International efforts to sequence cancer genomes now provide an overview of the major genetic alterations that occur in most human cancers. These studies have identified many highly recurrent alterations in specific cancer subtypes but have also identified mutations that occur at lower frequency and unstudied variants of known cancer-associated genes. To elucidate the function of such cancer alleles, we have developed several approaches to systematically interrogate genomic changes found in human tumors. In general, we have taken two complementary approaches. In the first approach, we focus on perturbing genes identified as mutated, amplified, or deleted by cancer genome annotation efforts, whereas in the second, we have taken an unbiased approach to identify genes that are essential for cancer cell proliferation or survival in cell lines that are extensively annotated to identify context-specific essential genes. These studies begin to allow us to define a cancer dependencies map.

Major advances in sequencing technologies over the last decade have led to an exponential increase in our ability to analyze and describe cancer genomes (Stratton et al. 2009; Garraway and Lander 2013; Vogelstein et al. 2013). The massive throughput of these technologies has facilitated international studies to identify genetic alterations and define gene programs in specific types of human cancers. Indeed, we now have a draft view of the major alterations that occur in most human cancers.

However, these studies have also made it clear that most adult human cancers harbor hundreds if not thousands of genetic alterations, probably as a consequence of past or ongoing genomic instability (Hanahan and Weinberg 2011; Vogelstein et al. 2013). In addition, efforts to profile human cancers as part of the clinical evaluation of tumors have led to the identification of a large number of cancer-associated alleles that have never been studied. Two major questions need to be answered for every genomic alteration found in cancer: (i) Does the gene initiate cancer or is it required for tumor maintenance? (ii) Does the cancer-specific variation yield a therapeutic opportunity? Yet, validating each and every observed allele, expression change, and copy number alteration is a dauntingly time-consuming task if studied one event at a time.

To complement the knowledge gained in these genome characterization studies, we and others have developed systematic methods to manipulate gene expression in human cells with the goal of elucidating the function of these cancer alleles (Fig. 1). Gain-of-function screens for increases in cell transformation, proliferation, and viability take advantage of expression libraries composed of cDNA or open reading frames (ORFs) (Gerhard et al. 2004; Rual et al. 2004; Yang et al. 2011; ORFeome Collaboration 2016). Identifying genes that, when overexpressed or mutated, drive an oncogenic phenotype allows for the prioritization of functional candidates from the many genes identified through genomic technologies.

Conversely, loss-of-function screens allow for the interrogation of candidate tumor suppressor genes. Depletion by RNA interference (RNAi) or inactivation with the clustered regularly interspaced short palindromic repeats (CRISPR)–Cas9 system mimics loss-of-function mutations observed in cancer. Unbiased screens using these technologies have discovered novel tumor suppressors both in vitro and in vivo (Berns et al. 2004; Westbrook et al. 2005; Zender et al. 2008; Bric et al. 2009; Cheung et al. 2011a; Hsu et al. 2014; Chen et al. 2015; Wang et al. 2015). In light of the high rates of passenger mutations, these loss-of-function approaches provide additional information that permits the identification of bona fide tumor suppressor genes.

Loss-of-function technologies also allow for the assessment of essential genes on which cancer cells harboring particular genetic alterations are dependent. An understanding of cancer dependencies, defined here as genes that when depleted by RNAi, inactivated by CRISPR–Cas9, or inhibited by a small molecule lead to decreased proliferation or viability of cancer cells, will help inform therapeutic decisions and future drug development.
Here, we review recent advances in the use of these technologies to decipher cancer gene function. We discuss genomic tools to identify functional alterations as well as technologies used to define cancer dependencies.

