New tools for old drugs: functional genetic screens to optimize current chemotherapy

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Abstract

Despite substantial advances in the treatment of various cancers, many patients still receive anti-cancer therapies that hardly eradicate tumor cells but inflict considerable side effects. To provide the best treatment regimen for an individual patient, a major goal in molecular oncology is to identify predictive markers for a personalized therapeutic strategy. Regarding novel targeted anti-cancer therapies, there are usually good markers available. Unfortunately, however, targeted therapies alone often result in rather short remissions and little cytotoxic effect on the cancer cells. Therefore, classical chemotherapy with frequent long remissions, cures, and a clear effect on cancer cell eradication remains a corner stone in current anti-cancer therapy. Reliable biomarkers which predict the response of tumors to classical chemotherapy are rare, in contrast to the situation for targeted therapy. For the bulk of cytotoxic therapeutic agents, including DNA-damaging drugs, drugs targeting microtubules or antimetabolites, there are still no reliable biomarkers used in the clinic to predict tumor response. To make progress in this direction, meticulous studies of classical chemotherapeutic drug action and resistance mechanisms are required. For this purpose, novel functional screening technologies have emerged as successful technologies to study chemotherapeutic drug response in a variety of models. They allow a systematic analysis of genetic contributions to a drug-responsive or – sensitive phenotype and facilitate a better understanding of the mode of action of these drugs. These functional genomic approaches are not only useful for the development of novel targeted anti-cancer drugs but may also guide the use of classical chemotherapeutic drugs by deciphering novel mechanisms influencing a tumor's drug response. Moreover, due to the advances of 3D organoid cultures from patient tumors and *in vivo* screens in mice, these genetic screens can be applied using conditions that are more representative of the clinical setting. Patient-derived 3D organoid lines furthermore allow the characterization of the "essentialome", the specific set of genes required for survival of these cells, of an individual tumor, which could be monitored over the course of treatment and help understanding how drug resistance evolves in clinical tumors. Thus, we expect that these functional screens will enable the discovery of novel cancer-specific vulnerabilities, and through clinical validation, move the field of predictive biomarkers forward. This review focuses on novel advanced techniques to decipher the interplay between genetic alterations and drug response.

1. Introduction

Anti-cancer drug resistance is the major cause of death of cancer patients with disseminated tumors (Borst, 2012). In some patients intrinsic (or primary) drug resistance is already observed from the start (i.e. prior to chemotherapy) and tumors grow in the presence of chemotherapy (Holohan et al., 2013). Such intrinsic drug resistance can be a cancer-type specific or caused by individual cancer features (Gottesman, 2002). Frequently however, resistance arises in two steps. The tumor initially responds, but not all tumor cells are eradicated. From the residual disease the tumor regrows and eventually becomes resistant to all available chemotherapeutic drugs (Borst, 2012). We have recently reviewed various mechanisms that may cause minimal residual disease (Blatter and Rottenberg, 2015). Although residual disease may already contain selected drug-refractory tumor cells, it is also possible that the residual tumors are only transiently resistant due to cell cycle characteristics (Pajic et al., 2017). Then, drug resistance is acquired during the course of treatment (Housman et al., 2014). This secondary resistance is often due to (epi-)genetic alterations arising during the treatment and lead to, for instance, the activation of alternative signaling pathways, increased drug efflux, altered drug target availability, or rewiring of the DNA damage response (Holohan et al., 2013, Borst, 2012, Bouwman and Jonkers, 2012). To attenuate the development of drug resistance, combinational therapies of several drugs with different molecular mechanisms are frequently given to cancer patients (Al-Lazikani et al., 2012). Another approach is to re-sensitize resistant tumor cells by drugs targeting the resistance mechanism or the tumor microenvironment (De Henau et al., 2016, Callaghan et al., 2014). Unfortunately, we often lack knowledge about the mechanisms underlying resistance and therefore we usually lack a personalized strategy how to treat patients with (relapsing) tumors.

In the past decades, progress in the treatment of disseminated cancers has reduced cancer-related mortality (Kort et al., 2009). In addition to classical chemotherapy, also targeted anti-cancer drugs further improved cancer remission (Motzer et al., 2015, Zhou et al., 2011). Despite these advances, treatment failure due to drug resistance remains a substantial challenge in the clinical management of cancer. Treating a non-responsive tumor causes side effects without providing a benefit for the patient. Moreover, it incurs unnecessary costs and may even decrease the likelihood of success of subsequent treatments with other regimens (Siddiqui and Rajkumar, 2012).

To improve cancer therapy outcome, precision oncology is a promising strategy. Through the assessment of a tumor's specific genetic or proteomic changes, *i.e.* its biomarkers (Mehta et al., 2010), an individualized best treatment regimen can be chosen. Prognostic gene expression signatures are clinically well established, because prognosis of tumor recurrence directly depends on the altered expression of a number of genes involved in tumor progression and metastasis (Reyal et al., 2008, Wirapati et al., 2008, Cardoso et al., 2016). Conversely, a tumor's response to a particular treatment can fail due to the alteration of a single gene, such as the drug target or drug entry transporter (Borst and Wessels, 2010). Such alterations may not reliably be picked up by standard gene expression profiling. Thus, it is not surprising that only few predictive biomarkers are established, and even those remain imperfect in predicting therapy success. Currently, biomarkers are only available for targeted therapies, which block or stimulate specific pathways of tumor cells

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(Twomey et al., 2017) and usually yield good initial response with a modest effect on overall survival (Fojo and Parkinson, 2010). In contrast, classical cytotoxic chemotherapy interferes with all rapidly dividing cells, does not rely on oncogenic protein or pathway alterations, but often results in long-term remission and even cures some cancer types, and reduces cancer-related mortality. Unfortunately, not all patients benefit from the treatment and many eventually become resistant to all drugs available. Hence, there is a lack of clinically validated predictive biomarkers for classical chemotherapy.

Regarding targeted therapy, an early example of a predictive biomarker is HER2 expression status for trastuzumab treatment in metastatic breast cancer, an anti-cancer drug approved by the FDA in 1998. In combination with classical chemotherapy, trastuzumab efficiently decreases disease progression in HER2-amplified metastatic breast cancer (Cobleigh et al., 1999). In contrast, trastuzumab provides no benefit in breast cancer patients lacking HER2. Unfortunately, only about 30% of all HER2-positive breast cancer patients respond to trastuzumab-containing chemotherapy (De Palma and Hanahan, 2012). Thus, there are additional factors that influence therapy response, such as the intertumoral heterogeneity among a cancer (sub-) type, reflecting variations in molecular profiles of cancers in different patients. Additionally, the intratumoral heterogeneity complicates predictions of drug response (Ng et al., 2014). Molecular and genetic profiling of tumors has become cheaper and is often readily available. For mutations in specific genes, for instance *BRAF*, the effect on therapy response has been well characterized, so that sequencing of the corresponding genomic region will directly yield a predictive marker for therapy response. Unfortunately, the number of such well-defined biomarkers is limited, and to date only a small fraction of cancer patients directly benefit from established biomarkers. This is aggravated by the fact that not all patients bearing *BRAF* mutations do respond equally well to targeted BRAF inhibitors (Corcoran et al., 2015, Long et al., 2014, Prahallad et al., 2012). Thus, even such well-defined biomarkers are not sufficient, and additional characterization of the tumor is needed.

Several approaches have successfully identified novel molecular peculiarities which serve as predictive biomarkers. Hypothesis-driven approaches have, for instance, resulted in the establishment of *BRCA1/2* mutational status in predicting a positive response upon PARP inhibitor treatment in breast and ovarian cancer (Farmer et al., 2005, Tutt et al., 2010, Bryant et al., 2005). Analyses of large, population-based clinical trials have also identified subgroups of responsive patients (Uryniak et al., 2011), *e.g.* leukemia patients with the Philadelphia chromosome responded better to imatinib treatment (Druker et al., 2001). Predictive markers based on clinical data have also been suggested for classical chemotherapeutics, including high HER2 or low tau expression as markers for paclitaxel sensitivity (Pusztai, 2007). Besides *BRCA1/2* status, these markers have not entered the clinic, however, and still require additional validating clinical studies (Schork, 2015).

In recent years, advances in experimental genetic screening techniques have linked many genotypes to novel phenotypes in mammalian cells (Chen et al., 2015, Brockmann et al., 2017, Zhou et al., 2014b, Blomen et al., 2015, Wang et al., 2015b, Hart et al., 2015). Furthermore, genome-wide screens have broadened our understanding of molecular mechanisms responsible for therapy

response ((Ruiz et al., 2016, Berns et al., 2016, Planells-Cases et al., 2015, Wijdeven et al., 2015), for instance; and Table 1. Thus, these screens are valuable tools which can reveal novel mechanisms of resistance or hypersensitivity towards drugs, and facilitate a better understanding of drug response which might ultimately result in novel predictive biomarkers (Figure 1). While most targeted anti-cancer therapeutics exploit gain-of-function alterations, *e.g.* in terms of oncogene addiction (Pagliarini et al., 2015), not all tumors bear targetable gain-of-function mutations. Inactivation of tumor suppressor genes is frequent, and cannot be directly targeted with a drug. However, as shown by the example of PARP inhibitor treatment in *BRCA1/2* mutated tumors, loss of a tumor suppressor can offer a treatment option with low side effects for healthy tissue. The study of synthetic lethality and context-dependent gene essentiality has been challenging in mammalian cells and was for long time limited to few model organisms. With the development of CRISPR/Cas9 genome editing and insertional mutagenesis in haploid human cells, it is now possible to efficiently study genetic interactions as well as the functional consequence of genetic mutations and possibly reveal new predictive biomarkers by linking drug-responsive phenotypes to genotypes.

