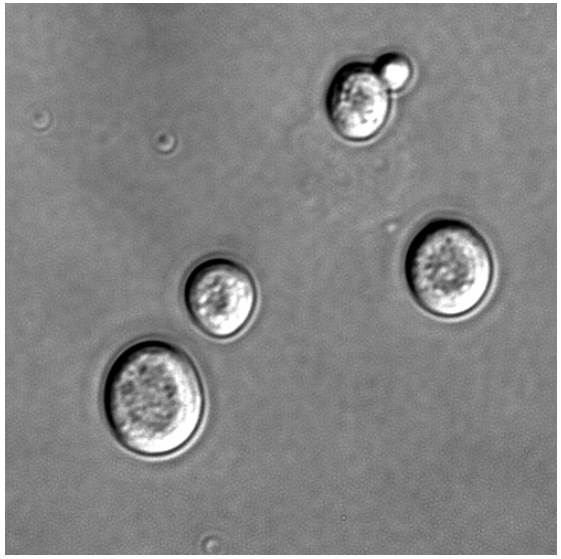
Recombinant DNA Technology

Studying protein interactions: Two Hybrid in yeast

•***Saccharomyces cerevisiae*** is a species of budding yeast. It is perhaps the most useful yeast owing to its use since ancient times in baking and brewing.

•It is one of the most intensively studied eukaryotic model organisms in molecular and cell biology, much like *E.coli* as the model prokarote.



•*Saccharomyces* cerevisiae cells are round to ovoid, 5–10 micrometres in diameter. It reproduces by a division process known as budding

- *S. cerevisiae* was the first eukaryotic genome that was completely sequenced. (April 1996)

- The genome is composed of about 13,000,000bp and 6,275 genes, although only about 5,800 of these are believed to be true functional genes.

Two-hybrid system

Il problema generale di caratterizzare interazioni proteina-proteina, identificando eventuali partner proteici di una proteina data, può venire risolto in vari modi, tra cui:

- co-immunoprecipitazioni (utilizzo di anticorpi e risoluzione su gel nativi)
- cross-linking (con reagenti, come la glutataraldeide, che formano legami covalenti tra le proteine che interagiscono)
- colonne di affinità con partner di interazione
- co-purificazioni in colonne cromatografiche
- screening di librerie di espressione
- utilizzo di phage display libraries

Il two-hybrid system si distingue perché arriva a **identificare e clonare contemporaneamente** il gene che codifica per il partner proteico senza bisogno di informazioni preliminari sui partner proteici o di strumenti precostituiti (per es. anticorpi)

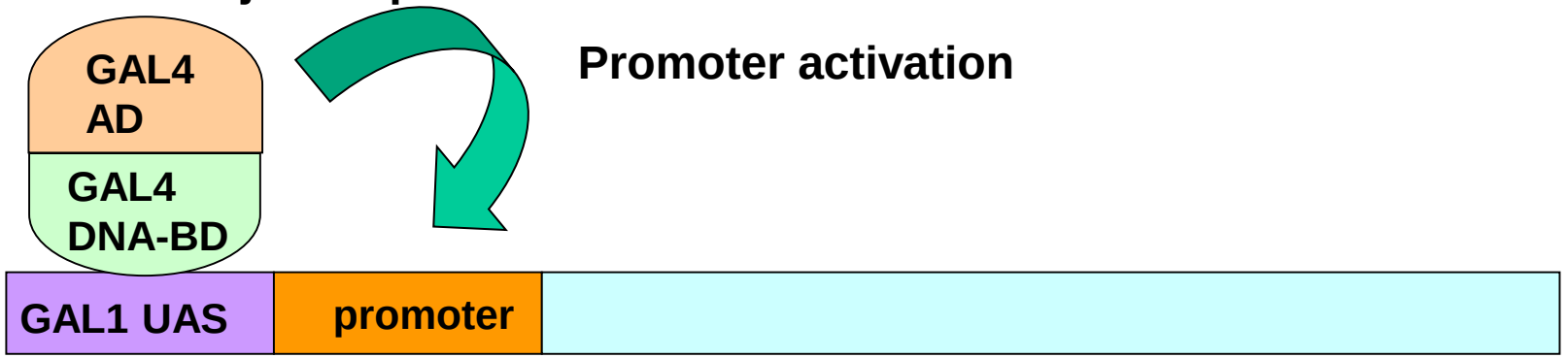
Yeast Two-Hybrid Analysis

- **Two-hybrid screening** is a technique used to discover protein-protein interactions by testing for physical interactions (such as binding) between two proteins .

