

SYLlCa přednáška

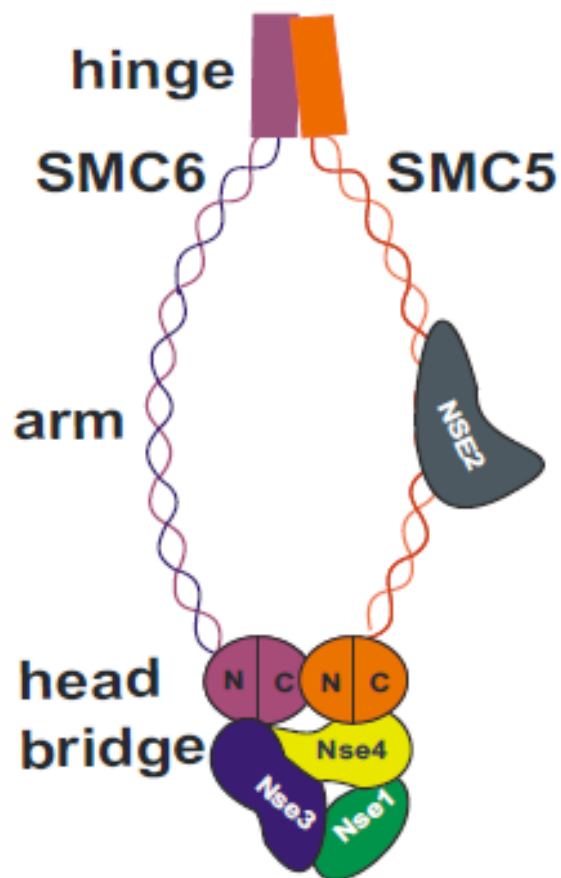
Johanne M. Murray

(Sussex University, Genome Damage and Stability Centre)

SMC5-6 complex and replication stress

24.4. v 10.00 – A11, 205

Povinná přednáška



Metody analýzy proteinových komplexů

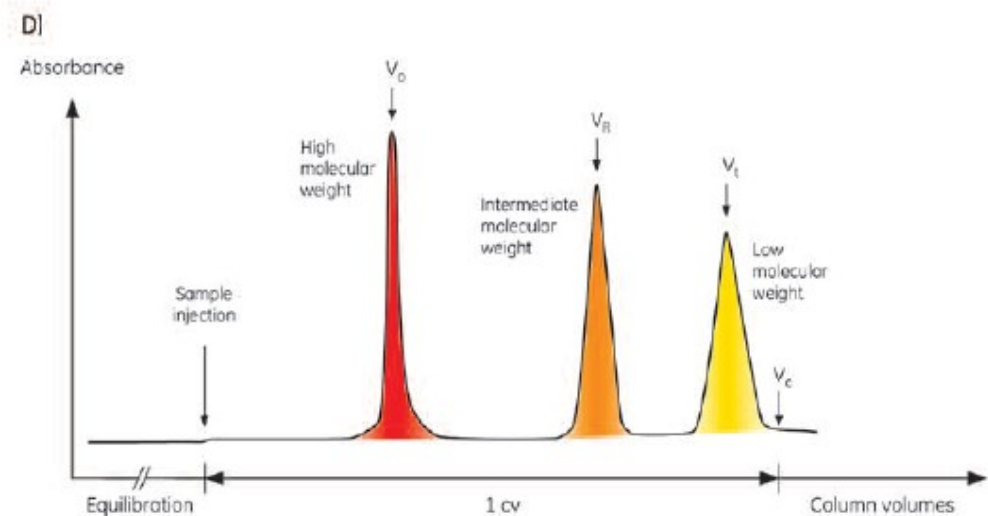
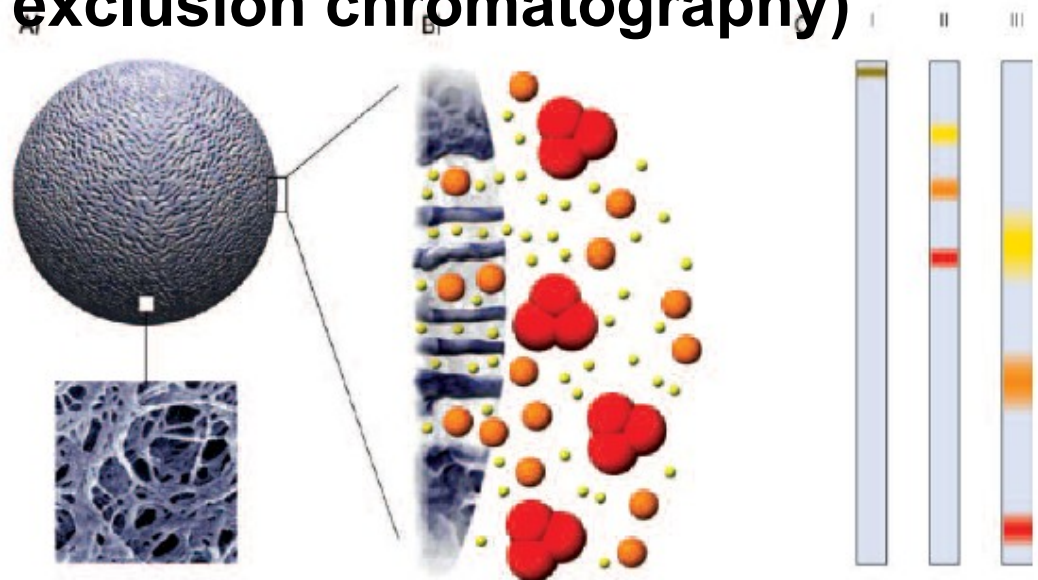
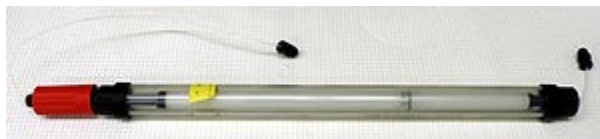
- Gelová filtrace, ultracentrifugace
- TAP-tag (a jiné tagy) purifikace a MS analýza

Metody analýzy protein-proteinových interakcí

- ko-imunoprecipitace, pull-down, ko-purifikace ...
- (kvasinkový) dvou-hybridní systém
- BiFC, FRET, ko-lokalizace, ko-exprese
- Flourescenční anisotropie, SPR, ITC ...
- ko-krytalizace, cryoEM ...
- databáze (interactom a komplexy ...)
- genetické metody (syntetická letalita, suprese)

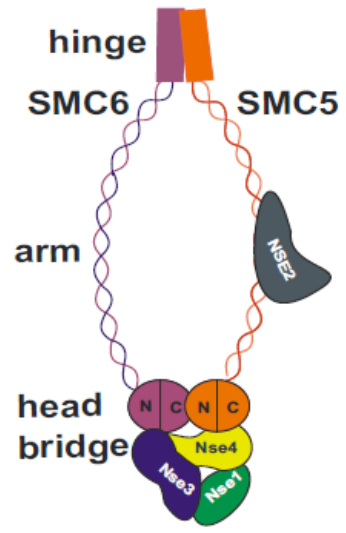
Metody analýzy proteinových komplexů

- Gelová filtrace (size exclusion chromatography)

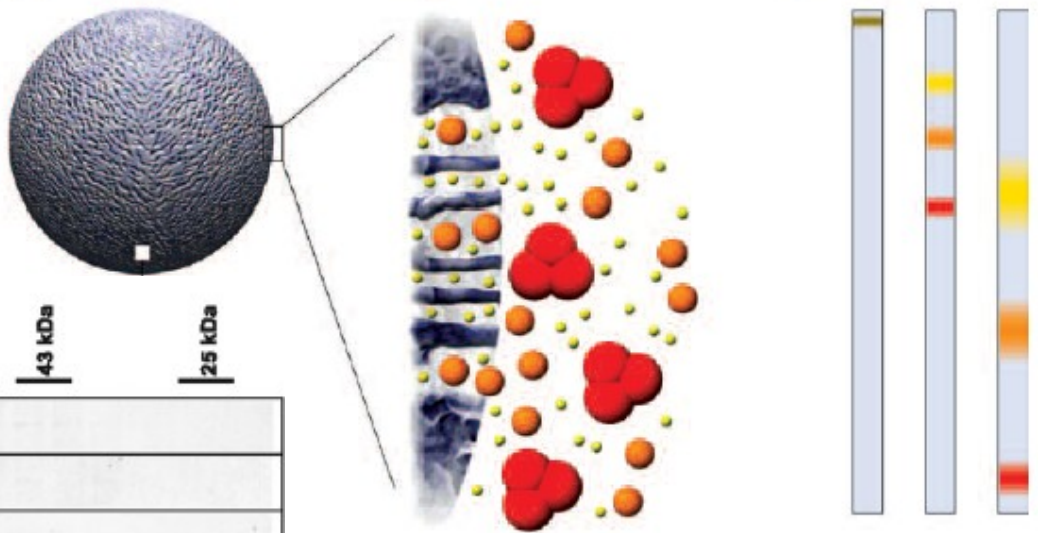


M

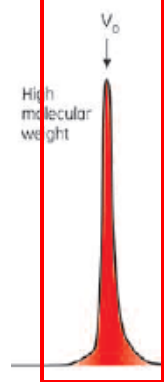
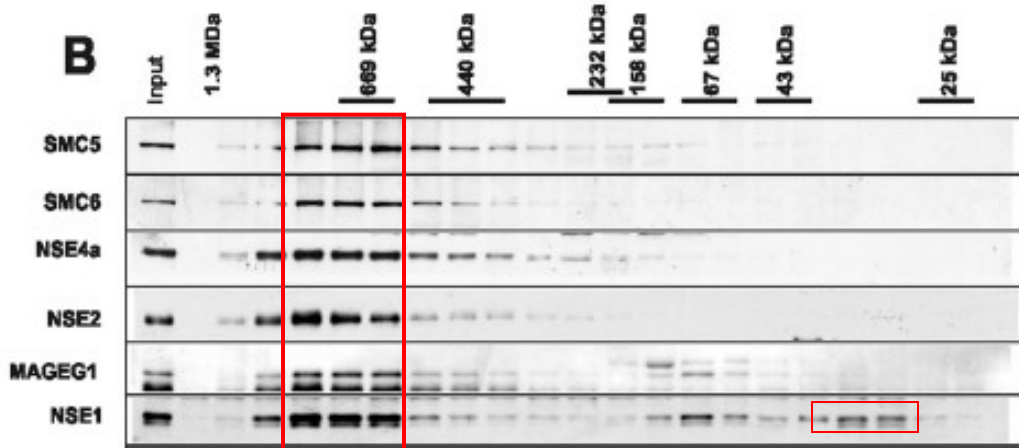
- G



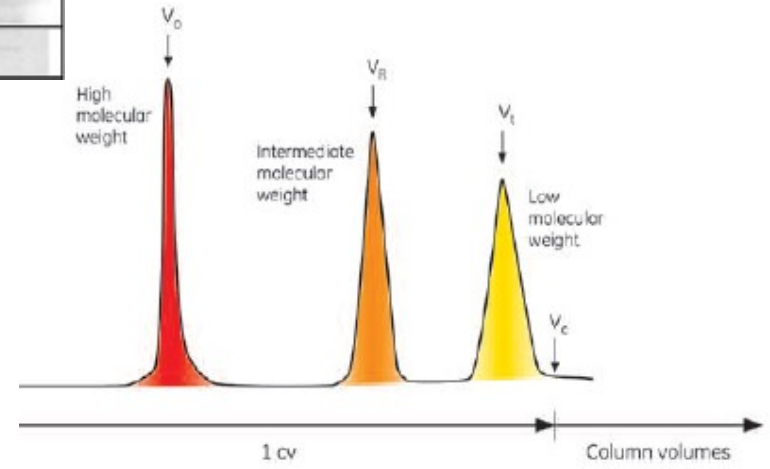
analýzy proteinových komplexů (size exclusion chromatography)



B

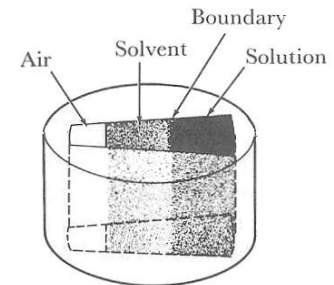
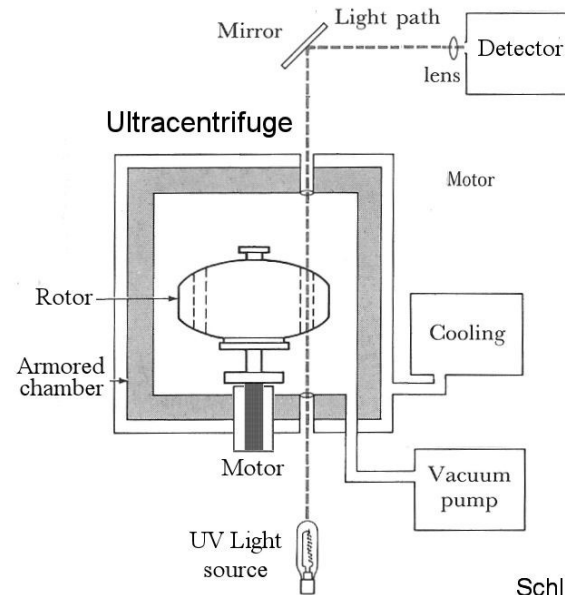


GF frakce lyzátu lidských buněk – podjednotky SMC5-6 komplexu identifikovány pomocí protilátek

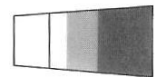


Metody analýzy proteinových komplexů

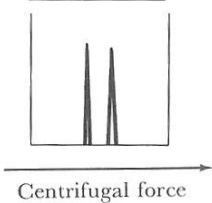
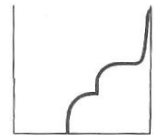
ultracentrifugace



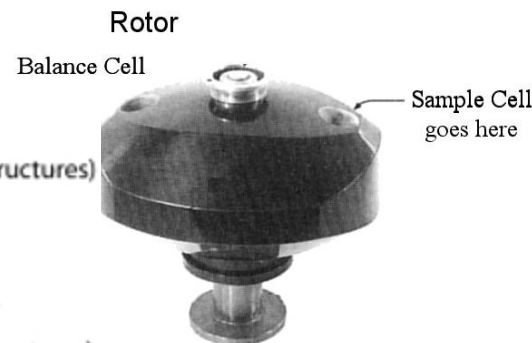
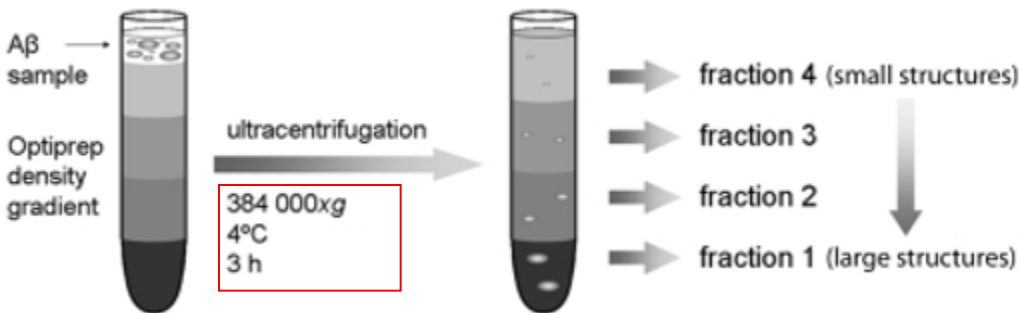
Sample Cell



Schleirin Optics (schematic)



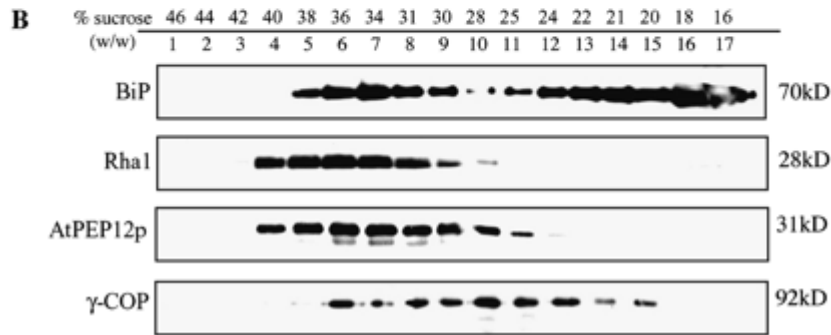
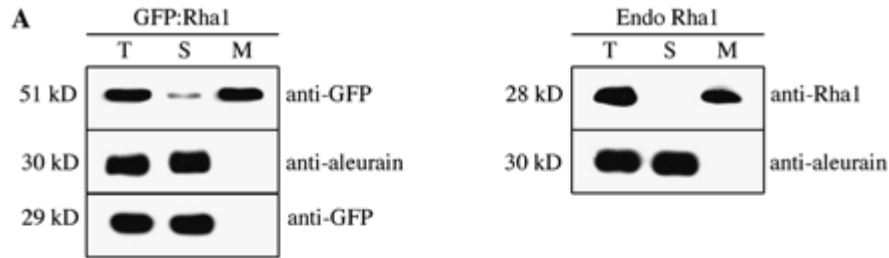
Cukerný/hustotní gradient



Je třeba přesně vyvážit!

