IN PERSPECTIVE

Cancer biology functional genomics: From small RNAs to big dreams

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Abstract

The year 2021 marks the 20th anniversary of the first publications reporting the discovery of the gene silencing mechanism, RNA interference (RNAi) in mammalian cells. Along with the many studies that delineated the proteins and substrates that form the RNAi pathway, this finding changed our understanding of the posttranscriptional regulation of mammalian gene expression. Furthermore, the development of methods that exploited the RNAi pathway began the technological revolution that eventually enabled the interrogation of mammalian gene function—from a single gene to the whole genome—in only a few days. The needs of the cancer research community have driven much of this progress. In this perspective, we highlight milestones in the development and application of RNAi-based methods to study carcinogenesis. We discuss how RNAi-based functional genetic analysis of exemplar tumor suppressors and oncogenes furthered our understanding of cancer initiation and progression and explore how such studies formed the basis of genome-wide scale efforts to identify cancer or cancer-type specific vulnerabilities, including studies conducted in vivo. Furthermore, we examine how RNAi technologies have revealed new cancer-relevant molecular targets and the implications for cancer of the first RNAi-based drugs. Finally, we discuss the future of functional genetic analysis, highlighting the increasing availability of complementary approaches to analyze cancer gene function.

KEYWORDS
cancer dependencies, functional genomics, RNA interference, RNAi, shRNA, siRNA

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1 | INTRODUCTION

Scientists worldwide routinely perform experiments in mammalian cells to assess gene function by inducing a gene-specific reduction in expression followed by measurement of a biologically relevant endpoint, often in as little as 48 h. The development of the reagents and methods that induce this targeted decrease in gene expression began with two reports in the spring of 2001 of sequence-specific silencing of gene expression in human and mouse cells.[1,2] These studies demonstrated that synthetic double-stranded RNA (dsRNA) molecules of 21 or 22 nucleotides (nts) transfected into mammalian cells could induce a substantial decrease in the levels of a transcript containing a sequence complementary to one strand of the synthetic duplex RNA. Importantly, the reduction in messenger RNA (mRNA) levels resulted in reduced levels of the protein encoded by the targeted transcript,[1] and the synthetic double-stranded RNA (dsRNA) did not trigger antiviral immune responses associated with exposure of mammalian cells to longer dsRNA molecules.[2] The rationale for testing whether dsRNA molecules of ~21 nts could target endogenous mRNAs in mammalian cells arose from previous studies conducted in model organisms. Specifically, studies performed in plants, fungi, *Caenorhabditis elegans*, and *Drosophila melanogaster* reporting a posttranscriptional gene silencing mechanism mediated by dsRNA that became referred to as RNA interference or RNAi.[3-10] Over the next few years, many groups defined the principal components—enzymes, substrates, and associated proteins—of the RNAi pathway.[11-31] Critical findings demonstrating RNAi's function in regulating endogenous gene expression soon emerged, building on earlier observations of the regulation of genes in *C. elegans* involved in development and differentiation by small RNAs.[32-42] The discovery of entire classes of regulatory noncoding RNAs, particularly microRNAs (miRNAs), that control gene expression via the RNAi pathway added additional levels of complexity to the regulation of the transcriptome,[43-47] leading to significant insights into how disruption of miRNA-regulated gene expression contributes to disease states, including cancer.[48-54]

Our ability to harness RNAi to study gene function represented a landmark achievement for the scientific community with immediate applicability across multiple biological contexts and disease phenotypes, especially cancer. Importantly, RNAi-based technologies enabled researchers to conduct large-scale loss-of-function (LOF) genomic screens that provided a means for unbiased discovery of drivers of carcinogenesis and drug development targets. Since 2001, hundreds of cancer-based studies have reported the employment of RNAi-based methods to study mammalian gene function, leading to the discovery of many genes with no previous links to cancer biology, and enhanced understanding of the functions of established oncogene and tumor suppressors. More recently, the extensive experience with RNAi and the global research efforts in optimizing screens and developing standardized pipelines for the analysis of LOF data across multiple cancer model systems have laid a foundation for the accelerated employment of CRISPR (clustered regularly interspaced short palindromic repeats)-based methods for functional genetic analysis. In this perspective, we will discuss how the discovery of RNAi and the development of functional genomic approaches that utilize small RNA-guided protein complexes have altered our understanding of carcinogenesis and furthered the identification of new cancer therapeutic targets (Figure 1). We will also consider the future impact of RNAi and other RNA-based technologies on our understanding of tumor biology and cancer treatment.

2 | TURNING BIOLOGY INTO TECHNOLOGY

The initial descriptions of RNAi in mammalian cells led rapidly to the generation of the resources that enabled researchers to deplete protein expression with relative ease. Specifically, the field saw the development of the tools we still use today: expressed short hairpin RNAs (shRNAs) and synthetic small interfering RNAs (siRNAs) that mimic precursor or mature miRNAs[55-59] (Figure 2). However, the design of artificial mediators of RNAi came with challenges. In particular, the prioritization of sequences in a transcript that favor target mRNA cleavage and degradation required extensive optimization. Over time, studies that defined miRNA-mediated gene regulation and iterative empirical studies that tested many different sequences corresponding to a specific transcript resulted in an enhanced understanding of the parameters that facilitate effective RNAi.[60-68] Based on these results, academic groups and commercial vendors developed computational models that could predict the sequence of active and specific siRNA or shRNA with some level of accuracy. Selection parameters included the nucleotide content at the 5′ ends of each strand of the dsRNA, with a high A/U nucleotide content at the 5′ end of one strand favoring its incorporation into the enzyme complex—the RNA-induced silencing complex (RISC), responsible for the interaction with the target mRNA.[69] Our group contributed to these efforts by demonstrating the impact of targeting the coding region of protein-coding genes versus regulatory 5′ or 3′ untranslated region (UTR) regions and sequence polymorphisms when selecting siRNA or shRNA sequences.[70,71]

Improved RNAi-effector design tools lead to the generation of siRNA or shRNA libraries initially targeting subsets of genes, such as gene families or genes with similar functions. These libraries progressed to become genome-wide in scale, paving the way for functional genomic studies previously impossible in mammalian cells.[72,73] The decision to use siRNA or shRNA screening platforms for functional genomic studies depended on many factors. However, in most cases, arrayed screens that involved the analysis of single or limited numbers of RNAi effectors per well of a multi-well plate typically used siRNA resources, while expressed shRNA resources frequently utilized a pooled approach (Figure 3). Parameters used to select a screening approach included the biological question that the screen aimed to address, the model system, the assay endpoint, and access to suitable infrastructure. As the scale of experimental RNAi-based workflows increased, so did the need to develop computational tools that facilitated assessment of data quality, the normalization of
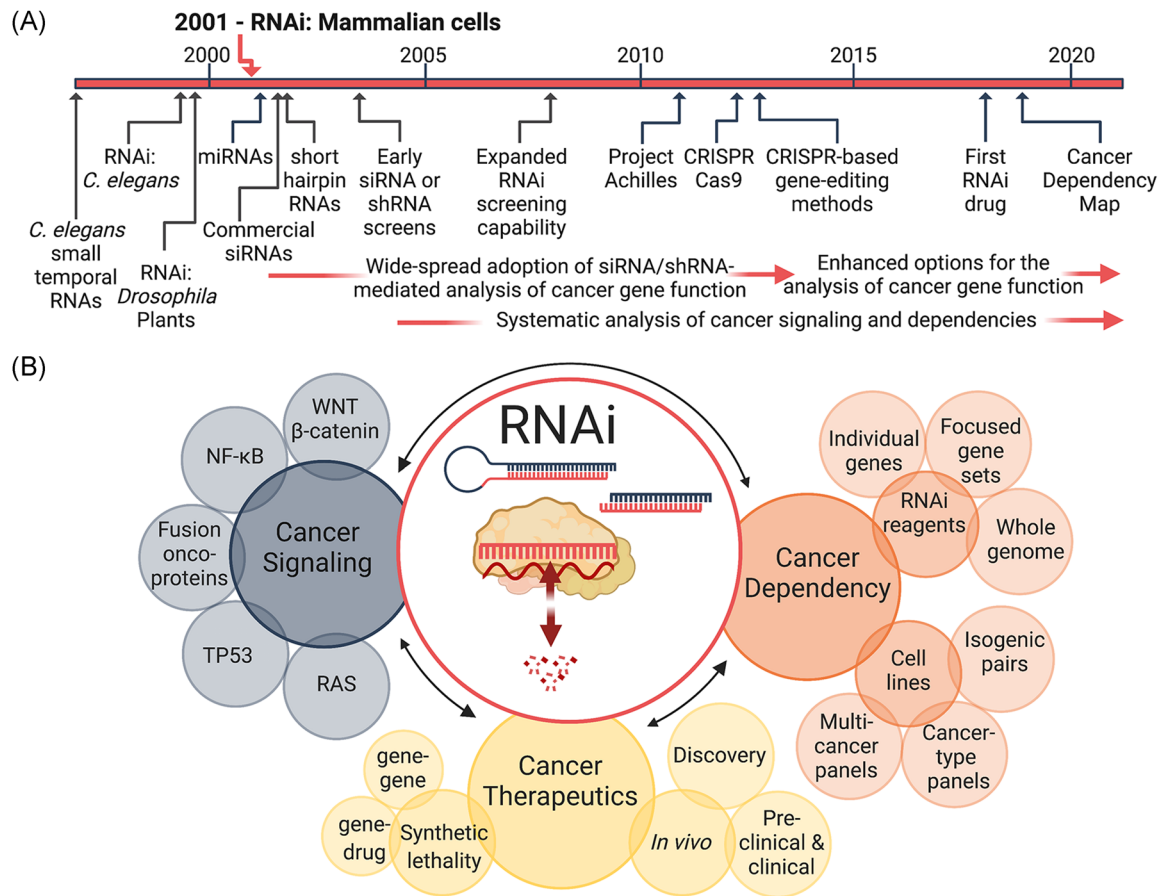
FIGURE 1 Cancer biology functional genomics. (A) Timeline highlighting the findings leading up to the identification of the RNAi pathway in mammalian cells and the subsequent development of RNAi-based methods for the study of cancer biology. (B) A schematic representation of the topics discussed in this perspective. The ability to manipulate gene expression via the posttranscriptional gene silencing mechanism, RNAi, has enabled interrogation of cancer signaling pathways and cancer-specific dependencies and further provided new avenues for the development of cancer therapeutics. *C. elegans*, *Caenorhabditis elegans*; CRISPR, clustered regularly interspaced short palindromic repeats; RNAi, RNA interference; shRNA, short hairpin RNA; siRNA, small interfering RNA