Although some novel anti-cancer drugs have been successful and have yielded improvements for cancer patients, they remain imperfect (Fojo and Parkinson, 2010, Groenendijk and Bernards, 2014), and have also become a financial burden for the health system (Kantarjian et al., 2013, Aggarwal, 2010, Prasad and Mailankody, 2017, Fojo and Parkinson, 2010). During the course of treatment, most patients sooner or later also received classical chemotherapy including platinum drugs, topoisomerase inhibitors, microtubule-targeting agents or antimetabolites as part of standard care (Gonen and Assaraf, 2012, Giovannetti et al., 2017). Their clinical use is based on empirical experience. However, these drugs are relatively cheap, effective and widely used. If clinical oncologists could be supported in their choice of classical chemotherapy based on molecular characteristics of a tumor, the therapeutic benefit of a standard treatment may increase and drugs to which the tumor is unlikely to respond would be avoided. To improve the proper selection of the treatment of choice and to expand our repertoire of drug response predictions, one needs to identify more molecular peculiarities of tumors which impact therapy response.

This review therefore elaborates on genome-wide screening techniques in mammalian cells with special emphasis on the response against classical cytotoxic drugs.

2. Forward genetic screens to improve our understanding of drug resistance and synthetic lethality.

Using forward genetic screens, genetic mutations can efficiently be linked to phenotypes of interest and identify the crucial genes for the selected phenotype. The success of a screen depends on the choice of the best model as well as efficient gene suppression. Genome-wide screens were made possible in mammalian cells with the discovery of RNA interference, which introduces small specific RNA molecules into cells and targets the corresponding host mRNA for degradation (Carthew

and Sontheimer, 2009, Brummelkamp et al., 2002). RNA interference screens have led to the discovery of novel predictive biomarkers for chemotherapy response which could be translated into the clinic. For instance, large-scale RNA interference screens identified loss of *PTEN* as a determinant of trastuzumab resistance in breast cancer (Berns et al., 2007) and feedback activation of EGFR as a resistance mechanism in *BRAF*-mutated colorectal cancer (Prahallad et al., 2012). However, for many applications RNA interference screens are imperfect, because this technique usually fails to inactivate gene function completely and remaining gene activity may mask the phenotype (Echeverri et al., 2006, Booker et al., 2011, Kaelin, 2012), although in some cases, such as the knock-down effect of essential genes on drug response, an incomplete gene inactivation might be required to detect an effect of the knock-down on drug response. Nevertheless, off-target effects complicate RNA interference screens through a high proportion of false positive hits. As RNA interference screens for biomarker discovery have been reviewed elsewhere (Mullenders and Bernards, 2009, Iorns et al., 2007, Swanton et al., 2008), we will focus here on more recent developments in screening technologies (Table 2 and Figure 2).

2.1 CRISPR/Cas9 screens in 2D cell lines

The discovery of the bacterial Cas9 endonuclease has revolutionized genome engineering. A 20-basepairs site-specific single guide RNA (sgRNA) directs the endonuclease to its corresponding target site and introduces a DNA break. This break stimulates repair by non-homologous end-joining (NHEJ) or homology-directed repair (HDR) by frequently introducing frameshifts by indel mutations, resulting in a premature stop codon or nonsense-mediated decay of the transcript. Consequently, gene inactivation is achieved at the genomic level by CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 by creating a gene knock-out which, for many applications, outperforms gene knock-down by RNA interference (Evers et al., 2016, Hart et al., 2015, Wang et al., 2015b). CRISPR/Cas9 also allows the study of inactivation of non-transcribed elements (Wang et al., 2014b).

2.1.1 Choice of cellular model

The universal applicability of CRISPR/Cas9 screens in a broad repertoire of models (Wang et al., 2015b) makes this technique a powerful tool to study cancer-type-specific genetic requirements. The most outstanding advantage of CRISPR/Cas9 is that genetic mutations can be introduced into versatile cellular models. Some mechanisms of drug resistance or hypersensitivity might only be present in a certain lineage or cancer type. Melanoma patients bearing the valine-to-glutamate change at residue 600 (V600E) of *BRAF*, for instance, respond well to a BRAF inhibitor in combination with a mitogen/extracellular signal-regulated kinase (MEK) inhibitor (Long et al., 2014), whereas the same treatment in colorectal patients bearing the same *BRAF* mutation is less efficient (Corcoran et al., 2015). It may therefore be necessary to study specific genetic requirements in several models to identify novel genetic dependencies originating from pathway rewiring following

genetic perturbations. Exploiting such lineage-specific vulnerabilities could identify targets for use in personalized medicine (Muller et al., 2012).

Despite this major advantage, the large genetic variation between tumor cell lines may also hinder the successful identification of genetic contributions to a phenotype. The efficacy of genetic modification of CRISPR/Cas9 depends on the number of target loci in the genome. Whereas most human cells are diploid, cancer cell lines often have increased numbers of chromosomes or individual gene amplifications due to genomic instability (Stratton et al., 2009). To inactivate genes in HeLa cells, for instance, which have up to five copies of a chromosome (Landry et al., 2013), Cas9 needs to cleave the locus with higher efficiency compared to a cell line with a diploid gene set. Screens performed in diploid cells will therefore likely yield more robust data with a higher signal-to-noise ratio compared to polyploid cells. Furthermore, cells adapt to genetic mutations with secondary genetic changes or other forms of compensation (Teng et al., 2013). This needs to be particularly considered when comparing studies in long-term cultured isogeneic cells (Housden et al., 2017). Due to the individual variation of genes affecting their genetic interactions, every cell line will have a different essentialome. Combining several screens from multiple cell lines can therefore identify the "core essentialome" of a lineage, while at the same time some information will be missed as a result of the variation in genetic context between cell lines. Hence, the properties of the chosen cell line directly impact screening results.

Moreover, the activity of DNA repair pathways in a cell line influences mutation efficiency by CRISPR/Cas9. The introduced DNA break can be repaired by HDR, which uses a homologous DNA template such as a sister chromatid. HDR is usually error-free, resulting in an unchanged genomic locus. The success of genetic inactivation using CRISPR/Cas9 therefore depends on the error-prone NHEJ repair pathway. The DNA cleavage site gets trimmed before re-ligation of the two DNA ends. This causes insertions or deletions of base pairs, resulting in a frameshift. If both HDR and NHEJ are active in a chosen cell line, one can expect a lower probability for complete gene inactivation in every locus (Miles et al., 2016). However, in cell lines lacking a functional HDR, CRISPR/Cas9-mediated gene knock-out has a high success rate (Miles et al., 2016).

2.1.2 Impact of the sgRNA library on screening success

To date, three major classes of CRISPR/Cas9-mediated genomic modifiers are available. The most common CRISPR libraries are knock-out libraries, but gene repression (CRISPRi) and activation (CRISPRa) libraries are also employed for specific research questions (Lopes et al., 2016, Miles et al., 2016, Gilbert et al., 2014, Dominguez et al., 2016). Libraries can either be genome-wide, targeting for example about 20,000 genes (Sanjana et al., 2014), or specifically designed to study one particular pathway or a selection of genes of interest (Zhou et al., 2014b). For instance, within a genetic region of interest, CRISPR/Cas9 tiling screens are able to dissect which genetic segments encode functional domains relevant for a phenotype (Korkmaz et al., 2016). Recent developments of

the CRISPR system for RNA targeting or base editing will further broaden the investigable research fields (Cox et al., 2017, Gaudelli et al., 2017).

The selection of the individual sgRNAs in a library directly impacts screening results. For loss-of-function screens, sgRNAs should preferably target an early, constitutively expressed exon of a gene of interest. Ideally, this leads to nonfunctional transcripts of all gene variants with no off-target activity on other genes. Most commercially available libraries contain 5-10 sgRNAs per gene designed to achieve high cleavage efficacy and low off-target activity. However, these libraries do not take potential genomic pleomorphisms of different cell lines into account, and for some genes, shorter sequence variants might not be targeted and retain some gene functionality.

Off-target effects arise from partial complementarity of the sgRNA target site with additional unintended target sites. Off-target effects can be reduced by the use of improved Cas9 nuclease variants, *e.g.* by a decreased binding efficacy and increased site specificity, by the use of alternative endonucleases or improved bioinformatics algorithms to design specific sgRNAs (Kleinstiver et al., 2016, Slaymaker et al., 2016, Kim et al., 2016, Zetsche et al., 2015, Doench et al., 2014). This, in turn, increases the need for a perfect alignment of the sgRNA sequence with the target site. A single nucleotide mismatch as a cell line-specific genomic variation or the introduction of a terminal G during sgRNA design could by itself prevent sgRNA alignment with the target site. Moreover, optimized PAM sequence variants may also improve site specificity (Kleinstiver et al., 2015).

Another crucial step in CRISPR/Cas9 screens is the efficacy of library transduction in a cell line. Typically, sgRNA libraries are transduced at a MOI of 0.4 to 0.6 to ensure that every cell contains only a single sgRNA (Shalem et al., 2014, Wang et al., 2014b). For successful screens, a single copy of each sgRNA needs to cleave both (or even more) copies of the target locus (Wang et al., 2014b). If more than one sgRNA is introduced into a single cell (as usually achieved through direct transfection), it is challenging to determine which sgRNA caused the phenotype due to the relatively low number of distinct sgRNAs. In contrast, less than one sgRNA per cell can lead to insufficient library coverage at the end of a screen.