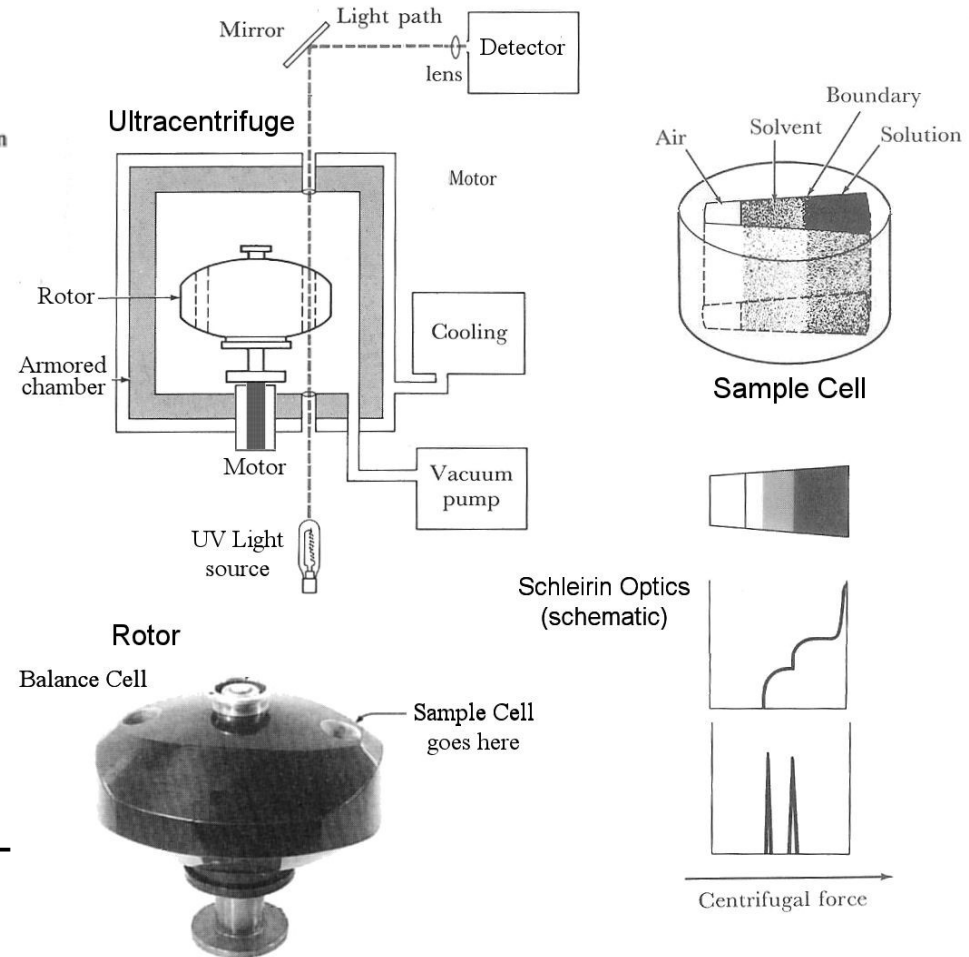
Metody analýzy proteinových komplexů

ultracentrifugace



A. Hrubší pouze rozdělí na kompartmenty/organely (S – soluble, M – membranové frakce ... jaderná ...)

B. Jemný - cukerný gradient



Metody analýzy proteinových komplexů

- Gelová filtrace, ultracentrifugace
- TAP-tag (jiné tagy a protilátky) purifikace a MS identifikace

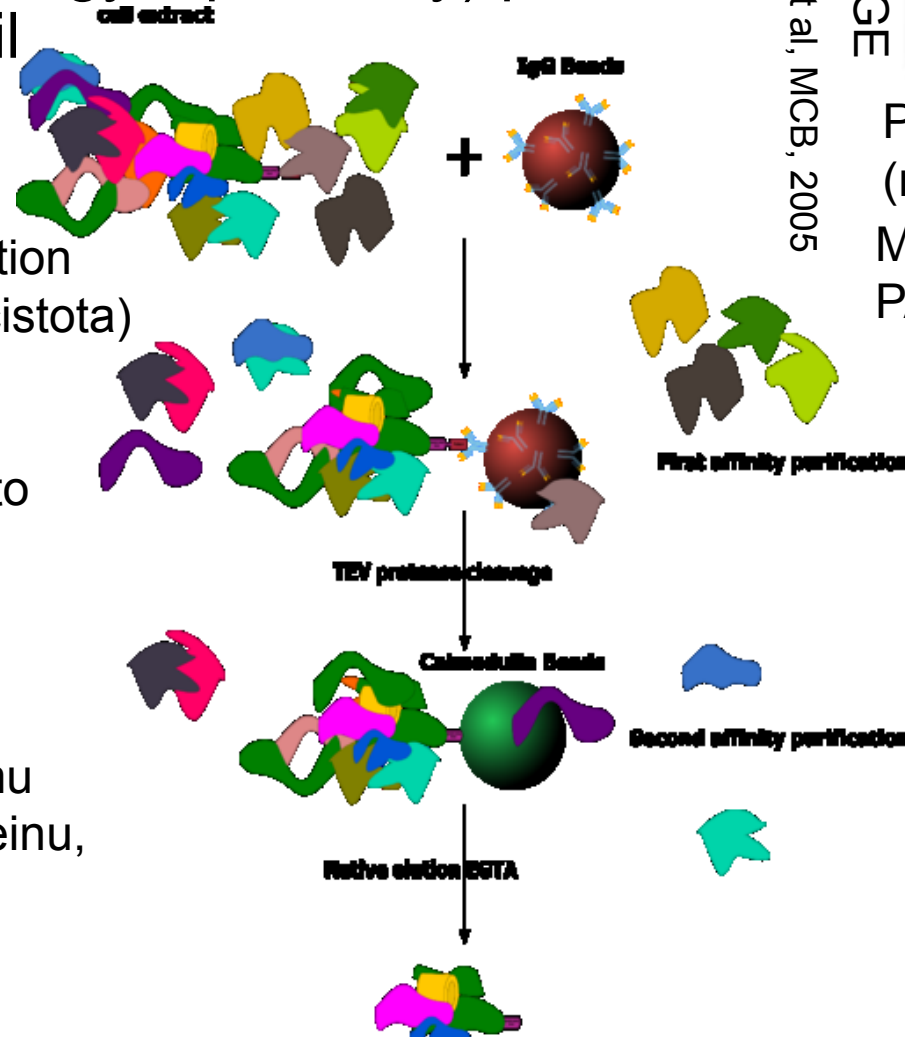


Tandem-affinity purification (vícestupňové – vyšší čistota)

1. calmodulin-binding (CBP)
2. TEV-proteasové místo (tobacco etch virus)
3. Protein A (váže IgG)

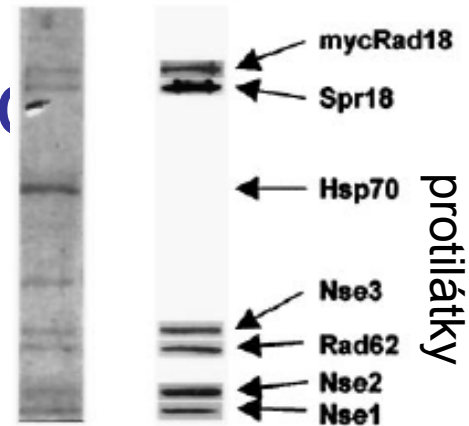
Tagy (myc, HaloTag ...)
Zaintegrované v genomu (přirozená hladina proteinu, přirozený výskyt partnerů/komplexu ...)

Puig et al, Methods, 2001

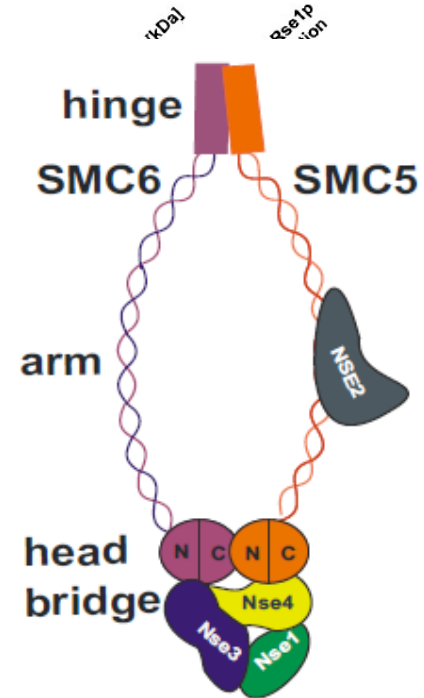


Sergeant et al, MCB, 2005

SDS-PAGE



Pozor na kontaminace (např. chaperony)
MS analýza SDS-PAGE nebo roztoku



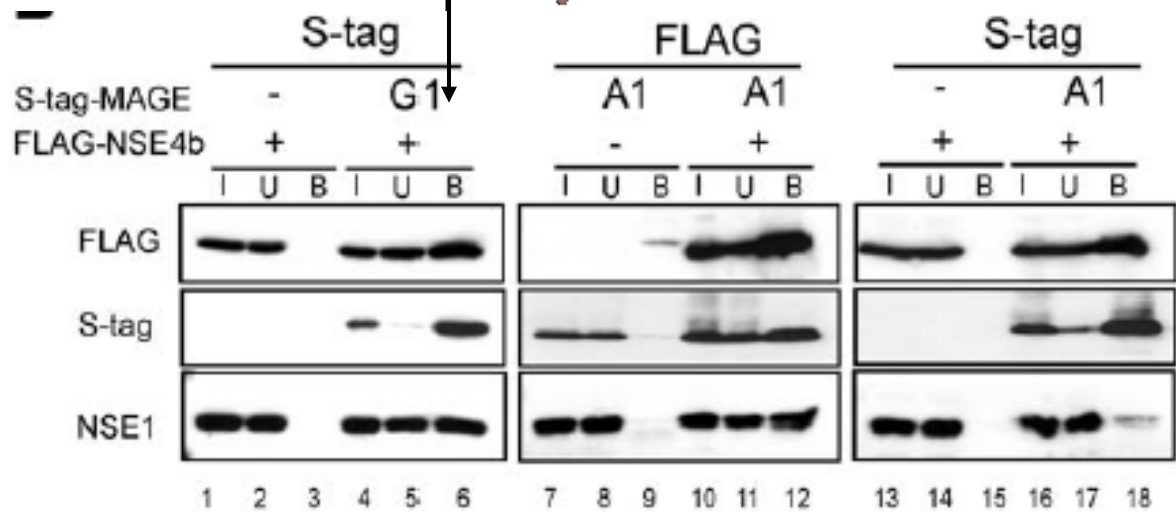
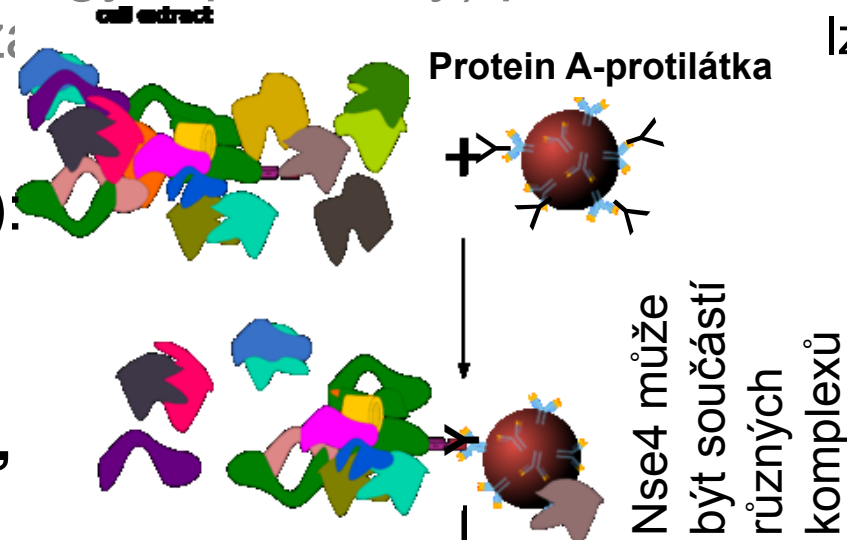
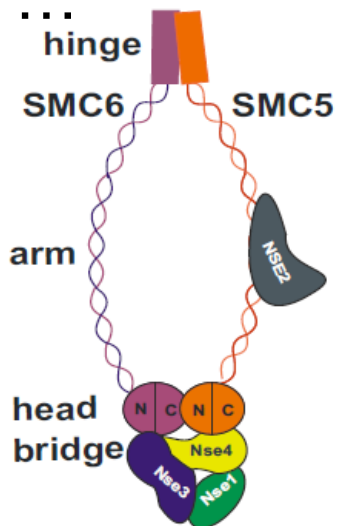
Metody analýzy proteinových komplexů

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Protein Tag

Tagy (a protilátky).

Myc, FLAG, V5, S-tag, GFP, GST, Streptactin, MBP



- **ko-imunoprecipitaci**
 lze využít i pro analýzu protein-proteinových interakcí – uměle vnesené konstrukty (např. transfekce plasmidů do buněk)
riziko nepřímých interakcí

Metody analýzy proteinových komplexů

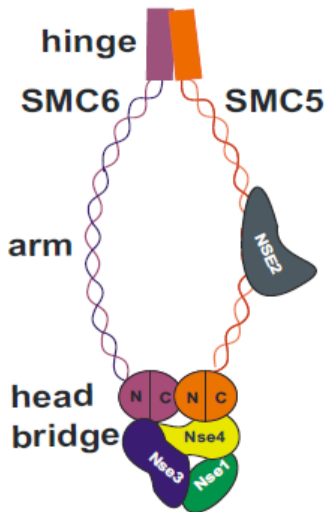
Pro stabilní komplexy – nelze použít pro charakterizaci struktury/architektury komplexů a pro analýzu slabých/přechodných interakcí

Metody analýzy protein-proteinových interakcí

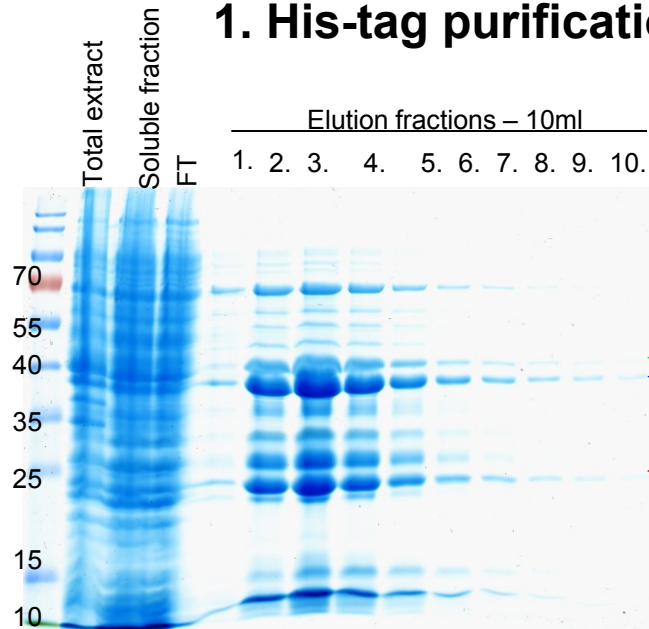
- **ko-imunoprecipitace, pull-down, ko-purifikace ...**
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- genetické metody (syntetická letalita, suprese)

Ko-purifikace

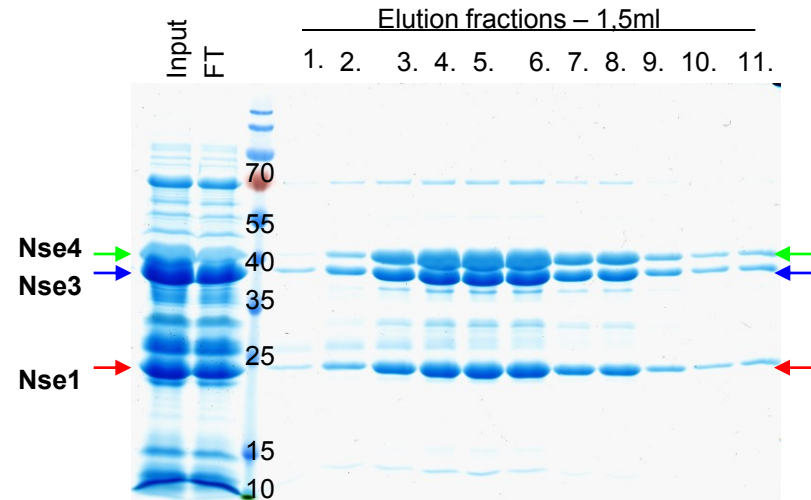
Silné interakce/komplexy – proteiny lze **ko-exprimovat** (může pomoci s jejich rozpustností) a následně **ko-purifikovat** (více Dr. R. Dopitová)



1. His-tag purification



2. Strep-tag purification

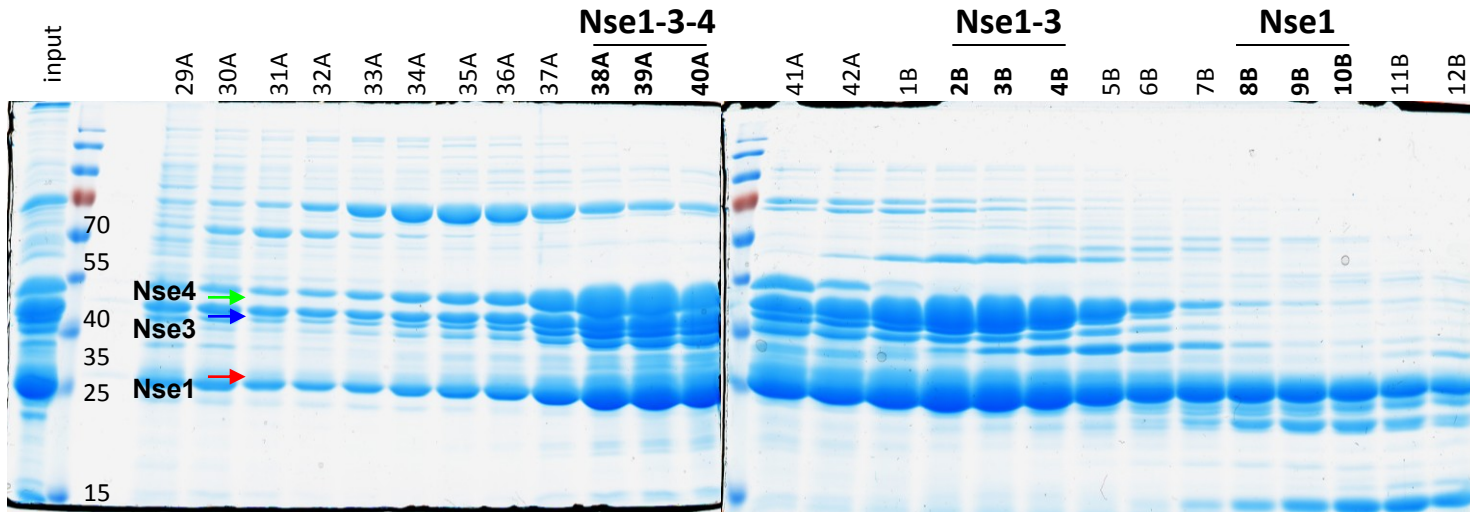
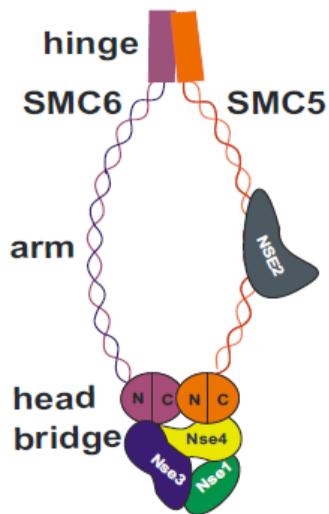
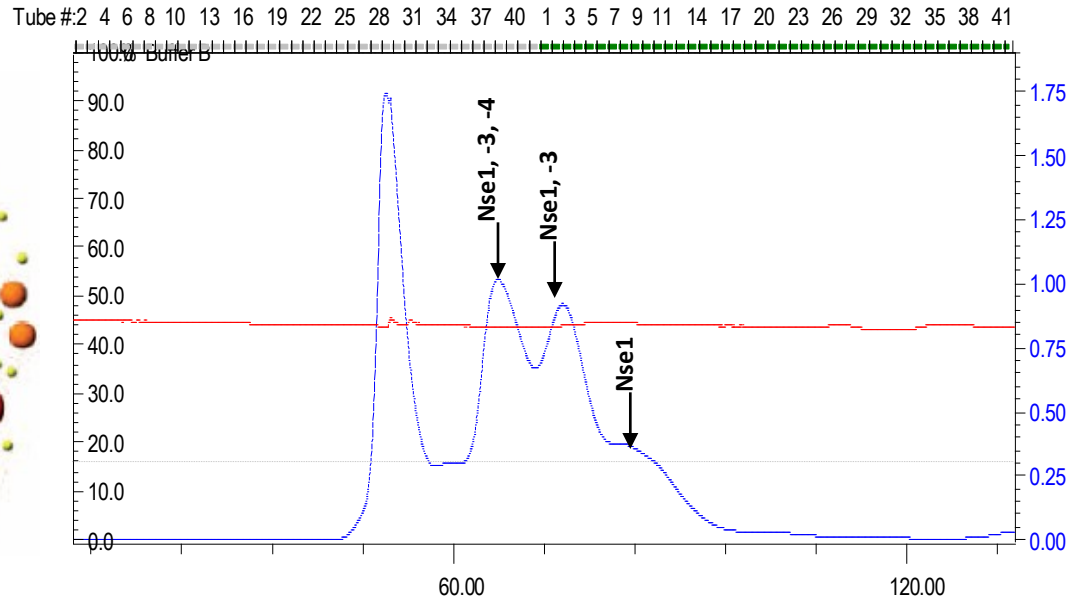
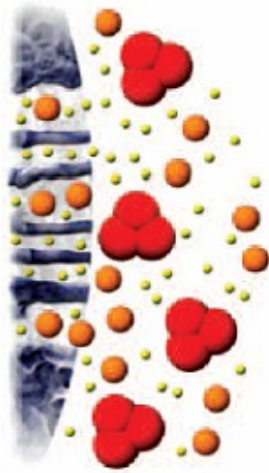


