Souhrn pŚedchozí pŚednáġky

- Genetické metody
  - mutageneze/ñscreenñ
  - komplementace
  - identifikace
- Bunŋ, ný cyklus
  - PrŢbŋh a regulace BC
  - Synchronizace bunŋk
  - Mechanismy regulace párování
  - Homothalické kmeny

Mechanismy opravy poġkozené DNA

Cviļ ení: 11.12., A7 (2.17) - pláġtŋ, psací a kreslící potŚeby

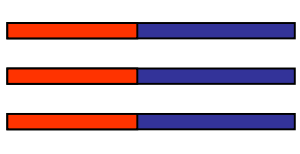
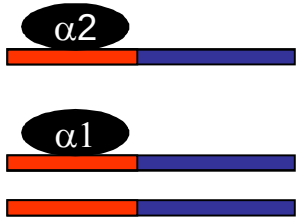
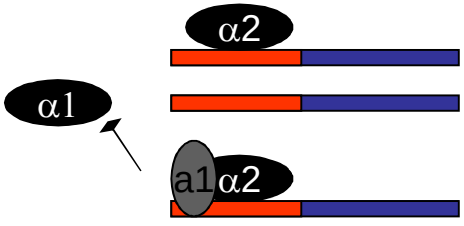
Osnova přednášky

- Regulace transkripce
  - Gal4 transkripční faktor
    - hybridní systémy
    - aplikace
  - modifikované kvasinkové hybridní systémy
- Kvasinkový chromatin
- Aneuploidie a rakovina
- Evoluce kvasinek

Regulace transkripce v haploidních buňkách (konstitutivní)

a1, a2 + α1, α2 - transkripční faktory, které ovlivňují transkripci 3 skupin genů

a-spec.= *MFA*1,2 (a-feromon), *STE*2 (α-receptor), *STE*6, 14 (úprava a sekrece feromonu)
α-spec.= *MFα*1,2 (α-feromon), *STE*3 (a-receptor), *STE*13, *KEX*2 (proteasy)
haploid spec.= *STE*4,18 (podjednotky G-proteinu), *RME*1 (inhibitor meiosy)

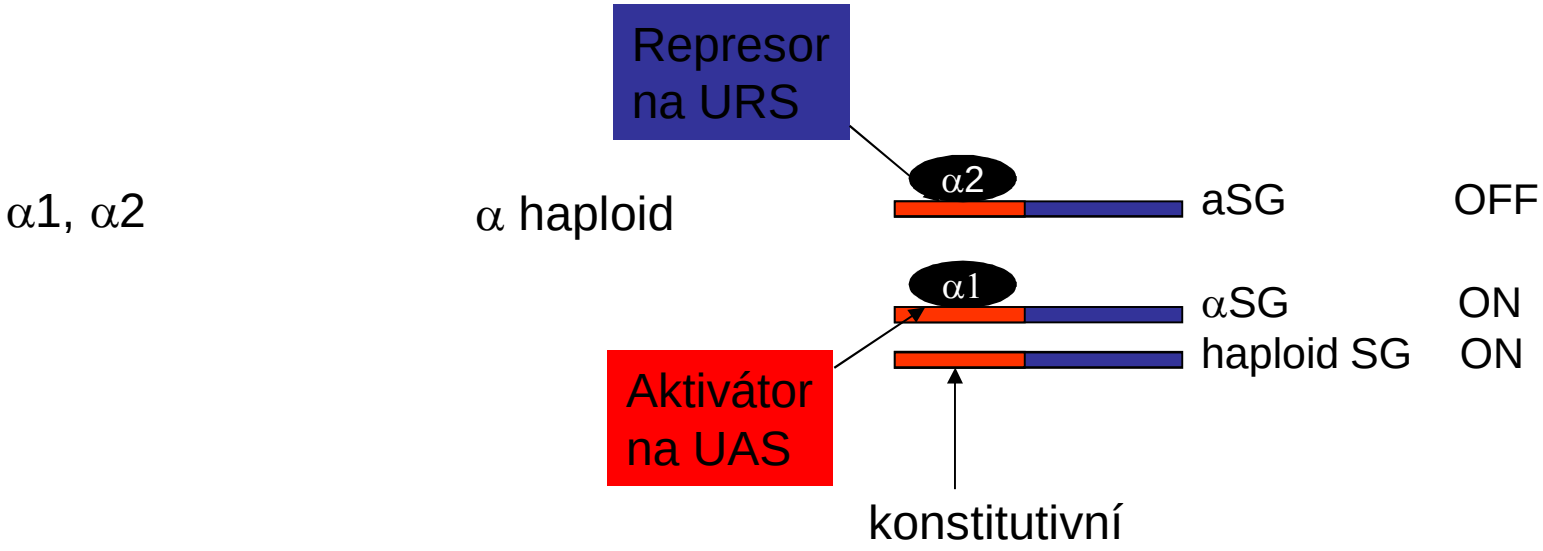
MAT lokus	Typ buňky	Geny kontrolované MAT lokusem		
a1, a2	a haploid		aSG αSG haploid SG	ON OFF ON
α1, α2	α haploid		aSG αSG haploid SG	OFF ON ON
α1, α2 a1, a2	diploid		aSG αSG haploid SG	OFF OFF OFF

Struktura promotorŢ

Kvasinkové promotory se liġí od bakteriálních a vyġġích eukaryot (kvasinky netranskribují z takových promotorŢ ï kvasinkové plasmidy é)

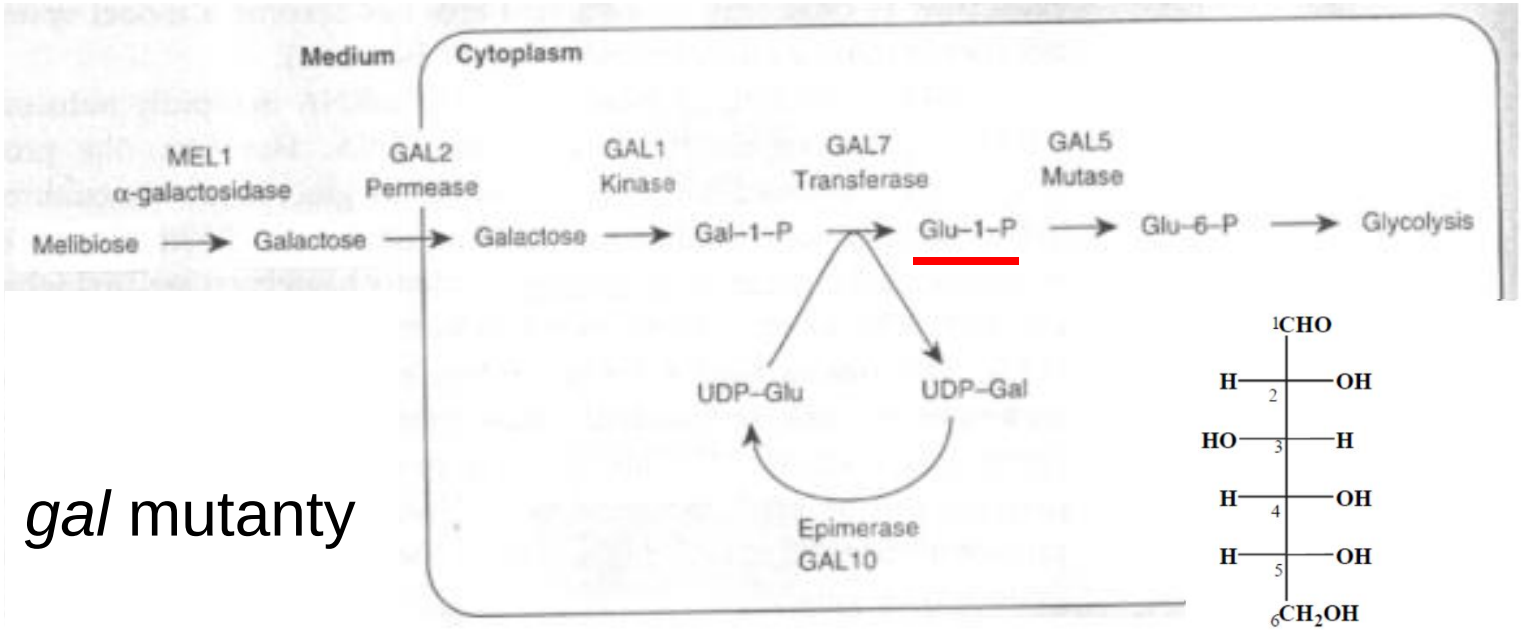
-VĎtġina míst pro iniciaci transkripce obsahuje TC(G/A)A a PuPuPyPuPu (specifické pro kvasinky)

- TATA box (TATAT/AAT/A) je 60-120bp od iniciaļního místa (podobné Pribnowovu boxu u bakterii)
- UAS (upstream activating sequences) a URS (upstream repressing sequences)
- DAS (downstream activating sequences ï pŚímo v sekvenci genu)



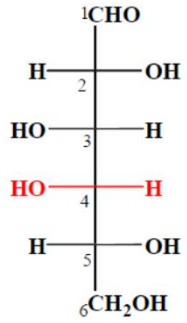
Regulace metabolické dráhy galaktózy

RŢzné kvasinky vyuģívají rŢzne cukry (viz pŚednáģka o urļování kvasinek)



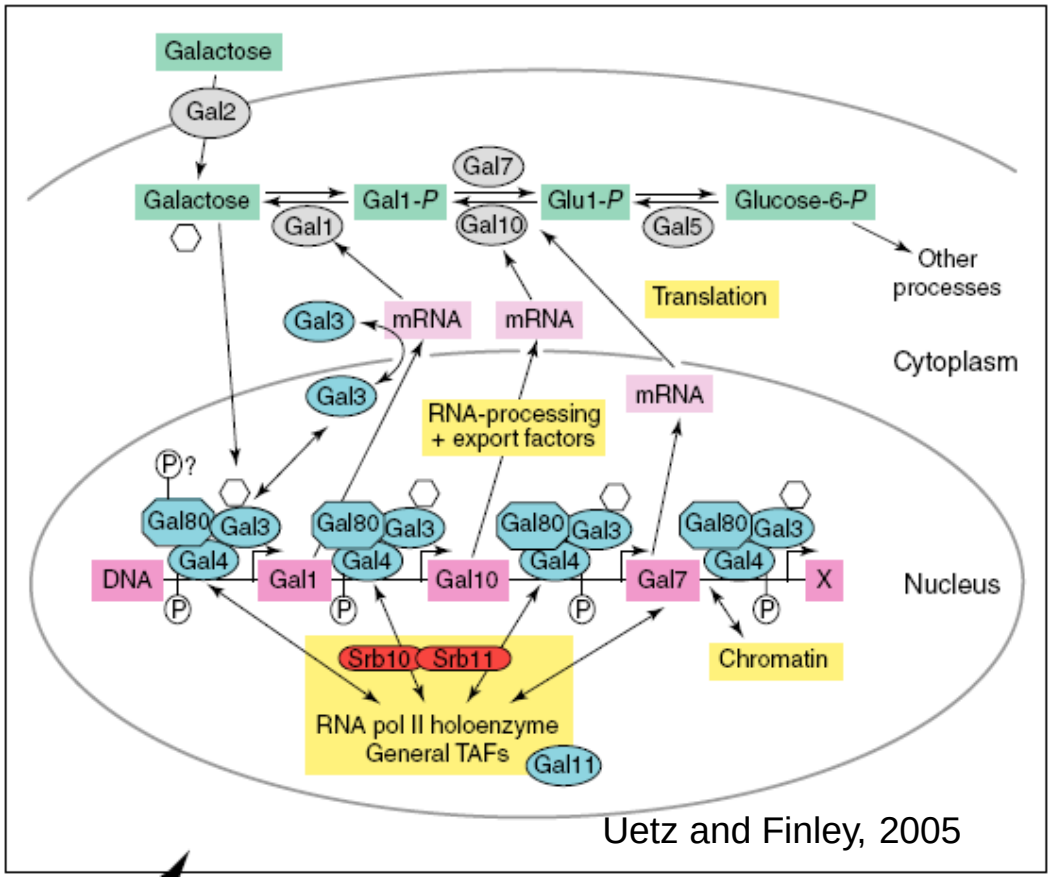
gal mutanty

D-glucose



D-galactose

- Pouze *GAL5* gen je konstitutivnŊexprimován (potŚebný pro metabolismus glukózy)
- vģechny ostatní jsou indukovány rŢstem na galaktóze a reprimovány glukozou
- *GAL1, GAL7 a GAL10* geny jsou v klastru na chromosomu 2
- ***GAL4*** gen kóduje transkripļní faktor (aktivátor), který se váģe na UAS tŊchto genŢ



Uetz and Finley, 2005

Regulace transkripce *GAL* genŢ

- *GAL1, GAL7 a GAL10* geny jsou v klastru na chromosomu 2
- *GAL4* gen kóduje transkripţní faktor (aktivátor), který se váţe na UAS tţchto genŢ

- Gal80p se váţe na Gal4p a reprimuje/inhibuje transkripci
- Gal3p pŚemţní galaktozu na induktor (váţe se na Gal80p a blokuje vazbu na Gal4p)

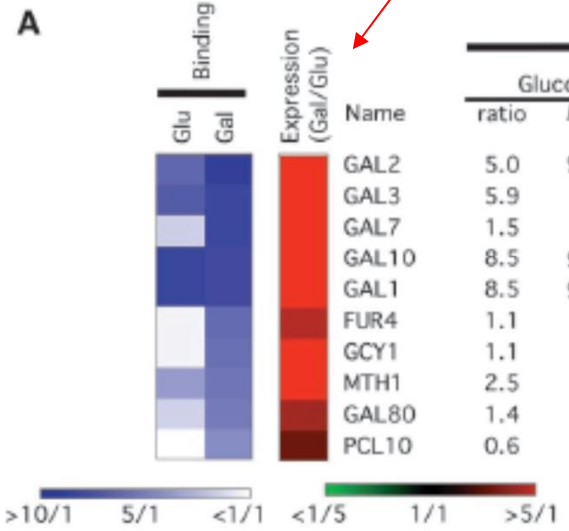
- *GAL1* promotor je rychle indukovaný a velmi silný ï 1000x se zvýšší mRNA (aţ 1%)
- pouţívá se pro overexprese/nadprodukce proteinŢ
- nesmí být pŚítomna glukosa !

