Reverse Genetics — Gene mutations

I) Gene Knockout (KO) / Gene Replacement via Recombinational Repair

A) Homologous Recombination (HR)
Recombination of chromosomal locus with an exogenous template — nature of template determines KO or gene replacement (or tag addition)

- Yeast, mouse (ESC) — Standard Practice
  Possible because of high recombination rate

B) Repair of induced Double Strand Break (DSB)
- HR repair of DSB by exogenous template to give KO or replacement.
- Nonhomologous End Joining (NHEJ) to give deletion

1) Transposable Element Excision
- Drosophila (Rong et al. 2002, Genet Dev 16: 1568-1571)
- C. elegans (Frohjaer-Jensen et al. 2010, Nat Meth 7: 951-953)
2) Site Specific Nuclease Mediated
   - CRISPR Cas9 *
   - TALENS
   - Zn-Finger nucleases

C. elegans, Drosophila, Zebrafish, mouse, rat, tissue culture cells
Non-standard organisms

C. elegans : Arribere et al., Genetics, 2014
papers Paix et al., Genetics, 2014

II) Screening Populations of Chemically Mutagenized Organisms for DNA sequence changes.

- C. elegan Deletions - detected by PCR
  G3, 2013 2:1415-1425

- Arabidopsis & Zebrafish point mutations
  TILLING
  Targeted Induced Local Lesions In Genomes
Issues to Address with "Targeted" Mutations

- Both Homologous Recombination mutations & those isolated in the PCR Pool Screening method

1) Does the Mutation Disrupt the Gene Such that NO Product is Produced?
   - Genetic Tests
   - Molecular Tests

2) Where any Extraneous Mutations Induced at the Same Time?
   - Surprisingly high rate of extraneous mutations induced in both yeast and ES cells following homologous recombination experiment.

3) Does the Mutation Effect Adjacent Genes?
   - For Mouse Knockouts, see Olser et al. (1996) Cell 85: 1-4

4) Strain Backgrounds - can modify phenotype
Million Mutant Project

EMS

\[ W \xrightarrow{1/4} p_0 \]

\[ \xrightarrow{1/2} F_1 \]

\[ \xrightarrow{1/2} F_2 \text{ (avoid strong phenotypes)} \]

\[ \xrightarrow{} F_{10} \text{ (inbred)} \]

2,000 lines

UG Sequencing

400 SNV/strain

21% Non-synonymous

1% Nonsense mutation

0.5% Splice site change

- Randomly distributed throughout genome

- Nonsense mutations reduced from expected, not found in known essential genes

Natural variation

40 wild Celgene strains

UG Sequencing

16,000 SNV/strain

10% Non-synonymous

0.1% Nonsense mutation

0.01% Splice site change

- Non-randomly distributed

- Nonsense and splice site mutations show very strong reduction from expected, in both essential and nonessential genes

- Mutagenesis samples the genome more fully than natural variation.

- Strong selection acting in natural environments on most genes
"Reverse Genetics" – Temporary or Transient "Knockout" of Gene Activity

1) RNAi
2) Antisense RNA (morpholino)
3) "Dominant-Negative" Products
4) Antibody Injection
5) Ectopic/Overexpression

- Interfering reagent must be delivered to the cells/organism.
- Assay for phenotypes at a later time.

- If there is a Negative Result - No Phenotype
  No Conclusion about Function - Redundancy Can be Made.

- If there is a Phenotype, is it specific to the Gene under test?
RNA Silencing Phenomena in Eukaryotes

Endogenous dsRNA
- homologous to mRNA
  - siRNA
    - Degradation of homologous mRNA
  - homologous to repeated DNA
    - siRNA
      - Chromatin Silencing (induced, repressive histone modifications)

Endogenous hairpin RNA
- miRNA
  - Translational Repression and/or
    - Degradation of mRNA containing homologous sequences
- What is the Mechanism by which RNAi Degrades Endogenous mRNA?

What is the Normal Role of RNAi in Organismal Biology?
- Protect against RNA (DNA?) Viruses?
- Suppress Transposable Element Mobilization?
- Regulate Endogenous RNA Levels?

How does RNAi move throughout the Organism?

In cases where RNAi is Heritable, what is the Mechanism of Transmission?