- The most common screening approach is the **yeast two-hybrid assay**. The method is based on the properties of the yeast **GAL4 protein**, which consists of **separable domains** responsible for **DNA-binding** and **transcriptional activation**. Plasmids encoding two **hybrid proteins**, one consisting of the GAL4 DNA-binding domain fused to protein X and the other consisting of the GAL4 activation domain fused to protein Y, are constructed and introduced into yeast. Interaction between proteins X and Y leads to the transcriptional activation of a **reporter gene containing a binding site** for GAL4.

The GAL4 Transcription Factor

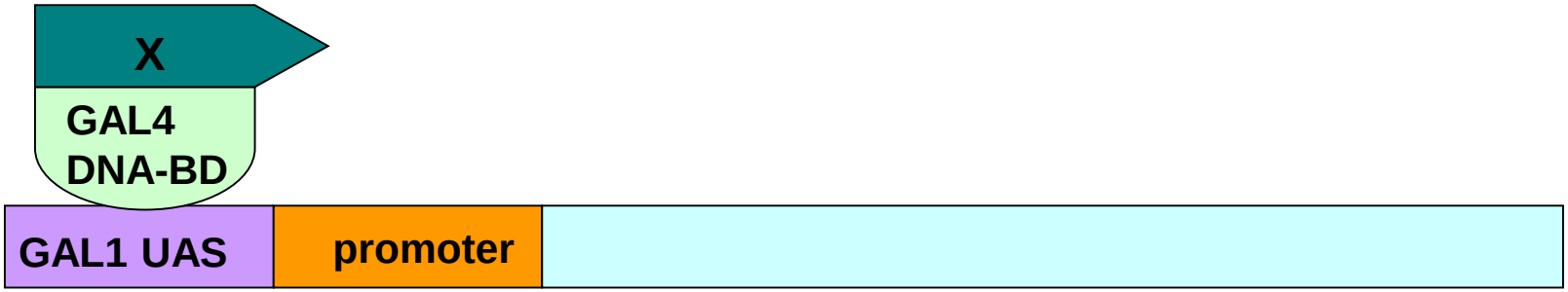
The yeast GAL4 transcription factor is a protein consisting of two major domains, a DNA binding domain (DNA-BD) and a transactivation domain (AD). Its normal role is to bind the GAL1 UAS (Upstream Activation Sequence) element and activate transcription from the adjacent promoter.



For the purposes of the Y2H system, the coding sequences for these two domains are separated and expressed from different plasmids.

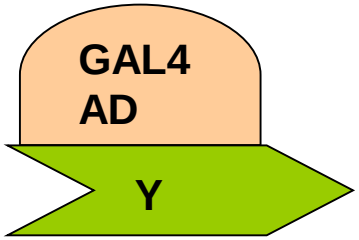
Yeast 2-hybrid

1. The hybrid of the GAL4 DNA-binding domain (BD) and protein X binds to the GAL1 UAS but cannot activate transcription without the activation domain.



