

Unraveling Tumor Suppressor Networks with In Vivo RNAi

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BMI1 is a known oncogenic transcriptional repressor in glioblastoma stem-like cells, but its downstream mediators are poorly understood. Recently, in *Cancer Cell*, Gargiulo et al. (2013) designed a rational in vivo RNAi screen based on BMI1 ChIP-seq from neural progenitors and identified functional tumor suppressor targets, including *Atf3* and *Cbx7*.

The discovery and characterization of genes implicated in tumor formation, progression, and drug resistance is critical for prognosis and advances in cancer therapy. A classical approach for cancer gene discovery is the search for gross genetic alterations in tumors, including chromosome translocations or copy number changes. The granularity of this analysis has recently been extended to the level of single base alterations by extensive whole-genome sequencing (Mattison et al., 2009). This approach has led to the discovery of many potential new players in oncogenesis, yet the mere identification of gene alterations does not definitively tie these changes to the process of tumor development. Functional screening approaches, including insertional mutagenesis, transposon screens, and RNA interference (RNAi), have sought to directly address the relevance of genetic alterations by evaluating the consequences of mutational insertions or genetic loss of function on cellular fate.

Insertional mutagenesis has been used for over 30 years as an effective tool for the identification of proto-oncogenes in mice. Specifically, exposure of mice to Moloney-based retroviruses results in the random insertion of proviruses that can lead to proximal gene activation (Uren et al., 2005). However, this approach has been less effective at inducing gene deficiencies, due to the low probability of biallelic gene disruption. In contrast, RNA interference has served as an effective tool for examining the cellular consequences of gene suppression, yet the adaptation of this technology to in vivo systems remains a challenging process. In the latest edition of *Cancer Cell*, Gargiulo et al. (Gargiulo et al., 2013)

have essentially coupled these two approaches, using results gained from insertional mutagenesis approaches as a foundation for an in vivo RNAi screen to examine the biology of glioblastoma.

B lymphoma Mo-MLV insertion region 1 homolog, or BMI1, a component of the polycomb repressive complex 1 (PRC1), was first identified in an insertional mutagenesis screen for genes that, when overexpressed, cooperate with c-Myc in promoting B cell malignancy (van Lohuizen et al., 1991). Substantial subsequent work has characterized BMI1 as a critical positive regulator of cellular pluripotency (Pietersen and van Lohuizen, 2008). *BMI1* depletion in brain tumors leads to a decrease in malignancy and is associated with later onset and less severe histological grading in mouse model tumor transplantation assays (Abdouh et al., 2009; Bruggeman et al., 2007). Gargiulo and colleagues applied ChIP-seq to identify *BMI1* target genes in neural progenitor, adult brain, and brain tumor cells. Even though a vast majority of identified target genes were cell type independent, cell-type-specific target genes also emerged from this analysis. The authors identified several developmental regulators, including *Tal1*, *Runx3*, *Pitx2*, and *Foxf1*, as well as several long noncoding RNAs, as *BMI1* target genes. In silico pathway analysis associated a substantial subset of *BMI1* target genes with TGF- β and BMP signaling pathways.

To validate the biological significance of their findings, the authors performed an in vivo RNA interference screen using data derived from ChIP-seq experiments. Specifically, they used RNAi-mediated suppression of *BMI1* shRNA to activate

BMI1-repressed transcriptional targets and then used a targeted RNAi library derived from ChIP-seq data to identify *BMI1* targets that impact tumor cell growth. Results from this screen showed that shRNAs targeting genes implicated in neural development were significantly enriched in resulting tumors. From this set of genes, they chose to focus on candidate tumor suppressor genes that had previously been described to be epigenetically silenced in human tumors: *Alx3*, *Atf3*, *Cbx7*, *Gfi1*, *Il5ra*, and *Ptprd*. In subsequent validation experiments the authors confirmed that suppression of *Atf3* and *Cbx7*, in a *Bmi1*-depleted background, leads to an acceleration of tumor progression and a negative impact on animal survival. Furthermore, the authors found a correlation of *Atf3* and *Cbx7* expression with patient prognosis for human glioblastoma.

