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To cite this article: Hendrik J Kuiken & Roderick L Beijersbergen (2010) Exploration of Synthetic Lethal Interactions as Cancer Drug Targets, Future Oncology, 6:11, 1789-1802, DOI: [10.2217/fon.10.131](https://doi.org/10.2217/fon.10.131)

To link to this article: <https://doi.org/10.2217/fon.10.131>



Published online: 09 Dec 2010.



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Exploration of synthetic lethal interactions as cancer drug targets

Hendrik J Kuiken¹ & Roderick L Beijersbergen^{1*}

¹Division of Molecular Carcinogenesis, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

*Author for correspondence: Tel.: +31 20 512 1960 ■ Fax: +31 20 512 1954
■ r.beijersbergen@nki.nl

In cancer research the quest continues to identify the Achilles' heel of cancer. The ideal cancer drug targets are those that are essential in tumor cells but not in normal cells. Such targets are defined as cancer-specific vulnerabilities or as synthetic lethal interactions with cancer-specific genetic lesions. The search for synthetic lethal interactions focuses on proteins that are frequently mutated but elude pharmacological inhibition, for example, RAS, or proteins that are lost in cancer cells and by definition cannot be targeted, such as the tumor suppressor genes *p53*, *APC* and *RB*. These genetic interactions could yield alternative, effective targets for cancer treatment. However, it remains very difficult to predict or extrapolate these synthetic lethal interactions based on existing knowledge. With the discovery of RNAi, unbiased large-scale functional genomic screens for the identification of such targets have become possible potentially leading to major advances in the treatment of cancers. In this review we will discuss the biological basis of synthetic lethal interactions in relation to existing targeted therapeutics, lessons taught by targeted therapeutics already used in the clinic and the implementation of RNAi as tool to identify such synthetic lethal interactions.

The need for cancer-specific treatment strategies

Tumor development is an evolutionary process driven by an accumulation of mutations allowing cells to acquire traits that can ultimately lead to a cancerous state [1]. As a consequence cancer is presented as a collection of diseases with high differential complexity that is not limited to differences in the location of origin and the stage of the disease, but extends to the genetic context and heterogeneity within one tumor type. Although surgery may be used to efficiently resect a primary tumor, it cannot clear the patient of metastatic cancer cells that might already reside at distant locations. Furthermore, the location of the primary tumor might not allow sufficient surgical margins to assure resection of infiltrating tumor cells in nearby tissue. Hence, in most instances, localized or systemic treatment with radiation, conventional cytotoxic or targeted molecular therapeutics is needed to prevent both recurrence of the primary tumor and metastasis.

Even after 70 years of cancer drug discovery, most of the molecular therapeutics in use today share the same mode of action: interference with the DNA replication processes by causing DNA damage, or interference with cell division through (de)stabilization of microtubules [2]. Their effectiveness is based on two general cancer hallmarks: increased proliferation and impaired DNA damage checkpoints,

both of which drive cancer cells to a certain death in the presence of additional damage. Despite their established effectiveness in a wide range of cancers, and some remarkable success stories, such as the use of cisplatin regimens in testicular cancer and combinations of conventional regimens in Hodgkin's lymphoma, these conventional cytotoxic drugs generally have low therapeutic indices as they cause many side effects at the cost of therapeutic efficacy [3,4]. Moreover, differences in sensitivity as well as intrinsic or acquired resistance to a treatment can cause patients with similar tumor types to display diverse response rates. For this reason, it is of equal importance to identify biomarkers that can predict the responsiveness to cancer therapeutics and which can guide personalized treatment strategies by selecting patients for specific therapies in order to develop novel cancer-specific therapeutics [5].

To develop new cancer-specific treatments with an improved therapeutic index two strategies can be followed: first, targeting a cancer-specific molecule or process; or second, targeting a noncancer-specific molecule or process for which the cancer cell has acquired a qualitative or quantitative requirement [6]. A major challenge in cancer treatment is the presence of dormant cells within the tumor, which rarely or never divide during the treatment, and as such are not killed by conventional molecular therapeutics. This population of cells can include

Keywords

■ chemical-genetic ■ network addiction ■ (non)oncogene addiction ■ RAS ■ RNAi
■ screen ■ shRNA ■ siRNA
■ synthetic lethal interaction

cancer stem or initiator cells, which can give rise to a recurrent tumor or metastasis [7]. Hence, there is a need for targeted therapies, which are highly selective for cancer cells, and do not rely on cell division for their toxicity. Ideally such therapies should also target and kill dormant cancer cells.

Targeted cancer therapies & network addiction

Advances in molecular and cancer biology have led to rational drug-design strategies, resulting in the development of so-called targeted therapeutics [2,8]. Among these are therapeutics that target driving oncogenes, such as the successful small-molecule inhibitor, imatinib mesylate, which targets the BCR–ABL fusion protein, c-KIT and PDGF receptor in chronic myelogenous leukemia, gastrointestinal stromal tumors, and hypereosinophilic syndrome, respectively [9–11]. Other targeted therapeutics such as gefitinib, an inhibitor of mutant or overexpressed EGF receptor (EGFR) in non-small-cell lung cancers, and the antibody trastuzumab, which blocks signaling from overexpressed HER2/neu in breast cancers, have also shown success in the clinic [12–15]. The mechanism of action for this class of targeted therapeutics is based on an acquired dependency on a specific oncogenic signaling pathway; a concept known as oncogene addiction [16]. The importance of sustained oncogenic signaling is also strengthened by the mechanisms by which tumor cells become resistant to these targeted therapeutics. In the case of acquired resistance to imatinib mesylate, these tumors harbor mutations in the kinase domain of BCR–ABL, or contain genomic amplification of the BCR–ABL locus [17]. However, the mechanisms of resistance do not always imply the target itself, but can also occur in downstream components of the signaling network. In the case of trastuzumab, mutations that result in the activation of the PI3K effector pathway, which is downstream of HER2/Neu, are a major determinant of resistance [18]. With respect to gefitinib, mutations or amplification of its target EGFR, but also amplification of MET confers resistance to gefitinib in non-small-cell lung cancers. However, MET is not downstream of HER2/Neu, but confers resistance to gefitinib by driving HER3-dependent activation of the PI3K pathway [19]. This, and the observation that introduction of a constitutive active PI3K mutant is sufficient to confer resistance to gefitinib, indicates that

these tumors have in fact become addicted to the activation of a specific molecular network, which is not mutated itself, but deregulated by distinct upstream genetic alterations [20–22].

