With a bit of history: The language of Genetics & the cAMP pathway in budding yeast
Extended notes

What is the role of cAMP in yeast?

I) Screen for Mutants defective for cAMP production – how? by identifying mutants that require exogenous cAMP for proliferation. Using a random mutagenesis approach, mutated yeast cells are plated in the presence of cAMP to form individual colonies and replica-plated on medium lacking cAMP: colonies unable to grow require cAMP i.e. the mutation prevents cells from making cAMP (Q: what could this be? a locus encoding the enzyme generating cAMP or an activator of the enzyme)

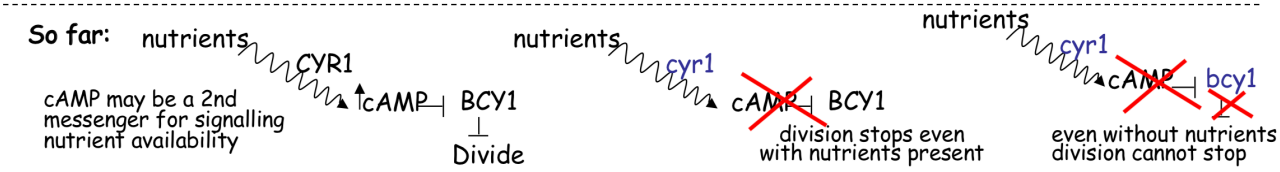
1) one essential locus identified: *CYR1* - Q: are there any phenotypical consequences to the genetically forced depletion of intracellular cAMP?; i.e. what is cAMP production promoting? let's see by observing the consequences of trying to grow these mutant cells *without* exogenous cAMP

a *cyr1* mutant arrest before bud emergence in the absence of external cAMP —same response as absence of nutrients in wild type cells

2) Isolate SECOND SITE SUPPRESORS allowing a cyr1 cell to grow without cAMP - follow up to identify further components of a pathway. Starting with the *cyr1* mutant >> 2nd random mutagenesis (or could also try isolation of spontaneous revertants...), select colonies that are able to grow without cAMP (Q: what could this mean? a mutation away from *cyr1* relieves the dependency e.g. by inactivating a negative regulator of the pathway, normally inactivated by the production of cAMP upon signalling.

one non-essential locus identified: *BCY1*, i.e. *cyr1* requires cAMP, *cyr1 bcy1* double mutant divides without exogenous cAMP. In the absence of nutrients, a *bcy1* mutant continues to divide and dies.

i.e. this mutation uncouples the presence or absence of nutrients and the decision to divide



II) Find mutants from unrelated screens with similar phenotypes -Q: any other genetically defined components of this pathway discovered by independent screens? e.g. more mutants arresting prior to bud emergence with similar appearance to *cyr1*. In an unrelated screen for ts conditional mutants blocking the cell cycle....

cdc35^{ts} was ALLELIC to *cyr1* (both mutations land on the same locus)
cdc25^{ts} can be rescued by exogenous cAMP but it is not allelic to *cyr1* (failure to bud is relieved by exogenous cAMP, i.e. this mutant fails to make cAMP like *cyr1* but maps elsewhere)

and with Molecular Biology-based tools.....

The availability of shuttle vectors that can be manipulated using bacteria as host and can be also introduced and maintained into yeast cells, makes it possible to isolate the culprit loci by screening genomic libraries for plasmids COMPLEMENTING/RESCUING the mutation in question. As we'll see repeatedly, ts alleles are invaluable for this purpose.

- A library was screened for clones COMPLEMENTING a *cdc35*^{ts}: sequence showed “similarity” to adenylyl cyclase (once the ORF of the complementing locus is sequenced, the predicted translation product can be compared to known proteins).

cdc35^{ts} at RESTRICTIVE TEMPERATURE had low levels of cAMP. (biochemical tests followed to confirm the suspicion that *CDC35* encodes adenylyl cyclase)

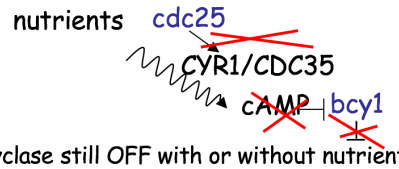
CDC25 was also cloned and mapped to a separate locus (i.e. something other than cyclase...).

- Based on the clones, the endogenous genes were DISRUPTED: both essential. (pre-genome project days, only after recovery of a gene, a true disruption could be constructed!!!!). Lethality could be rescued by exogenous cAMP or by a *bcy1* mutation. (confirming that the relevant loci were identified and that the original mutations from the screens resulted in LOSS OF FUNCTION alleles)

- *CDC35* encodes adenylyl cyclase while *CDC25* may encode an activator (presumed to operate upstream of cyclase, this turned out to be true, see below)

So far:

Cyclase OFF with or without nutrients, no cAMP is made, cells arrest



bcy1 is epistatic to *cdc25* and *cdc35*

Cyclase still OFF with or without nutrients, cells lack the cAMP-controlled brake, thus, cannot arrest even if cAMP is low

III) Finding more genetic interactors

- Screen for MULTICOPY SUPPRESSORS of a *cdc25^{ts}*-> a truncated clone encoding the catalytic domain of Cdc35/Cyr1 and 3 new loci: *TPK1, 2, and 3*.

