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Anti-cancer Drug Discovery: Update and Comparisons in Yeast, *Drosophila*, and Zebrafish

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Abstract

Discovery of novel cancer chemotherapeutics focuses on screening and identifying compounds that can target ‘cancer-specific’ biological processes while causing minimal toxicity to non-tumor cells. Alternatively, model organisms with highly conserved cancer-related cellular processes relative to human cells may offer new opportunities for anticancer drug discovery when combined with chemical screening. Some organisms used for chemotherapeutic discovery include yeast, *Drosophila*, and zebrafish which are similar in important ways relevant to cancer study but offer distinct advantages as well. Here, we describe these model attributes and the rationale for using them in cancer drug screening research.

Keywords

Model organism; yeast; *Drosophila*; zebrafish; anti-cancer drug discovery; HTS

INTRODUCTION

In the past decades, anti-cancer drug discovery has focused on major mutations in cancer cells that are genetically distinct from normal cells [1] such as gene point mutations, amplification, deletions and translocation, as well as some epigenetic changes [2]. Based on the known pathological underpinnings of carcinogenesis, scientists have searched for drugs that could target ‘cancer-specific’ biological processes to eliminate cancer cells, while offering minimal toxicity to normal cells. Among various robust technologies and methods for anti-cancer drug discovery, high throughput screening (HTS) is extremely powerful and consists of random screens of small molecule compounds to find “hits” that have activity in a representative disease model [3]. Model organisms are useful tools when they bear highly

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CONFLICT OF INTEREST

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conserved cancer-related cellular processes relative to human cells and use of these organisms for cancer drug discovery may hold promise [4] as data from these models are extrapolated to human cancers.

Combining model organism studies with chemical screening offers the ability to study small molecules in chemical libraries that induce a phenotype in the model organism that is engineered to reflect specific genetic or epigenetic changes in human cancers. Data from these studies can identify novel drug candidates that are bioactive in a model system or obtain essential structural information for further drug design. Popular organism models include yeast, *Drosophila*, and zebrafish which offer genetic amenability, low cost, and culture conditions compatible with large-scale screening [5, 6]. Fully mapped genomes and detailed phenotypic reports for mutants of these organisms provide a foundation for of large-scale screening protocols, rapid identification of molecular targets of screened compounds, follow-up, establishment of loss-of-function and gain-of function HTS. In the past decade, many tools have been developed to facilitate manipulation of gene expression. In zebrafish, for instance, nuclear transfer techniques for generating transgenic lines [7, 8], targeted mutations [9], and cloning [10] have been developed. Full length zebrafish cDNAs and DNA microarrays for expression profiling experiments are also available [11, 12]. These tools have made it possible for gene function studies to be performed rapidly in zebrafish. Such models also allow testing of context-specific anti-cancer therapeutics and offer unique applications for screen design that can be exploited for various research intentions (Table 1 depicts pros and cons of each model).

ANTI-CANCER DRUG SCREENING IN YEAST: A CELL-BASED STRATEGY

Yeast (*Saccharomyces cerevisiae*), is a proven model eukaryote widely used for investigating various molecular and cellular biological functions. Yeast can serve as a unique model for the study of fundamental cellular processes such as cell cycle, DNA replication, recombination, cell division, and metabolism [13]. Yeast is also genetically well defined: its entire genome sequence has been elucidated and the corresponding databases are generally accessible [14]. Comparison of yeast and human genomes indicates 30% of known genes involved in human disease have a yeast counterpart [15]. Specifically, most cancer-related pathways (such as DNA-damage checkpoint, replication checkpoint, and response to mitogenic stimuli) and corresponding genes are conserved between yeast and humans, yet yeast lack critical functions important to human cancers such as lack of p53 in cell cycle checkpoints. Without p53, yeast can only make binary decisions between arrest and cycling in response to DNA damage, whereas human cells can undergo other fates such as apoptosis [16]. Overall, as a simplified model to investigate cancer, yeast is an attractive system that can be exploited to screen novel anti-cancer compounds and indeed yeast-based screens have yielded lead compounds for therapeutic application against different types of human cancers [17].