The observations that cancer cells can acquire a dependency for a genetic alteration led to the oncogenic shock model [23]. Oncogenic signaling induces both pro-apoptotic signals as well as prosurvival signals. Upon inactivation of the oncogenic signal, the pro-apoptotic signaling is sustained longer than the prosurvival signal, thereby inducing apoptosis. An alternative explanation is based on the hypothesis that (epi) genetic mutations can occur, which are dependent on a pre-existing genetic alteration. In the absence of this alteration, these mutations would be lethal for the cells. Consequently, reversion of the genetic alteration results in a lethal phenotype caused by unmasking a secondary mutation. Rather than (epi)genetic events, these secondary events can be an accumulation of cell intrinsic or extrinsic events only tolerated in the presence of the genetic alteration to which the cancer cell is addicted. The addiction to specific genetic alterations is not restricted to oncogenes, as reintroduction of tumor-suppressor genes such as *P53*, *RB* or *APC* also cause cancer cells to arrest or undergo apoptosis [16,24]. This phenomenon is named tumor-suppressor hypersensitivity, illustrated by stabilization of wild-type p53 in human cancer cells. A large fraction of human tumors have retained expression of wild-type p53. However, the p53 pathway is inactivated due to other alterations, for example, increased expression of MDM2, a negative regulator of p53. Inhibition of the MDM2–p53 interaction by treatment with a MDM2 inhibitor known as Nutlin-3 results in stabilization of p53 and induction of apoptosis in cancer cells, but not in normal cells. The finding that knockdown of 53BP1 can rescue cancer cells from Nutlin-3-mediated effects suggests that these cancer cells exhibit intrinsic DNA damage signals, which are masked by the absence of p53 function, but which upon restoration of p53 expression causes activation of the p53 pathway resulting in apoptosis [25].

The mechanisms by which tumor cells can become resistant to targeted therapeutics have increased our understanding of mechanisms of drug action and the networks to which cancer cells are addicted. These detailed insights can guide the development of novel targeted therapeutics directed to crucial hubs in these networks. In addition to being more widely

applicable, such targets might be less prone to acquired resistance caused by additional mutations in upstream or downstream signaling molecules. These hubs might not be oncogenic by themselves and this form of dependency is often referred to as non-oncogene addiction [26]. The concepts of oncogene and non-oncogene addiction are extensively discussed by Sharma and Settleman, and Luo *et al.* [27,28]. Although the exploration of these concepts has just started, it is clear that these acquired cancer-specific dependencies are of great importance for the development of novel targeted therapeutics.

Molecular buffering & genetic interactions

As discussed above, the majority of the current targeted therapeutics are based on the concept of oncogene addiction. However, many oncogenes have proven to be difficult targets for small-molecule inhibition. For example, activating mutations in the *RAS* oncogenes are frequent in human cancer, but so far targeting *RAS* itself has remained a major challenge [29]. An analogous situation applies to *MYC*, a transcription factor that upon mutation or amplification acts as an oncogene. It is clear that cancer cells driven by oncogenic *RAS* or *MYC* can become addicted to these oncogenic signaling pathways. This is clearly illustrated in mouse models with inducible expression of mutant *RAS* or *MYC*, where inactivation of their expression in established tumors resulted in tumor regression [30–32]. Inhibition of non-oncogenic molecules acting up- or down-stream of these oncogenes may provide an indirect mean of targeting these genetic lesions. However, the tumor cells' acquired dependencies are not restricted to the linear network downstream of the oncogenic alteration *per se*. Different molecular networks can control or buffer overlapping cellular processes, and perturbations in one network can result in an acquired dependency for another. Such possible interactions are difficult to predict from existing knowledge, and require unbiased approaches for their identification. Examples of these genetic interactions include synthetic lethal interactions. Two genes have a synthetic lethal interaction when cells die if they have both genes mutated but can survive if either gene alone is mutated [33,34]. The identification of negative genetic interactions associated with genetic alterations specific for human cancer can be exploited directly as drug targets or provide anchors for further investigation of cancer-specific vulnerabilities [35,36].

One of the best characterized synthetic lethal interactions that is presently being investigated in clinical trials is between breast cancer (BRCA)1/2 and poly(ADP-ribose) polymerase (PARP)1 [37,38]. PARP1 is required for base excision repair, which is the preferred method of repairing modified bases in DNA prior to entry in S-phase. Loss of PARP1 function results in single-strand breaks, leading to the collapse of replication forks during S-phase. On the other hand, BRCA proteins are involved in homologous recombination repair and can resolve these single-strand breaks during S-phase by using sister chromatids as templates. In the absence of both PARP1 and BRCA1/2, error-prone repair mechanisms take over, which eventually lead to an accumulation of DNA damage resulting in cell death. Recently, a synthetic interaction between loss of PTEN and PARP was observed [39]. Cells that have lost expression of PTEN also lost the expression of RAD51, which like BRCA1/2 is involved in homologous recombination repair. The enhanced sensitivity to PARP inhibitors could be restored by introduction of either PTEN or RAD51. These are clear examples of two DNA damage repair networks having a genetic interaction. The BRCA1/2 interaction with PARP1 and other examples of synthetic lethal interactions based on DNA repair and checkpoint networks, including p53–ATM and p53–MK2, are reviewed by Rouleau *et al.* and Reinhardt *et al.* [40,41].