Depending on the plasmid system used to deliver the library, a stable expression of both sgRNA as well as Cas9 increases the genomic cleavage over time (Wang et al., 2014b). This may be on purpose, but it might also be problematic when screens are performed over a long time period, as not only on-target sites but also off-target sites will be cleaved repeatedly. DNA double strand breaks also result in genotoxic stress, causing a non-specific anti-proliferative effect which limits the use of CRISPR/Cas9 for highly amplified genomic regions (Aguirre et al., 2016, Munoz et al., 2016, Housden et al., 2017).

2.1.3 Data analysis

Owing to the popularity of CRISPR/Cas9 screens, a broad spectrum of resources for data analysis is available. To analyze a screen, sgRNA sequences are amplified by PCR before deep sequencing. The library sequencing reads may subsequently be analyzed using publically available algorithms, including HitSelect (Diaz et al., 2015) or MaGeCK (Li et al., 2014b). Although they require some programming experience, these algorithms are widely used, relatively user-friendly and provide a certain consistency in the analysis of many CRISPR/Cas9 screens.

When analyzing CRISPR/Cas9 screens, it is crucial to remember that sgRNA copy abundance is counted as an indirect measurement of mutations. Not every sgRNA necessarily creates a frameshift mutation, since the DNA break can also be repaired error-free by HDR or with an in-frame mutation retaining functionality of the transcript. When a sgRNA introduces both frame-shift and in-frame mutations, resulting in a mixed phenotype of this sgRNA, it is possible that this sgRNA will not score as being significant in the analysis (Shalem et al., 2015).

2.1.4 Applications to study drug response

CRISPR/Cas9 screening technology has enabled a systematic analysis of gene function in mammalian cells in terms of both positive (enrichment of mutants) and negative selection (depletion of mutants) (Wang et al., 2014b, Shalem et al., 2014, Zhou et al., 2014b, Wang et al., 2015b). Already at an early stage of development of the CRISPR/Cas9 technology, it has been shown that multiplexing with several sgRNAs can be achieved *in vitro* as well as *in vivo* (Horii et al., 2013, Wang et al., 2013, Cong et al., 2013, Mali et al., 2013).

In general, positive selection screens are less prone to alterations in library representation than negative selection screens which require a quantitative evaluation of sgRNA abundance. A good coverage and few false positives made CRISPR/Cas9 screens a valuable tool to study resistance phenotypes. In terms of anti-cancer drug resistance, for instance, genes were identified whose loss-of-function-mutations cause resistance to vemurafenib, cytosine arabinoside, 6-thioguanine and DNA topoisomerase II (Shalem et al., 2014, Kurata et al., 2016, Koike-Yusa et al., 2014, Wang et al., 2014b). As an example, Shalem et al., identified two known mediators of resistance against the BRAF inhibitor vemurafenib, *NF1* and *MED12* (Huang et al., 2012, Whittaker et al., 2013) in a genome-wide CRISPR/Cas9 library screen targeting 18,080 genes with 3-4 sgRNAs/gene (Shalem et al., 2014). Additionally, the authors found novel genes which had not been linked to BRAF inhibitor response so far, and validated their inactivation as resistance factors towards vemurafenib *in vitro*. Among those genes were members of the STAGA complex which recruit Mediator complex proteins including *MED12,* which negatively regulates TGF-β, to c-myc activating cell proliferation (Liu et al., 2008). Thus, CRISPR/Cas9 screens are able to identify novel mechanisms of anti-cancer drug resistance.

For the analysis of negative selection screens, the abundance of all sgRNAs needs to be quantified to identify the sgRNAs that were depleted from the population. As a consequence, gene knock-out efficacy needs to be close to 100% because every remaining non-modified cell or in-frame

modification may mask the phenotype. These screens are less robust compared to positive selection screens and thus depend on more extensive statistical analysis, which in turn could be complicated by poor sgRNA-mediated cleavage efficacy. Despite these technical hurdles, CRISPR/Cas9 screens have successfully identified essential genes in mammalian cells (Wang et al., 2015b, Hart et al., 2015), as well as synthetic lethal genetic interactions which could potentially serve as targets for cancer therapy (Toledo et al., 2015, Steinhart et al., 2017). For instance, a study focusing on synthetic lethal interactions with oncogenic Ras identified critical regulators of the MAPK pathway in acute myeloid leukemia cell lines (Wang et al., 2017).

Moreover, CRISPR/Cas9 screens have proven useful to study intracellular phenotypes by employing reporter- or antibody-labelled quantitative protein measurements (DeJesus et al., 2016, Parnas et al., 2015). This type of screen compares cell populations with high and low signal measurements, and couples genes to an intracellular phenotype which does not necessarily result in altered cell viability. Studying such intracellular phenotypes broadens the understanding of key biological processes, and may identify crucial genetic dependencies of a phenotype of interest.

2.2 Haploid insertional mutagenesis screens

Insertional mutagenesis screens are a powerful alternative to CRISPR/Cas9 screens. They provide the possibility of applying principles of classical genetics in mammalian cells to uncover fundamental biological processes in a highly comparative manner. In particular, recessive genetic screens in haploid yeast have substantially contributed to gene discovery and our understanding of development, basic physiology, and various diseases (Giaever and Nislow, 2014). It was therefore a great achievement of Thijn Brummelkamp and coworkers to establish insertional mutagenesis screens in haploid human cell lines and thereby increase the power of insertional mutagenesis in a mammalian system (Carette et al., 2009).

2.2.1 Haploid cell lines

Insertional mutagenesis screens crucially depend on haploid (or near-haploid) cell lines, because no second copy of the gene exists which could phenotypically mask the effect of gene inactivation. Thus, genetic manipulation is highly efficient in haploid cells. To date, only few haploid cell lines exist. The KBM7 cell line, derived from a chronic myeloid leukemia patient, is haploid except for chromosome 8 (Kotecki et al., 1999). Its non-hematopoietic derivate HAP1 is haploid for all chromosomes, except of a duplicated 30-megabase fragment of Chromosome 15 fused to Chromosome 19, which was excised to obtain the fully haploid eHAP cell line (Essletzbichler et al., 2014). With the isolation of haploid human, murine, rat and monkey embryonic stem cells, a broader repertoire of haploid cell lines is currently being established, also by further differentiating these cells

into several lineages (Elling et al., 2011, Leeb and Wutz, 2011, Sagi et al., 2016, Yang et al., 2013, Li et al., 2014a).

Compared to CRISPR/Cas9 screens, the restriction to a few haploid cell lines, limits the use of haploid screens in the study of lineage-specific biology. However, this limitation simultaneously provides an opportunity, exemplified by the achievements of yeast genetics where a well-characterized single-model organism unveiled numerous insights into diverse biological processes (Giaever and Nislow, 2014, Boone et al., 2007). By introducing genetic alterations into a clearly defined, controlled genetic background, effects of gene inactivation can be studied and compared with precision, facilitating the investigation of complex genetic effects and interactions (Costanzo et al., 2016, Brockmann et al., 2017). Subsequently, findings from haploid screens may be validated in a variety of cell lines to address lineage-specific variations.

2.2.2 Insertional mutagenesis

In contrast to CRISPR/Cas9 screens, which depend on a sgRNA library, mutations are usually achieved by random mutagenesis in haploid screens. The choice of the random mutagenesis technique is commonly determined by the ease of retrieving genetic mutation sites for analysis from a pool of cells, with transposon- or retroviral gene-trapping being the most frequent mutagenesis strategies to date. Mutagenesis performed by gene-trapping is highly efficient and delivers a molecular tag to identify mutations in the genome, by integrating an exogenous viral DNA section in the host genome which marks the mutation site (Carette et al., 2009). The gene-trap cassette introduces a splice acceptor site, followed by a polyadenylation signal, which prematurely terminates gene splicing and translation and thereby creates mutants resembling knock-outs (Carette et al., 2009). Transposon-mediated gene-trap mutagenesis is achieved by co-transfecting a gene-trap vector with a plasmid for transposase expression. The transposase stimulates insertion of the gene-trap in the genome, and by varying transposase expression, the gene-trap insertion frequency can be influenced (Mates et al., 2009, Pettitt et al., 2015).

In theory, gene-trapping mutation events occur all over the genome and this technique therefore provides a very high coverage. However, the gene-trap integration is not completely random, as preferred viral or transposon integration sites exist (Lee et al., 2007, Wang et al., 2014b, Carette et al., 2009, Blomen et al., 2015). Nevertheless, a genome-wide distribution of integration sites, for example integrations in more than 98% of expressed genes by retroviral gene trapping (Carette et al., 2011a), is achieved, and improved vectors can reduce integration bias (Schnuetgen et al., 2008).

Compared to the use of sgRNA libraries which employ a guiding RNA strand defining the target site and directly depend on sgRNA representation as well as cleavage success and error-prone DNA repair, insertional mutagenesis creates individual mutations at a higher frequency and efficiency (Blomen et al., 2015). The phenotypic effect of a gene is determined indirectly in CRISPR/Cas9

screens, usually by determining the abundance of DNA encoding the sgRNAs. In contrast, sensitive amplification of gene-trap insertion sites allows a direct count of hundreds of independent mutations in a gene, achieving a high resolution of the observed phenotype (Elling and Penninger, 2014).