results across a screen, correction for sequence-biases, the statistical classification of lead candidate genes for further analysis, and the need for appropriate experimental procedures to validate the results of large-scale screens.[74]

As we recognize the successes that turned RNAi biology into RNAi technology, it is also critical to acknowledge the problems of data reproducibility and inconsistency that emerged as RNAi-based studies became ubiquitous. The development of second- and even third-generation RNAi reagents helped address some of the data reproducibility issues associated with early studies. However, it is vital to consider the RNAi mechanism's intrinsic features that generate variation, including differences in the efficacy of even highly optimized reagents and that RNAi reduces gene expression; it does not eliminate it. Over time, the increased publication of results that emphasize consistency in the phenotypic effects induced by siRNAs or shRNAs targeting the same gene has helped reduce the likelihood of false-positive results. Nevertheless, many RNAi-independent factors also need attention when assessing the results of any functional genomic study. For example, the number of cell lines used to define a

tumor-type or mutation-specific effect. The signal-to-noise ratio of the endpoint assay used to identify candidate genes of interest can also influence the interpretation of a study's results. Finally, the failure to include appropriate secondary assays or orthogonal approaches to validate initial findings can contribute to the pursuit of nonspecific targets.[75] However, no technology is perfect, and it is essential to remember that the advent of RNAi-based protocols for the manipulation of gene expression initiated whole new ways of examining mammalian biology, including the study of carcinogenesis.

3 | UNRAVELING THE MISWIRING OF CANCER SIGNALING

Most tumor cells exhibit alterations in signaling pathways that regulate the many processes required for carcinogenesis, including cell proliferation or differentiation, and environmental responses. Thus, it is not surprising that the cancer community quickly adopted RNAi-based functional genetic studies, including unbiased screens to study
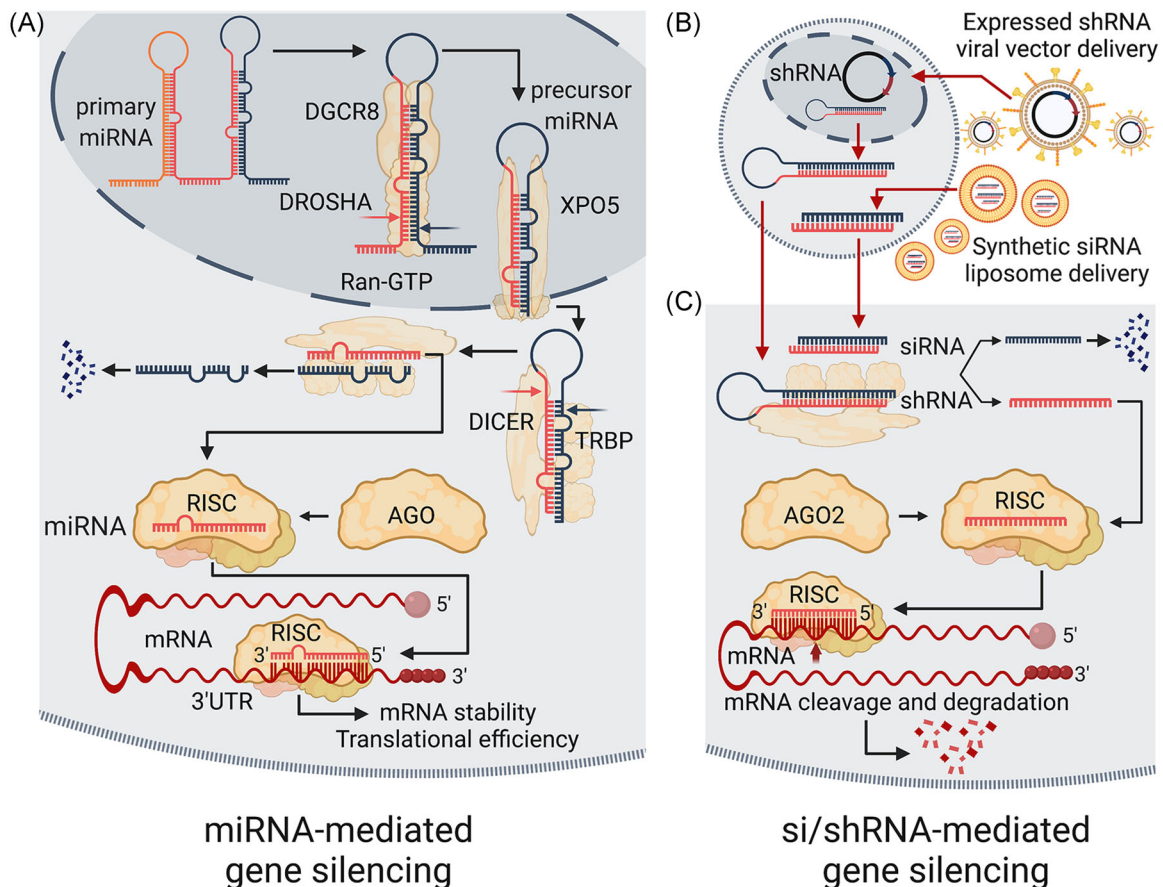
FIGURE 2 RNAi-mediated regulation of gene expression. (A) Simplified schematic of miRNA biogenesis and their subsequent effect on gene expression. In brief, the ribonuclease III enzyme DROSHA, together with the DGCR8–microprocessor complex subunit, processes the pol II transcribed primary miRNA transcripts to release the precursor miRNA (pre-miRNA). The translocation of the pre-miRNA to the cytoplasm requires the RAN–GTP binding protein (Ran-GTP)-dependent export protein Exportin 5 (XPO5). In the cytoplasm, a second ribonuclease III enzyme, DICER further processes the pre-miRNA and together with TRBP (TARBP2 subunit of RISC loading complex) facilitates the loading of the mature miRNA guide stranded into the RNA-induced silencing complex or RISC, with a member of the argonaute (AGO) family of proteins at its core. The miRNA-RISC binds one or more complementary mRNA sequences, predominantly in the 3′UTR of mRNAs with a tolerance of some nucleotide mismatches, altering mRNA stability and/or the efficiency of translation. (B) An outline of the principal methods used to deliver RNAi reagents (shRNAs or synthetic siRNAs) into cells. The delivery of shRNAs typically involves the cloning of the sequences corresponding to both strands of the duplex siRNA linked through a region that will form a stem-loop into a viral vector system (usually lentiviral-based) that will facilitate the expression of the shRNA in a mammalian cell. The delivery of chemically synthesized duplex RNA molecules, synthetic siRNAs, typically involves using a lipid-based reagent that facilitates uptake into the cytoplasm. (C) A simplified schematic of shRNA and siRNA-mediated targeting of a transcript for degradation via the RNAi pathway. Following the delivery or generation of the duplex RNA, the guide strand loads into RISC with argonaute RISC catalytic component 2 (AGO2) at its core. The RNA-loaded AGO2-RISC can interact with all regions of an mRNA, and through perfect or near-perfect base-pairing catalyzes the cleavage of the mRNA, resulting in transcript degradation. 3′UTR, 3′ untranslated region; miRNA, microRNA; mRNA, messenger RNA; RNAi, RNA interference; shRNA, short hairpin RNA; siRNA, small interfering RNA

the genetic and epigenetic basis of cancer, and the functional consequences of alterations in specific genes and pathways. Here, we discuss selected RNAi-mediated functional genetic studies of representative cancer-associated genes and pathways.