Most insights into genetic interactions have been obtained from the model organism *Saccharomyces cerevisiae*, in which a genome-wide deletion collection has been generated that can be used with high-throughput screening techniques [42]. An extensive review on genetic interactions and large-scale studies in model organisms is provided by Dixon *et al.* [43]. To identify genetic interactions in human cancer cells, one can take two approaches: an educated approach based on current knowledge of signaling pathways and data from model organisms, or a completely unbiased approach by means of functional genomic screening approaches with large gene sets. The first approach is based upon the assumption that the synthetic interactions are conserved during evolution. In addition to *S. cerevisiae*, large-scale genetic interaction screens have been performed in *Schizosaccharomyces pombe* and *Caenorhabditis elegans*, allowing assessment of the conservation of such networks between different species [43]. Comparative analysis of genetic interaction networks between *S. pombe* and *S. cerevisiae*

show that approximately 30% of the negative interactions are conserved [44]. However, it was shown that a very small percentage (~5%) of the genetic interactions are conserved between *C. elegans* and yeast [45,46]. A possible explanation for the higher conservation between *S. cerevisiae* and *S. pombe* compared with *C. elegans* is given by Dixon *et al.* [43]. Cellular-level redundancy in multicellular organisms might mask the detection of certain genetic interactions, which manifest themselves in a subtle way and require alternative phenotypic readouts. Yet another plausible explanation is the lack of selective pressure on synthetic genetic interactions and as a consequence low conservation [45,46]. During evolution, additional redundancies for a certain process can be acquired resulting in the loss of the genetic interaction without affecting the genes that displayed that interaction. One obvious example is that of gene duplication. Questions arising from these observations are: how much rewiring of such genetic interactions can occur in human cancer, and what are the implications for acquired resistance to cancer therapies based on such genetic interactions? With regard to the search for novel cancer-specific targets, the low conservation of synthetic genetic interactions between species directs us to the second approach, unbiased functional genomic screens to identify synthetic genetic interactions with genetic alterations in human cancer.

RNAi as a tool for functional genomics & identification of synthetic lethal interactions

Although the phenomenon of RNAi was first described in plants in 1990, the discovery of this mechanism in *C. elegans* stands at the basis of loss-of-function genomics in higher organisms, rewarded with the Nobel price in 2006 [47,48]. RNAi is the mechanism whereby introduction of dsRNA into a cell results in the degradation of a mRNA product that shares high complementarity with the dsRNA. Since its discovery, advances in the generation of tools that mediate RNAi have resulted in an extensive set of genome-wide RNAi libraries [49]. These libraries can be divided into two major classes: synthetic siRNA and vector-based shRNA libraries (FIGURE 1). The siRNA libraries can be subdivided into those derived from algorithm-driven design and chemical synthesis, and those which are enzymatically generated by RNaseIII digestion of dsRNA fragments of approximately 500 bp (endoribonuclease-prepared siRNAs). The wide availability and ease of use of synthetic siRNAs

have led to their extensive application in many studies [50]. Although knockdown efficiency of siRNAs is high, the knockdown is transient, restricting the use of siRNAs to short-term experiments. In addition, testing siRNAs across multiple cell lines can be challenging, as it may require optimization of transfection conditions for every individual cell line.

The development of vector systems expressing shRNAs, which are processed in the cell to siRNAs, have provided a tool for long-term experiments [51,52]. These libraries can be subdivided by the vector used (e.g., retroviral or lentiviral) or by the shRNA design (i.e., a shRNA or the shRNA embedded in a miRNA structure [shRNA-miR]) [49]. The introduction of the shRNA constructs through viral infection makes these libraries more suitable for testing in large panels of different cell lines. Similar to siRNA libraries, shRNA libraries can be used for screens in a single-well format (FIGURE 1). However, in contrast to siRNA libraries, shRNA libraries can be used in a pooled format (FIGURE 1). In this approach large numbers of shRNA vectors are combined and used to infect a single, large population of cells. After selection, the integrated shRNA cassettes can be recovered by PCR on genomic DNA and the relative abundance of each individual shRNA can be determined by hybridization to DNA microarrays or deep sequencing [53]. The barcode technology has been applied successfully for positive selection screens and, to a lesser extent, in negative selection screens. The latter approach can be affected by the under-representation of individual vectors due to library construction and virus production, the limits of the detection system to measure highly complex populations, or due to cross-hybridization and background issues associated with barcode arrays. However, owing to technical advances in the design and generation of the shRNAs and barcodes, the pooled-format approach has recently found its entry into negative selection screens [54,55].

Both RNAi platforms, as reviewed by Mullenders and Bernards, have led to the identification of novel modulators in signaling pathways, yielded insight into complex biological processes and will undoubtedly contribute to the discovery of potential cancer drug targets [50]. Several screens have selected genes based on their effects on proliferation or the induction of apoptosis. When such screens are performed in a pair of cell lines, one of which carries a specific genetic alteration, it is possible to identify genes that have a synthetic lethal interaction with that specific genetic alteration. Several