However, similar to the need of a sgRNA disrupting all gene variants simultaneously to abolish gene function, alternative splicing as well as gene-trap integrations near the 3' end of a gene can also maintain (partial) gene activity (Blomen et al., 2015).

2.2.3 Data analysis

Deep sequencing following PCR amplification retrieves millions of unique gene-trap integration sites which are mapped to the genome, allowing subsequent direct counting of individual knock-outs that each has contributed to the phenotype of interest. The large number of individual mutants permits powerful statistical analyses. This enables an improved distinction between hits and background noise compared to other screening methods (Elling and Penninger, 2014). Due to the high efficacy of gene disruption and the absence of second gene copies, haploid cells further improve the signal to noise ratio compared to screens performed in diploid cells, enabling the identification of subtle fitness defects or advantages (Wang et al., 2014b, Blomen et al., 2015).

To analyze insertional mutagenesis screens, basic statistical tests such as Fisher's exact and/or binomial tests are usually employed. However, for complex research questions, such as the study of a cell line's essentialome, more complex algorithms are used (Blomen et al., 2015). Recently, bioinformatics pipelines have been proposed for the analysis of gene-trap insertional mutagenesis screens (Yu and Ciaudo, 2017, Mayor-Ruiz et al., 2017).

2.2.4. Applications to study drug response

Initially, haploid insertional mutagenesis screens were predominantly employed to study resistance factors of host cells towards pathogens (Carette et al., 2009, Carette et al., 2011b, Guimaraes et al., 2011, Jae et al., 2013). Furthermore, haploid screens yielded novel insight into resistance against chemical compounds including chemotherapeutic agents like platinum drugs, topoisomerase II, PARP, and other chemical inhibitors (Wijdeven et al., 2015, Planells-Cases et al., 2015, Pettitt et al., 2013, Shen et al., 2016, Heijink et al., 2015). Regarding platinum drugs, it has been unclear which transporters are responsible for reduced drug uptake that may explain clinical drug resistance (Borst et al., 2008). Using a haploid screen, we identified volume-regulated anion channels composed of LRRC8A and D as a cellular uptake mechanism of cisplatin and carboplatin (Planells-Cases et al., 2015), providing a new lead to understand clinical resistance. Similarly, proposed resistance mechanisms against topoisomerase II inhibitors, such as doxorubicin, cannot explain all cases of therapy failure in the clinic (Pommier, 2013). Here, a haploid screen identified novel resistance mechanisms which reduce DNA double strand break formation or stimulate DNA

repair (Wijdeven et al., 2015). Although gene trap viruses should be inactivating, a gain-of-function mutation cannot be excluded. For example, we found a significant enrichment of integrations that induce the *ABCG2* gene expression upon topotecan selection (Guyader, Blomen, Gerhards, Brummelkamp and Rottenberg, unpublished results). Despite the technical differences between haploid and CRISPR/Cas9-positive selection screens, direct comparisons of both approaches showed high concordance (Marceau et al., 2016, Timms et al., 2016, Wang et al., 2015b).

The inactivation of genes at high efficiency makes haploid screens very effective for negative selections, as most sense integrations in an intron will lead to reproducible and complete depletion of a gene (Elling and Penninger, 2014, Burckstummer et al., 2013). Since negative selection aims at identifying mutations that are depleted from a population of mutants, which is easily influenced by environmental factors, a robust identification of depleted mutations of the pool of cells can be challenging. A unidirectional design of the gene-trap cassette overcomes this problem and has facilitated the identification of essential genes in human cells as well as genotype-specific gene requirements by using the distribution of sense and antisense orientations as a readout for gene essentiality (Wang et al., 2015b, Blomen et al., 2015, Haarhuis et al., 2017). Haploid screens have furthermore been employed to study genotypes which sensitize cells to chemical compounds (Pettitt et al., 2017). For instance, we have recently found that loss of the tumor suppressor *FBXW7* sensitize cells to *Vinca* alkaloids. This finding might aid the choice of microtubule-targeting chemotherapeutic drugs in patients (Gerhards et al., *submitted*).

Similar to CRISPR/Cas9, reporter- or antibody-based haploid screens have deciphered genetic modifiers of various intracellular phenotypes regardless of their effect on the viability of a cell (Brockmann et al., 2017, Lebensohn et al., 2016, Mezzadra et al., 2017, Timms et al., 2016, Lee et al., 2013). For instance, a reporter-based haploid screen for transgene silencing demonstrated the potential of this methodology to study epigenetic changes in human cells (Tchasovnikarova et al., 2015). Epigenetic mechanisms such as DNA methylation or histone modifications are considered to drive secondary drug resistance by altering the expression of genes involved in drug transport, DNA repair or apoptosis (Wilting and Dannenberg, 2012, Brown et al., 2014). As genetic reporters are not available for all intracellular processes, antibody-based screens in fixed mutagenized cells further expanded the scale of query phenotypes that can be studied (Brockmann et al., 2017). Hence, haploid screens can contribute to our understanding of the epigenetic drivers of drug resistance and potentially identify novel therapeutic strategies.

2.3 *In vivo* screens

Screens in 2D models are affected by assay conditions, the choice of cell lines as well as their growth on plastic in high oxygen. It has been shown that results vary between labs despite similar approaches (Scholl et al., 2009, Babij et al., 2011). Furthermore, it has been shown that tumors exhibit contrasting drug responses *ex vivo* and *in vivo* (Teicher et al., 1990) and that a tumor's microenvironment impacts drug response (Straussman et al., 2012). Additionally, comparisons of

patient-derived and control cell lines revealed that the variation rather reflects the genetic background, culture conditions and cell line history than a disease-relevant phenotype (Soldner and Jaenisch, 2012, Gillet et al., 2011). Thus, 2D cancer models encounter limitations regarding their clinical relevance and their application to study complex phenotypes such as metastasis formation or a host's immune response. *In vivo* screens can overcome these limitations and bridge the gap to clinically more relevant settings. They allow not only assessing tumor cell-intrinsic contributions to drug sensitivity or resistance, but also take a body's microenvironment and immune response into account.

2.3.1 Choice of model

In vivo screens may be conducted by using a large cohort of knock-out animal models (van der Weyden et al., 2017). However, this approach is not feasible for all laboratories, as the majority does not have an extensive collection of animal models. With the development of CRISPR/Cas9, powerful and fast alternatives became available. CRISPR/Cas9 can be employed for *ex vivo* gene editing of cells which are subsequently transplanted into recipient mice or for non-germline manipulation of tumors *in vivo*. Somatic delivery of Cas9-expressing cells can cause an immune response clearing those cells (Wang et al., 2015a). Therefore, Cas9-transgenic mice are frequently employed, which only require locally or systemic delivery of the sgRNAs, for instance through lentiviruses, into mice (Platt et al., 2014, Annunziato et al., 2016). Alternatively, lentiviruses encoding both Cas9 and sgRNAs or *ex vivo* CRISPR/Cas9-modified cells can be injected into immunodeficient or Cas9-tolerant mice for *in vivo* screening (Chen et al., 2015, Braun et al., 2016). A genome-wide screen, targeting for instance 20,000 genes with 6 sgRNAs per gene, would consist of 120,000 sgRNAs and therefore require millions of transplanted tumor cells. This is often not feasible, but a genome-wide screen is usually not necessary to study the phenotype of interest *in vivo*. For most *in vivo* screening approaches, a focused library targeting selected biological processes is sufficient to address the research question.

2.3.2 Applications to study drug response

The major benefit of *in vivo* over *in vitro* screens is the contribution of the animal organism to the phenotype. A solid tumor *in vivo* is a multicellular complex that interacts with its surrounding tissue, differing substantially from a clonal cancer cell line in a cell culture dish. The tumor microenvironment and tumor-stromal interactions gained increasing importance as modulators of drug response. For instance, stroma-induced drug resistance has been described in several preclinical models for chemotherapeutic drugs such as resistance to doxorubicin, vincristine or vemurafenib (McMillin et al., 2013).

Until today, most *in vivo* screens were performed using RNA interference editing (Gargiulo et al., 2013, Zhou et al., 2014a, Beronja et al., 2013, Meacham et al., 2015, Rudalska et al., 2014,

Schramek et al., 2014). *In vivo* CRISPR/Cas9 screens have been employed to assess the metastatic potential upon gene inactivation (Chen et al., 2015). Furthermore, novel immune modulatory factors which could serve as immunotherapeutic targets were identified in a CRISPR/Cas9-edited melanoma screen *in vivo* (Manguso et al., 2017). Additional biological processes for *in vivo* screening approaches include the role of tumor angiogenesis and hypoxia in drug response, and basic research topics such as tumor development and tissue regeneration. Screens for these complex phenotypes are still in the process of being optimized.

Nevertheless, limitations of pooled *in vivo* screens are encountered due to the complexity of the library, poor efficiency of virus delivery, loss of sgRNA representation and of diversity in the outgrown tumor after transplantation or injection, as well as the complex interactions of tumors with the host body (Chen et al., 2015). Furthermore, ethical concerns of performing *in vivo* screens may rise, as they require large numbers of experimental animals.

2.4 Screens using 3D cancer organoids

A promising model to recapitulate *in vivo* tumor behavior in an *ex vivo* setting is the use of 3D organoid cultures (Clevers, 2016). These have several advantages over 2D cell lines. Traditional cell lines are usually clonal, immortalized cells, genetically adapted to cell culture conditions and lacking a tumor's heterogeneity as well as its complex multilayered cell organization. In a solid tumor, a drug may not reach all cells at equal levels due to varying vascularization. This situation is mimicked to some extent in a 3D culture, as drugs do not freely penetrate the viscous biomaterial in which organoids are embedded. In contrast, all cells in a monolayer will receive equal concentrations of an administered drug. Furthermore, the composition and architecture of organoids correspond to the tissue they are derived from. Organoids can usually be expanded easily, cryopreserved and biobanked and efficiently genetically manipulated (van de Wetering et al., 2015, Schwank et al., 2013, Drost et al., 2017).

