Advanced Genetics 1 & 2

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Perturb a biological system (mutations, pharmaceutical drugs, etc)

↓ **Informative mutations (pros & cons)**

Examine the consequences or phenotype (assays or readouts)

↓ **Examples**

Interpret the phenotype (reconstruct wild-type situation)

↓ **Interpret mutants' relationship (principles)**

Propose hypothesis or conclusions

↓ **Integrative approach**

Test hypothesis & deepen the understanding (biochem, cell biol, struct, single mol)

Informative mutations (alleles)

- **Commonly used mutations** (null, hypo-morph..) pros vs. cons

- **How to identify primary effects of mutants** (avoid indirect phenotype)?

- **Conditional alleles** (on-off/reversibility; essential/non-essential genes)

Control at protein level

Classical approach - Temperature sensitive (ts) alleles

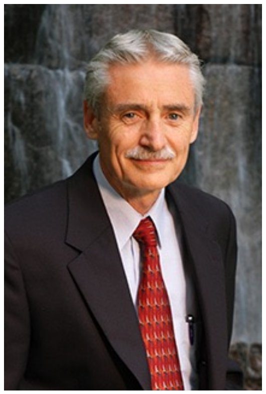
Chemically induced approaches:

1) Degrons - depletion at specific time to observe acute effects
2) Analog-sensitive alleles & small molecule inhibitors

Control at Transcription level - *Tet off vs Tet ON systems*

Control at Translational level – *RNA aptamer system*

Conditional alleles -1 : temperature sensitive

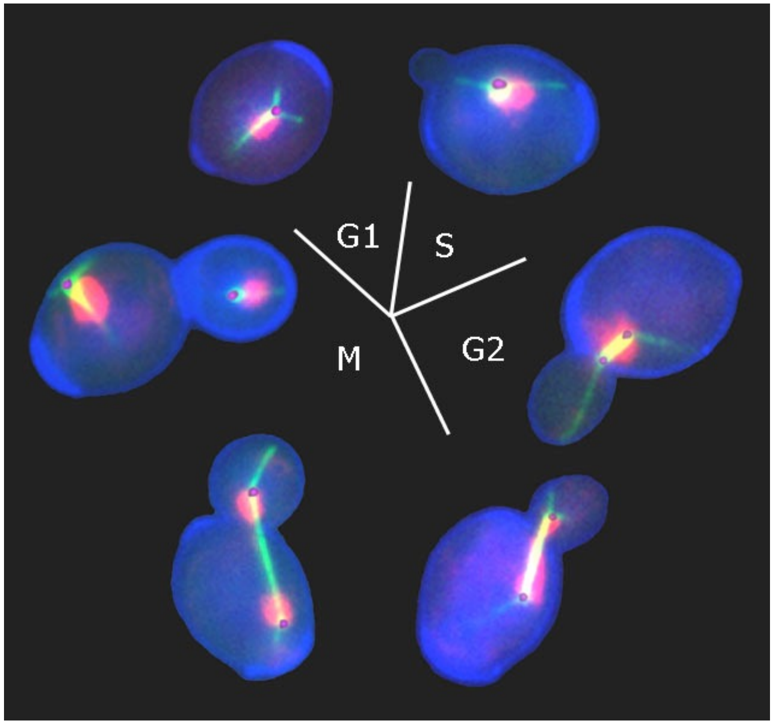


Lee Hartwell

Paul Nurse

- **Question:** how does cell cycle?
- **Hypothesis:** proteins may drive the progression of one cell cycle stage to the next.
- **Strategy:** ID mutants arresting at specific stages of the cell cycle.
- **Issues:** these mutants are probably “dead”.
- **Solution:** find ts alleles that arrest the cell cycle progression only at higher temperature.
- **Biological principle:** proteins tend to mis-fold at higher temperatures.