Nse4 protein se samostatně exprimuje málo a je nerozpustný

Ko-purifikace

Silné interakce/komplexy – proteiny lze ko-exprimovat (může pomoci s jejich rozpustností) a následně ko-purifikovat

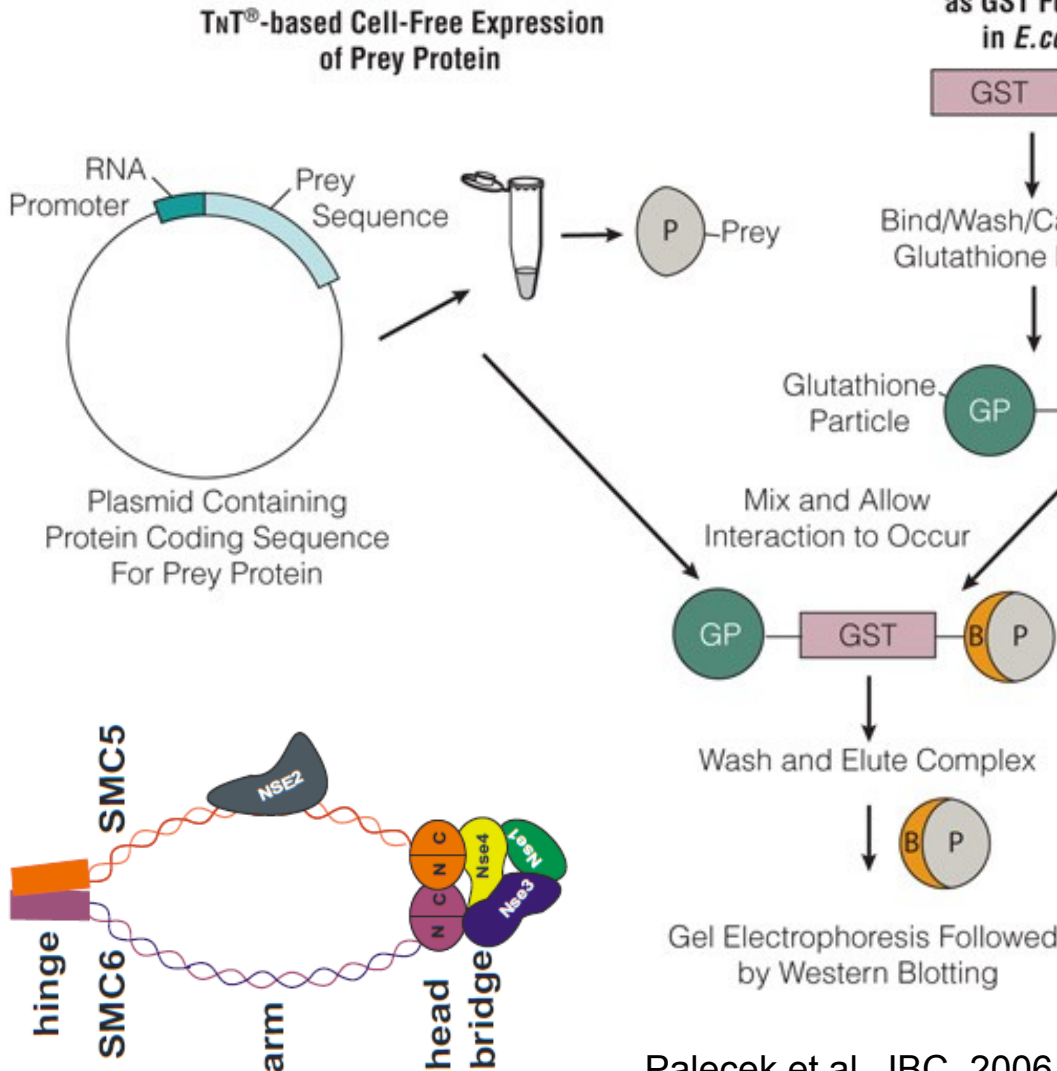
Gelová filtrace



Pull-down (ko-immunoprecipitace) **Silné interakce**

PROMEGA – *in vitro* TNT systém
methionin S³⁵ (pouze 2 partneri)

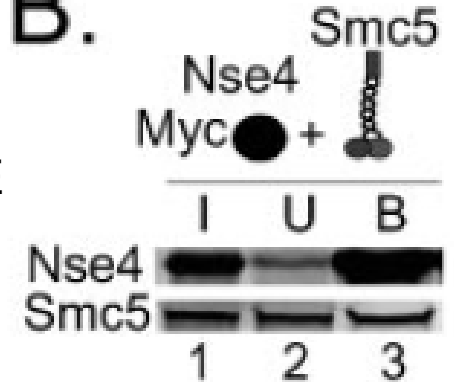
oba proteiny v TNT



Palecek et al, JBC, 2006

B.

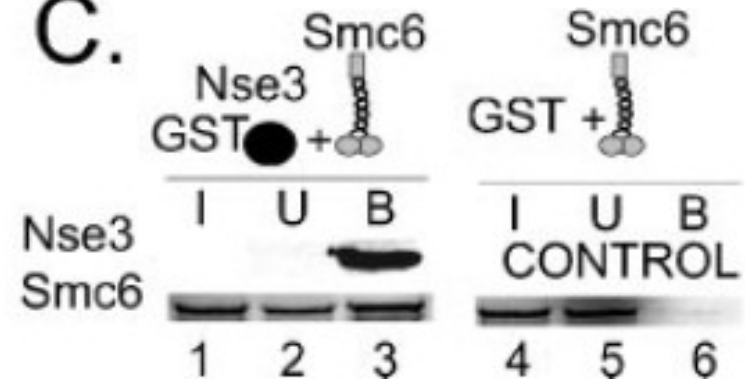
PAGE



Slabé interakce

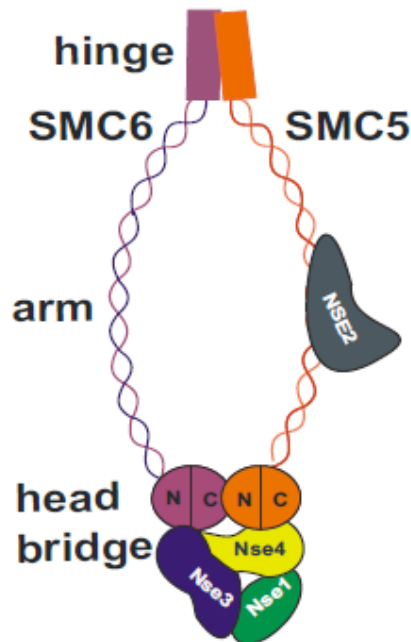
bait v přebytku
(bakt. exprese) a
prey v TNT

C.

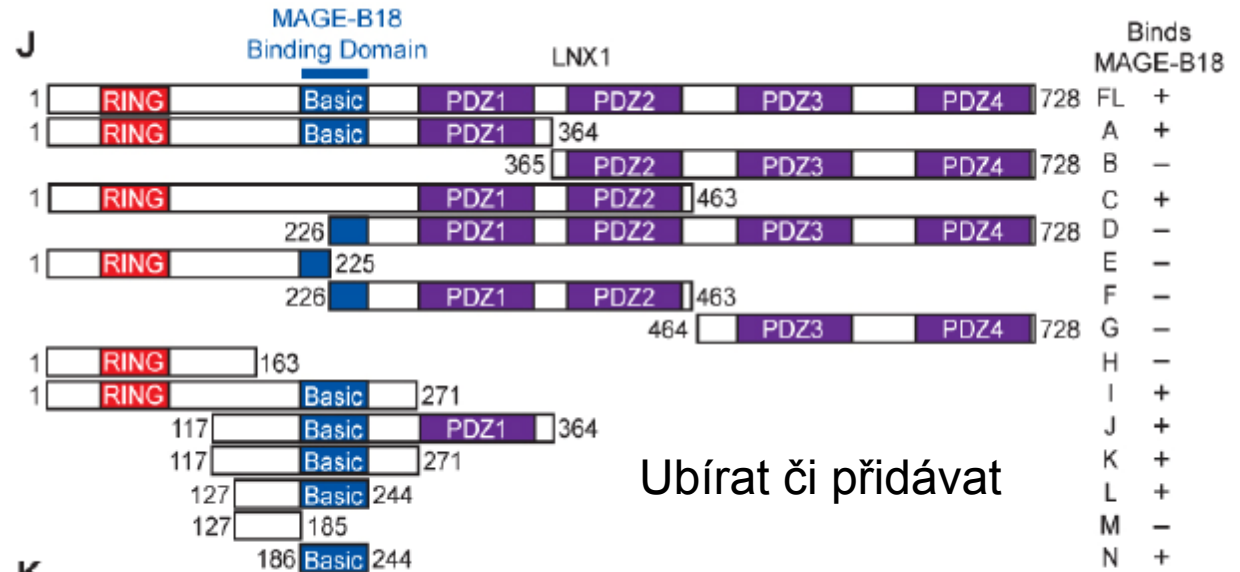


PAGE => Western (anti-GST)

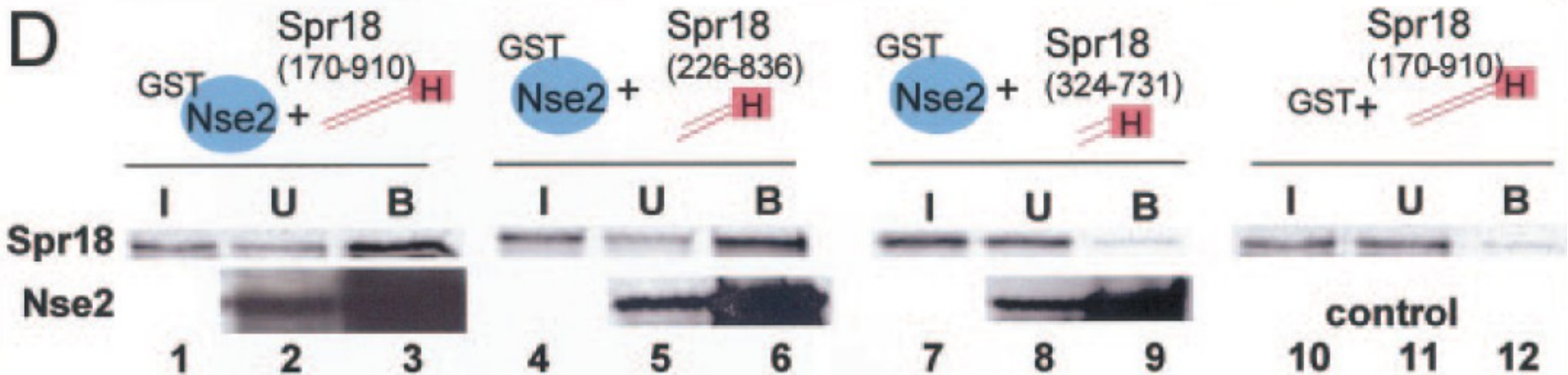
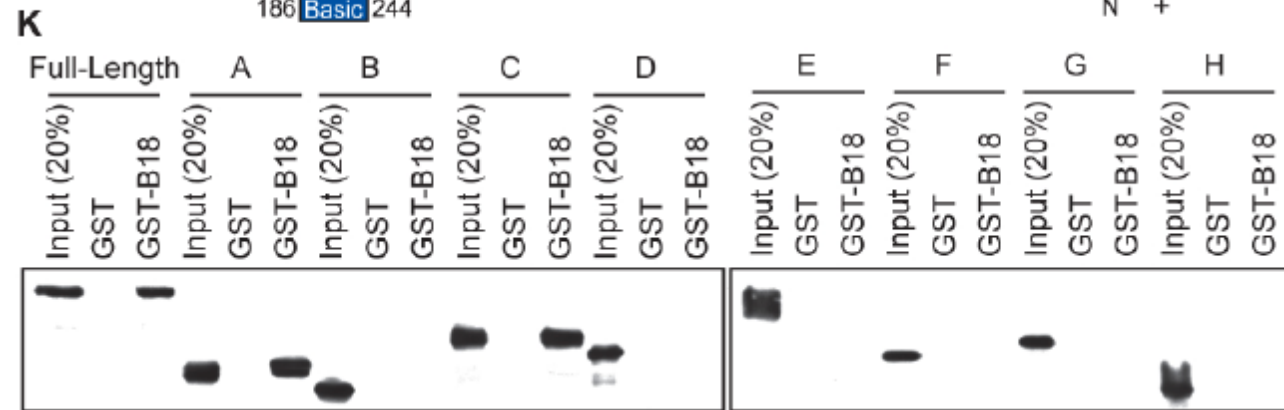
Charakterizace interakcí - domény



Sergeant et al, MCB, 2005
Doyle et al, Mol Cell, 2010

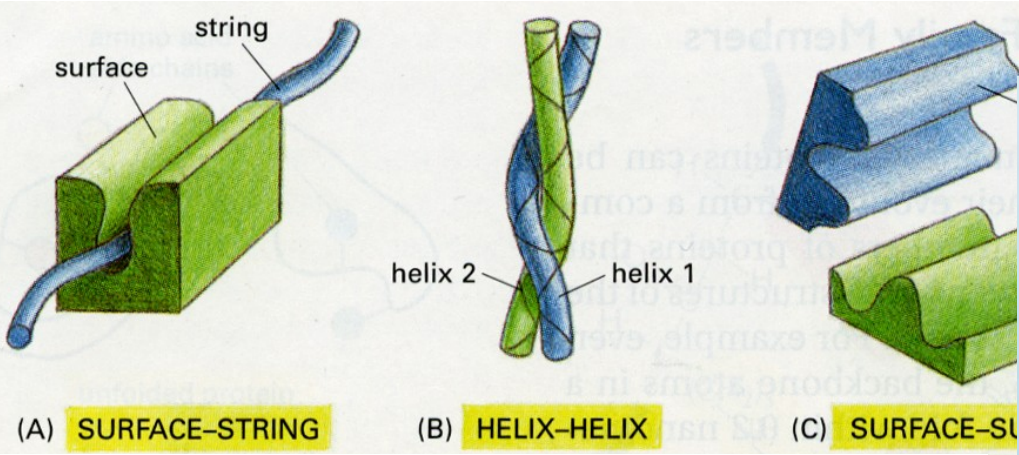


Ubírat či přidávat



Charakterizace interakcí – peptide

Podobně jako při ELISA jamky jsou potažené streptavidinem peptide se přes biotin ukotví



Epitope

G N F K S L L W E I V C S A Q E A H Q V

Length of protein segment: 24 residues

Length of epitope: 8 residues

G N F K S L L W

K S L L W E I V

L W E I V C S A

I V C S A Q E A

S A Q E A H Q V

Example 1

Length: 8 mer

Offset: 3

No. Peptides: 5

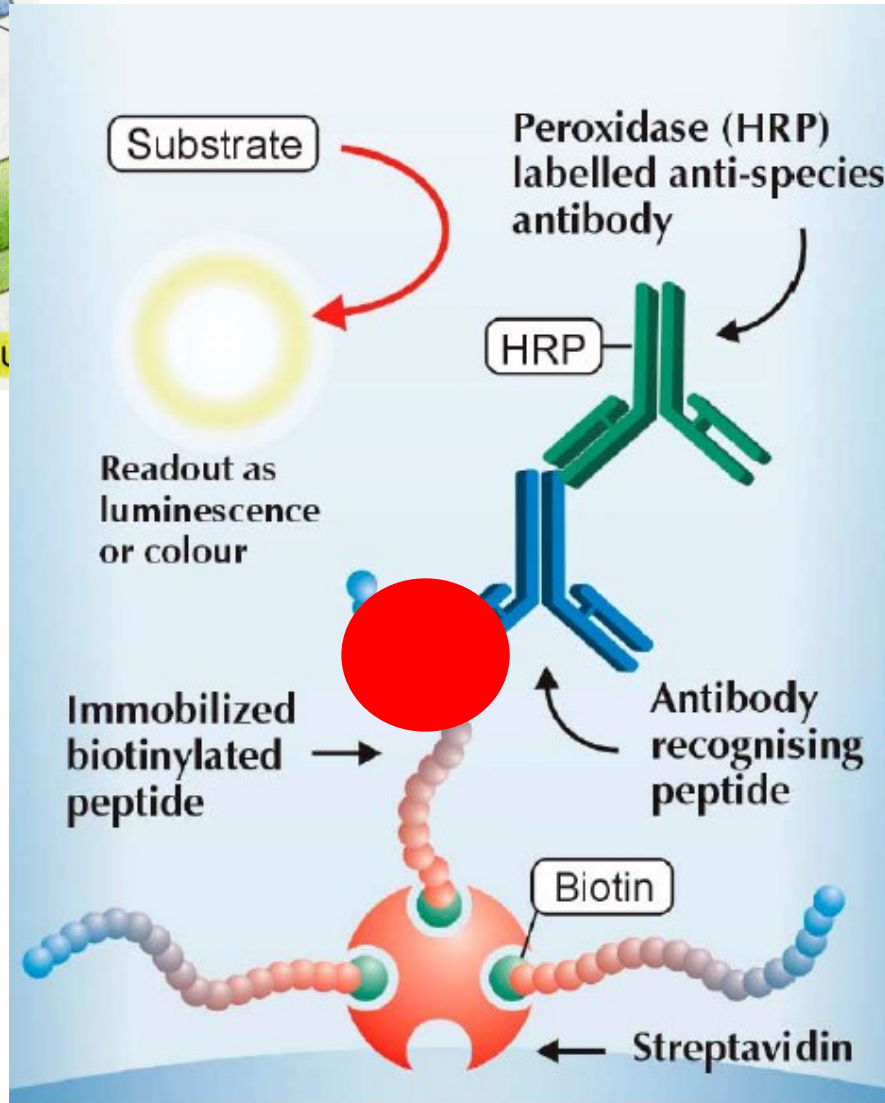


Figure 1: An ELISA using biotinylated peptides and coated plates

Lze mapovat epitop pro protilátky (vazbu)
Peptidy jsou na N-konci biotinylované

Charakterizace interakcí – peptide

Podobně jako při ELISA jamky jsou potažené streptavidinem peptide se přes biotin ukotví

