting the genetic properties and growth rate. Hence, we speculated that the disruption of all ABC transporters located on the plasma membrane that are not important for viability and genetic experiments or for the conditional expression regulation of the *ERG6* gene could increase the drug sensitivity without influencing the transformation, mating, or sporulation efficiency.
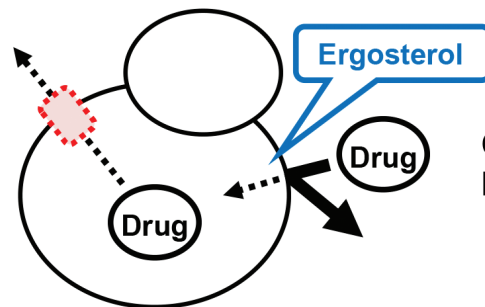
In this review, we discuss the construction of two multidrug-sensitive yeast strains, 12geneΔHSR [48] and 12geneΔHSR-iERG [49], which are available for genetic analysis. We also discuss the application of these strains in drug screening and target ID [50].
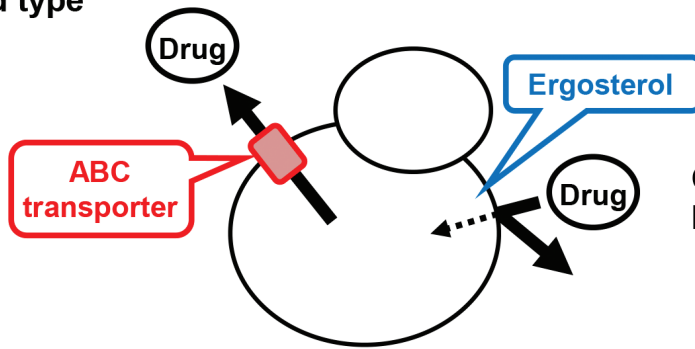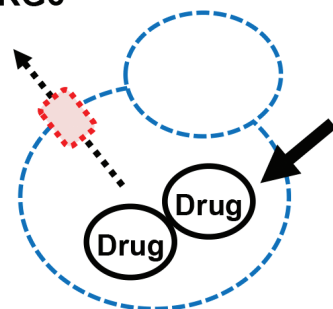
Figure 1. The work flow of the construction of multidrug-sensitive strains. (A) The parental strain, BY4741, possesses high genetic manipulation availability, but shows high drug resistance. (B) 12geneΔ0HSR, created by disruption of the drug efflux system and introduction of the *RME1(ins-308A)* mutation, achieves drug-sensitivity without compromising the genetic manipulation availability. (C) 12geneΔ0HSR-iERG was created by the insertion of a *gal1* promoter into *ERG6*. This strain shows high drug sensitivity but drastically decreased genetic manipulation availability under the glucose condition, because ERG6p expression is repressed. Instead, genetic manipulation is available under the galactose condition through enhancement of the ERG6p expression.

2. Construction and application of multidrug-sensitive yeast strains

2.1. Construction of multidrug-sensitive yeast strains

We constructed a multidrug-sensitive yeast strain by disrupting 12 ABC transporter-related genes and suppressing the *ERG6* gene. The work flow is shown in **Figure 1**. As a first step, we focused on drug efflux systems. The drug efflux system composed of ABC transporters confers resistance against a wide variety of compounds [45–47]. Therefore, it is difficult to predict which transporters will confer drug resistance against the drug of interest. We thus decided to construct the 12geneΔ0 strain through the disruption of all of the ABC transporters involved in drug export located on the plasma membrane and transcription factors involved in multidrug resistance specifically on a BY4741 background [48]. Gene disruption of eight gene-encoding ABC transporters (*AUS1*, *PDR5*, *PDR10*, *PDR11*, *PDR12*, *PDR15*, *SNQ2*, and *YOR1*) and four genes encoding transcriptional factors (*PDR1*, *PDR3*, *PDR8*, and *YRR1*) was carried out using a PCR-based markerless gene disruption method modified from the *delitto perfetto* method [52]. Because 12geneΔ0 leaves no marker genes in the genome, auxotroph markers which the parental strain originally possesses can be used for further studies. To use 12geneΔ0 for chemical genetics, it is important to show not only its multidrug sensitivity but also its transformation, mating, and sporulation efficiencies, which are necessary for genetic analysis. The transformation and mating efficiency of 12geneΔ0 were on the same order as those of the parental strain BY4741 (**Table 3**). However, the sporulation efficiency was drastically decreased in 12geneΔ0 (**Table 3**). It was reported that single-nucleotide polymorphisms of three genes (a noncoding regulatory region of *RME1(ins-308A)*, and two missense mutations in *TAO3* and *MKT1*) are involved in sporulation efficiency, and when these mutations were introduced in S288c, the parental strain of BY4741, the sporulation efficiency increased [53]. We therefore introduced the *RME1(ins-308A)* and *MKT1(D30G)* mutations into 12geneΔ0. Although both mutations increased the sporulation efficiencies, the *MKT1(D30G)* mutant formed petite colonies as reported previously [54]. Therefore, we decided to use the *RME1* mutant for our studies, and the strain created was named 12geneΔ0HSR (12geneΔ0 strain showing High Sporulation by *RME1(ins-308A)* mutation) [48]. 12geneΔ0HSR showed sporulation efficiency comparable to that of BY4741. By testing the drug sensitivities of the 12geneΔ0HSR, BY4741Δ*erg3*, and BY4741Δ*erg6* strains, we revealed that there are different spectrums of drug resistance conferred by the efflux and barrier systems (**Figure 2**) [48], suggesting that it is necessary to disrupt both the drug efflux and permeability barrier systems to make a strain with high sensitivity against a wide range of multiple drugs. To disrupt the permeability barrier system without affecting any of the genetic properties, we introduced the conditional expression promoter *GAL1p* in the *ERG6* gene in 12geneΔ0HSR (**Figure 1**) [49]. The constructed strain, 12geneΔ0HSR-iERG6, showed improved sensitivities to several compounds under the glucose condition (*ERG6* suppression), and it exhibited sufficient transformation and sporulation efficiencies under the galactose condition (*ERG6* expression) (**Table 3**). Because of its high sensitivities to several compounds, the 12geneΔ0HSR-iERG6 strain will be a useful tool in chemical biology studies.