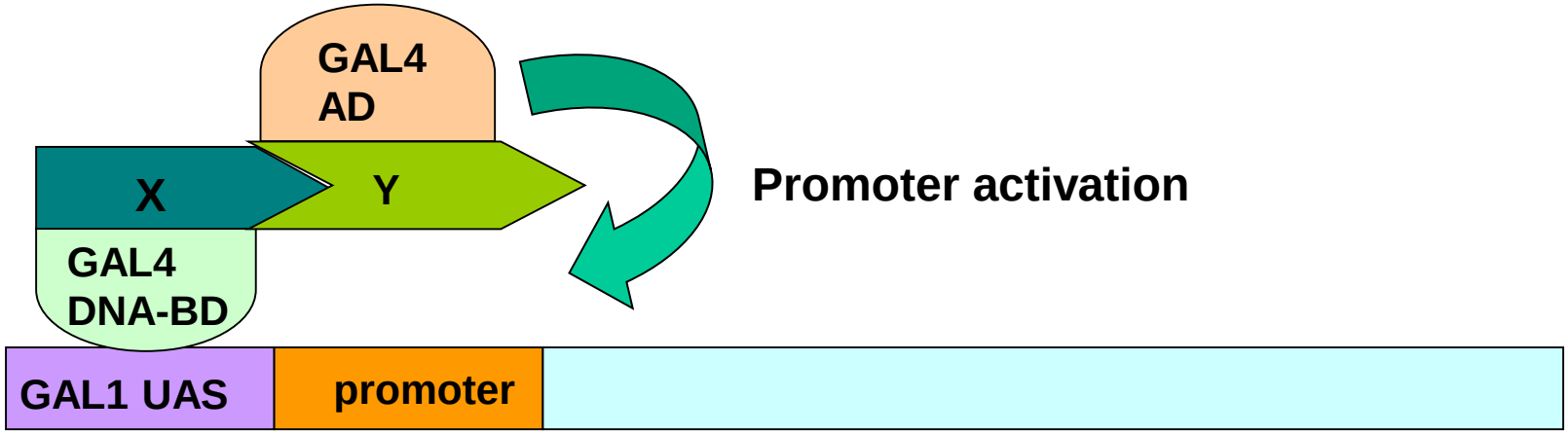
Yeast 2-hybrid

2. The hybrid of the GAL4 activation domain (AD) and protein Y cannot localise to the UAS by itself and thus does not activate transcription.



Yeast 2-Hybrid System: Summary

Interaction between the X and Y portions of two hybrid proteins *in vivo* reconstitutes GAL4 transcription factor function and results in expression of a gene.

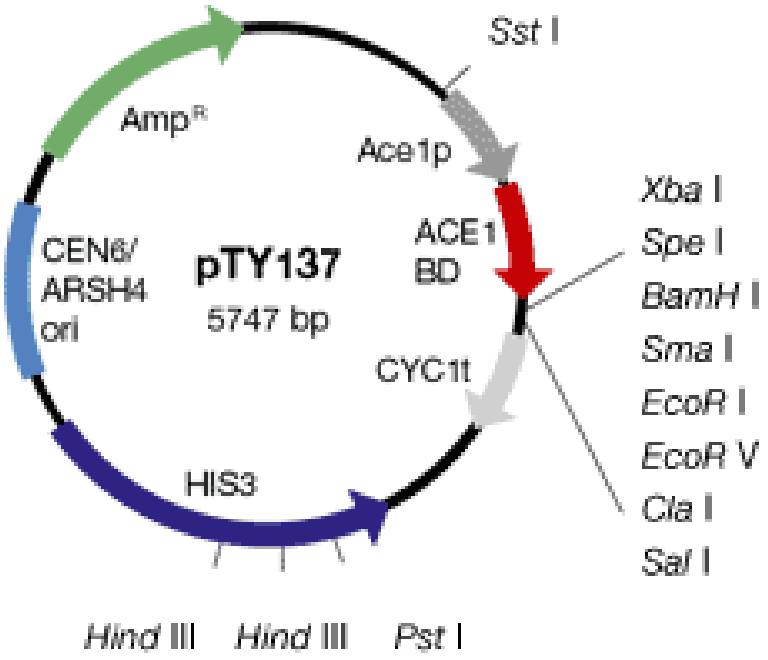


Yeast 2-Hybrid System: Summary

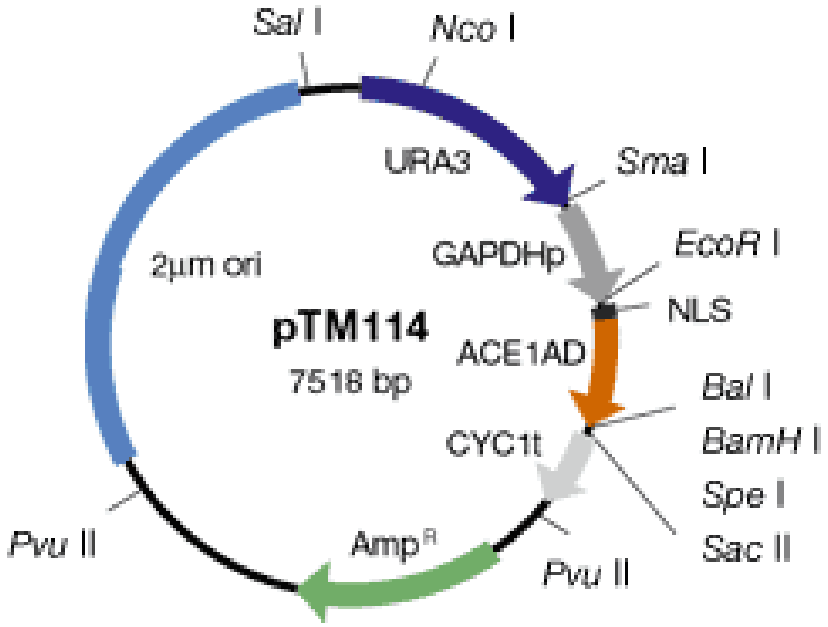
Interaction between the X and Y portions of two hybrid proteins *in vivo* reconstitutes GAL4 transcription factor function and results in expression of a reporter gene.

