Interazioni proteina-proteina

Metodi genetici basati su *split proteins*

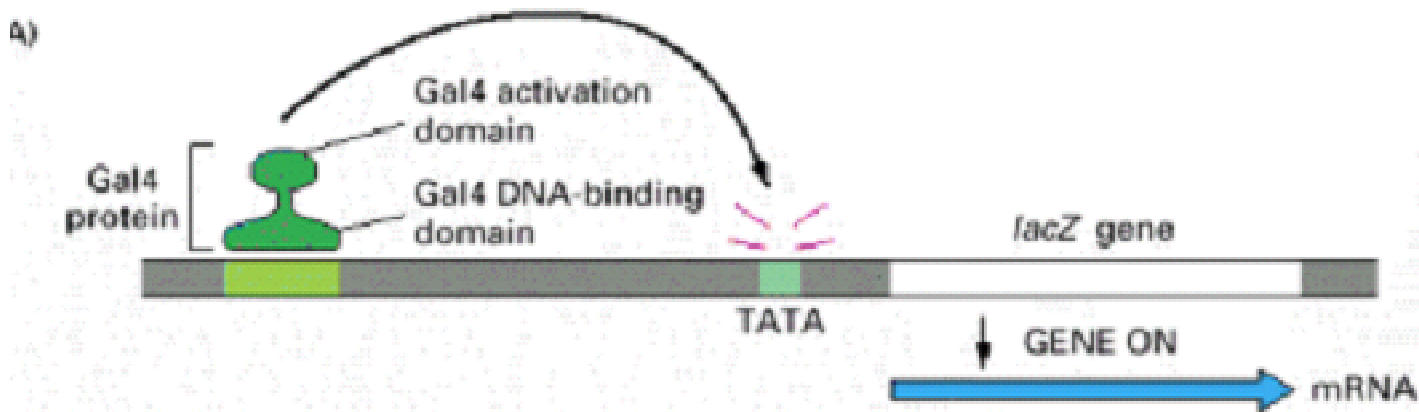
Sistema a doppio ibrido

1989 by Fields and Song

•E' un metodo genetico che usa l'attività trascrizionale come misura dell'interazione proteina-proteina *in vivo*

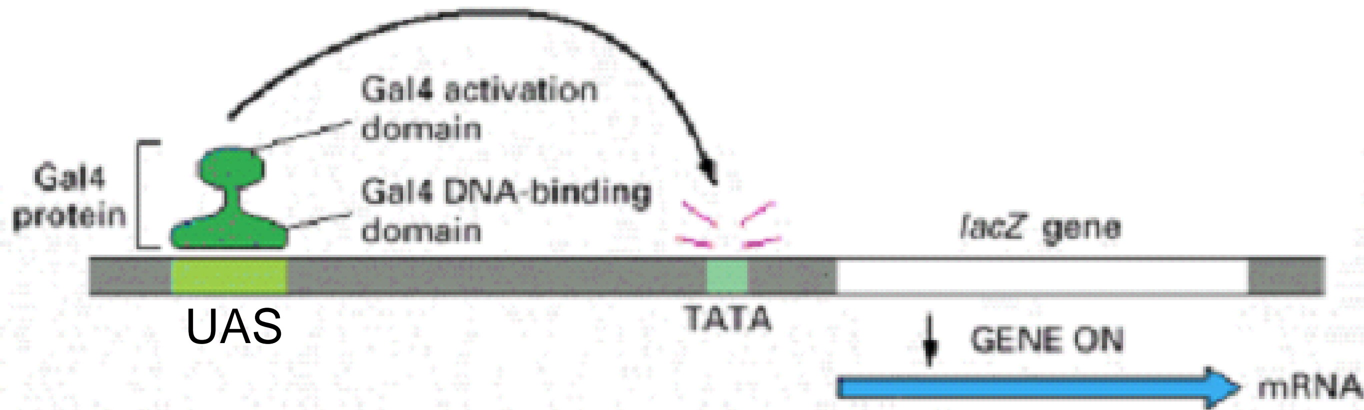
•Gli attivatori di trascrizione sono delle proteine caratterizzate da un dominio che lega il DNA **BD** e un dominio che attiva la trascrizione **AD**

•Il dominio che lega il DNA serve per indirizzare il dominio attivatore sul gene che deve essere trascritto mentre il dominio attivatore direttamente viene a contatto con il complesso trascrizionale



•Il fattore di trascrizione Gal 4 può essere separato in una unità capace di legare il DNA e una unità in grado di attivare la trascrizione.

147 residui N- terminali codificano per il DNA binding domain (DB) o "bait"
114 residui C-terminali codificano per un fattore che attiva la trascrizione (AD) "prey "



Per attivare la trascrizione, i due domini non devono necessariamente essere legati covalentemente ma possono essere messi a contatto dall'interazione di altre due proteine ad esempio X ed Y (Field e Song 1989)

Costruzione dei due ibridi

•il DNA del binding domain deve essere fuso al DNA della proteina X

• il DNA del dominio attivatore deve essere fuso al DNA della protein Y.

•Le due chimere devono essere espresse in una cellula in cui sono presenti uno o più geni reporter

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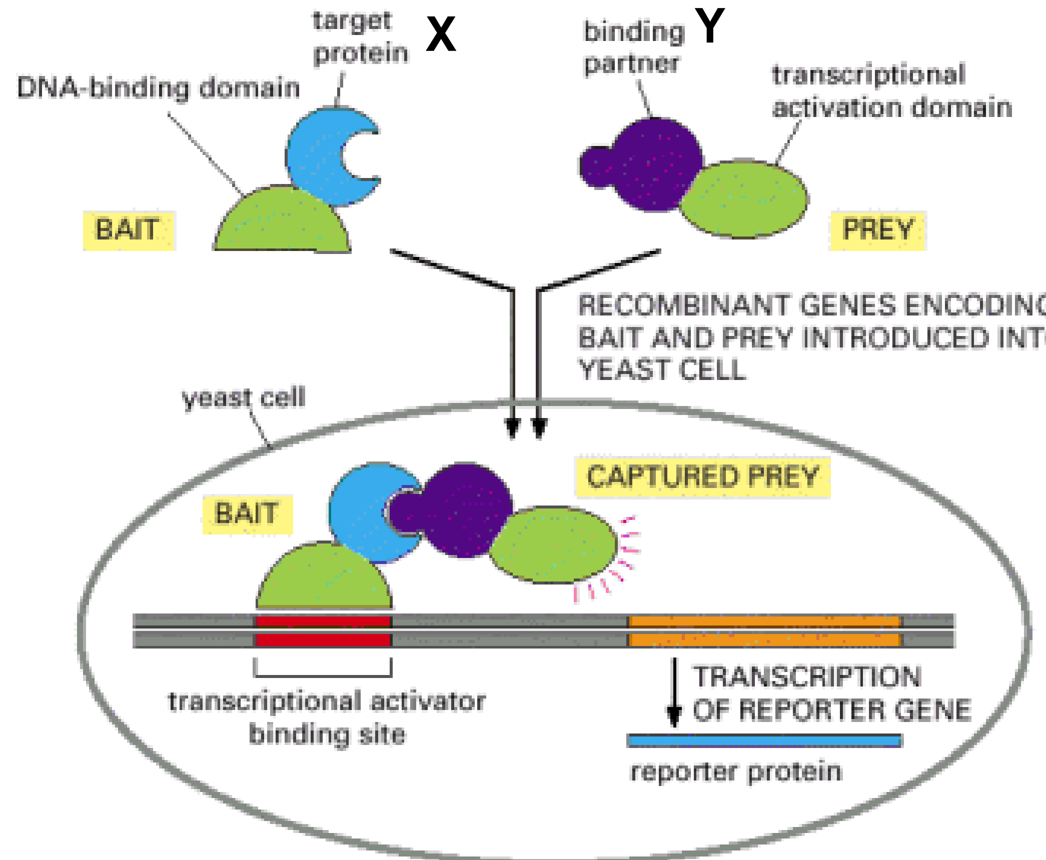
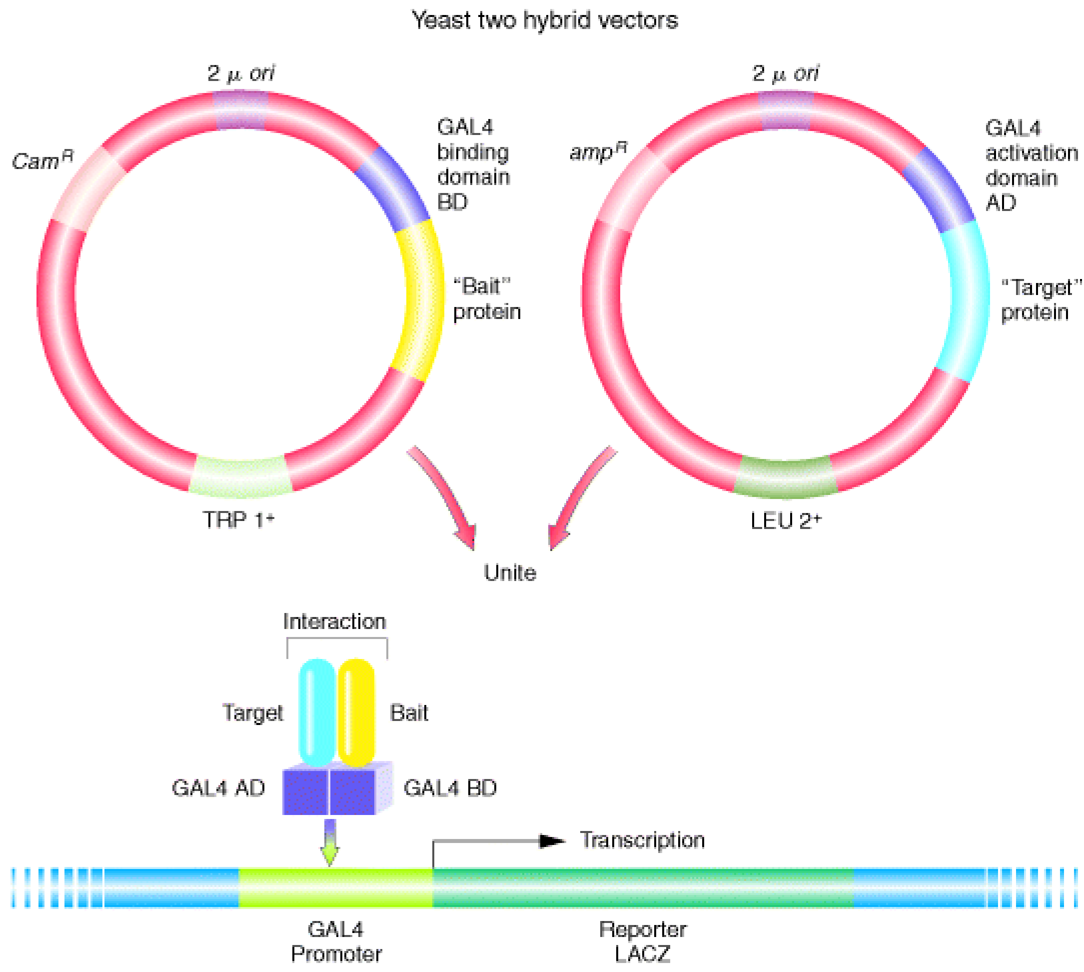