3.1 | WNT–β-catenin signaling and other developmental pathways

Several subtypes of cancer exhibit disruption of signaling pathways involved in embryonic development and cell differentiation. Because

of the crucial roles that developmental pathways, including Hedgehog (Hh) and WNT–β-catenin signaling, play in carcinogenesis, their characterization in healthy or tumor cells became the primary focus of many RNAi-based studies; an effort that continues using complementary functional genetic tools. Many of these RNAi studies used a reporter assay to measure the transcriptional output of a signaling pathway. For example, one of the first genome-wide RNAi screens conducted in 2003 used a *Drosophila* cell-based luciferase reporter assay of Hh signaling. The screen identified known components (e.g., Frizzled-1 and Frizzled-2 and GSK3-beta) and new regulators of the Hh pathway, including a link to WNT signaling
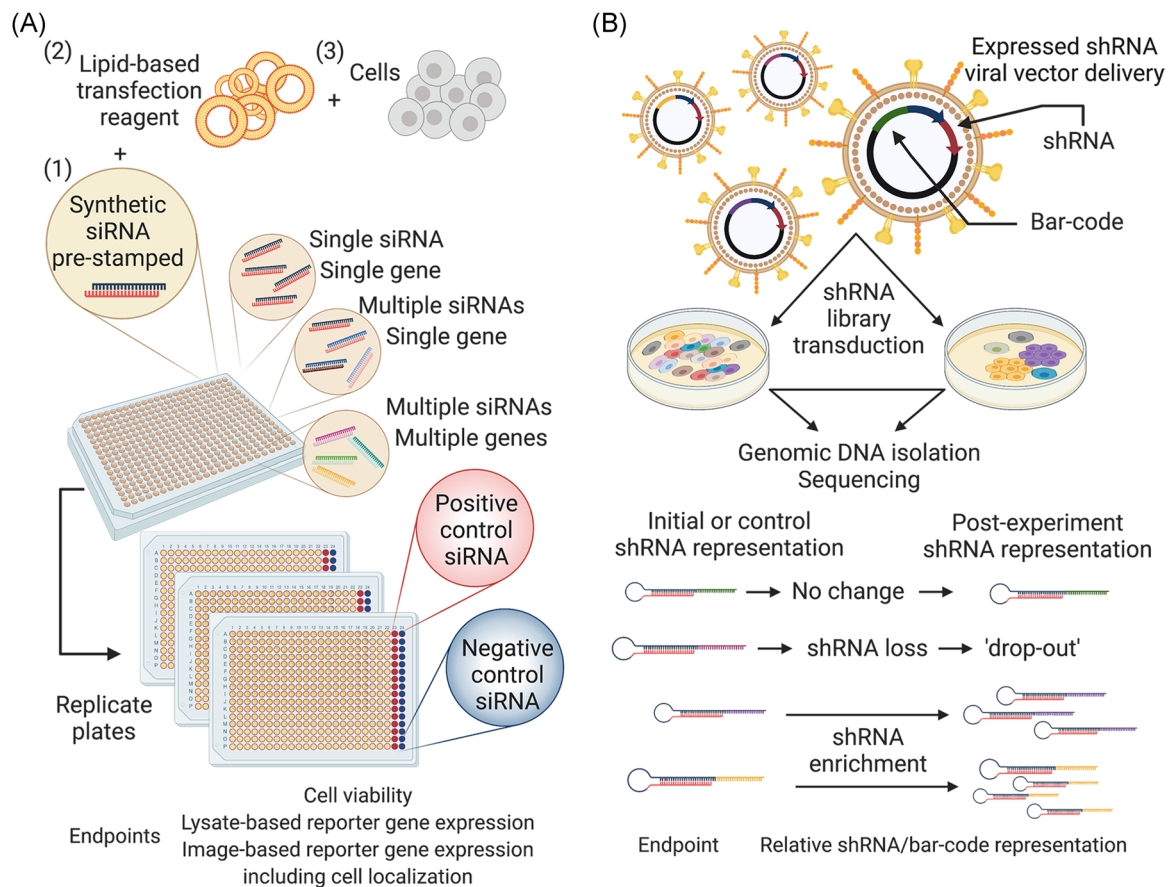
FIGURE 3 RNAi screening strategies. (A) A diagrammatic representation of plate-based arrayed siRNA screening strategies compatible with endpoints such as cell viability, and luminescence or fluorescence reporter assays. Synthetic siRNAs are pre-stamped into each well of a multi-well plate (1), and the siRNA–liposome complex formed by adding a pre-optimized amount of a lipid-based transfection reagent (2). Following the addition of cells (3) to the siRNA–liposome complex, the transfected cells grow for 24–96 h under standard cell culture conditions before assessing a relevant endpoint. (B) A simplified outline of bar-coded shRNA-based pooled screening using a lentiviral vector delivery system. Cell populations transduced using pooled libraries that retain a normalized representation of each shRNA form the basis of different experimental groups, for example, different time-points, Day 0 and Day 21, or two treatment conditions, vehicle or active compound. Following a selection period, the isolation of genomic DNA from each cell population allows for the assessment of the relative representation of each shRNA. RNAi, RNA interference; shRNA, short hairpin RNA; siRNA, small interfering RNA

through the activity of CK1α.[76] Another siRNA screening study performed in mouse cells expressing a similar reporter assay of the activation of the Hh pathway also resulted in further investigation of the links between the Hh and WNT pathways, highlighting the function of STK11 as a regulator of both pathways.[77]

Colorectal tumors frequently exhibit disruption of the WNT–β-catenin signaling that involves secreted WNT glycoproteins acting as activators of specific receptors, stimulating additional cellular responses, including gene expression changes regulated by β-catenin and members of the TCF/LEF family of transcription factors. To discover proteins that regulate the WNT-pathway, DasGupta et al.[78] used a *Drosophila*-based TCF-luciferase reporter assay of β-catenin transcriptional activity and identified over 200 putative regulators of WNT–β-catenin signaling. Critically, follow-up studies in zebrafish and mammalian cells confirmed the *Drosophila*-based results, including regulatory functions for the kinases LATS1, LATS2, and CDC2/CDK1. In mammalian cells, a 2008 publication also employed

a TCF-luciferase reporter assay in a genome-wide siRNA screen conducted in non-CRC cells, but that resulted in a focus on the function of the TCF/LEF family member, TCF7L2, in colorectal cancer (CRC).[79] A study published in the same year reported a genome-wide siRNA screen that also used a β-catenin–responsive luciferase assay, but this study used DLD1, colon adenocarcinoma cells, that harbor inactivating mutations in the tumor suppressor APC.[80] Follow-up analysis highlighted the function of AGGF1 that the authors linked to chromatin-remodeling, though to date, this finding's significance remains unclear. A third study published in 2008 reported the results of shRNA screens (targeting ~1000 genes) conducted in human colon cancer cells that assessed either cell viability or a readout of β-catenin-dependent transcription. Of the nine genes highlighted by these screens, one–CDK8–maps to a region of chromosome 13 frequently amplified in CRC. Subsequent studies showed that the function of CDK8 as part of the mediator complex contributes to the transcriptional activity of β-catenin.[81] We contributed

to another study that used RNAi to study candidate genes found within the region of chromosome 13 amplified in CRC. Interestingly, we found that silencing *LNX2* in CRC cell lines results in the down-regulation of NOTCH-regulated genes and TCF7L2-target genes. Furthermore, the examination of tumor-derived expression profiles found an *LNX2-TCF7L2* gene expression module in CRC samples.[82]

A further study of β-catenin transcriptional activity involved the correlation of data from a series of shRNA screens conducted as part of Project Achilles (discussed in further detail below in Section 4.4) with a cancer cell line's relative β-catenin activity.[83] This analysis and follow-up studies demonstrated the dependency of cells that exhibit high β-catenin on the function of the transcriptional coactivator YAP1.[83] Building on these RNAi studies, more recent CRISPR–Cas9 screens have revealed further vulnerabilities in cancer cells that depend on WNT-signaling. For example, a CRISPR-screen conducted in a pancreatic ductal adenocarcinoma cell line that demonstrated the importance of the Frizzled receptor FZD5 in cells harboring a mutation in *RNF43*.[84] The same group also recently described using a positive-selection assay to identify genes needed for β-catenin activity in cells harboring APC mutations.[85] The screen identified BCL9L, a known co-transcriptional activator of β-catenin, and IPO11, a protein not previously linked to β-catenin. Follow-up studies demonstrated that IPO11 regulates the nuclear transport of β-catenin in *APC* mutant cells that the activity of IPO11 could represent a mutation-specific vulnerability.

