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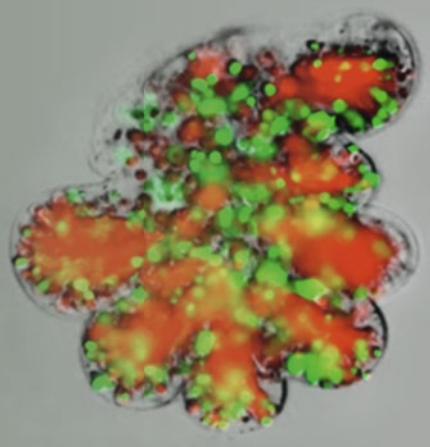

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Target Identification and Validation in Drug Discovery

Methods and Protocols

Second Edition

Humana Press

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Preface

Considering the incredible new developments in technologies over the last few years, this second edition of the book took care to cover breakthrough technologies in the field of drug target identification and validation. We are confident that the second edition will be as successful as the first, with the majority of chapters capturing recently emerging technologies. These new technologies include 3D cell culture and in particular revolutionary technologies such as CRISPR, which has a wide field of applications. Since new targets are still a valuable asset for drug development, an emphasis in this issue is on CRISPR-related screening technologies. Usage of haploid cell lines adds another breakthrough to exploit best CRISPR technologies. Other dynamic fields are "big data" and in silico approaches; hence we extended these topics compared to the previous edition. The in vivo applications of CRISPR and the best use of animal models in drug development complete the validation aspects covered by this book.

The quality of target identification and validation is a first and critical indicator for attrition rate in drug development. In a wider sense, target validation also includes aspects of efficacy and target patient population, which together define the drug properties and commercial aspects that determine the success of a drug development program. Humanized in vitro and in vivo models are instrumental to judge the probability of success, both of which are covered in this book.

This book contains a comprehensive collection of essential and state-of-the-art methods, contributed by internationally recognized experts in their specialized fields. The content of each chapter goes beyond pure protocol lists to also include useful hints, emphasizing the most critical steps and pinpointing typical pitfalls.

The chapters are organized by major categories covering methods of early drug development related to target identification and validation but also translational aspects, such as animal models and biomarker development.

This book is a valuable source of protocols for lab scientists; in addition, it represents a useful compendium for any "drug hunter" including molecular and cellular biologists, pharmacologists, pathologists, bioinformaticians, clinical researchers, or investigators, to name a few. Last but not least, any scientist who is not an expert in the field will get a quick overview on state-of-the-art technologies.

Most importantly, we thank all the authors for their valuable contributions. It was a real pleasure to interact with them in a highly professional manner. The result of these efforts is the second edition of a book of which all contributors can be proud.

Vienna, Austria

Jürgen Moll
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Part I

Identification of Novel Targets Using High Throughput Screening Assays

Chapter 1

Using Functional Genetics in Haploid Cells for Drug Target Identification

Jennifer C. Volz, Nicole Schuller, and Ulrich Elling

Abstract

Pooled genetic screens are a powerful tool to identify targets for drug development as well as chemogenetic interactions. Various complementary methods for mutagenesis are available to generate highly complex cell populations, including mRNA knockdown, directed genome editing, as well as random genome mutagenesis. With the availability of a growing number of haploid mammalian cell lines, random mutagenesis is becoming increasingly powerful and represents an attractive alternative, e.g., to CRISPR-based screening. This chapter provides a step-by-step protocol for performing haploid gene trap screens.

Key words Functional genomics, Genetic screen, Haploid, Gene trap, Stem cell

1 Introduction

Chemogenomic approaches can support drug development by rapidly uncovering functional interactions of small molecules and genes [1]. For example, overexpression of direct drug targets can result in partial resistance of cells to compounds and thus contribute to target deconvolution. Genetic screens can also shed light on resistance mechanisms of cytotoxic compounds such as chemotherapeutics. In addition, functional genomic studies can identify the genetic interactome of the drug target as well as the required enzymatic activity to activate prodrugs.

The use of transcriptional reporters coupled to a fluorescent protein combined with fluorescence-activated cell sorting (FACS) or an antibiotic resistance allows to expand the screenable range of phenotypes from lethal assays to any transcriptional event. Furthermore, immunolabelling of signaling events such as phosphoepitopes followed by flow cytometry allows to deconvolute the genetic interactome of drug-induced signaling. Importantly, genetic phenotypic screens are also a powerful tool to identify novel drug targets for subsequent targeted drug development, for

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example, for viral host factors or synthetic lethal interactions with tumorigenic mutations in positive or negative selection screening paradigms, respectively.