	Transformation efficiency (Cfu/µg)	Mating efficiency (%)	Sporulation efficiency (%)
BY4741	$9.6 \times 10^5 \pm 2.2 \times 10^5$	17.7 ± 7.5	21.9 ± 6.8
$\Delta erg6$	55.0 ± 51.3	4.8 ± 1.7	9.4 ± 4.7
12geneΔ0	$1.2 \times 10^5 \pm 2.0 \times 10^4$	15.7 ± 5.3	5.0 ± 2.9
12geneΔ0HSR	N.D.	N.D.	28.8 ± 4.6
12geneΔ0HSR-iERG6 (under glucose condition)	7.0 ± 8.2	6.4 ± 2.2	0.0 ± 0.0
12geneΔ0HSR-iERG6 (under galactose condition)	$3.0 \times 10^4 \pm 2.4 \times 10^4$	N.D.	10.7 ± 3.0

Values are mean ± S.D. calculated from three independent experiments. These data are edited from **Figure 1** of Ref. [48] for BY4741, *Δerg6*, 12geneΔ0, and 12geneΔ0HSR, or **Figure 2** of Ref. [49] for 12geneΔ0HSR-iERG6.

Table 3. Comparison of the efficiencies of transformation, mating and sporulation between BY4741, *erg6* disruptant and 12geneΔ0HSR.

2.2. Application 1: drug screening

2.2.1. Availability of 12geneΔ0HSR-iERG6 in drug screening

In general, *S. cerevisiae* exhibits high levels of drug resistance, which is an obstacle for drug screening. In fact, most of the compounds used for clinical or basic research show higher IC_{50} values against *S. cerevisiae* than against mammalian cells (**Table 2**). Therefore, multi-drug-sensitive strains of *S. cerevisiae*—for example, the *pdr1 pdr3 erg6* triple mutant or *pdr1 pdr3 yrs1 yrr1* quadruplex mutant—have been used for drug screening [43, 55]. To test the superiority of our strain, we screened mitochondrial inhibitors from microbial secondary metabolites and compared the hit ratio of 12geneΔ0HSR-iERG6 with that of BY25929 (*yrs1::HIS3 yrr1::TRP1 pdr1::hisG pdr3::hisG*), a multidrug-sensitive quadruplex mutant (**Tables 4** and **5**).
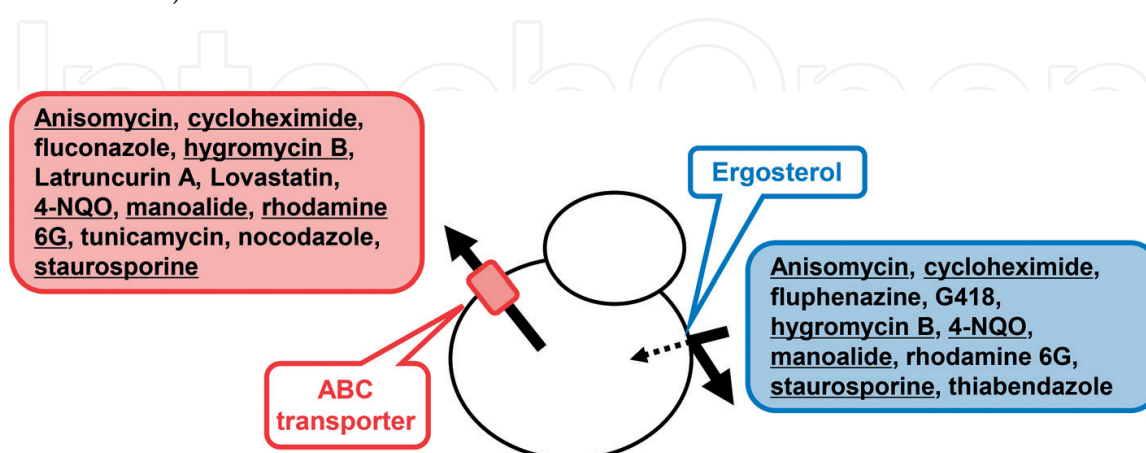

Figure 2. Drugs to which resistance was conferred by ABC transporters, ergosterol or both systems (indicated by underlining), respectively.

	Number of broth	Number of hit broth	Hit ratio (%)
Origin			
Fungus	2664	149	5.6
Actinomycetes	5617	289	5.1
Total	8281	438	5.3

Table 4. Hit ratio of screening of mitochondrial inhibitor using quadruplex mutant, BY25929.

	Number of broth	Number of hit broth	Hit ratio (%)
Origin			
Fungus	3144	270	8.6
Actinomycetes	3067	253	8.2
Total	6211	523	8.4

Table 5. Hit ratio of screening of mitochondrial inhibitor using 12geneΔ0HSR-iERG6.