3.2 | NF-κB signaling

The transcription factor complex NF-κB regulates various biological processes, including B-cell development and inflammation, and so alterations in proteins that form part of the NF-κB signaling cascade contribute to the initiation and progression of several tumor types. An early focused shRNA screen used a cell-based reporter of tumor necrosis factor (TNF) activation, an endpoint of NF-κB signaling, to identify negative regulators of NF-κB signaling by targeting members of the deubiquitinating family of proteins.[86] This study identified CYLD (CYLD lysine 63 deubiquitinase) as a negative regulator of NF-κB that removes ubiquitin from the TNF receptor-associated factor TRAF2; loss of CYLD drives a proliferative signal via the core NF-κB regulator, the IKK complex. Interestingly, individuals with cylindromatosis harbor mutations in *CYLD* that results in multi-focal skin lesions, and so this RNAi-based study prompted the initiation of a clinical trial of the IKKβ signaling inhibitor salicylic acid.[87] Overall, treatment with salicylic acid resulted in the complete remission of 2 out of 12 cylindromatosis tumors, with some response in 8 other tumors.

In a hematologic tumor-type, several RNAi screens performed by Staudt and co-workers have probed the NF-κB signaling pathway in a subtype of Diffuse Large B-cell Lymphoma (DLBCL). In an initial study using a pooled shRNA screening strategy targeting 2500 genes performed in cell lines representing the two subgroups of DLBCL-activated B-cell (ABC) and germinal B-cell (GBC) and an IKKβ

reporter assay, revealed the dependence of the ABC-DLBCL on NF-κB signaling.[88] Specifically, the screens identified CARD11 and two interacting proteins, MALT1 and BCL10, as critical regulators of the ABC-DLBCL. A follow-up shRNA screen in cells representing the ABC-subtype of DLBCL identified Myd88 as a consistent vulnerability across multiple ABC-DLBCL cell lines.[89] This same screening project also identified IRAK1 as an essential gene for the viability of these cancer cells. More recently, the same group has used CRISPR–Cas9 functional genomic screens to identify a multi-protein supercomplex in the DLBCL-ABC subtype that drives JAK-STAT3 signaling via NF-κB regulated IL-10 and MyD88.[90]

3.3 | Fusion oncogenes

Cancer genomes frequently show the presence of structural rearrangements. In some cases, genomic rearrangements result in the generation of a fusion oncogene that is the principal driver of tumorigenesis. One class of fusion oncogenes consist of a 5′ regulatory region and part of the protein-coding sequences of one gene and a portion of the protein-coding and 3′ regulatory sequences of a second gene. This class of fusion oncogenes includes those that encode the fusion kinase BCR-ABL and the fusion transcription factor EWS-FLI1, the principal oncogenic drivers of chronic myelogenous leukemia and Ewing sarcoma, respectively. The controlled depletion of fusion oncogene expression using RNAi has proven indispensable for assessing their function in carcinogenesis. Targeting a fusion transcript using an shRNA or siRNA can facilitate the systematic comparison of markers of cell state (e.g., the cell growth characteristics or differences in the transcriptome or the proteome) in the presence and absence of the oncogenic driver responsible for the disease. Here, as an example, we will discuss some of the RNAi-focused studies of the EWS-FLI1 fusion oncoprotein that is the primary oncogenic driver in most cases of the soft-tissue and bone tumor Ewing sarcoma (EWS).

The early studies that used RNAi to probe the biology of EWS examined the dependency of EWS cells on the expression of the fusion oncoprotein EWS-FLI1, which functions as an aberrant transcription factor. Some studies examined the effect of silencing the fusion transcript on the growth of EWS cells in vitro or in vivo,[91–96] while other early studies assessed the function of the EWS-FLI1 protein.[97] Several RNAi-based studies analyzed the function of EWS cell lines modified to express an shRNA targeting the *EWS-FLI1* fusion transcript in the presence of doxycycline.[98,99] Using the shRNA-inducible system and increasingly sophisticated 'Omic-scale methods to profile the transcriptome and epigenome of EWS cells following the silencing of EWS-FLI1 enabled the identification of many of the cell signaling and metabolism pathway that EWS-FLI1 deregulates as part of the tumorigenic process and the function of EWS-FLI1 as an aberrant transcription factor.[98–106] In complementary studies, our work used RNAi screens and follow-up gene-specific RNAi analysis to discover and validate the sensitivity of EWS cells to the loss of proteins that process the *EWS-FLI1* fusion pre-mRNA.[107] One aspect of this and subsequent studies highlighted the dependency of about

one-third of Ewing sarcomas that harbor chromosome 22 translocations in intron 8 of the 5′ partner gene, *EWSR1*. Specifically, our work demonstrated that EWS cells harboring *EWSR1*-intron 8 breakpoints depend on the RNA binding protein HNRNPH1 to generate the in-frame protein-coding fusion mRNA in these tumor cells.[107,108]

3.4 | The TP53 tumor suppressor gene

The transcription factor TP53 is the most frequently mutated gene in cancer.[109,110] Significant consequences of changes in TP53 function include disruption of the cellular response to DNA damage and the maintenance of genome stability. The importance of understanding the complexity of TP53 biology made it a prime target for early RNAi-based functional genetic studies. One of the earliest large-scale shRNA screens employed a ~24,000 shRNA library targeting ~7900 human genes (combined with RNAi depletion of p16^{INK4A}) and a modified human fibroblast line that enabled the identification of genes that resulted in a bypass of TP53-dependent cell arrest.[72] This screen identified five candidate genes of interest that follow-up experimentation linked to the regulation of p21 (CDKN1A) expression.[72] The same study also introduced the concept of molecular bar-coding of shRNA libraries to ease the assessment of the relative enrichment and depletion, initially using arrays, and more recently, sequencing to quantify the relative levels of an shRNA sequence in the genomic DNA extracted from different cell populations (Figure 3B). The group then performed a TP53-related shRNA screen using a bar-coded shRNA library that studied the MCF7 breast cancer cell line's response to nutlin-3, an MDM2 inhibitor.[111] MDM2 can bind TP53 and target it for proteasomal degradation. The inhibition of MDM2, therefore, stabilizes the TP53 protein, potentially activating the TP53 pathway. A comparison of the relative abundance of the shRNAs present in control and nutlin-3 treated MCF7 cell populations following 14 days of culture identified, as expected, significant enrichment of shRNAs targeting *TP53*, and shRNAs corresponding to *TP53BP1*, among others. Subsequent experiments showed that the function of TP53BP1 in the DNA damage response and the alterations in this response in some tumor cells explained its identification in the RNAi screen.

A seminal investigation, published in 2003, employed an RNAi-based strategy to investigate the effect of alterations in gene expression on in vivo tumor development focused on TP53 and its function in lymphoma.[112] The study by Hemann et al. described three shRNAs targeting *Tp53* that reduced protein expression each by a different amount relative to a control shRNA. The stable introduction of each of these shRNAs into hematopoietic stem cells (HSCs) isolated from the fetal livers of *Eμ-Myc* mice (a B-cell lymphoma model) generated a *Tp53*-epi-allelic panel of cells. Critically, the transplantation of the *Tp53*-shRNA expressing cells into irradiated recipient mice showed a correlation between the levels of Tp53 and a reduction in survival. Specifically, those cells expressing the lowest level of Tp53 showed an accelerated decrease in survival compared

with control mice, and histopathology consistent with that observed in mice bearing *Tp53*-null tumors. In contrast, the lymphoma generated by the HSCs expressing an intermediate level of Tp53 showed a different phenotype including, a longer time to lethality and less pronounced systematic disease. In Section 5, we will discuss additional TP53-related in vivo RNAi studies.

Since the publication of these early studies (pre-2006), hundreds of reports have described application of RNAi-based strategies to probe TP53 function. Here, we will highlight one further study, focused on the bone tumor, osteosarcoma (OS).[113] The genomes of OS tumor samples are complex, with almost no consistent genetic changes, except for mutations altering *TP53* expression or function.[114] As part of an integrated study of OS, Perry and co-workers conducted a pooled shRNA screen (40,000 shRNAs targeting ~8400 mouse genes) using tumor cells derived from a mouse model of OS (conditional deletion of *Tp53* and *Rb* in pre-osteoblasts). This screen identified *Pik3ca* as a lead candidate gene. Subsequent analysis demonstrated a dependence of mouse OS cells on the PI3K/mTOR pathway that non-tumorigenic mouse bone cells did not exhibit. Furthermore, Perry and co-workers reported that small-molecule inhibition of PI3K and/ or mTOR inhibited the growth of mouse and human OS lines, suggesting genetic alterations, including alterations in TP53 function, may sensitize OS cells to disruption of signaling through this pathway. To date, the rarity and genetic complexity of OS have meant clinical translation of this finding has proven a challenge, but this is a targetable pathway that, in the long-term, may have applicability for the treatment of OS.