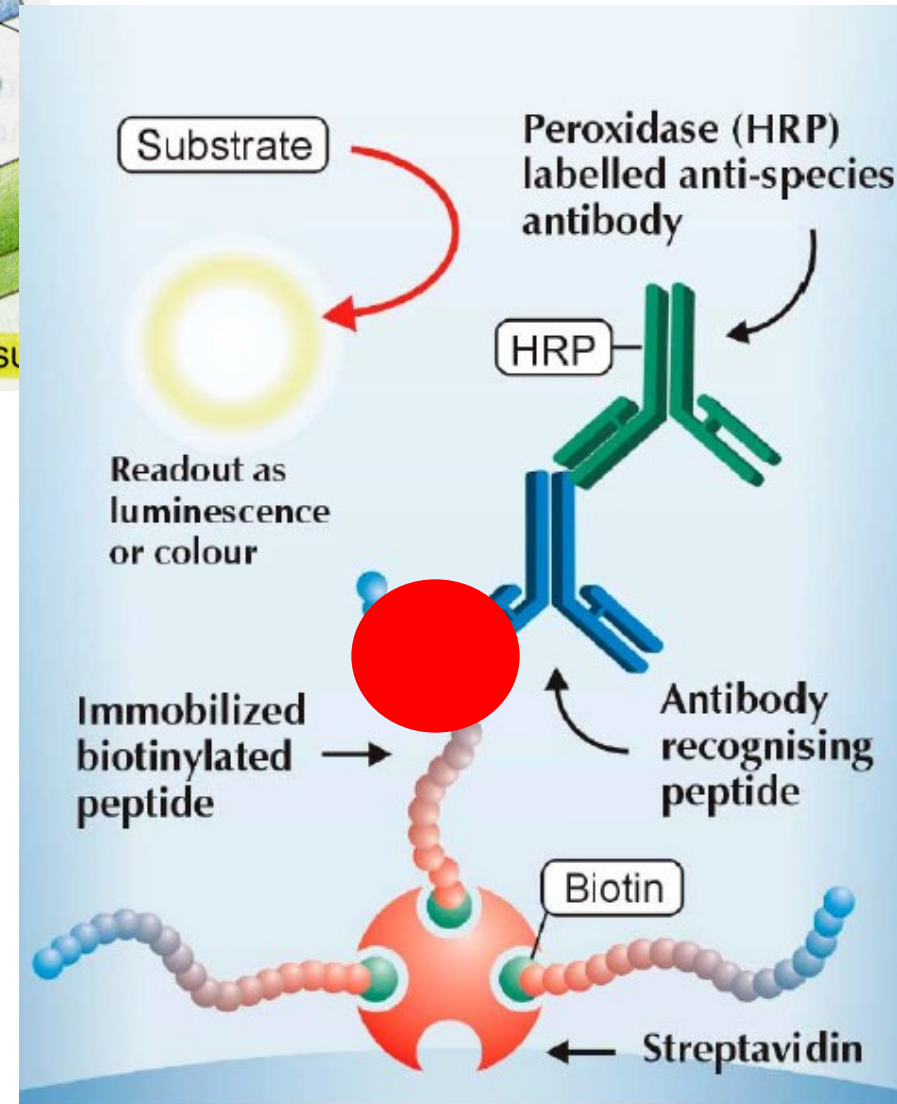
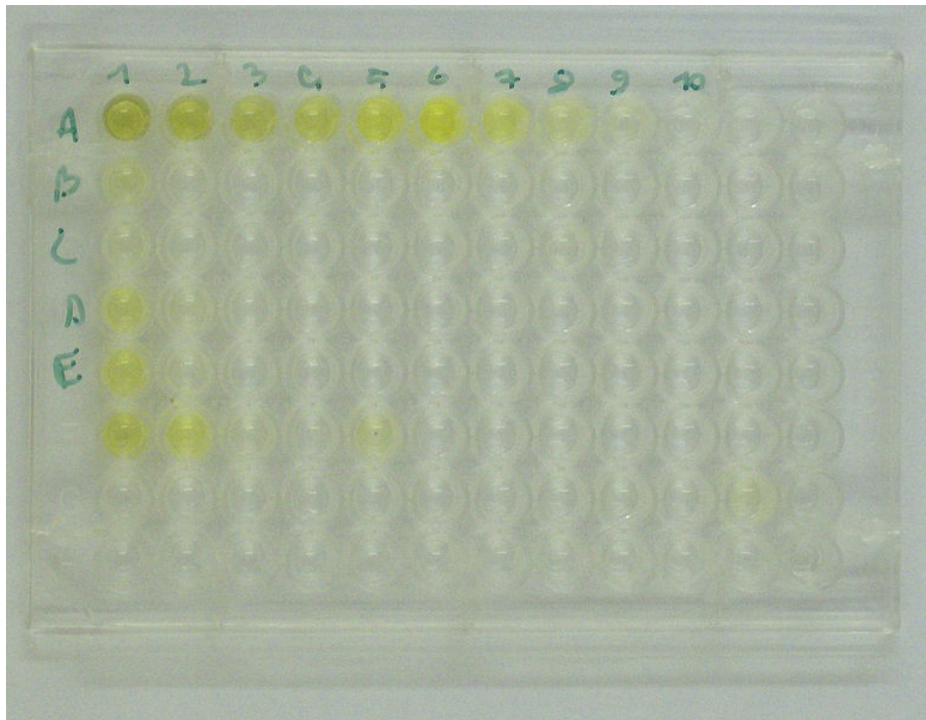
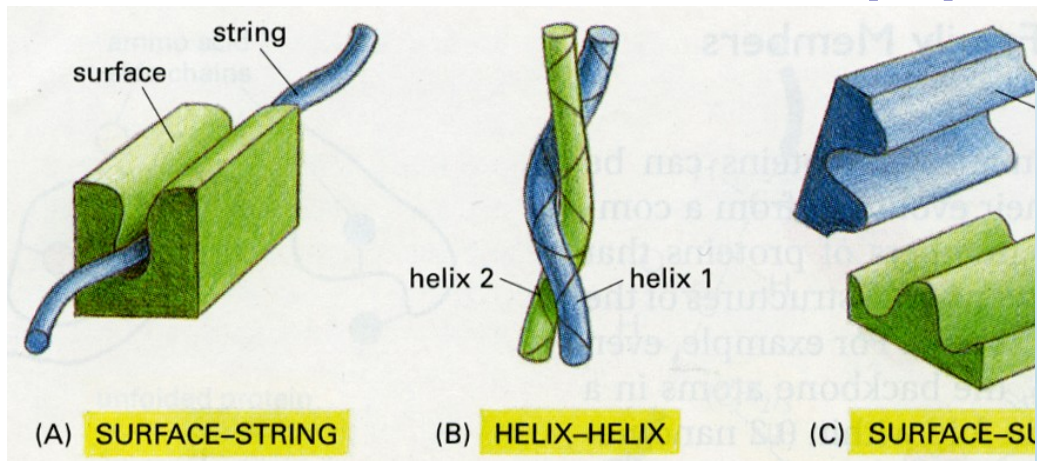


Figure 1: An ELISA using biotinylated peptides and coated plates

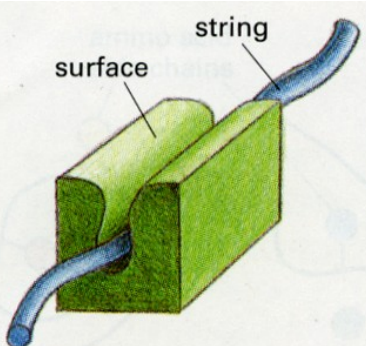
Charakteriz

C.

Kleisin motif

Nse3/MAGE-binding domain

peptide #7



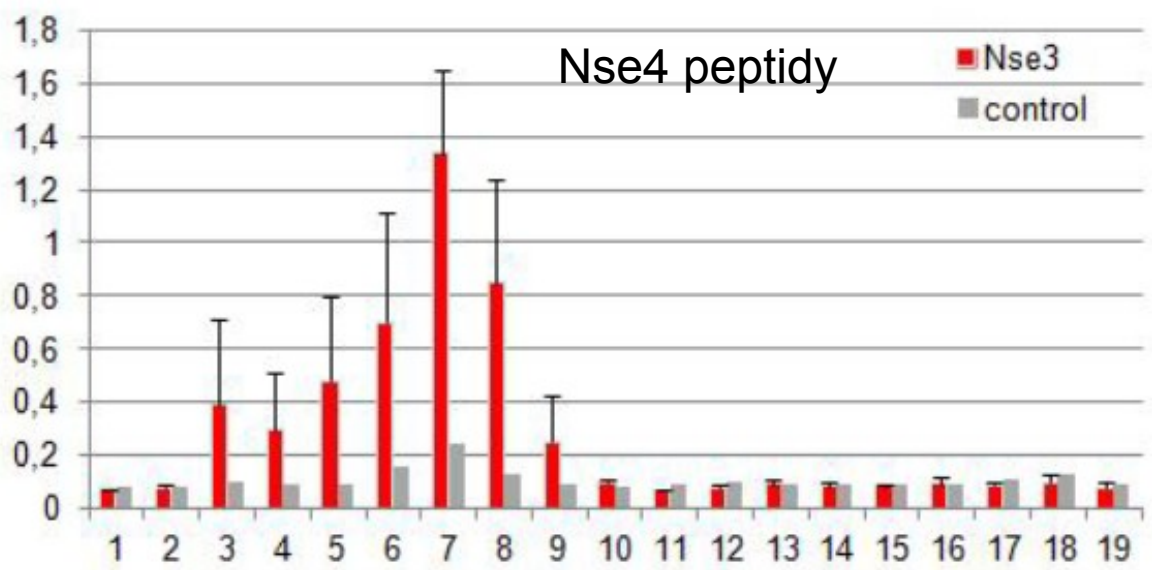
(A) SURFACE-STRING

Nse4/Nse4a

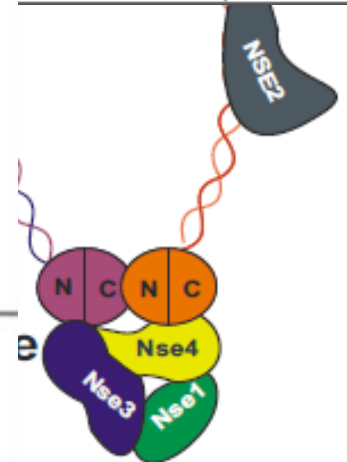
S.p.	YNDL	DNK	QES	L	LVDEENN	LY	IT	NDL	FSS	DAPT	ATL	A	LLL	X	T	V	L	AS	IR	AR	L	H	I	G	R	P	K	F	N	I	E	L	F	T	K	N	I	K	Q																								
D.r.	YADL	INE	VOQN	R	HLN	PANN	LT	VL	AN	KLFAN	RQ	AA	A	LL	V	L	A	T	IG	K	AS	OL	H	A	G	S	S	P	S	A	F	A	H	L	L	S	F	D	N	R	D																						
X.i.	YVDL	IRN	VOQN	R	HL	SSRT	DL	AL	Q	AN	KIFAG	V	SP	A	A	L	A	Q	L	L	V	L	A	S	I	G	K	AS	OL	H	A	M	T	V	P	T	S	T	A	D	L	L	S	F	G	L	N	R	SP														
G.g.	YREL	ICS	VOQN	R	HL	SSKS	N	L	T	AL	Q	N	L	F	S	G	V	SP	A	A	L	A	Q	F	L	V	L	A	S	I	G	K	AS	OL	H	S	R	S	I	T	T	S	E	T	A	D	L	L	S	F	G	L	N	R	T								
O.a.	-----	---	N	V	H	L	S	S	K	N	L	T	A	L	Q	N	L	F	T	G	V	SP	A	A	L	A	Q	F	L	V	L	A	S	I	G	K	AS	OL	H	S	R	S	I	T	T	S	E	T	A	D	L	L	S	F	G	L	N	R	T				
H.d.	YREL	THN	VOQN	R	HL	SSKS	N	L	T	AL	Q	N	L	F	N	R	V	SP	A	A	L	A	Q	F	L	V	L	A	S	I	G	K	AS	OL	H	S	R	S	I	T	T	S	E	T	A	D	L	L	S	F	G	L	N	R	T								
C.z.	-----	---	Q	E	N	S	I	L	N	A	S	D	E	L	T	V	L	E	A	N	T	L	F	N	G	V	SP	A	V	L	A	Q	F	L	V	L	A	S	I	G	K	AS	OL	H	S	R	S	I	T	T	S	E	T	A	D	L	L	S	F	G	L	N	R
H.m.	YRAL	INS	VOQN	R	HL	N	A	G	D	E	L	T	V	L	E	A	N	T	L	F	N	E	V	SP	A	V	L	A	Q	F	L	V	L	A	S	I	G	K	AS	OL	H	S	R	S	I	T	T	S	E	T	A	D	L	L	S	F	G	L	N	R			
H.s.	YRAL	INS	VOQN	R	HL	N	A	G	D	E	L	T	V	L	E	A	N	T	L	F	N	E	V	SP	A	V	L	A	Q	F	L	V	L	A	S	I	G	K	AS	OL	H	S	R	S	I	T	T	S	E	T	A	D	L	L	S	F	G	L	N	R			

peptide #6	SIKARQLHIGRPKFNIELFTKNIKQ
peptide #7	QRLHIGRPKFNIELFTKNIKQFLNY
peptide #8	IGRPKFNIELFTKNIKQFLNYPTSH
peptide #9	KFNIELFTKNIKQFLNYPTSHSNVT
peptide #10	ELFTKNIKQFLNYPTSHSNVTRIQE

A.



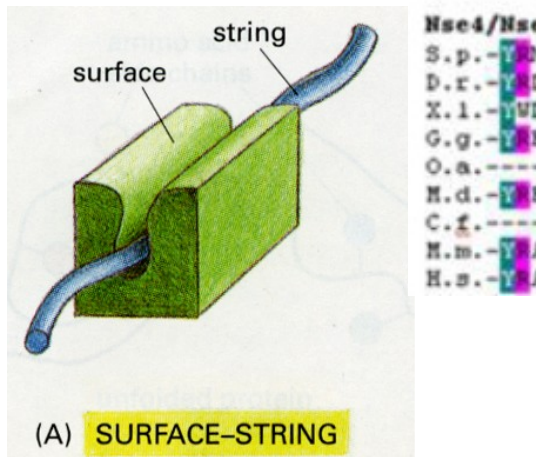
- L
- RLGKLA
- IRLGKLASNCE
- SRLGKLASNCEKQPA
- VSRLGKLASNCEKQPASLNL
- WSRLGKLASNCEKQPASLNL MVGP
- LGKLASNCEKQPASLNL MVGPLSFR



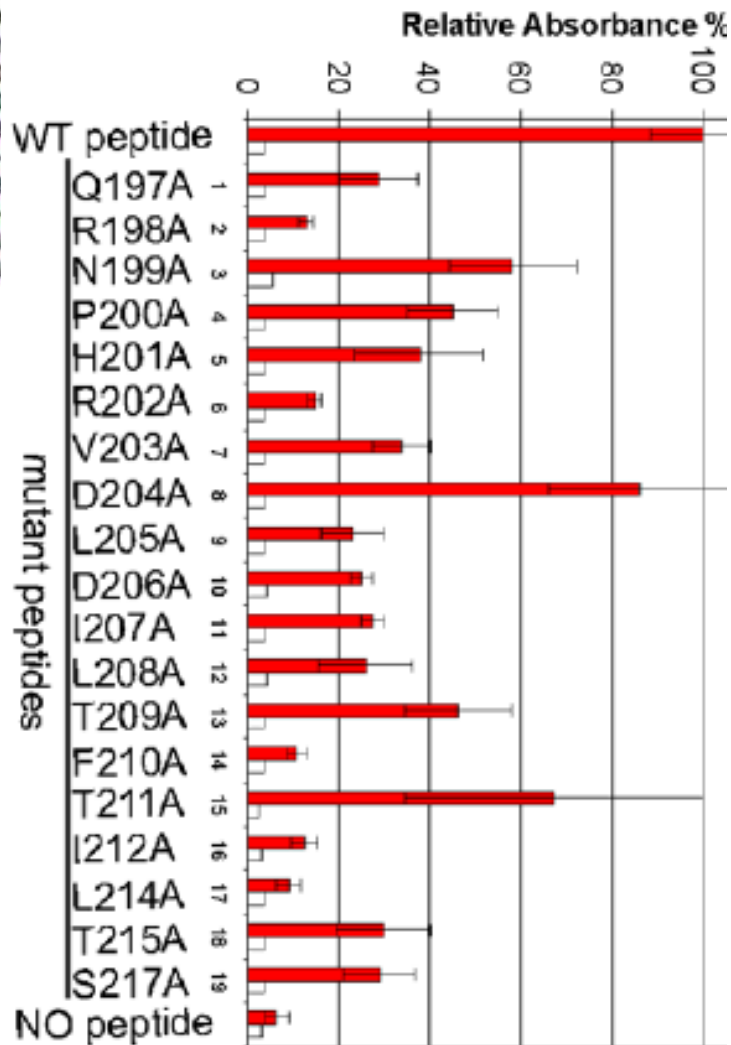
B.

#6 aa70-94	SIKARQLHIGRPKFNIELFTKNIKQ
#7 aa74-98	QRLHIGRPKFNIELFTKNIKQFLNY
#8 aa78-102	IGRPKFNIELFTKNIKQFLNYPTSH

Charakterizace interakcí – „alanin scan“

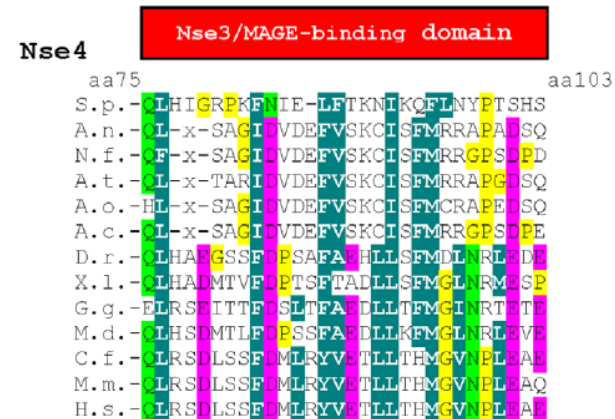


QRNPHRVLDILTFTIALTAS
ARNPHRVLDILTFTIALTAS
QANPHRVLDILTFTIALTAS
QRAPHRVLDILTFTIALTAS
QRNAHRVLDILTFTIALTAS
QRNPARVLDILTFTIALTAS
QRNPHAVLDILTFTIALTAS
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QRNPHRVDLAILTFTIALTAS

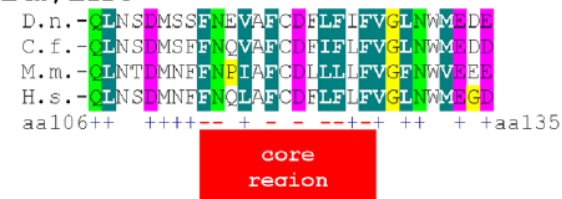


Zjistili jsme jak interaguje Nse4 s Nse3 na detailní úrovni

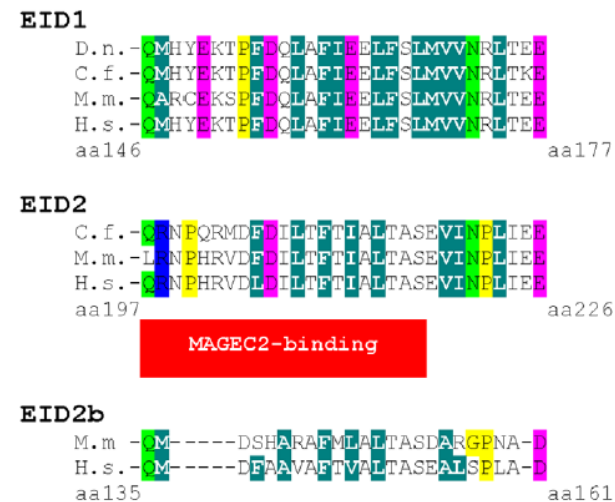
NSE4 subfamilies



NSE4b/EID3



EID subfamilies



Metody analýzy proteinových komplexů

Pro stabilní komplexy – nelze použít pro charakterizaci struktury/architektury komplexů a **pro analýzu slabých/přechodných interakcí**