Cautions!!
RNAi - RNA Interference

Fire et al., 1998  Nature 391: 806-811

Delivery Methods for C. elegans

Microinject dsRNA

Soak L1 or L4 Larvae in dsRNA

Feed Worms E. coli Synthesizing dsRNA

L4, Adult, L1

Score Po Phenotype

Score F1 Progeny Phenotype

Score Po Phenotype

Score F1 Progeny Phenotype

Score Po Phenotype

Score F1 Progeny Phenotype

Double Stranded RNA (dsRNA) is from either cDNA or Exons (+ Intron) of Gene to be tested (usually >500 bases)

Endogenous mRNA is depleted over time, so phenotype develops over time.
RNAi Mechanism

- Double Stranded RNA (dsRNA) is the Initial Active Component.

- The Homologous mRNA is the Target.
  Exonic dsRNA Works, Intronic dsRNA does not.

- RNAi is not Heritable in a Mendelian Sense.
  Endogenous Target Gene Shows No Sequence Change.
  Can have Epigenetic Changes (Methylation in Plants).

- Does not Require 1:1 Stochiometry of dsRNA to Endogenous Target mRNA.
  (Antisense RNA Requires >20-fold Excess)

- Endogenous Homologous mRNA is Degraded.

- RNAi Mechanism is Saturable.
21-25 nt Small Interfering RNA (siRNA) is the Active Component.

Exogenous or Endogenous Long dsRNA

\[ \downarrow \text{Dicer} \]

\[ \begin{array}{c}
\text{siRNA Duplexes} \\
\end{array} \]

\[ \downarrow \text{Helicase} \]

\[ \begin{array}{c}
\text{RISC} \\
\end{array} \] RNA Induced Silencing Complex

\[ \begin{array}{c}
\text{mRNA} \\
\end{array} \] Degraded
RNAi

For >1000 Genes where *lf* Phenotype is known from Classical Genetics, RNAi gives partial *lf*, or Hypomorphic Phenotypes, and in many cases, the Null Phenotype.

So can give *lf*, but not necessarily the Null Phenotype.

RNAi Phenotype is Gene specific, unless Gene under test has a Relative that is ≥ 90% Identical at the Nucleotide level.

But Genes Expressed/Functioning in the Nervous System are Refractory to RNAi

And >10 Genes whose *lf* Phenotype is known (Outside the Nervous System) Show No RNAi Phenotype.

So No Conclusion about Function/Phenotype Can be Drawn from a Negative Result.
- RNAi is a rapid method of providing information on wild-type gene function using a loss-of-function strategy.
  
gene-\(X\) (RNAi) has a cytokinesis defective phenotype. Conclude that gene-\(X\) (\(+\)) is necessary, either directly or indirectly for cytokinesis.

- Reduces maternal & zygotic gene activity (\(F_1\) screen)

- Can be scaled-up to give 1st pass functional information on all genes/ORFs in the genome (but caveats)

- Can be used to examine temporal aspects of gene function.
  
e.g.  
edcd homologue, \(ncc-1\) has \(L1\) lethal null phenotype  
\(\rho_0\) RNAi for \(ncc-1\) shows that the gene is essential for adult germ cell proliferation and meiosis I division.
- Can be used for examining gene families. 
  C. elegans has 4 B-type Cyclins - do RNAi on all 4 simultaneously.

- Can get functional information on alternatively spliced isoforms (≥22nt).

- Used for candidate gene testing in positional cloning experiment/deep sequencing.

- Used for biological relevance testing for genes (proteins) identified by other methods - 2-Hybrid Screen; IP-sequencing.

  RNAi of interacting gene should have phenotype related to the bait.
RNAi Interpretation Caveats.

- Conclusions Can Not be Drawn When No Phenotype is Observed with RNAi.

  Case 1  Gene Deletion $\Rightarrow$ No Phenotype $\Rightarrow$ Conclude No Essential Function

  Case 2  gene-$X$ (RNAi) $\Rightarrow$ No Phenotype  Can Draw No Conclusion

  - Gene may be Resistant to RNAi
  - Technical Failure

- Cross RNAi - genes that are $\geq$ 90% Identical at the Nucleotide level will show Cross-Interference.
  - If Genome is "Completely" Sequenced, then less of an Issue.

- As with "Classical" Gene Mutations, Determining if gene-$X$ has a Direct or Indirect Role in a Process that is Disrupted Requires Determining the 1st Phenotypic Deviation from Wild-type.
RNAi Caveats

- While RNAi does result in reduction of function and sometimes elimination (null) of function, one cannot conclude that the observed phenotype is null.