To identify the mitochondrial inhibitors, we used the difference in cell growth between the glucose medium and the glycerol medium. Yeast can use glycerol as a respiratory substance after the conversion to dihydroxyacetone phosphate via glycerol-3-phosphate by cytosolic and mitochondrial enzymes, GUT1p and GUT2p, respectively. Therefore, yeast could grow even in the presence of a mitochondrial inhibitor in glucose medium because of anaerobic respiration, but not in glycerol medium in which one of the metabolites in glycolysis, dihydroxyacetone phosphate, could not be produced. Therefore, we compared the growth inhibition induced by microbial broth samples on glucose medium (1% yeast extract, 2% polypeptone, 2% glucose, 1.5% agar) with that on glycerol medium (1% yeast extract, 2% polypeptone, 3% glycerol, 1.5% agar), and chose the broth which inhibited yeast growth on glycerol medium but not on glucose medium [55]. Growth inhibition activities of microbial broth samples were evaluated using the paper disc method on agar plates inoculated with recombinant *S. cerevisiae* strains. In detail, 6 mm sterile filter discs impregnated with each compound solution (10 μl) were placed on the agar plate using a forceps (medium volume; 30 ml/plate, cell number; 1.5×10^6 cells/plate, plate dimension; 144 × 100 × 14.5 mm, square shape), and the plates were incubated at 30°C for 48 h. After incubation, the diameters of the zone of inhibition were measured with a vernier caliper. As shown in **Table 4**, the hit ratio using the quadruplex mutant, BY25929, was about 5%. Because the hit ratio when wild-type yeasts (W303-derived yeast strains) were used in a similar screening system was 1.4% (fungus samples 0.5% (44 total hits among 8610 samples), actinomycetes samples 3.2% (125 total hits among 3912 samples), this result suggests that the quadruplex mutant is useful for drug screening with a high hit ratio. Indeed, a novel compound, decatamariic acid, was isolated as a mitochondrial inhibitor using the quadruplex mutant [55]. Moreover, the hit ratio using 12geneΔ0HSR-iERG6 increased to about 8% (**Table 5**).

To determine whether it is possible to isolate the novel compounds or not, we selected the microbial broths which were detected using 12geneΔ0HSR-iERG6 but not using the quadru-plex mutant. We found a total of 46 broths (fungus origin: 16 broths; actinomycetes origin: 30 broths) which inhibited the growth of 12geneΔ0HSR-iERG6 specifically. Among these broths, we selected two fungus broths for further purification of active metabolites, and iso-lated 4,6'-anhydrooxysporidinone (1, fusoxypyridone [56]), pestalotic acid A (2), and three novel compounds (manuscript in preparation) (**Figure 3**). 4,6'-Anhydrooxysporidinone has been isolated from *Fusarium oxysporum* in the course of the screening of anti-angiogenesis inhibitors [57], but showed weak cytotoxicity against mammalian cell cultures (IC$_{50}$ > 100 μM) and anti-MRSA activity (MIC = 100 μg/ml) [58]. Pestalotic acid A has been isolated from a *Pestalotiopsis* sp. as an antimicrobial compound containing a furylidine tetronic acid core [59]. Because of the lack of biological activity other than antimicrobial activities, the observation of antifungal activity is a novel insight. These results strongly suggest that 12geneΔ0HSR-iERG6 would be useful for drug screening.

2.2.2. Screening of readthrough compounds

Because the usefulness of our strains was confirmed, we next performed the preliminary screening of compounds that show readthrough activities. Readthrough compounds allow the translational machinery to skip nonsense mutations encoding premature termination codons (PTCs) and could become medicines for hereditary diseases caused by PTCs (**Figure 4**). To date, many small molecules have been developed as readthrough drug candidates. Several forms of aminoglycoside antibiotics, such as gentamicin (3), G418 (4), and its analogues, have been reported to show readthrough activities (**Figure 5**) [60]. Barton-Davis *et al.* revealed that the dystrophin expression in *mdx* mice, an animal model of duchenne muscular dystrophy (DMD) is increased after the administration of gentamicin (3) [61]. Novel aminoglycosides derived from gentamicin, which showed readthrough activity against four different nonsense DNA constructs underlying genetic diseases, were also recently reported [62]. However, long-term treatment with aminoglycosides showed serious side effects such as nephrotoxic-ity [63] and ototoxicity [64]. As a non-aminoglycoside readthrough compound, ataluren (5), which is a 1,2,4-oxadiazole derivative developed from a chemical library, promotes dystro-phin production in primary muscle cells from humans and *mdx* mice (**Figure 5**) [65]. It was also found that (+)-negamycin (6), which is a dipeptide-like antibiotic containing a hydrazide

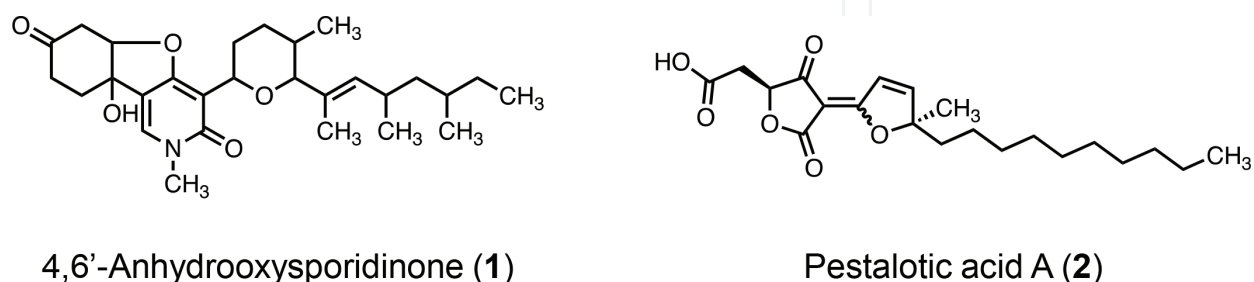4,6'-Anhydrooxysporidinone (**1**)   Pestalotic acid A (**2**)

