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 ES cells - standard practice possible because of high recombination rate

B) Repair of induced double stand break (DSB)
- HR repair of DSB from exogenous template to give KO or replacement.
- Nonhomologous end joining (NHEJ) to give deletion (or insertion)

1) Transposable element excision
- Drosophila (Rang et al. 2002, Genes & Dev. 16:1568-1581)
- C. elegans (Frøkjær-Jensen et al 2010, Nat Meth 7: 451-453)

II) Screening populations of chemically mutagenized organisms for DNA sequence changes.
- C. elegans deletions - detected by PCR (G3, 2013 2:1415-1425)
- Arabidopsis and zebrafish point mutations

TILLING:
- Targeted Induced Local Lesions In Genomes

Issues to address with “targeted” mutations

- For both homologous recombination mutations and those isolated with PCR pool screening methods.

1) Does the mutation disrupt the gene such that it is a null allele?
- Genetic tests
- Molecular tests

2) Were any extraneous mutations induced at the same time?
- Surprisingly high rate of extraneous mutation induced in both yeast and ES cells following homologous recombination experiment.

3) Does the mutation effect adjacent genes?
- For mouse knockouts, see Olsen et al 1996 Cell 85:1-4 for problems caused by position effects of targeted mutations.

4) Strain background - can modify phenotype.
Million mutation project  (Thompson et al. 2013 Genome Res 23:1749-1762)

- EMS
  - F0
  - F1
  - F2 (avoid strong phenotypes)
  - inbred
- ~2000 lines
- WG sequencing

Variation in ~2000 EMS mutant strains
- 400 SNV/strain
- 21% nonsynonymous
- 1% Nonsense mutation
- 0.5% splice site change
  - Randomly distributed throughout genome
  - Nonsense mutations reduced from expected, not found in known essential genes
  - Mutagenesis samples the genome more fully than nature variation.

Natural variation in 40 wild C. elegans strains
- 16,000 SNV/strain
- 10% nonsynonymous
- 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
  - Strong selection acting in natural environments on most genes.

Reverse Genetics -- Temporary Knockdown/ Perturbation of Gene Product Activity

RNAi - RNA interference  (Fire et al. 1998 Nature 391:806-811)

Delivery methods for C. elegans

Double stranded RNA (dsRNA) is from either cDNA or exons (+Introns) of gene to be tested (usually >500 bases). Endogenous mRNA is depleted over time, so phenotype develops overtime.
RNAi Mechanism

- Double stranded RNA (dsRNA) is the initial active component.
- The homologous mRNA is the target.
  - Exonic dsRNA works. Intronic dsRNA only 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 stoichiometry of dsRNA to endogenous target mRNA. (Antisense RNA requires > 20 fold excess.)
- Endogenous homologous mRNA is degraded.
- RNAi mechanism is saturable.

21-25nt small interfering RNA (siRNA) is the active component

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RNAi findings in C. elegans

- For >1000 genes where if phenotype is known from classical genetics, RNAi gives partial if, or hypomorphic phenotypes, and in some cases, the null phenotype.
  - Can give if, but not necessarily the null phenotype.
- RNAi phenotype is gene specific, unless gene under test has a paralog that is ≥ 90% identical at the nucleotide level.
- The Nervous System is refractory to RNAi.
  - For >50 genes, whose if phenotype is known (outside the nervous system) show no RNAi phenotype.
  - No conclusion about function/phenotype can be drawn from a negative result.
- The RNAi process in C. elegans is systemic.
RNA is a rapid method of providing information on wild-type gene function using a loss-of-function strategy. If gene-x(RNAi) has a cytokinesis defective phenotype, conclude that gene-x(+) is necessary, either directly or indirectly for cytokinesis.

- Reduced maternal and zygotic gene activity (F1 screen)
- Can be scaled up to give 1st pass functional information on all genes/ORFs in the genome (see High throughput genetics lecture)
- Can be used to examine temporal aspects of gene function. e.g. cdk-1 ortholog, ncc-1 has L1 lethal null phenotype. P0 RNAi for ncc-1 shows that the gene is essential for adult germ cell proliferation and meiosis I division.