Se X ed Y interagiscono si ha la ricostituzione funzionale dell'attivatore trascrizionale che attiva l'espressione del gene reporter

I due vettori contengono GAL4 binding domain fuso alla “Bait” protein e GAL4 activation domain fuso alla target protein



Applicazione

Capacità di isolare rapidamente nuovi geni .
E' possibile preparare delle" library" di cDNA fuse al dominio di attivazione da introdurre in ceppi che esprimono "DNA-binding domain" fusi alla proteina-esca.

Limiti

Non è possibile studiare l'interazione di proteine di membrana o di DNA binding protein che da sole abbiano la capacità di attivare la trascrizione

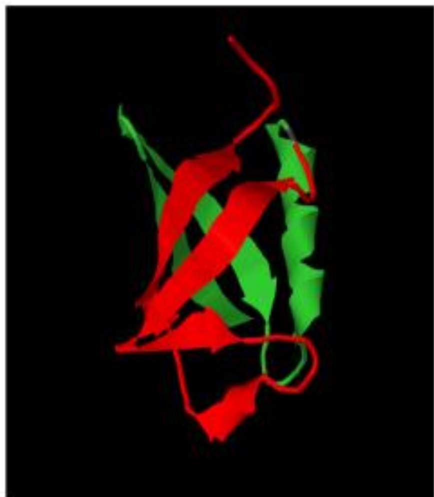
Protein Complementation Assay

- la proteina reporter è separata in due frammenti

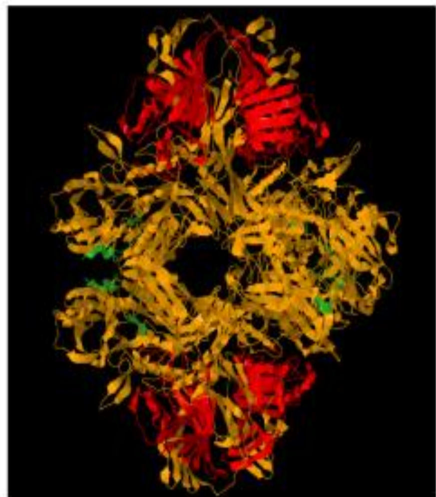
- i frammenti sono fusi geneticamente ai potenziali partners dell’interazione

- il ripristino della funzionalità della proteina reporter è mediato dall’interazione delle proteine partners

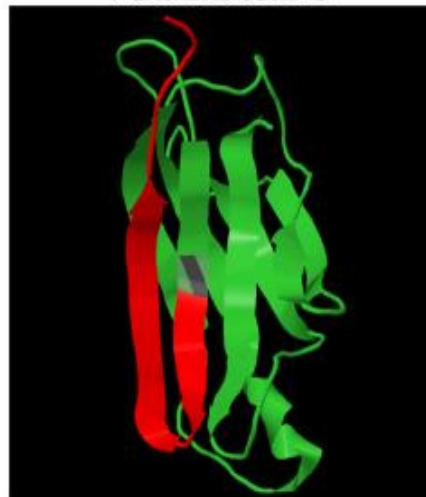
ubiquitin



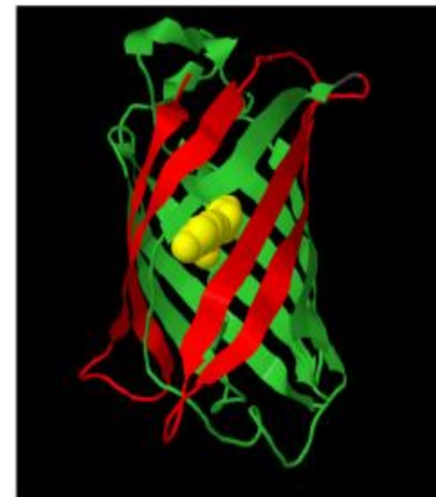
β-galactosidase



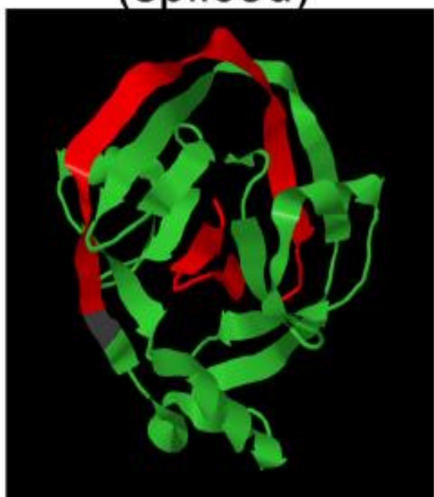
dihydrofolate reductase



GFP variants

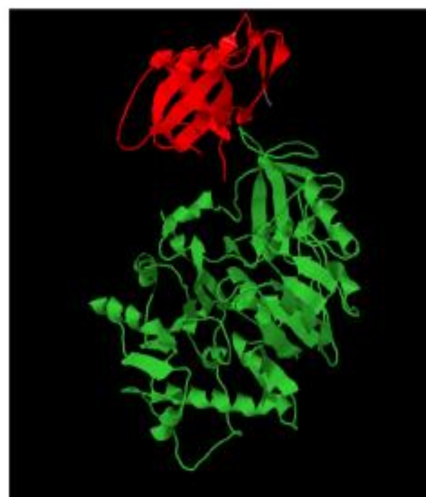


dnaE intein (spliced)

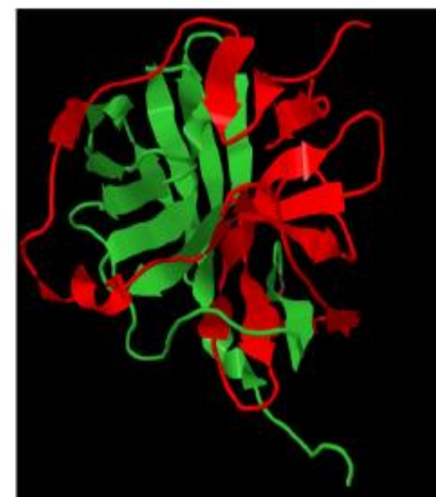


β-lactamase

firefly luciferase



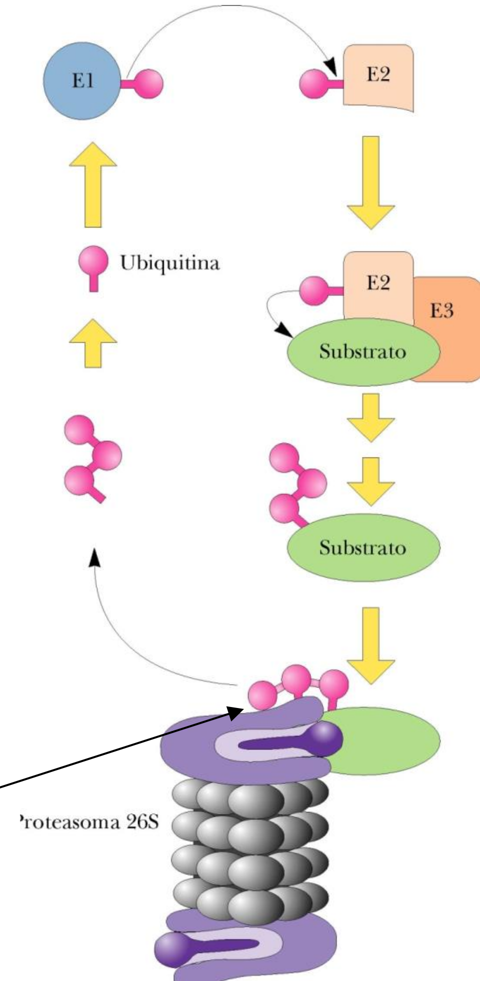
TEV protease



Split ubiquitin membrane-based yeast two hybrid system

L'ubiquitina è una proteina di 76 amminoacidi che partecipa al turnover proteico

L'estremita C- terminale dell'ubiquitina forma un legame ammidico con la proteina che deve essere degradata