Figure 3. Structure of 4,6'-anhydrooxysporidinone (**1**) and pestalotic acid A (**2**).
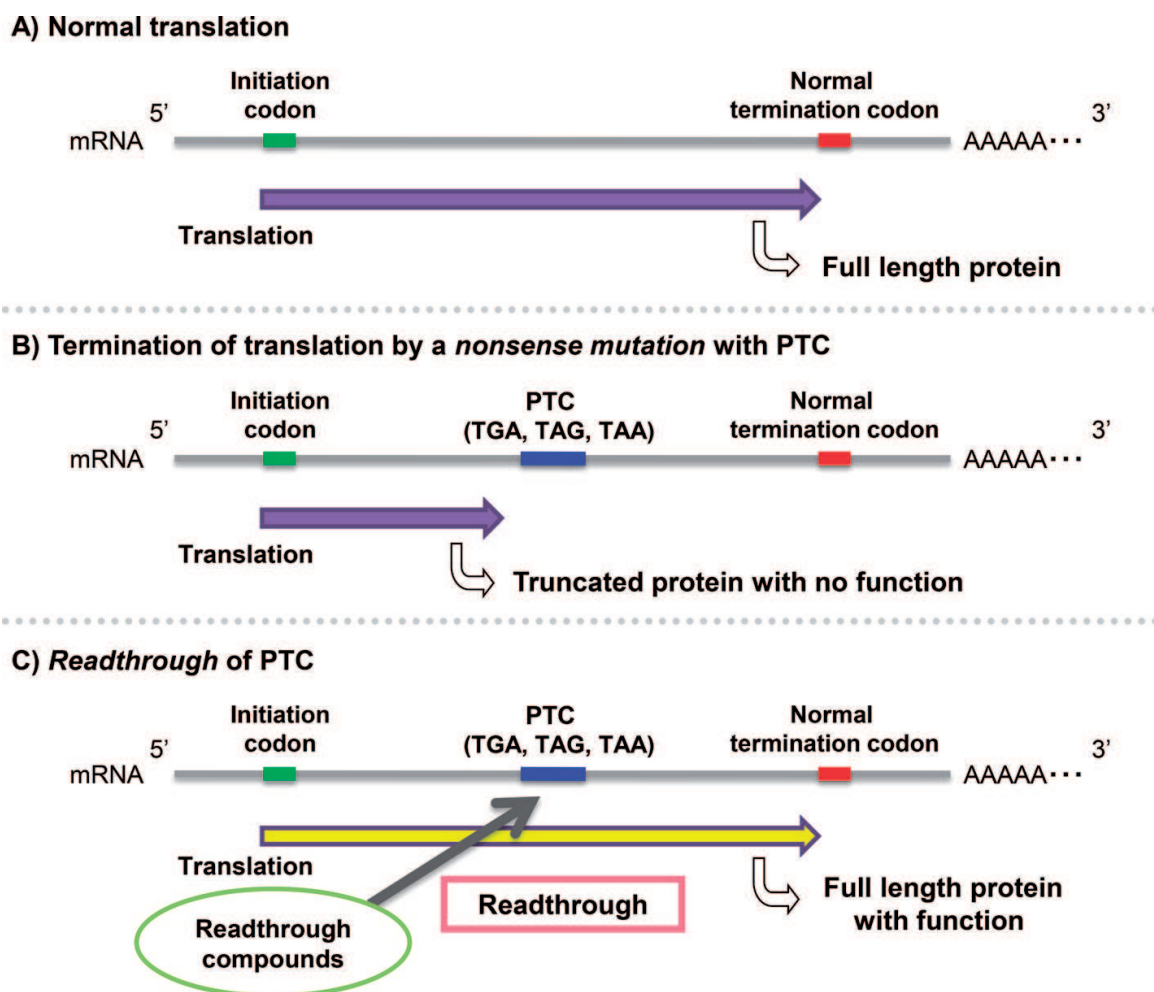
Figure 4. Nonsense mutation as a premature termination codon (PTC) and readthrough compounds. (A) mRNAs containing no PTC are translated into full-length and functional proteins. (B) In the case of mRNAs containing PTC, translation stops at PTC and non-functional truncated proteins are synthesized. (C) In the presence of readthrough compounds, even mRNAs containing PTC are translated into full-length and functional proteins.

structure [66], has readthrough activity and restores dystrophin expression in the muscles of *mdx* mice (**Figure 5**) [67]. In our structure-activity relationship study of (+)-negamycin, we discovered several more potent derivatives, including Leucyl-3-*epi*-deoxynegamycin (TCP-126, **7**) and TCP-112 (**8**) (**Figure 5**) [68, 69]. However, the activities of these compounds are not sufficient for medicine, and the mechanism of action of the readthrough activity remains to be elucidated.