As a unicellular eukaryote, yeast bridges cell- and organism- based assays. Screens with yeast cells are easily adapted for growth in microtiter plates and in HTS formats. Moreover, its 90-minute doubling time allows for short experiments examining drug exposure over many population doublings [17]. In addition, the versatile genetic malleability of this

organism, as well as abundant literature for yeast genetics, has contributed to the development of diverse experimental tools and unique genetic selection systems (including advanced plasmid systems, homologous recombination techniques, and readily scored markers) that can now be readily applied to drug screening [5]. With these features, screenings based on most cancer-relevant fundamental biological processes, such as cell-cycle progression, DNA replication, and segregation, can be performed in yeast systems. Rationales for yeast screens for anti-cancer drug discovery and identification of molecular targets/pathways are summarized in Table 2. These studies support the idea that all yeast screens exclusively utilize cell growth conditions as the read-out, chiefly because natural reflectivity of yeast growth conditions can directly lend itself to automation for high throughput assessment of compounds.

Generally, yeast-based screen for anti-cancer agent discovery can be grouped into two categories. In the first type, a human protein (potential target) is over expressed in yeast cells, and this can lead to a specific phenotype (such as growth defect) that can be suppressed by chemical-induced inhibition of this protein. These compounds can be further validated in mammalian cells that express the same target protein or by *in vitro* biochemical assay. A notable and unique advantage of this approach is that such screens provide a very direct determination of compounds with known targets, which is the primary challenge for all phenotypebased chemical genetic screens. Screening for inhibitors of poly (ADP-ribose) polymerases 1 (PARP1) is an elegant example of use of yeast cells for this purpose [18]. Because pharmacological inhibition of PARP1 contributes to improved efficacy of DNA-damaging agents [19], such inhibitors may serve as potential anti-cancer drugs. When the human gene encoding PARP1 is expressed in budding yeast possessing no PARP activity, yeast growth defects are observed. This phenotype has been used successfully to validate the action of a known inhibitor of PARP1. By performing a cell-based screen in this system, novel inhibitors of this protein were then identified. Finally, the *in vitro* biochemical assay shows that these inhibitors directly inhibit PARP1 activity [18]. Recently, another yeast cell-based screen has been designed to identify inhibitors of telomere-associated protein tankyrase 1 (a member of PARP family) for cancer therapy, especially against BRCA (breast cancer associated)-associated cancers. Similarly, this assay is based on the reversal of growth inhibition caused by the heterologous expression of tankyrase 1. HTS in this system finally yielded one hit—that is, a flavone has been identified as a compound that can restore growth of yeast cells overexpressing tankyrase 1. Subsequent assays indicated that flavone inhibits tankyrase 1 not only in yeast but also in mammalian cells [20].

The second type yeast screen to discover anti-cancer compounds is mainly based on a specific molecular context in yeast cells. These screens (e.g. signaling pathway-based screens and chemical synthetic lethal screens) take advantage of conserved biological processes between yeast and humans. TOR (target of rapamycin) proteins are phylogenetically conserved from yeast to humans [21], and are pivotal regulators of cell growth in response to nutrient signals [22]. The TOR-dependent network controls many aspects of metabolism, and its deregulation may lead to corresponding diseased states. With prevalent hyperactivation of the mammalian TOR (mTOR) pathway in human cancers, agents to enhance TOR pathway inhibition can be potentially used as anti-cancer drugs [23].

Jing and colleagues recently used a yeast-based screen to identify new small-molecule enhancers of rapamycin (SMERs) targeting cell growth control. With a yeast-based screen, more than 400 compounds were identified in the presence of a suboptimal rapamycin concentration to result in a 'no growth' phenotype, whereas subsequent unrelated screens identified 86 potential SMERs considered to be synthetic lethal with rapamycin but with little toxicity alone at concentrations used. Consequently, five structurally distinct molecules with differing effects on growth were selected for further analysis by genomic, genetic and biochemical assays, and data show that one of the SMERs (SMER3) inhibits Skp1-Cullin-F-box (SCF; Met30, a member of the SCF E3-ligase family) [24].