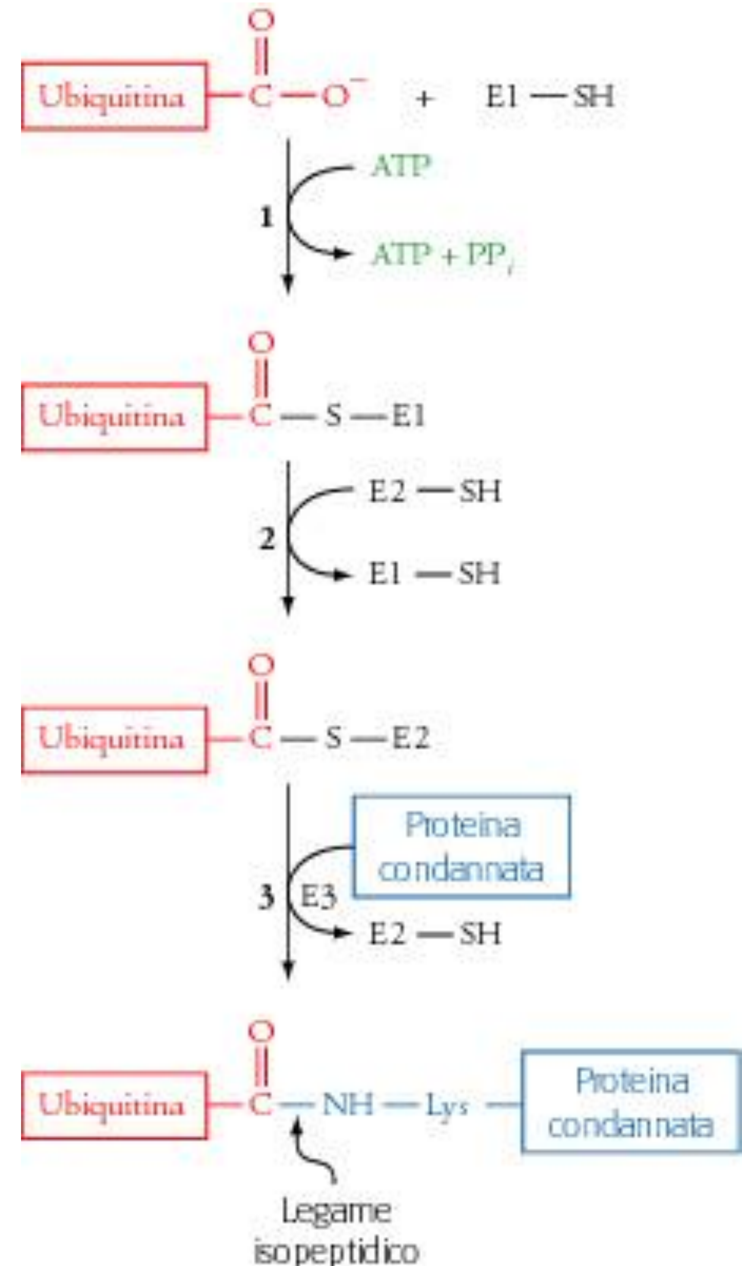
UBPs(ubiquitin- specific- proteases) idrolizzano il legame peptidico tra la proteina " condannata" e l'ubiquitina

La reazione avviene in tre tappe

•E1 attiva l'ubiquitina mediante legame tioestere che avviene con il consumo di ATP

• L'ubiquitina viene trasferita su E2

•E3 (PROTEINA-UBIQUITINA LIGASI)catalizza il trasferimento dell'ubiquitina su una lisina della proteina condannata



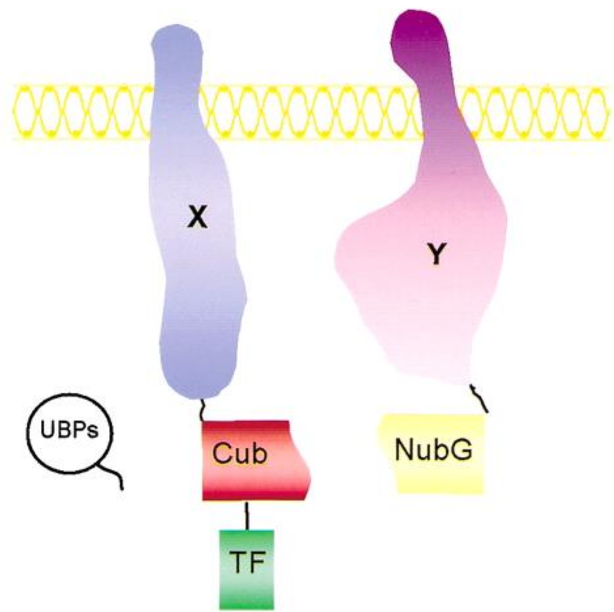
E3 svolge un ruolo nel riconoscimento della proteina condannata

•L'ubiquitina può essere espressa in due frammenti:

Nub (N terminal fragment 1-34)

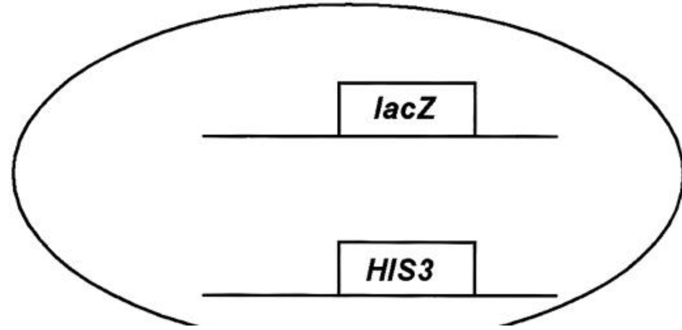
Cub (C-terminal fragment 35-76) che possono riassociarsi a formare l 'ubiquitina

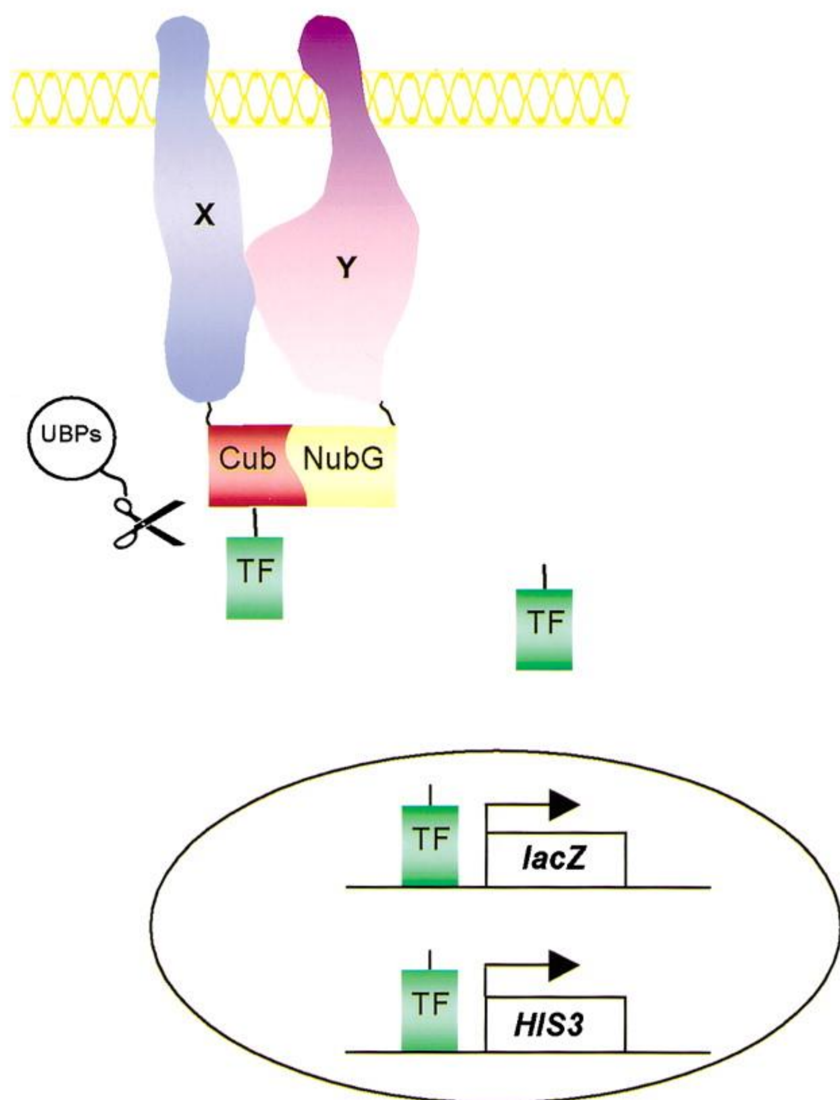
Costruzione dei due ibridi

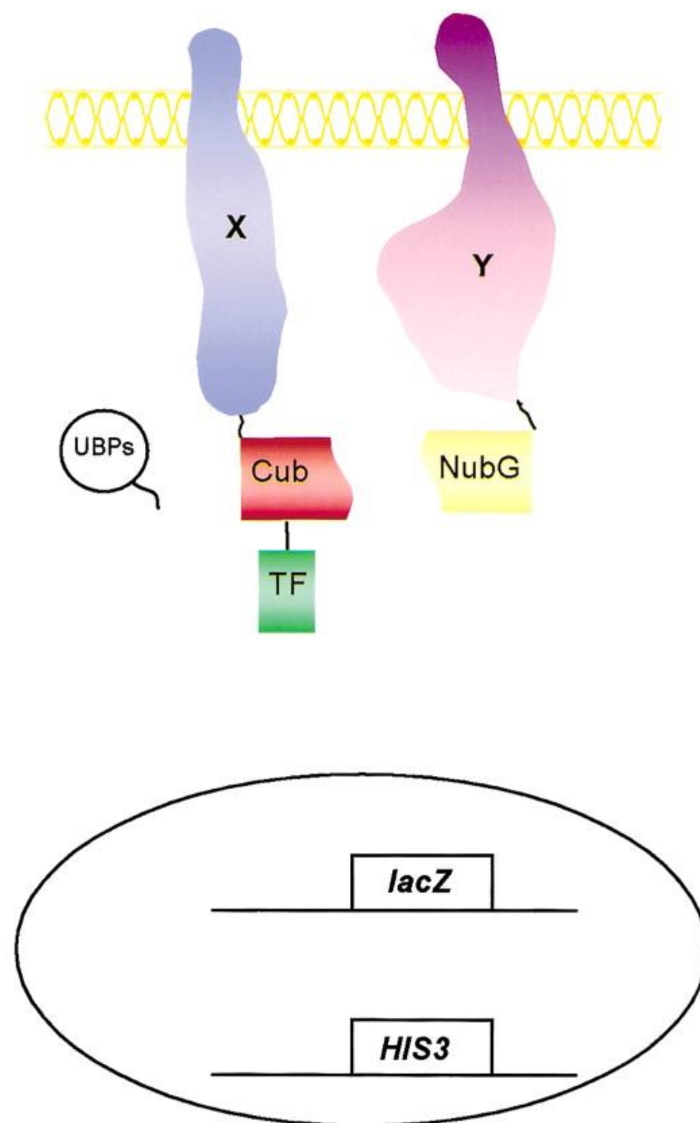


Al 5' del DNA di Cub è fuso il c DNA della protein X
al 3'di Cub è fuso il cDNA di TF
al DNA di Nub è fuso il DNA della protein Y.

