Random Homozygous Gene Perturbation (RHGP) as a Tool for Target Discovery and Validation

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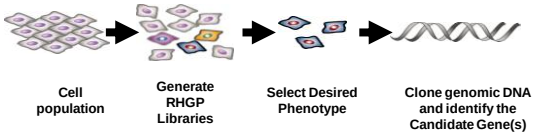
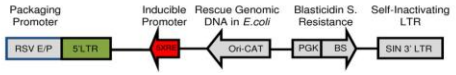
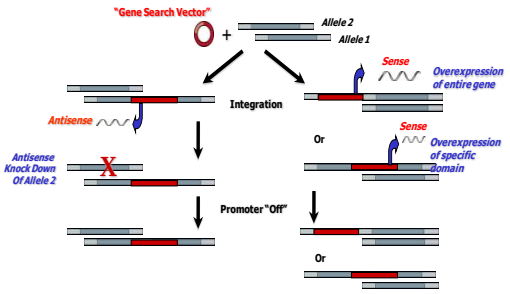
ABSTRACT
Random homozygous gene perturbation (RHGP) can identify and validate any host (cellular) gene target that directly causes a desired phenotype without requiring prior knowledge of the target. The central feature of RHGP is a unique lentiviral-based genetic element, known as a gene search vector (GSV) designed to interrogate the entire genome and identify target genes that cause the phenotype of interest. The GSV cassette contains an inducible promoter with an inducer response element (RE). The GSV is transduced into a target cell population that stably expresses the transactivator (TA). Transduction results in random integrations of the GSV into the genome of the target cells. In the presence of inducer, the TA binds the RE domain to activate the GSV promoter. Promoter activation triggers the transcription of the RNA extending into the host genome sequence flanking the 5´ LTR of the GSV. RHGP provides the ability to sample every gene in a cell for both over expression and loss of expression. When integrated in an antisense orientation, this event physically disrupts one allele, while producing antisense RNAs that knock down expression of the other allele. In the sense orientation, RHGP may overexpress the entire target gene when the vector is inserted upstream of the start site or overexpress the domains of the gene and produce a dominant-negative inhibitor of wild-type gene function. 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. With RHGP approach, FGI has successfully identified the host target genes involved in pathogenesis of cancer metastasis, drug resistance, Alzheimer's Disease and viral infections. Therapeutics against the first of three RHGP identified targets for infectious disease is now in clinic trial. Therapeutics against the other two targets will be entering preclinical development shortly.



Gene Search Vector (GSV)

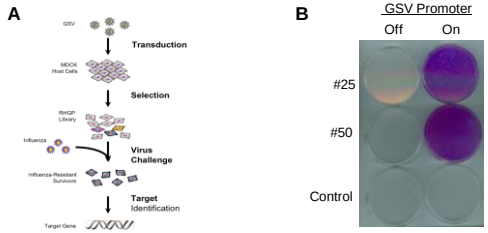


Potential Outcomes of GSV Integration

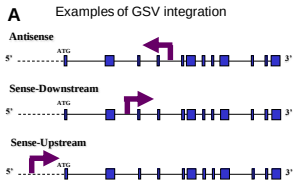


A schematic overview demonstrating how the random homozygous gene perturbation (RHGP) technology can perturb endogenous gene expression. In the example on the left, insertion of the GSV into the first allele disrupts expression from that allele. The orientation of the integration then allows the GSV 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. In the example on the right the GSV vector is inserted in the opposite orientation causing either an over expression of an entire gene (Sense orientation upstream of start site) or a dominant negative domains (Sense orientation downstream of start site).

Identification of Host Targets That Allow Cells To Survive an Otherwise Lethal Infection With Influenza Virus



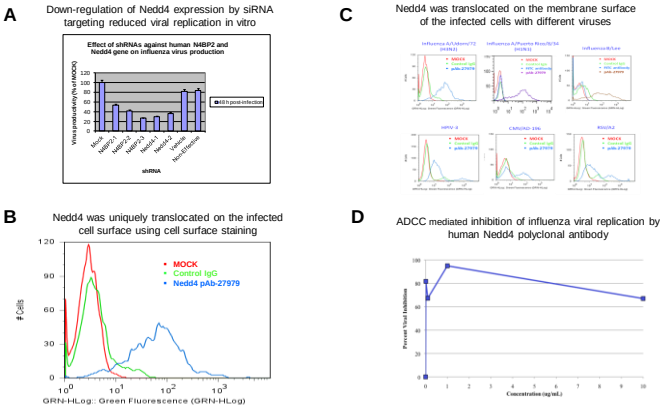
(A) Overview of the strategy to identify host genes necessary for influenza infection. Following transduction with the GSV and antibiotic selection to establish an "RHGP library" of perturbation, the host cells are challenged with a lethal infection from influenza virus. The influenza-resistant survivors were selected and the target genes are identified.
(B) Validation of influenza virus resistant subclones with reversibility assay. Two representative clones were challenged with a lethal infection in promoter "ON" or "OFF". The cells were assessed by crystal violet staining.