This century has seen several true revolutions in functional genomic approaches including small RNA-based methods to perturb gene function such as siRNA, shRNA, and sgRNA screening. Alternative methods include random mutagenesis induced by chemical mutagens or mobile DNA elements. However, random mutagenesis in mammalian cells was until recently limited to dominant phenotypes, as all mammals and established mammalian cell lines are diploid, i.e., carry two alleles for all autosomal encoded genes. In diploidy, the remaining healthy allele will mask any recessive phenotype. Meanwhile, yeast geneticists immersed in the awesome power of yeast genetics ("APYG") [2], where the power of haploid screens was already showcased in classic work half a century ago [3].

Chromosome loss and near-haploid chromosome sets are a peculiarity of acute myeloid and lymphoblastic leukemias [4, 5]. Based on the derivation of the near-haploid human myeloid leukemia cell line KBM-7 [6] with only chromosome 8 remaining diploid, seminal work by *Carette*, *Brummelkamp*, and *co-workers* established haploid genetic screens in mammalian cells in 2009 [7]. A partially reprogrammed adherent derivative of KBM-7, termed HAP-1, was developed shortly after [8]. These landmark studies led to the discovery of host factors of bacteriotoxins as well as viral life cycles [7, 9, 10].

As a direct consequence of sexual reproduction, all eukaryotes alternate between a haploid and a diploid stage in their life cycle [11]; however in most eukaryotic species including all mammals, the diploid stage is dominant, while the haploid stage is reduced to mature germ cells. Making use of the haploid stage of the life cycle in germ cells and induction of embryogenesis in the presence of only the maternal or paternal genome, the first fully haploid vertebrate cell lines were derived from frogs [12] and medaka fish [13]. In 2011, two groups reported derivation of the first truly haploid mammalian cell lines, mouse haploid embryonic stem (ES) cells from parthenogenic blastocysts [14, 15]. Shortly after, androgenic (male genome only) haploid mouse ES cells were reported [16, 17], followed by embryonic stem cells of rat [18], monkey [19], and recently human [20, 21]. Furthermore, haploid neural progenitor cells derived from rhesus monkey haploid embryonic stem cells extend the repertoire of haploid cell lines available for genetic screens [22]. Nevertheless, the availability of haploid cell lines still represents the major limitation of haploid screening technology. The fact that human haploid embryonic stem cells—unlike reported stem cell lines from other organisms—remain haploid also upon differentiation [20] opens the possibility of deriving a plethora of new haploid cell lines in the future.

Haploid screens are typically performed by targeting gene trap or polyA trap vectors randomly into the genome. This is accomplished by infection of cells with retro- or lentiviral vectors as well as transfection with transposons such as sleeping beauty, Tol2, or piggyBac. Integration of mobile elements can perturb gene expression by the presence of splice acceptors, which will trap mRNA transcripts if integrated into an intron. The advantage of insertional mutagenesis is the ease of identification of mutations in high throughput [9, 23]. However, hot and cold spots of mutagenesis, in particular of viral vectors, affect genome saturation, while transposons insert more evenly [23]. Thus, haploid insertional mutagenesis screens are mostly limited to positive selection and ideally performed based on at least 10^7–10^8 independently mutated cells. Under these conditions, positive selection screens identify often hundreds of biologically independent integrations within a gene of interest as causal for the phenotype of interest and can thus assign gene function with unprecedented certainty.

Insertional mutagenesis in haploid cells is ideally suited to uncover genetic interactions with small molecules in an unbiased manner based on loss of function phenotypes. Advantages of this approach are that (1) it does not require the generation of small RNA libraries based on gene predictions, (2) mutations are directly identified as opposed to being inferred indirectly by presence of shRNAs or sgRNAs, and (3) results are based on hundreds of biologically independent mutations resulting in unambiguous identification of hits [24]. Moreover, new gene trap systems carrying transcriptional enhancers have been developed to activate gene expression of nearby genes [25]. Thus, enhanced gene trap insertions can uncover direct drug targets also in diploid cells due to increased resistance [26] by increased drug target abundance.

Technically, a haploid genetic screen is similar to other pooled screens and consists of the generation of pools of mutagenized cells by infection or transfection with mutagenic elements followed by selection for gene trap insertions and subsequently phenotypes of interest. Mutations are then mapped to the genome by next-generation sequencing, and genes with clustered mutations are identified by bioinformatic pipelines. If mutated libraries of cells are already available, screens can be performed in less than 2 months, thereby making haploid insertion screens a powerful tool to identify chemogenetic interactions (Fig. 1). The following protocol provides a detailed step-by-step guide for successful performance of haploid genetic screens in positive selection paradigms.