FLP-FRT mediated mitotic recomb

$w^{hsf p}, m - FRT40a$

$w^{fert40a}$

1) Mutant clone - lacks GFP, $w^+$
2) Twin-spot - $2x$GFP, $w^+$
3) All other tissue - $1x$GFP, $w^+$

1) How easy is it to see clone?

2) What would you expect if the mutation had no effect on cell proliferat? 

3) What if the mutation decreased cell proliferat? 

4) What if mutation increased cell proliferat? 

Of interest? Why?
FLP-FRT F1 Genetic Mosaic Screen

A) w^+ x w^- 

\[ \text{EMS} \quad \text{y}^w \quad \text{y}^w \]

1) Mitotic recomb \( \Rightarrow \) eye
2) Essentially all eye cells undergo mitotic recomb \( \Rightarrow \) \( \sim \) or +/+  
   \[ \rightarrow \text{now - 2xw-} \text{no GFP} \quad 2x\text{GFP} \]

Assay: 1) More w- than w+ tissue?  
   Why?

B) Results from many groups

23 genes identified \( \rightarrow \)

Two classes

\[ \text{Some \# of cells in} \quad \text{Many \# more cells in} \]
\[ \text{mutant clone + twin-spot,} \quad \text{mutant clone} \]
\[ \text{but mutant cells are} \quad \downarrow \]
\[ \text{much bigger.} \]

4 genes identified

1. Salvador - 698 kb  
2. Warts (let's) - 1099 kb NDR-kinase  
3. Hippo - 699 kb - 518-20 family kinase  
4. Mohs/Mats - 219 kb novel protein  
   (Later: Expanded + Merlin (NF-2))
   \( \text{\n}\]
4) Superfamily of  
   adaptor proteins
Mutant phenotype:  
1) More cell proliferation (↑Cycle)
2) Less cell death (↓DIAP)

sav/hpo/wts/Mob
-ve → cell prolif
+ve → cell death

Identification of molecular nature of Salvador + Hippo

1) Salvador - 4 alleles
   a) Fine-scale genetic mapping localized sav to a 20kb region
      1. 5 genes in the region
      2. Sequenced all 5 genes in region in all 4 alleles
      3. Results: 4 of 5 genes → no A in any background
         1 of 5 genes → premature stop codon in each background.

2) Hippo - 8 alleles
   a) Fine-scale genetic mapping localized hpo alleles to small region.
      1. Sequenced all genes in region
         Result: Only one gene disrupted in each background.

2. cDNA of this gene rescued hpo mutant phenotype when driven under G418/UAS-control: Ubi-Gal4/UAS-GeneX; hpo

   Kinase domain  Autology  Dimerization Domain
How can you order the action of these genes?

1) exm/rn
2) Hpo
3) Wts
4) Sav
5) Mobs

all have the same genotype
more cell prolif.
less cell death.

1) Try to create gain of f-cx alleles that manifest the opposite genotype? But, how?

A) Gene over-expression of genotype
   1. UAS \( \rightarrow \) merlin \( \rightarrow \) No effect
   2. UAS \( \rightarrow \) expanded \( \rightarrow \) cell death \( \rightarrow \) p-way hyperactivation
   3. UAS \( \rightarrow \) hpo \( \rightarrow \) cell prolif
   4. UAS \( \rightarrow \) wts \( \rightarrow \) No effect
   5. UAS \( \rightarrow \) sav \( \rightarrow \) No effect
   6. UAS \( \rightarrow \) Mobs \( \rightarrow \) No effect

2) Create "double-mutants" b/w gain of f-cx alleles + loss of f-cx mutations in other genes.

Logic:

a) Loss of f-cx genotype of a given gene is epistatic to the gain of f-cx genotype of all genes upstream of it. (aka: the f-cx of the upstream gene requires that of the downstream gene)

b) The gain of f-cx genotype of a gene is epistatic to the loss of f-cx genotype of genes upstream of it.
Examples of Epistasis Experiments

1) Gain of fox allelic expanded + loss of fox allele of hpo (Too little growth) - (Too much growth) 

Result: Observe overgrowth genotype identical to hipo.
- hpo is epistatic to ex (ex→hpo)
- ex requires the func of hpo to mediate its effect (↓ cell polis↑ cell death)

2) Loss of fox in ex+mur + gain of fox allele of hpo (too much growth) - (too little growth) 

Result: Observe too little growth (hpo genotype)
- hpo is epistatic to ex (ex→hpo)
- hpo does not require ex→

3) Gain of fox for hpo + loss of fox for wts 

Result: Too much growth: wts genotype
The beginning of knowledge is some discovery of something we don't understand. F. Herbart

CRISPR Loci: 1989-93

1) Assess salt tolerance in microbes - found an unusual locus thru sequencing. ⇒ hypervariable CRISPR-associated genes (Cas)

- ORF ORF ORF ORF

- Direct repeats (28-37bp)

- Spacers: 12-36bp - homology to φ + phage DNA

Highly variable
- Spacers # and content
- Varies b/w closely related strains

1) What is the freq of the spacers + where do they come from?
2) What is the freq of the ORFs?
3) What is the freq of the direct repeats?