2.4.1. Choice of model

3D organoids have been generated from the eye (Eiraku et al., 2011, Nakano et al., 2012), brain (Lancaster et al., 2013, Pasca et al., 2015), intestine (Sato et al., 2009, Spence et al., 2011, Forster et al., 2014), kidney (Takasato et al., 2014, Takasato et al., 2015), liver (Takebe et al., 2013, Huch et al., 2013), lung (Dye et al., 2015), inner ear (Koehler et al., 2013) and other organs. Organoids have furthermore been derived from cancer tissues (so-called tumoroids) (Baker et al., 2016, van de Wetering et al., 2015, Schutte et al., 2017, Pauli et al., 2017), or were transformed into cancer organoids by genetic modification (Drost et al., 2015, Matano et al., 2015). 3D cultures allow

an organ-like architecture, facilitate interactions with the extracellular matrix and provide rudiments of functionality (Fatehullah et al., 2016).

Considering that organoids consist of primary cells, the passage number influences the organoid's geno- and phenotype. During culturing, some cells of the heterogeneous cell pool will be lost, leading to a larger discrepancy between original tumor and the organoid line. When larger amounts of starting material is available, *e.g.* from large excised patient or mouse tumors, a higher complexity of the heterogeneous cell pool can be maintained for longer time than with little starting material such as a biopsy. Additionally, organoid culturing medium is frequently supplemented with various factors such as Noggin or R-spondin which further select cells or alter gene expression in the organoid culture. Thus, organoids are less robust and less well-characterized than cell lines, but the heterogeneity resembles more the tumors observed in patients.

Gene editing components to modify organoids can be delivered by adenoviruses, retroviruses or lentiviruses (Wang et al., 2014a, Koo et al., 2012, Duarte et al., *in press*). The subsequently achieved modifications create a polyclonal pool of cells with mixed genotypes. To efficiently select modified cells, reporter genes can be added to the viruses. However, the introduction of genetic modifications will also cause additional selective pressure so that one should evaluate to what extent the original tumor is still fully represented in the modified organoid culture after some passages.

2.4.2 Applications to study drug response

3D organoid cultures are useful to study drug response of patient-derived material *in vitro*. They can be archived easily and used for high-throughput analyses of drug response linked to their omics profiles (van de Wetering et al., 2015). Using matched samples of a PARP-inhibitor-sensitive and -resistant BRCA2-mutated mammary tumor, we have recently shown that more sophisticated functional assays to measure drug response (*e.g.* replication fork stability) can be performed in tumoroids (Ray Chaudhuri et al., 2016). How reliable the *in vitro* drug responses are in predicting the drug response in the patient from whom the tumoroids were derived remains to be seen. When we tested several matched PARP-inhibitor-sensitive and -resistant BRCA1-mutated organoids *in vitro*, we found examples where the resistant tumors regained drug sensitivity in vitro, despite the stable *in vivo* resistance of tumors derived from these tumoroids (Duarte et al., *in press*). This illustrates that further optimization of the *in vitro* conditions is required to mimic the response of real tumors.

The CRISPR/Cas9 system facilitates rapid genome engineering as well as forward screening approaches in 3D organoids (Nie and Hashino, 2017). Given the heterogeneous nature of organoids, these screens are more complex, their statistical power is reduced compared to screens in well-defined genetic backgrounds and the variation of size and shape of organoids further affect phenotypic selections. However, the sampling error is low due to the cellular complexity of the drawn test sample from the given pool of cells in the tumoroid and thus 3D screening results are expected to

validate *in vivo*. So far, a CRISPR screen in lung organoids with a miniature focused library discovered genes involved in ciliogenesis and barrier function in the airway epithelium (Gao et al., 2015). The high number of cells, required to ensure an appropriate coverage, needs to be taken into consideration when organoid screens will be performed. These numbers of cells are usually only obtained after several passages which might conflict with the ambition of high similarity between the organoids and the original tumor. Additionally, the success of organoid screens strongly depends on the control of organoid size with clear pathophysiological gradients, as seen in a solid tumor, while preventing a too large area of central necrosis. Due to a batch-to-batch variation of the extracellular biomaterial, reproducibility and scalability of experiments is thus far limited. Thus, large-scale screens remain technically challenging in tissue-derived organoids.

However, we found that a major advantage of tumoroids is that they can serve as a rapid intermediate step to introduce genetic modifications, and subsequently transplant the tumors into mice to test drug response *in vivo* (Duarte et al., *in press*). Using tumoroids from BRCA1-mutated mouse mammary tumors we introduced *Trp53bp1* mutations by CRISPR/Cas9 and show that the tumors derived from the *Trp53bp1*-depleted tumoroids are resistant to PARP inhibition. Importantly, we also observed that tumoroids exhibit high clonal heterogeneity *in vivo* and give rise to tumors that preserve the cellular complexity of the parental organoid population (Duarte et al., *in press*). We therefore conclude that these models are suitable to study the effects of intratumoral heterogeneity *in vivo* and are useful for *in vivo* screening approaches to identify mechanisms of drug resistance.

Since tumoroids are more readily cultured from patient tumors than patient-derived 2D cell lines (Boj et al., 2015, van de Wetering et al., 2015, Duarte et al., *in press*), they also provide an opportunity to better follow a patient's disease development. Using genome-wide CRISPR/Cas9 mutagenesis, the essentialome of an individual tumor may be determined to provide information about specific vulnerabilities of this particular tumor. The patient could subsequently receive a treatment accordingly, and if the tumor relapses or stops responding, a new organoid line could be obtained and the essentialome re-analyzed by another functional genome-wide screen. This "sensitive-to-resistant-essentialome"-comparison approach may yield new insights into drug resistance mechanisms, directly received from clinical patients, and provide useful information for new vulnerabilities and treatment options.

In summary, organoid screens are still in the process of optimization and technical hurdles need to be resolved. Nevertheless, they provide a very promising approach to study complex contributions to a phenotype of interest *in vitro* that resembles treatment responses *in vivo*. It is very likely that novel factors influencing drug response will be discovered soon in tumoroid models, and they will further broaden our repertoire to study cancer specimens directly derived from patients.

3. Conclusions

In contrast to targeted anti-cancer treatment, predictive biomarkers for classical chemotherapy have been much more difficult to identify. This might be explained by the complex heterogeneity between tumors due to secondary mutations, epigenetic alterations or other mechanisms of drug resistance which are still poorly understood (Borst, 2012). Even in a well-characterized genetically homogeneous tumor system, such as syngeneic transplantable tumors from genetically engineered mouse models, the identification of genes contributing to drug response is limited (Rottenberg et al., 2012). Only if a gene is altered in most of the analyzed tumors, gene expression analysis will detect it as a predictive candidate, whereas drug response mechanisms present only in a subset of tumors will be missed. Given the fact that a variety of mechanisms, either single- or multi-genetic in nature, influence drug response, together with various mechanisms of action of classical chemotherapeutics, it is not surprising that we have not been very successful to establish predictive biomarkers for these drugs.

The advances in screening technologies however might provide the necessary starting point to develop novel hypotheses which might eventually translate into clinical predictive biomarkers. Forward genetic screens in mammalian cells have substantially broadened our understanding of cell biology, the mode of action of chemical compounds and anti-cancer drug resistance or hypersensitivity. These approaches offer crucial tools to discover novel tumor vulnerabilities, while at the same time highlighting context-specific dependencies and mechanistic complexities of cancer. A variety of cellular features determine drug sensitivity of a cell, and drug response varies between individual tumors and patients, which represents a major hurdle for clinical oncologists. Frequently, cancer patients do not benefit from a chosen treatment but predominantly experience the side effects, and better predictive biomarkers are urgently needed. Forward genetic screens allow modeling of the complex mutational landscape of human cancer and identifying similarities or differences between drug effects. For instance, studies performed on cellular platinum uptake showed that carboplatin and cisplatin, but not oxaliplatin, are entering cells through LRRC8A/D-containing anion channels (Planells-Cases et al., 2015). Thus, for patients with low *LRRC8A/D* expression, cisplatin and carboplatin might not be an effective treatment regimen. Such findings may be clinically useful and, thus, more screens using well-established, classical chemotherapeutic drugs may further contribute to improved precision oncology.

We expect that further technical improvements of existing screening technologies and the comparison of screens for various drugs will aid in the development of novel treatment regimens. The potential use of patient-derived tumoroids for drug response screens can additionally be exploited for personalized medicine. Future treatment concepts will increasingly be based on specific personalized tumor features and thus knowledge about specific tumor vulnerabilities is essential to move the field forward. Since classical chemotherapeutic agents are widely available, inexpensive and well-characterized in terms of dosage and toxicity, more effort should be made to unravel the full potential of these drugs and assess their benefit in clinical trials for patient subgroups characterized by particular genetic mutations. Furthermore, new drug combinations could be suggested when the impact of a genotype on drug response is better understood.