To discover novel readthrough compounds, we constructed yeast strains for the screening of readthrough compounds using 12geneΔ0HSR. *ADE2* is an enzyme that is essential to producing adenine in live yeast systems, and its mutation induced the accumulation of red pigment in vacuoles [70]. One of the *ade2* auxotroph markers, *ade2–101*, has a nonsense mutation (*ochre*) at 190 bp [71]. Therefore, we introduced PTCs at the same site as in the *ADE2* gene and inserted the *ADE2* loci of 12geneΔ0HSR by pop-in/pop-out. The resulting strains 12geneΔ0HSR *ade2-E64X* required adenine for growth and formed red colonies in adenine-limited medium (**Figure 6A**). In contrast, most of the colonies appeared white on
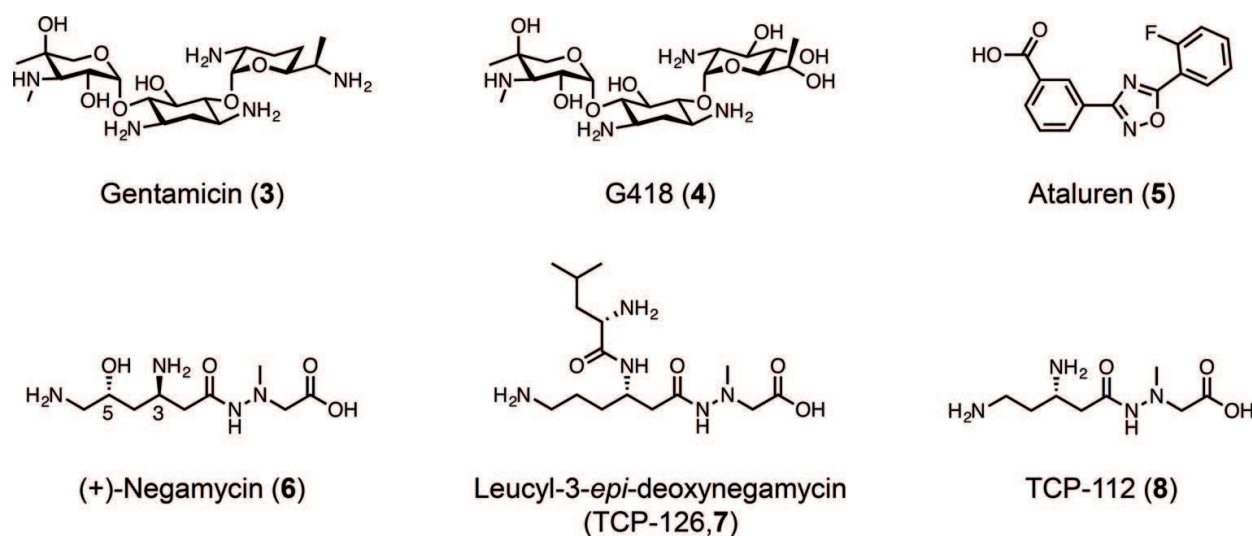
Figure 5. Structure of readthrough compounds. Gentamicin (**3**) and G418 (**4**) are aminoglycoside-type readthrough compounds. Aataluren (**5**), (+)-negamycin (**6**), and negamycin derivatives (Leucyl-3-*epi*-deoxynegamycin (TCP-126, **7**), TCP-112 (**8**)) are non-aminoglycoside-type readthrough compounds.

medium containing TCP-126 (**Figure 6B**), suggesting that TCP-126 evoked readthrough in *ade2-E64X*. In addition, DMSO solution (3 μl) containing readthrough compounds (G418 or negamycin analogues including TCP-126) induced the white halo on the 12geneΔ0HSR *ade2-E64X* strain-inoculated plate after 4 days incubation (**Figure 6C**). These results indicated that 12geneΔ0HSR *ade2-E64X* is suitable for use in the qualitative analysis of readthrough activity.

Next, we initiated a high-throughput screening of the readthrough compounds based on the halo assay using chemical library. This screening is underway, but already several hit compounds have been found, including rapamycin (**9**) [72], wortmannin (**10**) [72], and A23187 (**11**) [73] (**Figure 7**). These data provided further evidence of the usefulness of the 12geneΔ0HSR *ade2-E64X* strains for identifying and elucidating the mechanism of action of readthrough drugs.