3. thermophilus ⇒ Impt in dairy culture (yogurt) = Danon

Experiment: 1) Challenge a φ-sensitive S. thermophilus strain & two highly similar virulent φ + select φ-resistant bacteria

- φ-sensitive bacteria

φ-sensitive bacteria

⇒ φA and/or φB

Grow ⇒ Lys ± spin + plate ⇒ φ-resistant bacteria

2) Seq. CRISPR locus in parental + each φ-resistant bacteria

<table>
<thead>
<tr>
<th>φ resistant derivatives</th>
<th>φA</th>
<th>φB</th>
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<tbody>
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<td>Parental:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>L</td>
<td>L</td>
</tr>
<tr>
<td>2</td>
<td>S1</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>S2</td>
<td>L</td>
</tr>
<tr>
<td>4</td>
<td>L</td>
<td>S3</td>
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φ resistance

<table>
<thead>
<tr>
<th></th>
<th>φA</th>
<th>φB</th>
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<tr>
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<td>S</td>
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<td>R</td>
</tr>
<tr>
<td>4</td>
<td>R</td>
<td>R</td>
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</tbody>
</table>

Why?
Key results: 1) All \( \phi \)-resist. lines acquired new spacers
   2) Spacer content identical to \( \phi \) puA
      \[ \phi_A + \phi_B \approx \text{v. similar in sequence} \]
   3) Spacer number + sequence \( \approx \) w/ \( \phi \)-resistance

Where do spacers come from?
What do they do?

How do you assess necessity + sufficiency of spacers = resist?

\( \phi \)-resistant:

\[ \text{Cas5(G)}, \text{cas1}, \text{cas6, cas7}, \text{L...} S1 \text{S2} \text{S3} \ldots R S \]

Conclusions: 1) Spacers acquired during infect = confer resistance.

2) Cas genes required for resistance.

Model for CRISPR-mediated adaptive immunity

1) Adapted (Spacer addition)

2) Expression

- Cas7
- Cas9 loading
- CR RNA
- \( \phi \) DNA
How does the CRISPR/Cas system achieve self-non-self recognition?

- How does CRISPR/Cas9 specifically cleave the virus, but not the CRISPR locus?
Jinek et al 2012: Nobel Prize

crRNA + Cas9 → Cut DNA @ sites of complementarity?

What are the minimal elements required for CRISPR-med. site-specific DNA cleavage?

Experiment: Drop-out/Add-in Exps

<table>
<thead>
<tr>
<th>Purified Components</th>
<th>Cas9</th>
<th>crRNA-Sp2</th>
<th>tracrRNA</th>
<th>crRNA-Sp1</th>
<th>Mg²⁺</th>
<th>Cut?</th>
<th>Assay</th>
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<tbody>
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<td></td>
<td>+</td>
<td>-</td>
<td>+</td>
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<tr>
<td></td>
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<tr>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td>NOT cleaved</td>
</tr>
</tbody>
</table>

Conclusion: Created a simple in vitro system to assess Cas9/cRNA functionality

Is the PAM required for Cas9/cRNA DNA cleavage + binding?

Cleavage

- Cas9 + Sp4 → Cut
- Cas9 + Sp4 → ND

DNA-binding

- Cas9 + Sp4 → Enzymatically dead

PAM is required for DNA-binding + cleavage
Can CRISPR/Cas9 be programmed to target any DNA?

1) Design 5 sgRNA to target GFP

2) Exp. (Cas9 + sgRNA) + GFP → cut? not cut?

→ CRISPR-Cas9 can be programmed to target any DNA in vitro. **[Video]**

→ Cas9 a/k/a enzyme-dead.

Doorbell Analogy / Genetic Modifiers
Genetic Modifiers: Castle 1919

Hooded Rats: hooded phenotype = recessive trait
  = hooded mutant = hypomorphic mutant kit
  = phenotype: black hood on otherwise white rat,
    w/ black stripe running down middle of back
  = wt = all black/brown

Question: Does the nature of a gene mutant change?

selective breeding

hooded rat

selective breeding

Select for more black

- 10 generations
  - No white except on belly + a little on flanks

Select for more white fur

- 16 generations
  - Gene? Dead?
  - No, rt did

Cross each inbred line
  - to a third wt line,
  - selecting for hooded after
    in F2 gen. of each cross.

3-4 generations

Converge on same qtype.