Metody analýzy protein-proteinových interakcí

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- **(kvasinkový) dvou-hybridní systém**
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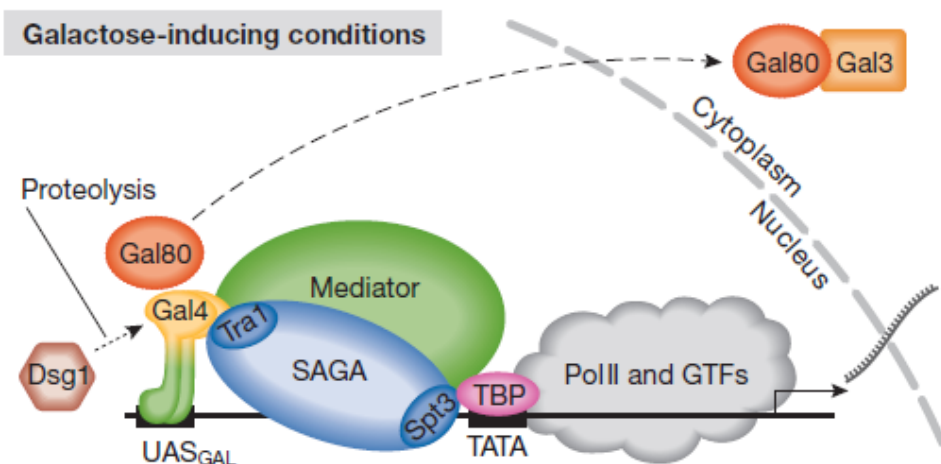
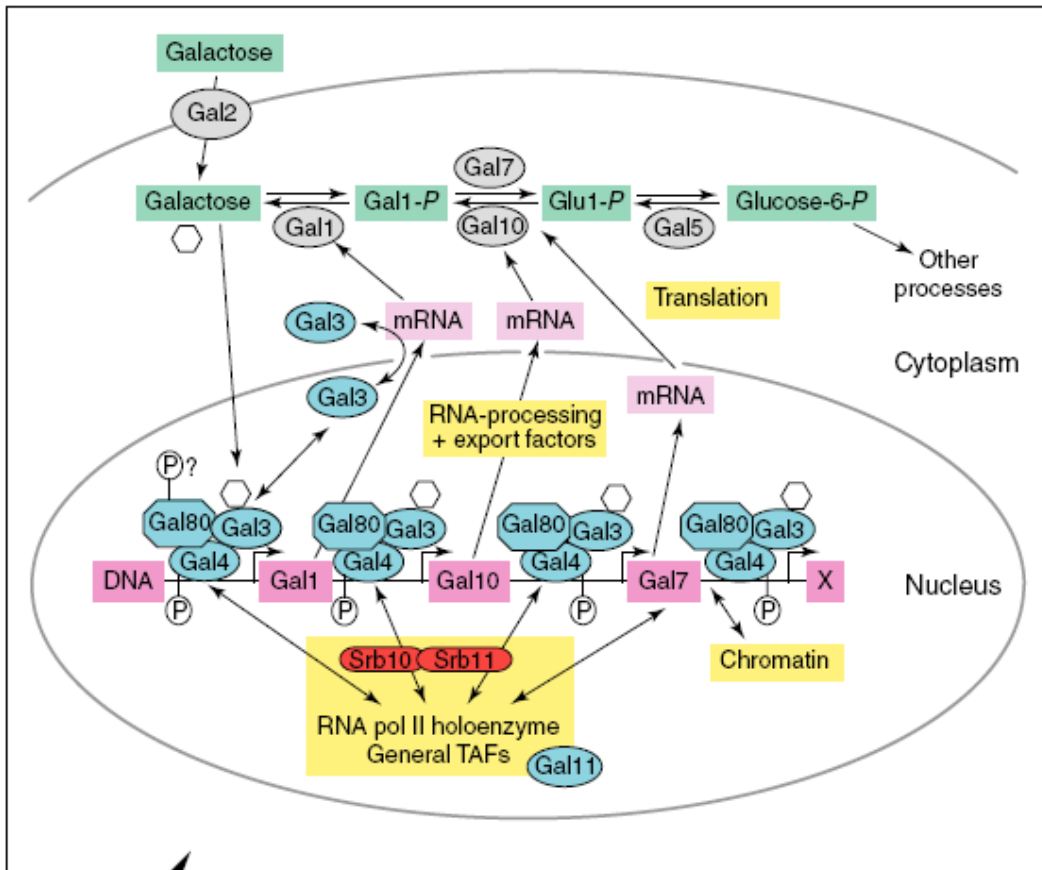
Dvou-hybridní systémy (kvasinkové)

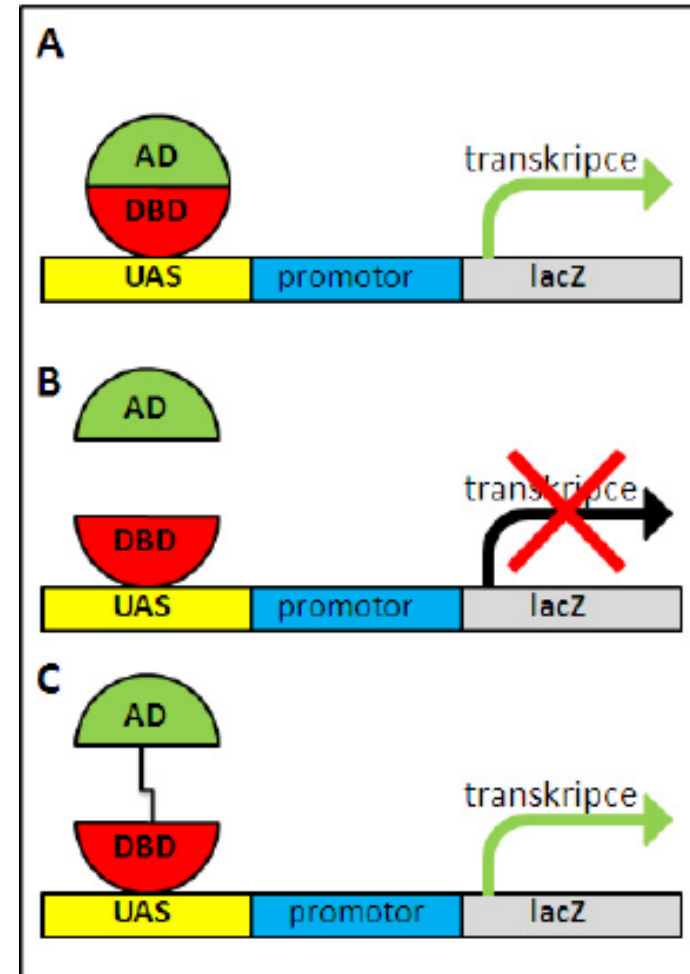
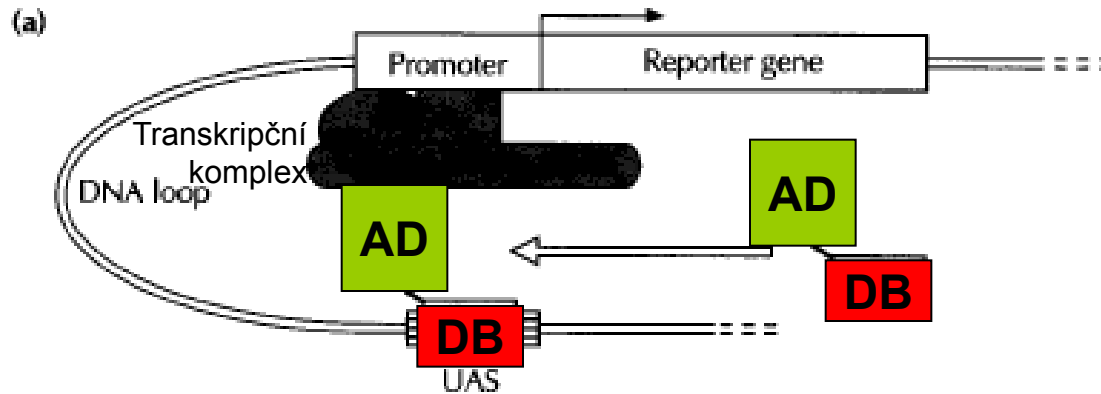
Při studiu mechanismů transkripce v kvasinkách *S. cerevisiae* byl vyvinut tzv. Y2H

Na spínání/regulaci metabolismu galaktosy se podílí transkripční faktor **Gal4p** – váže specifické sekvence v promotorech genů (Gal enzymů) a aktivuje jejich transkripci

Uetz and Finley, 2005

Traven et al.: EMBO Report, 2006

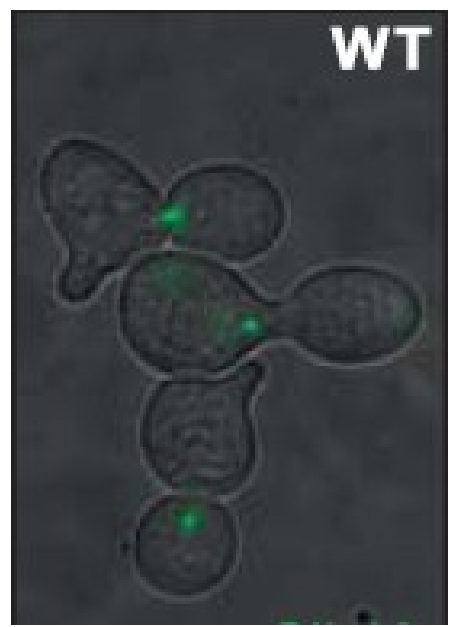
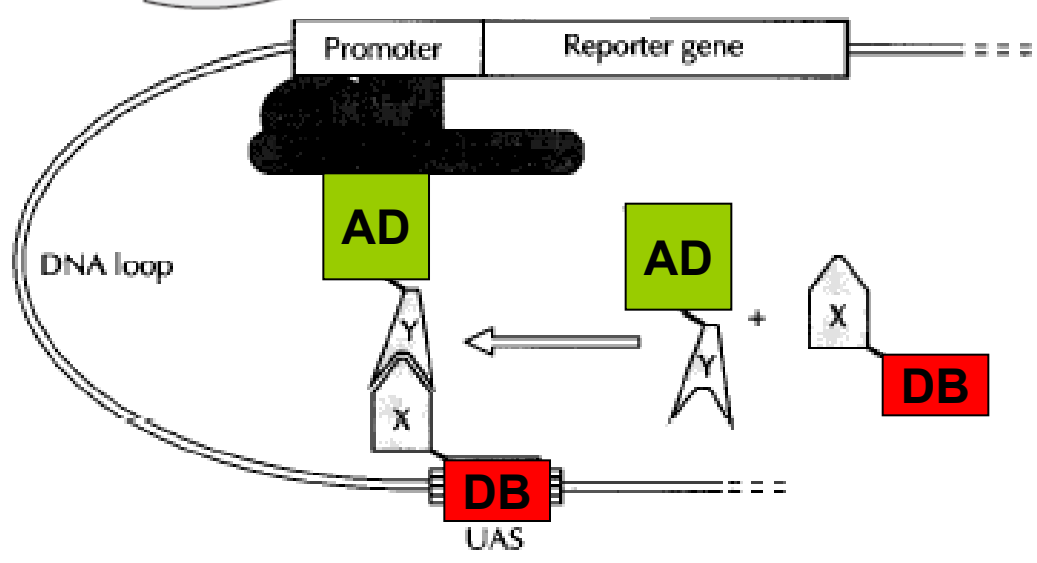
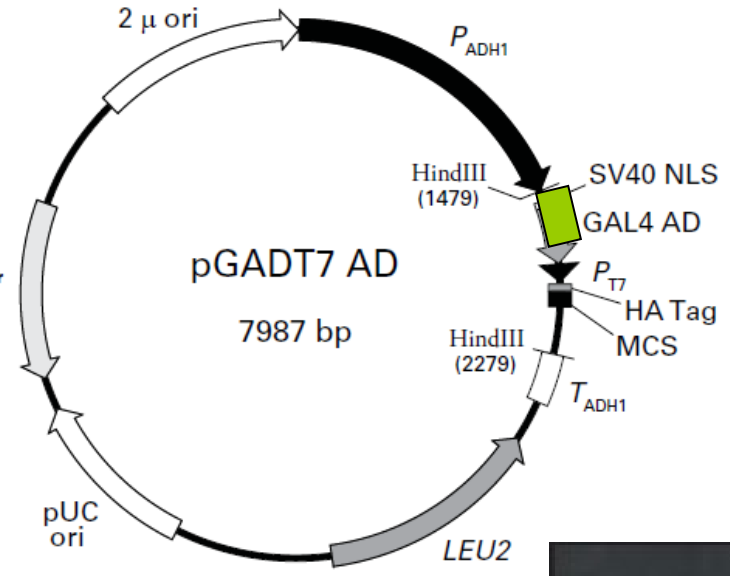
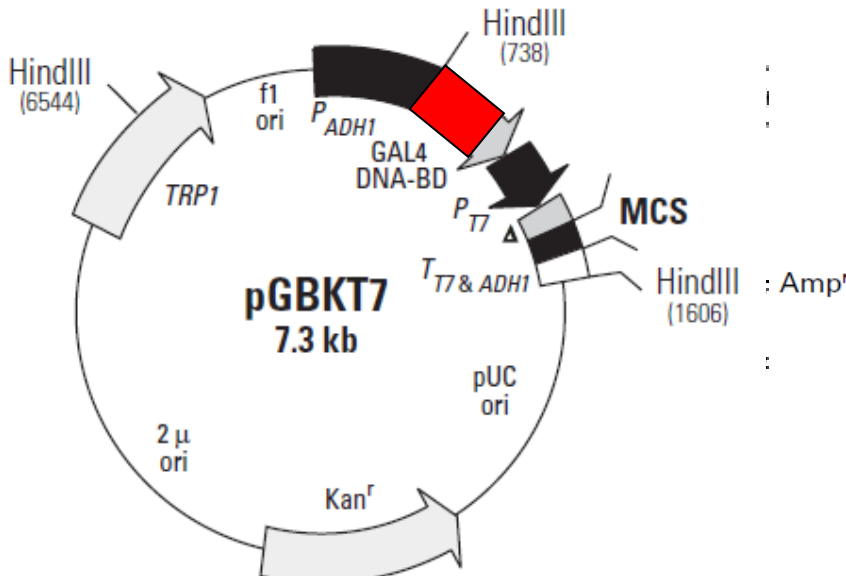




- DNA-vazebná doména (DB) bez aktivační domény (AD) není schopna aktivace transkripce
 Je možné **propojit domény** jakýmkoli linkerem a transkripci reaktivovat

GAL4

Gal4p



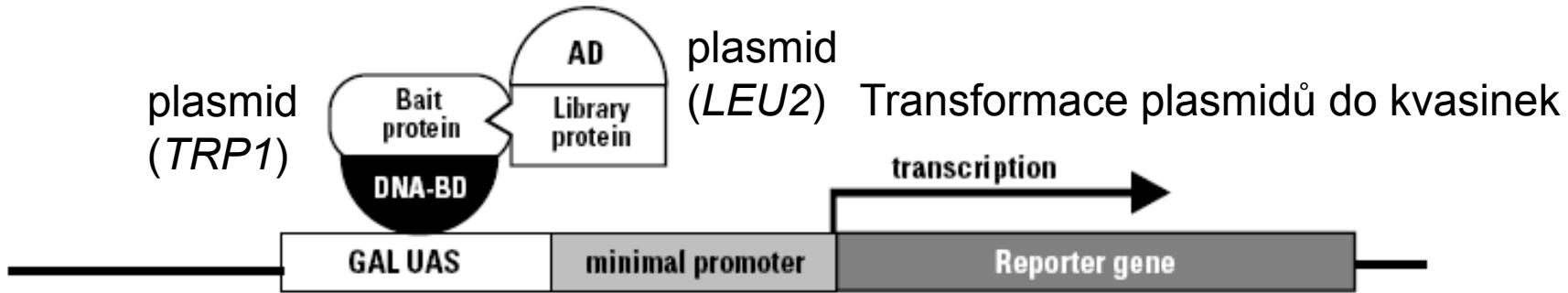


Figure 2. The two-hybrid principle. The DNA-BD is amino acids 1–147 of the yeast GAL4 protein, which binds to the GAL UAS upstream of the reporter genes. The AD is amino acids 768–881 of the GAL4 protein and functions as a transcriptional activator.

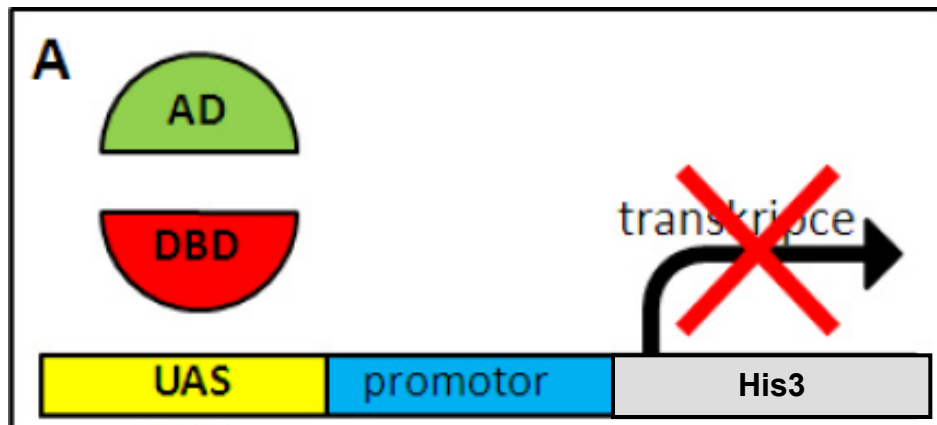
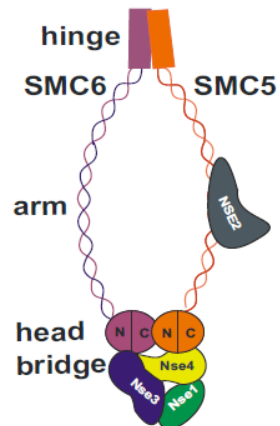
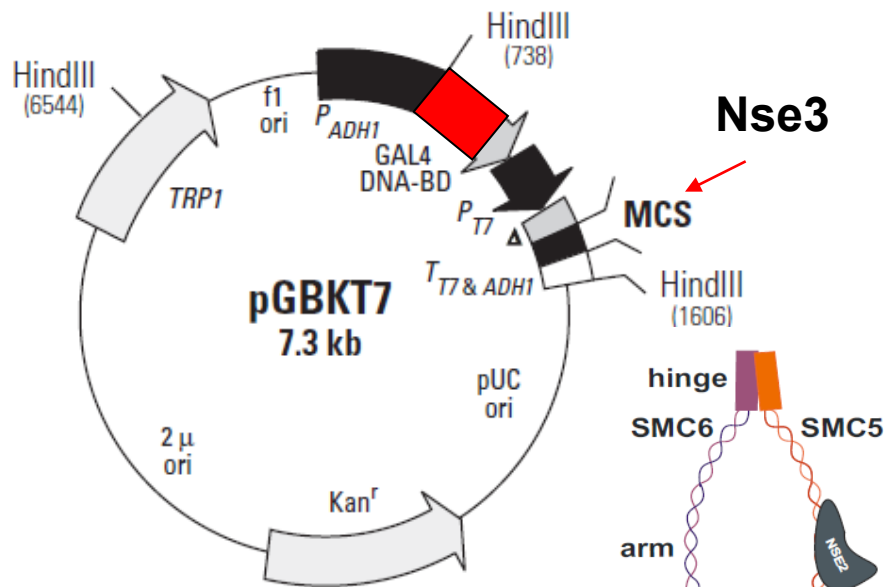
AH109
Kvasinkový
kmen

