

# Function-first approaches to improve target identification in cancer

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Target discovery for cancer is undergoing a sort of revival with an increasing need for improved therapeutics. Likewise, the strategies to discover new and better therapeutic targets have come full circle, with greater emphasis placed upon targets that are functionally relevant to the disease process. In this article, we review the evolution of cancer target discovery and discuss random homozygous gene perturbation, an emerging technology that combines the practicality of screening for new targets by emphasizing function as the primary criterion, with cutting-edge advances in gene-based screening of all potential targets in a cell.

The past decade has witnessed remarkable advances in the diagnosis and treatment of many life-threatening cancers. Much of this progress builds upon understanding of fundamental mechanisms that control cancer cell behavior. Such knowledge formed the foundation for the rationale and design of targeted therapies against molecular changes and regulatory mechanisms that are selectively utilized by cancer cells. Prominent successes include targeting HER2 on breast cancer cells with trastuzumab (Herceptin<sup>®</sup>), proteasomes in multiple myeloma with bortezomib (Velcade<sup>®</sup>) and EGFR on non-small-cell lung cancer cells with gefitinib (Iressa<sup>®</sup>) [1–5]. However, most rationally designed therapies under development today are focused upon a relatively narrow set of targets. This emphasis logically follows from increased knowledge about these targets and commercial success with existing drugs that stimulate the development of fast-follower drugs targeting the same, or similar, molecules. Nonetheless, these trends can stifle breakthroughs that might otherwise arise from underappreciated or unknown targets or mechanisms. The key, therefore, is to develop novel means of identifying targets that critically control cancer cells' drug sensitivity, metastatic potential and other hallmarks of the pathophysiology of the disease.

## Brief history of cancer target discovery

The history of oncology drug development has evolved with increasing understanding of cancer cell behavior. In the first period of drug development, compound libraries were analyzed for inhibition of tumor cell growth or survival (see TABLE 1 for an overview). These approaches were made possible as a result of advances in

the culture of tumor cells in the laboratory. Such improvements made it possible to develop experimental screens to identify compounds that killed or prevented the growth of cultured tumor cells. Many of these assessments utilized a function-first approach, which placed greatest value on the ultimate outcome and less emphasis on the identity of the target or mechanistic basis of anti-tumor activity. These approaches required and stimulated improvements in high-throughput screening procedures and fostered efforts to diversify the array of chemical structures via isolation of novel natural products by creating novel chemical libraries, and/or a combination of the two. The resulting cytotoxic therapies comprise much of our antineoplastic arsenal today, including alkylating agents (platinum compounds), antibiotics (doxorubicin), antimetabolites (methotrexate and 5-fluorouracil), alkaloids (vincristine) and taxanes (taxol). Often, the identity of the target for the therapeutic was unknown, or the mechanistic basis was identified later. Although effective, these cytotoxic antineoplastic agents were often accompanied by unwanted side effects, which could limit the use of the compounds over time. Compounding this, increased expression of the P-glycoprotein, coupled with the rapid mutation rate of tumor cells, tends to favor selection of drug-resistant tumors [6].

The ability to culture tumor cells in the laboratory also allowed investigators to begin understanding changes that distinguish benign and malignant cells, which heralded an era of targeted intervention. Fundamental advances in the understanding of oncogenes that contribute to malignant transformation and facilitated the identification of signaling pathways that govern

## Keywords

cancer ■ drug resistance  
 ■ random homozygous gene  
 perturbation ■ RHGP ■ siRNA  
 ■ target discovery

future part of **medicine** **fsg**

Table 1. Evolution of cancer drug discovery.

	Era of drug screening	Era of targeted intervention	'-omics'-based target discovery	Function-first target discovery
Function-first screening	Yes	No	No	Yes
Target identity known?	No (but later deduced)	Yes	Yes	Yes
Breadth of target types	Broad	Narrow	Broad	Broad
Target link to disease	Direct	Direct	Correlative	Direct
Enabling technologies	– HTS – Compound libraries	– Oncogene discovery – Mechanistic understanding	– Genome knowledge – Omics technology	– siRNA – RHGP

Shown is an outline of the rapidly evolving criteria that have been used to screen for cancer drugs and targets. Note that recent function-first target discovery strategies combine the upfront biology afforded by direct screening with understanding of tumor cell behavior and new technologies.

