BENG 183

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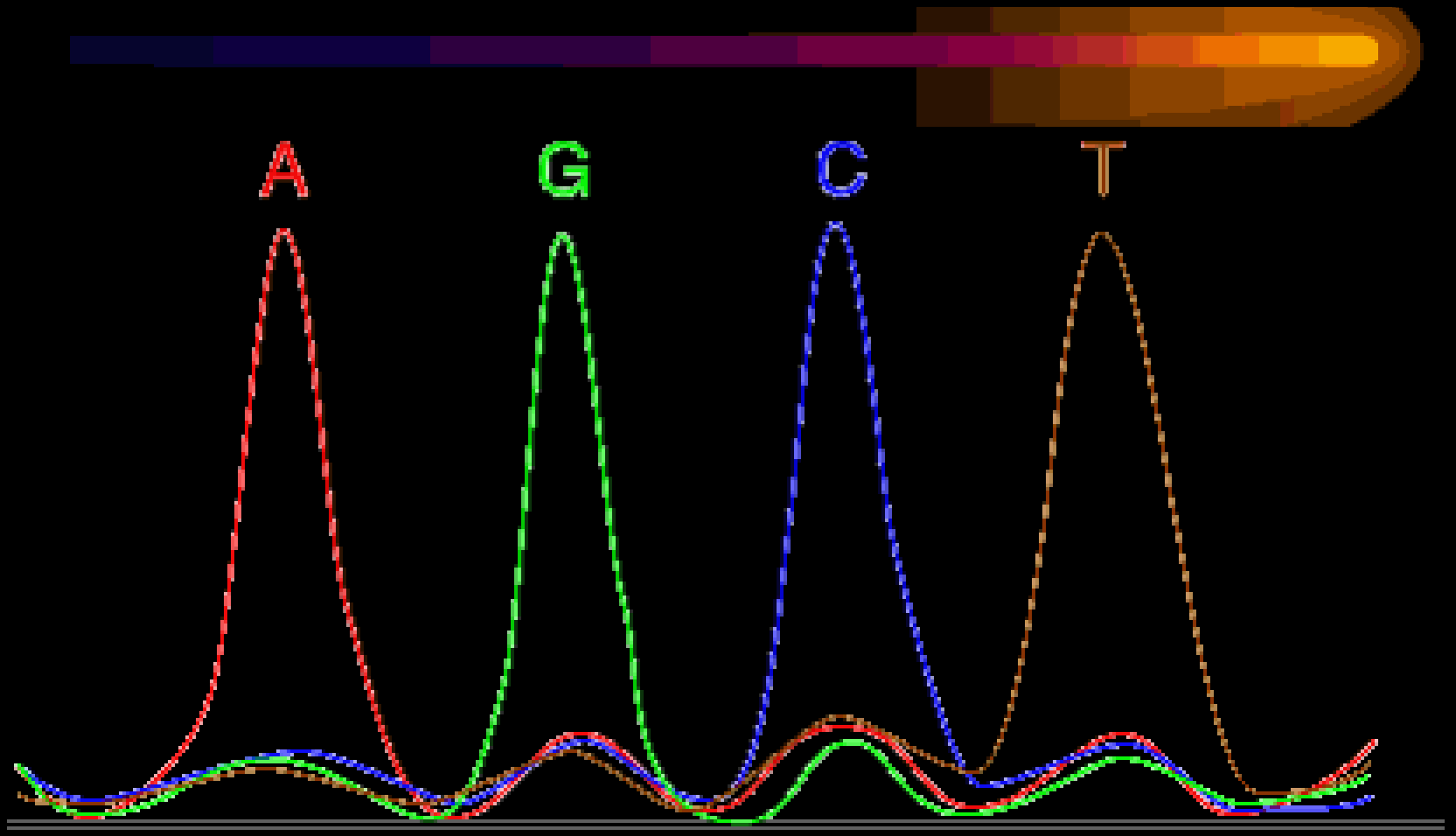
Comprehensive mutant libraries



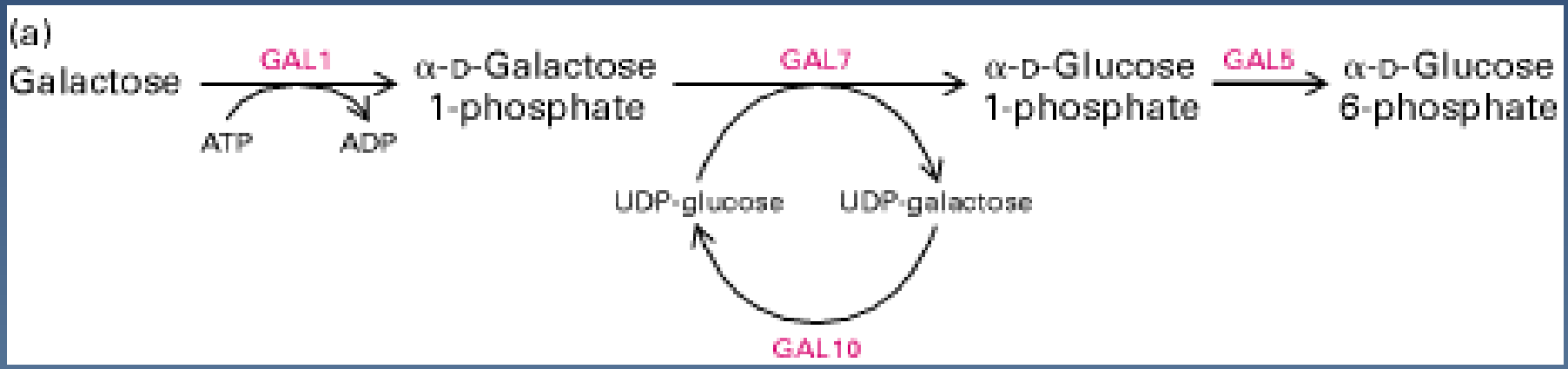
Forward vs. Reverse Genetics

- Forward genetics
  - Random mutagenesis by screening for mutants with impairment for a particular biological process
- Reverse genetics
  - Directed mutation of a gene to identify in which biological processes it functions
  - Assuming more and more importance, due to systematic strategies to mutate every gene
  - These efforts result in large mutant libraries