2.3. Application 2: target ID

Since our strains show multidrug sensitivity without a decrease in genetic availability, they should also be useful for performing target ID for drugs and the mechanism evaluation of compounds, especially those which are only available in limited amounts, such as natural products. Here we show an example of target ID [50]. Eudistomin C (EudiC, **Figure 8**), a natural product isolated from the Caribbean tunicate *Eudistoma olivaceum* [74, 75] shows broadspectrum antiviral activity [76]. Because of a unique structural feature, oxathiazepine ring attached to a tetrahydro-β-carboline, EudiC has attracted attention as a lead compound for antiviral medicines. However, several trials for its clinical development have failed due to the strong cytotoxicity of EudiC. To reveal the cause of the cytotoxicity of EudiC, it is important to identify the target molecule responsible for the cytotoxicity of EudiC. By using the yeast genetic approach, we found that a mutation in the RPS14A gene confers EudiC-specific
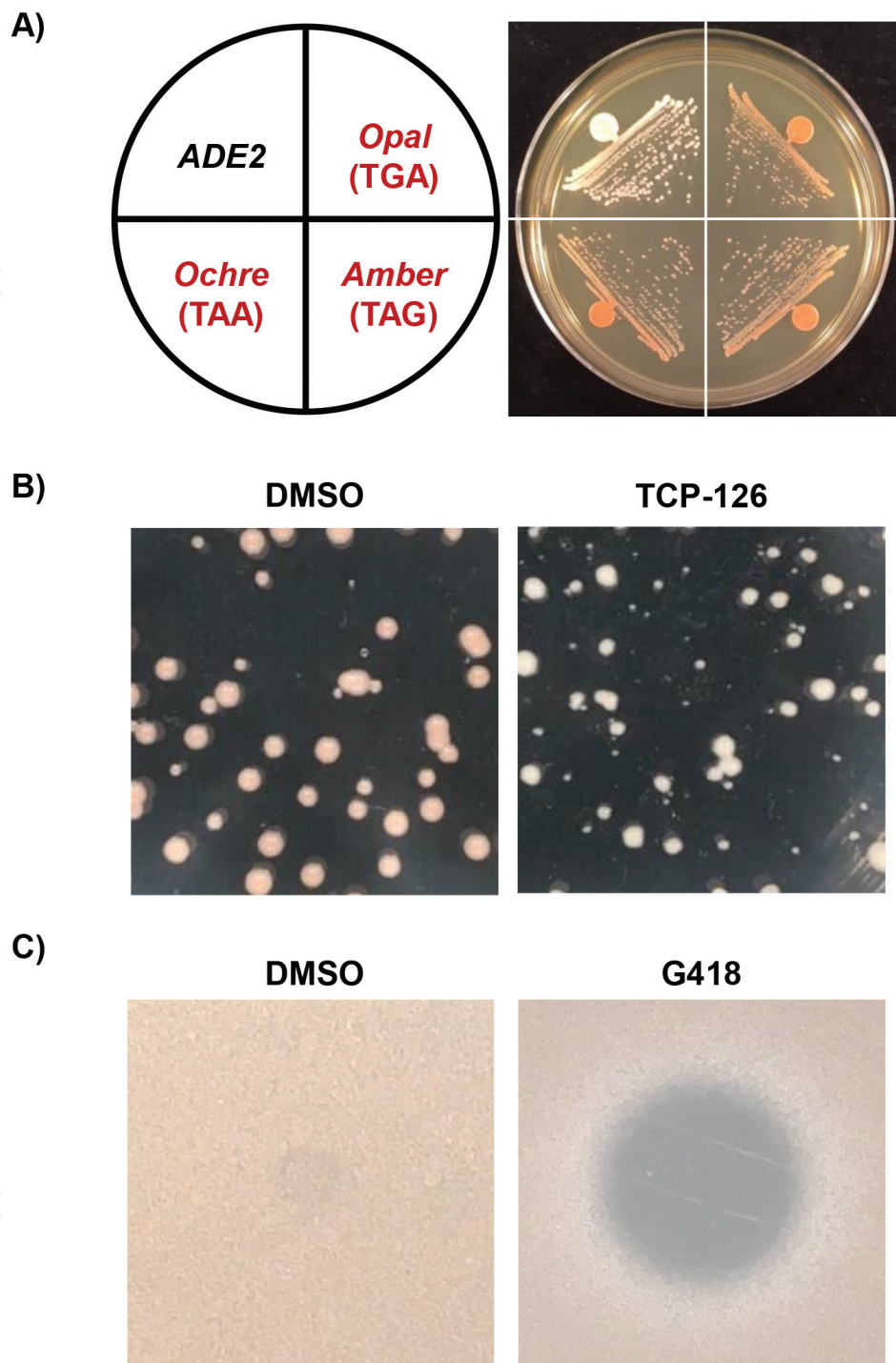
Figure 6. The color of 12geneΔ0HSR *ade2-E64X* strains turned from red to white in the presence of readthrough compounds. (A) 12geneΔ0HSR *ade2-E64X* strains were plated on YPD containing 0.0005% adenine for 4 days. The wild-type strain (12geneΔ0HSR) formed white colonies, but 12geneΔ0HSR *ade2-E64X* strains formed red colonies. (B) 12geneΔ0HSR *ade2-E64X* strains were plated on SC-ADE + 0.0045% adenine with or without luecyl-3-*epi*-deoxy-negamycin (TCP-126) for 4 days. The colonies formed on medium containing TCP-126 were white, suggesting that TCP-126 evoked readthrough activity in the 12geneΔ0HSR *ade2-E64X (TGA)* strain. (C) DMSO and G418 were spotted on 0.5% agar containing 12geneΔ0HSR *ade2-E64X* strains overlaid on YPD containing 0.0005% adenine. After 4 days incubation, the halo that formed around the G418 was white.
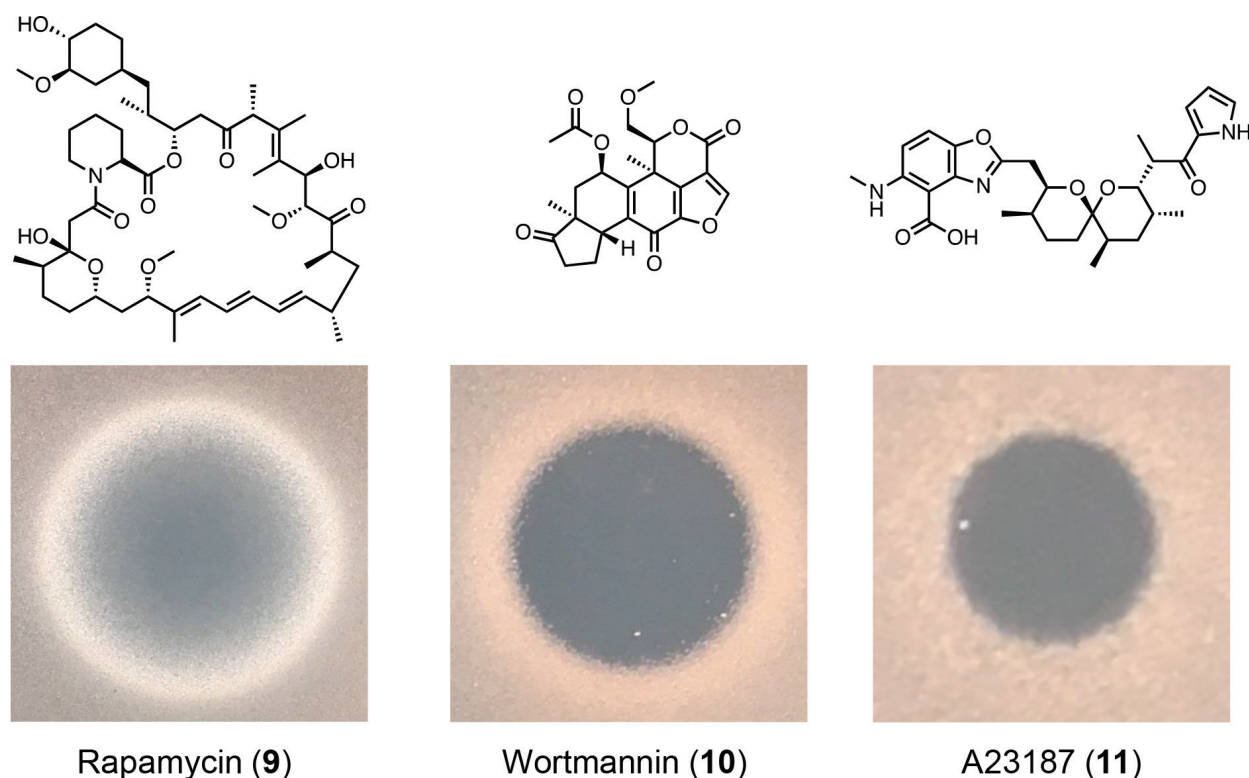
Figure 7. Compounds showing readthrough activities in our screening. Rapamycin (**9**), wortmannin (**10**), and A23187 (**11**) were found as readthrough compounds in our assay system. The structures and haloes of these compounds are shown.
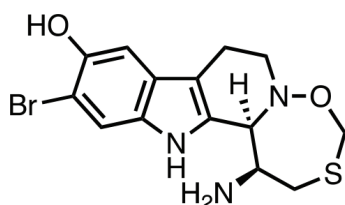