HTS: High-throughput screening; RHGP: Random homozygous gene perturbation.

cancer cell behavior (growth, survival and invasion) improved understanding and engendered new generations of chemotherapeutic agents. The resulting compounds impacted known targets, such as topoisomerases (podophylotoxins), and growth factors or their receptors. The improved selectivity for tumor cells resulted in more effective and selective antineoplastic drugs. Since targeted drugs were screened or engineered to selectively inhibit particular targets, the new molecular medicines were often accompanied by linked diagnostic agents, which might predict patient eligibility or treatment outcome. Although we remain in the midst of this era of targeted intervention, many of the same historical impediments remain, including unwanted side effects and the acquisition of drug resistance. Compounding this, many of the resulting drugs focus on a relatively narrow set of targets (e.g., tubulin, growth factors and kinases). This prioritization was a logical outcome of the need to intensively investigate validated targets. However, an unintentional outcome has been a steady narrowing of the types of targets under investigation.

With improved understanding of the genetic links to disease, increased appreciation of the genetic basis of cancer drove interest towards genomic and then proteomic sampling of tumor cells. The growth of '-omics' to identify potential targets for cancer cells coincided with dramatic technological advances in DNA sequencing and garnered much attention, leading academic and pharmaceutical communities to invest considerable infrastructure into the technologies. Genomics, proteomics and related strategies increased the number and breadth of targets. Thus, the field found itself awash in a wide array of targets and the perceived need for continued target discovery reached a nadir. However, it was increasingly understood that the correlative links identified using '-omics' often did not

distinguish targets that cause or contribute to disease pathology from those that arise as side effects of the disease process. This ambiguity has required considerable investment to resolve questions surrounding cause and effect, thus decreasing the general appetite for novel oncology targets, since many organizations had invested considerable infrastructure into the technologies needed to investigate '-omics' research.

#### Development & improvements of a function-first approach

Trends suggest a re-emergence of a function-first drug discovery that returns to the earlier modes of investigation. For example, improved technologies can sample and select for a desired phenotype (e.g., growth inhibition of tumor cells or decreased proliferation of angiogenic endothelial cells) in a population of tumor cells. These new approaches combine advances in genetic technology with improved fundamental understanding to identify more relevant and selective cellular behaviors (i.e., those that are unique to, or necessary for, malignant character). One promising new approach is the advent of genome-wide siRNA libraries to assist target discovery [7-10]. There now exist multiple commercial sources of siRNA libraries that target a variety of human or murine gene families (e.g., kinases and nuclear receptors). While the cost and availability of such libraries have improved, this technology remains largely restricted to relative experts in RNAi research because of the need for different transfection reagents, assay systems and limited durability of the siRNA knockdown of target expression. For example, gene silencing is often limited to 24-72 h post-transfection. Compounding this, not all gene targets are known, and thus may not be included in most libraries. Even if included, the efficiency of target knockdown can be highly variable among different genes. These difficulties have limited the application and outcomes of siRNA-based

discovery efforts. Finally, siRNA libraries are, by definition, limited to knockdown of target genes and do not provide the ability to upregulate genes that might be comparably interesting.

### Random homozygous gene perturbation

Random homozygous gene perturbation (RHGP) can provide an efficient, robust and high-throughput means to improve target discovery using a function-first approach. Unlike siRNA, RHGP does not require *a priori* knowledge of the target and seeks out any target that directly causes a desired phenotype (e.g., metastatic potential or modified tumor cell sensitivity to treatment). Another strength of RHGP is the capacity to sample every target in a cell, both for overexpression or loss of expression, and without any prior knowledge of target expression or function. RHGP not only facilitates exploration of known targets, but can also be used to identify novel targets that have not yet been annotated or for which no prior function has been attributed. Indeed, in one study, approximately 40% of the targets identified had not yet been annotated [11].