Forward genetics

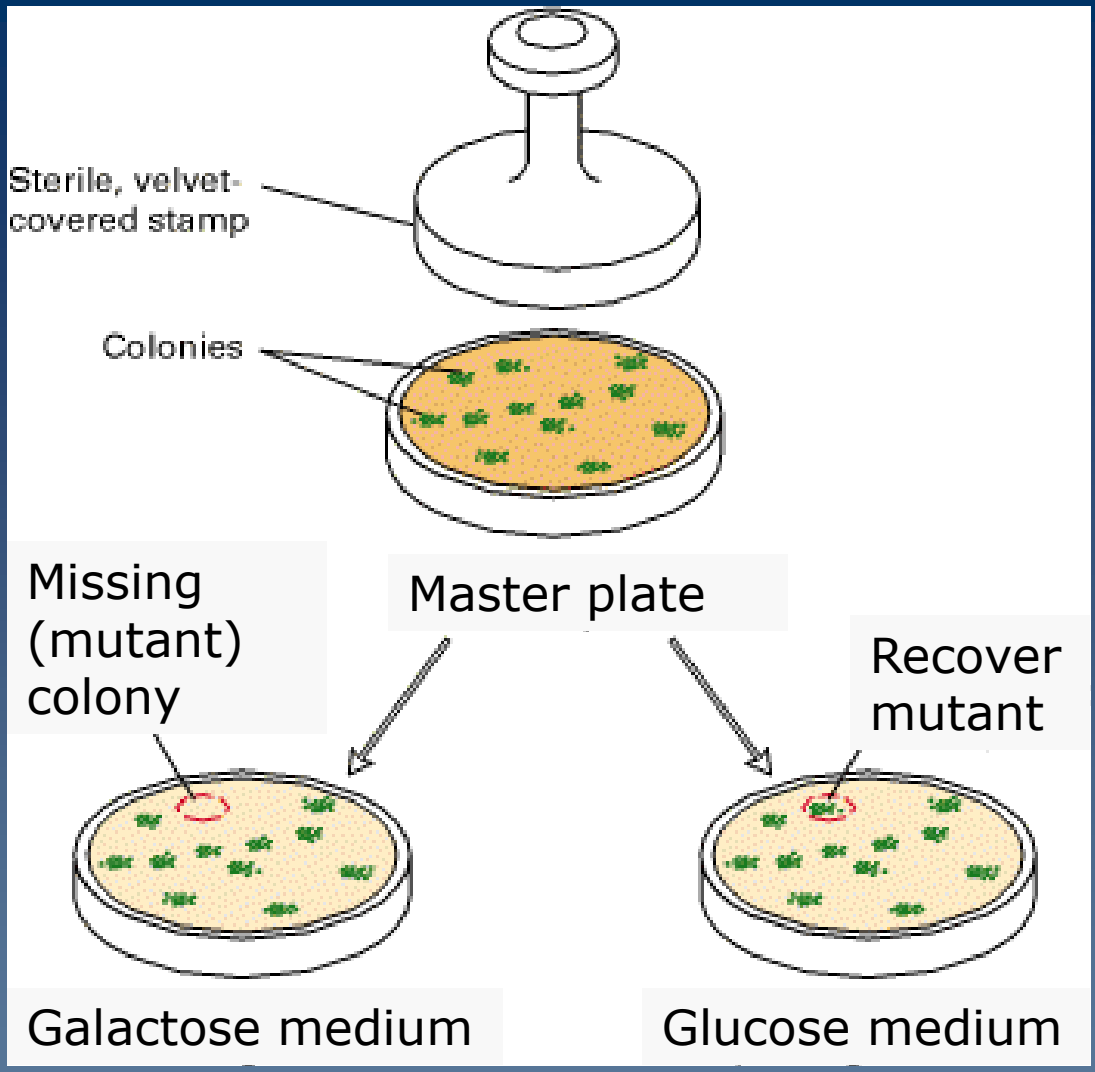
- Saturation mutagenesis is a classical approach for isolating mutants affecting a specific biological process
- Recover enough mutants so that it is likely that every gene in genome has been hit at least once.
- E.g., past screens have looked for:
  - Replication defective mutants in bacteria
  - Cell-cycle defective mutants in yeast
  - Developmental mutants in fly and zebrafish
- In the 'new' functional genomics all mutants are interesting and are being catalogued, not just those affecting a particular process.

Forward genetics



- Example: identifying genes that function in galactose metabolism

The basic mutant screen



1. Expose cells in culture to DNA mutagen such as EMS or MMS
2. Streak out to obtain single colonies on rich media
3. Replica-plate to restrictive media and screen for "lost colonies"