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

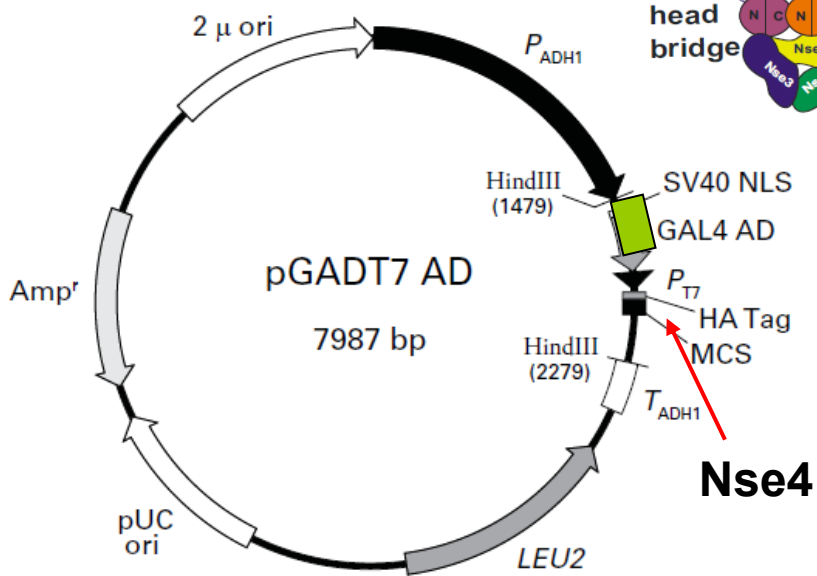
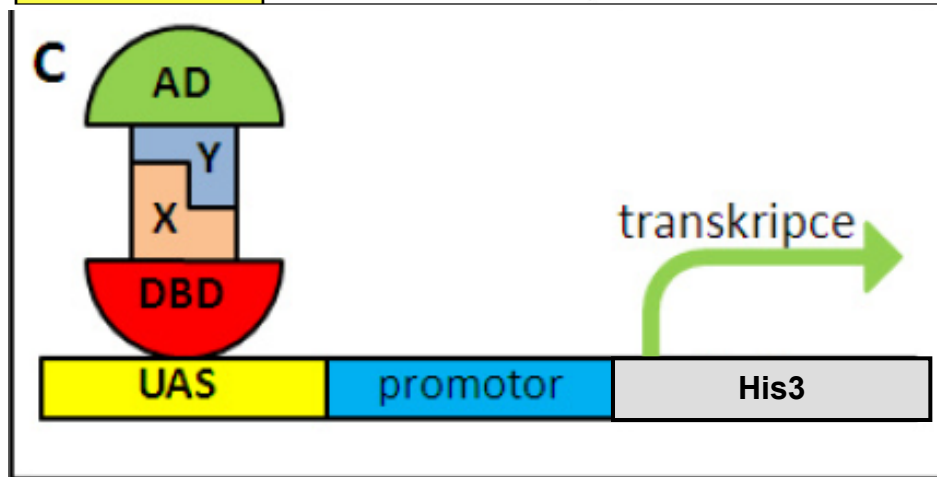
GAL1 UAS	GAL1 TATA	HIS3
GAL2 UAS	GAL2 TATA	ADE2
MEL1 UAS	MEL1 TATA	<i>lacZ</i>
MEL1 UAS	MEL1 TATA	MEL1

- Testuje se schopnost růstu kvasinek na médiu bez histidinu (nebo adeninu – červená/bílá)
 - lze použít i pro hledání proteinových interakčních partnerů (screen knihovny)

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



	Kontrola: (-Leu, -Trp)	5 mM 3-AT (-Leu, -Trp, -His)	10 mM 3-AT (-Leu, -Trp, -His)	15 mM 3-AT (-Leu, -Trp, -His)	20 mM 3-AT (-Leu, -Trp, -His)	30 mM 3-AT (-Leu, -Trp, -His)	60 mM 3-AT (-Leu, -Trp, -His)
BD-Nse3 + V2AD	●	●	●	●	●	●	●
BD-Nse3 + AD-Nse4	●	●	●	●	●	●	●
VBD + AD-Nse4	●	●	●	●	●	●	●



mutanty BD-Nse3 + AD-Nse4	kontrolní miska (-Leu, -Trp)			testovací miska (-Leu, -Trp, -His, + 20 mM 3-AT)		
mutanty	1	2	3	1	2	3
F212A	●	●	●	●	●	●
L213A	●	●	●	●	●	●
M214A	●	●	●	●	●	●
V216A	●	●	●	●	●	●
I217A	●	●	●	●	●	●
F219A	●	●	●	●	●	●
I220A	●	●	●	●	●	●
S223A	●	●	●	●	●	●
V227A	●	●	●	●	●	●
H229A	●	●	●	●	●	●
S230A	●	●	●	●	●	●
L232A	●	●	●	●	●	●
F235A	●	●	●	●	●	●
L236A	●	●	●	●	●	●
S255A	●	●	●	●	●	●
S257A	●	●	●	●	●	●



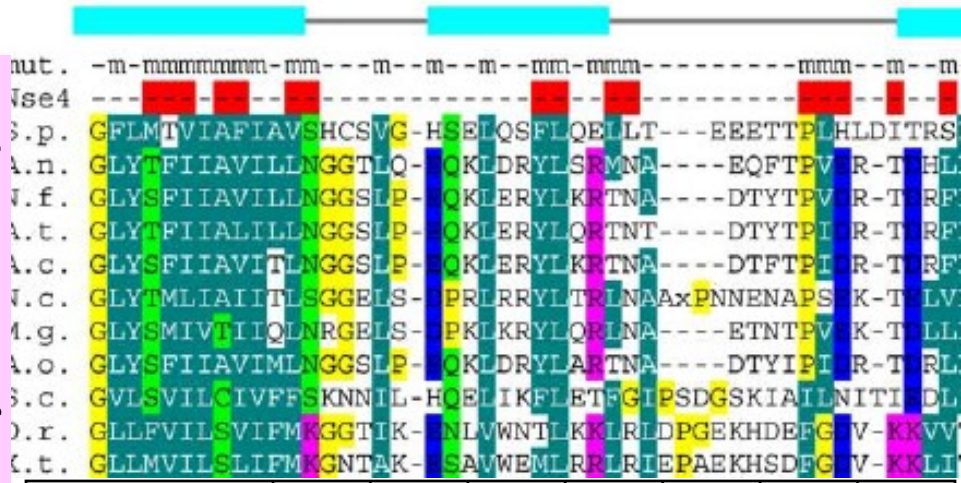
H5

L4

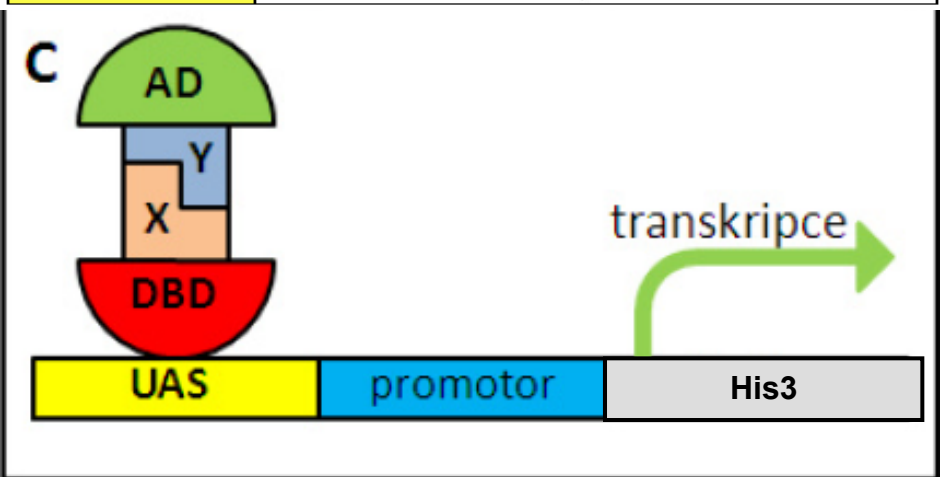
H6

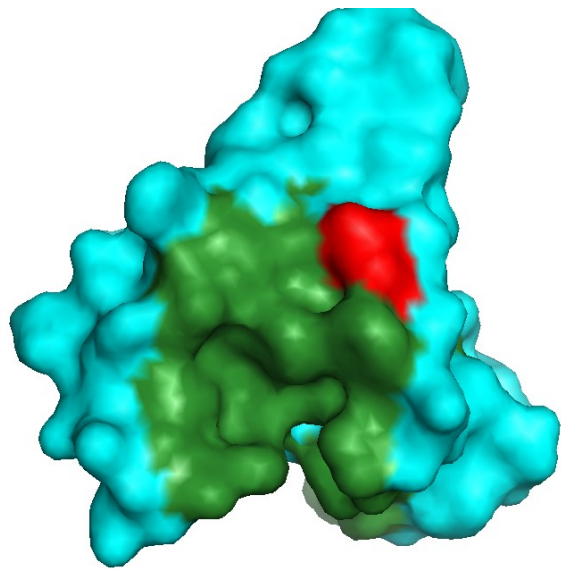
H7

“alanin scan” konservovaných AMK ukázal hydrofobní kapsu

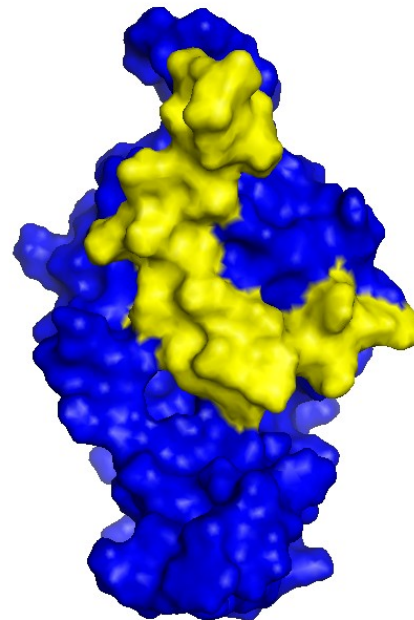


	Kontrola: (-Leu, -Trp)	5 mM 3-AT (-Leu, -Trp, -His)	10 mM 3-AT (-Leu, -Trp, -His)	15 mM 3-AT (-Leu, -Trp, -His)	20 mM 3-AT (-Leu, -Trp, -His)	30 mM 3-AT (-Leu, -Trp, -His)	60 mM 3-AT (-Leu, -Trp, -His)
BD-Nse3 + V2AD	●	●	●	●	●	●	●
BD-Nse3 + AD-Nse4	●	●	●	●	●	●	●
VBD + AD-Nse4	●	●	●	●	●	●	●





mut.	-m-	mmmmmmmmmm	mm	--m-	m-	m-	m-	mm	mmmm	mmmm	--m-	m-	m-																																			
Nse4																																																
S.p.	G	F	L	M	I	V	I	A	F	I	A	V	S	H	C	S	V	G	-	H	S	E	L	Q	S	F	L	Q	E	L	T	-	-	-	E	E	T	T	P	L	H	L	D	I	T	R	S	
A.n.	G	L	Y	T	F	I	I	A	V	I	L	L	N	G	G	T	L	Q	-	K	L	D	R	Y	L	S	R	M	N	A	-	-	-	E	Q	F	T	P	V	R	-	T	H	L				
N.f.	G	L	Y	S	F	I	I	A	V	I	L	L	N	G	G	S	L	P	-	K	K	L	E	R	Y	L	K	R	T	N	A	-	-	-	D	T	Y	T	P	I	R	-	T	R	F			
A.t.	G	L	Y	T	F	I	I	A	L	I	L	L	N	G	G	S	L	P	-	K	K	L	E	R	Y	L	Q	R	T	N	A	-	-	-	D	T	Y	T	P	I	R	-	T	R	F			
A.c.	G	L	Y	S	F	I	I	A	V	I	L	L	N	G	G	S	L	P	-	K	K	L	E	R	Y	L	K	R	T	N	A	-	-	-	D	T	F	T	P	I	R	-	T	R	F			
N.c.	G	L	Y	T	M	L	I	A	I	I	T	L	S	G	G	E	L	S	-	P	R	L	R	R	Y	L	T	R	I	N	A	A	x	P	N	N	E	N	A	P	S	K	-	T	L	V		
M.g.	G	L	Y	S	M	I	V	T	I	I	Q	L	N	R	G	E	L	S	-	P	K	L	K	R	Y	L	Q	R	L	N	A	-	-	-	E	T	N	T	P	V	K	-	T	L	L			
A.o.	G	L	Y	S	F	I	I	A	V	I	M	L	N	G	G	S	L	P	-	K	K	L	D	R	Y	L	A	R	T	N	A	-	-	-	D	T	Y	I	P	I	R	-	T	R	L			
S.c.	G	V	L	S	V	I	L	C	I	V	F	F	S	K	N	N	I	L	-	H	O	E	L	I	K	F	L	E	T	F	G	I	P	S	D	G	S	K	I	A	I	L	N	I	T	I	D	L
D.r.	G	L	L	F	V	I	L	S	V	I	F	M	K	G	G	T	I	K	-	N	L	V	W	N	T	L	K	K	L	R	L	D	P	G	E	K	H	D	E	F	G	V	-	K	K	V	V	
X.t.	G	L	L	M	V	I	L	S	L	I	F	M	K	G	N	T	A	K	-	S	A	V	W	E	M	L	R	R	L	R	E	P	A	E	K	H	S	D	F	G	V	-	K	K	L	I		

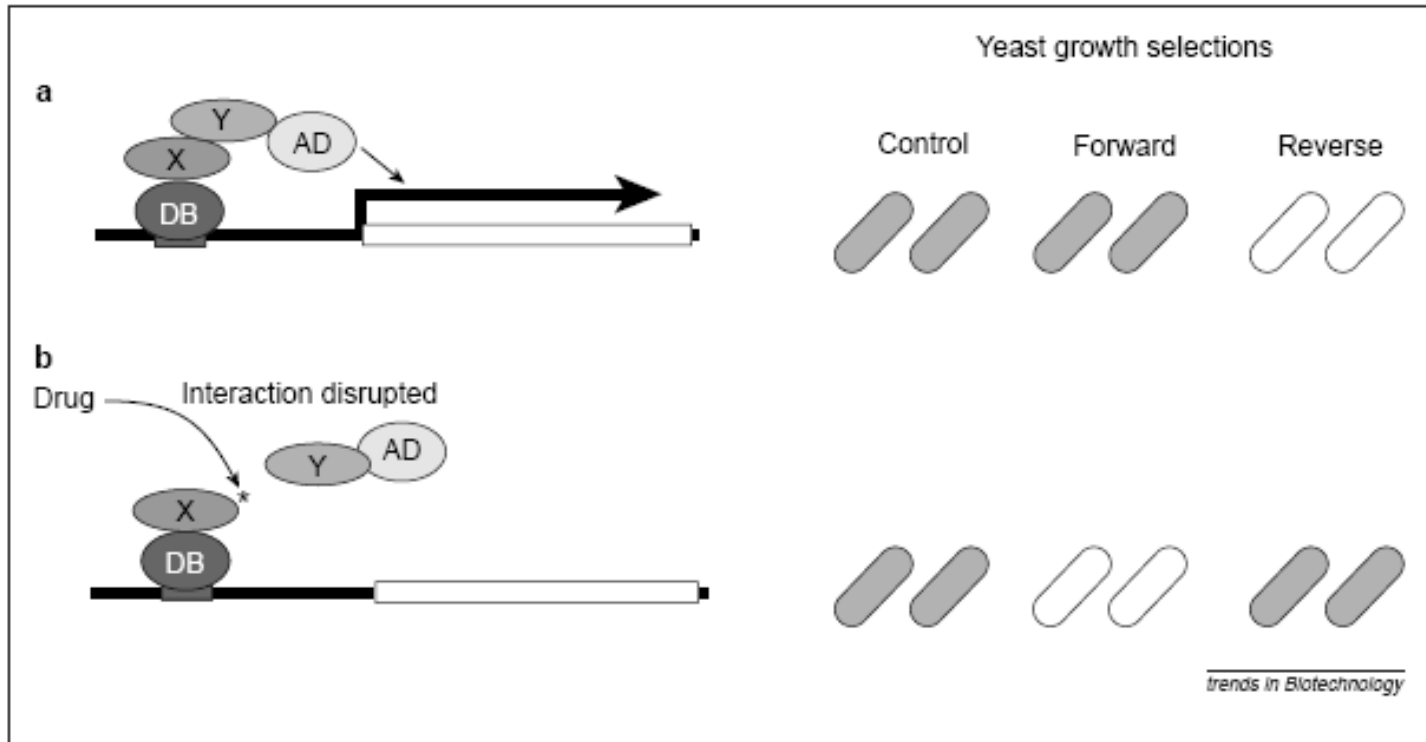


“alanin scan” konzervovaných AMK ukázal hydrofobní kapsu na povrchu Nse3

Do níž se váže hydrofobní šroubovice Nse4 proteinu

Pomocí *in silico* (MD) analýzy byl vytvořen model dimeru Nse3-Nse4 (docking)

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)

Split-hybrid systém

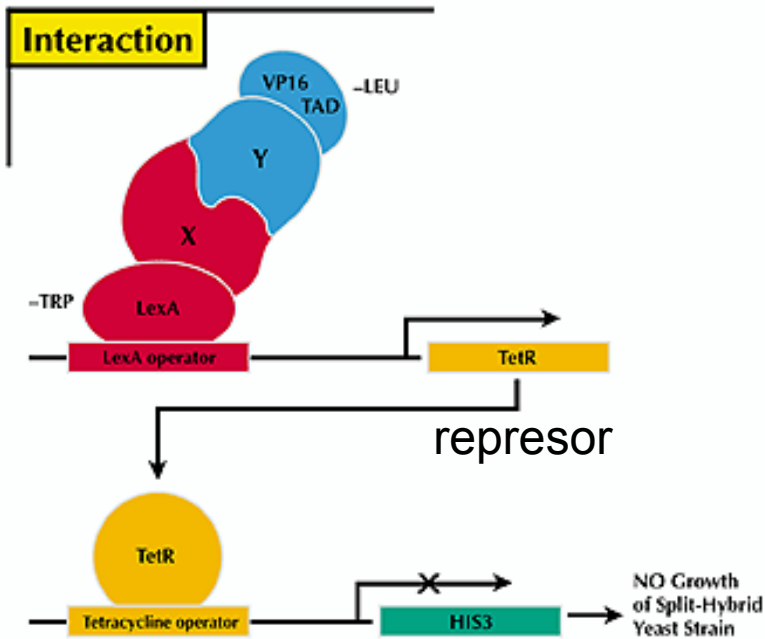


Fig. 1
Protein X is fused to the LexA DNA binding domain and Protein Y is fused to the transcriptional activator domain, VP16-TAD. Interaction between X and Y leads to the expression of the tetracycline repressor protein TetR. TetR expression prevents transcription of the HIS reporter gene making cells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