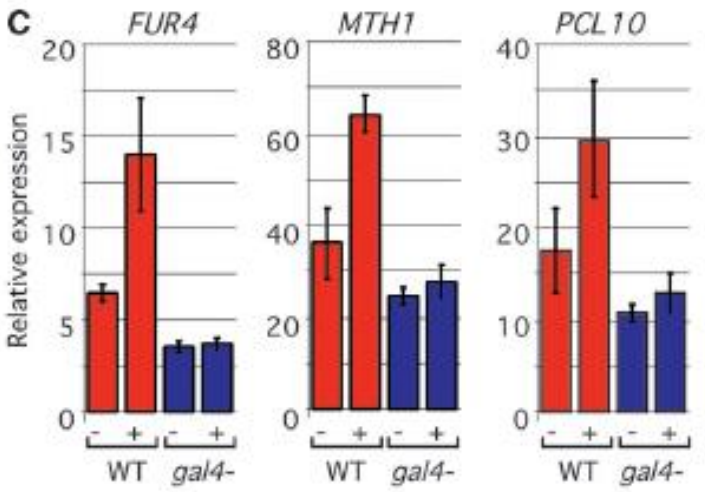
ChIP Gal4p

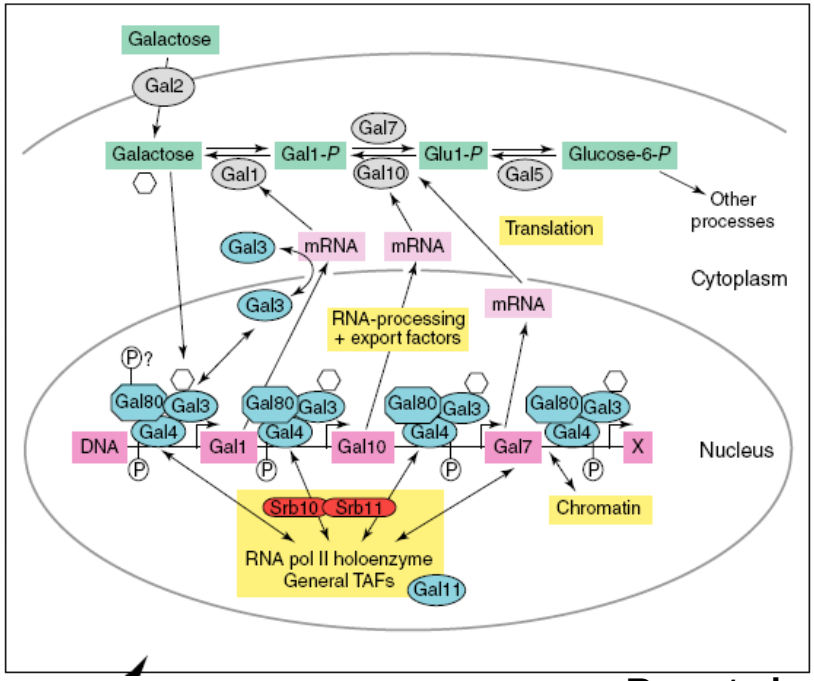
microarray po galaktose

A

Name	Binding: Glucose ratio	Binding: Glucose *P*-value	Binding: Galactose ratio	Binding: Galactose *P*-value	Expression ratio (Gal/Glu)	Description
GAL2	5.0	$9.6X10^{-4}$	9.6	$1.4X10^{-7}$	188.6	Galactose permease
GAL3	5.9	$3.2X10^{-4}$	8.3	$2.5X10^{-7}$	18.2	Regulatory protein required for rapid induction of galactose pathway
GAL7	1.5	0.29	8.4	$3.8X10^{-7}$	170.1	UDP-glucose--hexose-1-phosphate uridylyltransferase
GAL10	8.5	$9.5X10^{-5}$	7.9	$7.0X10^{-7}$	118.8	UDP-glucose 4-epimerase
GAL1	8.5	$9.5X10^{-5}$	7.9	$7.0X10^{-7}$	271.3	Galactokinase, first step in galactose metabolism
FUR4	1.1	0.86	4.7	$7.1X10^{-6}$	4.6	Uracile permease (adjacent to GAL1)
GCY1	1.1	0.74	4.3	$1.2X10^{-5}$	93.1	Galactose-induced oxidoreductase
MTH1	2.5	0.01	4.1	$2.1X10^{-5}$	21.6	Repressor of hexose transport genes
GAL80	1.4	0.39	3.7	$4.1X10^{-5}$	4.0	Negative regulator for expression of galactose-induced genes
PCL10	0.6	0.24	3.0	$2.6X10^{-4}$	2.6	Cyclin that associates with Pho85p, involved in glycogen accumulation

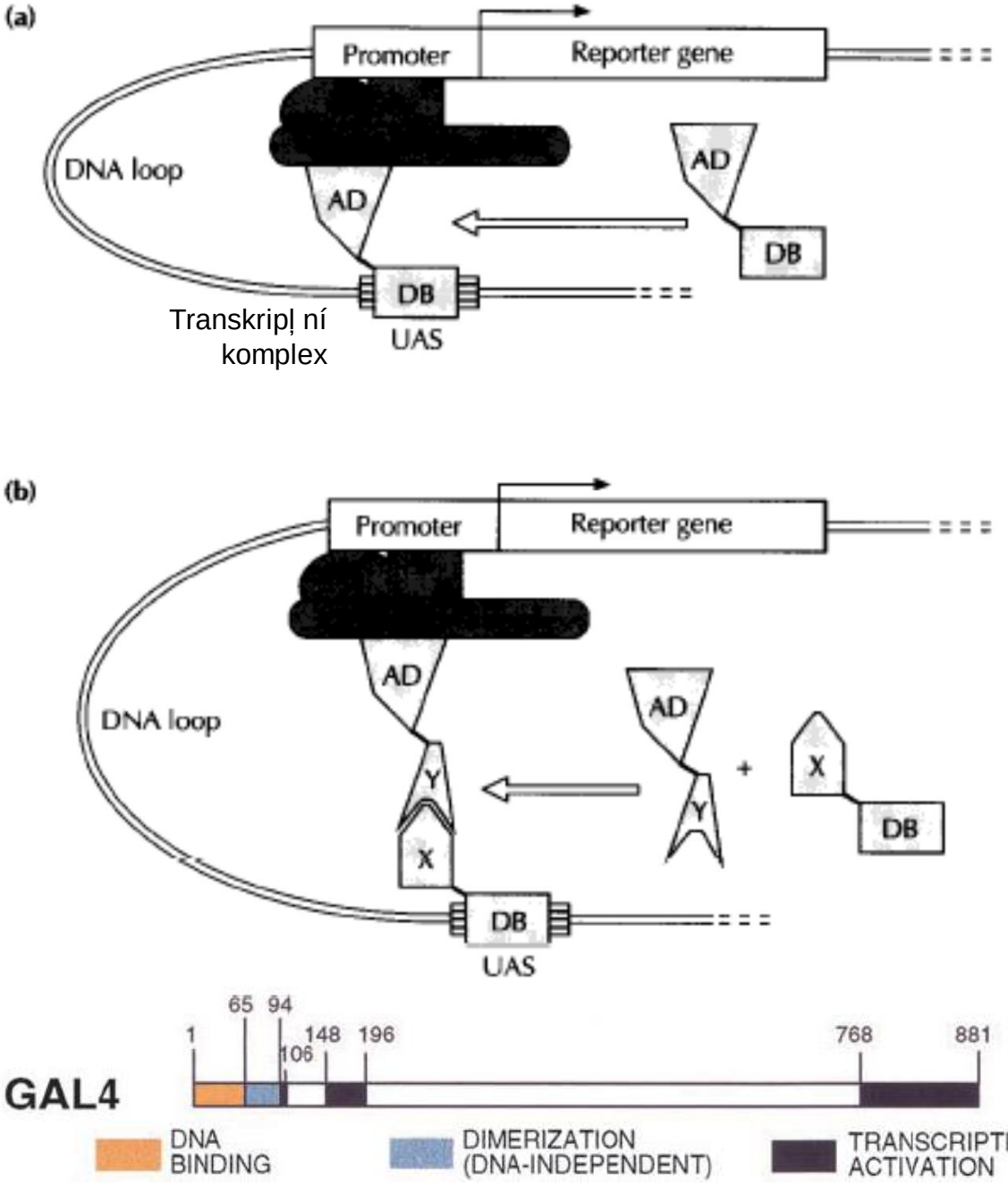


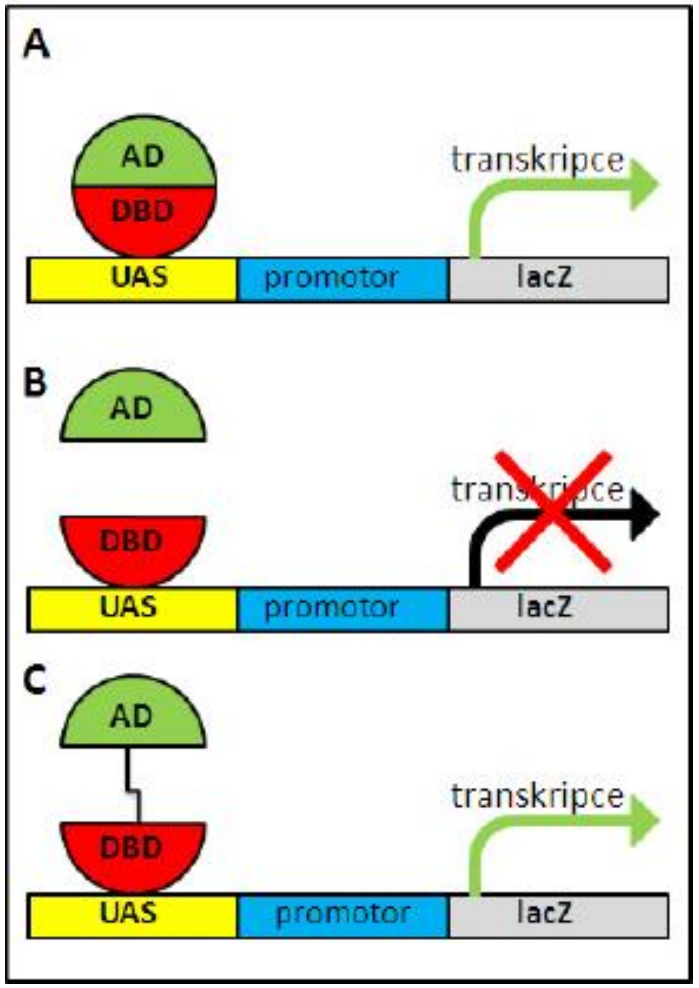




Ren et al., Science, 2000

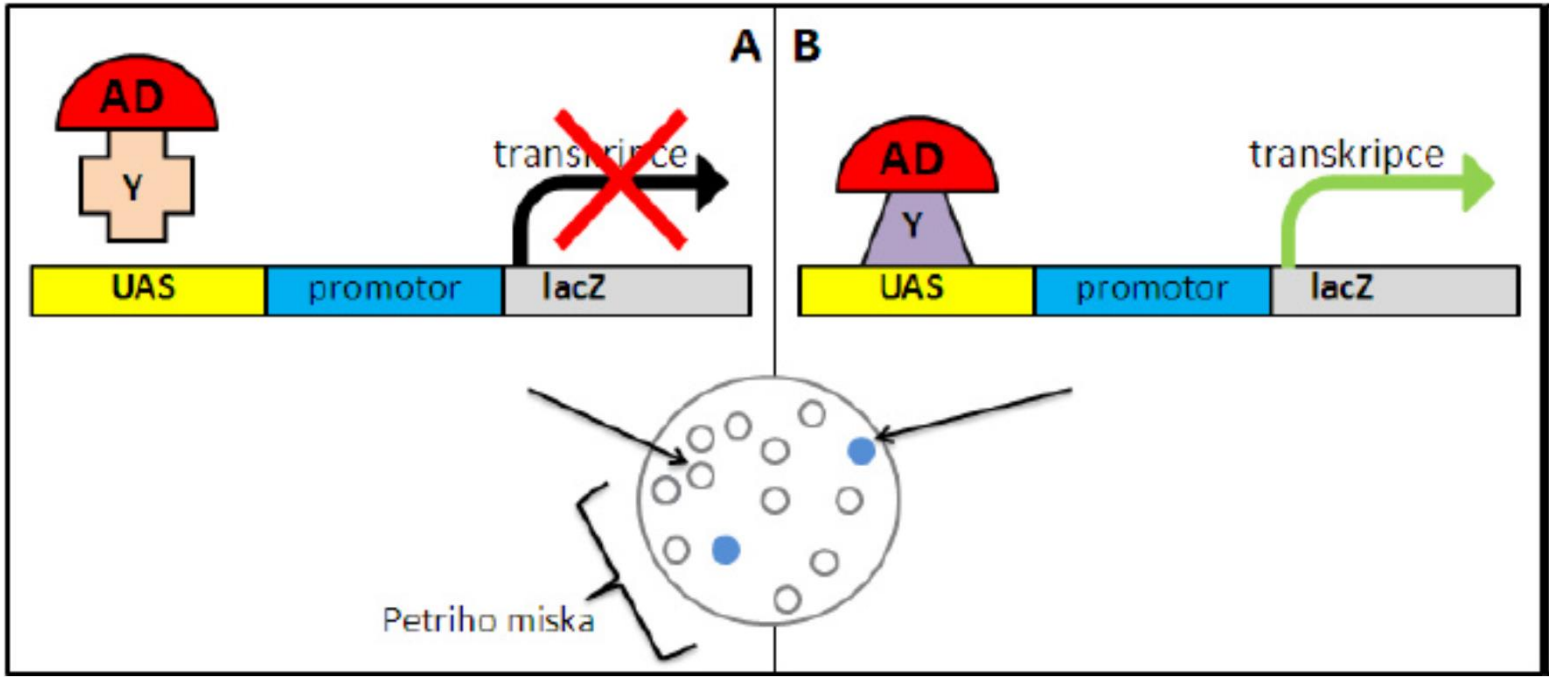
Transkripční aktivátor Gal4p





CO Biotech (1995) p. 59

Vznik 1-hybridních systémŢ

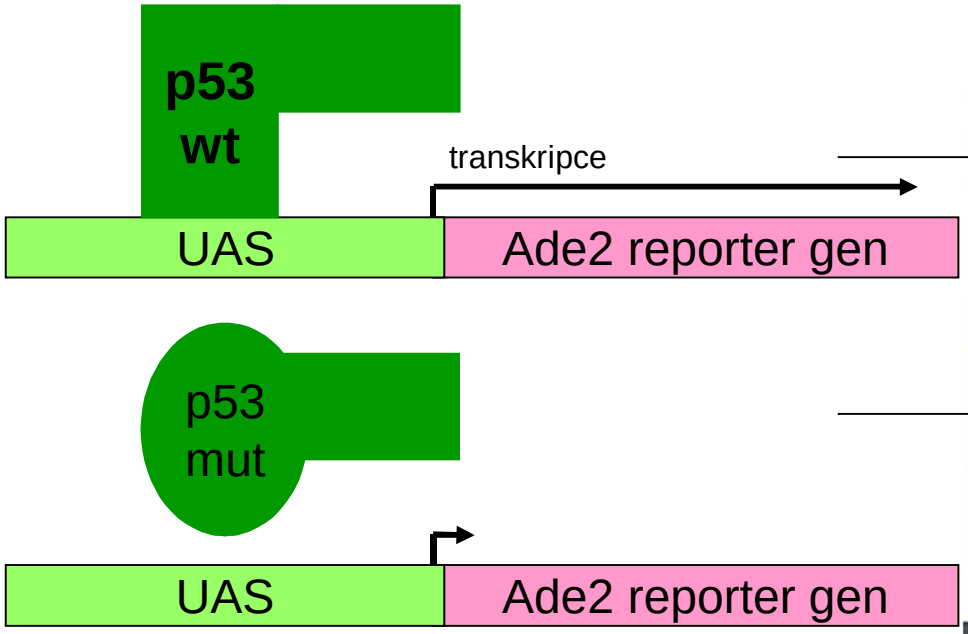


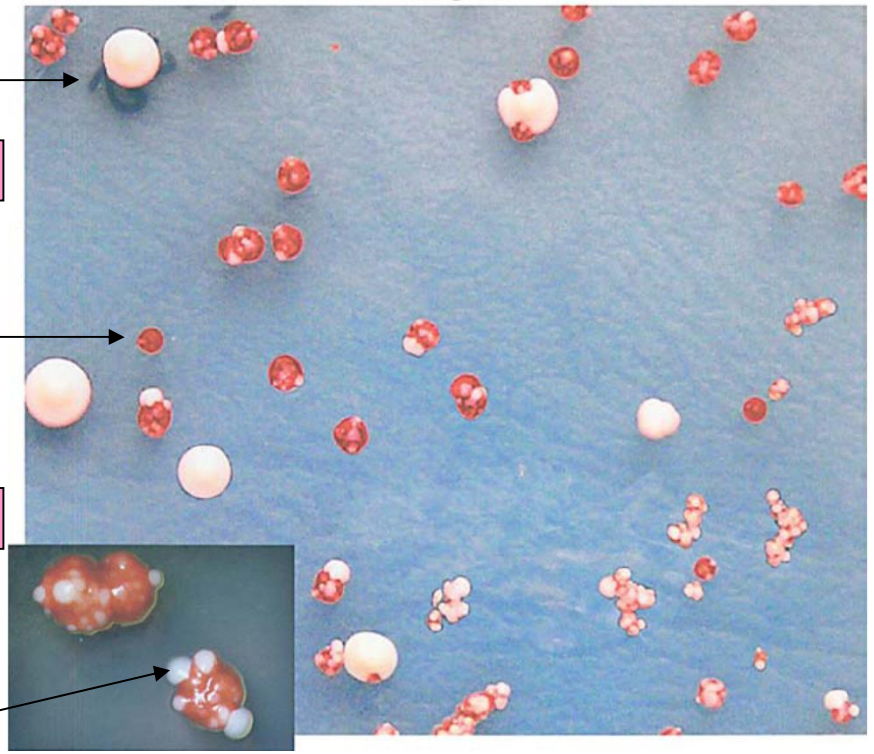
RŢzné transkripţní faktory mají podobné domény a lze je kombinovat é

Lze hledat DNA-vazebné proteiny pro danou UAS sekvenci (AD-hybridní knihovny)