Complementation testing

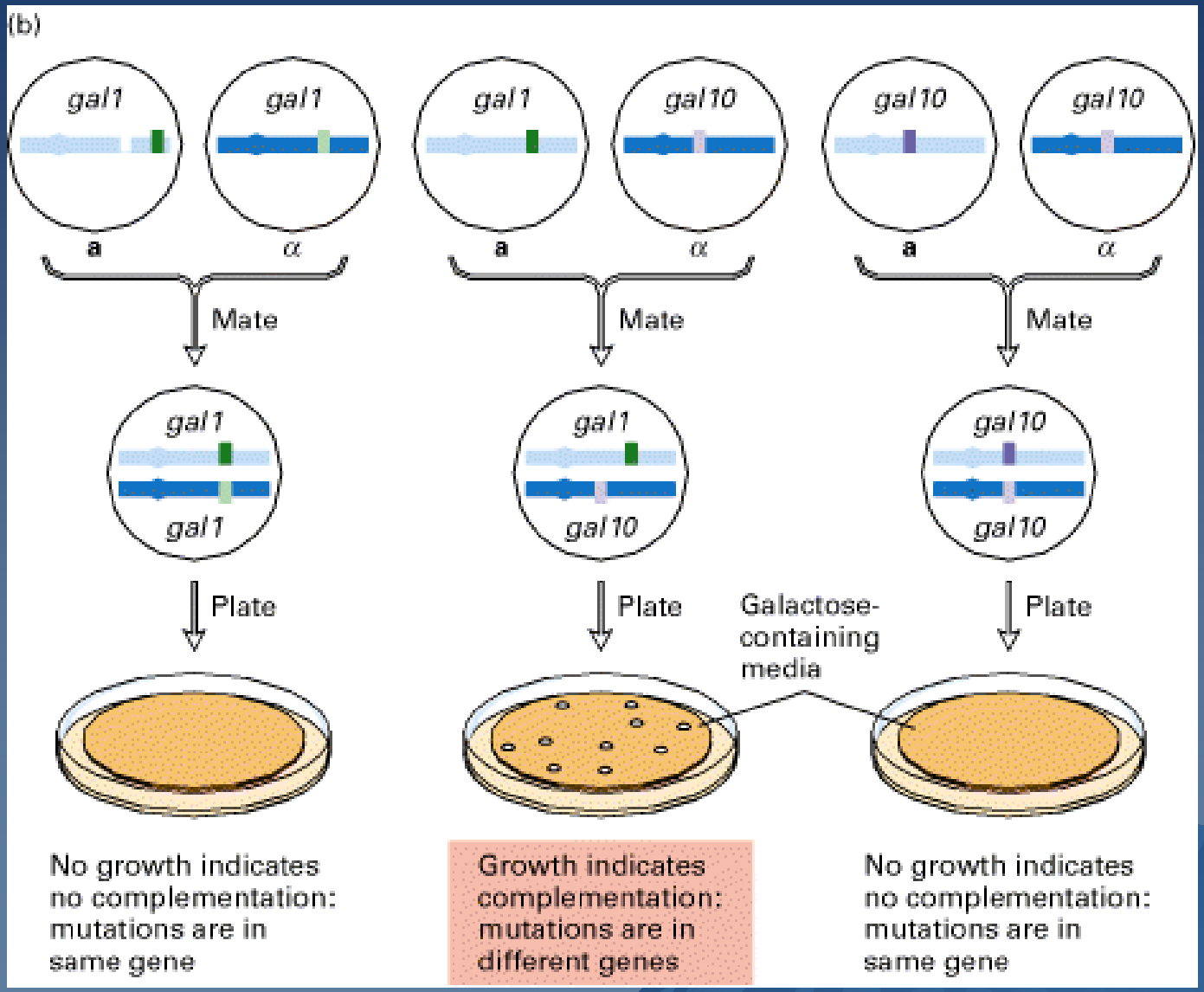


Fig. 8-11 from *Molecular Cell Biology*

Systematic “reverse” strategies

1) **Targeted mutation of every single gene one at a time—requires knowledge of genome sequence**
2) Random approach in which genes are mutated indiscriminately and then identified by sequencing around site of mutation—can be applied even if genome is not fully sequenced
3) *Phenocopy* of mutations without permanently modifying the genome of the organism