SCREENS FOR FUNCTIONAL CANCER ALTERATIONS

Interrogation of Copy Number Alterations

The two major types of expression libraries are composed of cDNA or ORF constructs (Boehm and Hahn 2011). cDNA libraries are generated from the reverse transcription of mRNA from a particular cell type or tissue. Thus, cDNA libraries are biased toward genes that are highly expressed in the template tissue and remain incomplete because of unexpressed genes and incomplete reverse transcription of large transcripts. In contrast, ORF expression constructs are produced by individually cloning polymerase chain reaction (PCR) products of cDNA to remove untranslated region (UTR) sequences. Thousands of clones are then pooled to obtain a library with equal representation across the genome. Even when ORF libraries are fully sequenced, these collections contain alleles that vary from database sequences, and the largest libraries, exceeding 17,000 genes, currently do not include all transcript variants and isoforms for many genes (Yang et al. 2011; ORFeome Collaboration 2016).

In the context of cancer, ORF overexpression can mimic copy number amplifications that increase the prevalence of driving oncogenes. Screens for genes that, when overexpressed, drive an increase in proliferation or transformation potential permits the identification of functional copy number changes from the large number of passenger alterations caused by genomic instability (Table 1). For example, Dunn et al. introduced a selected library of ORFs corresponding to genes frequently amplified in ovarian cancer, identifying GAB2 as a novel ovarian cancer oncogene (Dunn et al. 2014). Injection of liver progenitor cells overexpressing a number of candidates into mice identified two coamplified genes, BIRC2 (cIAP1) and YAP1, as capable of transforming cells (Zender et al. 2006). Given that large amplifications often include multiple genes, these studies can hone in on the specific genes responsible for oncogenesis or tumor maintenance, such as TLOC1 and SKIL in 3q26 (Hagerstrand et al. 2013), KCNK9 in 8q24.3 (Mu et al. 2003), and PPM1D in 17q23 amplified cancers (Li et al. 2002).

To complement ORF expression, one can also perform gene suppression studies using RNAi (discussed in detail below) to identify amplified genes that are essential for the survival of cells that harbor such amplifications (Table 1). Amplified genes required for survival are more likely to be drivers in those cells than nonessential amplified genes. RNAi screens in large numbers of cell lines identified PAX8 as oncogenic in many ovarian cancers (Cheung et al. 2011a), whereas in vivo RNAi delivery confirmed ID4 as a novel ovarian oncogene (Ren et al. 2012).

Independent validation by ORF expression and RNAi methods establish oncogene candidates as capable of both transforming normal cells and maintaining cancer cell survival. Targets confirmed by both experiments are high-confidence oncogenes and include CRKL in non–small cell lung cancer (Cheung et al. 2011b) and FRS2 in ovarian cancer (Luo et al. 2015).

Characterizing Variant Alleles

cDNA and ORF constructs can also express variant alleles of genes to assess their oncogenic potential (Table 1). For example, introduction of oncogenic PIK3CA into noncancerous cells drives an increase in proliferation and cancer-related phenotypes (Isakoff et al. 2005).
Table 1. Functional assessment of cancer genomic alterations

| Genomic observation                              | Functional genomics tool(s) | Model                              | Phenotype(a)                         | Conclusion                              |
|-------------------------------------------------|----------------------------|------------------------------------|--------------------------------------|-----------------------------------------|
| Copy number amplification; mRNA overexpression  | ORF library of wild-type alleles | Noncancerous, immortalized cells   | ↑ proliferation; ↑ transformation potential | Increased gene function drives cancer   |
| Hotspot point mutations; variants of unknown significance in validated oncogenes | RNAi, CRISPR–Cas9 targeting amplified genes | Cancer cell lines | ↓ proliferation; ↓ viability | Increased gene function required for survival |
| Inactivating mutations; copy number loss         | ORF library of variant alleles | Noncancerous, immortalized cells   | ↑ proliferation; ↑ transformation potential | Oncogenic driver mutations             |
| Mutation, gene expression, and copy number profiles | RNAi, CRISPR–Cas9 targeting wild-type alleles of mutated genes | Noncancerous, immortalized cells   | ↓ proliferation; ↓ viability | Loss of function drives cancer           |
|                                                   | RNAi, CRISPR–Cas9 targeting entire genome  | Cancer cell lines                 |                                      | Genomic alteration leads to dependencies on specific genes |

Although experiments with these tools have validated many of the most common oncogenic mutations, genome sequencing has revealed that a majority of variants found in cancer are present at low frequencies in the population. This finding makes it difficult to gauge functionality of variants even in known oncogenes and tumor suppressors (Garraway and Lander 2013; Vogelstein et al. 2013).