- Takto funguje napŚ i FASAY (**F**unctional **A**nalysis of **S**eparated **A**lleles in **Y**east) pro testování mutantních p53 (transkripţní faktor)

prof. Ġmardová, Oncology Reports (2008) p. 773



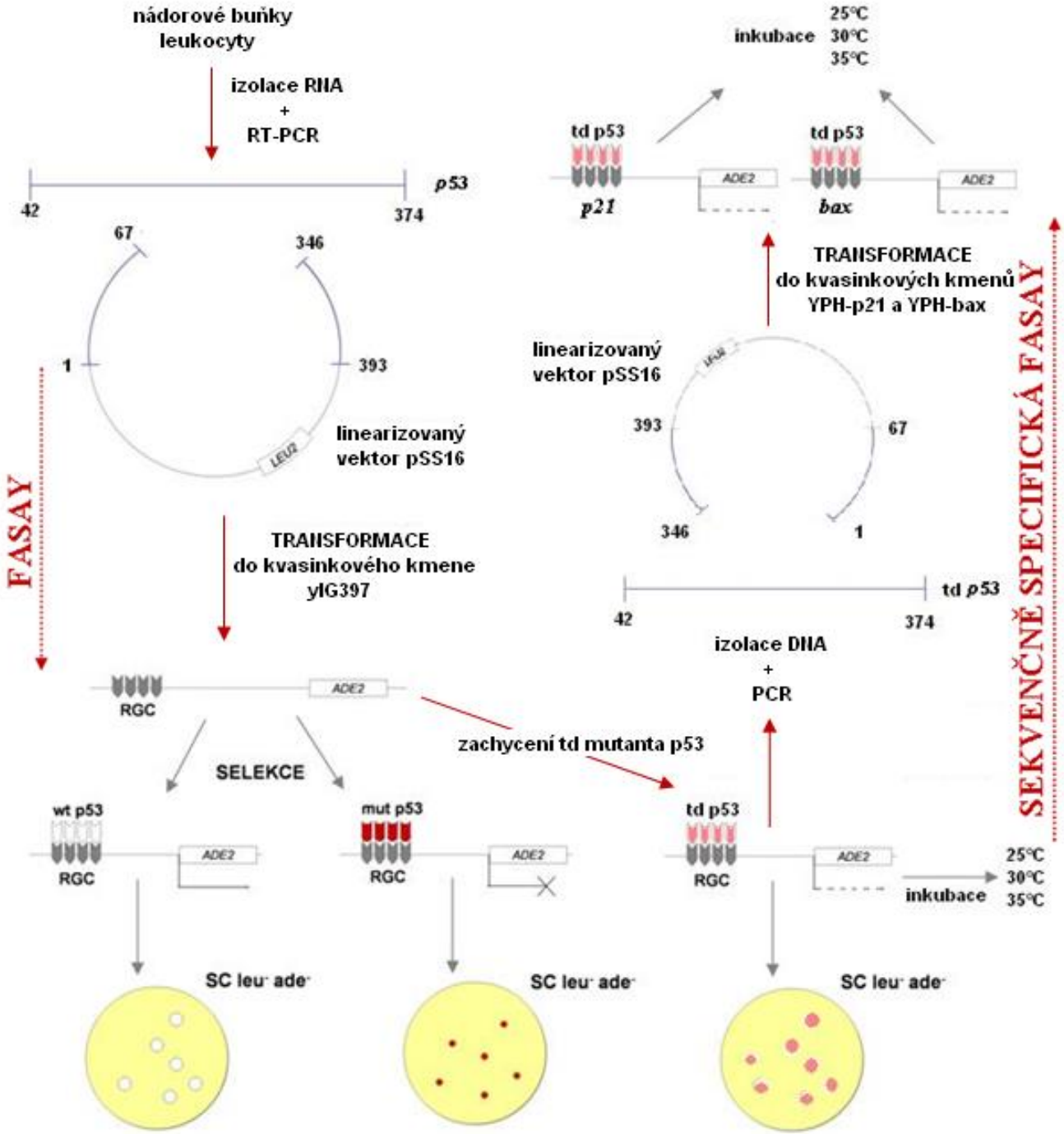


mut p53 (duplikace 30bp)
kvasinka opravila

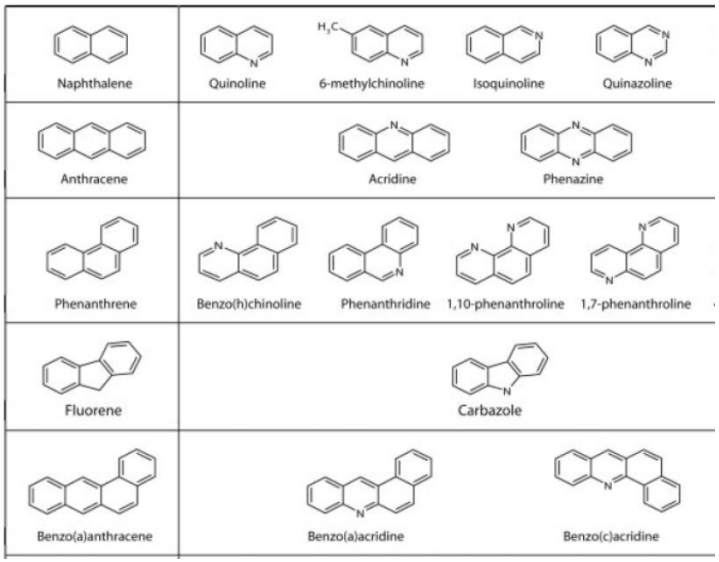
Analýza funkčních vlastností p53

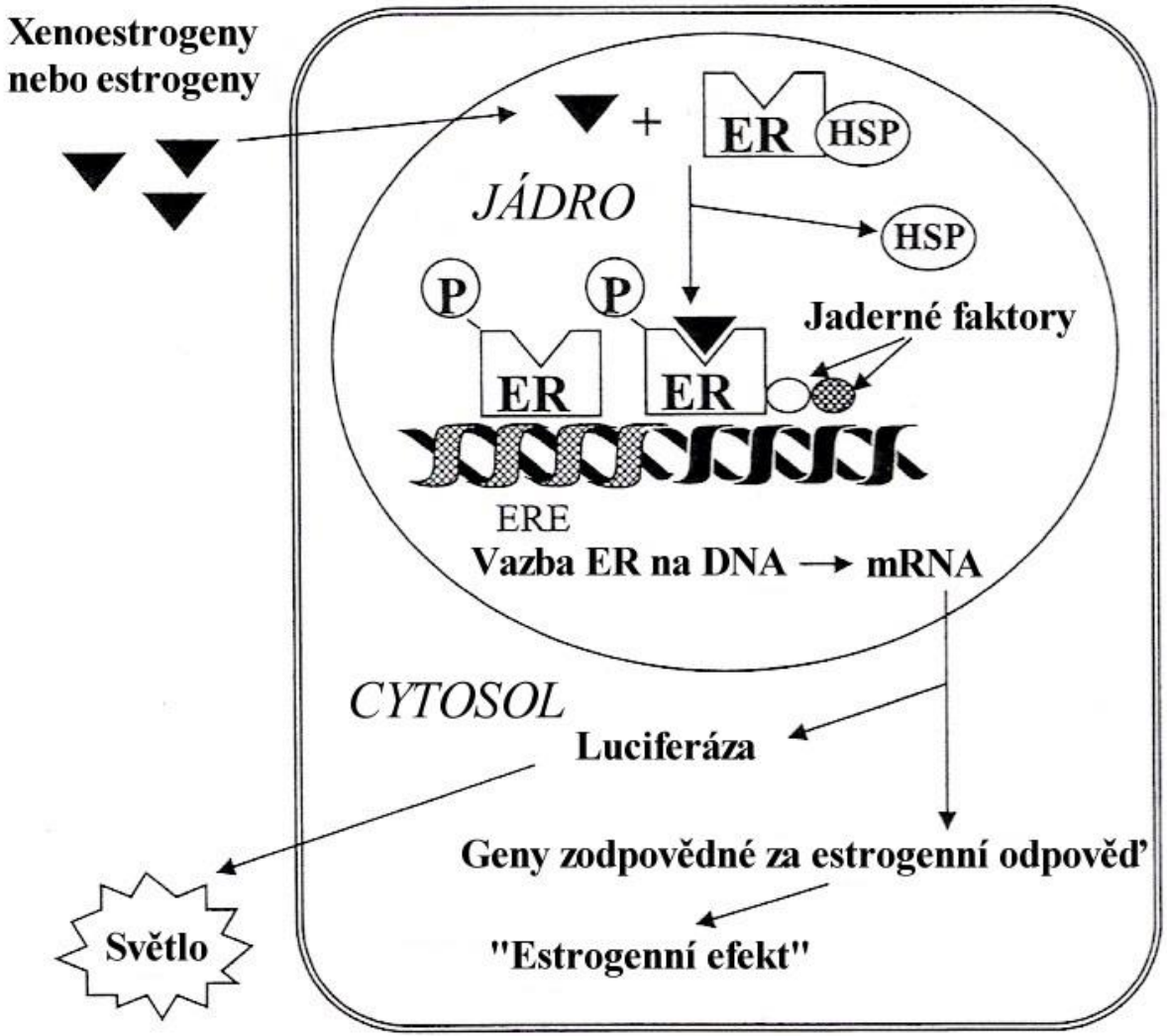
- stanovení aberací p53 v klinickém materiálu - imunoanalýza, FISH, sekvenace *TP53*

- urļ ení funkļ ního statutu - stanovení transaktivaļ ích schopností p53 metodou **FASAY (functional analysis of separated alleles in yeast)** - stanovení transaktivaļ ních vlastností p53 prostŚednictvím speciálnĎ upraveného kvasinkového kmene *Saccharomyces cerevisiae* yIG397


nádorové buňky
leukocyty
izolace RNA
+
RT-PCR
p53
42
374
67
346
1
393
LEU2
linearizovaný
vektor pSS16
FASAY
TRANSFORMACE
do kvasinkového kmene
yIG397
RGC
ADE2
SELEKCE
wt p53
mut p53
SC leu⁻ ade⁻
zachycení td mutanta p53
td p53
izolace DNA
+
PCR
inkubace
25°C
30°C
35°C
TRANSFORMACE
do kvasinkových kmenů
YPH-p21 a YPH-bax
p21
bax
linearizovaný
vektor pSS16
td p53
SEKVENČNĚ SPECIFICKÁ FASAY

Toxikologické aplikace



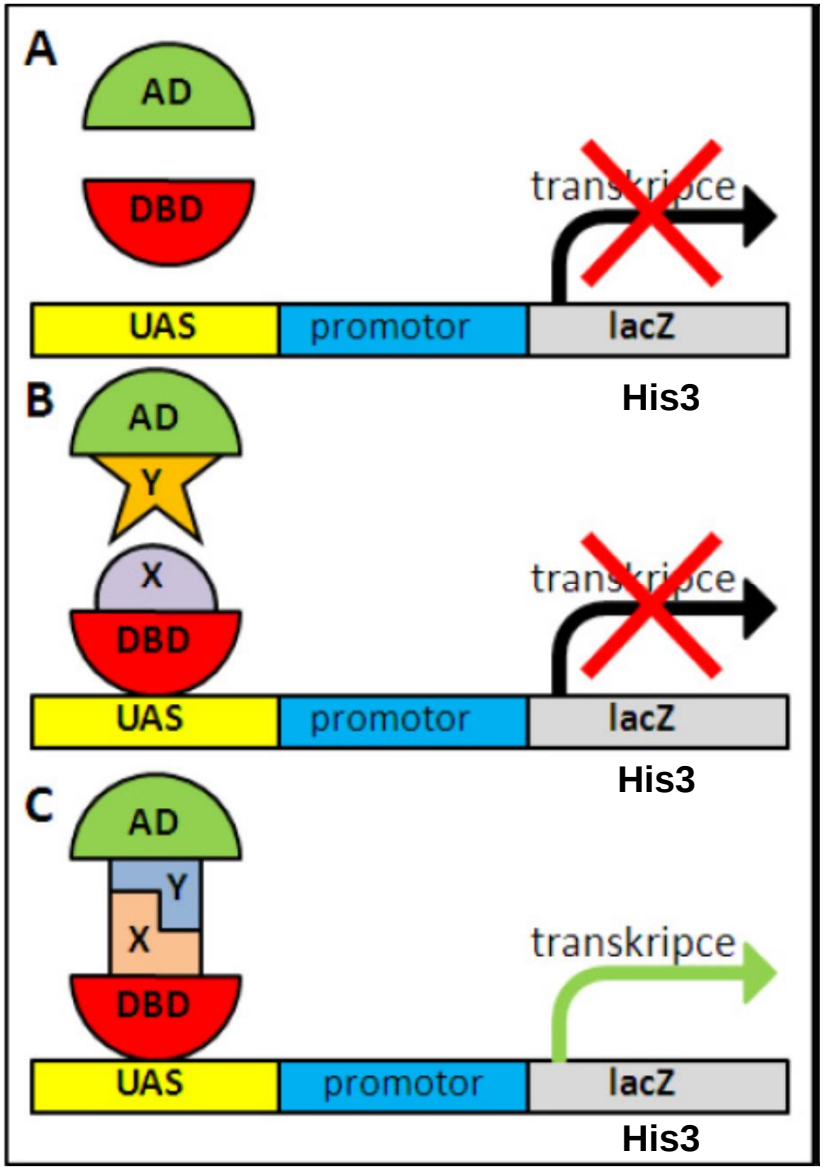


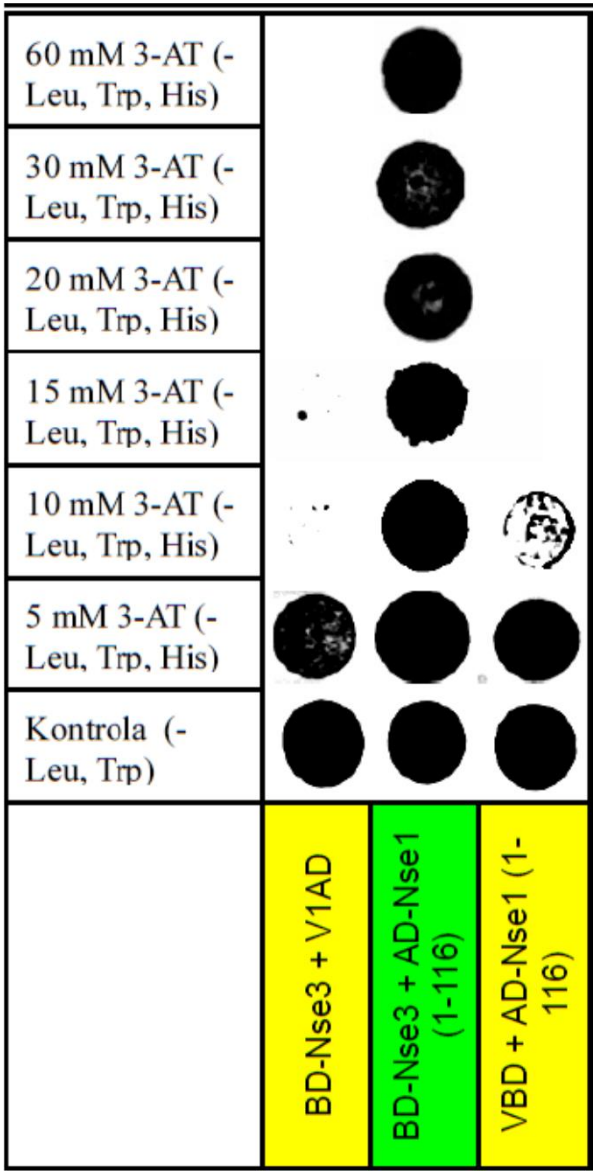
RECETOX/CETOCEON
(Dr. Ľupr/prof. Holoubek)