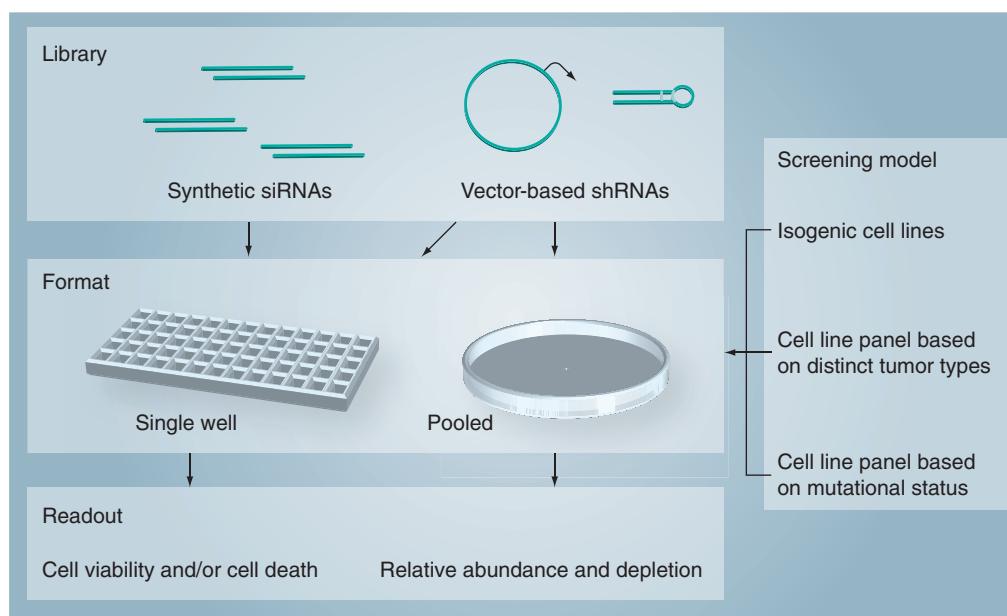


Figure 1. Screening systems and models for cancer-specific vulnerabilities. Advances in the field of RNAi have led to the generation of several distinct siRNA and shRNA libraries, which can be used in single-well, or in the case of shRNA libraries, in either single-well or pooled format screens. To identify cancer-specific vulnerabilities, three strategies can be taken: a RNAi screen in isogenic cell lines, a panel of cell lines based on their tumor type or a panel of cell lines for which the mutation status for a given gene is known. When the single-well approach is taken, the effect of the siRNA or shRNA on the viability of the cell can be measured with either cell viability or cell death assays. In the case of a pooled shRNA library approach, a microarray barcode or deep-sequencing readout can be used for measuring the negative selection against a shRNA over time.

labs have applied such screening systems, and those that have been completed are discussed below (TABLE 1). Although these screens have been productive and initial results are encouraging, we likely have only seen the beginning of the potential outcomes.

RNAi screening systems & models for the identification of genetic interactions

One of the first genetic interaction siRNA screens in human cells was aimed at the identification of genes that, upon knockdown, selectively sensitize MYC overexpressing cells to a DR5-agonistic antibody [56]. DR5 is one of the five receptors that can bind TNF-related apoptosis-inducing ligand (TRAIL) and like TRAIL, DR5-agonistic antibodies can stimulate DR5-mediated apoptotic signaling. The cytokine TRAIL and DR5-agonistic antibodies have received much attention as potential cancer therapeutics, and several tumors, including those with high expression of MYC, display increased sensitivity [57]. Rottmann *et al.* screened a library of 624 siRNAs and identified 13 hits including GSK3 β that upon knockdown differentially sensitizes a MYC overexpressing cell line [56]. It was previously shown that GSK3 β phosphorylates MYC resulting in SCF^{FBW7}-mediated

ubiquitination and subsequent proteasomal degradation [58]. As a consequence, knockdown of either GSK3 β or FBW7 results in increased levels of MYC and its downstream target DR5. Because DR5 is required in a dose-dependent manner for DR5-agonistic antibody-mediated apoptosis, high expression or stabilization of MYC results in increased sensitivity. Hence, MYC and proteins controlling the levels of MYC might act as indirect markers for the treatment response to TRAIL and DR5-agonistic antibodies. The use of GSK3 β inhibitors could be beneficial in combination with TRAIL or DR5-agonistic antibody therapies. The question remains whether GSK3 β inhibition by itself induces apoptosis in MYC overexpressing cells, as high levels of MYC are associated with apoptosis. If this is not the case this would suggest that the genetic interaction between GSK3 β and MYC is restricted to a context wherein the DR5 receptor is sufficiently activated.

Another RNAi screen was designed to identify lethal genes in specific cell types. Ngo *et al.* used an inducible shRNA library targeting approximately 700 genes selected on their relevance to cancer cell proliferation and survival [59]. Using this library, they identified several genes that are specifically required for

Table 1. RNAi screens for synthetic lethal interactions with genetic alterations in human cancer.

Author	Library type	Library size	Targets	Assay	System	Hits (n)	Validated hits	Ref.
Rottmann <i>et al.</i> (2005)	siRNA	624	Kinases	Single well (cell viability)	MYC isogenic cell lines	13	1 (GSK3 β)	[56]
Ngo <i>et al.</i> (2006)	Inducible shRNA	683	NA	Barcode	B-cell-like DLBCL vs germinal center B-cell-like DLBCL	15	1 (CARD11)	[59]
Shaffer <i>et al.</i> (2008)	Inducible shRNA	~2500	Genome wide	Barcode	Myeloma vs lymphoma	NA	1 (IRF4)	[61]
Naik <i>et al.</i> (2009)	siRNA	691	Kinases	Reporter	WNT/ β -cat luciferase reporter followed by validation in WNT/ β -cat addicted cell lines	11	5 (Cdc2L1, Lmtk3, Pank2, ErbB3 and VEGFR)	[62]
Bommi-Reddy <i>et al.</i> (2008)	shRNA	88	Kinases	Single well (cell viability)	Two pairs of VHL isogenic cell lines	28	5 (CDK6, HER4, IRR, MAP2K1 and MET)	[63]
Sarthy <i>et al.</i> (2007)	siRNA	~4000	Genome wide	Single well (apoptosis)	KRAS isogenic cell lines	3	1 (Survivin)	[64]
Luo <i>et al.</i> (2009)	shRNA	32293	Genome wide	Barcode	Two pairs of KRAS isogenic cell lines	50	13 (THOC1, DHX8, USP39, Ubc9, COPS3, COPS4, hMIS18 α , CASC5, borealin, SMC4, PLK1, APC1 and APC4)	[65]
Scholl <i>et al.</i> (2009)	shRNA	1011	Kinases, phosphatases and cancer-related genes	Single well (cell viability)	Panel of tumor cell lines with known RAS status	1	1 (STK33)	[67]
Barbie <i>et al.</i> (2009)	shRNA	957	Kinases, phosphatases and cancer-related genes	Single well (cell viability)	Panel of tumor cell lines with known RAS status	45	1 (TBK1)	[68]