Additional uses of RNAi

- Used in validation of causal gene identification from whole genome sequencing. RNAi of the gene should give a similar phenotype as loss of function of the gene being examined.
- Used for biological relevance/validation testing of genes (proteins) identified by other methods. Example: gene products identified from protein interaction experiments - yeast 2-hybrid screen or IP Mass spec.

RNAi of the gene IDed from protein-protein interaction predicted have a phenotype that is related to the Bait gene.

RNAi interpretation caveats

- Conclusions following observation of a wild type phenotype.
  - Case1: gene-x deletion ⇒ No phenotype ⇒ Conclude No essential function
  - Case2: gene-x(RNAi) ⇒ No phenotype. Can draw NO conclusion
    - gene or cell type may be resistant to RNAi
    - Technical failure
- Cross RNAi - genes that are > 90% identical at the nucleotide level can show cross-interference.
- As with gene mutations, determining if gene-x has a direct or indirect role in the disrupted process requires determining the 1st phenotypic deviation from wild type.
RNAi interpretation caveats
- While RNAi results in reduction of function and sometimes elimination (null) of function, one can not conclude that the observed phenotype is null. RNAi mediated depletion of the endogenous mRNA is time dependent and may not go to zero.
- RNAi cannot deplete protein.
- If not null, then use of RNAi in epistasis experiments can be misleading.

RNAi interpretation caveats
- Many gene products function in multiple processes where the amount of gene activating required for the different processes is distinct.
  gene-x(RNAi)  
  Process A disrupted  
  Process B occurs normally  
  Can not conclude that gene-x has no essential function process B

RNAi interpretation caveats
- Method to method/lab to lab variation in efficiency of obtaining RNAi phenotype for specific genes.
Possible reasons
1. Different methods of delivery, injection, feeding, soaking
2. Differences in regions of RNA being targeted, size of dsRNA, total concentrated being introduced.
3. Environmental factors (temperature, growth conditions)
5. Genotype of recipient.
6. Time of phenotypic analysis
7. Method of phenotypic analysis, expertise.
RNAi in mammalian cells

- siRNA transfected into cells (short term knockdown)
- Short hairpin RNA (shRNA), delivered by lentiviral vectors (persistent knockdown) (available through CDI/S. Stewart)

Cautions when interpreting results

1. Off-target affects a major concern (sequence dependent and 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. (2009) HIV protocols 485:257-270
Briere et al. (2006) Nature 441:537-541 (sequence independent Off target)
Buchard et al. (2009) Mol 15:308-315
Anderson et al. (2008) Mol 14:583-583 (sequence dependent Off target)

Morpholino gene product knockdown (Antisense method)

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

- Morpholino oligonucleotide (~25 bases)
- 4 bases + morpholino ring + phosphorocliamidate linkage
- Does not cause mRNA degradation
  - steric blockage of target region in the mRNA
  - translation initiation
  - splice sites
  - must be introduced in excess

Cautions
1. Toxicity
2. Off-target affects
3. Previous caveats
Reverse genetics using expression of "Dominant negative" gene products (primarily used in cell culture)


- However, lots of caveats to this method:
  - Biochemical mode of action of Dn-product, in most cases, is unknown; so don't know if poisoning the corresponding wild type gene product (definition of dominant negative) or another gene product.
  - If the gene product has multiple activities, are they affected equally?
  - Was enough Dn gene product expressed to cause inactivation?

Ectopic gene expression (basically a sufficiency test)

This strategy attempts to deduce gene function by asking 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.

Example 1

MyoD expression

10T½ cells

Remove Growth Factors

Muscle cells

MyoD was interpreted as having an important role in muscle cell type specification.

Problem: MyoD gene knockout has a wild-type phenotype (in mice).

∴ While MyoD is sufficient for muscle development, it must function redundant with other gene(s) as it is not necessary.
Example 2

Hairy is a helix-loop-helix (HLH) transcription factor involved in proper segmental identity in the Drosophila embryo.

Hairy was overexpressed in the early embryo to examine the effect on segmental identity.

- Early ectopic expression of hairy caused ♀ specific lethality.
- Hairy binds to another HLH protein that functions to activate dosage compensation and sex determination and causes it to be inactivated.
- The early overexpression results in poisoning of another pathway (sex determination & dosage compensation) rather than changing anterior-posterior segmental organization.