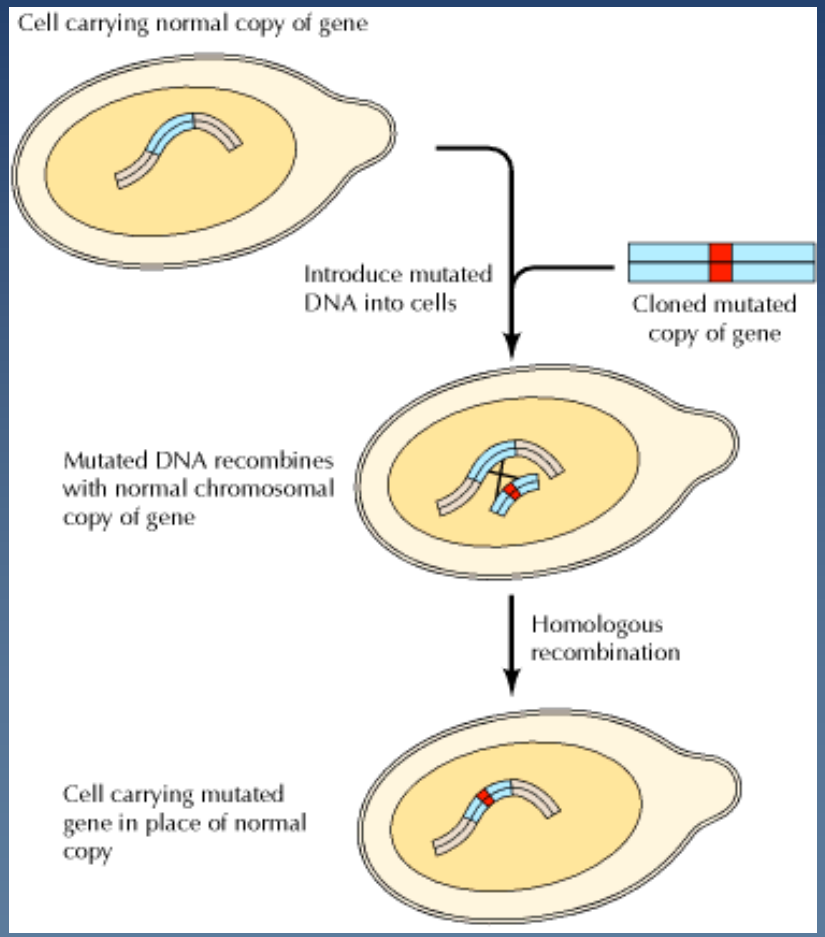
1) Systematic targeted mutation

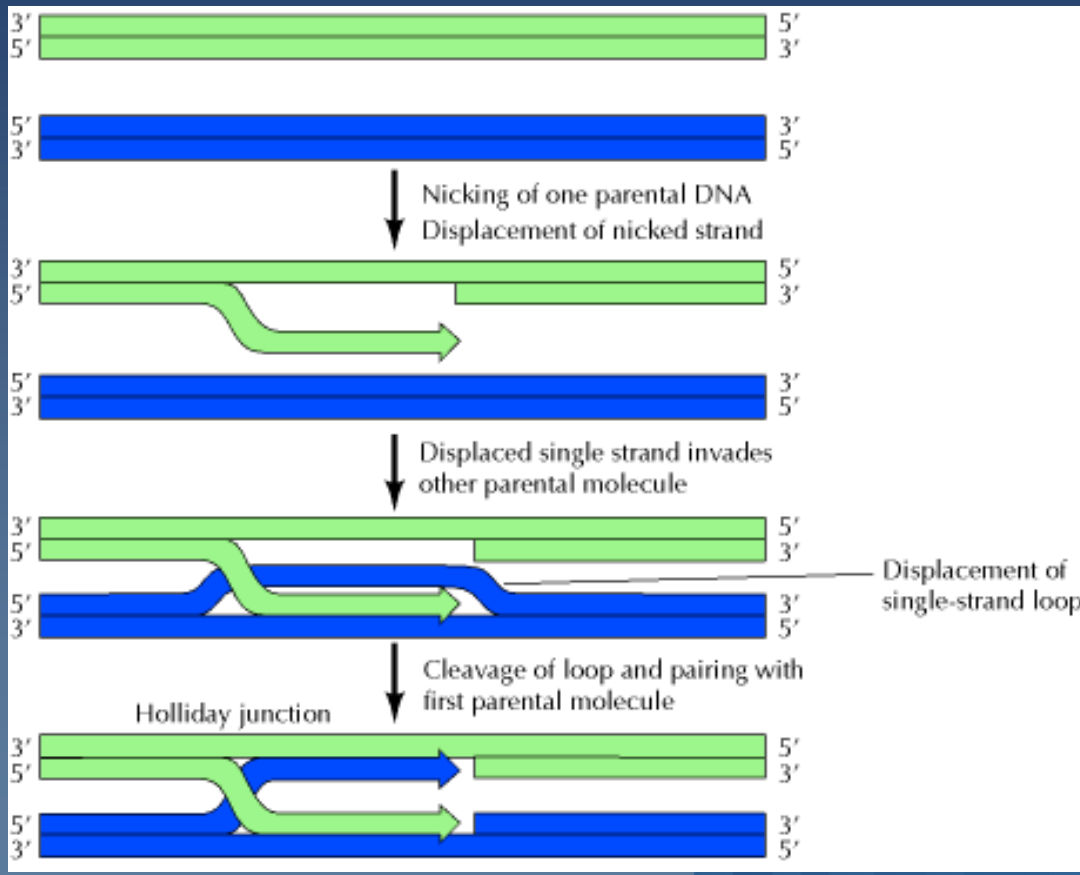
- The term *gene knockout* is used when the goal is to completely inactive the gene
- Gene targeting involves process of *homologous recombination* (HR), which is highly efficient in bacteria and yeasts.
- Inefficient in higher animals/plants which are typically 10^5 times more likely to recombine randomly than with the homologous target
- However, mouse embryonic stem cells (*murine ES cells*) have an unusual propensity for HR.

Homologous recombination (HR)

- The *Holliday* model proposes that recombination is initiated by a nick (in one strand) or a double stranded break (DSB) in a parental DNA molecule.
- The nicked strand is then displaced, and the resulting single strand invades the other parental molecule by homologous base pairing.
- Holliday junctions have been imaged directly using electron microscopy (EM).
- Enzymes involved in recombination have been identified by analysis of recombination-defective mutants of *E. coli*.
  - RecA catalyzes strand exchange for formation of Holliday junctions (Rad51 in yeast and other eukaryotes).
  - Other enzymes RecB/C/D can catalyze the single stranded breaks