Bartos et al, Env Tox, 2006

D-luciferin + O_2 —*luciferáza*→ oxyluciferin + světlo

2-hybridní systém





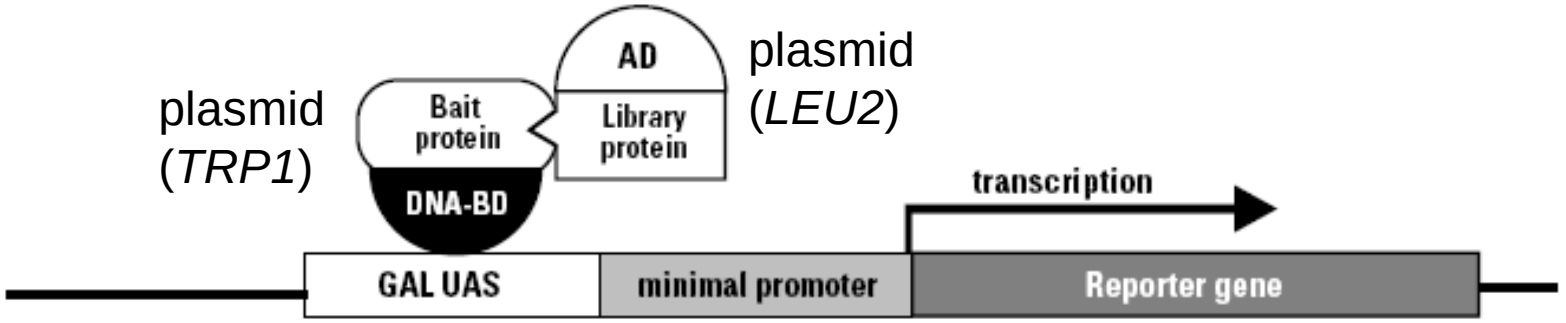
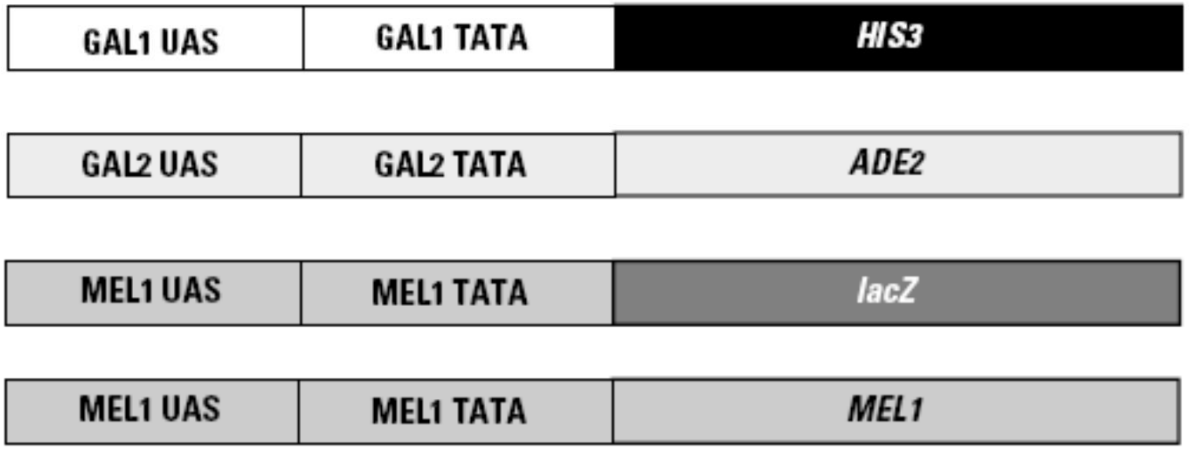


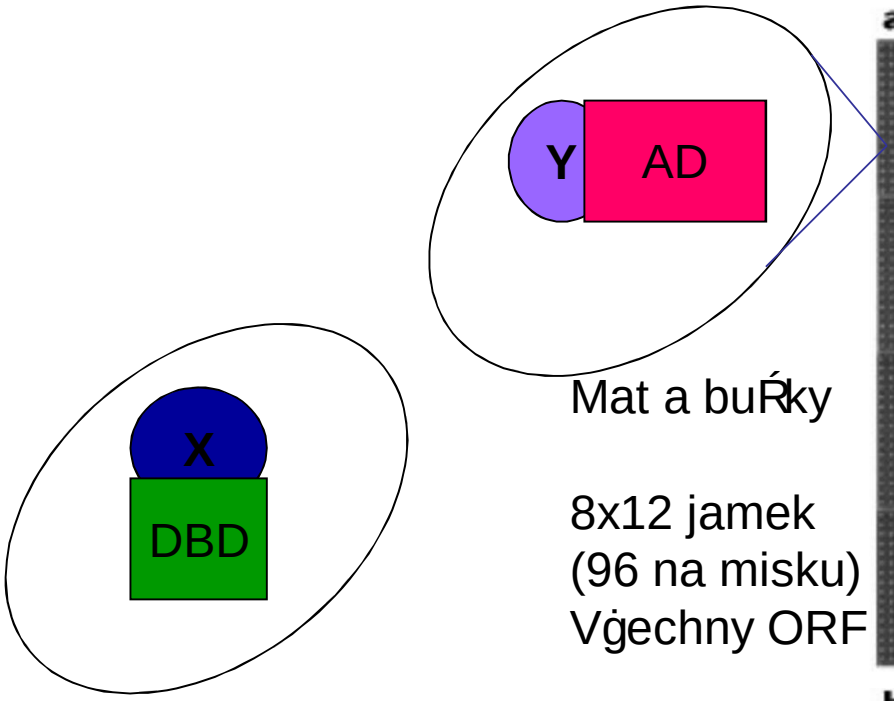
Figure 2. The two-hybrid principle. The DNA-BD is amino acids 1–147 of the yeast GAL4 protein, which binds to the GAL UAS upstream of the reporter genes. The AD is amino acids 768–881 of the GAL4 protein and functions as a transcriptional activator.

AH109

MATa, trp1-901, leu2-3, 112, ura3-52, his3-200,
gal4Δ, gal80Δ, LYS2 : : $GAL1_{UAS}$-$GAL1_{TATA}$-HIS3,
$GAL2_{UAS}$-$GAL2_{TATA}$-ADE2,
URA3 : : $MEL1_{UAS}$-$MEL1_{TATA}$-lacZ



MaV203 kmen navíc obsahuje *URA3* reporter gen ï lze tedy selektovat na uracilovou auxotrofii + reversní systém tj. mutanty disruptující interakce (na FOA)

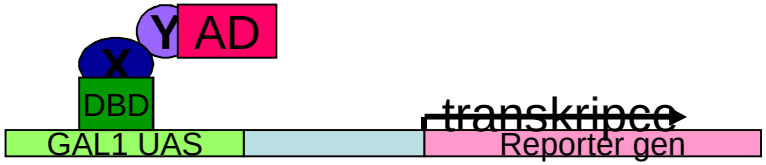


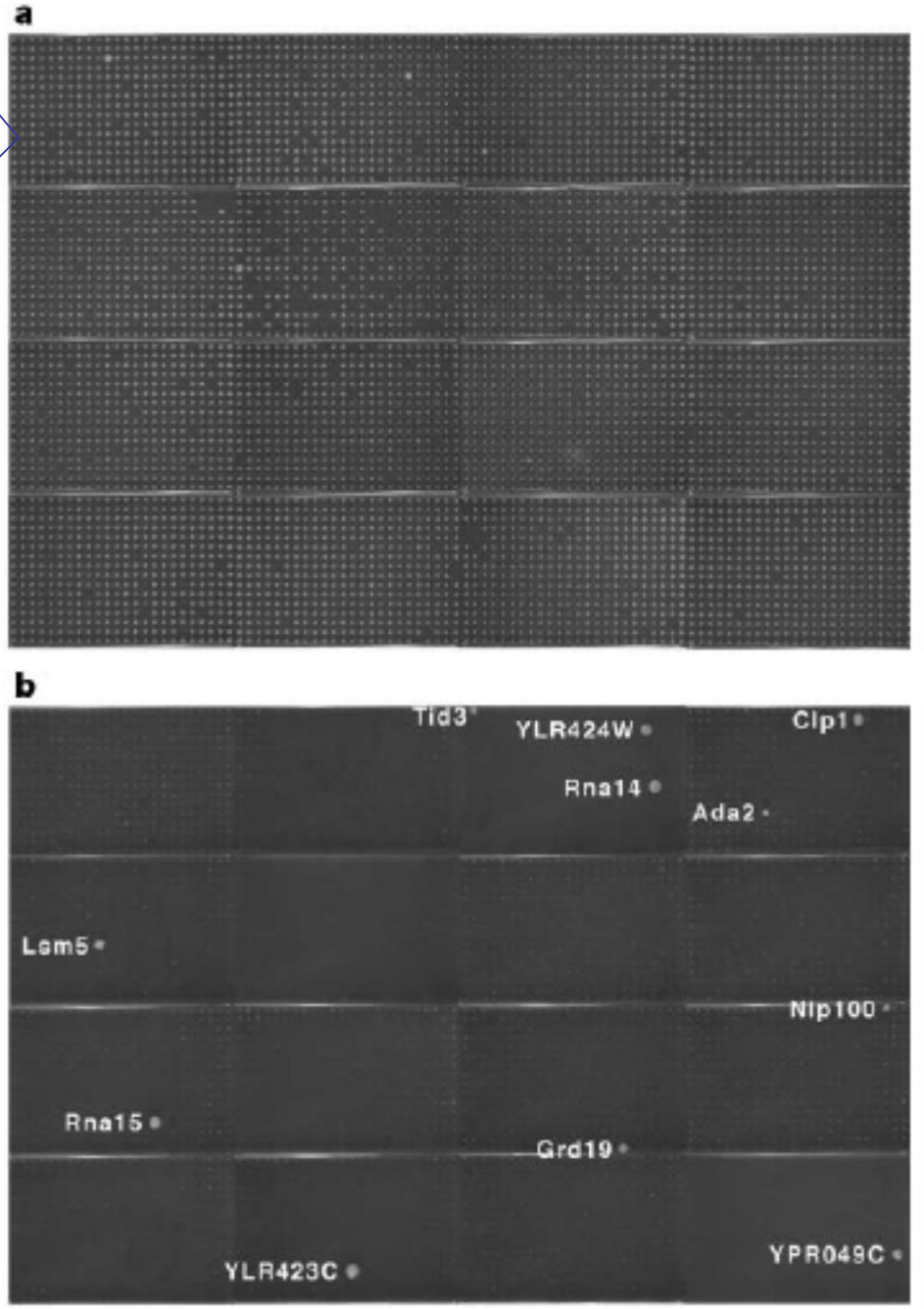
Mat a buŘky

8x12 jamek
(96 na misku)
Vġechny ORF

Mat α buŘky

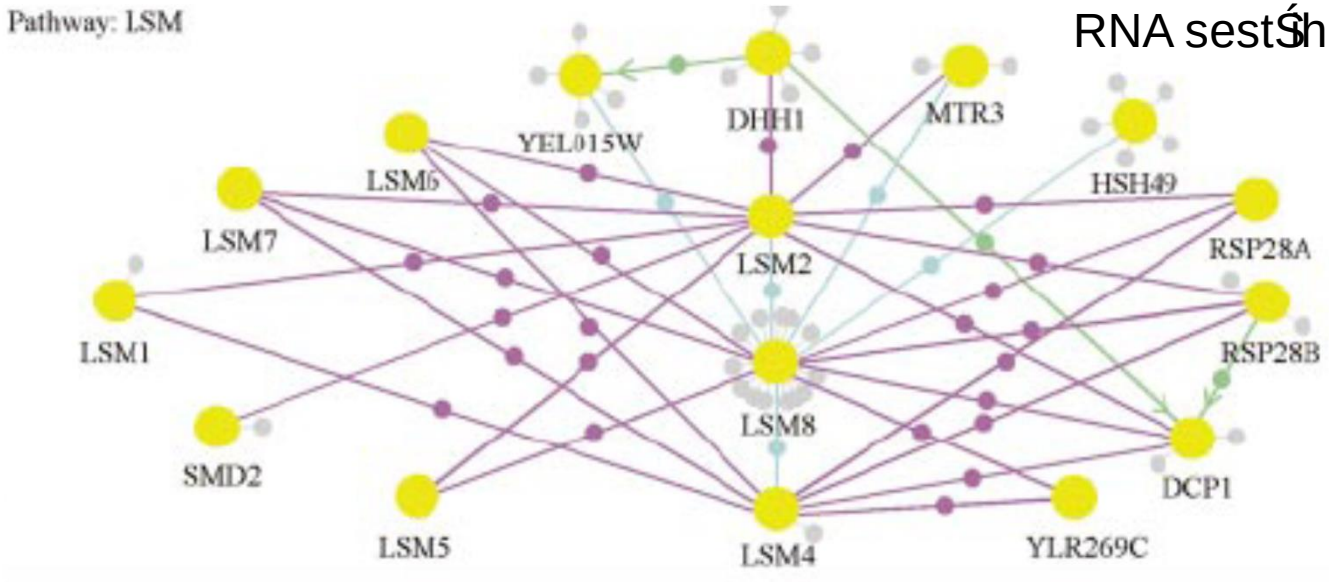
Kvasinkový ĂINTERACTOMEń



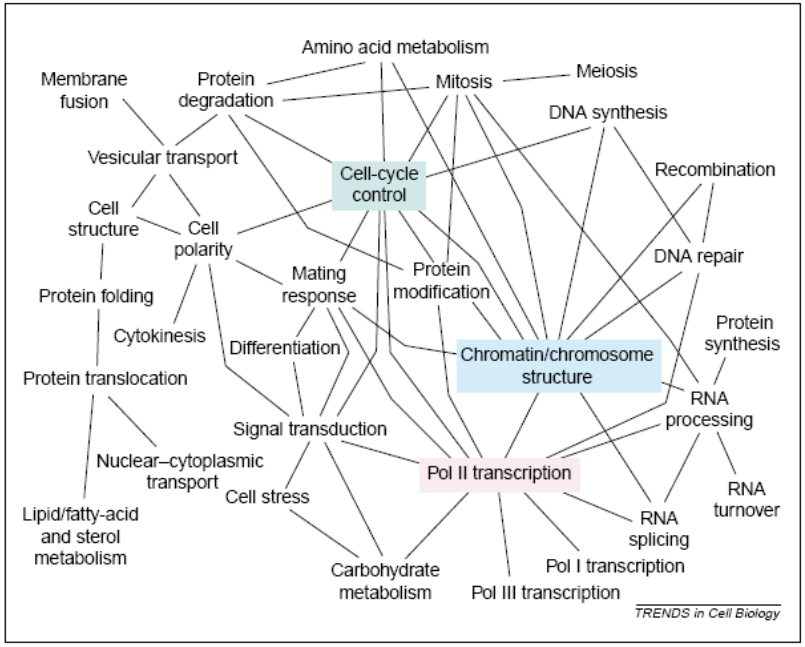


Nature (2000) p. 623

Protein Ănetworksñ



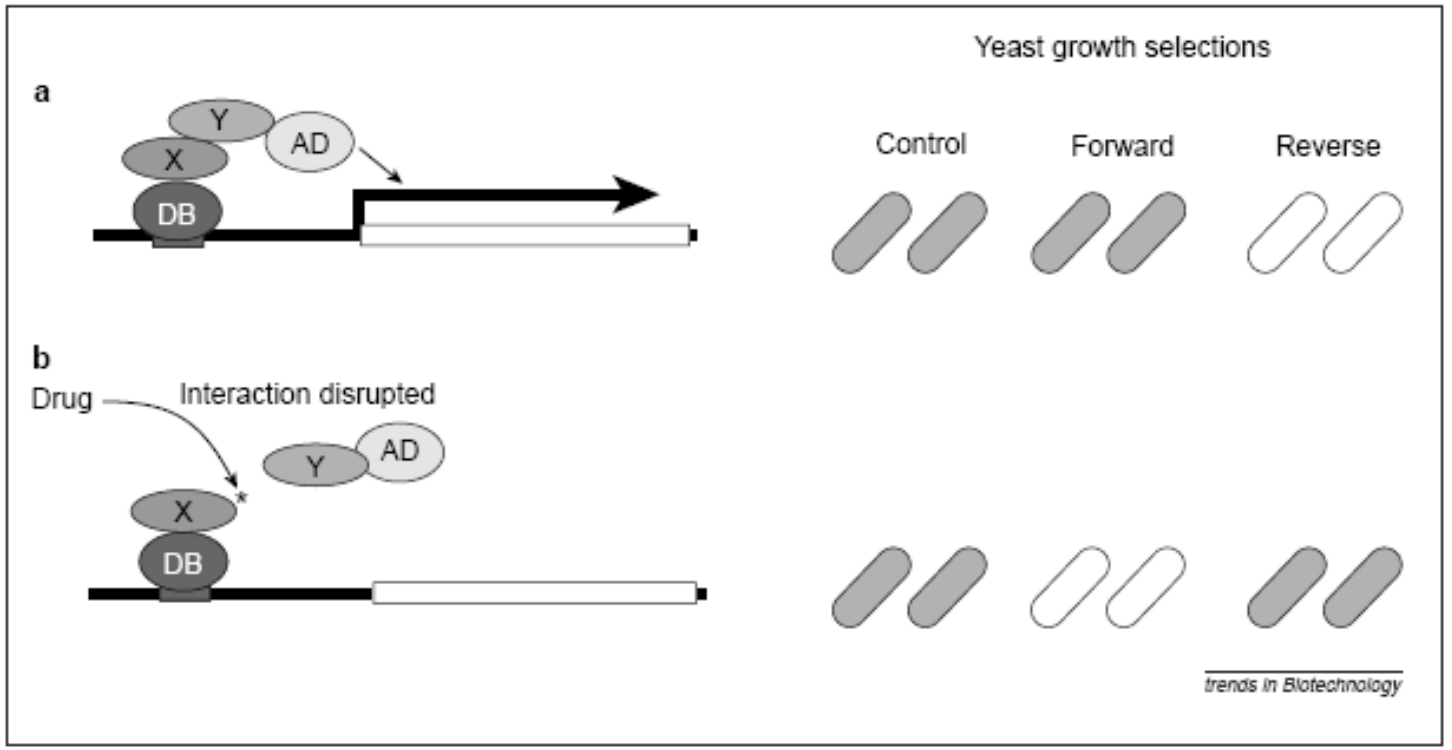
RNA sestŠh



Network/síŠnaznaļ uje funkļ ní vztahy
Tucker et al, TiCB, 2001

- Ănigh-throughputñscreen - interaktom *S. cerevisiae* >30 000 interakcí (~6000 proteinŢ)
- podobný Ănigh-throughputñscreen pro lidské proteiny é