Cell Division Cycle (CDC) ts alleles



- **G1:** **no bud,** only 1 centrosome
- **S:** **small bud;** 2 centrosomes (close by), DNA level increase,
- **G2:** **bigger bud;** 2 centrosomes separated, DNA at bud neck,
- **M:** **budding cells separated from mother cell;** centrosomes & DNA separated to two cells

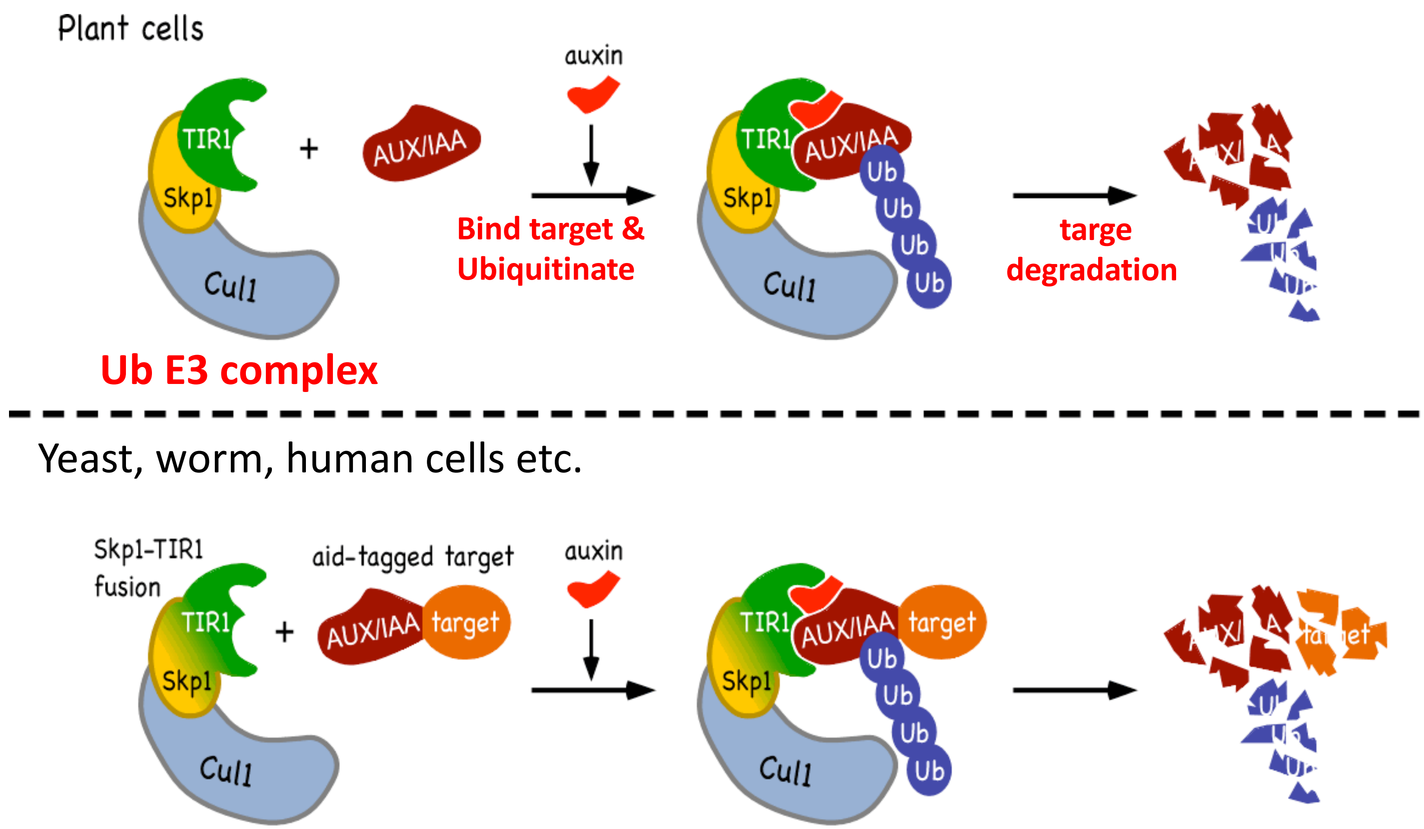
Conducted in haploid budding yeast cells