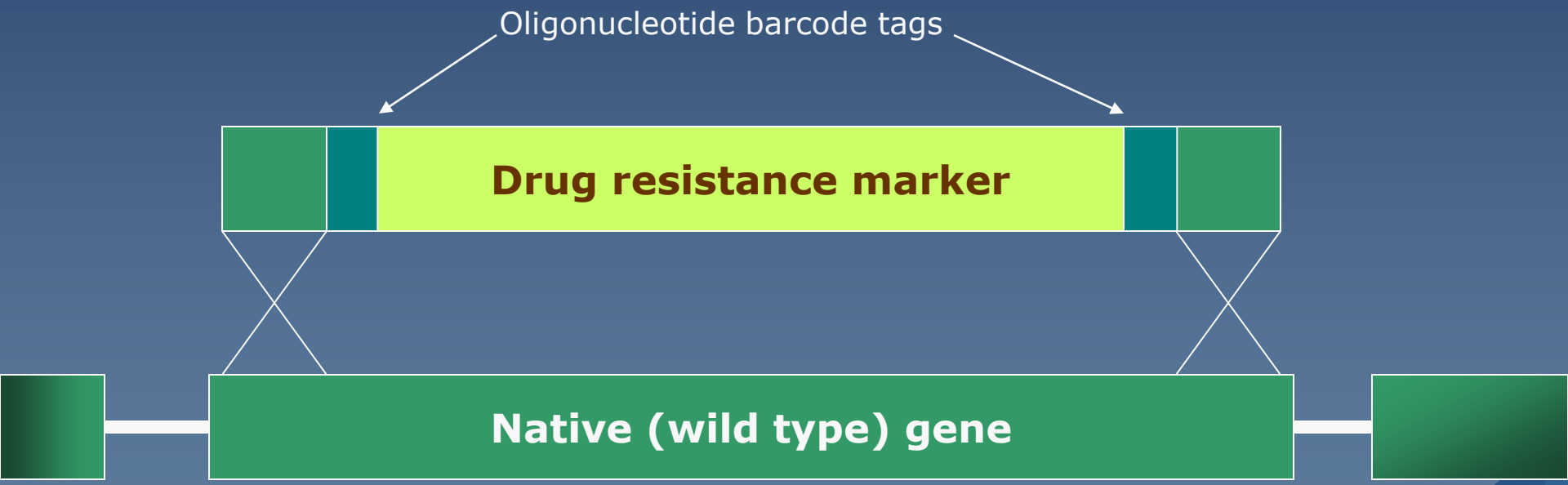
Diagrams of HR





Figs. 3.42 and 5.34 The Cell *A Molecular Approach*

HR for systematic knockouts in yeast and mice



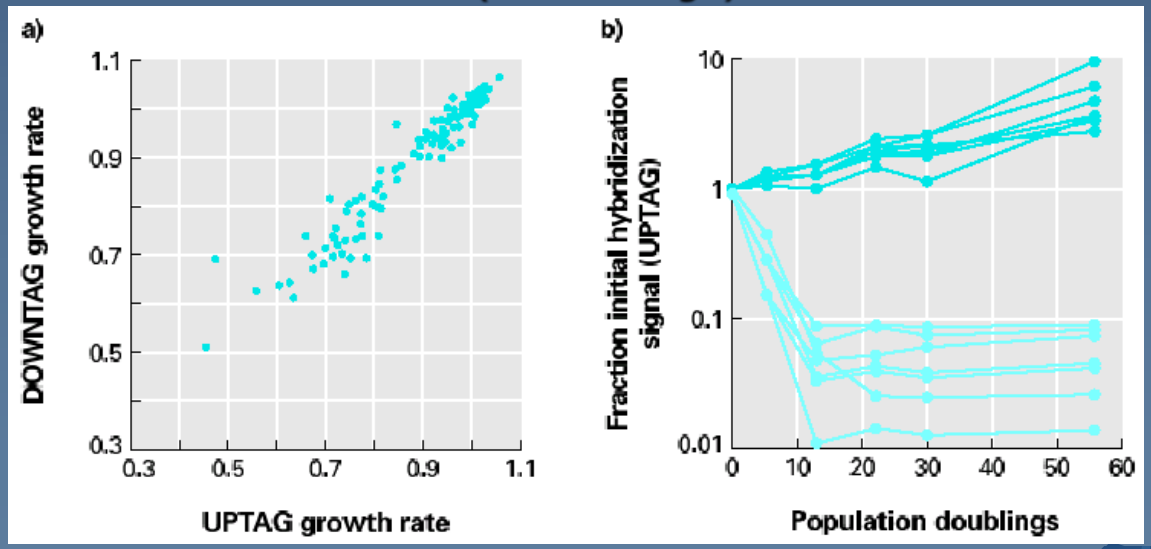
Systematic phenotyping with a barcode array

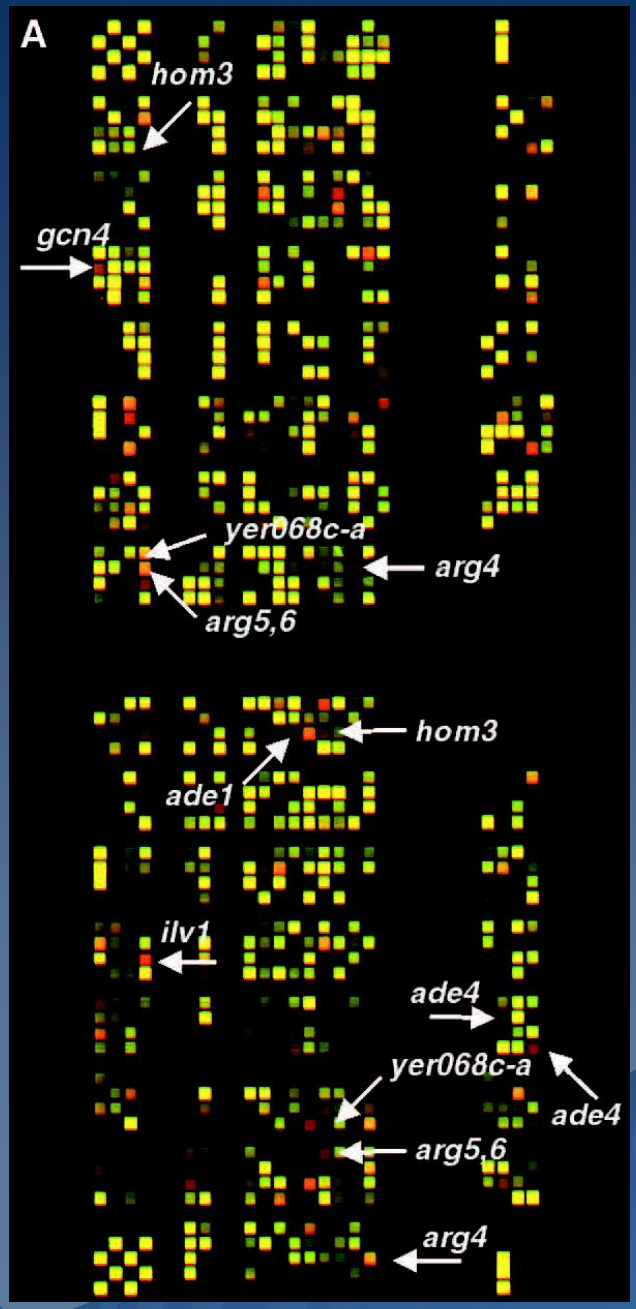
Ron Davis and friends…

These oligo barcodes are also spotted on a DNA microarray

Growth time in minimal media:

- Red: 0 hours
- Green: 6 hours (3 doublings)





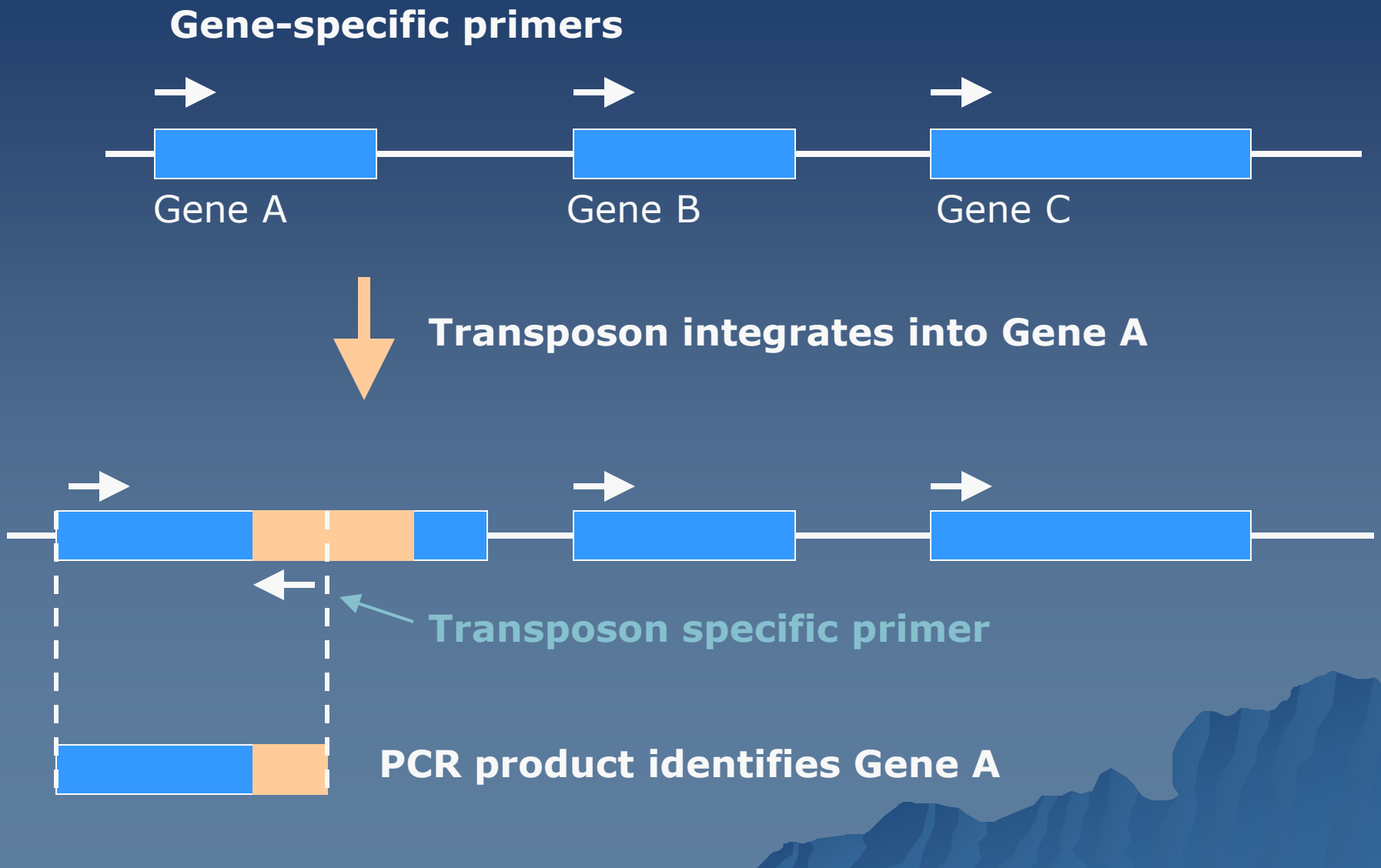
2) Genome-wide random mutagenesis

- *Insertional mutagenesis* is one popular random strategy, in which a piece of DNA is randomly inserted into the genome leading to gene disruption and loss of function.
- The piece of DNA can be a foreign (exogenous) sequence or a transposable element (naturally occurring seq. that jumps from site to site via transposase enzyme);
- PCR or hybridization techniques can be used to identify the DNA flanking the point of insertion, and thus the disrupted gene.
- The insertion is also associated with any observable phenotypes it generates.

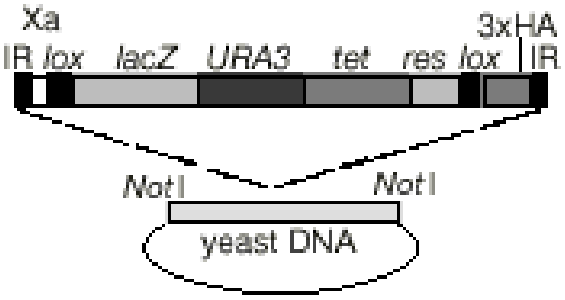
Insertional mutagenesis tricks

- Plasmid rescue vector: The insert contains origin of replication and antibiotic resistance marker. DNA from the mutagenized organism is digested and the resulting fragments ligated to form circular DNA. Only those circles with the insert will propagate and amplify in bacteria.
- Gene trap vector: The insert contains a visible marker gene such as lacZ, activated only if the insert falls within a transcribed gene.
- Activation tagging: Like above, but the insert contains a strong outward-facing promoter. Any nearby gene will be strongly overexpressed by the presence of the insert. Unlike other mutagenesis which typically inactivates a gene function.