Although yeast-based chemical screening has been successful for identifying potential drugs for cancer therapy, limits of this unicellular organism prevent it from modeling all aspects of cancer (which is inherently a disease of metazoans; see Table 1). In this case, an alternative system screening for anti-cancer compounds maybe *Drosophila* which is evolutionarily closer to humans.

DROSOPHILA AS A CANCER MODEL FOR DRUG DISCOVERY

Drosophila has been typically used for genetics, molecular and cellular biology studies [25, 26]. The entire *Drosophila* genome has been sequenced and annotated, and more than 60% of human genes have functional orthologs to *Drosophila* [27]. Moreover, more than 75% of the human disease genes in the Online Mendelian Inheritance in Man database have strong protein sequence conservation with *Drosophila* genes [28]. Accordingly, this model metazoan is gaining attention for cancer studies. However, some cancer-relevant mechanisms that are different in *Drosophila* and human remain. Telomere protection, for instance, is a critical distinction. Differing from telomere maintenance in human, *Drosophila* telomeres are maintained by transposition of specialized retrotransposons rather than by telomerase activity, and their stability is independent of the sequence of DNA termini [29]. *Drosophila* biologists with an interest in translational medicine are trying to exploit this model to screen chemical compounds for novel cancer therapeutics, and some success has been had [30]. Table 2 depicts features of typical *Drosophila* screens designed thus far to identify anti-cancer therapeutics and molecular targets/pathways.

One use of *Drosophila* for anti-cancer screening is a cell- and luciferase-based drug model. This strategy is carried out in *Drosophila* cell lines to find modifiers targeting a conserved cancer-related pathway. Individual compounds from a small-molecule library are normally assessed using either transcription factor-dependent luciferase reporter activity or oncogenic gene promoter-driven luciferase reporter activity. Thus, such screens can be employed to identify drug candidates that affect specific transcriptional activity and gene expression. This way, transcription factor-dependent luciferase reporter activity serves as the read-out, and *Drosophila* screens can be designed to search for compounds targeting a particular transcription module in a signaling pathway. Then, identified compounds could be identified that perturb fewer junction pathways or functions. An example of this type of identification is the recent search for inhibitors that target misregulated β -catenin responsive transcription (CRT) in *Drosophila* cells. β -catenin plays distinct roles at the plasma membrane and in the nucleus (functioning as transcriptional coactivator), and so would be ideal for identifying

compounds that target CRT in the nucleus without disturbing functions of β -catenin at the plasma membrane. With this in mind, a highly targeted suppressor screen (which used Wnt responsive luciferase reporter-dTF12 activity as the read-out) was devised to identify leads that specifically modulate the CRT. Eventually, three hits were generated, and as expected they were verified to be CRT inhibitors that can directly inhibit interactions between β -catenin and TCF4 [31]. These compounds can therefore be further developed for therapeutic intervention of cancers with Wnt-associations [31]. Compared to yeast-cell-based screening, *Drosophila* cell-based screening benefits from the following aspects. Unlike yeast cells, they do not have a cell wall, which might cause false negatives due to permeability of some compounds, and *Drosophila* cell plasma membrane composition is more similar to mammalian composition than yeast cell composition is; protein divergence between yeast and vertebrates is more pronounced such that screened hits from yeast might not recognize orthologous targets in mammals.

In addition to *Drosophila* cell-based anti-cancer screens, whole organism-based screening is emerging as a promising approach. In principle, *Drosophila* screens are carried out by first engineering the organism (introducing a disease form of a human gene or a *Drosophila* gene mutant into the organism), and then screening for chemicals that can modify phenotypes is performed. This strategy, compared to yeast screens and cell-based *Drosophila* screens, offers a powerful tool for identifying anti-cancer compounds that are bioactive in an intact organism. This permits assessment of drug absorption, distribution, metabolic stability, and toxicity, suggesting that the *Drosophila* whole organism model is suitable for identifying drug candidates and reducing false positives. The *Drosophila* whole organism model has been used to screen small molecules that curtail oncogenic RAS-driven tumor over-growth. The GFP transgene was used to label RAS^{V12}-expressing cells so that tumor development within larvae could be monitored *in situ* by microscopically viewing GFP fluorescence. The anti-tumor chemical efficacy in this screen was represented quantitatively by GFP signal intensity. To test the suitability of this *Drosophila* tumor model for chemical intervention, five known inhibitors of mitogen-activated protein kinase (MEK) were used. And these five MEK inhibitors could down regulate ERK phosphorylation (a downstream target of MEK) in *Drosophila* cells. However, only one MEK inhibitor was capable of reducing GFP-positive tumor burden due to the better bioavailability, whereas its counterpart with poor solubility, lower cell permeability, and less biostability, was not effective [32]. These data support the idea that *Drosophila* screening in the whole animal context is useful for identification of compounds with specific pharmacokinetic profiles.