DLBCL: Diffuse large B-cell lymphoma; KRAS: Kirsten ras protein; VHL: Von Hippel-Lindau.

the survival of activated B-cell-like diffuse large B-cell lymphoma (DLBCL) cells, but not germinal center B-cell-like DLBCL cells. Among the hits were genes involved in NF- κ B signaling, including *CARD11*. The identification of NF- κ B components validates the screen as it was previously shown that DLBCL cells are dependent on NF- κ B activation for their survival [60]. Similarly, Shaffer *et al.*, using an inducible shNRA library, screened for genes that, upon knockdown, selectively kill myeloma cell lines but not lymphoma cell lines [61]. They validated IRF4 as a crucial transcription factor for myeloma cells. IRF4 controls the expression of a large number of genes including *MYC*, which implied that the selective sensitivity to IRF4 knockdown can be the result of changes in expression of one of many, or even a combination of target genes. Hence, additional research will be required to fully understand the mechanism behind this selectivity. Despite the fact that these screening models wherein two or more tumor types are used as a query panel do not allow pinpointing of the gene associated with the genetic interaction, the genes identified can be of great interest as tumor-type-specific drug targets.

Naik *et al.* took a different approach to identify synthetic lethal interactions [62]. They tested a siRNA collection targeting kinases for genes involved in the WNT pathway as measured by a WNT/ β -catenin luciferase reporter. As a fraction of colon cancers have constitutive activation of the WNT pathway, and have become addicted to this pathway activation, the identification of genes that upon knockdown reduce the WNT/ β -catenin luciferase reporter activity represent interesting drug targets. This screen led to the identification of five genes that upon knockdown reduced the WNT/ β -catenin reporter activity, including *VEGFR1*. Follow-up experiments showed that knockdown of VEGFR1 is only toxic to colon cancer cell lines, which have constitutive activation of, and are addicted to the WNT pathway. Knockdown of VEGFR1 results in decreased phosphorylation of β -catenin, but does not affect the stability or nuclear accumulation of β -catenin. Thus VEGFR1-mediated direct or indirect phosphorylation of β -catenin might be required for β -catenin-dependent transcription. Although the approach taken in this screen led to the identification of a specific vulnerability to VEGFR1 inhibition for cells with an addiction to an activated WNT/ β -catenin pathway, it was restricted to the identification of genes directly involved in β -catenin-mediated activity

of an artificial reporter, and did not allow identification of vulnerabilities connected to altered expression of genes as a consequence of activation of the WNT/ β -catenin pathway. Nevertheless, VEGFR1 might be an interesting candidate for the treatment of WNT/ β -catenin-addicted colon cancers.

Bommi-Reddy *et al.* performed a true synthetic genetic interaction screen for genes that specifically affect the viability of cells that have lost the expression of Von Hippel–Lindau (VHL) [63]. They infected two pairs of isogenic cell lines with 100 shRNAs targeting 88 kinases, representing a preselected set based on viability screens executed in HeLa and HEK293 cell lines. One of the five validated hits was *CDK6*. It was shown that VHL^{-/-} cells are more sensitive to a CDK4/6 inhibitor than the wild-type VHL reconstituted cell lines. Although the mechanism behind these synthetic genetic interactions remains to be elucidated, these observations do suggest that patients with clear cell renal carcinomas that have frequently inactivated VHL might benefit from targeted molecular therapeutics for CDK6.

RNAi screens for synthetic lethal interactions with oncogenic RAS

Several groups have undertaken RNAi screens aimed at the identification of synthetic genetic interactions with oncogenic RAS, a protein that until now has escaped direct inhibition by small molecules. Sarthy *et al.* screened a siRNA library targeting 4000 genes for selective toxicity with mutant KRAS and found that KRAS cells are more sensitive to depletion of survivin. They showed that survivin knockdown results in a G2/M arrest followed by resynthesis of DNA and polyploidy due to a failure to undergo cell division [64]. In another effort, Luo *et al.* screened a genome-wide shRNA library in a pooled format and identified several genes, which upon knockdown show selective growth impairment for KRAS-mutant cells [65]. These genes included components of the anaphase promoting complex (*APC/C*) and *PLK1*, suggesting an increased dependency of KRAS-mutant cells on mitotic checkpoints and progression. Interestingly, these KRAS-mutant cell lines are also more sensitive to treatment with a PLK1 inhibitor. Furthermore, xenograft models in mice using these cell lines show regression upon treatment with the PLK1 inhibitor. This and many other studies suggest that PLK1 inhibitors might be effective in treating a broad range of tumors. A comprehensive discussion on PLK1 and the therapeutic potential of PLK1 inhibitors is given by Strebhardt and

Ullrich [66]. Although these KRAS-mutant cells show an increased mitotic index and a higher fraction of lagging chromosomes, the mechanism behind the mitotic-specific vulnerabilities is still unclear. PLK1 inhibitors are currently in clinical trials and it will be very interesting to see whether RAS tumors show an increased sensitivity in a clinical setting.