Insertional mutagenesis with transposons

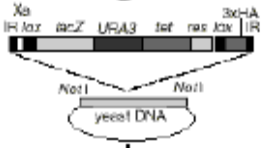


Multifunctional Tn3 cassette for *E. coli* mediated random mutagenesis of yeast



Library of mTn insertions in yeast DNA, generated in *E. coli*

mini-Transposon Mutagenesis



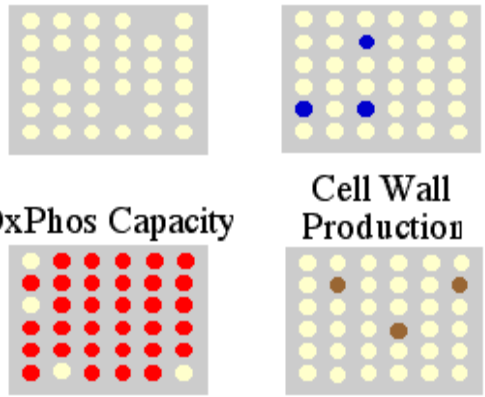
Phenotype Macroarrays

Selective Growth

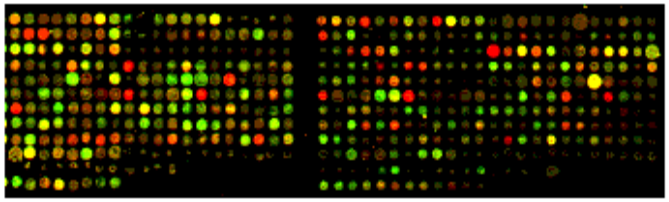
Gene Expression

OxPhos Capacity

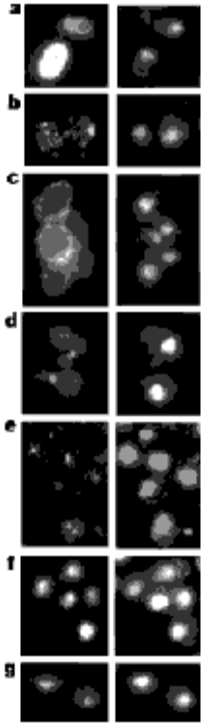
Cell Wall Production



Expression Microarrays



Epitope Tagging



Mutagenesis carried out in surrogate environment of *E. coli* cell.

Tn3 is not subject to tRNA gene bias as is yeast Ty element.

Mutagenized yeast DNA on plasmid is transfected back into yeast.

Transpsn also contains a reporter gene and an epitope tag

Mutational genomics strategies

1) Targeted mutation of every single gene one at a time—requires knowledge of genome sequence
2) Random approach in which genes are mutated indiscriminately and then identified by sequencing around site of mutation—can be applied even if genome is not fully sequenced
3) ***Phenocopy* of mutations without permanently modifying the genome of the organism**

3) Phenocopy libraries

- A phenocopy has the same appearance and effect as a mutation, but without modification to the DNA
- Typically generated by interfering with gene expression (e.g. RNAi) or protein expression (e.g. antibodies)
- Sometimes called gene *knockdown* as opposed to gene *knockout*.

Virus-induced gene silencing (VIGS)

- Mechanism originally observed in plants.
- Thought to result from a plant defense system which specifically recognizes viral nucleic acids and targets them for degradation.
- Almost all plant viruses use dsRNA, which is not found in the native plant. This dsRNA is targeted specifically so that only viral DNA is degraded.
- This may also be the explanation for why RNAi evolved in animals (see next slide).
- If the viral genome is engineered to contain a plant transgene, the plant defense system degrades any RNA with that sequence—thus the gene's endogenous RNA will also be degraded.

RNA interference (RNAi)

- First documented in *C. elegans.*
- Also based on the ability of dsRNA to induce potent and specific post-transcriptional gene silencing.
- *C. elegans* is also a good model organism for functional genomics:
  - Small multicellular organism with small genome
  - Known developmental progression: 1 cell → 959 cells !!!
  - Can be stored as frozen stocks
  - Bacteria expressing RNAi can be fed to the worms!!!
  - RNAi can also be directly injected—this is still the most effective means of gene knockdown.