Aside from acquiring information on pharmacokinetics from whole organism-based screening, whole-body phenotypes in *Drosophila* could afford significant insight about drug candidates. An emerging *Drosophila* whole-animal screen for therapeutics against multiple endocrine neoplasia type 2 (MEN2) revealed that subtle changes of unique phenotypic traits could contribute to understanding polypharma cological profiles of anti-tumor agents, thereby facilitating drug optimization [33]. Briefly, the *Drosophila* tumor model was established by targeting oncogenic dRet^{MEN2B} to multiple developing epithelial tissues, and the primary read-out for the screen was the survival of pupae and adults (these developmental stages were blocked by expression of oncogenic dRet^{MEN2B}). With the

screen, AD57 was identified to be a potent anti-MEN2B compound. Interestingly, not only could AD57 rescue the developmental blockade but also it could suppress ectopic wing veins induced by dRet^{MEN2B} expression. Given that RAS/ERK pathway activity is positively associated with ectopic veins in adult wings [34, 35], it is possible that AD57 could affect RAS pathway activity, and suppressing RAS pathway activity would improve the overall efficacy of AD57. Similarly, another compound (AD58) in the screen was shown to be highly toxic and suppressed *Drosophila* development compared to tumor controls. Intriguingly, AD58 treatment consistently enhanced the phenotype of ectopic wing veins, whereas this enhancement was suppressed by the knockout of a functional copy of *erk*. These data indicated that AD58 toxicity might be due to elevated RAS pathway activity. Considering that AD58 is a known mTOR inhibitor and mTOR can provide feedback inhibition of the RAS pathway [33, 36, 37], further experiments were conducted to assess whether AD58 toxicity was partially due to inhibition of the *Drosophila* target of rapamycin (dTOR). Data suggest that its toxicity is indeed mediated by dTOR inhibition. Building upon such information on polypharmacological profiles of tested agents, two anti-MEN2B compounds with improved profiles (greater efficacy/lower toxicity), were eventually designed [33].

Although *Drosophila* screening systems are excellent for anti-cancer drug discovery, the model also has limitations (Table 1). Limiting factors for *Drosophila* HTS include their relatively large size, uptake issues through the cuticle, and the need to administer chemicals by feeding, which poses problems for standardization. In addition, *Drosophila* is anatomically and physiologically different from humans. Accordingly, *Drosophila* can produce only a partial picture of human cancer. Thus, zebrafish may present an alternative means for studying these aspects.

THE DEVELOPING ZEBRAFISH: A VERTEBRATE MODEL FOR ANTI-CANCER SCREENS

During the past decades, zebrafish have been almost exclusively employed to study organ and embryonic development [38, 39]. With a large zebrafish genome database available at the Zebrafish Information Network (ZFIN) and at the FishMap website [40, 41], important details are now available to the research community. Additionally, optical clarity of zebrafish embryos and larvae allows visualization of expression of fluorescent protein-tagged transgene and *in vivo* observation of morphological defects. Zebrafish have multiple features that encourage their use in cancer biology and drug screening [42] (Table 1).