- ***haploid*** *(1N) and diploid (2N) yeast cells grow mitotically*
- *Grow* ***rapidly*** *mitotically (90 min); meiosis fast (3 days)*

Key steps:

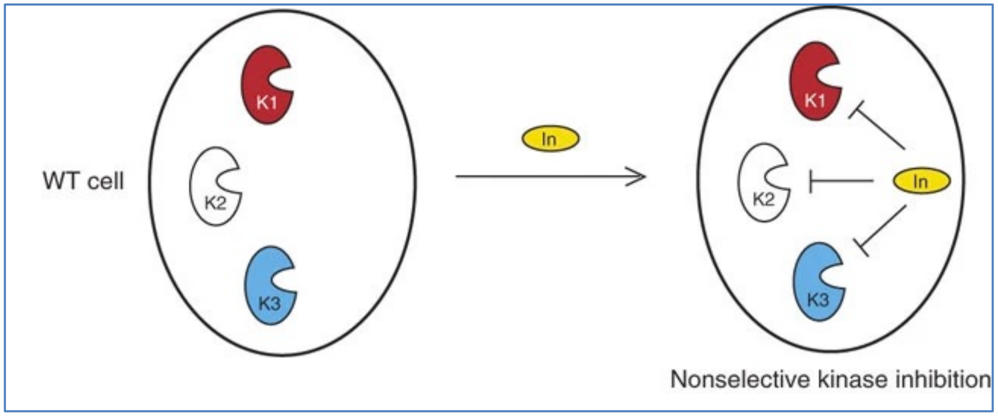
- random mutagenesis of 1 million yeast cells by genotoxins (EMS etc).
- let each mutated cell form a single colony at 25ºC
- shift cells to 37ºC - WT cells can still grow
- ID mutants arrested in G1, S, G2, or M at 37ºC.
- Identify genes affected by mutations using cells kept at 25ºC.

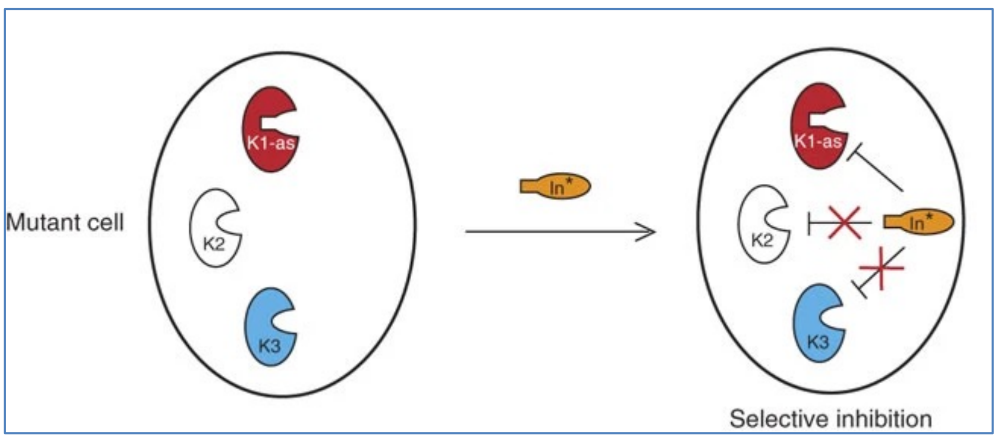
Conditional alleles-2: chemically induced degrons



- Deplete at specific time (G1, G2, S or M phase), specific tissues, or conditions.
- Degradation speed & Reversibility & Specificity (minimize the effect on other proteins)
- *Disease applications - Proteolysis targeting chimeric (**PROTAC**) technology*

Conditional alleles -3: Analog sensitive alleles





- **A conventional small-molecule inhibitor** (In) typically blocks multiple kinases (human has > 500 kinases & many are highly related.)
- **Mutating a residue in a protein kinase (K1)** creates a pocket where an enlarged inhibitor (In*) can bind.
- **The new inhibitor** cannot bind to wild-type kinases nor other kinases, allowing specific inactivation of the mutant kinase; reversible upon IN washout.