- These loci encode the catalytic subunit of cAMP-dependent protein kinase Q: what was known about PKA (aka cAMP-dependent protein kinase?

PKA contains two types of subunit

OFF state , binding of the regulatory subunit inhibits the catalytic subunit (the one carrying out phosphorylation of relevant targets)

TPK1, 2, 3

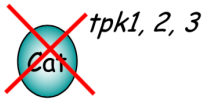
ON

Production of cAMP promotes activation of PKA: cAMP binding to the regulatory subunit releases the catalytic subunit. Once free, it will be active as a kinase toward relevant targets.

Multiple copies of *TPK* genes will override the inhibition through excess of free catalytic subunits (titration of the regulatory subunit renders the kinase INDEPENDENT of cAMP)

- Triple deletion is lethal (redundancy). Cannot be rescued by adding cAMP or a *bcy1* mutation

with or without cAMP, the absence of a catalytic subunit of the kinase prevents division

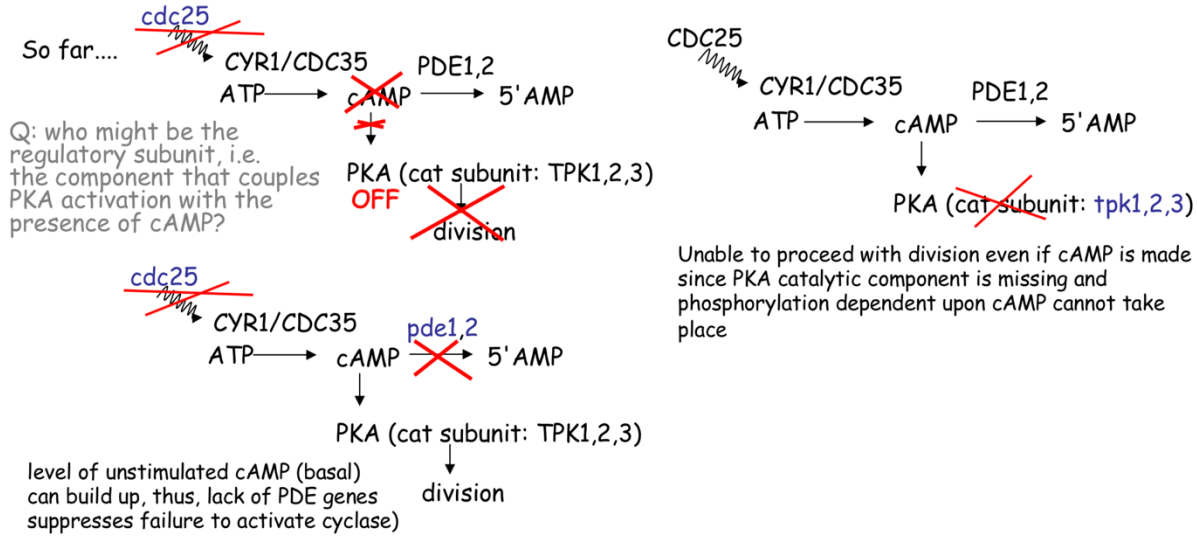


the active kinase is DOWNSTREAM BCY1 or cAMP production

tpk1,2,3Δ is epistatic to *bcy1*

-Screen for SECOND SITE SUPPRESSORS of a *cdc25^{ts}*: isolated 4 additional loci- *PDE1, 2* and *IRA1* and *2*.

- The sequence of the *PDE* genes suggested similarity to phosphodiesterases (known enzymes that convert cAMP into 5'AMP to terminate or limit cAMP-mediated signalling)



Q: is activation of adenylyl cyclase by the product of *CDC25* direct or are there intervening components?

IV) The missing link: identification of yeast homologues of human *RAS*

- Using the cDNA of the protooncogene Harvey-*ras* as a probe, two loci were identified in yeast by Southern blot analysis.

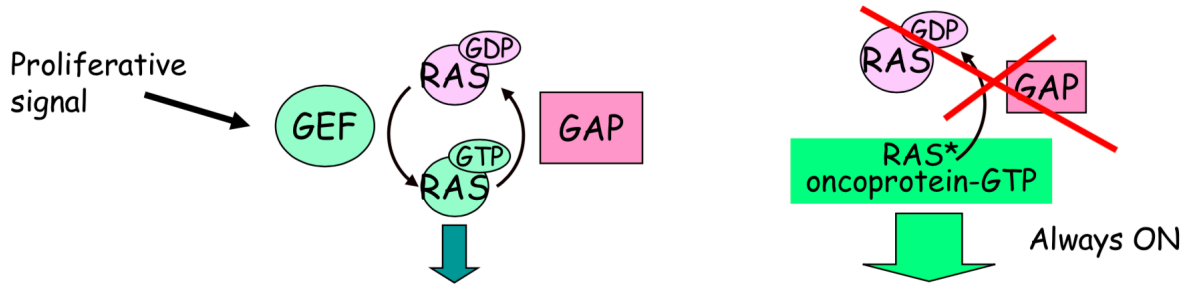
- The genes were cloned by colony hybridisation using the same probe: *RAS1 & 2*.