NubG ha una mutazione puntiforme (Ile13→ Gly13) che non permette l'associazione spontanea con Cub. **L'associazione avviene solo quando le due proteine fuse (X e Y) interagiscono**



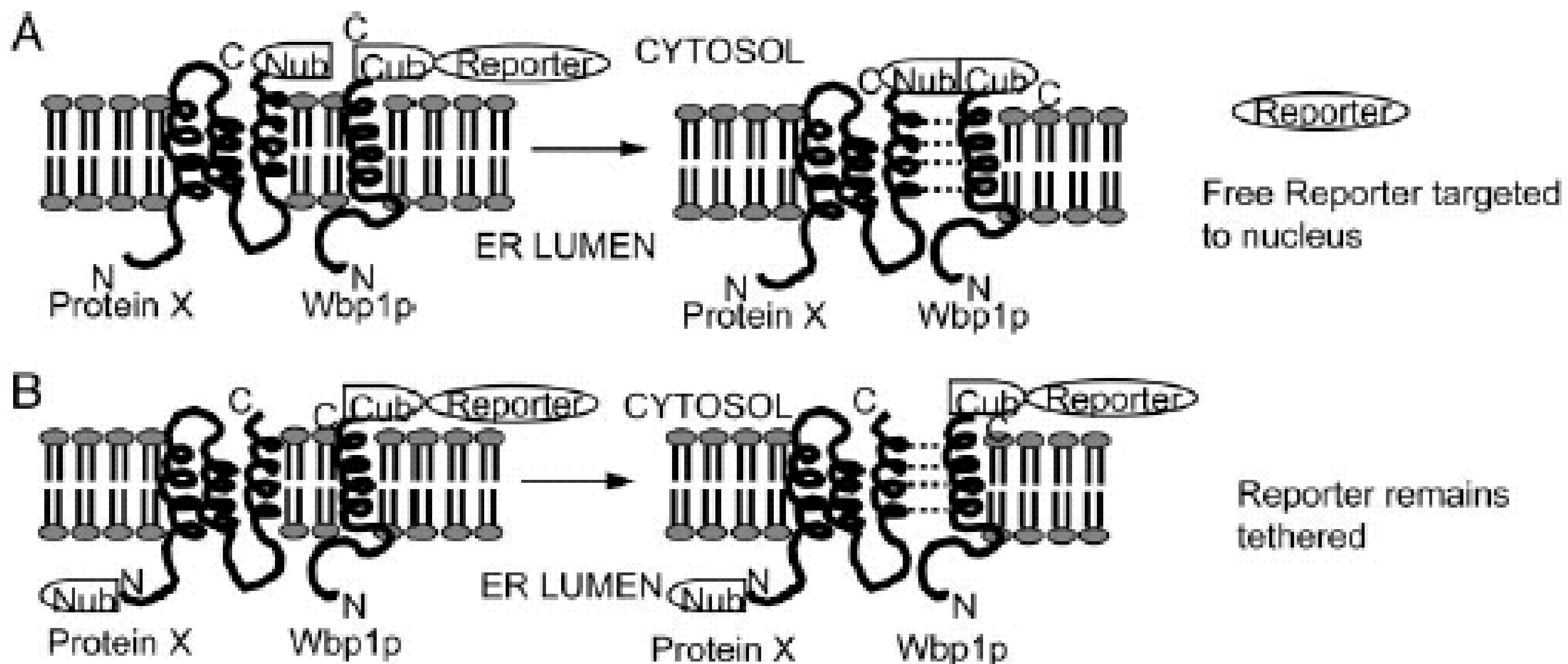

A
X
Y
UBPs
Cub
NubG
TF
TF
TF
lacZ
TF
HIS3


B
X
Y
UBPs
Cub
NubG
TF
lacZ
HIS3

Nub e Cub devono essere legate alla regione della proteina di membrana che si localizza nel citosol dato che la proteasi è localizzata nel citosol

Pertanto non si può studiare l'interazione tra due proteine che hanno entrambe le regioni C terminali nel lumen del reticolo endoplasmatico

E' possibile avere informazioni sull'orientamento della regione N e C terminale delle proteine di membrane



PNAS

Studies of yeast oligosaccharyl transferase subunits using the split-ubiquitin system: Topological features and *in vivo* interactions

Aixin Yan, Elain Wu, and William J. Lennarz*

Department of Biochemistry and Cell Biology and Institute for Cell and Developmental Biology, Stony Brook University, Stony Brook, NY 11794

Contributed by William J. Lennarz, March 31, 2005

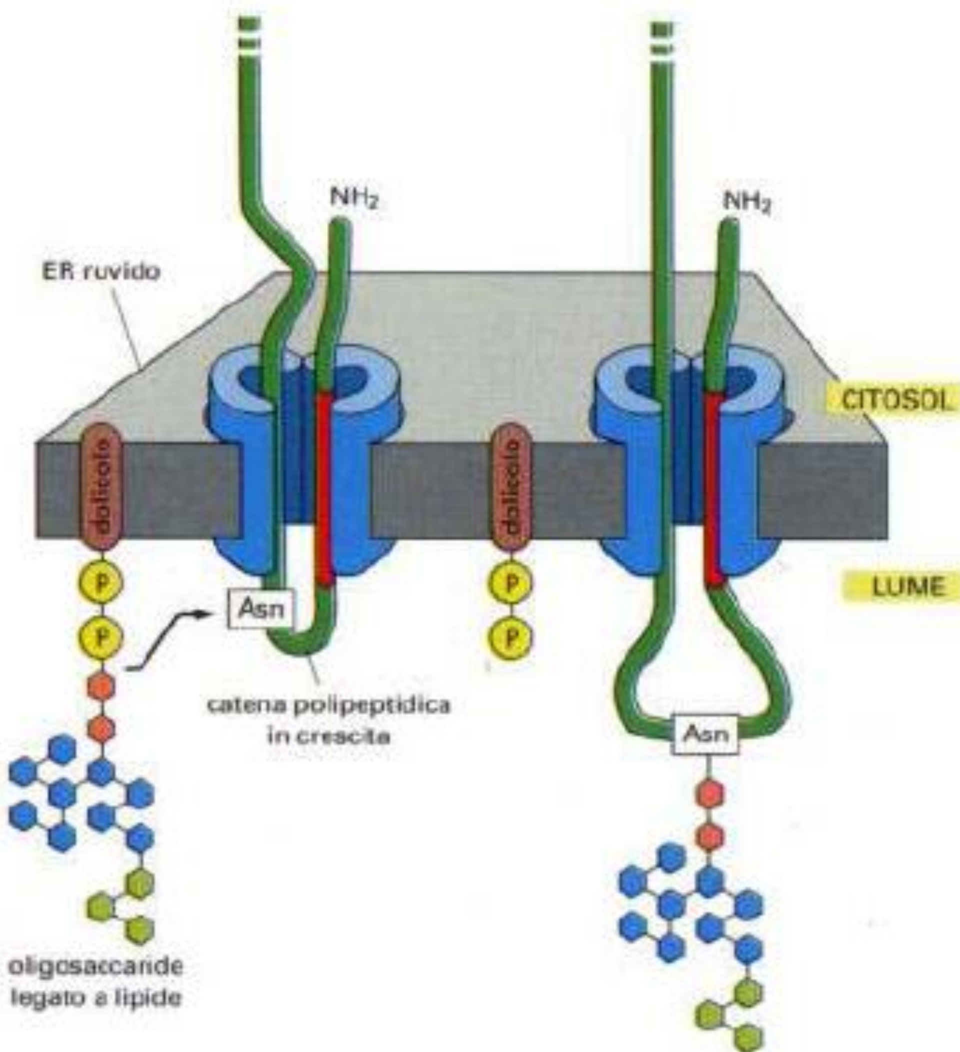
Oligosaccharyl transferase (OT) catalyzes the cotranslational N-

specifically applies to membrane proteins, the split-ubiquitin

Oligosaccaril transferasi (OT) catalizza la N-glicosilazione sull'asparagina di una sequenza consenso Asn-X-Thr / Asn-X- Ser) quando il polipeptide nascente è traslocato nel reticolo endoplasmatico

D

b



.
Nel lievito sono stati identificati nove geni che codificano le subunità di questo complesso proteico di membrana OST1,OST2, OST3, OST4, OST5, OST6,WBP1, SWP1, STT3

In questo lavoro mediante "split-ubiquitin system" sono stati chiariti due aspetti:
l'orientamento in membrana di tutti i componenti
L' interazione *in vivo* delle diverse subunità