Thus far, most successful zebrafish screenings have been performed with embryos and larvae. Two major approaches are used for cancer models: (1), xenografts of cancer cells from other organisms or allografts from zebrafish donors with cancer; and (2), construction of zebrafish cancer mutant lines or transgenic oncogene lines [43]. Akin to yeast and *Drosophila*, zebrafish as a vertebrate system can be used to screen for anti-cancer compounds based on fundamental biological processes and an intact developing organism contributes to fewer false-positive results. For example, zebrafish embryo genetic screens for compounds have permitted the identification of novel cell-cycle inhibitors [44]. In another study by Le *et al.*, the authors performed a chemical genetic screen of a *KRAS*^{G12D}.

induced zebrafish embryonal rhabdomyosarcoma and found two inhibitors to have antitumor activity. They verified the importance of activated RAS pathway in both embryogenesis and oncogenesis [45]. Transgenic zebrafish embryos (*mitfa:BRAF^{V600E};p53^{null}*) were used in a chemical genetic screen to identify small-molecule suppressors of the neural crest lineage, and the group of compounds that showed the best effects was assessed further for effects on melanoma [46]. One member of this class of compounds, leflunomide, appeared to be a potent inhibitor for transcription of elongation of genes that are required for neural crest development and melanoma growth.

More importantly, zebrafish have recently acquired value as models for mimicking tissue-specific tumors in humans, particularly hematopoietic malignancies and several lead compounds have been found, including those which target human acute myelogenous leukemia (AML), therapy-refractory B-cell acute lymphoblastic leukemia (B-ALL), chronic myelogenous leukemia (CML), and T-cell ALL (T-ALL) [47–48]. Zebrafish anti-cancer screens and molecular targets/pathways of identified compounds are summarized in Table 2.

A boutique zebrafish *in vivo* screen has been used to identify compounds to treat human AML. Instead of concentrating on leukemia cell death, compounds that could reverse leukemic oncogene AML1-ETO (AE) were studied with regard to leukemic stem cell differentiation. The rationale for this approach was that leukemic stem cells in most AML patients and posterior blood island of the embryonic zebrafish have similar AE-induced cell fates and aberrant differentiation [49–51]. The screen was devised in a transgenic zebrafish line Tg (*hsp: AML1-ETO*) which expresses AE under the control of the *hsp70* heat shock promoter as the platform and the reversal of *gata1* downregulation (which marks the changes in hematopoietic cell fate) was the read-out. In particular, embryos were processed for *in situ* hybridization of *gata1*, which guarantees that *gata1* staining was amenable to a high-throughput format. As a result, embryos exposed to 22 compounds showed strong *gata1* staining, and 15 hits were confirmed after re-testing. Importantly, the mechanism of antagonism of nimesulide (a compound identified) on AE's effects unveiled unexpected roles of COX-2- and β -catenin-dependent pathways in AE-mediated hematopoietic differentiation, implicating inhibitors of these pathways which may provide therapeutic benefits for treating human AML [47].

Apart from the discovery of compounds for hematopoietic malignancies, zebrafish embryos can be applied in anti-angiogenesis screens for cancer therapy. Physiologic angiogenesis is key to cell development, reproduction and repair, whereas pathologic angiogenesis occurs in tumor formation and metastasis [52]. Thus, compounds with anti-angiogenic activity may offer anti-cancer properties. In a study by Tran *et al.*, the LOPAC1280 compound library (Sigma) was screened on fluorescent-labeled 1-dpf Tg(VEGFR2:GRCFP) zebrafish embryos in 384 well plates to assay for antiangiogenic compound. In addition to two compounds (SU4312 and AG1478) with known antiangiogenic activity, the authors also observed a previously unknown compound, indirubin-3 β -monoxime (IRO) which showed strong antiangiogenic activity in the zebrafish angiogenesis assay. IRO also scored positive in a HU-VEC tube formation and proliferation assay supporting, indicating that it is an antiangiogenic compound [53]. In another study by Xiao-Yan and colleagues, the 20-hpf transgenic zebrafish line Tg (*kdrl: EGFP*) was plated in 96 well plates for anti-angiogenic

drug screens to identify compounds targeting cancer angiogenesis and seven compounds were identified that inhibit growth of zebrafish intersegmental vessels. Subsequent mechanistic studies indicated that rosuvastatin (an identified compound) could inhibit angiogenesis by targeting endothelial cell function *in vitro*. Furthermore, this compound can significantly suppress prostate cancer (PCa) growth in a mouse xenograft tumor model offering preclinical evidence for the therapeutic potential of rosuvastatin to treat human PCa [54].