Recently, Kim et al. (2016) reported a method to assess variants of unknown significance (VUSs) through introduction of a pooled variant allele ORF library into immortalized cells and testing for transformation potential in mice (Fig. 2). This proof-of-concept experiment reported a dozen previously unknown transforming alleles, including some that were present at very low frequency, demonstrating that even rare alleles may contribute to cell transformation (Kim et al. 2016). Complementing the results of transformation potential, gene expression signatures were derived for each variant allele to firmly identify VUSs that resemble profiles of known oncogenic alleles of the same genes (Fig. 2; Kim et al. 2016). Expansion of this technology would inform both basic research and clinical decision-making as genomic characterization of patients becomes more prevalent.

**LARGE-SCALE CANCER DEPENDENCY SCREENS**

**RNAi**

RNAi can be mediated by two types of reagents: small interfering RNAs (siRNAs), which are transiently introduced into cells, and short-hairpin RNAs (shRNAs), which are stably expressed following genomic integration via viral vectors (Rao et al. 2009). In both cases, binding of si/shRNA to complementary messenger RNA (mRNA) transcripts of interest leads to a decrease in mRNA levels and their protein products. This depletion, suppression, or knockdown of the target gene then allows for functional assessment of a phenotype. Genome-scale RNAi experiments can be performed in an arrayed or pooled format (Echeverri and Perrimon 2006). In an arrayed experiment, a small number of cells are individually exposed to each reagent, usually in a 96- or 384-well plate format. siRNA work best in arrays, as they induce potent depletion over 2–4 d, allowing for the assessment of short-term phenotypes (Rao et al. 2009). In a pooled experiment, a population of cells is exposed to a library of reagents targeting many genes in a massively parallel manner. shRNA are preferable for pooled experiments, as the DNA encoding each shRNA is incorporated into the infected cells’ genomes, serving as a barcode for later detection of relative changes in representation (Echeverri and Perrimon 2006). If an shRNA decreases proliferation of cells, it will be relatively depleted in the final pool after a growth period, whereas an shRNA that increases proliferation will be relatively enriched in the final pool (Cowley et al. 2014). Additionally, shRNA expression is permanent, allowing for observation of long-term phenotypes (Rao et al. 2009).

RNAi has been used to determine whether loss of a particular gene can drive proliferation and transformation, allowing for the assessment of the function of inactivating mutations observed in patients (Table 1; Berns et al. 2004; Westbrook et al. 2005; Bric et al. 2009). In addition, RNAi can be used to assess dependencies on a genome scale (Luo et al. 2009). Project Achilles is an effort to screen hundreds of cancer cell lines with pooled, genome-wide shRNA libraries to determine which genes are required for the growth and viability of many cancer cell lines (Cheung et al. 2011a; Cowley et al. 2014). The large number of cell lines is designed to cover a wide spectrum of cell lineages and genomic alterations. All cell lines screened through Project Achilles are also profiled as part of the Cancer Cell Line Encyclopedia (CCLE) (Barretina et al. 2012), which facilitates the integration of genomic and transcriptomic information with dependency findings.

Although genome-wide RNAi experiments have identified several gene dependencies (Barbie et al. 2009; Luo...
et al. 2009; Scholl et al. 2009), this technique has some important limitations. First, the vast majority of RNAi reagents induce only partial loss of gene expression, preventing the identification of dependent genes that require full inactivation. Furthermore, there is a significant variation in the quality of reagents even with advanced design tools, creating many false-negative results due to ineffective suppression (Booker et al. 2011).