B

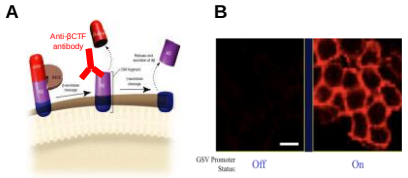
Perturbation	Number of Unique Targets (99 total)
Antisense	56
Sense-downstream	35
Sense-upstream	9

(A) Overview of possible orientations and sites of GSV integration into the genome.
(B) A summary of the host target genes identified from a single RHGP campaign (Sui, B., et al., *The use of Random Homozygous Gene Perturbation to identify novel host-oriented targets for influenza.* Virology, 2009. **387**(2): p. 473-81).



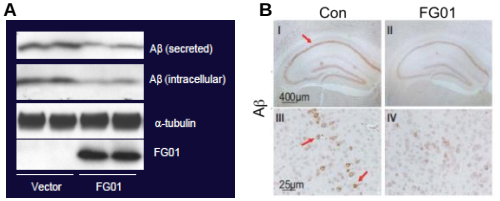
(A) Validation of N4BP2 and Nedd4 gene via shRNA targeting of HEK293 cells. The shRNAs of N4BP2 (N4BP2-1, 2 and 3) and Nedd4 (Nedd4-1 and 2) were used to transfect HEK293 cells. Viral titers were measured by plaque assay.
(B) Cell surface staining assay. MDCK cells were infected with Influenza A/Udorn/72 at MOI of 0.1. After 18h post-infected, cells were stained with polyclonal antibody.
(C) Cell surface staining assay with different viruses. MDCK, HEK293, Hep-2 or MRC-5 cells were infected with viruses at MOI of 0.1 or 1.0. The cells were stained after 18h (influenza) or 48h (HPIV, CMV and RSV) PI.
(D) Inhibition of viral replication by an ADCC mechanism. MDCK cells were infected with influenza virus and incubated with anti-Nedd4 polyclonal antibody in presence of purified human NK cells. Viral titers were measured by RT-PCR.

Identification of Target Gene, FG01 (Rps23r1) Involved In Alzheimer's Disease



(A) A screening assay was developed to detect the phenotype using anti-ß-CTF antibody. ß-CTF accumulates on the cell surface due to decreased Aß protein release from its precursor(left). Surface exposure of ß-CTF (red on right panel) on cells expressing Aß precursor protein (APP) indicated accumulation of ß-CTF and lack of Aß production.
(B) The identification and reversibility of FG01, a gene that interrupts the final step in Aß production. When up-regulated, there is no Aß production, and thus Anti-ßCTF binds to the cell surface (On). When the FG01 upregulation is reversed by turning off the GSV promoter, Aß is produced once again.

Target validation in vitro and in vivo



(A) FG01 or control vector (Con) were transfected into mouse N2aSwe or human HeLaSwe cells. The levels of Aβ and FG01 were observed by Western Blot.
(B) Brain tissues from FG01-transgenic 3XTg AD mice and controls (Con) at 11 months of age were analyzed by immunohistochemistry for Aβ. Panels III and IV are higher magnifications of cortical regions from I and II, respectively. Red arrows indicate positive immunoreactivity (Zhang, Y.W., et al., *A Functional mouse retroposed gene Rps23r1 reduces Alzheimer's beta-amyloid levels and tau phosphorylation.* Neuron, 2009. **64**(3): p. 328-40).

Representative Successes of RHGP

Indication	Criteria for Selection	Selection methods
Cancer	Chemo- or Hormone-Resistance	Survival
Cancer	Metastatic Character	Survival or Adhesion Changes
Alzheimer's Disease	Decreased Aβ Production	Flow Cytometry
Host Targets for Influenza	Resistance to Influenza	Survival
Host Targets for HIV	Resistance to HIV Challenge	Survival
Enhanced Antibody Production	Increased Antibody Levels	Flow Cytometry