Given the advantages and limitations of the screening techniques, the chosen approach should be carefully evaluated. Screening output might depend more on experimental design than on the technology itself, as shown by a study comparing CRISPR/Cas9 with optimized RNAi screens concluding that both techniques perform equally well (Morgens et al., 2016). In general, the most comprehensive picture will be achieved by combining several approaches. Clinical data from patients remain essential to validate the findings and assess the predictive potential of identified gene-drug dependencies. Thus, studies would benefit from more than one approach to answer the same question and their combination into a therapy response map might promote the translation into clinically predictive markers and effective treatments with improved patient life quality and overall survival.

4. Acknowledgements

Figure legends

Figure 1: Exemplary workflow to discover novel predictive biomarkers based on forward genetic screens.

Figure 2: Simplified layouts of the screening technologies presented in the current review. **A.:** Screens in 2D cell lines using CRISPR/Cas9 or insertional mutagenesis to study genetic contributions to a phenotype of interest upon drug treatment. 2D cell lines are modified with either CRISPR/Cas9 or insertional mutagenesis before drug exposure. Depending on the research question of interest, the screen can be analyzed for enrichment (positive selection, potential drug resistance genes) or depletion of mutants (negative selection, potential drug hypersensitivity genes) or an intracellular phenotype by employing antibody- or reporter-based assays. **B.:** Mice bearing CRISPR/Cas9-modified tumors can be treated with a drug of interest and efficiently analyzed to study complex phenotypes such as metastasis formation. **C.:** Patient-derived organoids can be modified with CRSIPR/Cas9 and used for both rapid *in vivo* testing of a gene panel of interest and identification of the tumor's 'essentialome'.

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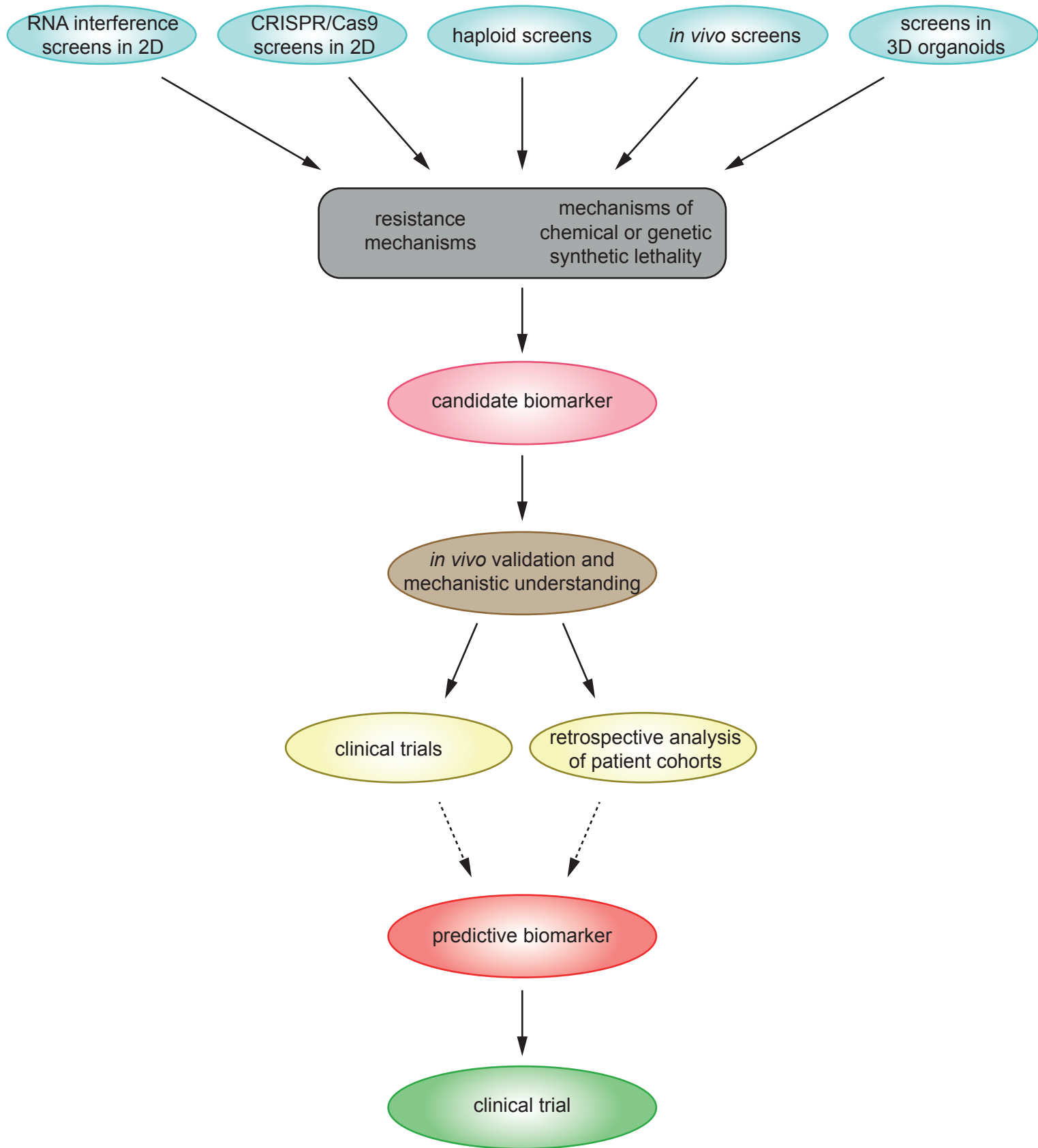
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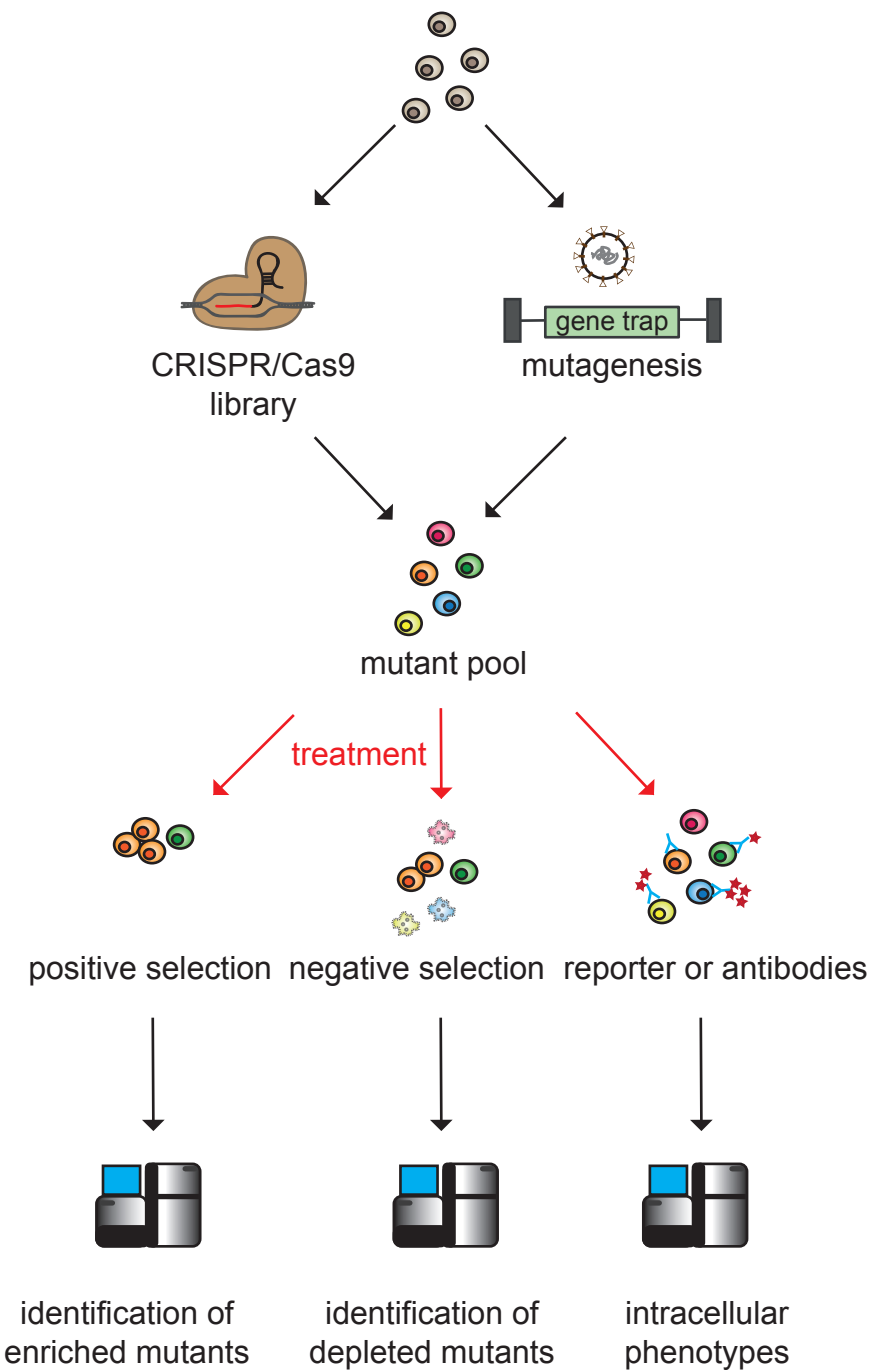
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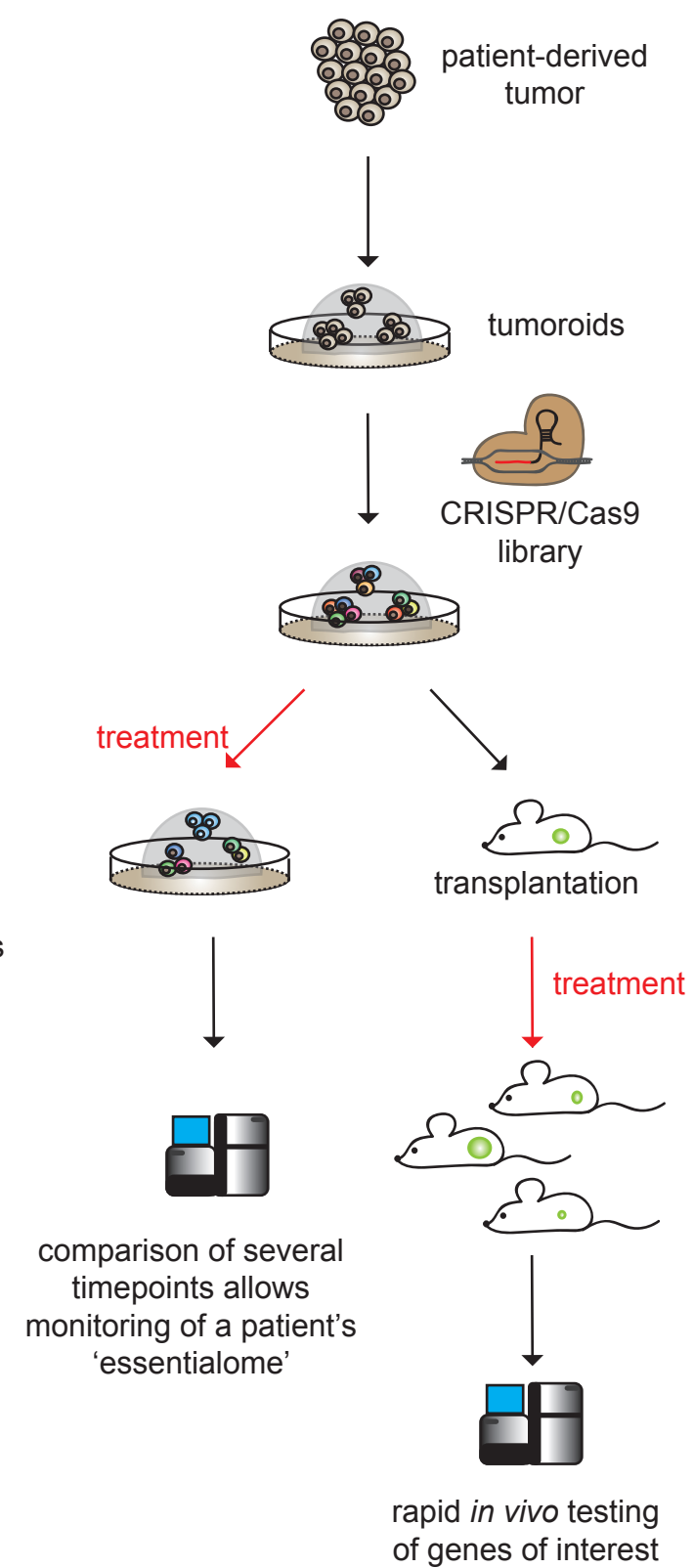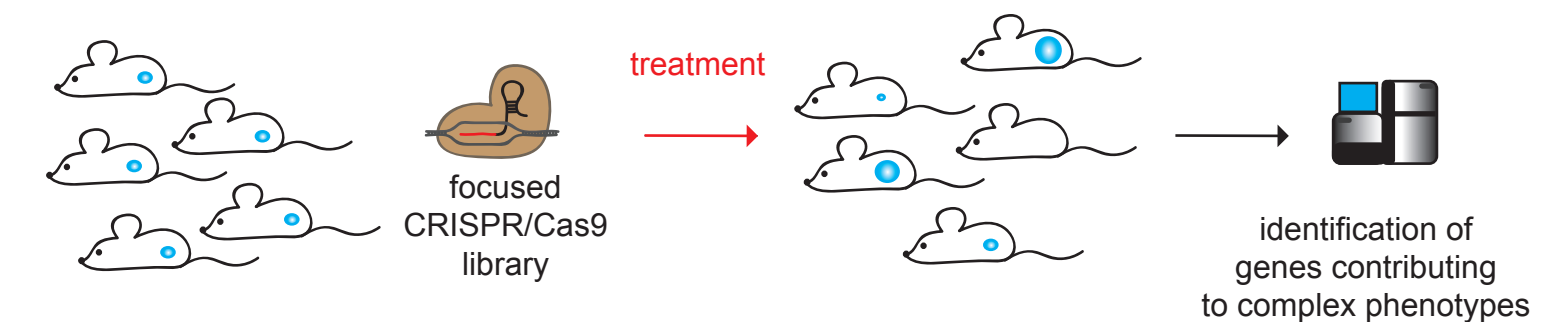
A. **Screens in 2D cell lines**