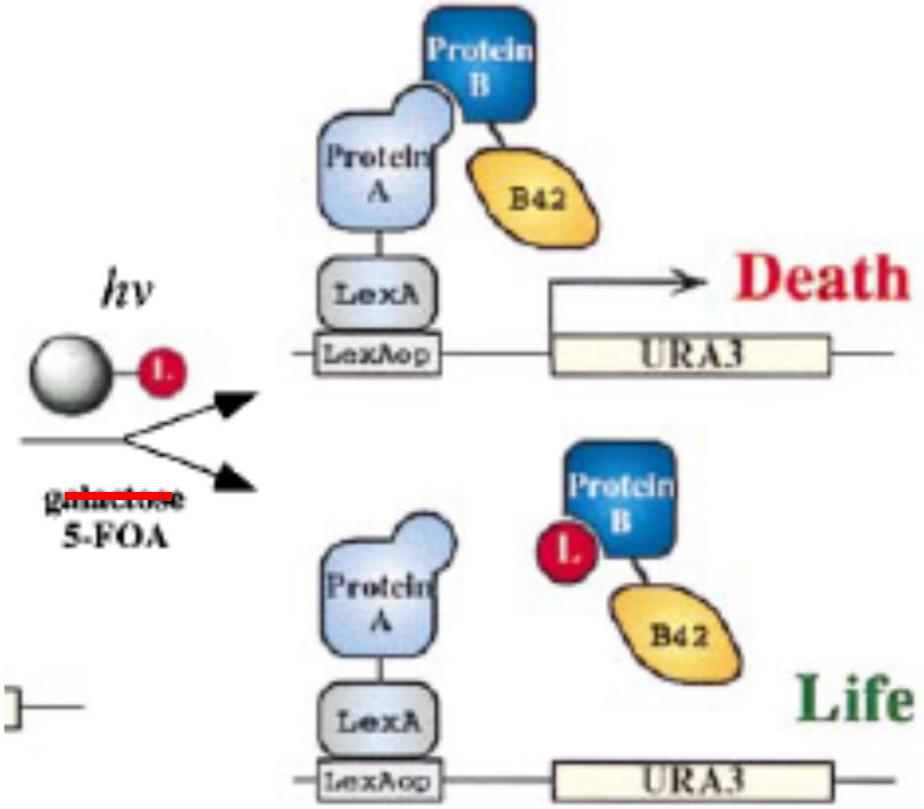
Reversní systém (Y2H)

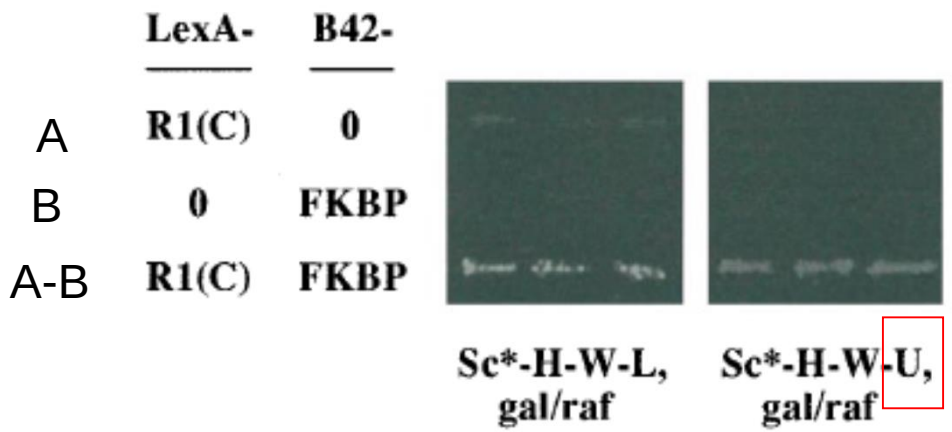


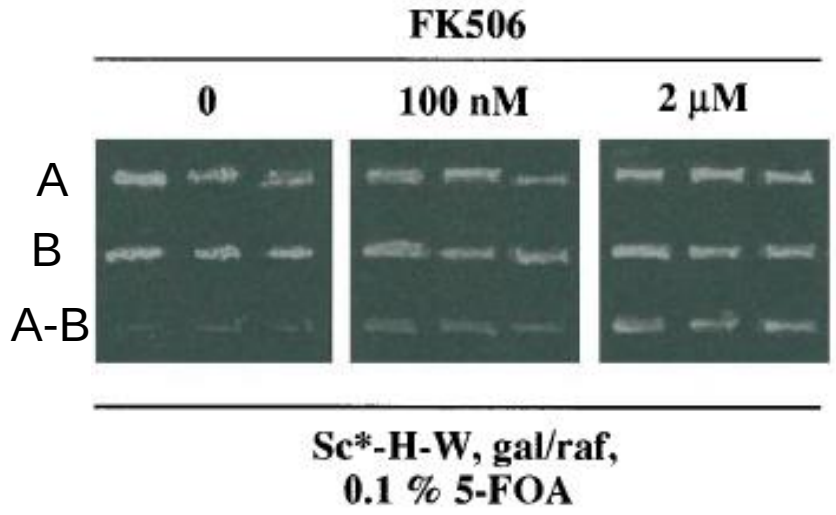
- při použití *URA3* reportéru lze použít toxickou 5-fluoro-orotátovou kyselinu (5-FOA) k negativní selekci tj. interakce povede k záhubě kvasinek, zatímco mutanty neschopné interakce na FOA plotnách porostou (mutanty nebo syntetické látky)

TIBTECH (1999) p. 374

Inhibitory proteinových interakcí







FK506 inhibuje vazbu proteinu FKBP12 na TGFβ-receptor
(ģivotaschopnost na FOA plotnách)

Huang a Schreiber, PNAS, 1997

Split-hybrid systém

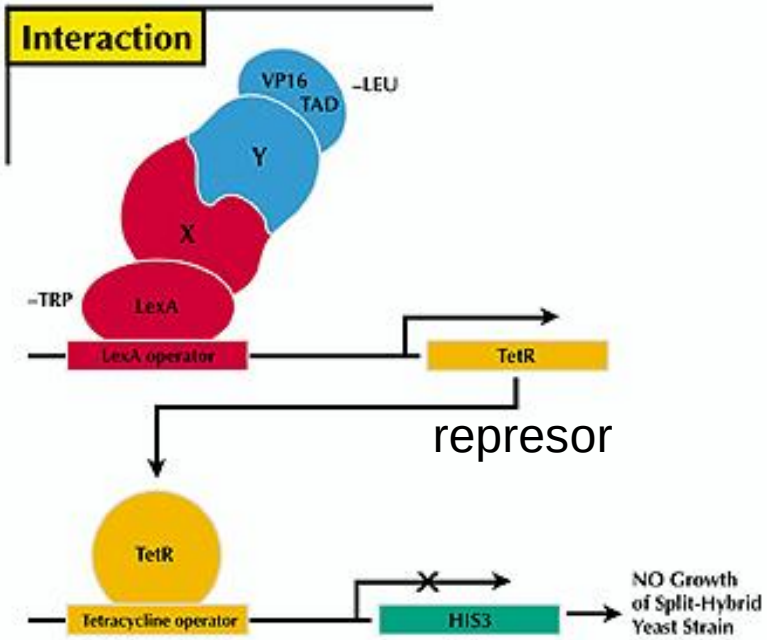


Fig. 1
Protein X is fused to the LexA DNA binding domain and Protein Y is fused to the transcriptional activator domain, VP16-TAD. Interaction between X and Y leads to the expression of the tetracycline repressor protein TetR. TetR expression prevents transcription of the HIS reporter gene making cells unable to grow on media lacking histidine.

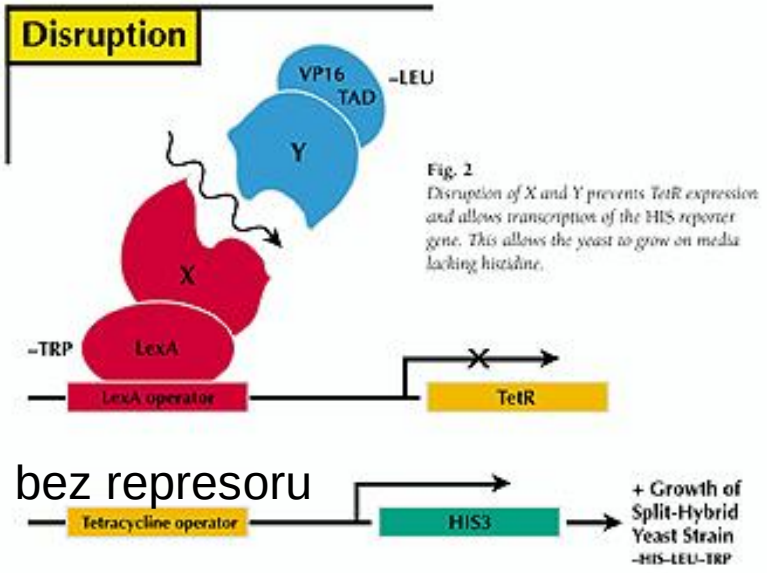
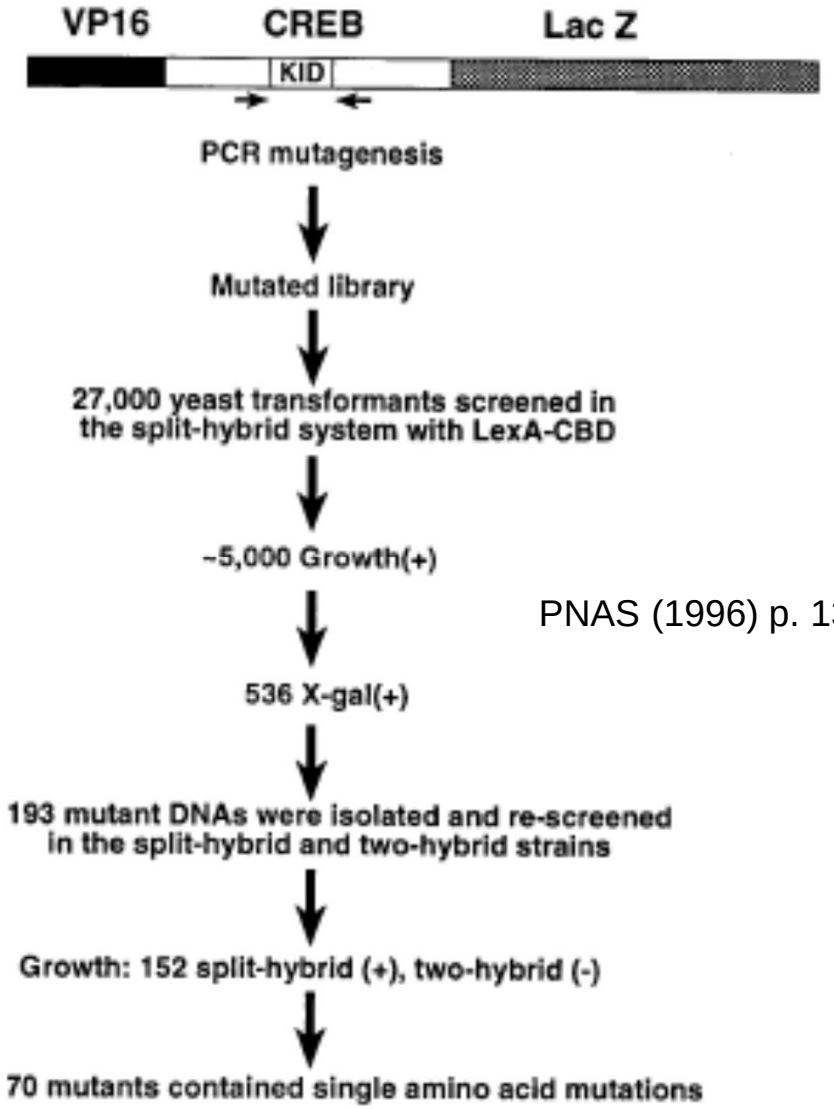
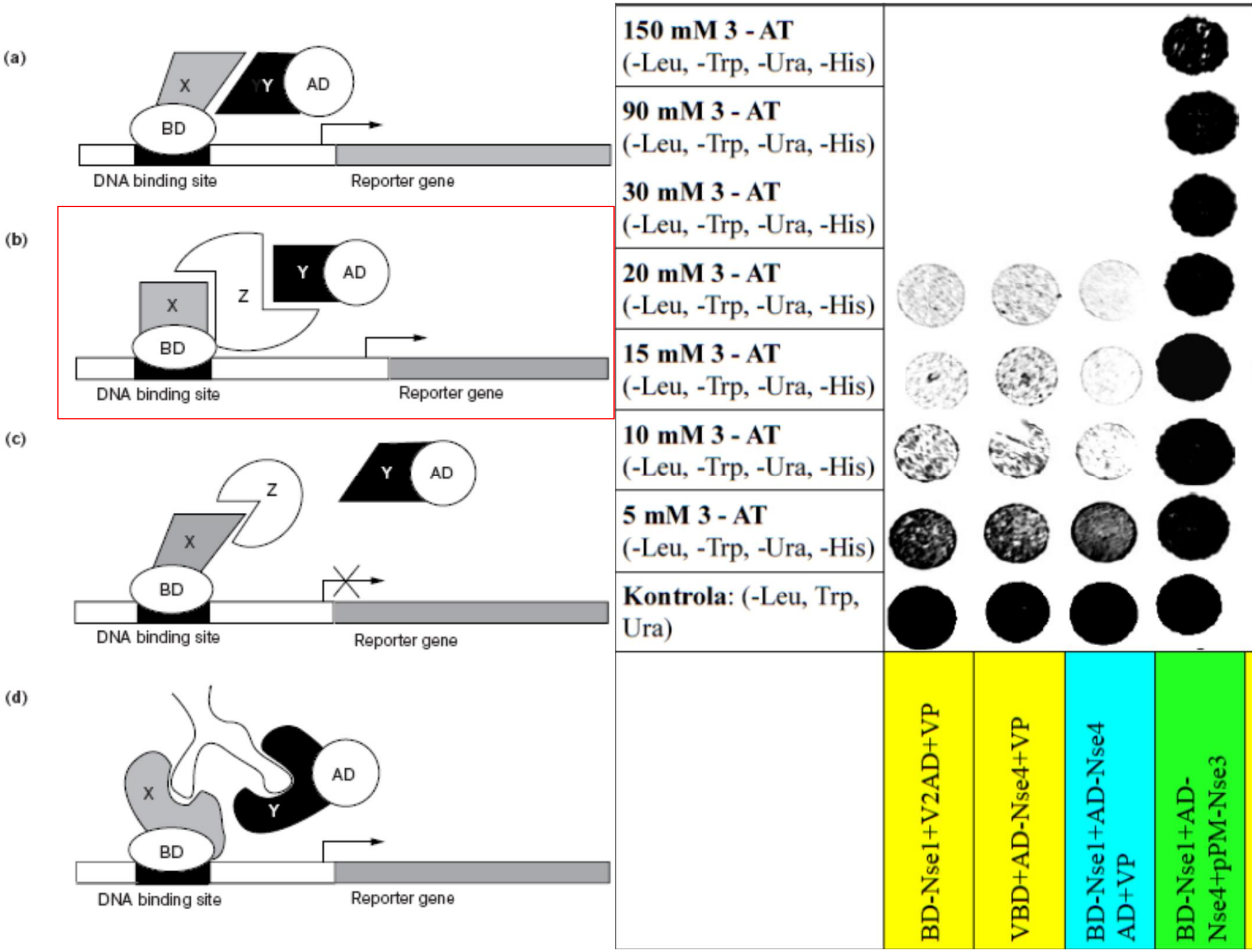