RNAi-mediated depletion of the endogenous mRNA is time dependent and may not go to zero.

RNAi can not deplete protein.

- If not null, then use of RNAi in epistasis experiments can be misleading.
Many gene products function in multiple processes — where the amount of gene activity required for the different processes is distinct.

\[ \text{gene-}X (\text{RNAi}) \rightarrow \begin{array}{l}
\text{Process A Disrupted} \\
\text{Process B Occurs Normally}
\end{array} \]

Can not conclude that gene-\(X\) has no essential function in process B

\[ \begin{array}{c}
\text{(\% Gene Activity)} \\
\text{Amount of mRNA}
\end{array} \]

- Process A requires a high level of WT gene activity, disrupted below 50%.
- Process B requires a low level of WT gene activity, is disrupted only when less than 50% gene activity remains.
RNAi Caveats

- Method to Method, Lab to Lab Variation in RNAi Phenotype for Specific Genes.

Possible Reasons

1. Different Methods of Delivery
   Injection, Feeding, Soaking

2. Differences in Region of RNA being Targeted,
   Size of dsRNA, total concentration being Introduced.

3. Environmental Factors (Temperature, Growth Conditions).


5. Timing of Phenotypic Analysis.

RNAi in mammalian cells

- siRNA transferred into cells (short-term knockdown)

- short hairpin RNA (shRNA), delivered by lentiviral vectors (persistent knockdown) (available through CDI/S. Stewart)

Interpretation of results

1. Off-target affects a major concern (sequence dependent or independent)

- Show that targeting other sequences in the gene gives the same phenotype.

- Rescue of phenotype with transgene containing a modified target gene (synonymous changes) or ortholog (mouse → human) that is resistant to RNAi.

2. All previous caveats apply

Henriksen et al. (2011) Meth Mol Biol 703: 189-204

Llano et al. (2007) HIV Protocols 485: 257-270

Grimm et al. (2006) Nature 441: 537-541

Buchard et al. (2009) RNA 15: 308-315

Anderson et al. (2008) RNA 14: 583-861
Morpholino gene product knockdown

Used to study gene function in zebrafish and Xenopus following delivery by microinjection.

Morpholino oligonucleotide (~25 bases)
- 4 bases + morpholino ring + phosphorodiamidate linkage
  that is stable in vivo.

Does not cause mRNA degradation
- Steric blockage of target region on mRNA
- Translation initiation
- Splice sites
  thus must be introduced in excess

1. Toxicity
2. Off-target effects
3. Previous caveats
"Reverse" Genetics using Expression of Dominant Negative - Antimorphic Products

- Should act in the same phenotypic direction as loss-of-function - So can potentially Deduce Wild-type Gene Function


- However, lots of Caviates to this method.
  - Biochemical Mode of Action of Dn-Product, in most cases, is Unknown.
  - Is the Gene A-Dn Product Poisoning the A+ Product in the Cell, or is it Binding to and Poisoning another Gene Product (B)? If so then experiment is not Accessing only A+ Gene Function.
  - If the A+ Product has Multiple Activities, are they Affected Equally?
  - Can Enough A-Dn Gene Product be Expressed (spatially & Temporarily) to Inactivate A+?

=A Negative Result (no phenotypic effect) is Inconclusive.
Ectopic Gene Expression: (Sufficiency Test)

This strategy asks whether a given cell type can respond to the ectopic gene expression.

- Not a good method of learning about gene function without considerable additional genetic and molecular data.

- One does not know, a priori, whether gene product action will be positive or negative, or in what process(es) it will act.

2 Examples of Misleading results.

1) [Myo D] excess → I0T½ Cells → Remove Growth Factors → Muscle Cells

MyoD assumed to have an important determinative role in muscle cell type specification

Problem: MyoD gene disruption has a wild-type phenotype (Mice).

° If MyoD has an important role in muscle development, it must be redundant to another gene(s).
2) hairy is a helix-loop-helix (HLH) transcription factor involved in proper segmental identity in the Drosophila embryo.

Early ectopic expression of hairy results in inhibition of Sex-lethal (a sex determination gene) transcription, causing specific lethality.

- the hairy HLH protein binds to positive regulators of Sex, transcription. These are themselves HLH proteins, resulting in their inactivation.

Poisoning of another pathway (sex determination) rather than affecting anterior-posterior segmental organization.