Sickle Cell disease = Population
  - severe
    - why?
      - i.d. suggest
  - mild
Doorbell analogy: “sensitized” background

Genetic Modifier Screen: Simon + Rubin - 1991

Known Observations:

1) EGF Rec = RTks one of first oncogene id.
   = downstream q-way thru
   + down stream b-telia
   + which it transduces signal unknown.
   = Model: RTks = signal via same
     d-stream media

2) sevenless = RTks = required for R7 spec.
   in fly eye.
   = homozygous viable

a) Bride of bride of sevenless = ligand of sev
   = required for R7 (homoz. viable)
   ⇒ SNT = seven in absentia = nuclear protein

b) Saturating mutagenesis ⇒ failed to identify any
   other genes ⇒ mutants that when homoz. ⇒ loss of...

Model:

\[ \frac{Y^{sev}}{a \cup b \cup c} \]

- genes downstream of sev
- likely act downstream of
- over RTks + are essential
- for embryogenesis?

But, how do you identify them? ⇒ saturate mut?
⇒ ? Dominant genetic modifier sc.
How do you create a sensitized gen. background?

1) Create a sev genomic rescue construct

2) Assess its ability to rescue sev phenotype
   WT
   sevd2/+  sevd2/-; P[sev] +

3) engineer potential temp. sens. mut. in sev.
   - How? Use known t.s. mut. in src as guide
   - assay at dev. @ dif. temps

Rescue of P7

\[
\text{sevd2} ; P[sev +] \quad 24.3^{\circ}C \quad 22.7^{\circ}C
\]

- (+
  - sevd2 ; P[sev +] 90\% of facts
  - lack P7
  - P7 will 50\% reduce in src of downstream gene cause loss of P7 @ 22^\circ C
Dominant Genetic Modifier Screen

\[ \frac{\text{sev}^{d_{2}}}{\text{TM3} \text{ SB P}[^{\text{sev}^{d_{2}}}] \text{ C x P}} \]

\[ \sqrt{22.7^\circ C} \]

\[ \left( \frac{\text{sev}^{d_{2}}}{\text{TM3} \text{ SB P}[^{\text{sev}^{d_{2}}}] \text{ C x P}} \right) \text{ screened 30,000 F1 flies for loss of RT} \]

20 dominant enhancers

Genetic Mapping

7 complementation groups (most recessive likely)

Now, what?

1) Do they act downstream of other RTs?
   \* \( \text{Clp} \): dom. allele of EGF-rec
   \* \( \text{Clp}^+/+ \): moderately rough eye
   \* \( \text{Clp}^+/- \): severe " + "

2) Identify a more affected gene that when mutated yields the relevant phenotype

D) Deficiency mapping - narrow down to specific region of DNA
D) Gene rescue
C) Allele sequencing
D) RNAi/sgn. of additional alleles (Crispr)

Dominant Genetic Interaction:
- Nature of interactant provides information on direct of gene for loss of f(?) mutation - genes act in same direction
- Suppress loss of f(?) mutation - genes act in opposite direction
- Enhance gain of f(?) mutation - genes act in opposite direction
- Suppress same " + " same direct
E(sev3C): Case Study

1) 2 alleles: E(sev3C)\(^{e1b}\) + E(sev3C)\(^{e2f}\)
   - Recessive lethal mutations: E(sev3C)\(^{e1b}\)/E(sev3C)\(^{e2f}\) = \(\text{dead}\)

2) Genetic mapping places E(sev3C) on 3R, near to curled.

3) Deficiency mapping: localize E(sev3C) to restricted DNA region
   - Df(3R) by 62

\[\begin{array}{ccc}
85D8-11 & Df(3R) by 10 & 85E10-13 \\
85P17-22 & Df(3R) by 62 & 85F18-16
\end{array}\]

1. \(\frac{E{(sev)3C}^{e1b}}{TM3} \times \frac{Df(3R) by 10}{TM3}\)

Genotype:
- \(\frac{E{(sev)3C}^{e1b}}{TM3} \times \frac{Df(3R) by 10}{TM3}\)
- \(\frac{TM3}{TM3}\)
- \(\frac{E{(sev)3C}^{e1b}}{Df(3R) by 10}\)

\(\emptyset\) ? - (absent)

- \(Df(3R) by 10\) fails to complement either E(sev3C) allele
- \(Df(3R) by 62\) complements both E(sev3C) alleles
- Where is E(sev3C)?

4) Gene Rescue:
   - Ras - mapped to this region; has been implicated in
   - Generate 12kb ras transgene:
   - \(\frac{P[ras^{+}, w^{+}]}{TM3} \times \frac{E{(sev)3C}^{e1b}}{TM3}\)
   - \(\emptyset\)

Genotype:
- \(\frac{P[ras^{+}, w^{+}]}{TM3}\)
- \(\frac{E{(sev)3C}^{e1b}}{TM3}\)
- \(\frac{\emptyset}{E{(sev)3C}}\)

\(\emptyset\) type: WT if viable.