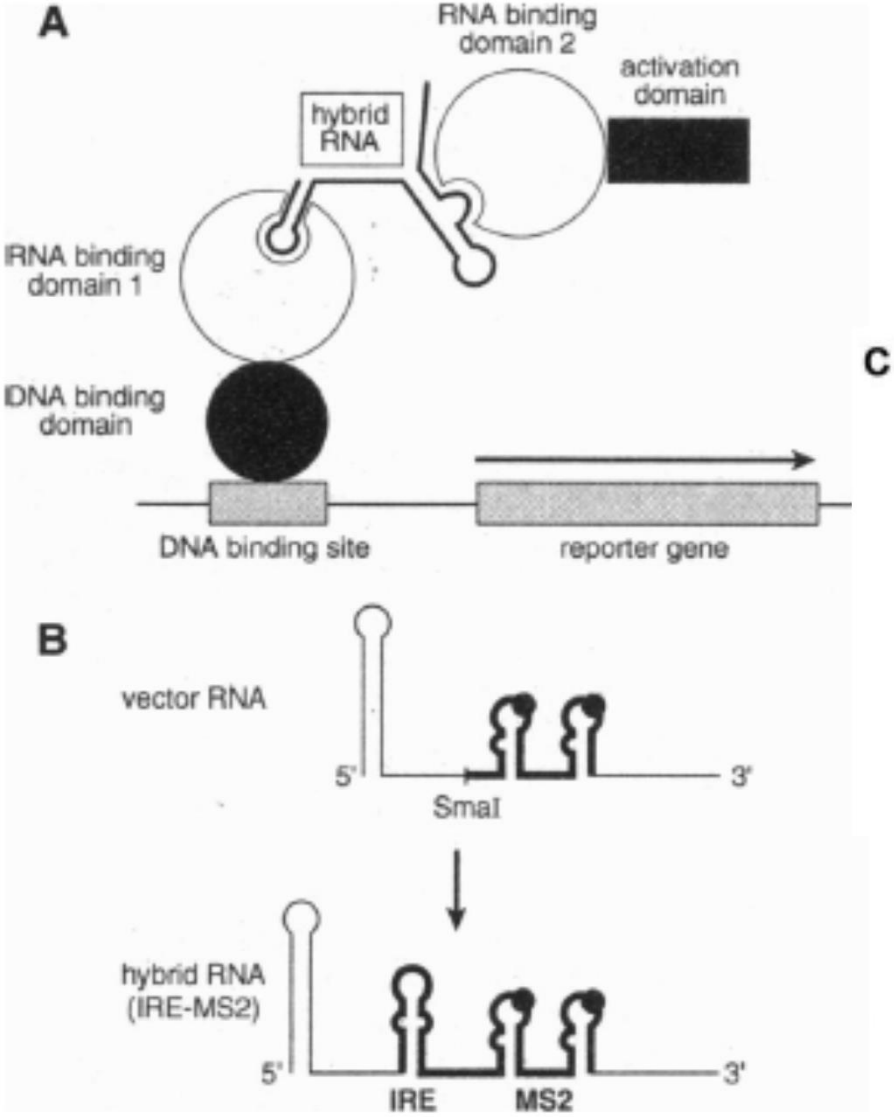
Fig. 2
Disruption of X and Y prevents TetR expression and allows transcription of the HIS reporter gene. This allows the yeast to grow on media lacking histidine.



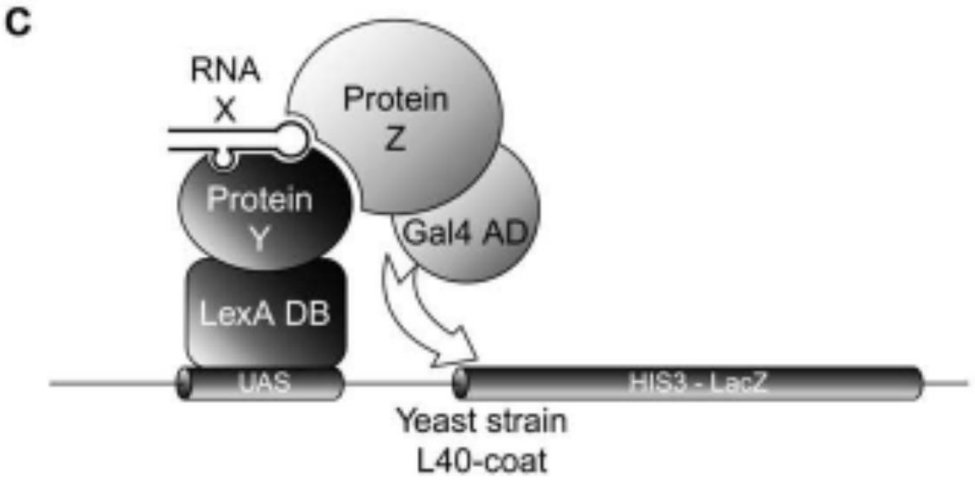
PNAS (1996) p. 13896


(a)
X
Y
AD
BD
DNA binding site
Reporter gene
(b)
Z
Y
AD
X
BD
DNA binding site
Reporter gene
(c)
Z
Y
AD
X
BD
DNA binding site
Reporter gene
(d)
X
AD
Y
BD
DNA binding site
Reporter gene
150 mM 3 - AT (-Leu, -Trp, -Ura, -His)
90 mM 3 - AT (-Leu, -Trp, -Ura, -His)
30 mM 3 - AT (-Leu, -Trp, -Ura, -His)
20 mM 3 - AT (-Leu, -Trp, -Ura, -His)
15 mM 3 - AT (-Leu, -Trp, -Ura, -His)
10 mM 3 - AT (-Leu, -Trp, -Ura, -His)
5 mM 3 - AT (-Leu, -Trp, -Ura, -His)
Kontrola: (-Leu, Trp, Ura)
BD-Nse1+V2AD+VP
VBD+AD-Nse4+VP
BD-Nse1+AD-Nse4 AD+VP
BD-Nse1+AD-Nse4+pPM-Nse3

Analýza vazby protein-RNA (Y3H)



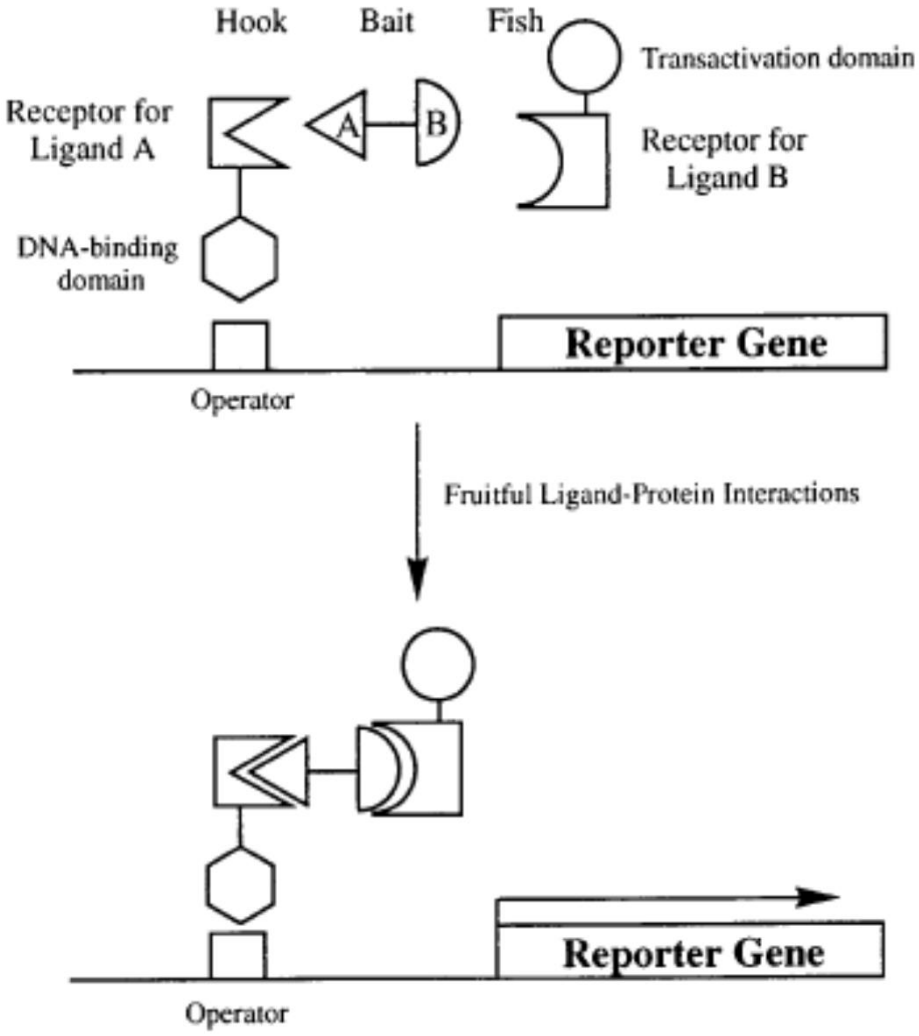
PNAS (1996) p. 8496

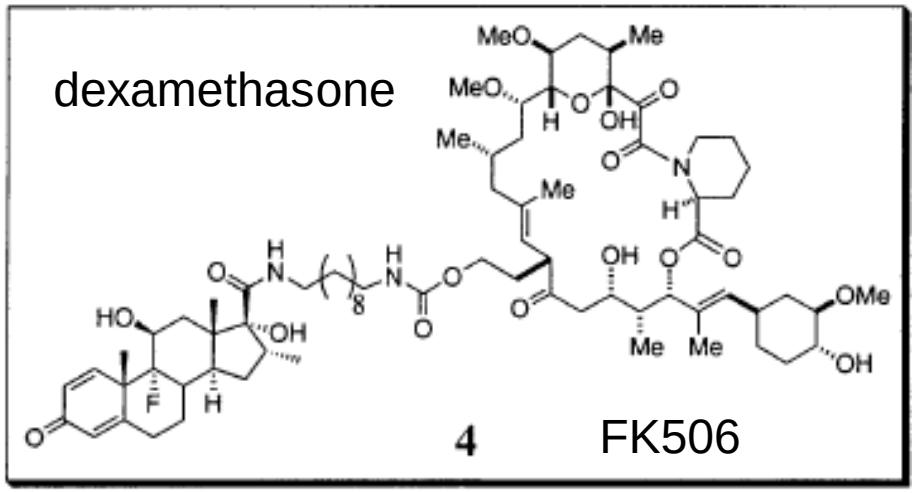


FEBS letters (2004) p. 7

Vazba ligand-receptor (Y3H)

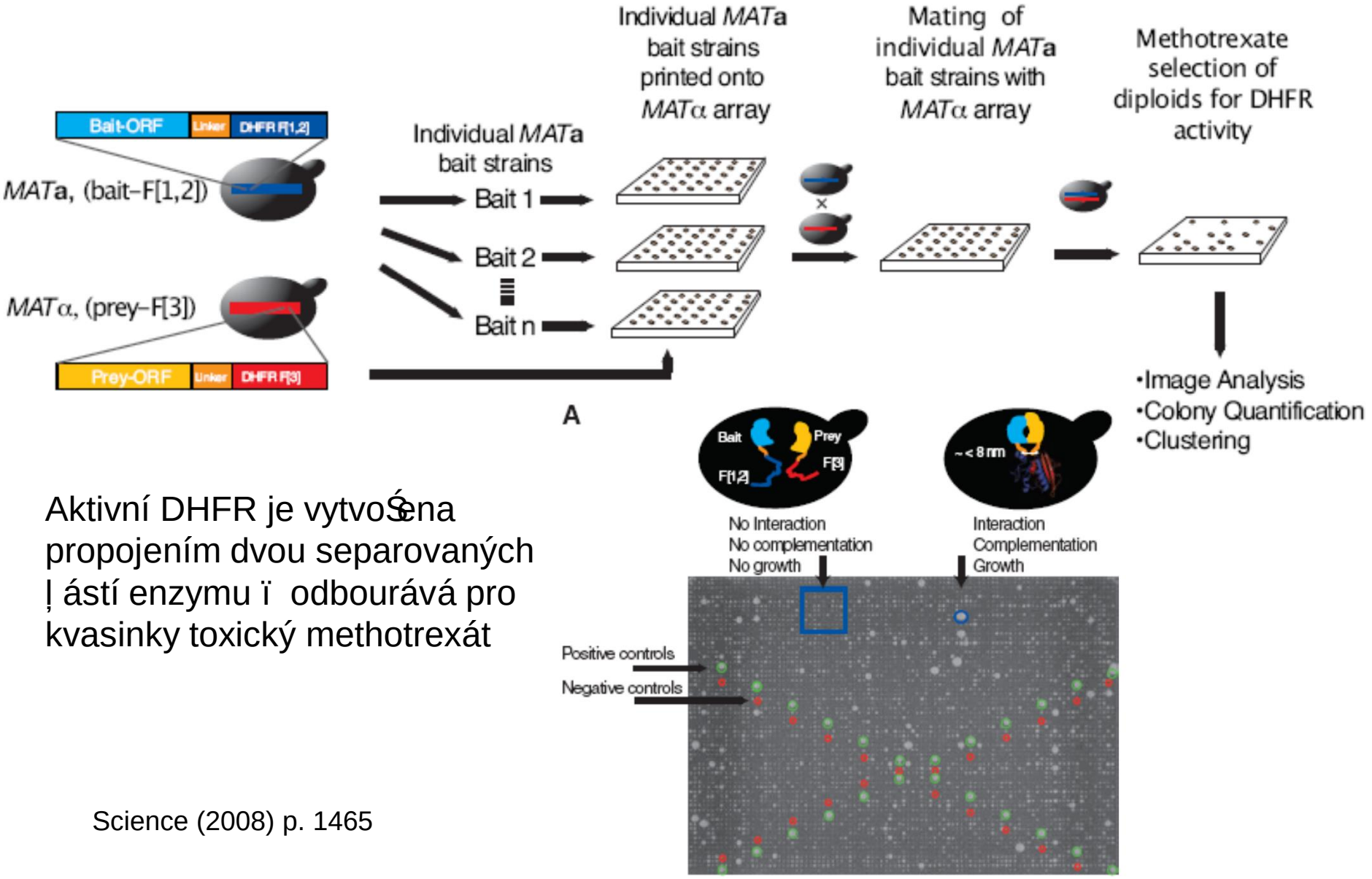
glucocorticoid receptor - FKBP12





PNAS (1996) p. 12817

Dihydrofolát reduktása/methotrexát

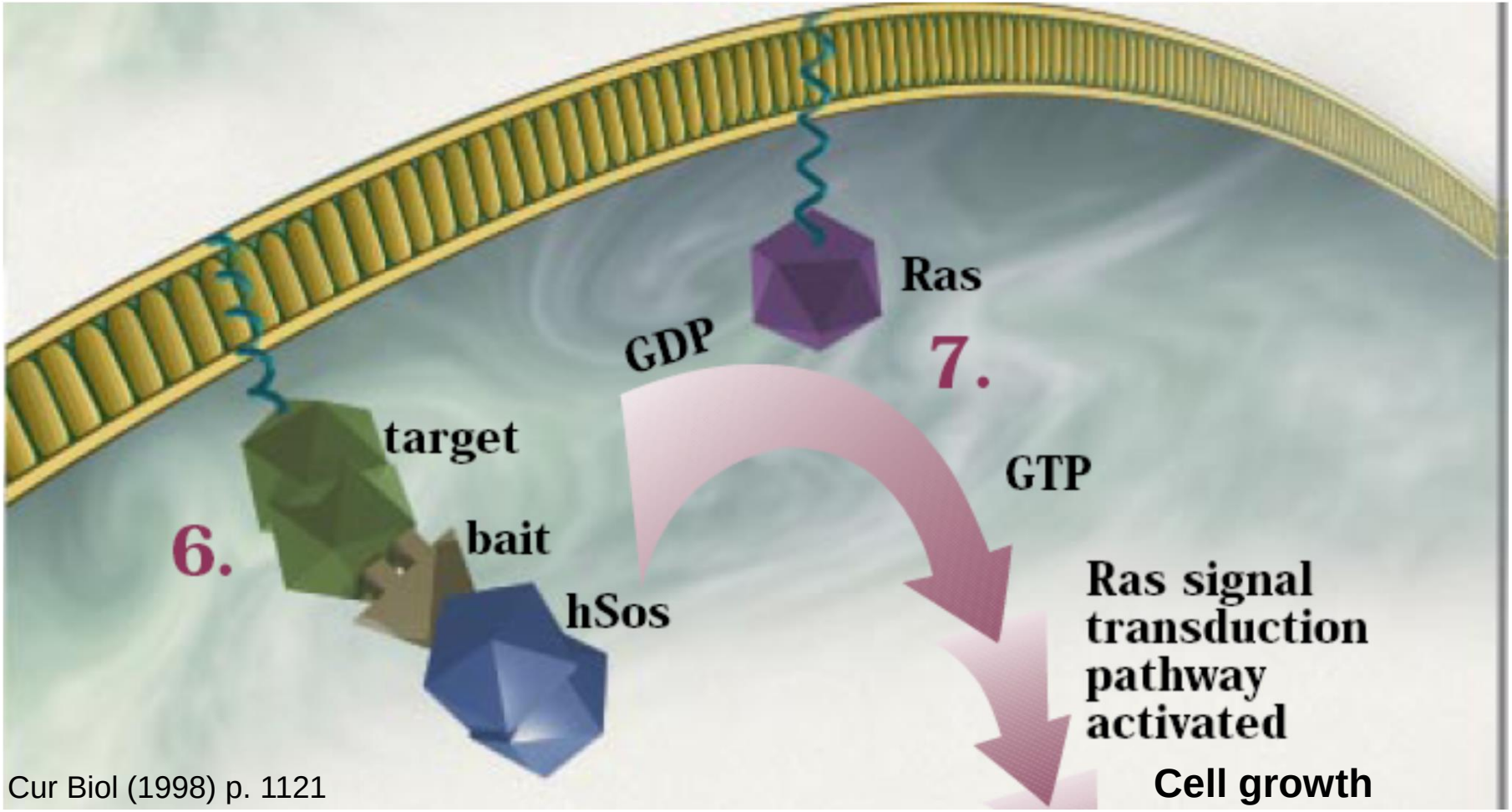


Aktivní DHFR je vytvoŚena propojením dvou separovaných ļ ástí enzymu ï odbourává pro kvasinky toxický methotrexát