Interestingly, no impact on differentiation or “stemness” could be seen after knockdown of *Atf3* and *Cbx7* in vitro, suggesting that performing this screen in vivo was critical for uncovering biology relevant to the in situ disease. Thus, the importance of performing screens in relevant physiological contexts is clear (Bric et al., 2009; Meacham et al., 2009). However, the adaptation of screening technology for in vivo use in solid tumors is not trivial, and the approaches used by Gargiulo, Van Lohuizen, and colleagues can serve as a valuable guide for best practices in performing such experiments. Of paramount importance is the choice of an appropriate model system that allows RNAi loss-of-function screens to be both robust and reproducible. The authors addressed a number of critical criteria in their glioblastoma model before

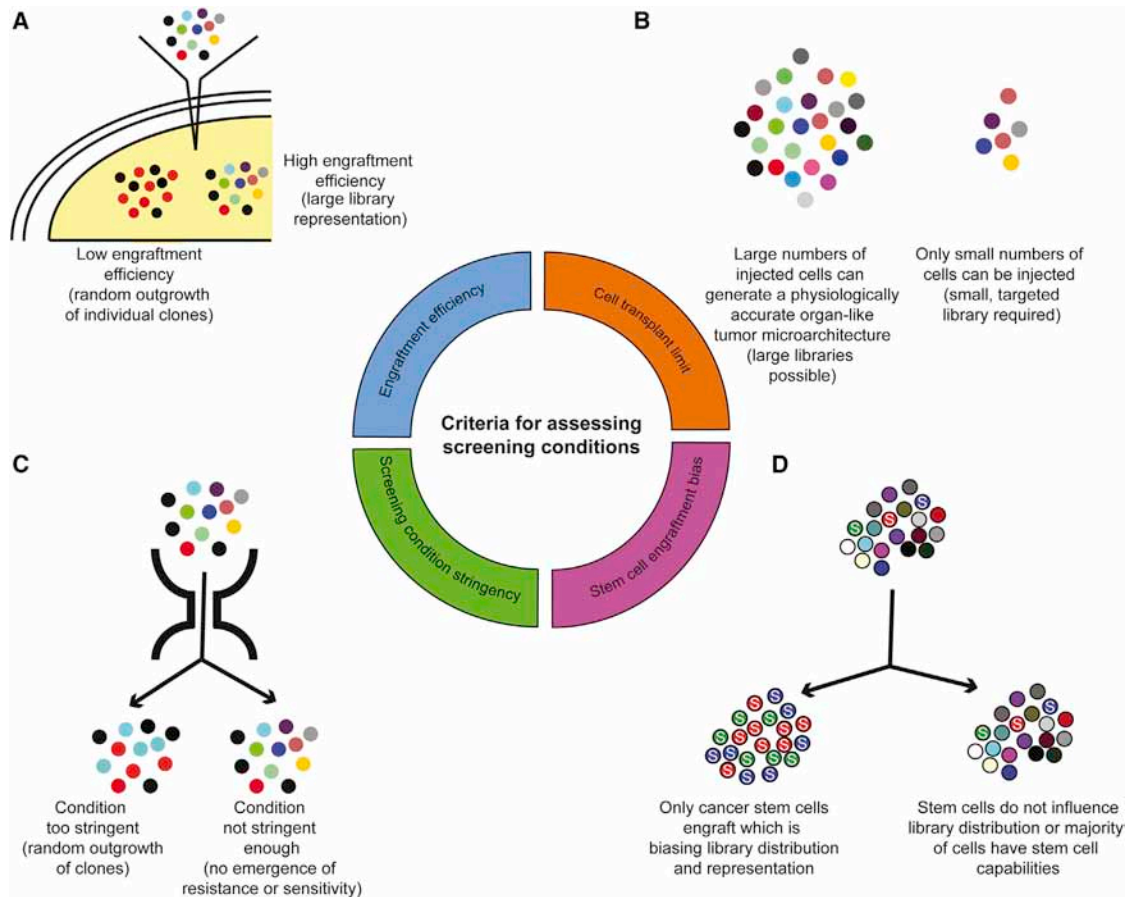


Figure 1. Criteria for Optimal In Vivo RNAi Screens

Retaining maximal shRNA representation promotes screen reproducibility and decreases stochastic cell behavior. When performing an in vivo RNAi screen, four main questions should be considered. (A) What is the tumor cell engraftment efficiency? Losing cells during injection and engraftment means a lower shRNA representation and reduced screening capacity. (B) Are the injected cells forming a pathologically accurate tumor? In some cases, injection of large numbers of cells results in the ectopic formation of cell masses that fail to recapitulate the normal tumor architecture. (C) What is the screening stringency? If the screening condition is too stringent, most cells will not survive and random clones will stochastically emerge. If the condition is not stringent enough, library representation will not change significantly over time. (D) What is the role of tumor “stem cells” in tumor transplantation? If tumors are derived from only a few cells with stem cell features (shown with an S), the shRNA library representation will be substantially reduced.