More importantly, off-target effects of si/shRNAs, defined as phenotypic consequences of interactions with mRNAs other than the intended complementary sequence, can lead to frequent false-positive results (Echeverri et al. 2006). Screens for cancer dependencies, where the output phenotype is simply a loss of cellular viability and proliferation, are particularly susceptible to false positives given the many housekeeping genes that, when inadvertently depleted, could copy this phenotype (Kae-lin 2012). Off-target effects are mediated by seed sequence complementarity, defined as bases 2–8 of an si/shRNA that can mimic the native miRNA pathways (Jackson et al. 2003, 2006). Paired control reagents with changes of bases 9–11 (C911) abrogate on-target effects, which require perfect complementarity but retain most off-target effects through the seed sequence (Buehler et al. 2012). Although these controls are very helpful in targeted screens and validation experiments, paired controls are not feasible at the genome scale necessary for cancer dependency screens.

For large-scale experiments, the most practical method to correct for off-target effects is to employ redundant reagents designed to target the same mRNA at independent locations and then computationally correct for outliers. Multiple si/shRNAs that target the same gene and produce the same phenotype are more likely producing on-target effects than a single reagent or those that produce disparate phenotypes (Echeverri et al. 2006). One algorithm, ATARiS, considers shRNA dependency data (approximately five shRNAs per gene) across many cell lines, identifying the reagents that behave similarly to each other in most contexts as on-target and eliminating shRNAs that do not cause the same phenotypes (Shao et al. 2013). Dependency data of approved shRNAs are then collapsed into a gene solution that reports the relative dependency of a particular cell line on that gene.
The CRISPR–Cas9 bacterial immune system has been adapted for genomic editing in mammalian cells (Jinek et al. 2012, 2013; Cho et al. 2013; Cong et al. 2013; Mali et al. 2013; Hsu et al. 2014). Small-guide RNAs (sgRNAs) direct the Cas9 endonuclease in a sequence-specific manner to genomic sites, allowing Cas9 to induce a double-strand DNA break (Cong et al. 2013; Hsu et al. 2014). These breaks are generally repaired in an error-prone fashion, creating inactivating mutations with high frequencies at target sites (Shalem et al. 2014; Wang et al. 2014). Pooled sgRNA screens are performed similarly to pooled shRNA screens, with a program such as ATARiS or MAGeCK collapsing multiple sgRNAs targeting the same gene into a single score (Shao et al. 2013; Li et al. 2014).

Gene editing with CRISPR–Cas9 has several advantages over RNAi (Shalem et al. 2014; Wang et al. 2014, 2015). First, biallelic gene inactivation at the DNA level bypasses issues in which partial depletion of mRNA is insufficient to cause a phenotype (Shalem et al. 2014; Wang et al. 2015). Second, genome editing with the CRISPR–Cas9 system leads to substantially fewer off-target effects when compared to RNAi (Shalem et al. 2014; Wang et al. 2014). Even when using first-generation libraries, editing was observed at likely alternative sites at very low frequencies (<2.5%) (Wang et al. 2014). Overall, the increased depletion strength and specificity by CRISPR–Cas9 gene editing should reduce both false-negative and false-positive results from genome-wide loss-of-function screens.

CRISPR–Cas9 screens are not limited to the total inactivation of protein coding genes (Fig. 3). Shi et al. used sgRNAs targeting individual protein domains to identify preferentially important protein domains and potential drug targets (Shi et al. 2015). At a genome-wide level, CRISPR–Cas9 has been used to disrupt miRNAs in myeloid leukemia, identifying specific dependencies in these cells that would be invisible to RNAi (Wallace et al. 2016). As a proof of concept, CRISPR–Cas9 was used to identify functional p53 and estrogen receptor enhancer elements from a large set of expected binding sites determined by chromatin immunoprecipitation with sequenc-
ing (ChIP-seq) and consensus motif analysis (Korkmaz et al. 2016). Using all possible sgRNAs to achieve saturated mutagenesis, Canver et al. determined key functional bases in the BCL11A enhancer (Canver et al. 2015). The creation of activation (Konermann et al. 2015) or catalytically inactive (Braun et al. 2016) Cas9 complexes allows for gene expression perturbations in both positive and negative directions on a genome scale. All of these advances extend our ability to functionally interrogate cancer genomes and expand the cancer dependency map.