Expression of β-galactosidase can be assayed using X-GAL

Bait-Plasmid



Prey-Plasmid



Reporter Genes

- LacZ reporter - Blue/White Screening
- HIS3 reporter - Screen on His+ media
  LEU2 reporter - Screen on Leu+ media
- ADE2 reporter - Screen on Ade+ media
- URA3 reporter - Screen on Ura+ media

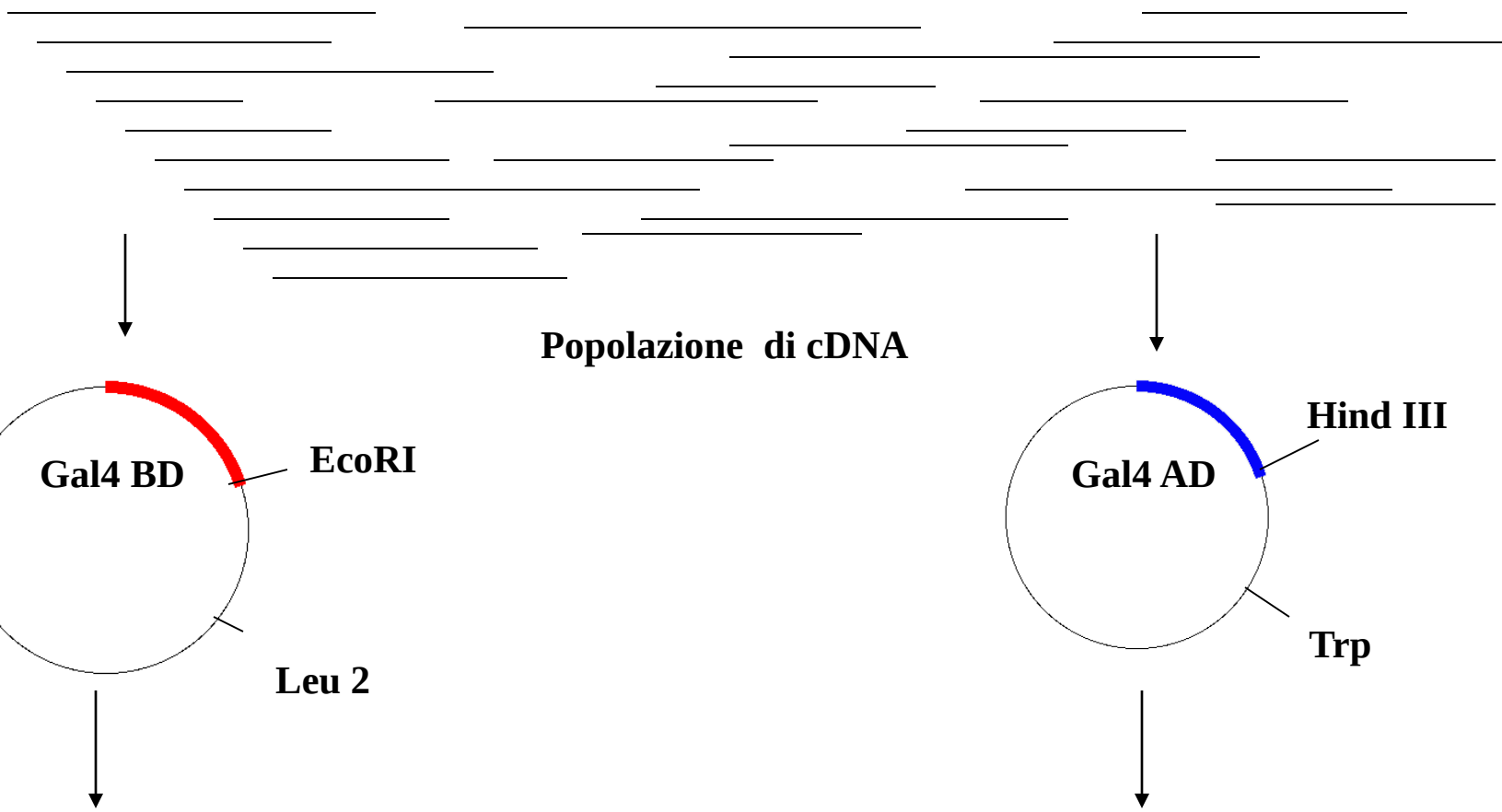
Screening for an interacting protein

1. The cDNA for the “bait” protein (or X in the following diagrams) is inserted into an expression vector to generate a hybrid protein consisting of the DNA binding domain of the GAL4 transcription factor (TF), fused to the “bait” protein. (The bait protein is the one for which we wish to isolate interacting proteins).

2. A cDNA library is created in a second expression vector which will express the product of the cloned cDNA (Y in the diagrams) as a hybrid protein with the activation domain of the GAL4 TF.

3. If X and Y interact, this will bring the two halves of the GAL4 TF together and therefore able to transactivate transcription from a promoter containing a GAL4 binding site.