3.5 | The Ras oncoprotein family

Activating mutations in *KRAS*, *NRAS*, and *HRAS*, which encode for members of the RAS family of proteins, occur at high prevalence in many cancer types, particularly subtypes of lung and pancreatic cancers.[115] One of the earliest shRNA screens first focused on identifying genes that may alter RAS activity in the absence of a mutation using modified primary fibroblasts, with enhanced potential to transform. The screen identified the transcription factor PITX1 as a putative suppressor of RAS activity,[116] a finding subsequent studies of hepatocarcinogenesis confirmed.[117] Another study conducted a genome-wide RNAi screen in K-Ras-transformed NIH-3T3 cells to investigate the suppression of a proapoptotic pathway by RAS proteins.[118] The screen implicated over 25 candidate genes in the regulation of FAS expression, a proapoptotic molecule. A follow-up study by the same group described how several of these candidate genes form a RAS-regulated gene network and suppresses FAS expression by altering DNA methyltransferase, DNMT1 activity.[119]

An early attempt to identify proteins that mutant RAS cells are selectively dependent on for cell survival employed a 4000 gene siRNA library to screen the colon cancer cell line DLD-1 that harbors one mutant and one wild-type *KRAS* allele.[120] The screen identified 75 candidate genes that Sarthy et al. re-screened using isogenic *KRAS* cell lines. From this follow-up screen, they

selected to examine the relative dependency of the *KRAS*-mutant line on the function of the negative regulator of apoptosis BIRC5 (Survivin). Three independent shRNA-based RNAi screening studies, published in 2009, used similar approaches to discover mutant RAS dependencies.[121–123] Each used slightly different model systems, isogenic cancer cell lines, multiple KRAS mutant and wild-type cell lines, or cell lines exhibiting different dependencies on mutant KRAS activity, and different shRNAs libraries. Given these differences, it is unsurprising that while there was some limited overlap in these screens' results, each group focused on different lead candidate genes. Unfortunately, none of these leads proved to be a critical breakthrough, illustrating the difficulty of these types of large scale functional studies,[124] particularly when dealing with the analysis of an oncoprotein that influences many interconnected signaling pathways.

Besides the complexity RAS signaling, there is also the possible impact of functional redundancy, where, under certain circumstances, members of a family of proteins can substitute, at least in part, for each other's function. To determine if the depletion of more than one gene within the KRAS-signaling pathway and its associated pathways could reveal KRAS-co-dependencies, Lee et al.[125] used previously validated siRNAs designed using a third-generation sequence algorithm and a screening workflow that pooled siRNAs targeting multiple genes.[71,126] This study demonstrated that the combined targeting of RAF-kinase signaling (BRAF and CRAF/RAF1) and autophagy (autophagy E1 ligase ATG7) induces G1-arrest and apoptosis of KRAS-mutant cells while mediating minimal effects on nonmutant cells. The study by Lee et al.[125] also showed evidence that RAC1 function inhibition could act as a co-target of the RAF kinases. Interestingly, two studies also published in 2019 broadly supported the concept of parallel targeting RAF signaling and autophagy as an approach for the treatment of RAS-mutant tumors, particularly RAS-driven pancreatic adenocarcinoma.[127,128]

Enhanced shRNA screening efforts conducted in large numbers of cancer cell lines (discussed in further detail in Section 4.4) have also generated additional potential modulators of KRAS signaling.[129,130] For example, McDonald et al.[130] reported the susceptibility of a subset of KRAS-mutant lung cancer cell lines that lack dependency on KRAS. Instead, they found that cells harboring mutations in *KEAP1* exhibit a dependency on NFE2L2, and cells that have alterations in the expression of *SMARCA4* showed a dependency on *SMARCA2* function.[130]

The publication of these comprehensive shRNA screening efforts came at the same time as reports of the first wave of genome-wide CRISPR–Cas9 screens designed to examine RAS biology that broadly used the same approaches as used for earlier RNAi studies.[131,132] For example, Wang et al.[131] highlighted five genes—*RCE1* and *ICMT* that encode proteins required for the generation of the mature membrane-associated form of RAS; two genes encoding proteins that function within the MAPK signaling cascade—*RAF1* and *SHOC2*; and *PREX1*, encoding an RHO, small GTP-binding protein that binds and activates RAC1 and thus Rac signaling. Interestingly, the dependency on PREX1 proved specific for AML cell lines, due to the lack of a

paralog of this gene in these lines, versus other cancer cell lines. Martin et al.[132] used a similar screening strategy to find genes that altered the growth of *KRAS*-mutant DLD1 cells and not *KRAS*-wild-type HCT-116 cells and a follow-up screen in LS513 cells, another *KRAS*-mutant line. The results of these screens highlighted a new area of vulnerability in *KRAS*-mutant cells, that of dependence on mitochondrial pathways. Additional studies, including the use of other *KRAS*-mutant/wild-type isogenic cell line pairs, demonstrated that *KRAS*-mutant cells exhibit selective dependency on proteins involved in mitochondrial oxidative phosphorylation or translation. Importantly, *KRAS*-mutant cells proved more sensitive to the use of inhibitors of these processes; however, it is still too early to know if any of these new putative mutant-*KRAS* vulnerabilities are reproducible and will prove tractable as drug targets. These, and other large-scale functional genomic screening studies conducted in isogenic, or cancer or mutation-specific cell lines, highlight the importance of such approaches to find new cancer-specific dependencies. In the next section, we will discuss the cancer research community's efforts to use RNAi and other LOF approaches to discover cancer dependencies systematically.

4 | MAPPING CANCER DEPENDENCIES—MORE REALLY IS BETTER

As RNAi technology's capabilities improved, cancer researchers began to assess the feasibility of conducting systemic large screen studies across multiple cancer cell lines, initially representing a single tumor type, and then an increasingly diverse variety of cancers. The predominant rationale for developing these efforts was the need to conduct functional genetic screens in cell lines representing the genetic and histopathologic heterogeneity of human cancer. Crucially, the scale of these studies needed the statistical power to identify the effect of a perturbation in the context of another variable, for example, an oncogenic mutation. Most such studies used cell survival as an endpoint. Well-based arrayed siRNA screens typically used the quantification of a metabolite as a surrogate for cell viability measured 48–96 h posttransfection (Figure 3A), while screens using pooled retro- or lentiviral shRNA libraries assessed the relative enrichment or depletion (or, "drop-out") of a cell harboring a given shRNA, usually 14–21 days post-transduction (Figure 3B). If performed at scale, both approaches can identify: genes coding for proteins with essential functions; proteins that most cancer cells, irrespective of tumor type, depend on for cell growth—pan-cancer dependency genes; and those proteins, that when depleted, alters the survival of a specific subtypes of cancers. A protein that a particular subtype of cancer depends on for survival may reflect differences in cell lineage or mutational state. Early studies typically involved the publication of large-scale LOF data sets generated using screening approaches as supplementary information or deposition of results in data repositories (e.g., PubChem: https://pubchem.ncbi.nlm.nih.gov/; GenomeRNAi: http://genomernai.org[133]). However, with time we have seen more structured efforts develop, including

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web-based sites that enable the community to search standardized and regularly updated LOF data sets and retrieve gene-specific or whole-genome data.

4.1 | Early siRNA screens

An early study that tested the hypothesis that RNAi screens conducted in a panel of cell lines could identify pan or mutation-specific gene dependencies employed siRNAs targeting 700 kinases and kinase-related genes.[134] Screens performed in breast, lung, and cervical cancer cell lines (five in total) demonstrated a cell line-specific dependency on the expression of PIK3CA and the presence of a *PIK3CA* mutation. This study also identified correlations between protein expression, including the mitotic regulator WEE1, and sensitivity to its depletion by RNAi. Interestingly, a study published in the same year reported the results of siRNA screens conducted in the breast cancer cell lines MDA-MB-231, BT-20, and HCC-1937 also described the sensitivity of these lines to the loss of WEE1 function.[135] These screens and others employing siRNAs targeting subsets of human genes conducted in human cancer cell lines began to establish the infrastructure needed to study many cell lines in parallel, for example, siRNA screens performed in a panel of 34 breast cancer cell lines.[136] This study of over 30 breast cancer cell lines reported that over 90% exhibited dependence on the mitosis-associated kinases PLK1, WEE1, and AURKA. This screening effort also highly ranked kinases previously linked to breast cancer, such as PI3KCA, ERBB2, and CSK1, and the examination of a subset of lines showed the relative dependency of PTEN-deficient cells on the function of MPS1, a mitotic spindle checkpoint protein. The same study also revealed that the activity of the ADCK2 kinase influences estrogen receptor (ER) signaling and the proliferation of ER-positive breast cancer cells.[136]