Wbp1 interagisce con Ost2p Ost6p Stt3p

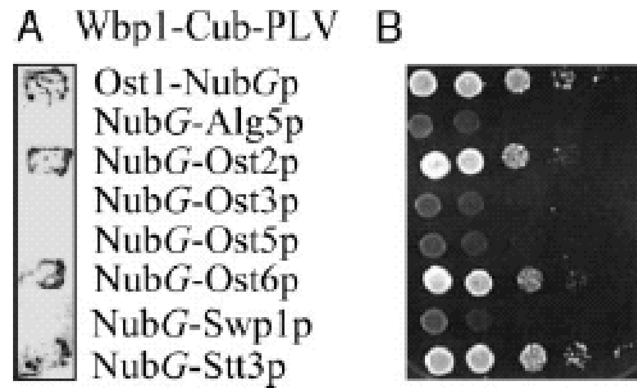
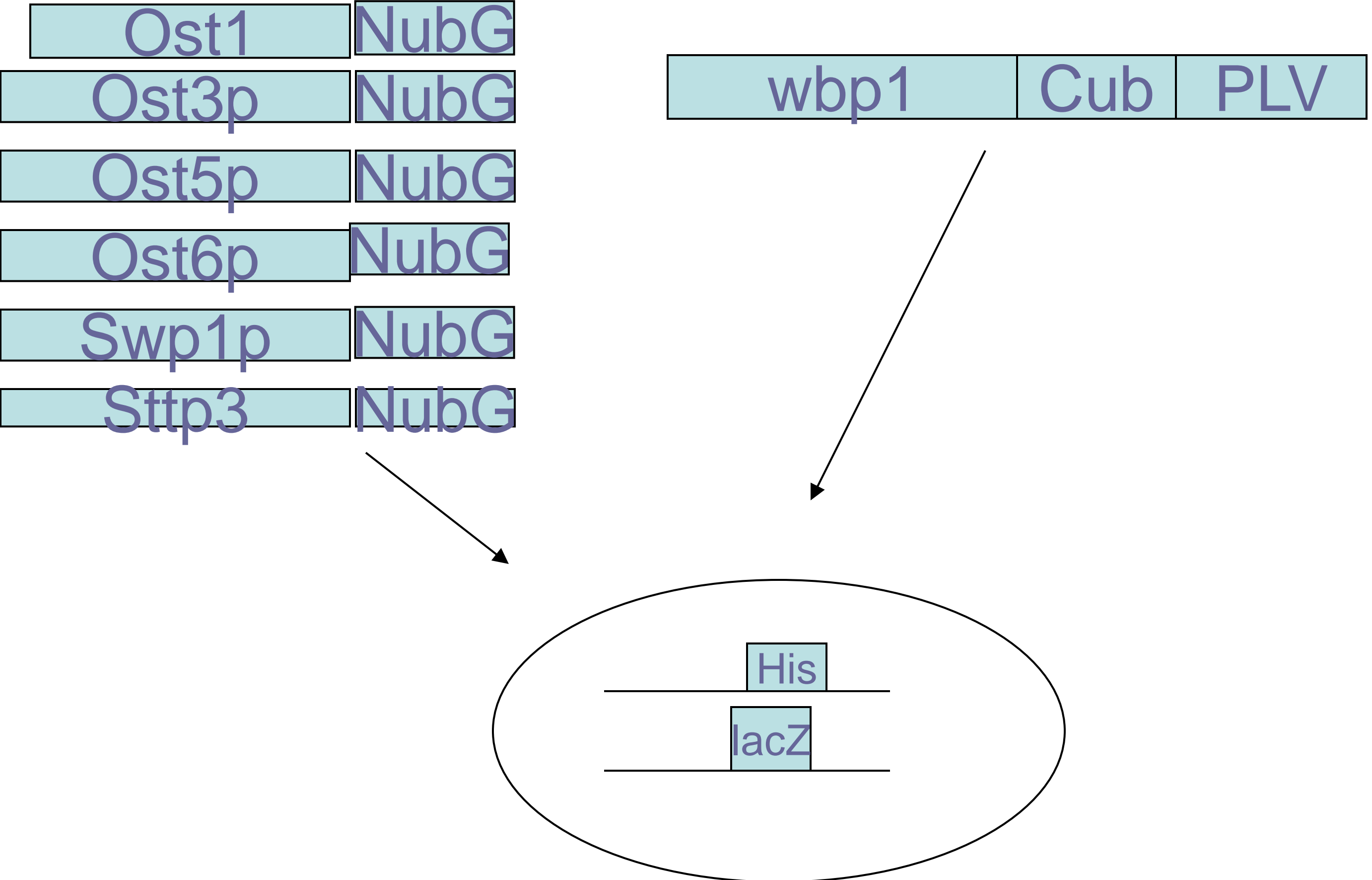


Fig. 2. Split-ubiquitin analysis between Wbp1-Cub-PLVp and NubG-Xp (X represents Ost2p, Ost3p, Ost5p, Ost6p, Stt3p, or Swp1p). (*A*) β-Gal activity of the transformants expressing Wbp1-Cub-PLVp together with the NubG-Xp fusion proteins. Cells were grown on plates lacking tryptophan and leucine, transferred to Whatman filters, permeabilized, and incubated in the presence of X-Gal. (*B*) Growth of the cells expressing Wbp1-Cub-PLVp with various NubG-Xp on agar plates lacking histidine. Cells were grown to logarithmic phase, 7 μl of 1:10 serial dilutions was spotted on the SD plates lacking tryptophan, leucine, and histidine and incubated at 30°C for 3 days. As a positive control, YG0673 was transformed with the plasmid expressing Ost1-NubGp, and, as a negative control, YG0673 was transformed with the plasmid expressing NubG-Alg5p.

Ostp2 Ost6p Stt3p hanno l’ N terminale orientato nel citosol

NubG è stato fuso al 3' dei cDNA delle proteine dell'Oligosaccariltransferasi



Ost3 Ost6 Swp1 presentavano attività β galattosidasica e potevano crescere in assenza di His

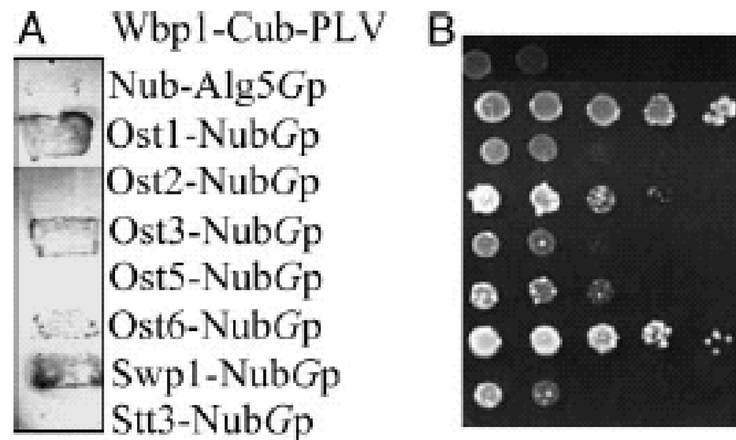


Fig. 3. Split-ubiquitin analysis between Wbp1-Cub-PLVp and X-NubGp (X represents Ost2p, Ost3p, Ost5p, Ost6p, Stt3p, or Swp1p). Shown are β-Gal activity (*A*) and growth of cells coexpressing Wbp1-Cub-PLVp together with X-NubGp fusion proteins on agar plates without histidine (*B*). The procedures, as well as the negative and positive controls, are as described in the legend to Fig. 2.

L'Nterminale di Ost3 e Swp1 era localizzato nel lumen del reticolo

La coespressione di sia NubG Ost5p che di Ost5 NubGp con WbpiCubPLV portava a cloni che non complementavano

Ciò poteva significare:

1. Assenza di interazione in vivo con Wbp1
2. Localizzazione sia dell’N-terminale che il C-terminale nel lumen del reticolo

Ost5 ha sia l’N-terminale che il C-terminale nel citosol e non interaisce con Wbp1

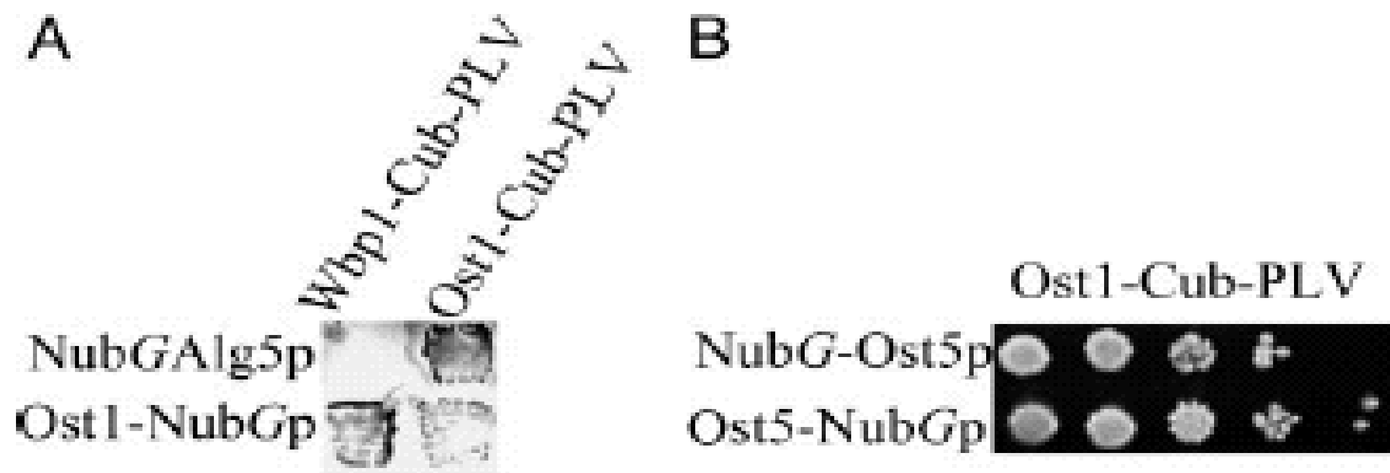
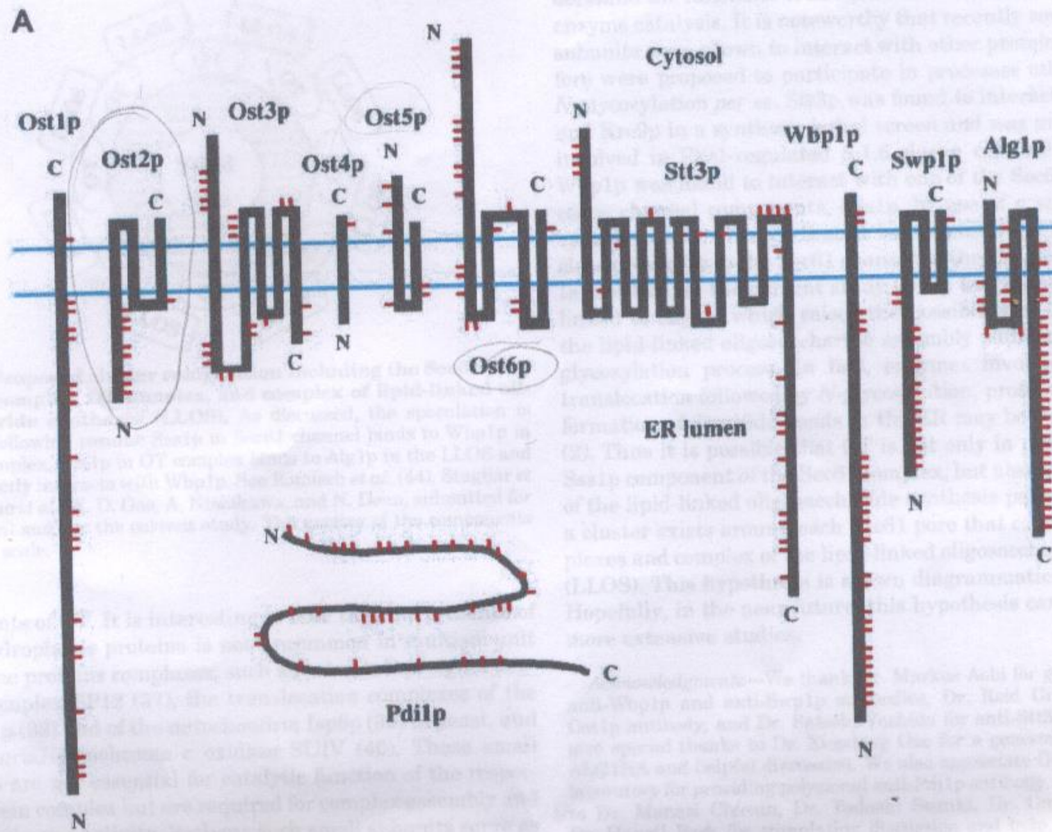