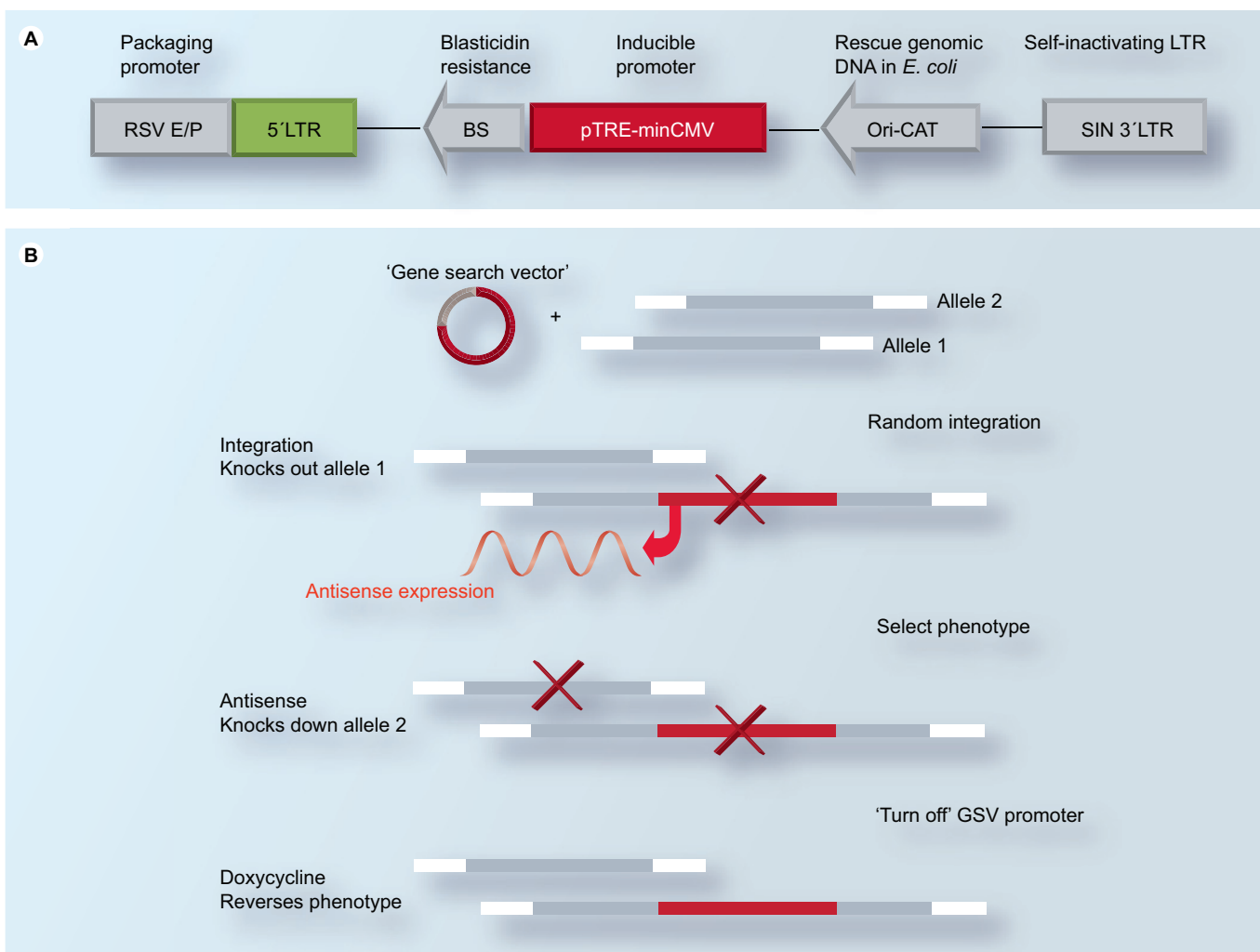
Originally known as random homozygous knockout (RHKO), the initial approach was developed to overcome barriers arising from the fact that eukaryotic cells generally have two sets of chromosomes [12]. The diploid nature of eukaryotic cells precluded simple knockout-based evaluation of target genes that have proven to be so powerful in our understanding of bacterial pathways. Unlike siRNA, which is limited to the knockout of known targets, RHGP-based approaches include the ability to simultaneously knockdown both copies of any target gene, independent of any prior knowledge or annotation of that gene. The potent inhibition is possible because the integration event itself is sufficient to knock out the first copy of the gene, while antisense expression of the target gene knocks down the second allele (FIGURE 1B). RHGP can also upregulate gene expression, including whole genes or individual domains (FIGURE 2A). To date, approximately one-half of the targets identified using RHGP have represented integration in a sense orientation, with the remaining half representing antisense integration events. Consequently, RHGP provides a means to interrogate the entire genome for any genetic change that is causative of the phenotype under investigation.