Science (2008) p. 1465

CytoTrap 2-hybridní systém

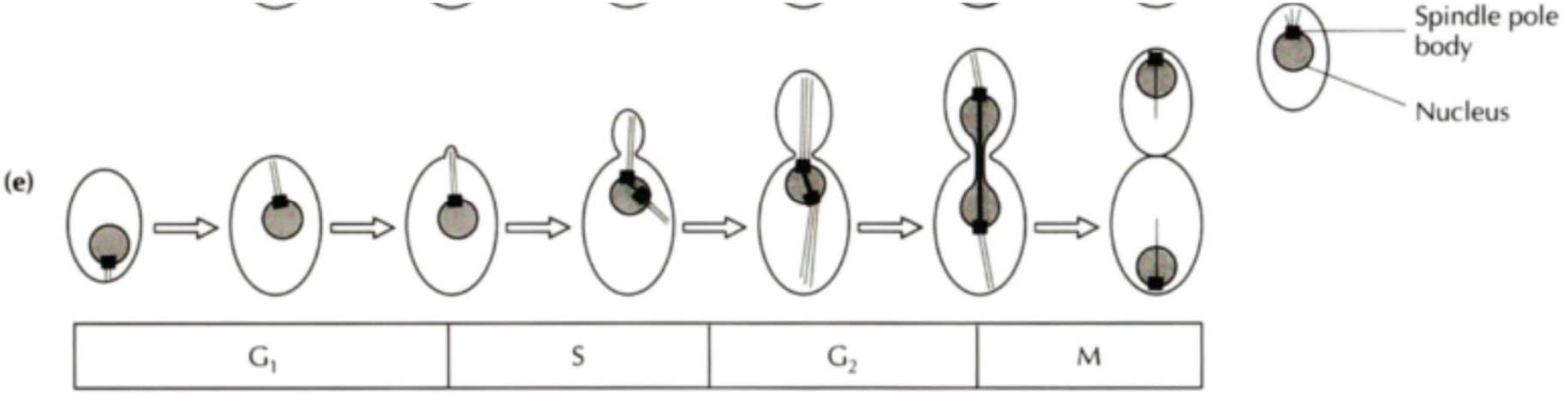
Kvasinkový *cdc25-2* ts mutant - hSOS (guanine exchange factor) aktivuje RAS pokud je ukotven na membránu v jeho blízkosti
- jeden partner je myristylován a ukotven na membránu a druhý (interakční) partner je fuzován k hSOS



Cur Biol (1998) p. 1121

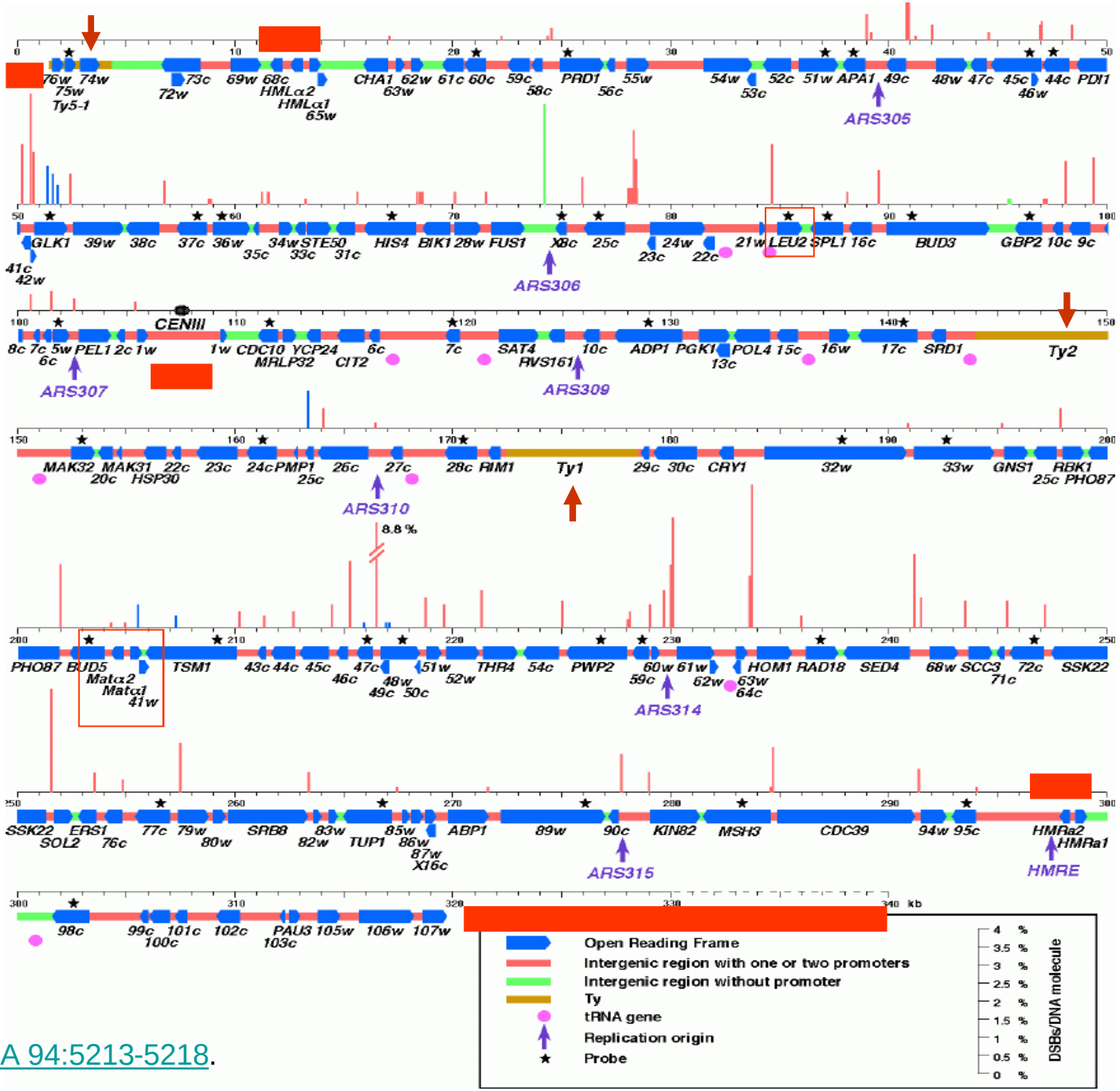
Základní prvky kvasinkového genomu *Saccharomyces cerevisiae* (vs *S.p.*)

- haploidní genom - 12Mbp, 16 chromosomŢ (chrI=0.22 ï chrXII=1.6Mbp)
- délka chromosomu XII se u rŢzných *S.c.* liġí dle poļ tu (aġ 200) kopii rDNA v repetici (9kbp), 262 tRNA, 40 snRNA,
- Krátké centromery a ARS (100bp)
- Geny (cca 6500) reprezentují 75% celkové sekvence (kompaktní)
- Redundantní (2000 genŢ duplikováno) ï cca30% genomu vzniklo duplikacemi
- <5% genŢ (220) obsahuje introny (0.5% genomu),
- 3% Ty1-5 transposony (46% u ļ lovĎka)
- Kondenzovaný/tichý heterochromatin: centromery, telomery a HMR/HML



Chromosom III
CEN=centromera
ARS=autosomal replicating sequence
TEL=telomery
tRNA
Ty transposony
MAT a HML/HMR lokusy

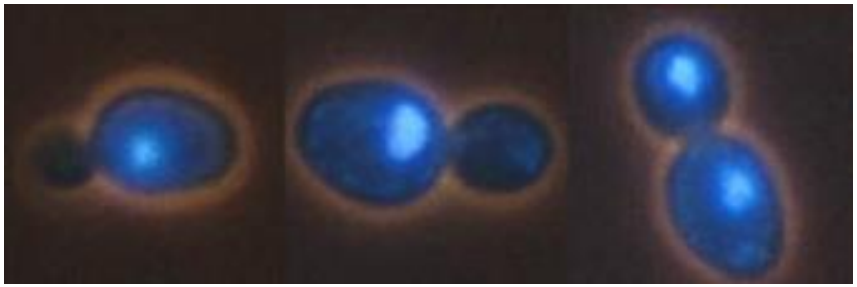
Heterochromatin:
centromera
telomery
HMR a HML
(MAT je aktivní
urļ uje haplotyp)



Proc. Natl. Acad. Sci. USA 94:5213-5218.

Pozorování DNA/chromosomŢ u kvasinek

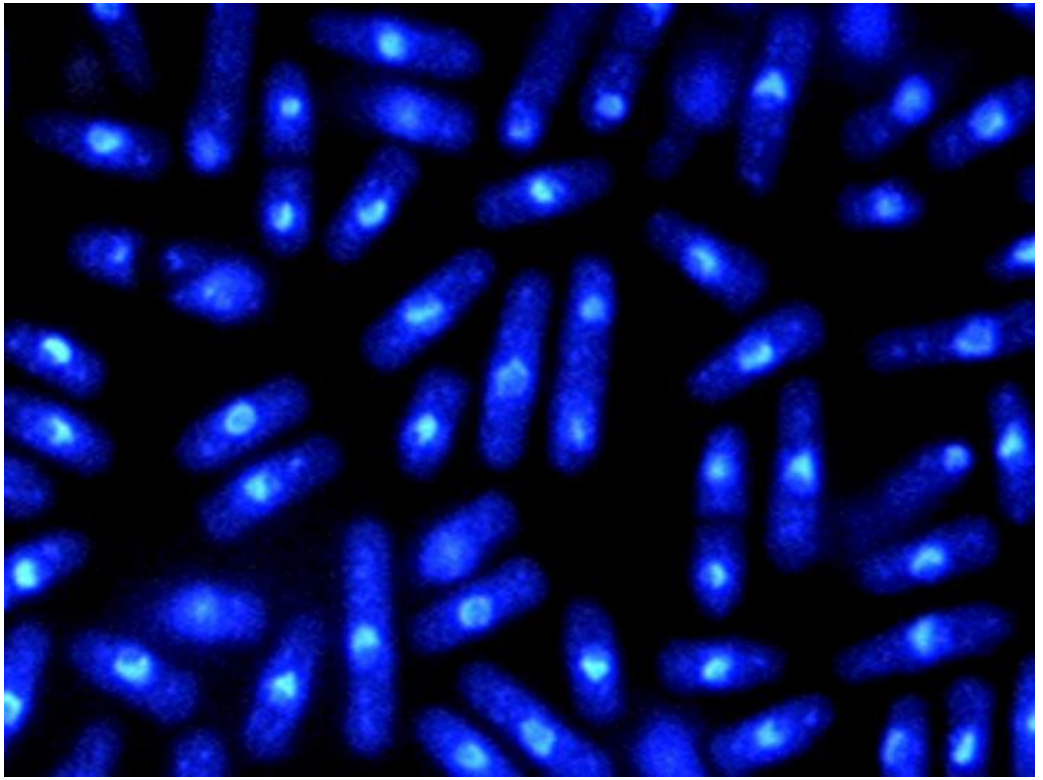
- Chromosomy jsou u kvasinek malé a tŊžko pozorovatelné ï barvení DNA na fixovaných preparátech pomocí DAPI (4´,6-diamidino-2-phenylindole)
- Použití fŢzních proteinŢ-GFP (green fluorescence protein) pro studium dynamiky chromatinu (H2A, kinetochora-centromera)
- TetR-GFP represor se váže na TetO sekvence (operon) zaintegrované v přesnŊ definovaném lokusu
- ChIP (chromatin immune precipitation) ï specifické sekvence, ChIP-seq nebo ŘChIP on CHIPř