Fig. 4. Split-ubiquitin analysis between Ost1-Cub-PLVp and NubG-Ost5p or Ost5-NubGp. (*A*) β-Gal activity of cells coexpressing Ost1-Cub-PLVp together with NubG-Ost5p or Ost5-NubGp fusion proteins, respectively. (*B*) Growth of the cells coexpressing Ost1-Cub-PLVp with NubG-Ost5p or Ost5-NubGp fusion proteins, respectively, on agar plates without histidine. The procedures, as well as the negative and positive controls, are as described in the legend to Fig. 2.


A
Cytosol
ER lumen
Ost1p
Ost2p
Ost3p
Ost4p
Ost5p
Ost6p
Stt3p
Wbp1p
Swp1p
Alg1p
Pdi1p
N
C

Interazione tra tutte le subunità di OT

- Ost1 e Stt3p interagiscono con diverse subunità
- Ost3 Ost5 e Ost6 prendono contatti con un numero limitato di componenti
- Ost2 e Ost4 interagiscono *in vivo* con molte subunità cosa che non era stata evidenziata esperimenti di crosslinking

Table 1. ***In vivo*** **interactions detected between OT subunits by the split-ubiquitin system**

Cub*	Ost1p	Ost2p	Ost3p	Ost4p	Ost5p	Ost6p	Stt3p	Wbp1p	Swp1p
Ost1p		+	+	–†	+	+	–†	+	+
Ost3p	+	+‡		+	–	–	+	+	–
Ost4p	–†	+	+		+‡	+	+	+‡	+‡
Ost5p	+	+‡	–	+‡		–	–	–†	–
Ost6p	+	+‡	–	+	–		+	+	–
Wbp1p	+	+	+	+‡	–†	+	+		+
Swp1p	+	+‡	–	+‡	–	–	+	+	

+, Interactions detected by split-ubiquitin system; –, interactions not detected by split-ubiquitin system. Blanks are shown because the pairs could not be tested.

*In the case of Ost2p and Stt3p, since their C termini are localized in the ER lumen, the Cub-PLV is not applicable.

†Negative results that do not agree with cross-linking studies in isolated microsomes (14).

‡Positive results that do not agree with cross-linking studies in isolated microsomes (14).

In vitro *In vivo*

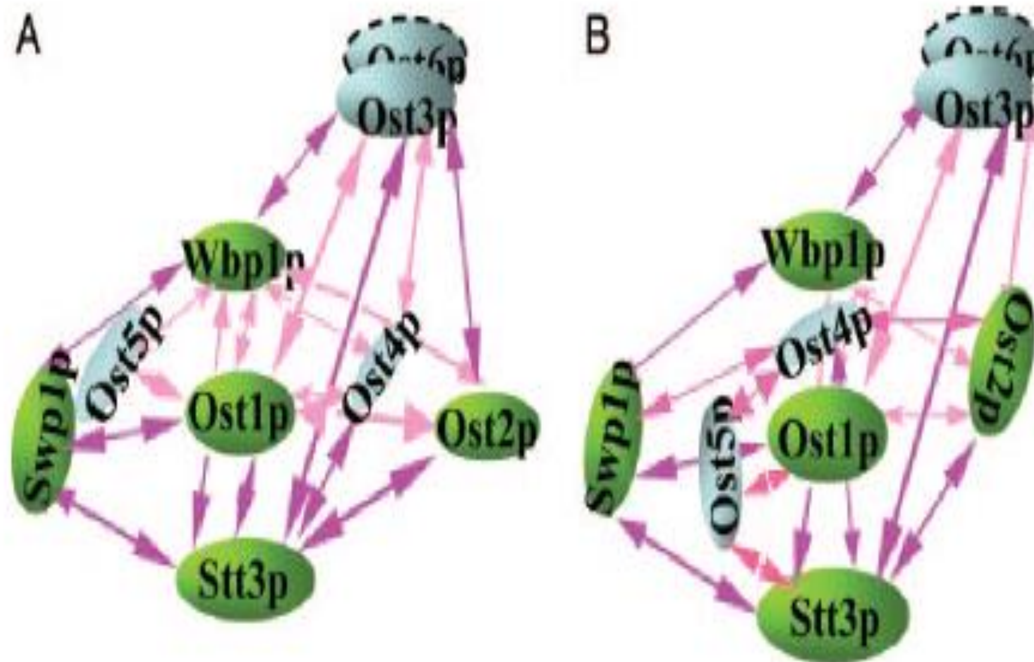


Fig. 5. Comparison of the structural organization of OT complex in a nonworking state (*in vitro*) (*A*) and a working state (*in vivo*) (*B*). The nonworking model was developed based on previous cross-linking studies in isolated microsomal membranes, and the working model was developed based on the *in vivo* interaction analysis as summarized in Table 1. In both of the models, Ost3p and Ost6p were proposed to be present in two distinct isoforms of the OT complex.

Enzimi

Il folding indotto dall’interazione delle proteine partners porta al ripristino dell’attività enzimatica

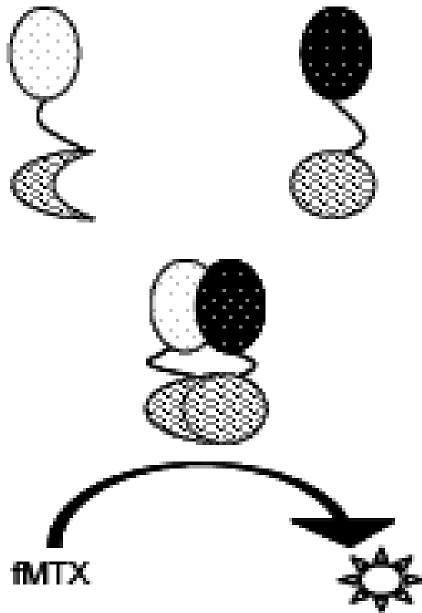
- Beta galattosidasi ,
- Deidrofolato reduttasi (DHFR)
- Beta lattamasi

- Disponibilità di substrati permeabili cromogenici che permettono di rilevare facilmente l’attività enzimatica
- Saggi basati sulla sopravvivenza

La didrofolatoreduttasi catalizza la riduzione dell’idrofolato in tetraidrofolato importante per la biosintesi dei nucleotidi

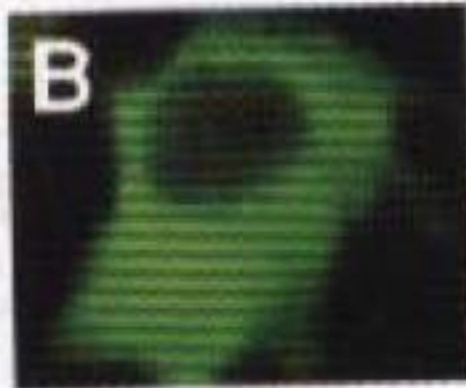
L’interazione tra le proteine partners è evidenziata:
1sopravvivenza delle cellule seminate su terreni privi di nucleotidi
2 saggio fluorescente in presenza di f MTX(METHOTREXATE)

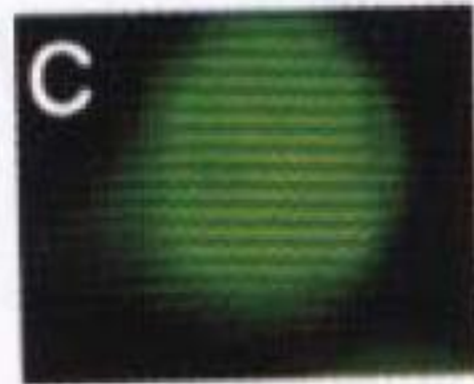
Dihydrofolate Reductase



f MTX lega con elevata affinità DHFR
Kd = 540 pM

Dihyrofolate reductase (DHFR) is a small
monomeric protein which catalyses the

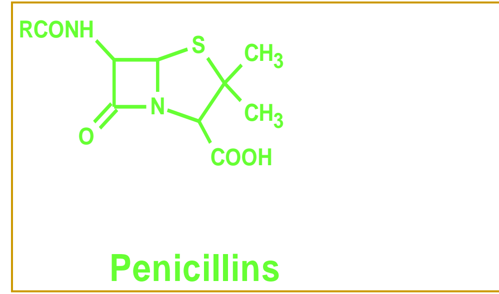


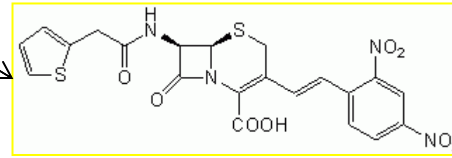


A B C CELLULE COS
D E F localizzazione di NPR1-DHFR E TGA2-DHFR trattate con acido salicilico (E ed F)