CONCLUSION

Model-organism-based chemical screens are powerful approaches for anti-cancer drug discovery. From the evolutionary standpoint, Yeast, *Drosophila*, and zebrafish are three representatives of drug screening systems with unique characteristics. Due to their small size, short life cycle, and simple growth conditions, these organism models are low-cost and have been used extensively in large-scale screening. Most screens have been carried out in *Drosophila* and zebrafish but few drugs have been identified. Thus, more screen types are needed for us with these organisms to target different cancer-related biological processes. A recent established zebrafish model that overexpressed Hedgehog in the skin, for example, could be a drug screening platform in the future for instance [55]. Furthermore, with the establishment of novel cancer models, especially in *Drosophila* and zebrafish, chemotherapeutic screens in these two system scan be expected to emerge.

Drugs directed against multiple targets would be useful for overcoming cancer drug resistance [56] and synergistic drug combinations using ‘combination chemical genetics’ may offer these attributes [57]. In fact, a dedicated cancer drug screening platform has identified multiple synergistic agents as potential combination therapeutics [58] and these await studies in model organisms. Discovery of single anti-cancer agents with multiple targets is another alternative and this idea has been successfully implemented in *Drosophila* [33]. Thus, development and use of model organisms offers expanded possibilities for identification of novel small bioactive molecules that could be anti-cancer therapeutics.

Resources for studying these model systems have been developed extensively and exhaustive database information has been compiled for each system relative to its genetics and molecular biology. This information is available online. The *Saccharomyces* Genome Database (<http://www.wormbase.org> SGD, www.yeastgenome.org) contains comprehensive annotated data on the yeast genome. The FlyBase database (flybase.org) contains sequenced genomes for 12 *Drosophila* species. The zebrafish genome is also sequenced and freely available at the Fishmap website (Meli *et al.*, 2008). Thousands of *Drosophila* strains can be obtained from the Bloomington Stock Center (Matthews *et al.*, 2005), and the Zebrafish International Resource Center maintain numerous zebrafish strains that can be obtained upon request (Henkenet *et al.*, 2004).

In conclusion, these organism models are valuable complementary alternatives to cellular and *in vitro* screening systems. Their genetic amenability, low cost, and culture conditions compatible with large-scale screening make them useful models for drug discovery. *Drosophila* and zebrafish models allow high-throughput screening in a whole-animal

context. This might help the selection of compounds with an improved safety profile at an early stage of drug discovery.

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Table 1

Comparisons of three model organisms for anti-cancer drug discovery

	Yeast	<i>Drosophila</i>	Zebrafish
Pros	<ul style="list-style-type: none"> Established wealth of experimental tools and unique genetic selection systems Short population doubling time Easy adaptation to HTS formats Suitable for screening compounds targeting most cancer-relevant fundamental biological processes Yielding compounds with known targets (first type screening) 	<ul style="list-style-type: none"> Relatively simple genetic manipulation Relatively easy adaptation to HTS formats Suitable for identifying compounds that affect transcriptional activity (cell-based screening) Multi-cellular context contributes to identifying drug candidates within the proper physiological and pathophysiological context (whole organism-based screening) 	<ul style="list-style-type: none"> Less expensive and not limited to use in as small a number of experiments as large vertebrate animals including rodents Relatively easy adaptation to HTS formats The developing organism contributes to identifying drug candidates within the proper physiological and pathophysiological context Providing vertebrate models to mimic some cancer-related tissue-specific disorders, particularly hematopoietic malignancies, in human for drug screening Embryos and larvae are transparent, which allows <i>in vivo</i> observation of morphological changes Various phenotypic traits can potentially provide additional information on effects of drug candidates
Cons	<ul style="list-style-type: none"> Unicellular system to model human cancer Compounds might not recognize the orthologous target. Protein divergence between yeast and vertebrates is high. 	<ul style="list-style-type: none"> The anatomy and physiology are significantly different from those of humans and can only produce a partial picture of human symptoms Cannot be grown in liquid medium which limits its use in HTS 	<ul style="list-style-type: none"> The anatomy and physiology are different from those of mammalian models It is not clear exactly how predictive zebrafish toxicity will be of human toxicity

Table 2

Summary of typical anti-cancer screens in three model organisms.