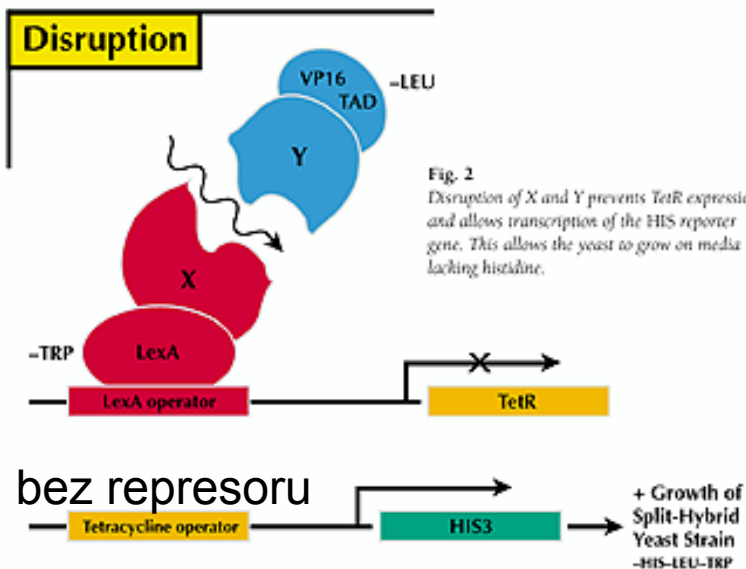
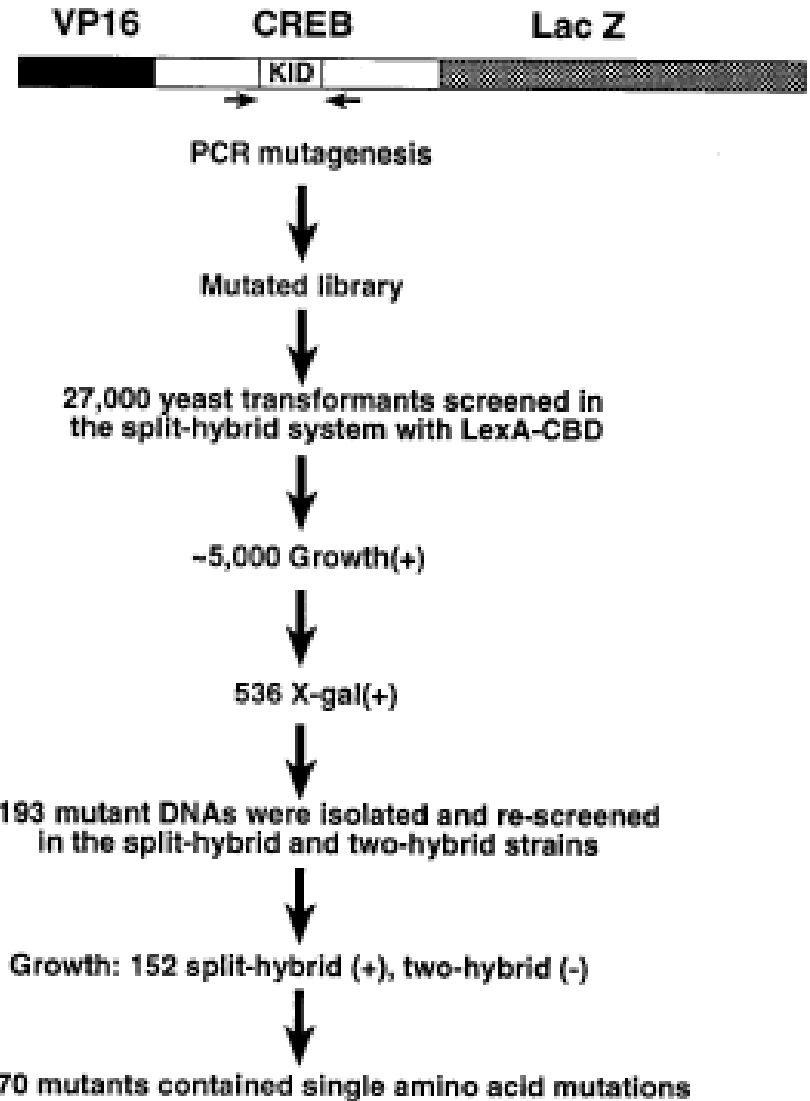
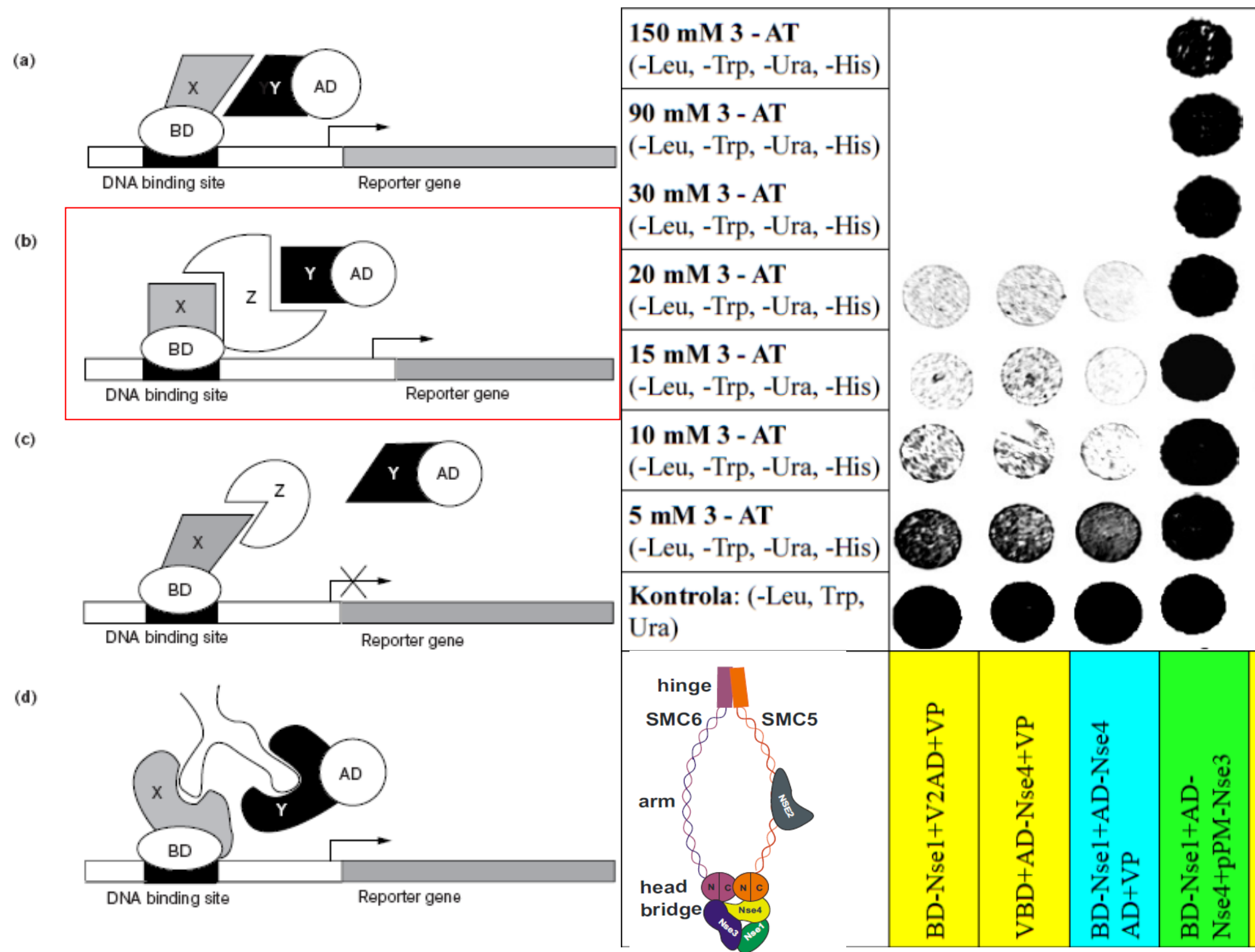
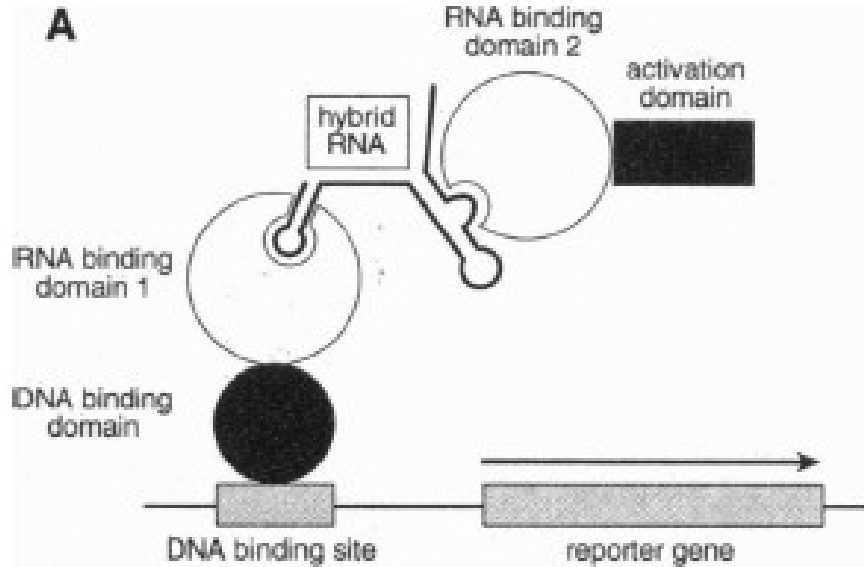


Fig. 2
Disruption of X and Y prevents TetR expression and allows transcription of the HIS reporter gene. This allows the yeast to grow on media lacking histidine.

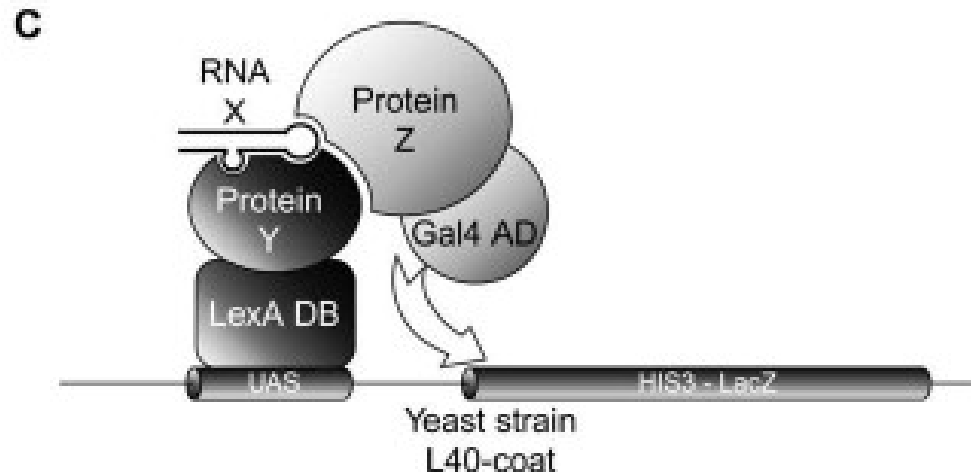
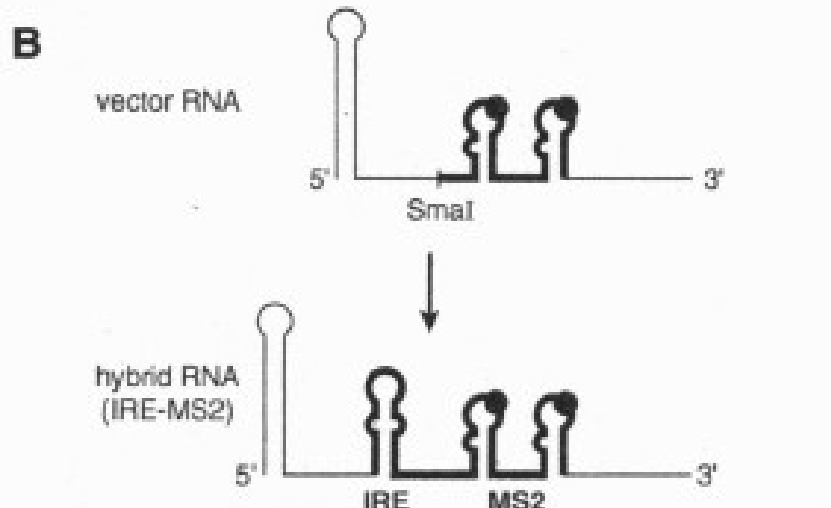




Analýza vazby protein-RNA (Y3H)

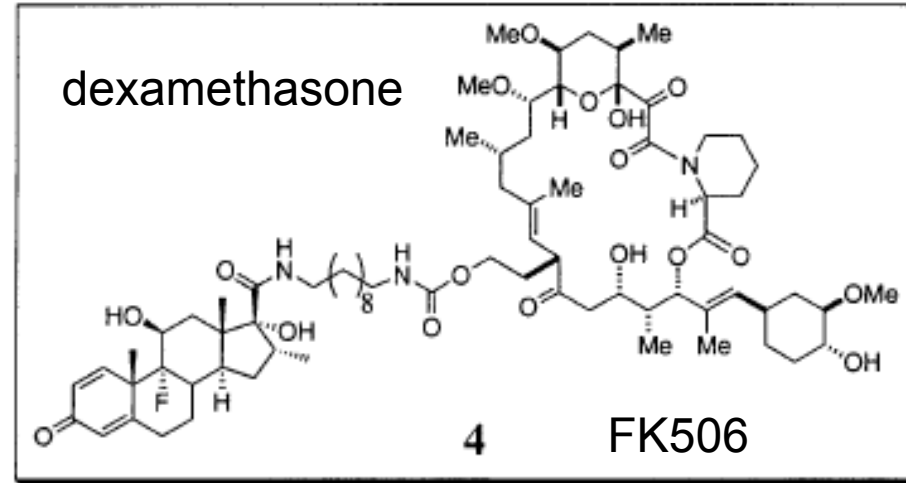
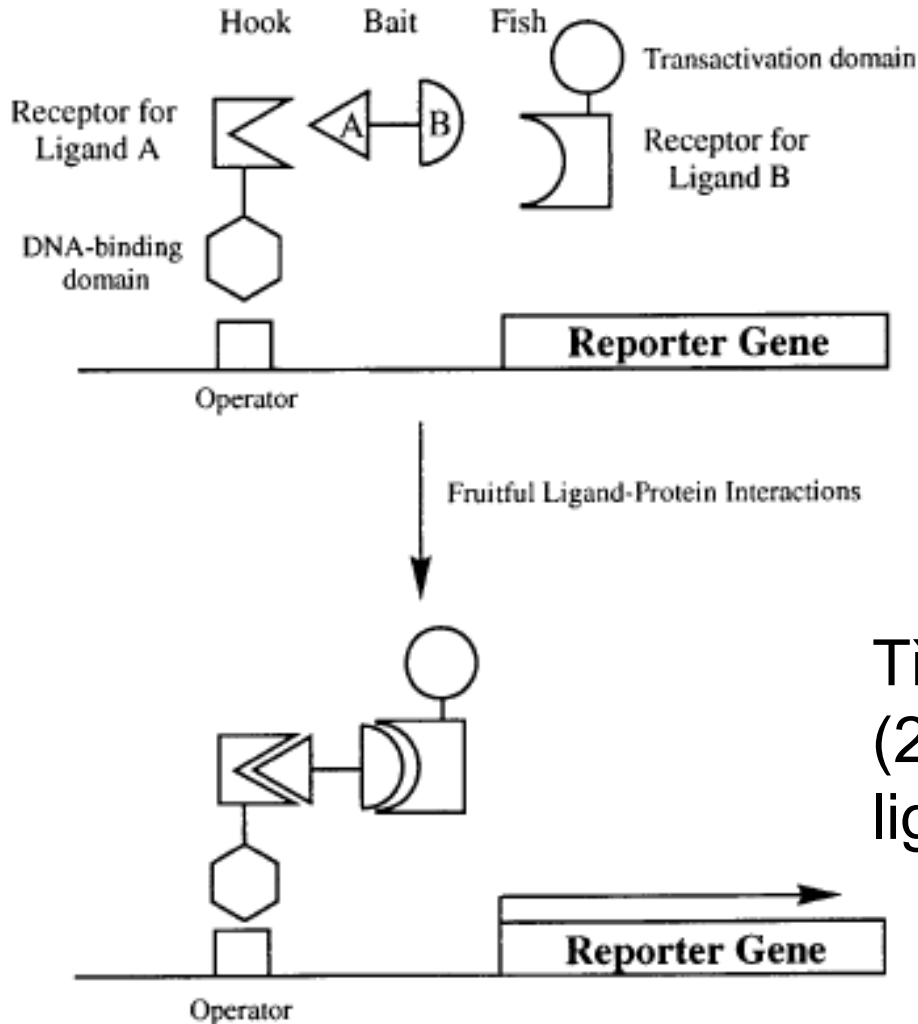


Tři fúzní makromolekuly
(2x protein a 1x RNA)



Vazba ligand-receptor (Y3H)

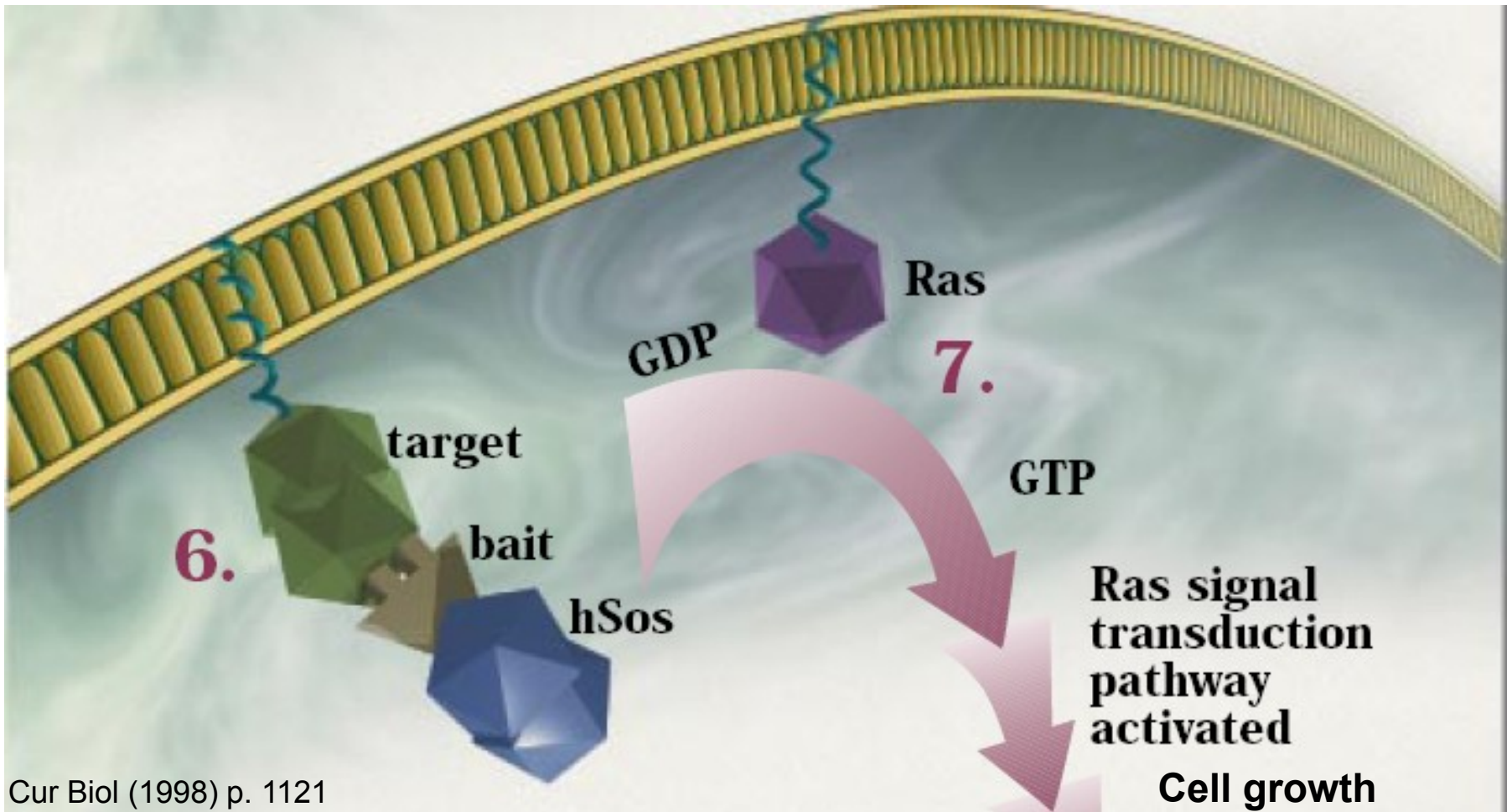
glucocorticoid receptor - FKBP12



Tři fúzní makromolekuly
(2x protein a 1x nízkomolekulární
ligand)