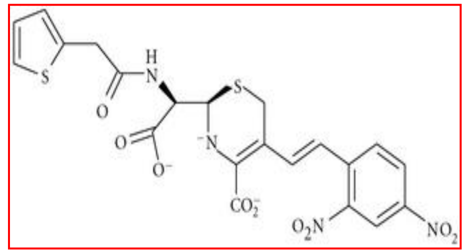
Beta lattamasi enzima monomerico di 29kD
Può essere scisso in due frammenti 24~215 e 216~286 che possono ricostituirsi
L'interazione tra le proteine partners può essere evidenziata

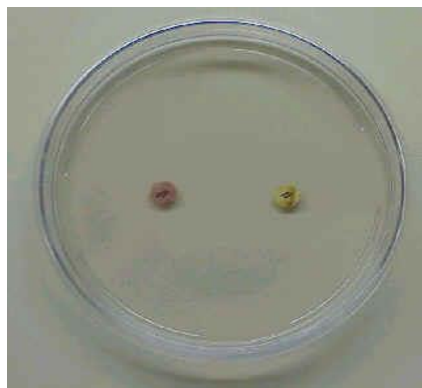
- misurando la resistenza all'ampicillina
- Saggio colorimetrico con nitrocefina

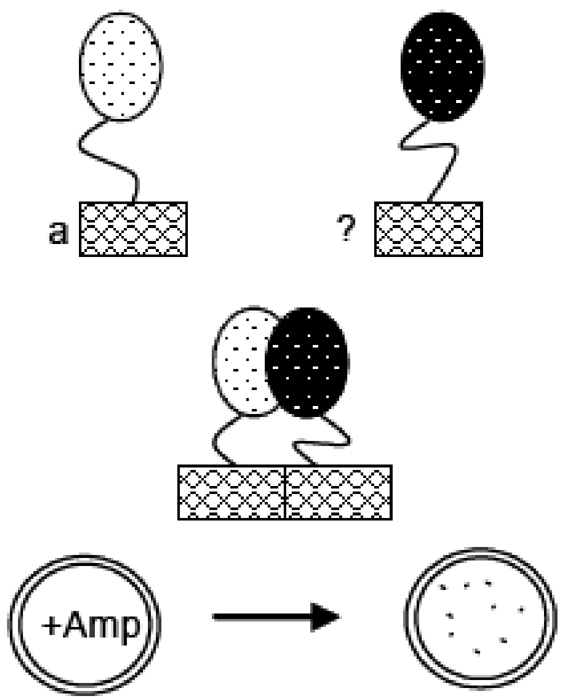




Beta lattamasi

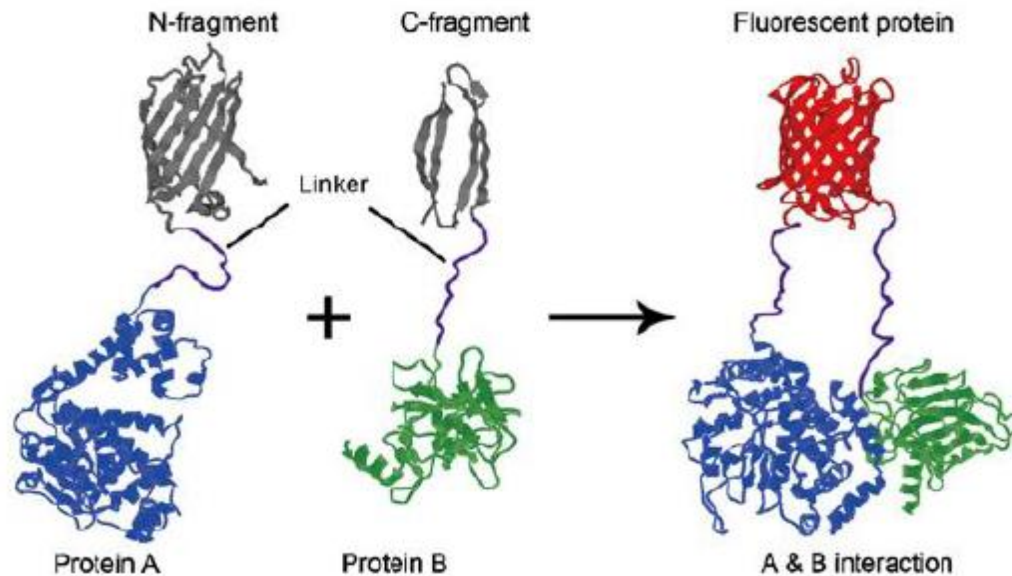






Bimolecular Fluorescent Complementation (BiFC)

Il folding della proteina fluorescente indotto dall'interazione delle proteine partner porta alla formazione del cromoforo



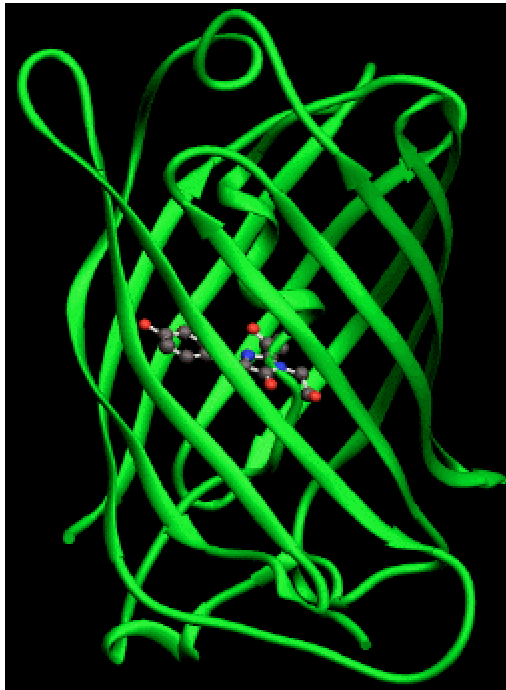
- L’interazione può essere visualizzata in vivo

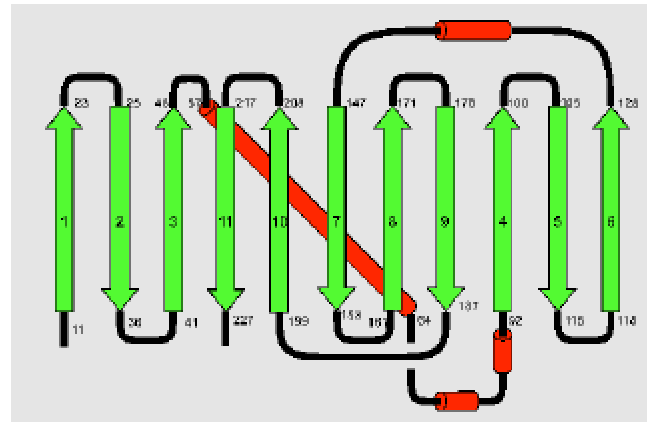
- Non è necessario sovraesprimere le proteine

- E’ possibile visualizzare l’interazioni deboli e transienti
- La visualizzazione dell’interazione proteina-proteina non è in tempo reale

- La multicolor bimolecular complementation assay permette la simultanea visualizzazione di complessi proteici nella stessa cellula

Struttura 3D della GFP





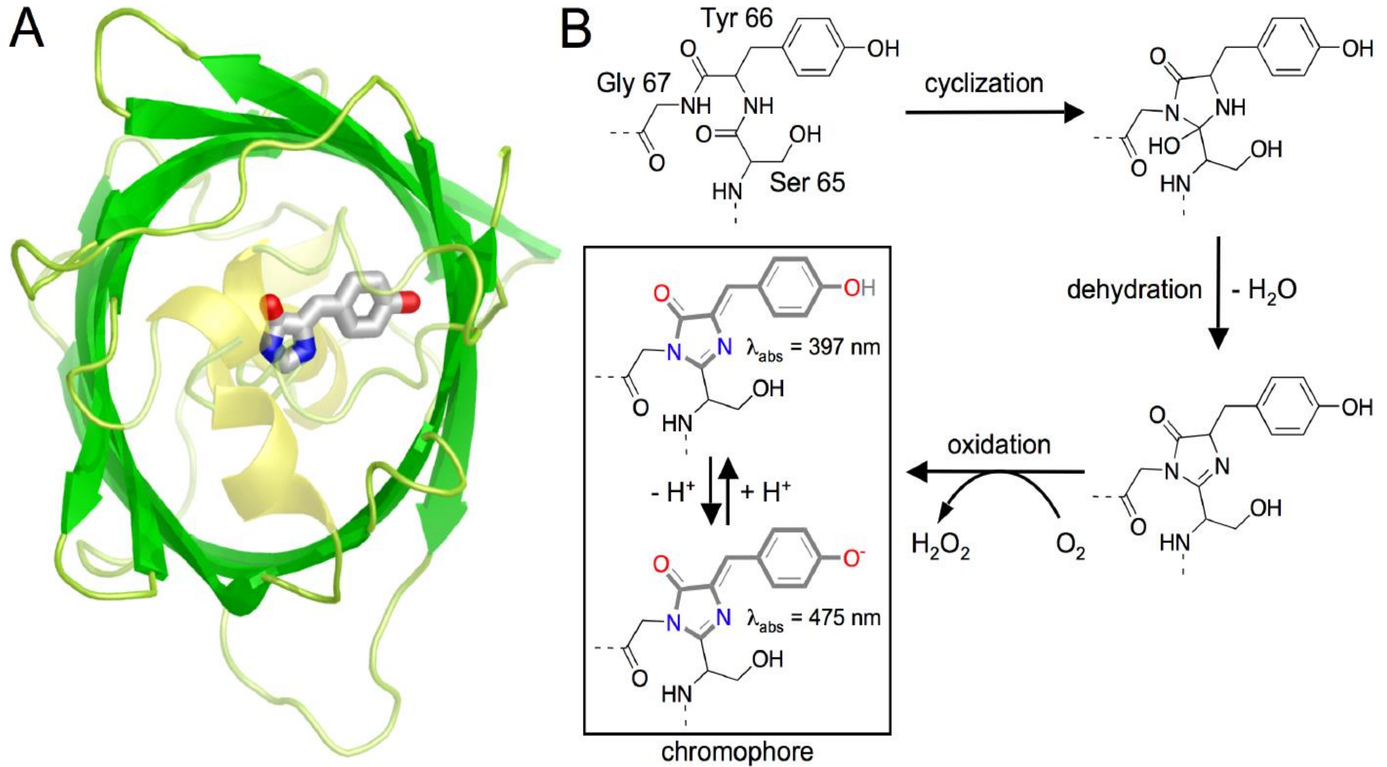
GFP è una proteina di 238 aminoacidi formata da 11 foglietti beta (Prasher *et al*.,1992) isolata dall' *Aequorea victoria*