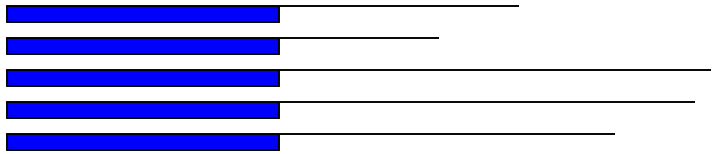
Costruendo una libreria di espressione in Gal4 AD e cotrasformandola in un lievito contente Un reporter sotto il controllo di una UAS si possono identificare proteine che interagiscono tra loro



Fusione tra Gal4BD e il gene codificante la proteina “esca” da testare

Gal4 BD **DNA ‘esca”**

Libreria di fusioni Gal4AD-cDNA



Screening for an interacting protein - overview

Expression plasmid pBD: coding sequence for GAL4 DNA-BD fused to coding sequence for "Bait" protein.

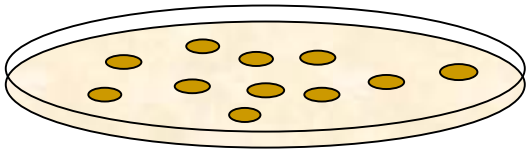
Expression plasmid pTA: coding sequence for GAL4 TA fused to sequences from cDNA library.

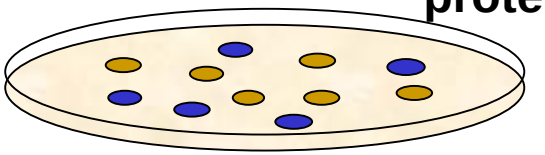
co-transform plasmids into yeast strain Y190 (*his3*–)

expression of both hybrid proteins in the same cell

DNA-BD / "bait" protein + **AD / cDNA library protein**

plate culture on appopriate medium to find cotransfectants in which the two hybrid proteins interact