performing the actual screen (Figure 1). First, they determined that the tumor cell engraftment efficiency was sufficiently robust to guarantee a large enough representation of the shRNAs in vivo (Figure 1A). Second, they used a well-characterized glioblastoma model system (Figure 1B). Thus, the tumors produced closely resembled the autochthonous malignancy following transplant. Third, they demonstrated that *BMI1* depletion, itself, does not significantly reduce library representation (Figure 1C). If the selective pressure to bypass BMI deficiency is too great, then even shRNAs that can theoretically bypass BMI1 depletion may do so inefficiently—leading to stochastic outgrowth of cells. Finally, they started with a set of glioma-initiating “stem cells”

(Figure 1D). If only a small proportion of the total transplanted cells contribute to tumor development, then the number of shRNAs that can be screened is limited to those present in the tumor-initiating population. Together, this combination of state-of-the-art screening technologies and a variety of clever approaches to overcome technical limitations make this work an exciting starting point for future in vivo RNAi screens in solid tumors.

This work also highlights ongoing challenges for future high-throughput screens. One complication is our limited understanding of how the behavior of diverse populations of shRNA-transduced cells present in an initial screen differs from single shRNA-transduced populations used for screen validation.

In this study, shRNA pools containing screen “hits” (like shRNAs targeting *Cbx7*) accelerate disease almost as rapidly as pure populations of cells expressing the scoring shRNA alone. Understanding this population dynamic will be critical for setting thresholds for what are considered screen hits in future studies. Another complication relies in processing the large amount of data emerging from such screening efforts. Here, the authors wisely focused on a prevetted orthogonal data set, namely data emerging from a ChIP-seq approach. Thus, the study was “prefocused” on an area of biology. Additionally, they applied stringent criteria for their definition of an interesting hit (including the requirement of being

epigenetically silenced in human tumors). With increasing amounts of data emerging from unbiased studies, it will be important to tie novel in silico analysis technologies to primary biological in vivo screening data.

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A Sexy Spin on Nonrandom Chromosome Segregation

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Nonrandom chromosome segregation is an intriguing phenomenon linked to certain asymmetric stem cell divisions. In a recent report in *Nature*, Yadlapalli and Yamashita (2013) observe nonrandom segregation of X and Y chromosomes in *Drosophila* germline stem cells and shed light on the complex mechanisms of this fascinating process.

To maintain tissue homeostasis, stem cells must strike a balance between self-renewal and differentiation. One mechanism for achieving this balance is asymmetric cell division, a phenomenon in which mitosis produces sister cells that adopt different fates: one cell acquires the stem cell characteristics of its mother and the other begins on a path to differentiation. During asymmetric cell divisions, unequal segregation of proteins and RNA can drive, or occur in parallel with, sister cells' decisions to self-renew or differentiate (Neumüller and Knoblich, 2009). Another form of asymmetry involves the nonrandom segregation of sister chromatids according to the identity of their template DNA strands. Because DNA is replicated semiconservatively, sister chromatids differ, intrinsically, by the

sequences, and relative ages, of their template strands. The intrinsic asymmetry of sister chromatids led to the “immortal strand hypothesis,” which posited that chromatids bearing the oldest template strands would be segregated to the self-renewing stem cell daughter based on the assumption that this strand would bear fewer replication-induced DNA mutations (reviewed in Rando, 2007). In a new study in a recent issue of *Nature*, Yadlapalli and Yamashita (2013) identify nonrandom segregation of individual chromosomes in *Drosophila* male germline stem cells (GSCs) seemingly based upon the sequence identity of the template strands and exploit this system to advance our understanding of the mechanisms by which nonrandom chromosome segregation occurs.

Despite earlier indications from their own work that the bulk of chromosomes do not segregate asymmetrically in dividing *Drosophila* GSCs (Yadlapalli et al., 2011), Yadlapalli and Yamashita revisited the issue of nonrandom chromosome segregation with a new experiment to study the segregation patterns of individual chromosomes. This analysis was accomplished using chromosome orientation fluorescence in situ hybridization (CO-FISH), in which newly synthesized DNA strands that have incorporated the nucleotide analog 5-bromo-2'-deoxyuridine (BrdU) are selectively degraded, enabling the identification of template strands with chromosome- and strand-specific fluorescent oligonucleotide probes. *Drosophila* GSCs are well suited for such studies of asymmetric cell