-Double disruptant is dead but can be rescued by cAMP or a *bcy1* mutation (Q:again, do these phenotypes relate to mutants identified in other screens?)

ras1 ras2 mutants share the phenotypes of *cdc25* and *cdc35* mutants, placing *RAS1* and *RAS2* in the cAMP pathway. Where? Necessarily, at a point upstream of formation of cAMP - at least, upstream cyclase....

What is RAS? Briefly, the RAS protooncogenes encode small GTPases that act as molecular switches. Ras is "ON" when bound to GTP and displays a weak intrinsic GTPase that converts the bound nucleotide to GDP, the "OFF" mode. Activation upon signalling takes place by EXCHANGE of the bound GDP to new GTP. This cycle is controlled by guanyl nucleotide exchange factors or GEFs (positive regulators) and GTPase-activating proteins or GAPs that stimulate the intrinsic GTPase (thus, negative regulators of RAS).

RAS ONCOGENES usually contain single point mutations that inactivate the intrinsic GTPase, rendering the protein insensitive to GAP and constitutively active. SIGNAL transduction is always ON irrespective of an external input, thus resulting in uncontrolled proliferation.



- *ras1Δ ras2Δ* lethality was also rescued by Harvey-ras expressed in yeast!!!

- A mutation analogous to the oncogenic "activated" H-*ras*^{Val12} was introduced in *RAS2*: *RAS2*^{Val19}.

Activated *RAS2*^{Val19} was oncogenic in mammalian cells!!!!!

These are very interesting findings demonstrating that *RAS* regulation IS CONSERVED between yeast and humans emphasizing the relevance of working the mechanisms for RAS activation by exploiting genetics in lower eukaryotes. This was a bonus of the experiments outlined below...

- A $RAS2^{Val19}$ strain contains high cAMP levels and fails to stop dividing in the absence of nutrients (like *bcy1* mutants)

This means, when Ras is constitutively bound to GTP (ON) cAMP production is very high and uncoupled from the presence or absence of nutrients.

Epistasis analysis is an important tool to determine "ORDER OF GENE FUNCTION" in a genetic pathway

- $RAS2^{Val19}$ bypasses a $cdc25^{ts}$ BUT NOT a $cdc35^{ts}$ mutation

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So: CDC25 → RAS → CYR1/CDC35 ⊣ BCY1

"Oncogenic" RAS is independent of *CDC25* suggesting that *CDC25* may activate RAS by normally promoting exchange (unnecessary in the oncogenic variant)

Active Ras, however, still requires cyclase to promote the formation of cAMP (the oncogenic form cannot bypass absence of its effector- Cdc35 unlike a *bcy1* mutant).

(the *BCY1*-encoded negative regulator must be downstream cyclase but upstream TPKs based on the **epistasis analysis** mentioned above)

-The sequence of the IRA1 and 2 genes predicted products related to human GAP (GTPase activating protein). Ira1 and Ira2 were shown to act as GAPs for yeast RAS.

By this time you should be able to suggest appropriate epistasis tests to confirm the above statement:

Mutant	Relevant phenotypes		
	Division in response to nutrients	intracellular cAMP levels	Suppression by exogenous cAMP
ira1 ira2	cannot arrest	high	n/a
$RAS2^{val19}$	cannot arrest	high	n/a
bcy1	cannot arrest	low	n/a
cdc25	cannot bud	very low	yes
ras1 ras2	cannot bud	very low	yes
cdc35	cannot bud	undetectable	yes
tpk1,2,3	cannot bud	high	no
cdc25 $RAS2^{val19}$	cannot arrest	high	n/a
cdc35 $RAS2^{val19}$	cannot bud	undetectable	yes
cdc25 ira1 ira2	?	?	?
cdc35 ira1 ira2	?	?	?
ira1 ira2 ras1 ras2	?	?	?
ira1 ira2 ras1 ras2 bcy1	?	?	?
ira1 ira2 tpk1,2,3	?	?	?

With the help of the diagram for the cAMP pathway shown in the following page (and the handout appendix on *S. cerevisiae* genetics) answer these questions:

A dominant allele $TPK1^{W}$ was isolated as a second site suppressor of a $cdc35^{ts}$.

a) What might be the mechanism of rescue by this mutant?

b) Suggest a simple experiment involving plasmid-based manipulations to confirm your

hypothesis.
A dominant allele *BCY1*^{C} was isolated as second site suppressor of *RAS2*^{Val19} lethality upon starvation.

c) What might be the mechanism of rescue?
d) Would your hypothesis be consistent with *BCY1*^{C} being suppressed by *TPK1*^{W}?