Figure 8. Chemical structure of eudistomin C (EudiC).

resistance [50]. The work flow is shown in **Figure 9**. We used dTC033, one of the multidrug-sensitive yeast strains which lacks 12 genes of the drug-efflux system. The sensitivity of dTC033 against EudiC was 25-fold higher than that of the parental strain BY4741. We isolated the 59 spontaneous mutants that show EudiC resistance. We then crossed these 59 EudiC-resistant strains with OTA014, which has the same genotype as dTC033 (except for the mating type and *RME1(ins-308A)* mutation), and confirmed that 34 of the strains showed dominant resistance. Dominant resistance is predicted to be the mutation in target molecules which inhibits drug-target interaction rather than a lack of cell death signals activated by EudiC treatment (**Figure 9**). These 34 strains were further tested for their EudiC resistance under a higher concentration of EudiC, and 11 strains were selected as strongly resistant mutants. To confirm that the EudiC-resistant mutations of these mutants were not related to multi-drug-resistance mechanisms such as drug efflux pump up-regulation, we checked the sensitivity
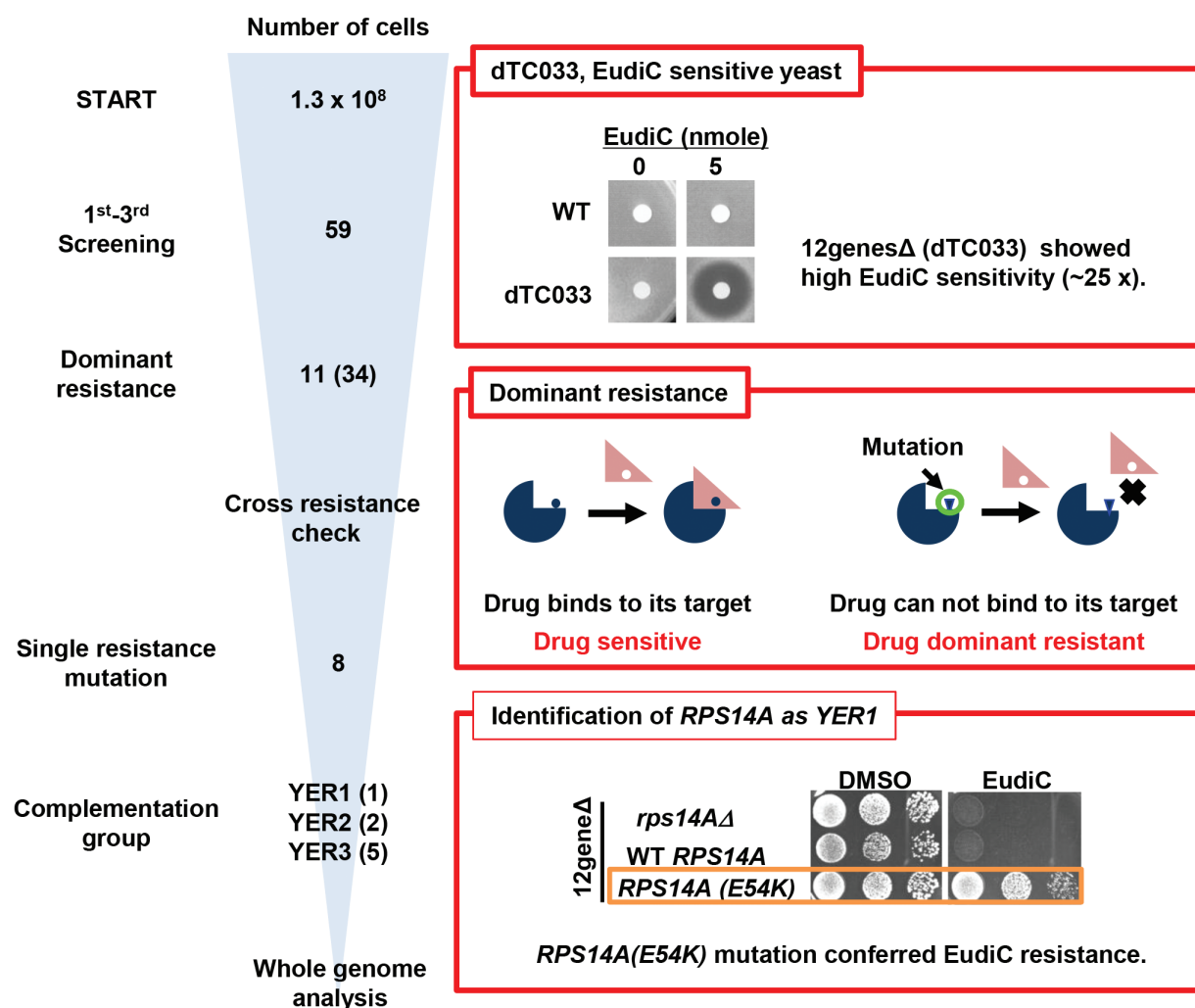
Figure 9. The work flow of the identification of RPS14A as a target of EudiC.