Although CRISPR–Cas9-mediated gene editing is quite specific, we and others have identified a gene-independent effect induced by CRISPR–Cas9. In multiple cell lines, sgRNAs targeted to amplified regions tend to score as dependencies, even if the sgRNAs match intergenic sequences or nonexpressed genes (Wang et al. 2015; Aguirre et al. 2016). Additionally, sgRNAs from first-generation, imperfect genome-wide libraries predicted to target multiple locations throughout the genome also scored as false positives (Aguirre et al. 2016). Further investigation discovered an additive loss of cellular viability with every Cas9-induced cut, independent of the inactivated target (Aguirre et al. 2016). The decrease in viability appears to be independent of the type of structural rearrangement, although multiple cuts made on independent chromosomes have a greater impact than the same number of cuts on a single chromosome (Aguirre et al. 2016). Importantly, this dependency on generally amplified regions is not seen in RNAi screens of the very same cell lines (Aguirre et al. 2016). Thus, alterations in copy number—the most frequent structural abnormalities in human cancer—have a significant impact on results from genome-wide CRISPR–Cas9 screens where the output phenotype is a loss of viability.

Overall, these initial findings highlight the importance of considering copy number alterations when analyzing CRISPR–Cas9 screening results. In fact, genomic copy number variation data are a requirement for any CRISPR–Cas9-based screen in cancer cell lines. For low number alterations, a computational correction should suffice to filter out false positives (manuscript in preparation). For high number alterations (>10–20), any signal due to gene impacts is likely obscured by the cutting effects, necessitating that these genes be excluded. Well-designed negative-control sgRNAs are important to set a baseline for filtering. Finally, even though CRISPR–Cas9 technologies have many advantages, it is important to recognize that additional weaknesses will likely be discovered as the technology continues to be implemented more broadly.

Small-Molecule Screens

Although not a true functional genomics tool, small-molecule screens are useful in complementing the technologies introduced above. The major advantage of compound-based screens is that drugs already approved by the U.S. Federal Drug Administration (FDA) or in clinical trials can more easily be repurposed for a newly investigated cancer type or mutational profile, shortening the time from discovery to treatment. The major disadvantage is that most small molecules have off-target effects and many inhibitors target a number of related proteins, making it more difficult to assess mechanism. A number of large small-molecule screening projects exist (Seashore-Ludlow et al. 2015; Rees et al. 2016; Yu et al. 2016), and comparisons with genome-wide RNAi and CRISPR–Cas9 screens can reveal important similarities, with hits present in all three screening modalities being the most promising (Hong et al. 2016).

Types of Cancer Dependencies

Findings from Project Achilles and other genome-wide screens have identified a number of cancer dependencies. Of particular interest are genes that are preferentially dependent in limited cancer contexts, such as cancers driven by a particular mutation or from a single-cell lineage as opposed to dependencies ubiquitous to all cells, such as on housekeeping genes, as there is more likely to be a therapeutic window that could be exploited in patients.

The simplest dependencies are upon the driving oncogenic mutations underlying that particular cancer. Unsurprisingly, a number of well-characterized oncogenes have been found as preferential dependencies, including KRAS in pancreatic (Cheung et al. 2011a) and lung cancer (Sunaga et al. 2011) and BCR and ABL1 in chronic myelogenous leukemia (Luo et al. 2008; Wang et al. 2015).

These genetic findings mimic clinical observations in patients, where therapies targeting oncogenes have been effective treatments for cancers that depend on these driving mutations. However, many of these mutations are currently thought to be “undruggable,” such as oncogenic KRAS and amplified MYC (Verdine and Walensky 2007; Cox et al. 2014). Furthermore, resistance to oncogene-targeted inhibitors, as has been observed in both BCR-ABL (Branford et al. 2003) and BRAF V600E (Shi et al. 2014) driven cancers, highlights the importance of finding additional dependencies even in cancers that harbor “targetable” mutations.

In these cases, there is a need to discover synthetic lethal dependencies, in which inactivation of one gene in a cancer context can sensitize to loss of a second gene (Kaelin 2005). Whereas mutation or loss of expression of a single gene has no impact, loss of both genes is lethal. From a therapeutics perspective, a patient’s noncancerous tissues should retain function of the gene mutated in the tumor, allowing for a preferential dependency in the cancer on the second gene.