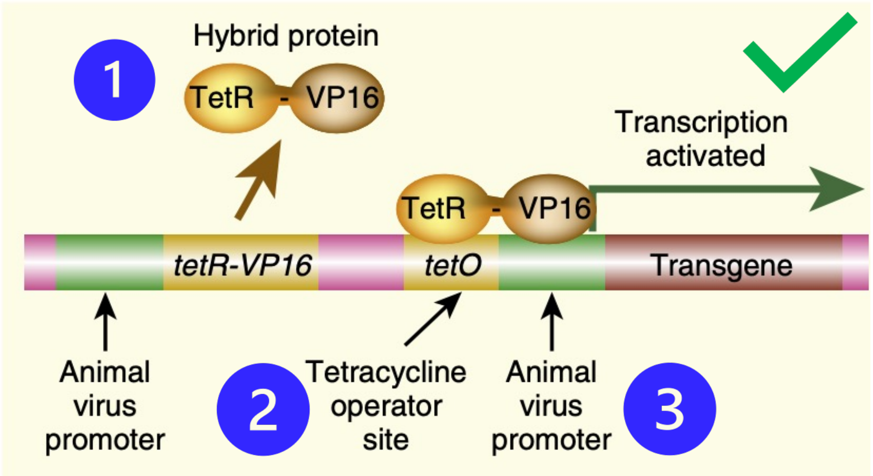
Conditional alleles-4: transcriptional regulation

Tet-OFF system

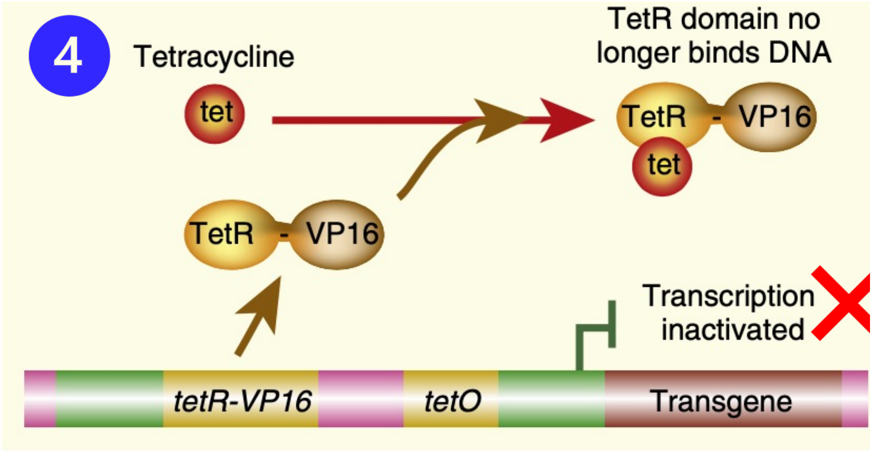
- ***tetO: promoter sequences for TetA* in *E. coli*** *(encodes a tetracycline pump protein*)
- ***TetR*: *repressor protein*** binds to tetO to repress TetA transcription.
- ***Tet (tetracycline)****:* binds to TetR, changes TetR conformation - dissociate TetR from TetO.

1. **TetR-VP16 fusion**: tetR DNA binding domain + VP16 transcription activator.

***2-3 tetO* & promoter bound by VP16** replace the promoter of gene of interest (transgene), so that tetR-VP16 can bind to this locus to turn on transcription ON