	Designed context for the screen	Read-out	Molecular targets/pathways	Ref.
<i>Yeast</i>	Yeast deficient in double strand break repair	Selective inhibition of cell growth	DNA-mediated processes	[59]
	Heterologous expression of either human PARP1 or PARP2	Rescue of growth defects caused by the heterologous expression of either PARP1/ PARP2	PARP1/2	[18]
	Heterologous expression of human tankyrase 1	Rescue of growth defects caused by the heterologous expression of tankyrase 1	Tankyrase 1	[20]
	Heterologous expression of mammalian cAMP phosphodiesterase (PDE) 2A, PDE4A, PDE4B, PDE8A	Enhanced growth in medium lacking uracil (activators); enhanced growth in 5FOA medium (inhibitors)	cAMP PDEs	[60]
	Yeast cells with rapamycin-sensitive functions of TOR	Visual inspection of cell growth (primary screen)	Skp1-Cullin-F-box (SCF) ^{Met30} ubiquitin ligase	[24]
	Expression of a temperature-sensitive topoisomerase	Growth inhibition measured by colony survival	DNA topoisomerases	[61]
	Yeast strain with a reporter gene (URA3) inserted near a telomere, where it is transcriptionally silenced by a Sir2-dependent pathways	Rescue of cell growth defects by inhibiting the activity of pathways composed of Sir2	Sir2-related pathways	[62–63]
	Yeast cells with methylthioadenosine phosphorylase (MTAP)-deficiency	Growth inhibition of MATP-deficient cell	Pathways synthetic lethal with MTAP inactivation	[64]
<i>Drosophila</i>	Heterologous expression of human APC	Rescue of eye defects caused by overexpression of human APC	Unknown	[65]
	Knockdown of Axin by RNAi, and expression of Wnt responsive luciferase reporter-dTF12	Inhibition of the activity of Wnt responsive luciferase reporter-dTF12	β-catenin-TCF4 complex	[31]
	Expression of STAT92E luciferase reporter gene	Inhibition of the activity of the JAK/STAT luciferase reporter	JAK/STAT signaling pathway	[66]
	Ectopic expression of activated human Ras (<i>Ras</i> ^{V12}) oncogene	Suppression of GFP signal in GFP-labelled tumor tissue per larva or pupa	CTP synthase (a possible crucial target)	[32]
	<i>Drosophila</i> larvae that are irradiated with a dose of X-rays to produce partial lethality	Survival of larvae (the fraction of animals that successfully emerged as viable adult flies)	Microtubule polymerization, DNA metabolism, protein synthesis	[67]
	Expression of oncogenic dRet ^{MEN2B} in multiple developing epithelial tissues	Suppression of dRet ^{MEN2B} - induced developmental block (i.e. survival of pupae and adults)	Activity of a panel of kinases, including Ret, Raf, Src, Tor, and S6K	[33]
<i>Zebrafish</i>	Wild-type zebrafish embryo	Developmental abnormalities, i.e. reduced	P53-dependent apoptotic pathways	[68]

	Designed context for the screen	Read-out	Molecular targets/pathways	Ref.
		pigment development, developmental delay, severely disrupted development, and death		
	Zebrafish embryo with the expression of oncogenic AML1-ETO (AE)	Strong <i>gata1</i> staining due to the reverse of the downregulated <i>gata1</i> expression	COX-2 and β -catenin-dependent signaling pathways	[47]
	Zebrafish larvae carrying the T-cell specific (lck:EGFP) transgene (whose expression fluorescently labels all T cells)	Visual inspection of green fluorescence	Mitosis and PI3 kinase/AKT/mTOR pathway	[48]
	Wild-type zebrafish embryo	Alteration of serine-10-phosphorylated histone H3 (pH3) level detected by antibody staining	Cell cycle control	[44]
	Zebrafish embryo carrying the endothelial-specific (flk1:EGFP) transgene (whose expression fluorescently labels endothelial cells)	Visual inspection of green fluorescence	Cell cycle control and apoptotic pathways	[54]