CRISPR/Cas9 library

gene trap
mutagenesis

mutant pool

treatment

positive selection

negative selection

reporter or antibodies

identification of enriched mutants

identification of depleted mutants

intracellular phenotypes

B. **Screens *in vivo***

focused CRISPR/Cas9 library

treatment

identification of genes contributing to complex phenotypes

C. **Screens using 3D cancer organoids**

patient-derived tumor

tumoroids

CRISPR/Cas9 library

treatment

transplantation

treatment

comparison of several timepoints allows monitoring of a patient's 'essentialome'

rapid *in vivo* testing of genes of interest

Table 1
Examples of screens performed to identify unknown factors of drug response, to suggest potential therapeutic strategies or to exploit novel screening concepts.

Selection	Screening method	Model	Identified genes - proof of concept	Identified genes - novel findings	Proposed mechanism	Remarks	Reference
Trastuzumab (HER2-targeting antibody)	shRNA screen (7,914 genes), positive selection	HER2-amplified breast cancer cell line BT474		PTEN	Loss of PTEN activates PI3K/AKT signaling	PI3K pathway activation as predictive marker	(Berns et al., 2007)
Vemurafenib (PLX4032, BRAF inhibitor)	Kinome shRNA screen (535 genes), positive selection	Colorectal cancer cell line WiDr		EGFR	BRAF(V600E) inhibition activates EGFR and stimulates proliferation (feedback activation)	Melanoma cells express low levels of EGFR and are thus sensitive to BRAF inhibition; BRAF-mutant colon cancer might benefit from combination of BRAF and EGFR inhibitors	(Prahallad et al., 2012)
Trastuzumab (HER2-targeting antibody)	shRNA screen (7,914 genes), positive selection	HER2-amplified breast cancer cell lines BT474, SKBR3 and HCC1954	PTEN	ARID1A	ARID1A loss activates ANXA1 which in turn activates AKT and causes resistance	High ANXA1 expression suggested as predictive marker	(Berns et al., 2016)
Vemurafenib (BRAF inhibitor)	Genome-wide CRISPR/Cas9 (18,080 genes), positive selection	BRAF(V600E)-mutated melanoma cell line A375	NF1, MED12	NF2, CUL3, TADA1, TADA2B	TADA1 and TADA2B (member of STAGA complex) recruit MED12 to c-myc to activate proliferation; MED12 activates TGF-βR signaling and MEK/ERK		(Shalem et al., 2014)
Cytosine arabinoside (antimetabolite)	Genome-wide CRISPR/Cas9 (18,080 genes), positive selection	Acute myeloid leukemia cell line U937	DCK	SLC29A	associated with nucleotide salvage pathway and required for the uptake and activation of Ara-C		(Kurata et al., 2016)
6-thioguanine (antimetabolite)	Genome-wide CRISPR/Cas9 (19,150 genes), positive selection	Male mouse ES (JM8) cells	Mismatch repair genes (Mlh1, Msh2, Msh6, Pms2)	Hprt, GM15293, Letmd1, Olfr815, Prkg1, Tmem8c		Unknown candidate genes did not validate in subsequent in vitro experiments	(Koike-Yusa et al., 2014)
6-thioguanine (antimetabolite)	Genome-wide CRISPR/Cas9 (7,114 genes), positive selection	Human near-haploid chronic myeloid leukemia cell line KBM7	Mismatch repair genes (MSH2, MSH6, MLH1, PMS2)				(Wang et al., 2014b)
Etoposide (DNA topoisomerase II inhibitor)	Genome-wide CRISPR/Cas9 (7,114 genes), positive selection	Human pseudo-diploid leukemic HL60 and near-haploid KBM7 cell lines	TOP2A	CDK6	G1-cyclin dependent kinase involved in etoposide cytotoxicity		(Wang et al., 2014b)
ATR inhibitor	Genome-wide CRISPR/Cas9 (19,150 genes), positive selection	Mouse ES cells (KH2)		CDC25A	CDC25A prevents cells from premature entry into mitosis	CDC25A levels could serve as criterion for patients more likely to respond; rationale to combine ATR and WEE1 inhibitor treatment	(Ruiz et al., 2016)
Phenotypic selection	Genome-wide (18,543 human and 18,986 mouse genes) and focused (132 Ras-associated genes) CRISPR/Cas9 library, negative selection	12 acute myeloid leukemia cell lines and NRAS-engineered mouse CGN Ba/F3 cell line		Several genes involved in Ras maturation or downstream of MAPK signaling pathway	Cancers driven by oncogenic Ras require Rac/PAK signaling to activate MAPK signaling	PAK inhibition as potential synthetic lethal therapeutic strategy in Ras-driven cancers	(Wang et al., 2017)
Phenotypic selection	Genome-wide CRISPR/Cas9 (18,080 genes), negative selection	Glioblastoma stem-like and neural stem/progenitor cell lines		PKMYT1	PKMYT1 essential to inhibit cyclin B-CDK1 activity is lost in glioblastoma	PKMYT1 inhibition as potential synthetic lethal therapeutic strategy in glioblastoma	(Toledo et al., 2015)
Phenotypic selection	Genome-wide CRISPR/Cas9 screen (17,232 genes), negative selection	RNF43-mutant pancreatic ductal adenocarcinoma cell line	components of Wnt pathway	FZD5	FZD5 encodes the main receptor for Wnt-β-catenin signaling in this context	FDZ5 inhibiton as a potential synthetic lethal therapeutic strategy in RNF43-mutated pancreatic cancer	(Steinhart et al., 2017)
Phenotypic selection	Genome-wide (18,360 genes) and mini-pool (300 genes) CRISPR/Cas9 screen, quantitiative protein measurement of SQSTM1 modulators	Human neuroglioma H4 cell line	MTOR complex 1 and canonical macroautophagy components	ufmylation components	Ufmylation induces SQSTM1 expression		(DeJesus et al., 2016)