The single-well shRNA screen described by Scholl *et al.* led to the identification of *STK33* [67]. The oncogenic KRAS-dependent effects were tested in a large panel of tumor cell lines from different origins and showed that the dependency on STK33 correlated with addiction to oncogenic KRAS expression. In addition to a reduction of cell viability *in vitro* under adherent conditions, knockdown of STK33 reduced the number of colonies in soft agar, and impaired tumor growth of KRAS-mutant cell lines in mouse xenograft experiments. Knockdown of STK33 causes reduction of the levels of phosphorylated S6K1, resulting in decreased phosphorylation of S6K1's target BAD. As a consequence, BAD is stabilized and promotes apoptosis. Knockdown of BAD rescues cells from apoptosis induced by STK33 knockdown, indicating that BAD-mediated apoptosis is required for the STK33 dependency. However, BAD knockdown cannot rescue KRAS-addicted cells from KRAS knockdown suggesting that, in addition to BAD-mediated apoptosis, loss of mutant KRAS expression results in additional, BAD-independent, pro-apoptotic signaling. Finally, Barbie *et al.* performed a single-well shRNA screen in 19 different cell lines to identify synthetic genetic interactions with oncogenic KRAS [68]. They identified a synthetic lethal interaction between KRAS and TBK1, and were able to validate this interaction in an isogenic cell line panel and an independent panel of lung cancer cell lines. The impaired growth upon knockdown of TBK1 in this cell line panel correlated with the lethal effect of KRAS knockdown, indicating that the dependency on TBK1 is correlated with addiction to oncogenic KRAS. Analysis of the expression profiles derived from the isogenic cell lines suggested that oncogenic KRAS activates the NF- κ B pathway resulting in a prosurvival signal. This is concordant with the observation that upon knockdown of TBK1, total and nuclear levels of the NF- κ B family member c-Rel decrease, as well as the c-Rel target BCL-XL, an antiapoptotic protein. Interestingly, in the absence of KRAS addiction, knockdown of either STK33 or TBK1 does not result in a synthetic lethal phenotype.

Similar to the WNT/ β -catenin and VEGFR1 interaction, the observations by Scholl *et al.* and Barbie *et al.* suggest that STK33 and TBK1 do not have a direct genetic interaction with KRAS, but are linked to characteristics associated with the addiction to oncogenic KRAS signaling. Together with a biomarker that classifies tumors that are addicted to oncogenic KRAS signaling, STK33, TBK1 and similar genes can be exploited therapeutically.

Lessons learned & challenges ahead

It has become clear that many approaches can be taken to screen for novel drug targets. Whether the screen is aimed at the identification of synthetic lethal interactions or genes for which a differential dependency exists between tumor types, they will undoubtedly provide us with novel candidate drug targets for cancer therapy. Although it is less effort to screen a pair of isogenic cell lines, synthetic genetic interactions that show a high degree of context independency are more likely to be clinically relevant. On the other hand, synthetic lethal interactions identified based on a number of cell lines should also be validated in a relevant isogenic cell line pair to exclude the effects of confounding mutations and adaptations contributing to the synthetic genetic interaction. Technical advances related to screening with pooled shRNA libraries, particularly those allowing for hit selection based on negative selection, can be used to identify genes that, upon knockdown, are only lethal in a specific subset out of a large and diverse set of different cancer cell lines [54,55]. Although several screens discussed above have yielded interesting candidates for cancer-specific drug therapy, additional research is required to fully understand the molecular basis of the context-specific dependency. Of specific interest are the screens that identified genes for which a dependency exists that correlate with the addiction of a cell to a certain oncogenic lesion (e.g., STK33 with KRAS). Further research will undoubtedly increase our understanding of these network addictions, and provide an explanation for the fact that only a fraction of tumor cell lines harboring such genetic lesions displays an addiction.

It is clear from published work that the percentage of hits that hold true in a larger panel of cell lines can be quite low. Despite the fact that this approach will help the researcher to narrow down the hit list to the most relevant candidates to pursue, he or she might falsely discard a relevant hit. Controlling for comparable

knockdown efficiency when testing in multiple cell lines might reduce this risk, but will also make the validation more laborious.

Many of the RNAi-based screen publications contain follow-up on hits that have previously been linked with the phenotype or pathway of interest. When hit lists are long, pathway analyses or queries in interaction databases are frequently performed to narrow down the list to a manageable number of hits for which hypotheses can be raised and tested with a higher confidence of success than would be applicable to hits for which no link has been established yet, or for which no biological information is available. It is questionable whether this undermines the unbiased screening approach as it implicates neglecting true novel hits. One way to prevent this is by performing high-throughput follow-up studies with secondary assays to pinpoint the approximate level of action for signaling pathways. Another approach is to combine the analysis with that of clinical data, including therapy responses. This will lead to a more rapid identification of those hits that are clinically relevant and can be explored for therapy.

Despite ample examples of RNAi screens in identifying novel targets, novel biomarkers, and detailed insights in complex molecular networks controlling essential aspects of tumor cell behavior and survival, it is clear that the elaborate validation and follow-up of RNAi screening results still pose a significant barrier in the interpretation of the genes identified. The results of RNAi experiments can be influenced by the alteration of the expression of genes other than the intended target, known as off-target effects. As a consequence, multiple individual siRNA or shRNA sequences are required to display the same phenotype. In addition, one should confirm the knockdown phenotype correlation for the individual reagents and where possible rescue experiments should be performed with cDNA sequences that are resistant to the RNAi effects. These validation steps are both complicated and time consuming and as a result most screening efforts have only validated a very limited set of genes rather than generating completely validated gene lists. Technical advances in the field of RNAi reagents that reduce off-target effects and the generation of matching RNAi-resistant cDNA rescue libraries might bring some change to this.