The central feature of RHGP is a unique lentiviral-based genetic element, known as a gene search vector (GSV), which was designed to interrogate the entire genome and identify target genes that cause the phenotype of interest. The GSV

cassette contains a promoter with a tetracycline-response element (TRE) (e.g., PminCMV promoter of the Tet-off system; FIGURE 1A) [13,14]. The GSV is transduced into a target cell population that stably expresses the tetracycline-controlled transactivator (tTA). Transduction results in random integrations of the GSV into the genome of the target cells. In the absence of tetracycline or doxycycline, the tTA binds the TRE element to activate the GSV PminCMV promoter. Promoter activation triggers the transcription of the RNA extending into the host genome sequence flanking the 5' LTR of the GSV (FIGURE 1B). When integrated in an antisense orientation, this event physically disrupts one allele, while producing antisense RNAs that knock down expression of the other allele. The inducible promoter of the GSV allows us to validate the candidates and eliminate false-positives that arise as a result of spontaneous mutation or other artifacts. The vector itself encodes for a self-inactivating lentiviral LTR, which prevents the GSV from re-emerging from a transduced cell. One potential pitfall of the reversibility of the RHGP technology could arise if the promoters for the GSV or the target gene are modified in an epigenetic manner. Such a modification could impair the ability of the promoter to be reversed using doxycycline, and thus cause the investigator to eliminate the targets based on an artificial lack of reversibility. To date, such an outcome appears to be rare, since only a small fraction of targets have demonstrated an inability to be reversed in the presence of doxycycline.

The GSV can integrate into the genome in either a sense or an antisense orientation. In the antisense configuration, the integration event itself inactivates one allele and facilitates expression of an antisense construct, which knocks down genes encoded on the other allele (FIGURE 2A). When integrated in the opposite (sense) orientation, RHGP can facilitate overexpression or unsilencing of target genes. This outcome could extend beyond simple overexpression of an entire gene (e.g., insertion upstream of the start site) or trigger overexpression of particular domains when integrating downstream of the start site, which could produce a dominant-negative inhibitor of wild-type gene function. As such, RHGP allows for interrogation of the entire cell genome to identify different types of targeting events.

RHGP requires construction of a 'library' of GSV-transduced cells (FIGURE 2B). A single integration per cell is controlled by transducing cells at a relatively low multiplicity of infection. Using mathematical models, we have established that  $10^7$  independent events are sufficient to cover