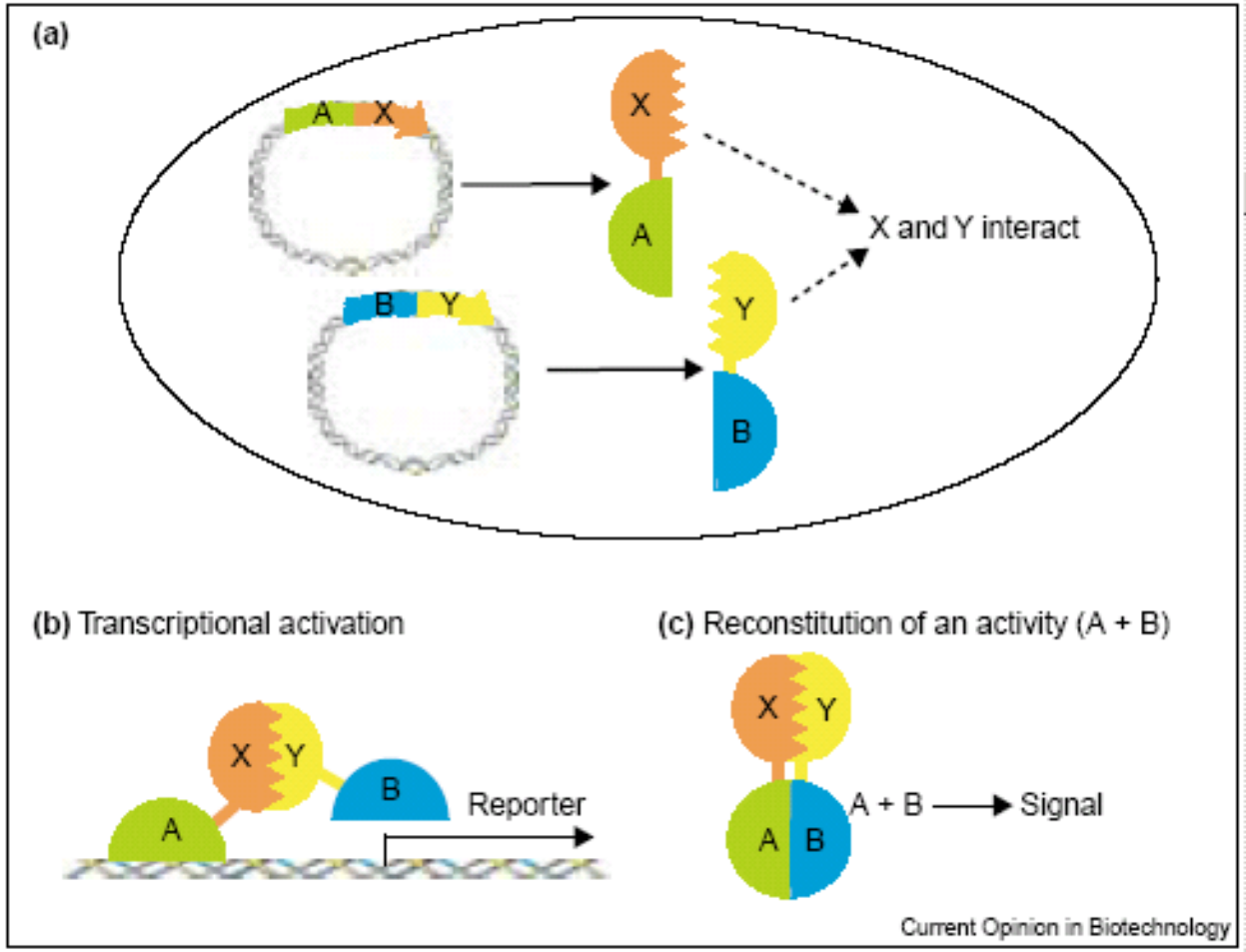




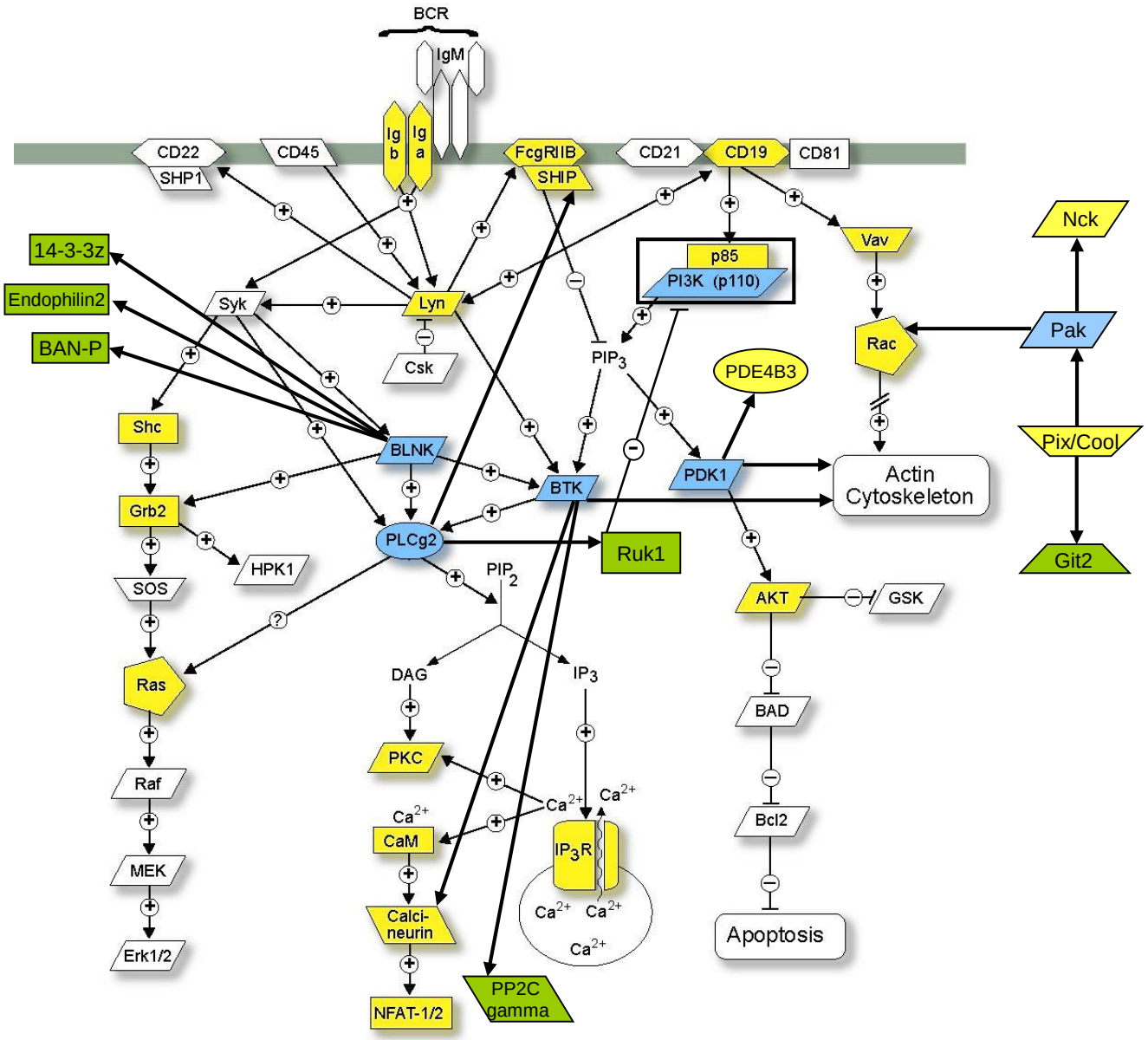
assay for HIS3 reporter gene activity (growth on histidine-deficient medium)

assay for β-galactosidase reporter gene activity (*lacZ* expression: blue colonies)

In vivo selection systems. (a) DNA encoding the target (X, red) and bait (Y, yellow) proteins is fused to either part (A or B) of the reporter system. Co-expression in a cell yields the fusion proteins A–X and B–Y. (b) The yeast two-hybrid system. Interaction between X and Y results in functional pairing of the DNA-binding domain (A) and the transcriptional activator (B) allowing transcription of a reporter gene and leading to a detectable phenotype. (c) The protein fragment complementation assay. Interaction between X and Y results in reconstitution of an active enzyme (A–B), which gives rise to a detectable signal such as fluorescence or biosynthesis of an essential growth factor.



B Cell Receptor Pathway



Problems with Two-Hybrid Screens

While two-hybrid screens can be very useful, they suffer from fairly high false-negative and false-positive rates

What are some potential sources of false-positive (proteins that appear to interact in the assay, but don't in living cells) and false-negative (proteins that interact in living cells but not in the two-hybrid assay) results?

Two-Hybrid False Negatives

- Target protein not in library
- Proteins do not fold properly or interact in the conditions used in the screen (e.g. human proteins in yeast cells)
- Proteins only interact in the presence of other proteins
- Proteins interact in ways that do not permit activation domain to function (multimerization)

Two-Hybrid False Positives

- Non-specific
  - Bait proteins that activate without target
  - Target proteins that activate without bait
  - Target/Bait proteins that are “sticky” and interact with many things
- Specific
  - Interactions between proteins that are never expressed together in living cells
  - Interactions between proteins that are normally inhibited by the presence of other proteins/conditions

Elimination of False Positives

- Sequence Analysis
- Plasmid Loss Assays
- Retransformation of both strain with bait plasmid and strain without bait plasmid
- Test for interaction with an unrelated protein as bait
- Two (or more) step selections