Most of the RNAi screens and follow-up experiments are performed in cell lines grown as a monolayer *in vitro*. The extrapolation of these results to an *in vivo* situation and even clinical responses remains to be investigated. Several

studies have addressed this issue, in part, for the identified hits, including the studies of Scholl *et al.* and Luo *et al.* [65,67]. Luo *et al.* confirmed the dependency on PLK1 in a mouse xenograft model with a PLK1 inhibitor rather than PLK1 knockdown [65]. It is worth noting that most of the small molecules act by impairing a protein's function and therefore do not necessarily mimic the effect of reduced protein levels upon RNAi-mediated knockdown. However, PLK1 inhibitors are currently being investigated in the clinic, and this might provide clinical confirmation of this RAS-specific vulnerability.

RNAi drug enhancer, resistance & chemical genetic screens

RNAi screening technology can also be applied to identify genes that upon knockdown sensitize cells to a specific cancer drug. Such genes can be used as targets in combination therapy, but can also act as potential biomarkers of drug response. Drug-enhancer screens have been performed using conventional cytotoxic drugs, including paclitaxel, gemcitabine, cisplatin, doxorubicin and fluorouracil [69–74]. However, there are also some examples of RNAi screens for drug-enhancing effects with targeted molecular therapeutics, such as TRAIL and PARP inhibitors [75,76]. Turner *et al.* performed a siRNA screen to identify kinases that upon knockdown sensitize cells to a PARP inhibitor [76]. Among the four validated genes that upon knockdown enhance the sensitivity is *CDK5*, a gene that has not been linked to DNA damage repair or DNA damage response before. The sensitizing effect of *CDK5* suppression is not limited to PARP inhibition but is also associated with enhanced sensitivity to treatment with camptotecin and cisplatin. Although the molecular mechanism behind the increased sensitivity to these therapeutics remains to be elucidated, these findings demonstrate the power of drug-enhancer screens in identifying novel drug response modulators. Furthermore, drug-enhancer screens with targeted therapeutics can result in the identification of genetic interactions between the identified gene and the drug target, such as the *CDK5*–PARP interaction.

In addition to drug-enhancer screens, RNAi can be used for the identification of genes that upon knockdown confer resistance to a specific drug treatment [18,25,77–79]. Iorns *et al.* describe a siRNA screen compromising the human kinome to identify kinases involved in the response to tamoxifen in estrogen receptor-positive breast cancer [79]. They subsequently showed that one of

their validated hits, *CDK10*, regulates the expression of c-RAF. The increase of c-RAF expression results in activation of the downstream p42/p44 MAPK pathway, which has previously been implicated in tamoxifen resistance. As reduced levels of *CDK10* expression confer resistance to tamoxifen, Iorns *et al.* analyzed whether *CDK10* expression can be used to predict the response in patients treated with tamoxifen. From this analysis, they concluded that low expression of *CDK10* was associated with a statistically significantly shorter time to disease progression, and significantly shorter overall survival. This and other examples clearly illustrate the potential of large-scale RNAi screens to identify novel mechanisms of drug resistance.

In addition to synthetic genetic interaction screens and RNAi drug-enhancer screens, several chemical–genetic screens have been performed in which chemical compounds are screened for selective toxicity in isogenic cell lines [80–82]. Although both RNAi and a specific inhibitor would impair protein function, they are not completely complementary to each other. The partial or complete loss of protein expression by RNAi can be dissimilar to the consequence of treatment with a chemical agent. For example, loss of expression can result in both the loss of a protein's activity as well as disruption of protein complexes, while binding of an inhibitor can specifically affect either protein activity or protein–protein interactions. Hence, chemical–genetic screens will complement the synthetic genetic screens in the identification of novel cancer-specific drug targets and therapeutics.

Conclusion

The recent developments in RNAi-based screening tools have provided a powerful combination of target identification in a functional manner, and on a genome-wide scale. The implementation of this strategy in an unbiased search for synthetic interactions in mammalian cells has resulted in a number of potential targets. Although these targets still need further validation *in vivo* and inhibitors should be developed to test their value in clinical settings, they do show the promise of RNAi-based screening in cancer drug target discovery. In addition to further validation as drug targets, additional research is required to fully understand the molecular basis of the context specific dependency. Of specific interest are the screens that identified genes for which a dependency exists that correlate with the addiction of a cell to a certain oncogenic lesion (e.g., *STK33* with *KRAS*).

Further research might increase our understanding of these network addictions, which are only present in a fraction of tumor cell lines harboring such genetic lesions. Moreover, the identification of biomarkers for such addicted tumors will help selecting the patients that are most likely to respond to this type of targeted therapies.

Future perspective

Since its discovery, RNAi made it possible to interrogate the role of individual genes in complex cellular processes. In combination with the unraveling of the complete human genome, genome-wide RNAi screens can be performed that can identify, in an unbiased way, genes involved in biological and clinical relevant processes. Developments in RNAi-based screening technologies have fuelled the expectation of new discoveries. These include the identification of novel targets, novel biomarkers and detailed insights in complex molecular networks controlling essential aspects of tumor cell behavior and survival. It is to be expected that in the coming years improvements in not only RNAi

technologies, but also in screening models and validation strategies, will increase the output of RNAi screens. We will see an expansion of RNAi screening reagents, including large collections with validated knockdown and a better understanding of potential off-target effects and strategies to exclude or prevent them. These developments will be followed by an increase in throughput, miniaturization and high-throughput validation reagents and methods. One major development will be the routine implementation of spotted RNAi reagents on glass slides to enable high-throughput, high-content screens in living cells. Further development of more complicated phenotypical readouts will increase the relevance of the cellular screening models, resulting in more relevant hits. At the same time, rather than end-point assays, the developments in kinetic measurements will more specifically address the questions of the role of individual components in complex cellular networks. This will undoubtedly lead to a better and more complete understanding of the regulatory networks and more importantly

Executive summary

The need for cancer-specific treatment strategies

- Despite the established effectiveness of conventional cancer therapeutics, their shared mode of action – interference with cell division or DNA replication processes by causing DNA damage – is generally associated with low therapeutic indices.
- As the conventional cancer therapeutics rely on cell division, they do not target dormant tumor cells, which might include cancer stem or initiator cells.
- Hence, there is a need for cancer-specific therapeutics that do not solely rely on cell division for their effects.