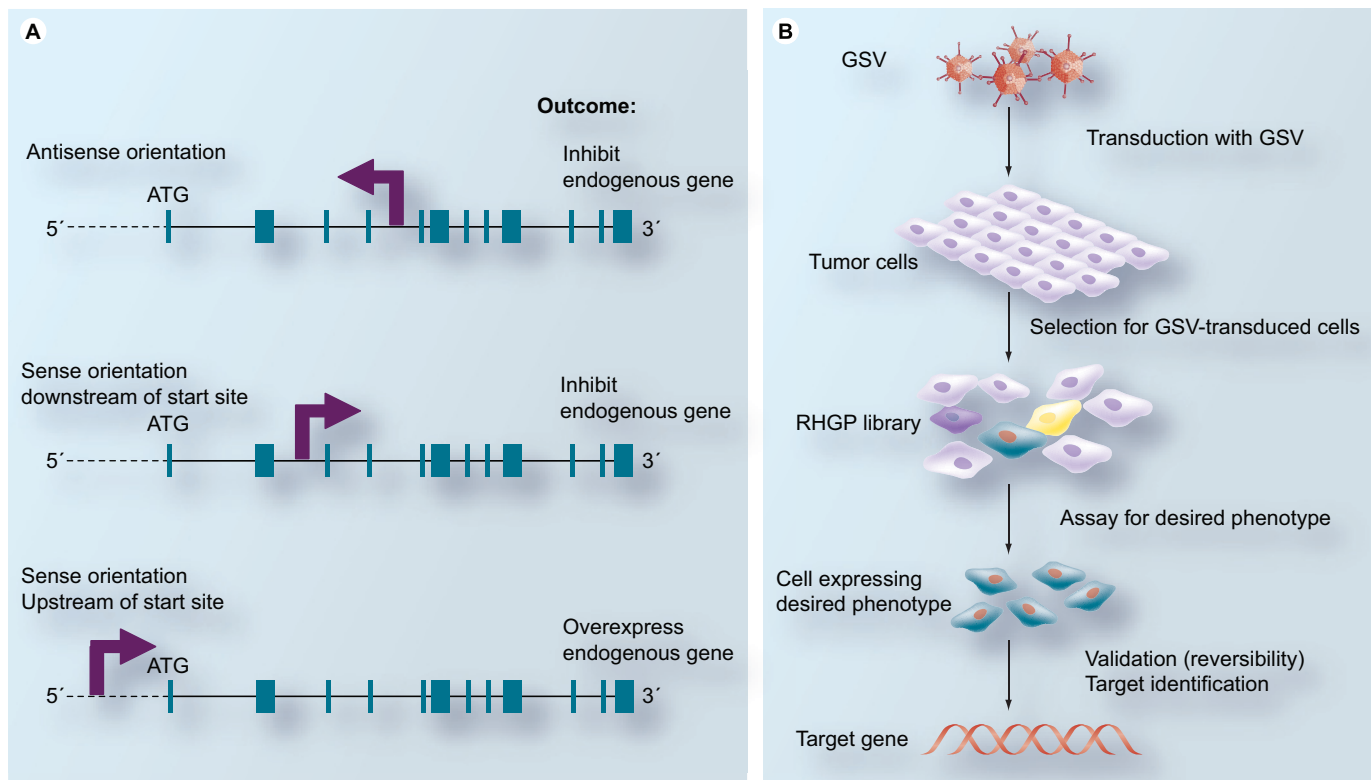


**Figure 1. GSV integration to inhibit endogenous gene expression.** A schematic overview demonstrating how the random homozygous gene perturbation (RHGP) technology can block endogenous gene expression. In the example, insertion of the GSV into the first allele disrupts expression from that allele. The orientation of the integration then allows the GSV to express an antisense RNA that knocks down expression from the remaining allele. This antisense expression is regulated by an inducible promoter, and thus the phenotype can be reversed by shutting down expression from the GSV. It should be noted that while this example demonstrates RHGP-based inhibition of endogenous gene expression, insertion of the GSV is random and when inserted in the opposite orientation, the GSV facilitates overexpression of the gene product.

BS: Blastidicin S resistant gene; GSV: Gene search vector; LTR: Long terminal repeat; Ori-CAT: Replication origin-chloramphenicol acetyltransferase gene; pTRE-minCMV: Tetracycline response element-minimal SMV promoter; RSV E/P: Rous sarcoma virus transcription enhancer/promoter; SIN: Self inactivating.

the entire genome for all possible perturbations (gain or loss of expression). RHGP is also flexible and not biased by the type of cells to be analyzed (suspension or adherent), species under investigation (any eukaryotic cell can be used) or transformed character (primary or immortalized or transformed). The RHGP strategy excludes GSV transduction events that are intrinsically toxic. For example, RHGP transduction events that disrupt the expression of a vital gene will be toxic to the host cells, and thus these clones will be eliminated from the RHGP library. Once transduced, the same library can be archived and used for other screens.

The library of transduced cells is then subjected to a selection for the desired phenotype. The success of RHGP screening is dependent on the assay that is used to screen the phenotype. Similar to other screening procedures, the desired phenotype must be unambiguous and robust. Once these minimal criteria are satisfied, RHGP can be applied to virtually any application in which the desired phenotype allows the cells to be physically isolated. As in one example, our laboratory and others have utilized RHGP to identify targets that cause breast or neuroblastoma tumor cells to become resistant to chemotherapeutic challenge with