of these mutants against several compounds. These strains did not show cross-resistance against 4-nitroquinoline 1-oxide, digitonin, cycloheximide, or rhodamine 6G, suggesting that these mutants obtained specific resistance against EudiC. We speculate that our strain cannot obtain cross-resistance easily due to its lacking all ABC transporters on the plasma membrane. To select mutants which have a single mutation responsible for EudiC resistance, we performed a tetrad analysis of the spores derived from the diploid of the 11 selected mutants and confirmed that 8 of the strains showed a 2:2 segregation pattern for EudiC resistance. These eight strains were classified into three complementation groups, which we named YER1 (1 strain), YER2 (2 strains), and YER3 (5 strains). "YER" stands for Yeast Eudistomin C Resistance. Whole-genome sequence analysis of the YER strains and further confirmatory analyses, including the disruption of mutated genes in YER strains and the re-introduction of identified mutations into wild-type strains (**Figure 9**), revealed that YER1 is *RPS14A(E54K)*. Unless we checked all of the gene mutations found in the coding region, we failed to identify the mutations in YER2 and YER3, suggesting that the YER2 and YER3 mutations were located on the noncoding region or repetitive sequences—for example, rDNA. *RPS14A* encodes a

component of the 40S ribosome, uS11, which participates not only in protein translation but also in 18S ribosomal RNA (rRNA) maturation (20S to 18S processing) in ribosome biogenesis with Fap7p [77]. To distinguish the effect of EudiC on uS11, we performed biochemical analysis using biotinylated EudiC and purified ribosome complexes. Because biotinylated EudiC failed to pull Fap7p down and no effect on 18S maturation processes was observed, it was confirmed that EudiC targets the matured 40S ribosome and inhibits protein translation but not rRNA maturation [50].

Collectively, our target ID studies of EudiC suggested the mode of action of EudiC cytotoxicity and indicated that our sensitive strains would be quite useful for performing drug target IDs in a relatively short period.

3. Conclusions and perspective

In the field of chemical biology, several model organisms, including yeast, worms, flies, and mice, have been used. Yeast is one of the most-used model organisms due to its ease of handling and its genetic availability, but its drug resistance is sometimes an obstacle to investigation. To overcome this problem, we constructed two multidrug-sensitive yeast strains, 12geneΔ0HSR and 12geneΔ0HSR-iERG6. These strains not only show a broad spectrum of drug sensitivities against compounds for which resistance is shown by both ABC transporters and ergosterol without influencing transformation, mating, or sporulation efficiency, but they are also useful for drug screening. Indeed, we performed a screening of antifungal compounds and protein translation regulators which skip stop codons and found some promising candidates. Using 12geneΔ0HSR-iERG6, we succeeded in improving the hit rate of drug screening from microbial broth. The screening of microbial broth which inhibits the growth of 12geneΔ0HSR-iERG6 but not of the quadruplex mutant identified novel compounds suggested that our multidrug-sensitive strain-based screening using previously tested chemical sources in yeast screening could identify new bioactive compounds. Furthermore, as our screening system for readthrough compounds, genetically modified multidrug-sensitive strains can be applied for several types of screening such as a yeast 2-hybrid system-based protein-protein interaction modulators screening. Recently, a yeast 3-hybrid system has been applied for drug-protein interaction analysis [78]. In this study, the *pdr5 snq2 yor1* triple mutant was used to increase the sensitivity of the system [78]. Our multidrug-sensitive yeast strain was thus shown to be useful for this kind of analysis. Moreover, we expect that the 12geneΔ0HSR and 12geneΔ0HSR-iERG6 strains will also be useful tools for genome-wide chemical biology studies such as synthetic lethal/sick genetic interaction analyses [19, 20], genome-wide overexpression screening [21], and haploinsufficiency-chemical sensitive assays [22]. In addition, the genetic approach using our strains identified the 40S ribosome component uS11 as a target molecule of the cytotoxicity caused by the antiviral compound EudiC. Because it has been reported that protein translation is one of the targets for antiviral agents [79–81], the effect on the 40S ribosome and the inhibition of translation by EudiC may cause both the cytotoxicity and the antiviral activity. In contrast, it has also been reported that the uS11 protein interacts with the eS1 and eS26 proteins, which form part of the mRNA exit tunnel [82], and that the

eS1 protein is one of the contact sites for hepatitis C virus internal ribosome entry sites (IRES) [83, 84]. These reports might suggest that EudiC decreases the interaction between ribosomes and some of the viral IRES, and efficiently inhibits the translation of viral proteins compared to that of host mRNA. Elucidating the detailed inhibitory mechanism of EudiC on protein translation and its effects on IRES-dependent translation might promote the development of EudiC as a novel antiviral medicine.

Recently, it has been reported that RNAseq combined with Crisper/Cas9-based genome-editing technologies is useful for target ID in mammalian cells [25]. Identification of the drug target using our multidrug-sensitive strains and confirmation of the identified mutation in mammalian cells by Crisper/Cas9-based genome editing will reveal the mechanisms of drugs in more detail. Our multidrug-sensitive strains have the potential to facilitate chemical genetic studies and contribute to the development of medicines in the future.

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