4.2 | Early shRNA screens

Complementary efforts, over the same period, focused on the employment of large-scale shRNA libraries, with three of the earliest studies published in 2008 employing libraries of increasing complexity, ranging from ~3000 genes (8000 shRNAs) to 9500 genes (45,000 shRNAs), and a wider variety of cell lines, beginning with three cancer cell lines (two colon lines and one breast line) and one non-transformed line, and by the third study, a screen of 12 cancer cell lines.[137–139] The late 2008 study published by Luo et al.[139] of 12 cancer cell lines screened using a subset of a genome-wide shRNA library[140,141]; the RNAi Consortium (TRC) enabled the authors to report that 268 genes had essential functions across all the cell lines studied. Overall, this finding reflected the activity of the proteins encoded by these genes in processes such as translation, mRNA biogenesis, including splicing, and protein degradation via the proteasome. The study by Luo and co-workers also examined their data to identify genes that, when silenced, decreased the growth of cell

lines representing one tumor-type versus another, identifying 63 genes that non-small-cell lung cancer (NSCLC) exhibited greater dependency on for cell survival than other cell lines. Among cell-line specific genetic vulnerabilities, this study also identified the sensitivity of the chronic myelogenous leukemia cell line K562 that harbors the *BCR–ABL* fusion gene to the shRNAs targeting the *BCR* or *ABL* genes. Luo and co-workers also confirmed the utility of RNAi screens to identify oncogenes within amplified regions, such as the identifying *CRKL* as a putative oncogene in NSCLC, a finding supported by follow-up studies by the same group.[142]

4.3 | Statistical analysis of RNAi screens

An essential part of developing the workflows for large-scale RNAi screening efforts (siRNA and shRNA) was the need for robust statistical analysis of the data these efforts generated. Such data analysis requires consideration of many technical and biological variables, including, though not limited to, the following: (1) The definition, quantification, and application of basic quality control metrics. (2) A means for appropriately handling inter- and intra-screen variation to facilitate valid comparisons within and between screens. (3) The consideration of RNAi-specific variables, including differences in the efficacy with which an siRNA or shRNA silences the expression of a target transcript or miRNA-like off-target effects. (4) The method used for the normalization and ranking of screening results and the criterion used to define a candidate gene for further analysis. (5) The ability to integrate functional genomic data sets and other relevant large-scale profiling results, including gene copy number and expression data.

Over time, several groups have reported statistical approaches designed to reduce the false-negative or positive rates of RNAi screens. For example, a statistical analysis of siRNA data used the median and the median of absolute deviations (±3) coupled with assessing the normalized data in quartiles, to center and identify candidates of interest.[143] This approach proved more robust than the mean ± three standard deviations more typically used to analyze small molecule screening data. Other statistical methods incorporated consideration of the consistency of phenotype generated by siRNAs targeting the same gene. For example, if an RNAi library contains four to six siRNAs targeting each gene, then there is enough redundancy in the data set to determine if the clustering of the results of these siRNAs is significant (Redundant siRNA Activity—RSA score) and more likely to represent a high confidence data point.[144] The Luo study mentioned above[139] also used a ranking-based approach called RIGER—"RNAi gene enrichment ranking" score method to identify lead candidate genes. Like the previous study, RIGER also considers the ranked position of the shRNAs targeting a given gene, but in this case, applied gene-set enrichment methods and Kolmogorov–Smirnov statistical analysis to guide the selection of genes for follow-up analysis. Other statistical methods assessed the consistency between the effects of siRNAs targeting each gene and considered the impact of miRNA-like off-target

effects. For example, one study used a genome-wide RNAi screening data set to determine the effect of siRNAs with identical seed sequences, developing a common seed analysis (CSA) score for each siRNA in the screen. Such analysis allows for the de-prioritizations of siRNAs with a high CSA score due to a promiscuous seed sequence.[145] An extension of this study used the heptamer seed sequence at the 5′ end of the guide strand to identify previously unidentified candidate genes of interest within genome-wide siRNAs screens.[146] Finally, with the increased availability of large-scale RNAi screening data using the same reagents in different screens, it was possible to devise computational methods that encompassed several features of these earlier efforts, in an easier to apply fashion. One such example, ATARiS—Analytic Technique for Assessment of RNAi by Similarity—used the large data sets developed as part of Project Achilles (discussed below) to develop gene and RNAi reagent level scores. By comparing the phenotypic effects of individual reagents across multiple screens, ATARiS facilitated the removal of reagents with significant off-target effects, while retaining RNAi effectors that mediated on-target suppression of gene expression.[147] ATARiS proved to be a starting point for a series of subsequent algorithms used to analyze RNAi data, such as DEMETER[129] and DEMETER2[148] and the analysis of CRISPR–Cas9 gene editing screens conducted in cancer cell lines[149] that must take account of gene copy number effects.[150,151]

4.4 | Building a cancer dependency map

The profiling of the genome, epigenome, and transcriptome of human tumors has revolutionized our ability to identify causally relevant changes in cancer cells that can assist both the diagnosis and treatment of cancer. However, while these high-resolution methods can detect molecular alterations in an individual tumor, we still lack an understanding of the impact of many of these changes on the affected gene's function. Enhancing the scale of cancer LOF screens and integrating these data sets with other data, including gene copy number, mutation status, and expression patterns, has the potential to address this deficit. In 2011, Cheung et al.[152] published the results of a systematic study of over 100 cancer cell lines employing an shRNA library targeting 11,194 human genes (54,020 shRNAs in total). This effort, part of a broader program called Project Achilles, aimed to identify cancer cell-type-specific genetic vulnerabilities across multiple cancer cell lines representing ovarian, colon, pancreatic, esophageal, and NSCLC, or glioblastoma. The initial study confirmed the dependency of colon cancer cell lines on the expression of *KRAS*, *CTNNB1*, and *BRAF*, and as discussed above, the differences in the sensitivity of wild-type and mutant *PIK3CA* cell lines to the targeting of *PIK3CA* by RNAi. Next, to aid the discovery of new cancer-type specific dependencies, Cheung et al.[152] integrated gene copy number and RNAi data, identifying PAX8 as a dependency in ovarian cancer cell lines that harbor amplification of the region containing this gene. Another study, published in 2012, employed an shRNA library targeting 16,000 genes to screen breast, pancreatic,

and ovarian cancer cell lines (72 lines in total).[153] In this case, the integration of expression profiles that correlate with different subtypes of breast cancer and RNAi screening data identified potential breast cancer subtype-specific vulnerabilities; findings supported and extended by a follow-up study that included the results of shRNA screens performed in 77 breast cancer cell lines.[154] Most of these studies used statistical analysis to reduce the false-negative and positive rates, but others also took the approach of increasing the number of RNAi effectors per gene. For example, a study by Hoffman et al.[155] showed that applying the RSA method[144] using data from ~17 shRNAs per gene allowed the identification of high confidence lead candidate genes. In this study, the shRNA library targeted genes coding for proteins involved in the regulation of chromatin state, and its employment in screens of 58 cancer cell lines identified the dependence of SMARCA4-deficient cells on the function of another SWI/SNF complex member, SMARCA2.[155]

By 2014, Project Achilles included a data set of shRNA screens targeting 11,000 genes (5 shRNAs per gene) performed in 216 cancer cell lines,[156] and by 2017 this resource had expanded to over 500 cell lines.[129] A complementary effort, also published in 2017, reported shRNA screens targeting 7837 genes (~20 shRNAs per gene) in 398 cancer cell lines.[130] Referred to as Project DRIVE, this study noted many of the previously defined dependencies, including cell lines harboring *NRAS*, *BRAF*, *KRAS*, or *PIKSCA* mutations. Integration with other data sets, such as gene expression profiles, revealed other vulnerabilities. For example, McDonald et al.[131] reported the dependence of several cancer cell lines on the expression of lineage-specific genes, particularly transcription factors such as SOX10. Critically, these and other efforts supported the development of web interfaces that facilitate the ability of the research community to search or download data sets and associated software packages, now harmonized within a single portal—the DepMap portal—https://depmap.org/portal/. Today, the DepMap portal integrates the data from RNAi and several CRISPR–Cas9 LOF large-scale screening projects encompassing over 700 cancer cell lines, along with many other data sets, including the multiparametric analysis of the cell lines that make up the Cancer Cell Encyclopedia (CCLE),[157] and drug sensitivity data.[158] Importantly, this represents an ongoing collaborative effort involving multiple institutions and funding sources that continues to employ pooled CRISPR–Cas9 gene-editing screens to define cancer-specific vulnerabilities.[149,159,160] The reader is encouraged to explore the DepMap portal interface and make use of the regularly updated results available at https://depmap.org/portal/download/. However, it is essential to remember that any hypotheses generated using these, and other large-scale cancer cell line data, will always need extensive experimental investigation and validation using orthogonal approaches.