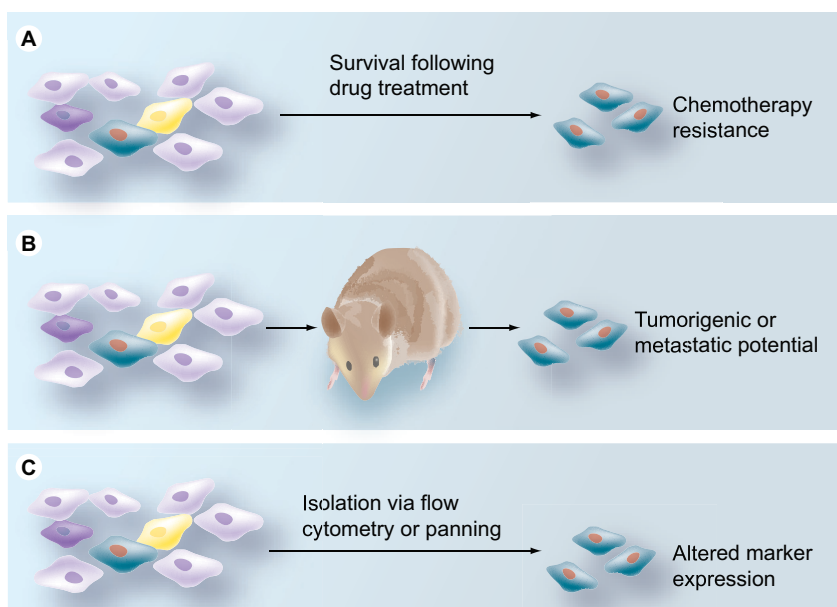


**Figure 2. Random homozygous gene perturbation for target discovery.** (A) Integration of the GSV into genomic DNA can occur in either a sense or antisense orientation. Note that certain integration events are predicted to inhibit endogenous gene expression, whereas others cause overexpression. (B) Shown is an overview of the RHGP-based experimental strategy to identify novel targets. The key to RHGP is a novel GSV, which can integrate into the genome and perturb (overexpress or inhibit) gene expression. After generating a library of cells with individual GSV integration events that cover the entire genome, the cells are subjected to robust assays that facilitate isolation of cells with the desired phenotype. The specificity of the RHGP in causing the phenotype can be validated through the reversible nature of GSV-based gene expression and validated targets can be rapidly identified using specific sequences in the GSV. GSV: Gene search vector; RHGP: Random homozygous gene perturbation.

cytotoxic agents (rapamycin), or that allow breast cancer cells to become insensitive to an antihormone such as tamoxifen ([15] and [Li W-B, Kinch M, Functional-Genetics, Inc. Unpublished Observ.]) (Figure 3A). Likewise, our collaborators have utilized a related approach to identify genes that regulate taxane resistance in prostate cancer cells [16]. In each case, drug-sensitive cells perished, while a subset of RHGP-perturbed cells survived. Unlike the correlative findings that typify ‘-omics’-based approaches, survival in the face of drug challenge was directly caused by the RHGP transduction event. The selection of a desired phenotype is not limited to cell-based assays, and RHGP can likewise be used for applications *in vivo*. For example, one can isolate cells that gain tumorigenic or metastatic potential in animal models (see Figure 3B). Beyond survival as an outcome, RHGP can also select for other properties, such as affinity-based isolation of cells that express particular markers (Figure 3C). Such outcomes are possible when the expression of a specific

marker (as the desired phenotype) can be used to physically isolate cells using flow cytometry or panning procedures.

Considerable efforts have been invested to optimize the robustness and efficiency of RHGP. Another important feature of RHGP is the ability to validate candidates by taking advantage of the GSV inducible promoter. Since the inducible GSV promoter is inactivated in the presence of doxycycline, false-positives, such as those that adopt the desired phenotype as a result of spontaneous mutation, or other artifacts not related to RHGP, can be eliminated. After the desired phenotype is obtained, the experiment can be repeated with a silenced GSV promoter to reverse the phenotype. If the phenotype persists using a silenced GSV, that clone would be deprioritized, since the phenotype might have arisen as the result of an unrelated artifact. As such, once an RHGP library has been constructed, it is possible to select for a phenotype of interest and validate the reversibility of the phenotype, all in a single study.



**Figure 3. Examples of RHGP application for cancer target discovery.**

Different strategies for random homozygous gene perturbation (RHGP)-based cancer target discovery are outlined. **(A)** A selection strategy for mechanisms that facilitate tumor cell resistance to chemotherapy has been applied to identify targets that cause resistance to paclitaxel [15], rapamycin [14] or tamoxifen [Li W-B, Kinch M, Functional-Genetics, Inc. Unpublished Observ.]. **(B)** RHGP can also be used to select for desired phenotypes *in vivo*. Shown is an example, where RHGP-transduced cells uniquely gain tumorigenic or metastatic potential. The tumors can then be isolated and targets identified using thermal asymmetric interlaced-PCR assays. **(C)** Beyond survival-based assays, RHGP can be used to identify targets that alter tumor cell expression of particular markers (using flow cytometry) or that gain the ability to bind particular substrates (by panning).