4. Tetracycline (tet): dissociates tetR-VP16 from DNA - transcription OFF.

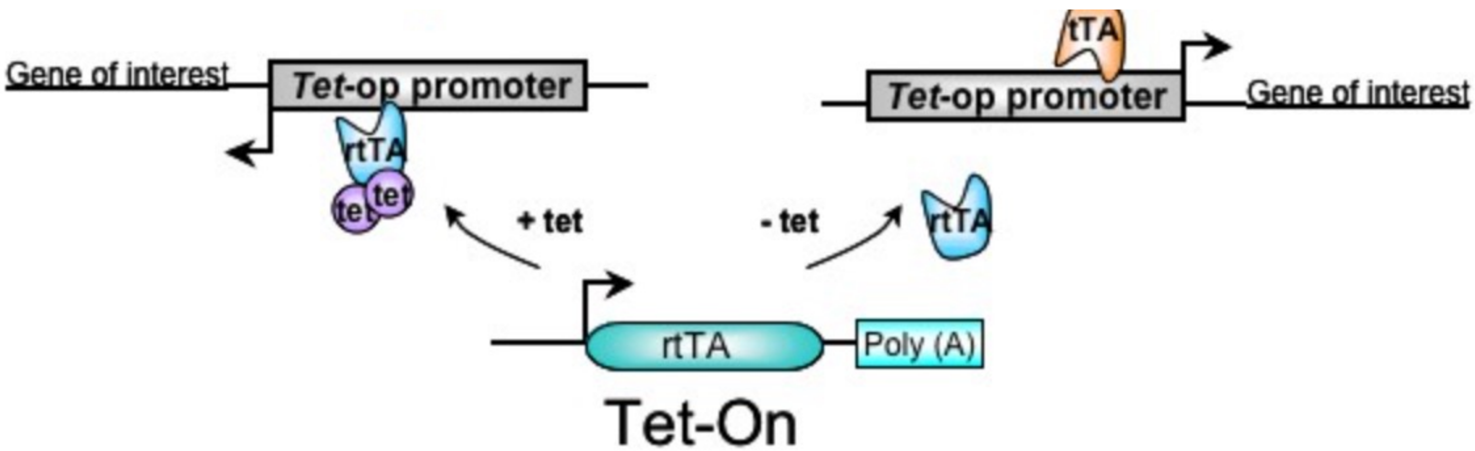


Conditional alleles-4: transcriptional regulation

Tet-ON system

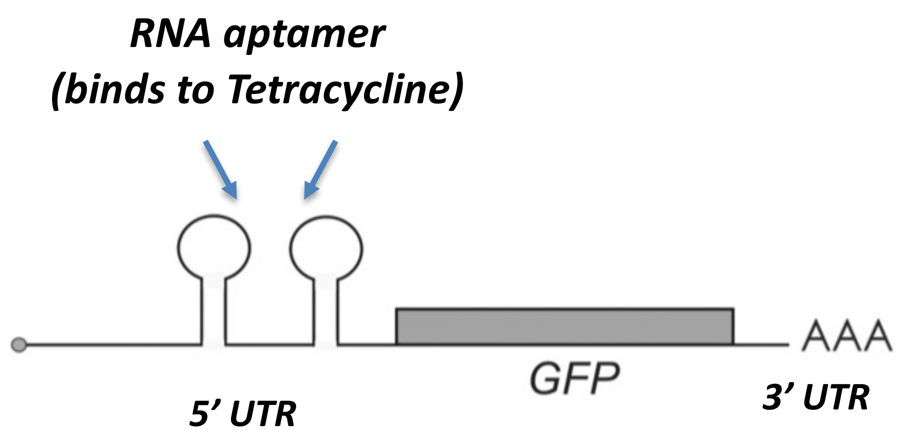
rtTA: a mutated version of **TetR** fused to the **VP16** transcriptional activation domain

rtTA differs from TetR by a few point mutations, which result in a reversal of tetracycline responsiveness, such that tetracyclines binding is a prerequisite for rtTA to associate with tetO.



Other inducible promoters: Galactose promoter, Cu+ promoter

Conditional alleles-5: translational shut down



- ***RNA aptamer:*** synthetic sequences used to screen for binding to small molecules with high affinity and specificity (similar to how an antibody binds to an antigen).
- Upon binding to Tetracycline, RNA aptamer inserted at 5' UTR can prevent ribosome binding
- In this is system, tetracycline can reduce protein translation up to to 40-fold.