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 fúzován k hSOS

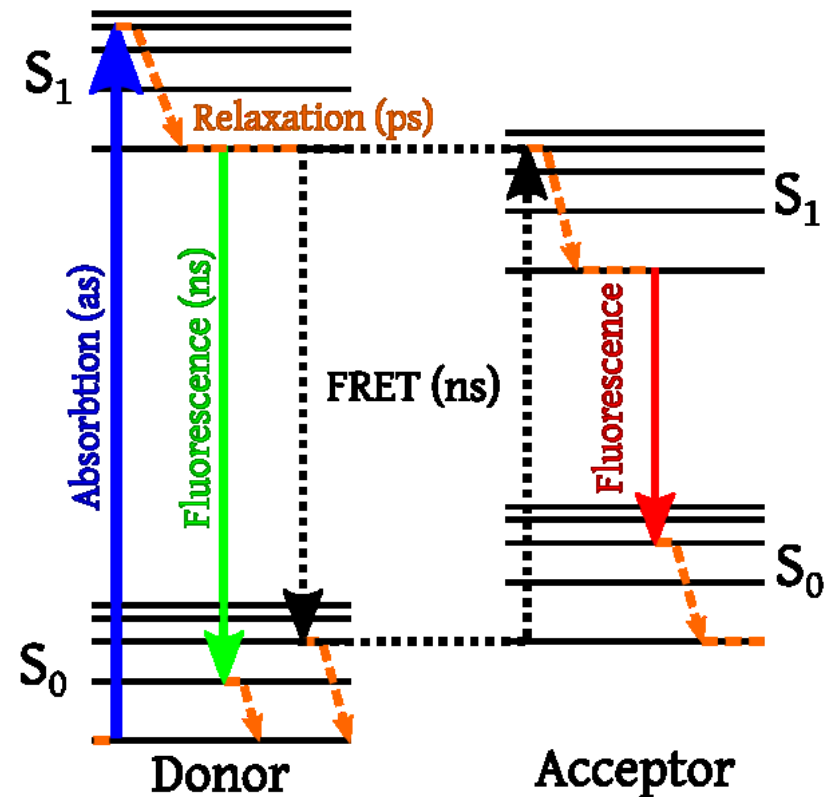
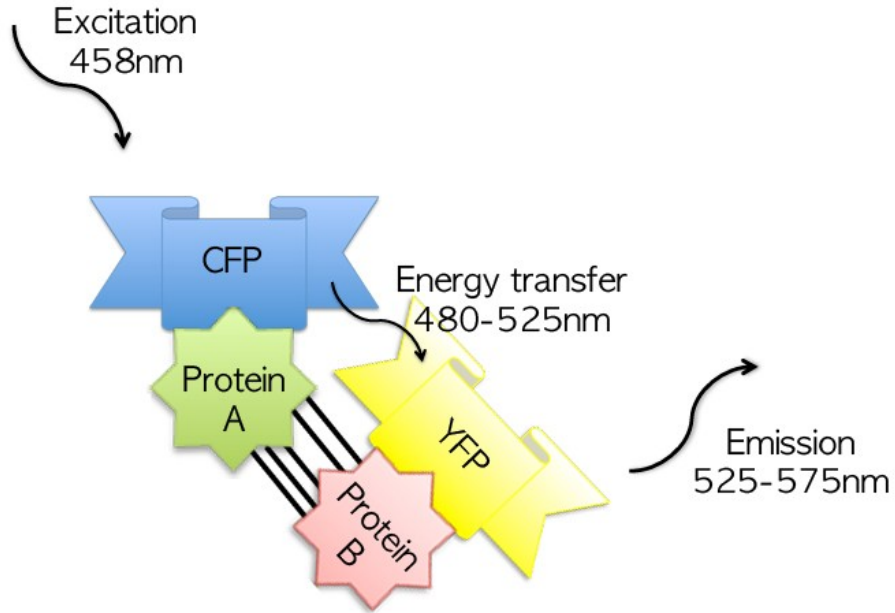


- Y2H systémy mají výhodu selekce – přežití kvasinek – je možné je využít pro hledání nových partnerů

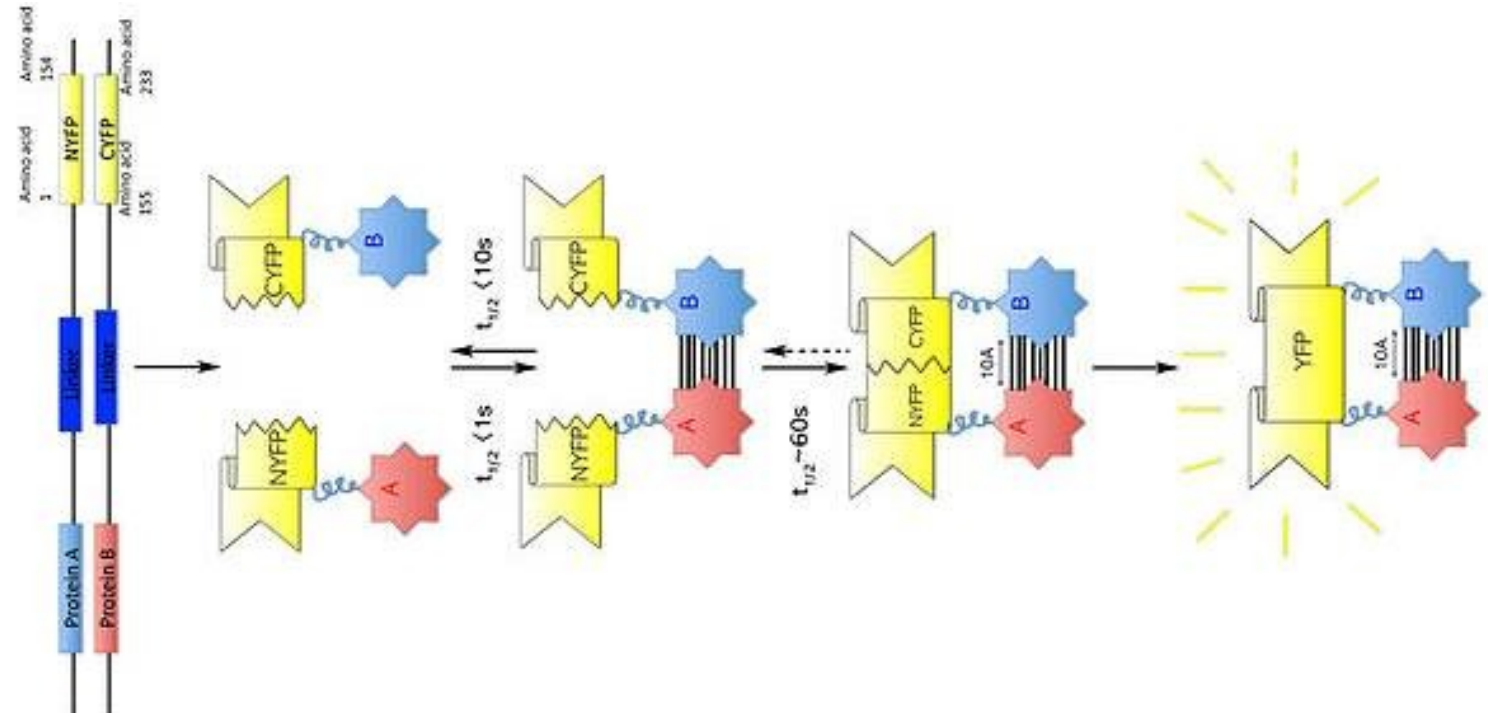
Metody analýzy protein-proteinových interakcí

- ko-imunoprecipitace, pull-down, ko-purifikace ...
- (kvasinkový) dvou-hybridní systém
- **FRET, BiFC, ko-lokalizace, ko-exprese**
- Flourescenční anisotropie, SPR, ITC ...
- ko-krytalizace, cryoEM ...
- databáze (interactom a komplexy ...)
- genetické metody (syntetická letalita, suprese)

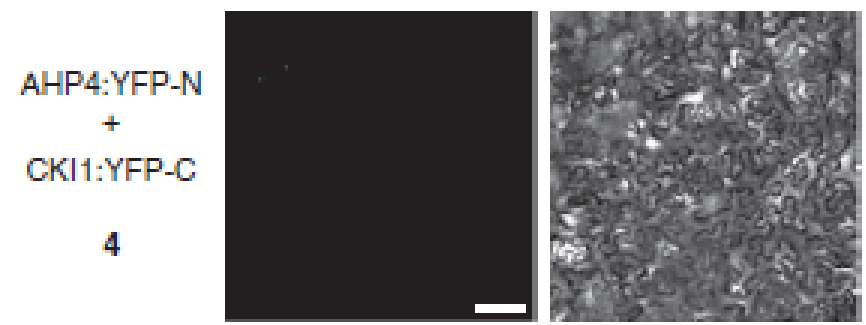
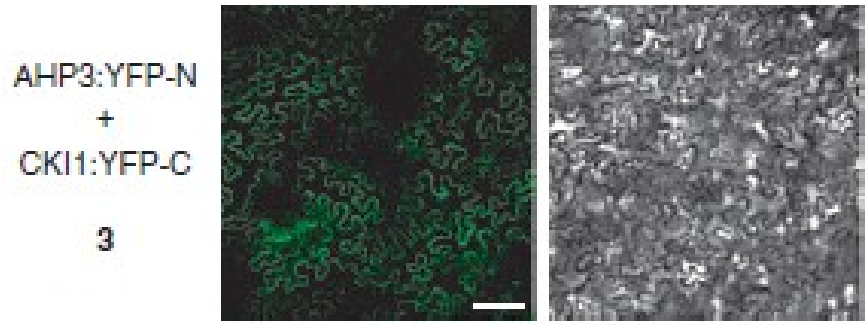
Förster/fluorescence resonance energy transfer



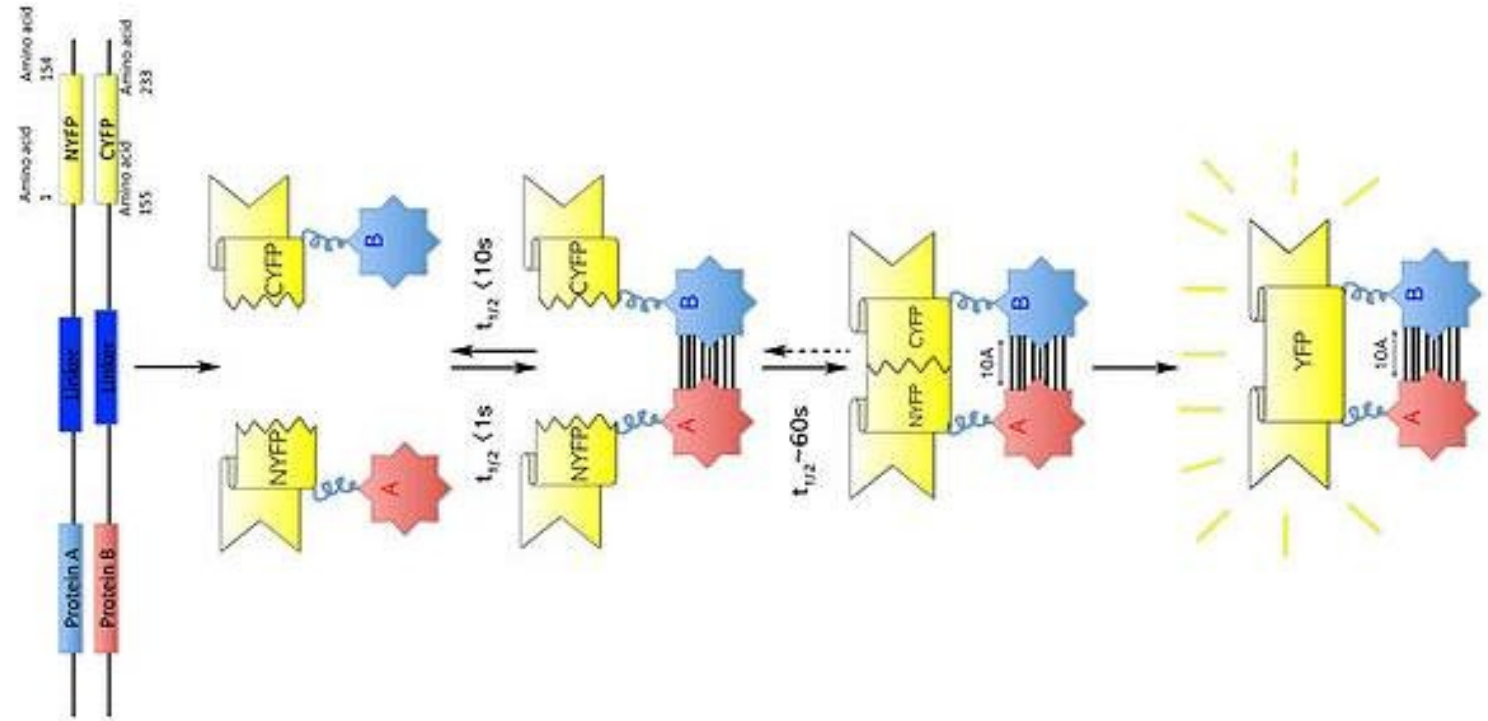
Bimolecular fluorescence complementation - BiFC



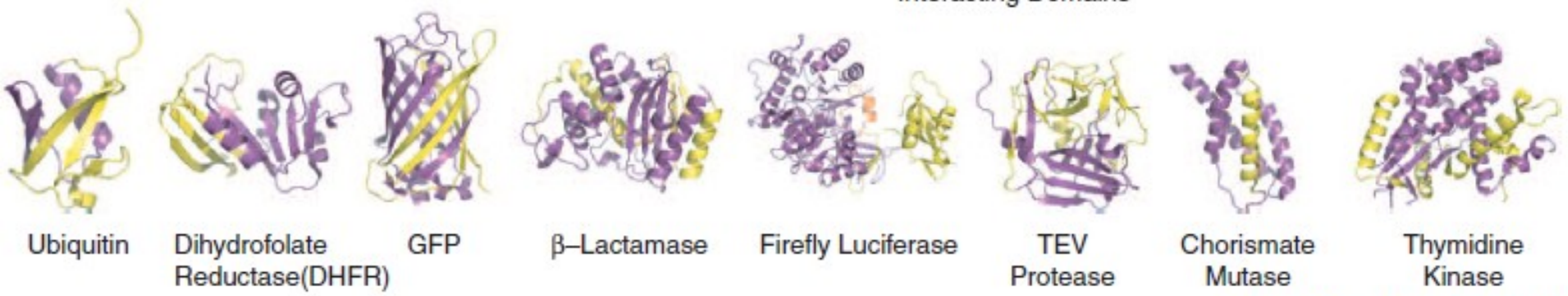
Propojuje se zpět fold/struktura nikoli 2 domény jako u Y2H (lokalizace proteinů do tkání, buněčných kompartmentů ...)



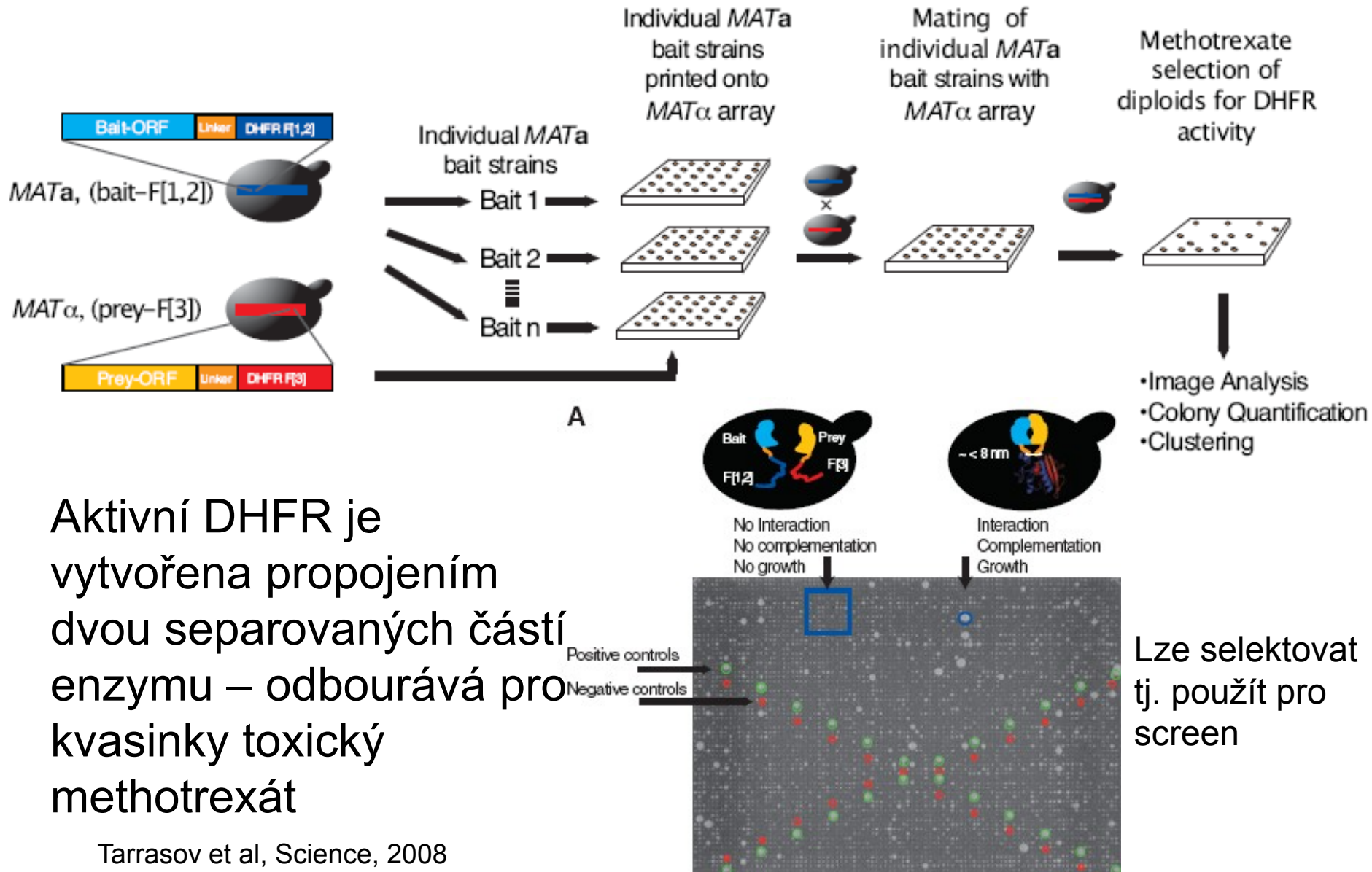
Bimolecular fluorescence complementation - BiFC



Protein-fragment complementation



Dihydrofolát reduktáza/methotrexát



Aktivní DHFR je vytvořena propojením dvou separovaných částí enzymu – odbourává pro kvasinky toxický methotrexát

Lze selektovat tj. použít pro screen