Screens have identified a number of such dependencies. For example, the mammalian SWI/SNF (BAF) chromatin-remodeling complex is made up of more than a dozen subunits, a number of which are incorporated into the complex in a mutually exclusive fashion (Wilson and Roberts 2011). Two sets of these pairs have been identified as synthetic lethal partners: Mutations in ARID1A, the most frequently mutated subunit of the complex, sensitize to loss of ARID1B (Helming et al. 2014b); similarly, mutation or loss of expression of SMARCA4 sensitizes to loss of SMARCA2 (Oike et al. 2013; Hoffman et al. 2014;
Wilson et al. 2014). Cells require either ARID1A or ARID1B and either SMARCA2 or SMARCA4 to build functional complexes, but cannot survive loss of both paired subunits (Helming et al. 2014a).

Finally, deletion and loss of heterozygosity of tumor suppressor genes can delete other proximal genes. In some cases, the resultant hemizygosity of essential genes creates a preferential dependency for further depletion of those same genes. Termed CYCLOPS (copy number alterations yielding cancer liabilities owing to partial loss), these dependencies tend toward components of essential complexes, such as the ribosome, proteasome, and spliceosome (Nijhawan et al. 2012). In a related case, hemizygous deletion of MTAP, frequent given its genomic proximity to the tumor suppressor CDKN2A, confers dependency on an independent gene, PRMT5, because of accumulation of an inhibiting metabolite (Kryukov et al. 2016).

REMAINING CHALLENGES

All of the screening efforts described above are dependent on cell lines as models for cancer. Despite decades of significant advancements driven by research in cell lines, two major questions regarding established cell line use remain. First, how well do established cell lines reflect cancer biology seen in patients? Comparisons of cell lines characterized as part of the CCLE and patient tumors from The Cancer Genome Atlas (TCGA) have shown a significant variation in genomic markers between some cell line models and primary tumors of the same lineage (Domecke et al. 2013). Cells grown in culture for extended periods of time tend to genomically resemble each other more than primary cancers from the same tissue, suggesting both clonal selection for growth on plastic and evolution due to a variety of pressures not present in vivo (Gillet et al. 2011). Second, do we have enough cell lines available to fully encompass the extraordinary diversity in cancer lineages, mutations, and changes to cellular homeostasis observed in patients? Rare cancers (incidence of <6/100,000/year) account for almost 25% of all cancers, highlighting the need to develop as many models as possible to study cancer broadly (Gatta et al. 2011).

The establishment of new, recently derived cell lines aims to overcome some of the disadvantages of established lines while building on model diversity (Fig. 4; Hong et al. 2016). A pediatric undifferentiated sarcoma cell line recapitulated the histology, copy number profile, and genomic fusion events detected in a relapsed tumor from the same patient and was amenable to high-throughput RNAi, CRISPR–Cas9, and small-molecule screens (Hong et al. 2016). Although it is impossible to apply genomic technologies to compare older cell lines to their original tumors, freshly established cell lines can be validated as accurate models through genomic sequencing. Increasing both the number and quality of models will greatly improve our ability to establish a comprehensive cancer dependency map.

CONCLUSION

By intersecting genomic characterization with functional screens, we are approaching an era in which sequencing information from patients’ tumors can be used to accurately predict dependencies and inform treatment for that specific cancer. The continued use of CRISPR–Cas9 screens to supplement the dependency data already produced by RNAi will discover many valuable dependencies. It is crucial that more cancer types, with a wide range of mutations and genomic features, continue to be screened using functional genomics technologies in order to capture all of the variation that we observe in patients’ tumors.

Although the last decade has been defined by massive advancements in cancer genome characterization, we envision that the next decade will build on this knowledge with high-throughput functional studies to understand the clinically actionable information held in the cancer genome. With continued resources and effort, a cancer dependency map could usher in an unprecedented era of directed medicine for cancer treatment.

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