Conditional alleles strategies - summary & comparison

Considerations:

1. Specificity (only affects YFG – your favorite gene).
2. Avoid Leakiness (ie. Causing defects before switch)
3. Fast switch
4. Reversibility and Practicality
5. Minimal effect on transcription (txc) and translational (txl) regulation.

	Pros	Cons
TS alleles	- Great specificity; relatively fast - Minimal changes at genes (No tag/txc/txl)	- can be leaky; - Not always reversible for all proteins
Kinase (inhibitor)	- Mostly specific; Fast; often reversible - Minimal change (No tag/txc/txl)	- can be leaky; - Mostly for enzymes.
AID-degron	- Excellent specificity; fast - Excellent reversibility; most proteins - No changes of txc/txl	- Tag may interfere with function - may not be effective for low abundant protein

How to study the biological roles of multi-functional proteins

Separation of functional (SOF) alleles

- Definition

- Basic Rules: mutations should be precise & specific in perturbing a particular function without affecting other properties of the protein

- Often need to make multiple point mutations at once – efficiency is great in yeast, but still an issue in other organisms.

How to generate SOF alleles

- Contain an enzymatic domain – catalytic site mutation
- Structural proteins or structural domains – ligand binding site (protein/DNA/RNA etc):

  Alpha-fold

- Post-translational modification (PTM) – change the conjugation site, e. g. K to R
- Cellular localization signal – e.g. nuclear importing and export signals

Some examples can be seen in the paper to be discussed

- ◊ Nuclear envelop contacts genomic DNA and control its functions in both yeast and human cells.
- ◊ Both human and budding yeast proteins are extensively regulated by modifications such as ubiquitination, sumoylation, and neddylation.
- ◊ Yeast serves as a model system to study diseases such as cancer, aging, neurodegenerative disorders, genome instability syndromes, and skin disorder.

1. Human genes make up 1-3% of its genome and yeast genes make up 70% of its genome.

2. Human cells have ~4000 genes that are homologous to yeast genes.

3. The number of distinct yeast chromosomes are half of that of human chromosomes.

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↓ Interpret mutants' relationship (principles)

Interpret mutational relationship

- Suppression (positive interaction)
- synthetic lethal/sick interaction (negative interaction)

Biological principles; discover new factors, pathways, mechanisms

Suppression: Reversal of the mutant phenotype

- Reversion of the starting mutation.

- Mutation of another site of YFG (e. g. ub-site mut for unstable proteins)

- Intergenic suppressors: genes functionally related to YFGs

1) Protein partners,
2) Acting in parallel pathways
3) in the same pathway – upstream or downstream of each other.

- Overexpression (dosage) suppressors (cdc28-4 and CLNs).

Dosage suppression

- increased amount of another, compensating gene product

High-copy suppression of *cdc28*^{ts}

Background: Cdc28p is a protein kinase required for the G1/S transition. However, Cdc28 protein level does not change during the cell cycle, though its activity peaks in G1/S transition.

Hypothesis: maybe a co-factor enable Cdc28 to act specifically in G1-S transition.

Question: Which protein(s) regulates Cdc28p activity during G1- S phase of the cell cycle?