Lipopolysaccharide	Genome-wide CRISPR/Cas9 screen (21,786 genes), quantitative measurement of Tnf expression	Mouse bone-marrow derived dendritic cells	*Tlr4, Myd88* (signal high), *Zfp36* (signal low)	components of OST complex, ER translocation pathway, PAF complex			(Parnas et al., 2015)
Doxorubicin (DNA topoisomerase II inhibitor)	Viral gene-trap haploid screen, positive selection	Human haploid cell line HAP1	*ABCB1, Keap1*	SWI/SNF subunits, *C9orf82, Eif4a1*	SWI/SNF regulates Topoisomerase II activity, C9orf82 negatively regulates DNA repair	Patients with low SWI/SNF expression should not be treated with doxorubicin but rather aclarubicin or topotecan	(Wijdeven et al., 2015)
Carboplatin (platinum drugs)	Viral gene-trap haploid screen, positive selection	Human near-haploid chronic myeloid leukemia cell line KBM7		Components of volume-regulated anion channel (*LRRC8D/LRRC8A*)	50% of cellular platinum drug uptake mediated via LRRC8A/D channels	Downregulation of LRRC8 subunits could have an impact on platinum resistance	(Planells-Cases et al., 2015)
6-thioguanine (antimetabolite)	piggyBac transposon haploid screen, positive selection	Mouse haploid ES cells	Proof of concept: DNA mismatch repair pathway genes (*Msh2, Msh6, Mlh1*)			Validation of loss-of-function screen	(Pettitt et al., 2013)
Olaparib (PARP inhibitor)	piggyBac transposon haploid screen, positive selection	Mouse haploid ES cells		*Parp1*	Parp1 is a drug target and required for drug toxicity	Inhibited PAPR1 enzyme forms a toxic DNA lesion	(Pettitt et al., 2013)
6-thioguanine (antimetabolite)	Viral gene-trap haploid screen, positive selection	Human near-haploid chronic myeloid leukemia cell line KBM7		*HPRT*	Enzyme converting 6-thioguanine to a toxic metabolite		(Carette et al., 2009)
Imatinib (tyrosine-kinase inhibitor)	Viral gene-trap haploid screen, positive selection	Human near-haploid chronic myeloid leukemia cell line KBM7	*NF1, PTPN1*	*PTPN12*	Tyrosine phosphatase negatively regulates c-abl		(Carette et al., 2009)
Formaldehyde	Viral gene-trap haploid screen, positive selection	Human near-haploid chronic myeloid leukemia cell line KBM7		12 candidate genes		6 out of 12 candidates validated	(Shen et al., 2016)
Imatinib (tyrosine-kinase inhibitor)	Viral gene-trap haploid screen, positive selection	Human near-haploid chronic myeloid leukemia cell line KBM7		*CASP10, CUX1, NF1, LYRM9, ZPBP, CEBPG*		Only *LYRM9* validated; only *NF1* was also identified by Carette et al., 2009	(Shen et al., 2016)
MK-1775 (Wee1 inhibitor)	Viral gene-trap haploid screen, positive selection	Human near-haploid chronic myeloid leukemia cell line KBM7		*SKP2,CUL1, CDK2* (among others)	Inactivation of S-phase can overcome Wee1 inhibitor resistance	Activity of DNA replication machinery could serve as selection criterion for Wee1 inhibitor treatment	(Heijink et al., 2015)
Talazoparib (PARP inhibitor)	piggyBac transposon haploid screen, negative selection	Brca2-mutated mouse haploid ES cells (H129.2)		*Ewsr1*			(Pettitt et al., 2017)
Phenotypic selection	Viral gene-trap haploid screen, quantitative protein measurement of AKT signaling	Human haploid cell line HAP1		*KCTD5; GNB1* and other genes encoding Gβγ subunits	KCTD5 negatively regulates GPCR signaling by triggering proteolysis of dissociated Gβγ subunits		(Brockmann et al., 2017)
Phenotypic selection	Viral gene-trap haploid screen, quantitative protein measurement of WNT signaling	Human haploid cell line HAP1 with 7TG-WNT reporter	several known regulators	genes linked to WNT receptor complex, CTNNB1 destruction complex and others	Other processes than CTNNB1 protein levels , e.g. truncation of domains, might regulate WNT signaling		(Lebensohn et al., 2016)
Interferon-γ	Viral gene-trap haploid screen, quantitative protein measurement of PD-L1 abundance	Human haploid cell line HAP1	IFNγR-pathway, *IRF1, CD274*	*CMTM6*	CMTM6/4 enhances PD-L1-mediated T-cell inhibition, stabilizes PD-L1 protein level	Novel potential target for immune-suppressive cancer therapy	(Mezzadra et al., 2017)
Phenotypic selection	Comparison of genome-wide CRISPR/Cas9 (19,050 genes) and viral gene-trap haploid screen, quantitative protein measurement of ER-associated degradation of MHC class I molecules	Human near-haploid chronic myeloid leukemia cell line KBM7 with MHC-I-ERAD reporter		*TXNDC11*	TXNDC11 encodes an EDEM2/3-associated disulphide reductase		(Timms et al., 2016)

Phenotypic selection	Genome-wide CRISPR/Cas9 screen (20,611 genes), positive selection	mouse non-small-cell lung cancer cell line transplanted into immunocompromised mice		several candidate genes enriched in late primary tumors, high overlap of candidate genes in metastases	Enrichment of mutations in anti-apoptotic or other tumor suppressive pathways		(Chen et al., 2015)
Monoclonal PD-1 antibody	Focused CRISPR/Cas9 screen (2,368 genes), positive selection	Mouse B16 melanoma cell line	CD47	*Ptpn2*, and several genes involved in four distinct biological processes	loss of Ptpn2 sensitize tumors to immunotherapy through increased antigen presentation and T-cell stimulation	Inhibition of Ptpn2 as a therapeutic strategy to increase the effect of anti-PD-1 immunotherapy	(Manguso et al., 2017)
Phenotypic selection	Mini CRISPR/Cas9 screen (10 genes), positive selection	3D mucociliary epithelial organoids from primary human basal cells		*GRHL2*	GRHL2 plays a key role in apical-basal cell polarity and epithelial morphogenesis		(Gao et al., 2015)

Table 2
Comparison of recent functional screening technologies.

CRISPR/Cas9 in 2D cell lines	
Advantages	Disadvantages
• Variety of cell lines or models can be used	• Cell line-specific genetic pleomorphisms, adaptations or genetic alterations might impair screening results
• Easy to study cancer-type- of cell lineage-specific genetic determinants	• Activity of DNA repair in the model impacts CRISPR/Cas9 cleavage success
• Several distinct libraries and systems for various purposes	• Dependence on representative library with efficient sgRNAs
• Ongoing developments of novel CRISPR systems for various applications	• Off-targets effects on unintendend genomic sites
• Publically available data analysis algorithms	• In-frame mutations can mask the phenotypes
• Some consistency in data analysis between different laboratories	• sgRNA abundance as indirect measurement of mutations

Haploid insertional mutagenesis	
Advantages	Disadvantages
• Application of principles of yeast genetics in mammalian cells	• Limitation to few haploid cell lines, more challenging to study lineage-specific biology
• Every genetic region with good coverage can be studied	• Integration bias of transposons or viruses
• Individual genomic mutations are directly measured	• Few publically available data analysis algorithms
• High statistical power	• Alternative splicing can maintain a functional transcript

in vivo screens	
Advantages	Disadvantages
• Contribution of the host's microenvironment, tumor-stromal interactions and immune response to the phenotype of interest	• Large number of animals needed for complex libraries
• Closer to the situation in patients	• Diversity of outgrown tumors
• Study of complex phenotypes such as metastasis formation or angiogenesis	• Interaction with microenvironment can mask effects of genetic manipulation
	• Virus delivery or tumor outgrowth rate increases experimental variation
	• Ethical concerns

3D cancer organoids	
Advantages	Disadvantages
• Multilayered complex 3D organization	• Loss of similarities with original tumor over time
• Resemble the original *in vivo* organ	• Growth factors supplemented in medium alter gene expression of organoids
• Heterogeneity resembles patients	• Genetically less robust, poorly characterized
• Patient-derived material can be studied easily	• Low statistical power