Once a phenotype has been validated using the reversible promoter, the GSV encodes for motifs to identify the target genes and determine the orientation of the GSV integration (i.e., sense or antisense). Early versions of RHGP required physical isolation of the host genomic DNA followed by conventional sequencing. This procedure required the outgrowth of clones to sufficient numbers to isolate enough genomic DNA for bacterial transformation. Individual bacterial colonies were then isolated and the plasmid isolated for sequencing of the GSV integration event. This process required weeks of effort with limited throughput. We recently improved target identification using a modification of thermal asymmetric interlaced (TAIL)-PCR. We incorporated TAIL-PCR to implement high-throughput identification of GSV insertion sites, since small numbers of cells are needed to isolate genomic DNA. These improvements facilitate high-throughput screening and yield information about the GSV insertion site within days rather than weeks.

TAIL-PCR is a method used to identify unknown sequences adjacent to known sequences in a given genome and with high specificity. In

a brief summary, TAIL-PCR was originally developed to identify gene insertion events in plants [17]. More recently, it has been used to identify gene insertion events in the mouse genome [18]. The method uses two different classes of primer (a series of nested long specific forward primers, and a short random reverse primer), and alternates between high- and low-temperature cycles. The high-temperature cycles favor binding of the long specific primer, thereby producing a single-stranded DNA product (asymmetric PCR) from the desired locus. The low-temperature cycles favor binding of both the long specific primer and the short random primer, allowing for normal symmetric PCR amplification to occur. In this fashion, the specific locus of insertion is preferentially amplified over nonspecific products, which rapidly becomes the predominant product in the PCR reaction after using nested specific primers. These TAIL-PCR reactions can then be sent directly for sequencing to identify the unknown sequences bordering the known locus. Since TAIL-PCR does not require any DNA manipulation or cloning, and since the interlaced cycling between high and low temperatures provides extremely high specificity for identifying sites of insertion, TAIL-PCR is very amenable to high-throughput identification of sites of genomic insertion by RHGP vectors. This approach facilitates target identification in days, which would ordinarily take weeks using conventional methodologies.

#### Future perspective

The combination of recent advances in the fundamental understanding of tumor cell biology, high-throughput screening procedures and advances in genomic and proteomic information, combined with new discovery technologies such as RHGP and siRNA, should dramatically increase our ability to identify safe and effective targets for cancer treatment and prevention. These advances in target discovery could then foster a new generation of targeted therapeutics that are designed to specifically target cellular processes that are essential for cancer morbidity and mortality. Based on ongoing research, we anticipate that new therapies for drug-resistant cancers might be the first tangible outcomes of this new investigation, to be rapidly followed by drugs that selectively target metastasis and other behaviors that uniquely characterize malignant cells.

New strategies for target discovery have focused upon the identification of targets that are directly responsible for important functions of malignant cells. Our laboratories have developed

an improved discovery technology, RHGP, which can interrogate the entire genome for any target (whether overexpressed or inhibited) that causes a phenotype of interest. The technology can identify and validate targets (based on the reversibility of the GSV), all in a single experiment. We have applied RHGP to identify targets that cause human or rodent tumor cells to become resistant to chemotherapy. RHGP can also be used for other applications, cell types or species where cells bearing the desired phenotype can be unambiguously isolated. As such, RHGP could provide a much-needed means to

the mechanistic causes of malignant behaviors or pathways that might be targeted to improve cancer survival and quality of life.

#### Financial & competing interests disclosure

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#### Executive summary

- The discovery of new therapeutics has evolved from a function-first screen for biological activity to target-based intervention.
- Recent technological advances (such as siRNA and random homozygous gene perturbation [RHGP]) combine the strengths of target-based intervention with a function-first screening of fundamental biological mechanisms that uniquely identify malignant cells.
- RHGP can sample all protein and gene targets in a cell, both for overexpression or loss of expression, regardless of prior knowledge of the targets.
- RHGP has been used to identify targets that cause tumor cell resistance to chemotherapy or hormone therapy.

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