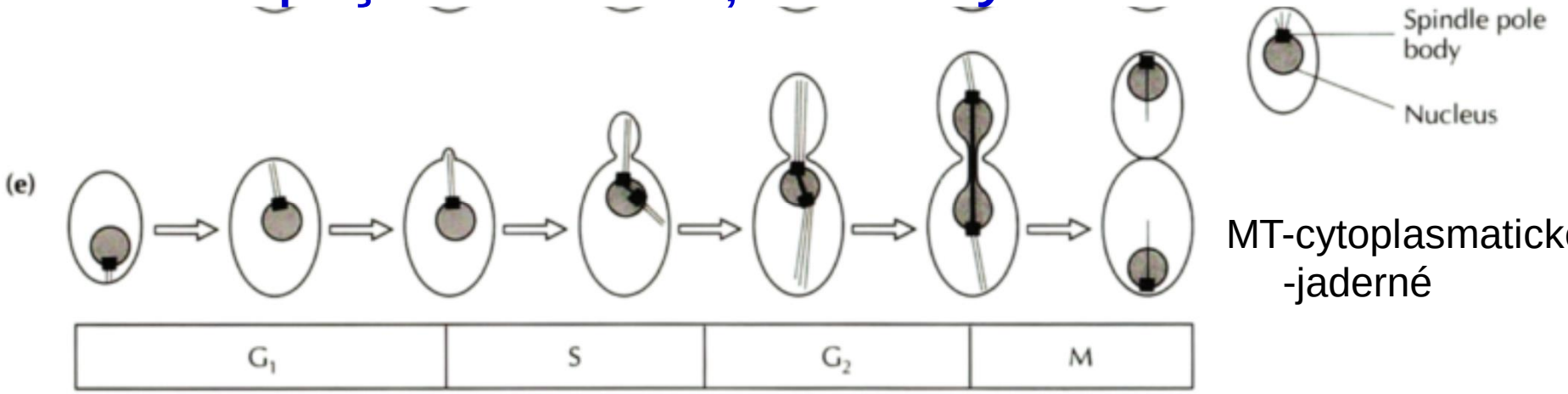
Saccharomyces cerevisiae

Schizosaccharomyces pombe →

Pozadí = mtDNA



DNA v prŢbŊhu bunŊ ného cyklu *S.cerevisiae*

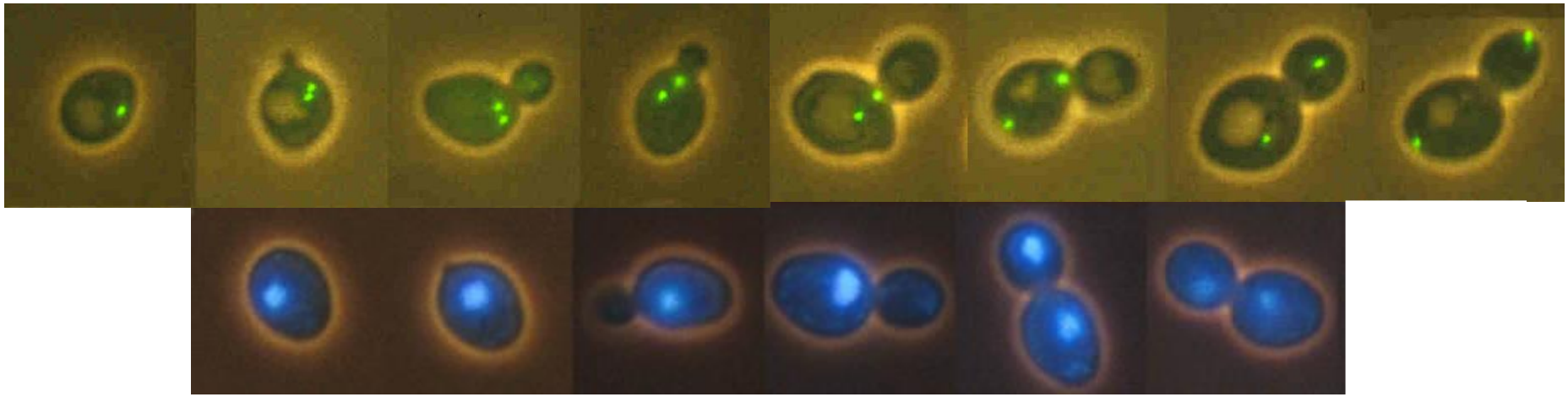


MT-cytoplasmatické -jaderné

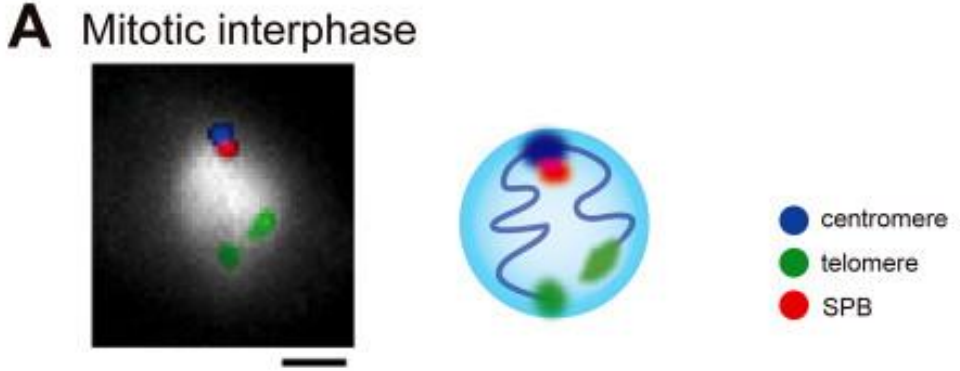
- zahájení tvorby pupene a duplikace SPB ï zaļ átek S fáze tj. replikace
- rozchod jaderných plakŢ na opaļ né póly ï pŚechod z S do G2 fáze
- jádro se protahuje ï zaļ átek M fáze (u kvasinek se jaderná membrána nerozpadá)
- na zaļ átku anafáze dochází k oddŊlení sesterských chromatid a jejich segregaci

Marco et al, Cell, 2013

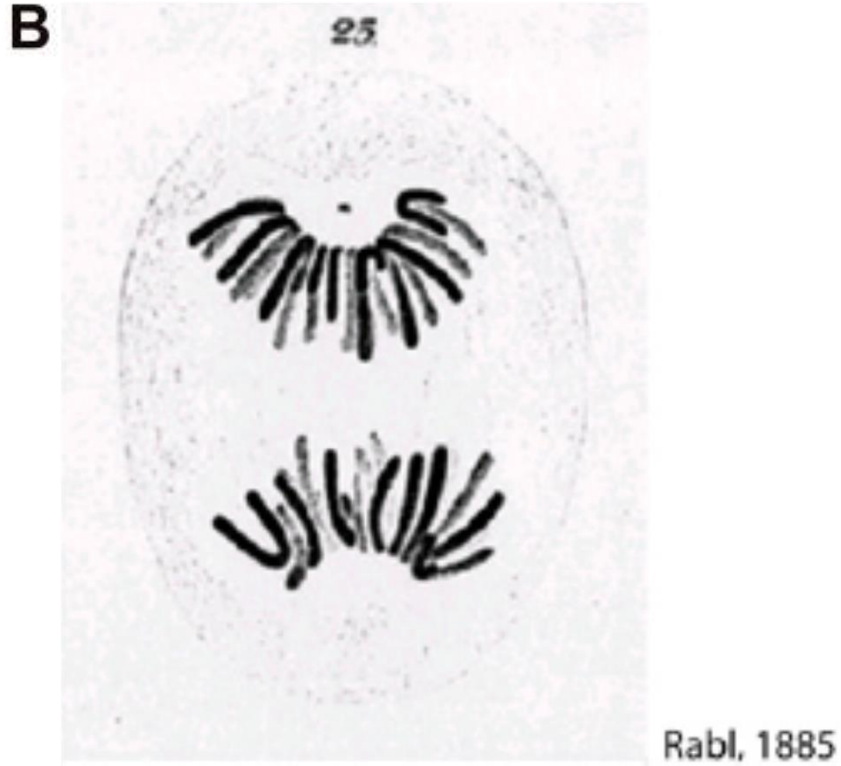
SPB-GFP barvení

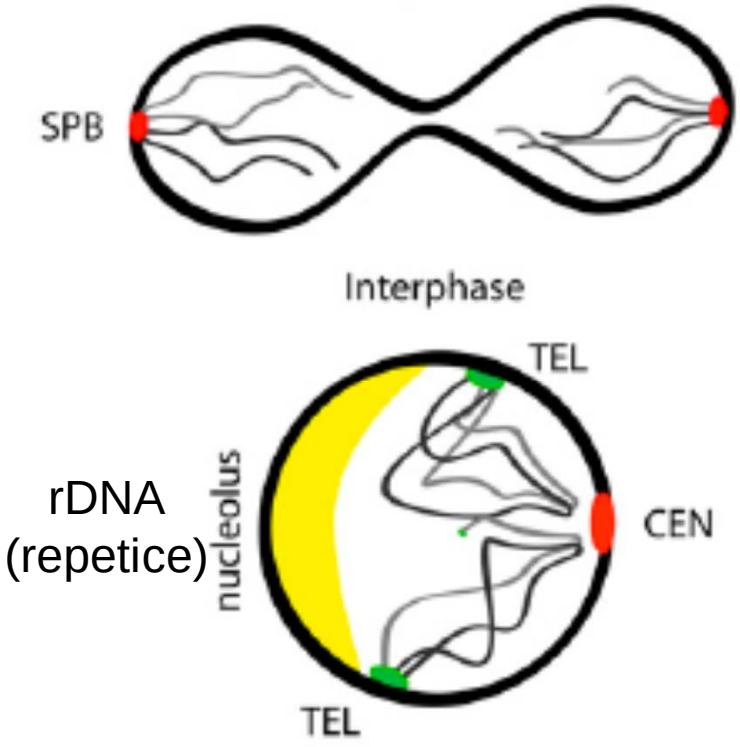


Orientace chromosomŢ v mitotickém jádŚe



FISH ï fluorescence *in situ* hybridization (1992)



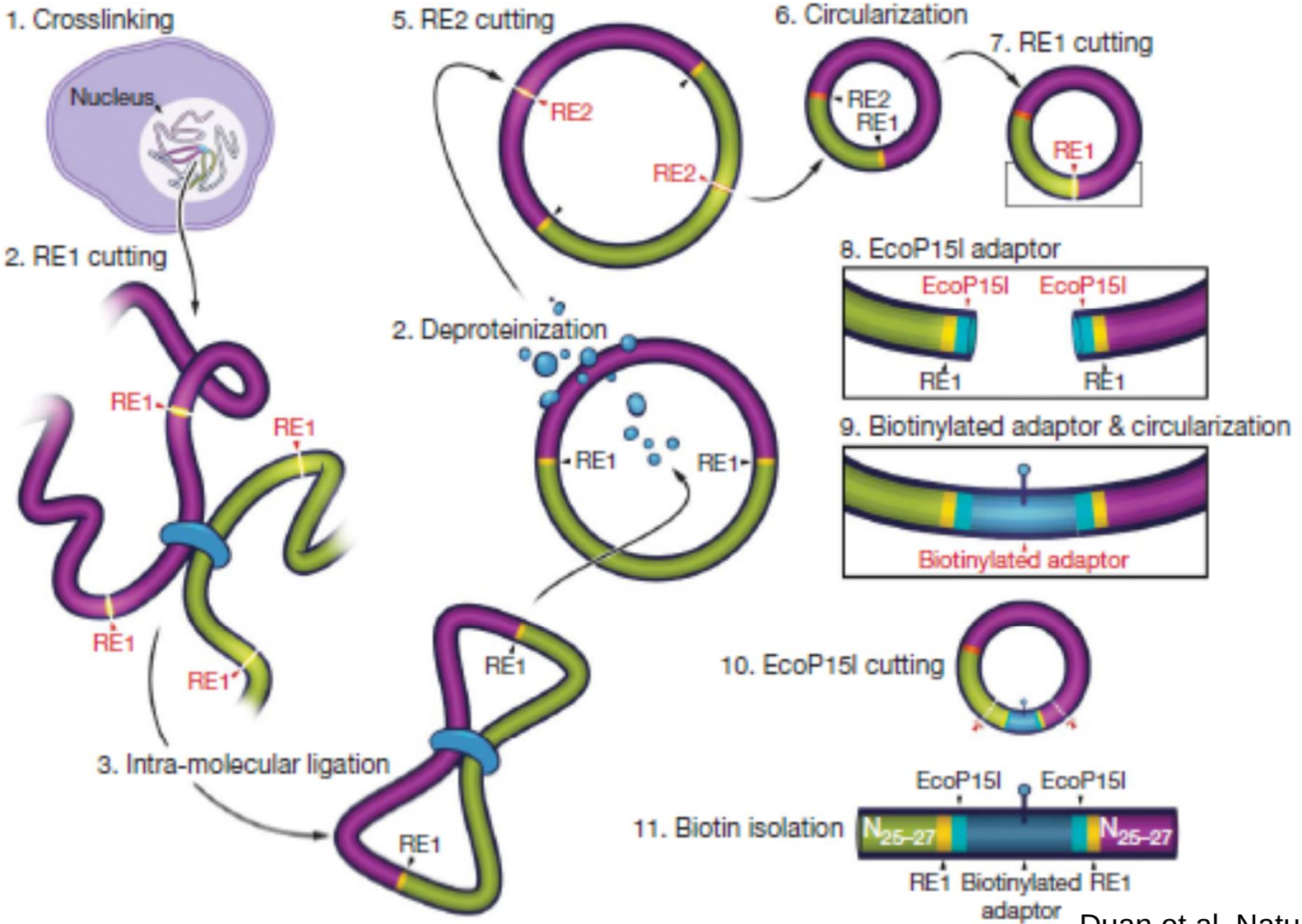


rDNA (repetice)

Tadei a Gasser, Genetics, 2012

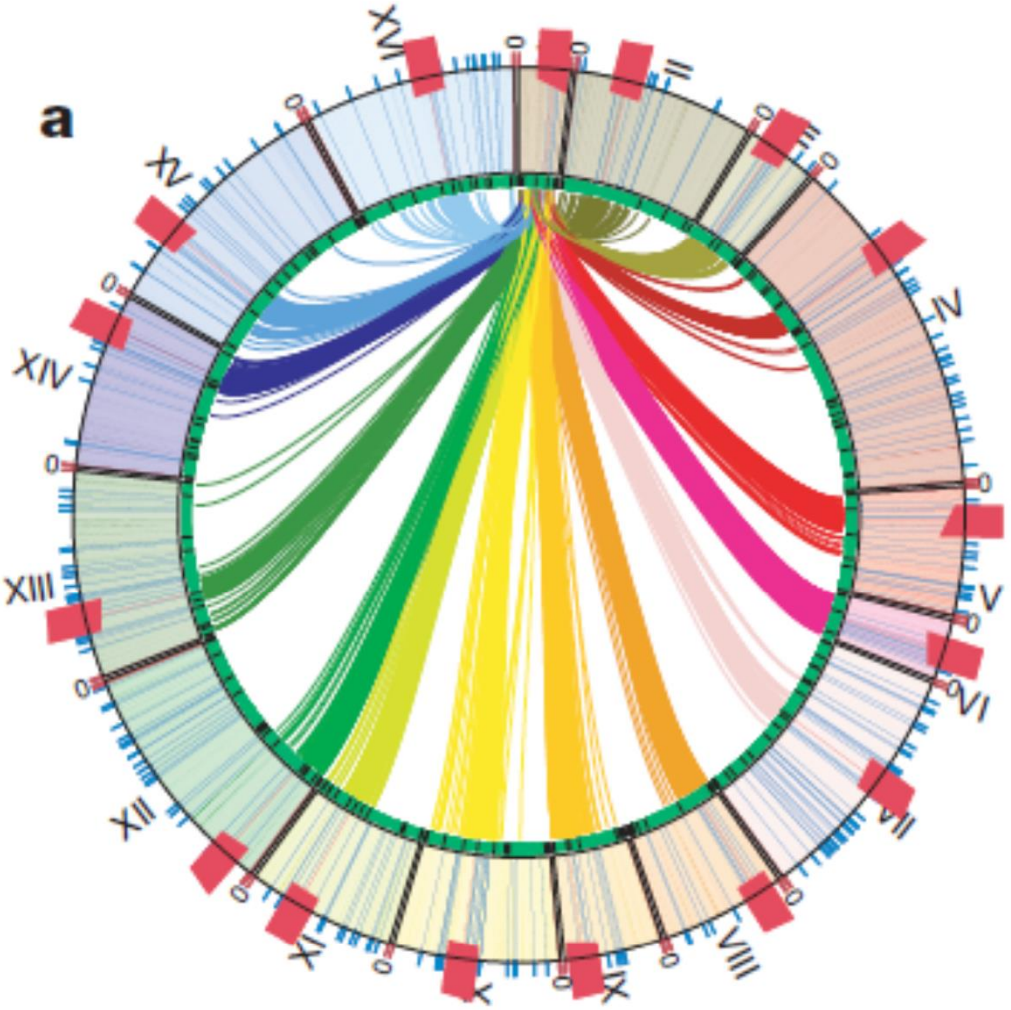
3D organizace chromosomŢ v *S.c.*

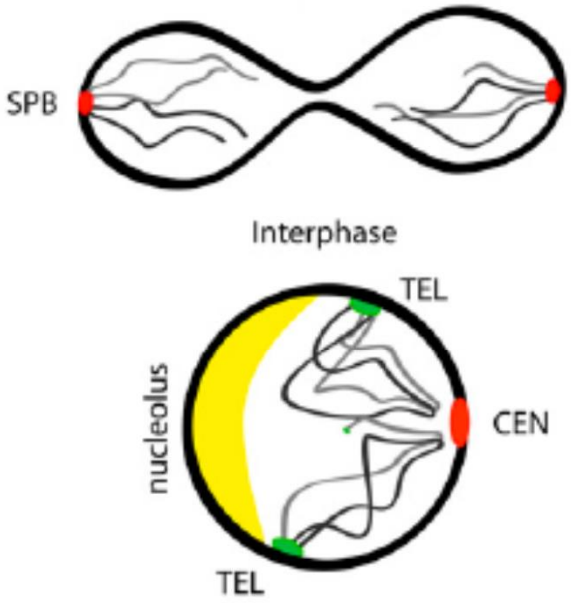
3C ï chromosome conformation capture



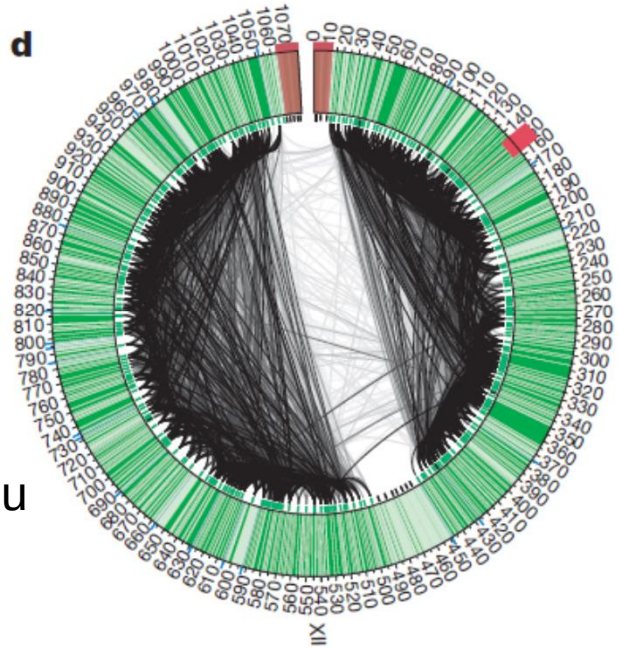
Duan et al, Nature, 2010

Vġechny centromery jsou blízko sebe





Chromosom XII



rDNA je mimo jádro ï v jadérku

Duan a spol, Nature, 2010