Mechanism of RNAi

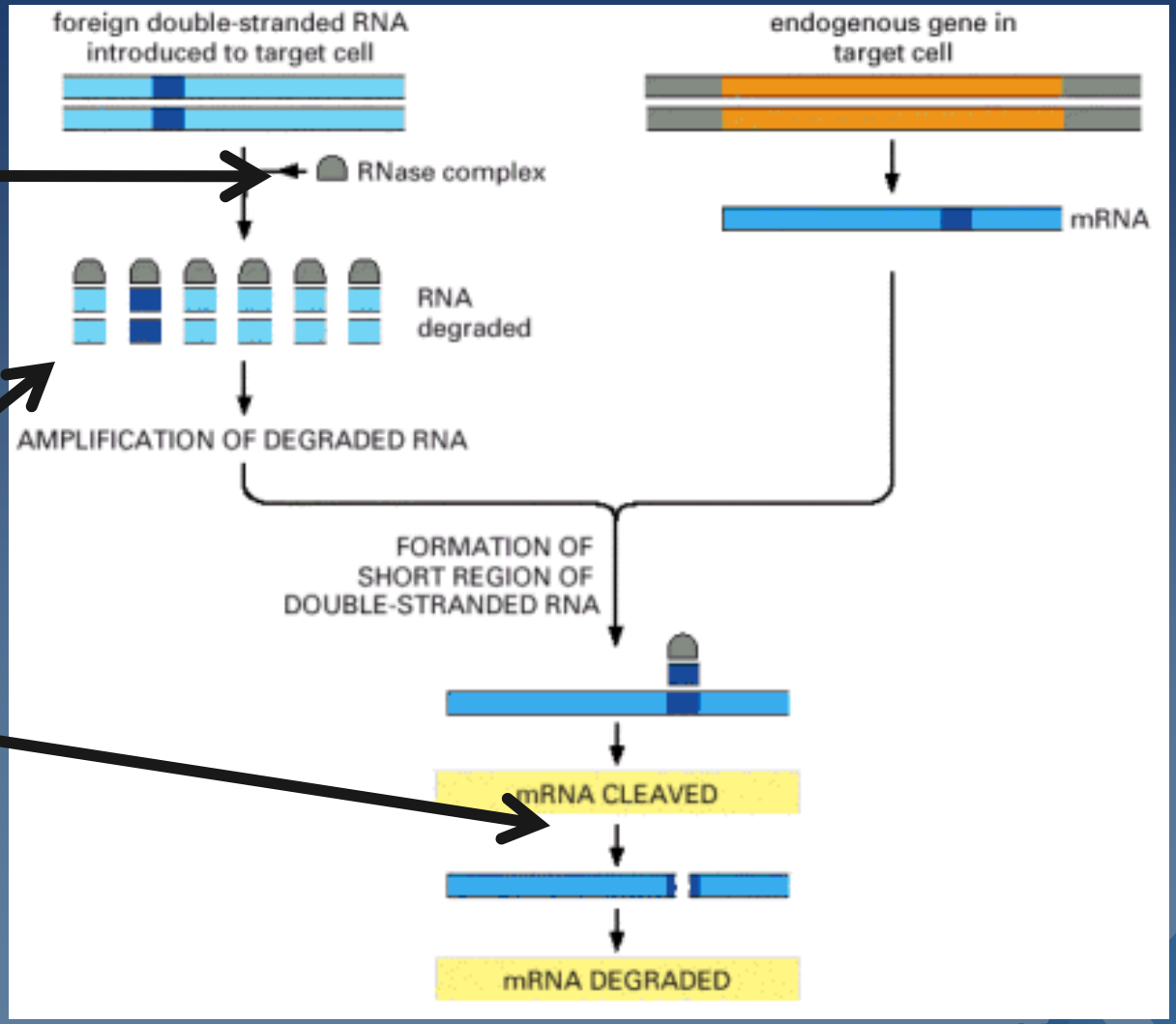
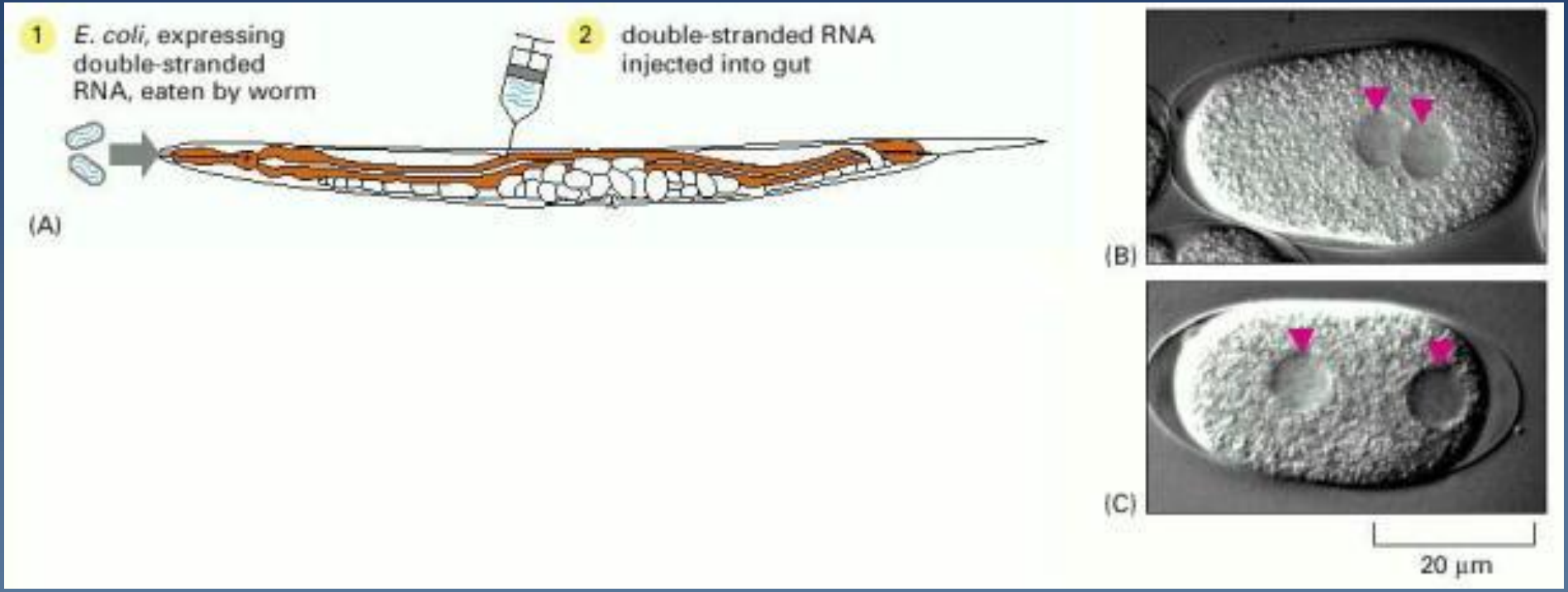


Fig. 7-107 Molecular Biology of the Cell

Examples of RNAi

- Gonczy *et al.* (2000) synthesized dsRNA molecules corresponding to over 2,200 worm genes (out of ~15,000 genes total) and found 133 phenocopies affecting the first two cell divisions during development
- In Maeda *et al.* (2001) success rate for neuronal gene targeting is low (why?) This can be circumvented by expressing hairpin dsRNA from a transgene, although this is a complex procedure.
- Large frozen stocks of RNAi molecules (and C. elegans mutants) are now available.

Example from Gonczy et al. 2000



Worm embryo in which a gene involved in cell division has been inactivated by RNAi. The embryo shows abnormal migration of the two unfused nuclei of the egg and sperm.