5 | RNAi SCREENING IN THE MOUSE

The examination of gene function through the application of small RNA-guided functional genetic tools has accelerated our ability to delineate the activity of complex transcriptional and signaling

cascades and identify new candidate target proteins for the treatment of cancer and other diseases. However, most such studies have involved the use of cell lines established years or even decades ago and grown as monolayers on treated plastic. While informative, such experiments lack the biological context that a whole-organism study can offer. For this reason, many groups sought to adapt RNAi and now CRISPR-based technologies for application in vivo. *C. elegans* proved a remarkably straightforward organism for the study of gene function in vivo by RNAi because there is no need for a carrier molecule to introduce the dsRNA,[161–163] but mouse models have and remain the principal whole-organism used for the study of cancer biology. As discussed above, an early in vivo RNAi study that investigated the function of TP53 in B-cell lymphomagenesis used shRNAs targeting *Tp53* to generate an epi-allelic cell series and showed a correlation between the levels of Tp53 protein and disease state.[112] However, difficulties in predicting and controlling the exact level of silencing by a given RNAi effector that emerged over the next few years meant that few others adopted this approach. The study by Hemann et al.[112] did though, along with others (e.g.,[164–166]), spur the use of ex vivo manipulated cells that expressed a gene-specific shRNA (constitutive or inducible expression) to study the consequence of targeting the candidate gene of interest on the initiation or maintenance of tumor growth, through the reintroduction of the modified cells to mice as transplants or xenografts. Here, we focus on the discovery potential of in vivo RNAi screens, typically conducted using pooled shRNA libraries. For an expanded discussion of the study of cancer in vivo using complementary gain- or loss-of-function functional genomic approaches, we direct the reader to a recent review by Weber et al.[167]

One early in vivo RNAi screen interrogated ~300 mouse genes for potential tumor suppressor function using $p53^{-/-}$; *Myc* hepatocytes (a liver tumorigenesis model) transduced ex vivo and transplanted subcutaneously into immunocompromised mice.[168] By analyzing the relative depletion or enrichment of specific shRNAs assessed by analysis of genomic DNA harvested from the resulting xenografts, Zender et al. identified the known tumor suppressor PTEN and several previously unknown putative tumor suppressors, including XPO4, that follow-up studies implicated in the regulation of proteins involved in TGF-β signaling or translation initiation. Another study from the same group used an analogous RNAi screening approach, but targeting ~1000 genes and HSCs from *Eµ-Myc* mice to identify B-cell lymphoma-associated tumor suppressors.[169] This screen identified several putative tumor suppressors, including a member of the DNA damage response machinery, Rad17, a finding corroborated by further studies, including a recent analysis of homozygous deletions in over 2000 primary tumors.[170] A complementary in vivo RNAi study discovered proteins involved in cell motility using shRNAs targeting about 1000 genes and the *Eµ-Myc* B-cell lymphoma model, focusing on proteins that modulated the growth of cells grown in vivo but not in cell culture.[171] Subsequently, the same group reported a genome-scale shRNA screen designed to identify modulators of B-cell leukemia progression, again comparing in vivo versus in vitro growth effects. This screen identified several

genes where growth in vivo generated specific dependencies, including a dependency on the function of the transcriptional regulator Phf6.[172] As an example of RNAi in vivo studies focused on a solid tumor, one study, by Iorns et al.[173] used modified nontransformed human mammary epithelial cells transduced with an shRNA library ex vivo and grown as xenografts in the mammary fat pads of severely immunocompromised mice. The screen identified established tumor suppressors such as TP53, and several additional tumor suppressor candidate genes, including the leukemia inhibitory factor receptor,[173] that more recent studies have shown may influence the metastasis of breast cancer cells to bone.[174]

Another highly informative series of in vivo RNAi studies used lentiviral packaged shRNAs injected into mouse embryos in utero.[175–177] This approach facilitates the genetic modification of the progenitor cells that will form the mouse epidermis and, depending on the embryo's genetic background, can enable examination of normal and oncogenic growth. Fuchs and co-workers first assessed this method by analyzing the effects of silencing individual genes and then conducting large-scale pooled shRNA screens using a similar strategy. One of the shRNA screens conducted in the context of a mutated HRAS demonstrated dependency of the mutant cells on the function of Ctnnb1 or Mllt6, findings the authors successfully replicated in cell line models of human squamous cell carcinoma (SCC).[176] A separate shRNA-screen (347 mouse genes) also used an in utero approach, but this time using embryos derived from TGFβ-Receptor-II conditional knockout mice, the same group identified Myh9 as a putative tumor suppressor in SCC.[177] Further studies suggested that Myh9 acts as a regulator of Tp53 stability and cellular localization.[177]

6 | WHEN TWO (OR MORE) ARE BETTER THAN ONE

Combination chemotherapy remains the backbone of cancer care and is likely to remain so for many tumor-types for years to come. For this reason, determining the optimal genetic background in which a drug acts or identifying more effective drug combinations was an early focus of RNAi studies and screens. Many of these studies built on the concept of synthetic lethality that had derived from results in fruit flies and yeast, which showed that in some cases, the function of one gene could compensate for the LOF of another, resulting in no discernable phenotype after genetic disruption of one or other of these genes. However, mutation of both genes results in organismal demise, or synthetic lethality.[178–181] In cancer, synthetic lethality typically refers to a gene that is nonessential when depleted in a nontransformed cell, but if depleted in a transformed cell harboring a tumor-specific mutation or combination of mutations, the cancer cell dies. Several studies described above illustrate the use of RNAi screens to identify synthetic lethality interactions, and the cancer dependency map also aims to identify such interactions by integrating LOF phenotypic information with mutational status or gene copy number. Here, we will highlight two further examples of

the identification of potential synthetic gene–gene interactions revealed by RNAi studies of *MTAP* and *PRMT5*, and *ARID1A* and *ARID1B*. We will also discuss another aspect of synthetic lethal interactions of interest to the cancer community, that of gene–drug interactions, focusing on genes that modulate chemotherapy agents.

6.1 | MTAP and PRMT5

The protein 5-methylthioadenosine phosphorylase (MTAP) catalyzes the breakdown of methylthioadenosine (MTA) to adenine and methionine. The proximity of the *MTAP* gene locus to *CDKN2A* (p16) results in the frequent loss of this gene in several cancer types. Three separate studies published in 2016 combined genetic information and the results of shRNA functional genetic studies to identify MTAP synthetic lethal interactions, all of which identified PRMT5 as a lead candidate.[182–184] Two groups used the shRNA screening data from the cancer cell lines that are part of the CCLE and identified the subset of genes, that when depleted, resulted in the loss of viability of MTAP-deficient cell lines,[182,183] while the third performed shRNA screens in HCT116-*MTAP* wild-type and homozygous deleted isogenic cell lines.[184] In addition to identifying protein arginine methyltransferase 5 (PRMT5), the screens also identified several known PRMT5 cofactors or proteins that function within the same pathway, including MEP50/WD77 and MAT2A. Biochemical studies demonstrated that the accumulation of MTA due to loss of MTAP resulted in the structural reordering of the cofactor binding pocket of PRMT5, decreasing its catalytic activity; however, tumor cell lines remain viable but sensitive to further disruption of PRMT5 function.[182–184] These studies highlighted a potential therapeutic strategy in MTAP deficient tumors where molecules that competitively bind to and inhibit PRMT5 in the same manner as MTA would lead to selective lethality in tumor cells. Building on this concept and other evidence,[185] several PRMT5 inhibitors (GSK3326595, JNJ-64619178, and PF-06939999) are now the subjects of current early phase clinical trials in patients with different subtypes of leukemia or lymphoma or advanced solid tumors (NCT02783300, NCT03614728, NCT03573310, and NCT03854227).

6.2 | ARIDIA and ARID1B

Another cancer-relevant synthetic lethal interaction that emerged from an analysis of the results of Project Achilles screens involved the examination of the shRNA screens conducted in *ARID1A*-mutant cell lines (18 lines) versus non-*ARID1A*-mutant lines (>100 lines).[186] Multiple cancers types exhibit mutations in *ARID1A*, a gene that encodes a member of the SWI/SNF chromatin-remodeling complex. Analysis of Project Achilles shRNA screening data showed *ARID1B* expression as essential for the viability of cell lines harboring *ARID1A* inactivating mutations. Follow-up studies demonstrated tumor cells harboring *ARID1A* mutations depend on the ARID1B, a close homolog of ARID1A, for the assembly of the SWI/SNF chromatin-

remodeling complexes. In the absence of both ARID1A and ARID1B, the disruption to the generation of SWI/SNF complexes results in cell death.[186] To date, there is no published ARID1B inhibitor; however, a recent analysis of inhibitors of other epigenetic regulators revealed that inhibition of EZH2, a methyltransferase member of the polycomb repressive complex 2, has efficacy in *ARID1A*-mutant ovarian clear cell carcinoma cell lines in vitro and in vivo.[187] Clinical trials with EZH2 inhibitors in various cancer types are ongoing, including the recruitment of patients with tumors associated with mutations in members of the SWI/SNF complex, such as rhabdoid tumors and synovial sarcoma (NCT02601950 and NCT02601937).