Targeted cancer therapies & network addiction

- Targeted therapeutics, including those based on pathway-targeted therapeutics, are based on the addiction of a tumor to certain cancer-specific cellular signaling pathways, and target the driving oncogenic event.
- Resistance mechanisms to these targeted therapeutics confirm the importance of oncogenic network addiction, and can direct us to critical nodes in cancer signaling pathways.
- In addition to addiction to oncogenic signaling, tumor cells can become hypersensitive to the reactivation of tumor-suppressor genes.

Molecular buffering & genetic interactions

- Not all cancer-specific lesions that lead to an addiction or acquired hypersensitivity can be targeted, however, up- or down-stream effectors can provide an indirect means of targeting these lesions.
- In addition, distinct networks that buffer overlapping cellular processes can contain valuable drug targets in the context of untargetable genetic lesions.
- Comparison of genetic interaction data from *Caenorhabditis elegans* with that of *Saccharomyces cerevisiae* has taught us that these genetic interactions are either not well conserved or masked by cellular-level redundancy in multicellular organisms.
- Consequently, unbiased functional genomic screens are required to identify negative genetic interactions with oncogenes or tumor-suppressor genes in human cancer.
- The identification of such interactions can yield novel drug targets and guide further investigation of cancer-specific vulnerabilities.

RNAi as a tool for functional genomics & identification of synthetic lethal interactions

- RNAi is the mechanism whereby introduction of dsRNA into a cell results in the degradation of a mRNA product that shares high complementarity with the dsRNA.
- Advances in the field of generating tools that mediate RNAi have yielded an extensive set of genome-wide RNAi libraries.
- Use of RNAi libraries in combination with a wide variety of assays have allowed the identification of novel modulators in signaling pathways and yielded insight into complex biological processes.
- Screens designed for the identification of genes involved in proliferation and apoptosis, or that have synthetic lethal interactions with oncogenes or tumor-suppressor genes will undoubtedly contribute to the discovery of novel cancer drug targets.

Executive summary

RNAi screens for synthetic lethal interactions with oncogenic RAS

- RAS-mutant cells are more sensitive to perturbations in mitosis as reflected by an increased sensitivity to inhibition of survivin, APC/C or PLK1 function.
- Oncogene-addicted Ras mutant cells are also more sensitive to inhibition of STK33, which is required for the suppression of BAD's function as a pro-apoptotic factor.
- In addition, RAS-mutant cells are specifically sensitive to inhibition of TBK1, which is involved in NF- κ B signaling and controls the expression of BCL-XL, an antiapoptotic protein.
- Both TBK1 and STK33 have a genetic interaction with mutant RAS that is limited to cells that display an addiction to oncogenic RAS signaling.

Lessons learned & challenges ahead

- Several RNAi synthetic lethal screens have been completed and resulted in the identification and subsequent validation of a small number of hits, most of which had a previously established link. Unbiased follow-up strategies might increase the list of validated hits.
- Synthetic lethal interactions identified in screens encompassing a panel of cell lines with known mutational status, require validation in isogenic cell lines to exclude interactions with confounding genetic events, and *visa versa*, hits from screens in isogenic cell lines need validation in other cell lines to exclude context dependency.
- Technical advances in the field of RNAi reagents that reduce off-target effects and the generation of matching RNAi resistant cDNA libraries will simplify the currently elaborate validation experiments.
- *In vivo* experiments, preferably with small-molecule inhibitors, are needed to investigate the potential of the identified genetic interactions as drug targets.

RNAi drug enhancer, resistance & chemical genetic screens

- In addition to genetic interaction screens, RNAi drug enhancer, resistance and chemical genetic screens can contribute to our understanding of network addiction and tumor-specific vulnerabilities.
- Furthermore, enhancer and resistance screens can lead to the identification of valuable biomarkers that may help guide selection of patients that will benefit most from a certain therapy.
- Chemical-genetic screens complement RNAi genetic screens in that they have distinct modes of action regarding the suppression of a protein's function; hindering protein-protein interaction, protein stability or catalytic activity versus suppression of gene expression.

Conclusion

- The recent developments in RNAi-based screening tools have resulted in a number of potential cancer drug targets based on synthetic lethal interactions.
- Further validation of these hits *in vitro*, but especially *in vivo*, and preferably with small-molecule inhibitors, can further pave the road to clinical implementation.
- More research is required to elucidate mechanisms of network addiction and to identify biomarkers for tumors that are addicted to specific network signaling. This will help select patients that are most likely to benefit from this type of targeted therapy.

the crucial nodes within these networks. Finally, the integration of other large-scale efforts with RNAi screening results, such as expression profiling, genomic alteration, complete genome sequencing, protein-protein interaction networks and the aforementioned complex, kinetic cellular network models, will definitely aid better hit stratification and result in the identification of novel cancer-specific vulnerabilities. Combination with the analysis of clinical data, including therapy responses, will lead to a more rapid identification of those hits that are clinically relevant and can be explored for therapy. The increase in screening speed and scale will result in more detailed understanding of network addiction and resistance to targeted therapies. This will further aid the selection of crucial nodes in cellular signaling networks relevant for tumor cell behavior and clinical response. Targeted therapies for such nodes might be less prone to acquired resistance, and applicable to a wider range of tumors that share a network

addiction driven by distinct genetic lesions. Based on these results, we will be able to predict which tumors are vulnerable to the inhibition of specific genes, and as such we will be able to select those patients that will benefit most from the targeted therapy. All together, these developments in RNAi screening technologies and interpretation of the results will lead to the identification of more relevant genes that can be explored for more specific and effective cancer treatment in a personalized manner.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

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