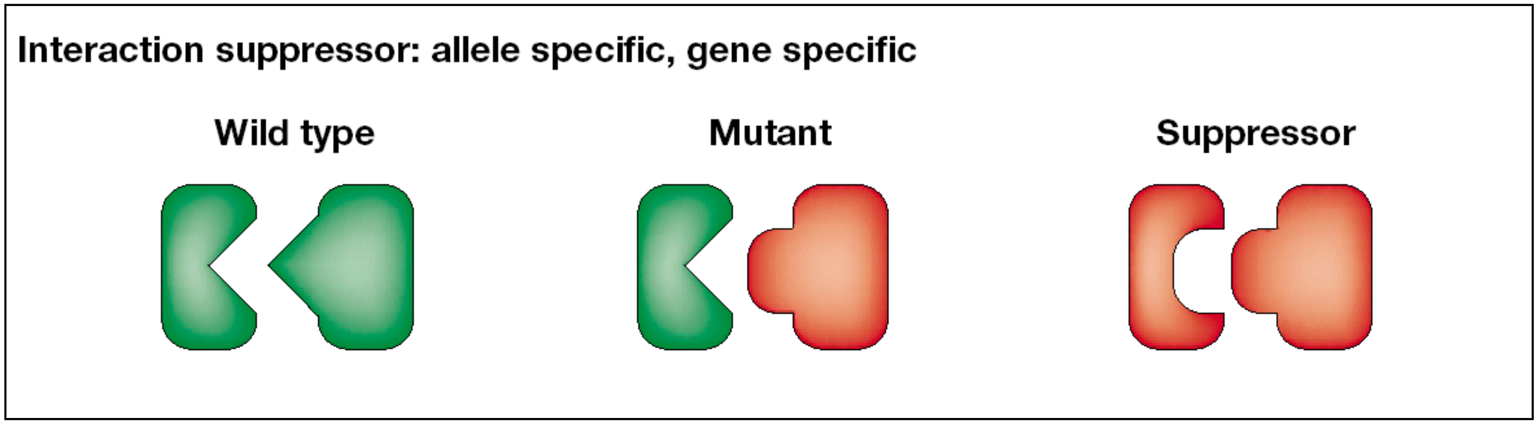
Method: *cdc28-4* mutant was transformed with a high-copy plasmid library (each clone overexpress a particular gene) and selected for restoration of TS defects.

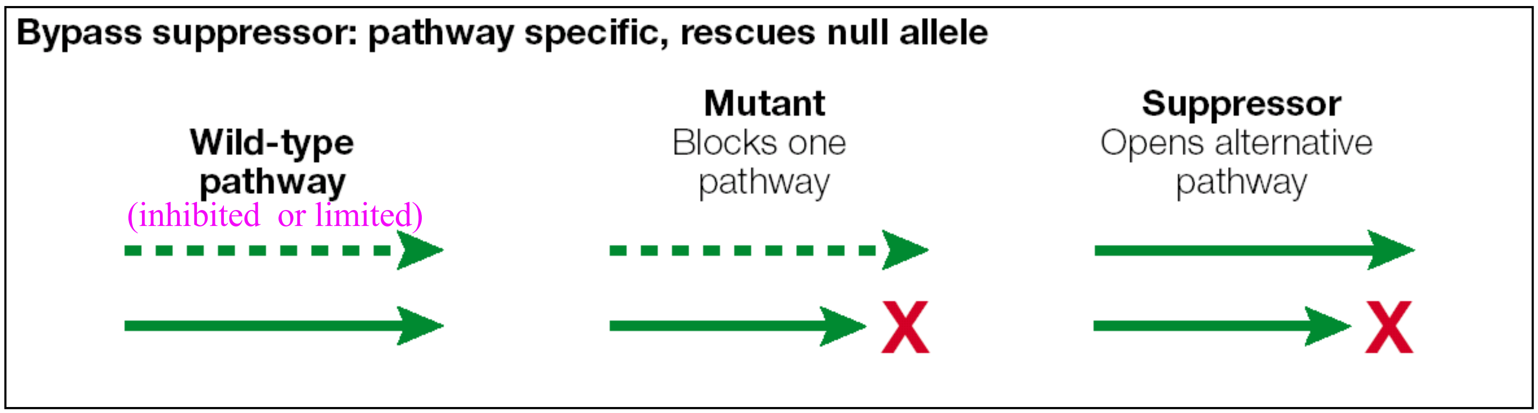
Results: Three plasmids were isolated: *CDC28*, *CLN1* and *CLN2*. The latter two encode cyclins that peak in G1 and activate Cdc28p during G1-S transition of the cell cycle.

Interpretation: *cdc28-4* may reduce its binding to CLN1/2, so that overexpressing CLN1/2 can compensate this defect.

Intergenic suppressors & mechanisms - part 1

(Protein partners OR proteins acting in parallel pathways)





How to distinguish between the two scenarios?