6.3 | Chemotherapeutic agents plus one

The applications of RNAi screening to find synthetic lethal gene–drug interactions relevant to cancer treatment began in 2006 with a siRNA screen targeting 20,000 genes conducted in HeLa cells.[188] This study compared the effects of each siRNA alone or combined with either the nucleoside analog pro-drug, gemcitabine, the microtubule stabilizer, paclitaxel, or the DNA cross-linker, cisplatin.[188] In this study, the silencing of genes coding for members of the BRCA family of proteins or the RAD6/RA7D18 DNA repair pathways significantly enhanced cisplatin activity. The authors, Bartz et al., attributed this finding to the silenced cells' inability to mitigate cisplatin-induced DNA damage in the context of mutant TP53 and loss of BRCA1 or other members of the BRCA1/2 network. Their findings contributed to the understanding of BRCA1-deficient and BRCA1-like tumors and their response to DNA-damaging agents. Subsequently, in 2007, Whitehurst et al.[189] performed siRNA screens in combination with paclitaxel, but in the NSCLC cell line NCI-H1155. The study identified 87 candidate paclitaxel-sensitizing genes that included multiple genes encoding components of the proteasome or the mitotic spindle apparatus. Our siRNA–chemotherapeutic drug combination studies included several examining the topoisomerase 1 (TOP1) inhibitor camptothecin (CPT). TOP1 inhibitors create drug-stabilized cleavage complexes that result in DNA damage, leading to their use as part of chemotherapeutic regimens to treat ovarian, lung, and colon cancers. For example, the treatment of ovarian and colon cancers often involves administrating CPT's clinical analogs—topotecan and irinotecan, respectively. However, TOP1 inhibitors have not performed successfully in clinical trials testing their efficacy to treat advanced breast cancer patients.[190] To determine if combining TOP1 inhibition with the inhibition of a second target could broaden the employment of TOP1 inhibitors, we conducted several siRNA screens to find proteins, that when inhibited, increase the efficacy of the TOP1 inhibitor, CPT, using different breast cancer cell lines representing the hardest to treat subtype, so-called triple-negative breast cancer.[191,192] One screen identified ataxia telangiectasia and Rad3-related protein (ATR) as a sensitizing target of CPT,[191] which prompted a follow-up RNAi screen and follow-up study of an ATR inhibitor.[193] Together, these studies contributed to the rationale of two multicenter clinical

trials sponsored by the National Cancer Institute that are ongoing (NCT02487095 and NCT02595931) testing the combination of ATR inhibition and the TOP1 inhibitor topotecan. Recently, the results of a phase one clinical trial combining topotecan with the ATR inhibitor M6620 (previously VX-970) demonstrated that the maximum dose of the combination was tolerable and showed promise in refractory small-cell lung cancer.[194]

7 | INTO THE CLINIC

The previous section discussed how RNAi studies guided the development of clinical trials testing traditional small-molecule drugs, but can we use the RNAi mechanism directly as a therapeutic approach? Groups began testing the potential of employing RNAi effector molecules to treat human diseases within a year of the initial studies reporting its presence in mammalian cells, targeting viral pathogens, including HIV, and mutant transcripts, including those associated with neurodegenerative diseases or cancer.[91,195–198] However, it took almost two decades for these initial efforts to bear fruit, with the approval of the first RNAi-based therapy in 2018 for the treatment of hereditary transthyretin amyloidosis (hATTR) based on the completion of a successful Phase III trial of Patisiran, a siRNA targeting the *TTR* transcript.[199] In late 2019, another siRNA-based drug, Givosiran—a siRNA targeting hepatic delta-aminolevulinic acid synthase 1 (*ALAS1*)—received approval for the treatment of acute hepatic porphyria (AHP), in part, because of the results of a recently published Phase III trial.[200] Other RNAi therapies at an advanced stage of development include a siRNA targeting of *PCSK9*, the subject of several recent clinical trials[201–204] involving patients with heterozygous familial hypercholesterolemia or atherosclerotic cardiovascular disease. However, building on these successes for the treatment of cancer has proved a challenge, and to date, the effective delivery of RNAi effector molecules into enough tumor cells to mediate a clinically relevant response remains the most significant obstacle. Several recent reviews have discussed the development of nucleic acid-based therapies and RNAi therapeutics, and for further details, we direct the reader to these.[205–209] Here, in brief, we will discuss two clinically focused efforts testing the use of siRNA-mediated gene silencing to treat solid tumors—the targeting of mutant-*KRAS* transcripts (siG12D-LODER) and the silencing of the gene encoding the EPHA2 receptor tyrosine kinase.

As discussed in Section 3.4, mutations in *KRAS* are present in many tumors, and several early RNAi studies assessed the feasibility of selectively targeting the transcripts encoding mutant KRAS.[210–214] The last of these studies, authored by Zorde Khvalevsky et al., described an investigational agent named siG12D-LODER™ that consists of a siRNA targeting the *KRAS* transcript present in tumors harboring the KRAS-G12D mutations encapsulated within a biodegradable polymeric matrix. When implanted intratumorally siG12D-LODER™, Zorde Khvalevsky et al.[214] observed reduced KRAS expression (measured by antibody staining) and decreased growth of orthotopic human pancreatic tumor xenografts in vivo. A subsequent open-label

Phase 1/2a trial of siG12D-LODER™ assessed the safety of intratumoral insertion of the encapsulated siRNA in 15 patients with advanced pancreatic cancer and receiving standard chemotherapy (NCT01188785). An article reporting the findings from this trial stated that patients tolerated the combination of the siRNA-based intervention and chemotherapy.[215] As of 2020, a multicenter Phase 2 study of siG12D-LODER™ in patients with advanced pancreatic cancer, in combination chemotherapy, remains active (NCT01676259), with an abstract submitted to the 2020 ASCO Annual Meeting reporting completion of trial accrual by December 2020.[216] Another mRNA target that is the subject of an early-stage clinical trial encodes the receptor tyrosine kinase EPHA2. A variety of solid tumors, including ovarian cancer, overexpress EPHA2, and several studies performed by Sood and co-workers, have demonstrated the reduction of *EPHA2* expression by RNAi (alone or in combination with another intervention) decreases the growth of several different xenograft tumor models.[217–222] An ongoing clinical trial is assessing the intravenous administration of a nanoliposome (DOPC)-encapsulated *EPHA2* siRNA (EPHARNA) in patients with solid tumors (NCT01591356). Hopefully, promising results from these two ongoing trials, and other similar efforts investigating the in vivo or ex vivo application of siRNAs (see Das et al.[223] for a recent summary), will accelerate the further assessment of RNAi as a treatment approach for cancer.

8 | CONCLUSION

Twenty years on from the first descriptions of RNAi in mammalian cells and the rapid development of the functional genetic tools that could target every gene in the human genome, the cancer biology research community now has available a remarkable array of approaches that perturb gene expression in a controlled and accurate fashion. Building on both the successes and limitations of RNAi-based technologies, the advent of approaches that make use of methods that employ CRISPR-based resources has expanded our options tremendously. We are now able to effectively edit DNA directly through the application of sequence-specific guide RNAs and the Cas endonucleases (e.g., Cas9 or Cas12 and modified variants), including the ability to make increasingly precise single-nucleotide changes (please see the following recent review[224]). Alternatively, we can perturb gene expression using either modified Cas9 (e.g., the catalytically inactivated version of Cas9 and dCas9) or an alternative enzyme such as Cas13 to inhibit gene expression (CRISPRi) or, through a further modification of dCas9, activate expression (CRISPRa). For further descriptions of CRISPR-based functional genetic tools, we direct the reader to recent in-depth reviews or commentaries and the citations and links therein.[225–231] We also see the emergence of systems that mediate the selective degradation of a protein.[232] The access to this diversity of functional genetic tools offers the cancer community the opportunity to address myriad questions. As a starting point, gene-editing tools may enhance our ability to study the early steps of carcinogenesis by generating model systems that better mimic the accumulation of mutations observed

in many tumors. It may also be possible to generate better metastasis models that require the concurrent activation of some genes and the inactivation of others. Furthermore, we now have the potential to examine the temporal effects of depleting genes over a broader time frame, from minutes using protein degradation tools through to hours or days using CRISPR- or RNAi-based approaches and the ability to reverse this effect. Employment of sophisticated temporal control over gene expression may also allow us to mimic better the dynamic and heterogeneous patterns of gene expression present in a growing tumor and thus improve insights into how we can target a higher proportion of cells within a tumor.

Twenty years ago, few of us would have dreamed that the discovery of gene expression changes mediated by small RNAs transfected into mammalian cells would trigger the technological revolution that continues to impact how many of us conduct cancer research. It is clear that small RNAs have enabled us to dream big, and it will be exciting to see what the next 20 years of cancer-focused functional genetic studies yields.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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