E’ fluorescente anche a T di 65°C e pH 11

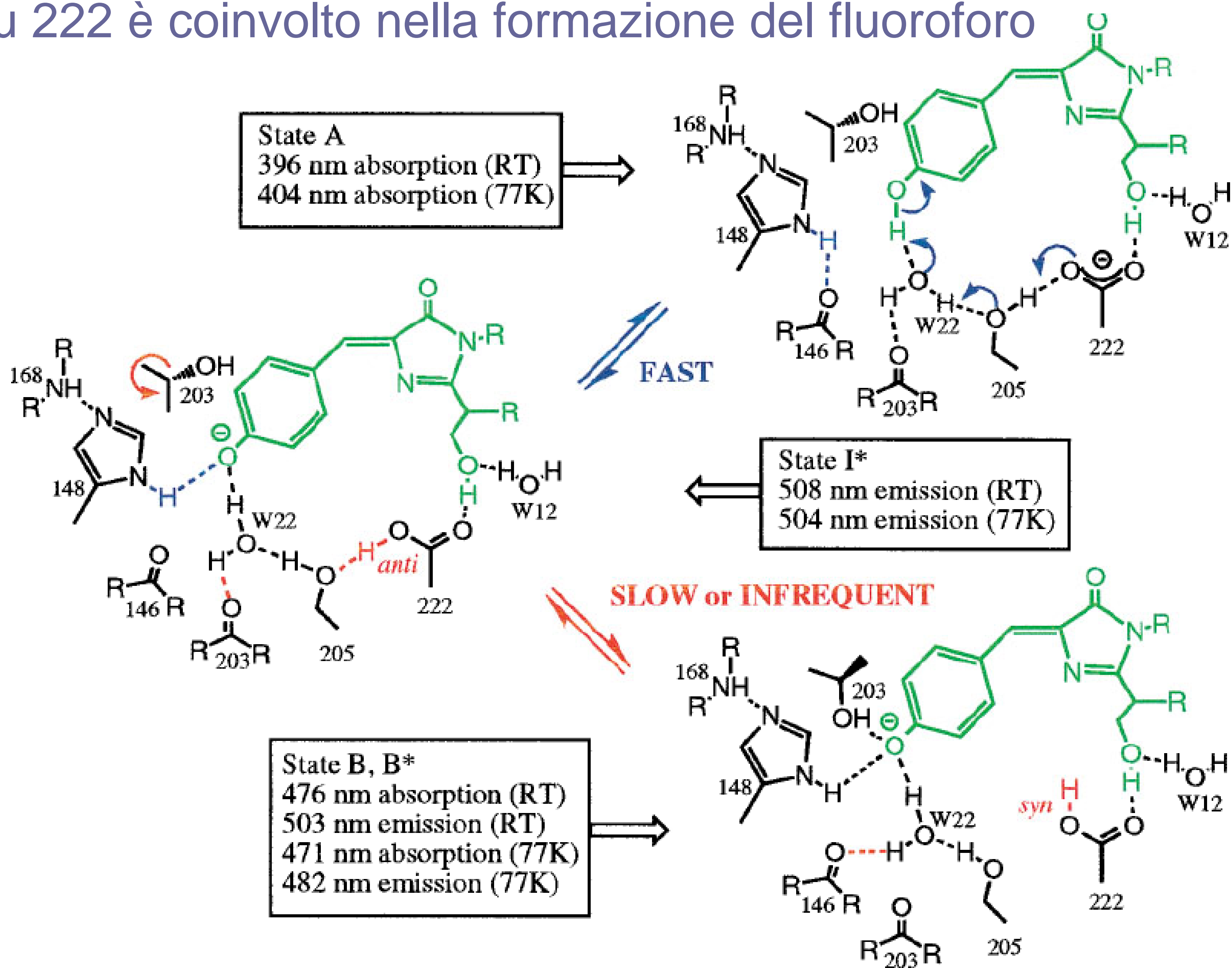
S65T produce il fluoroforo 4 volte più velocemente del wt

EGFP (Enhanced GFP) **F64L-S65T** 35 volte più fluorescente del wt

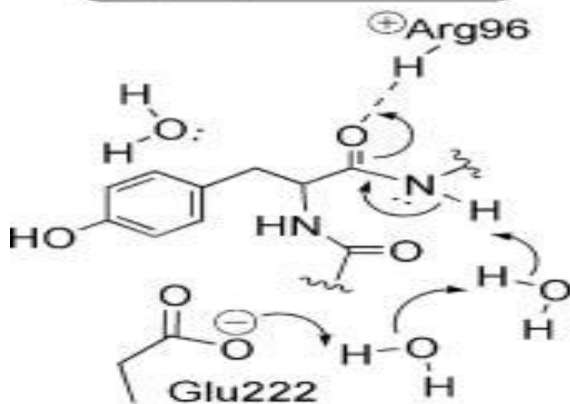
Formazione del cromoforo

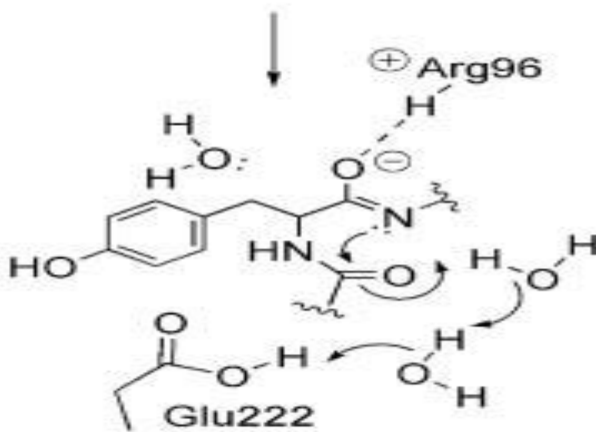


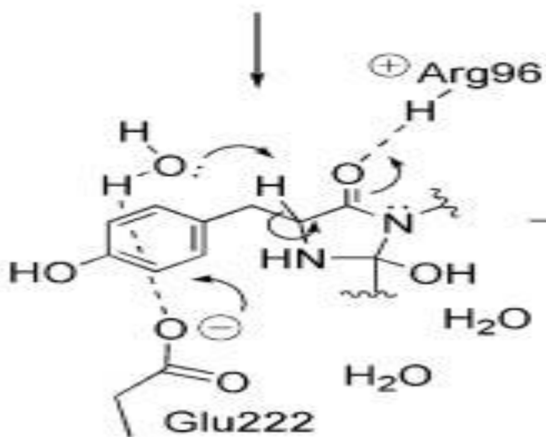
Glu 222 è coinvolto nella formazione del fluoroforo



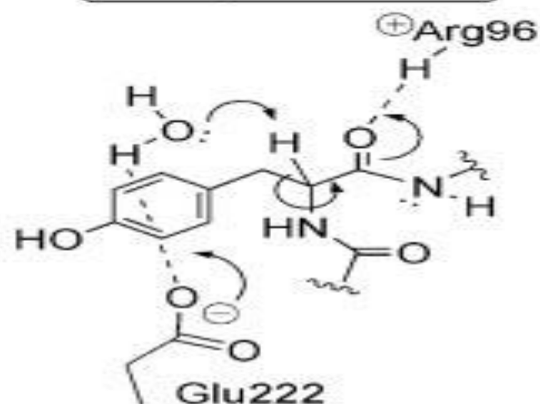
N-deprotonation followed by C_α-deprotonation

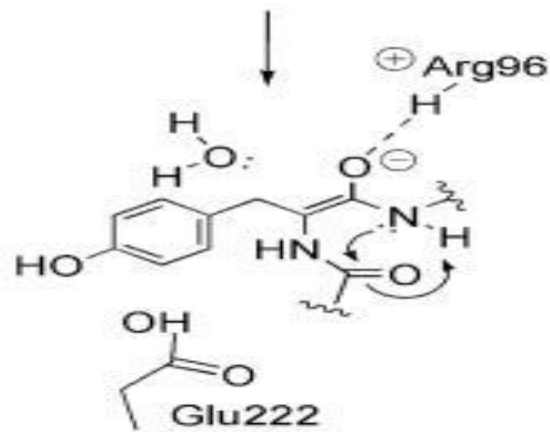


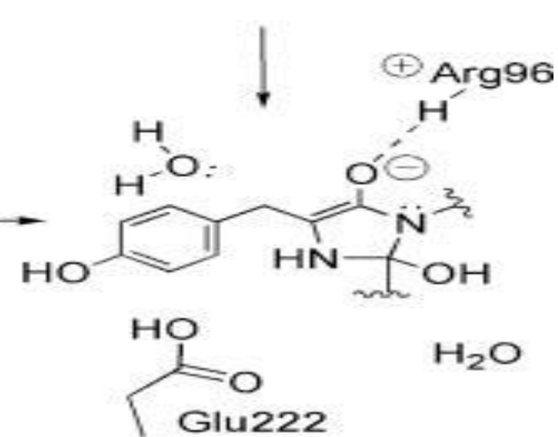




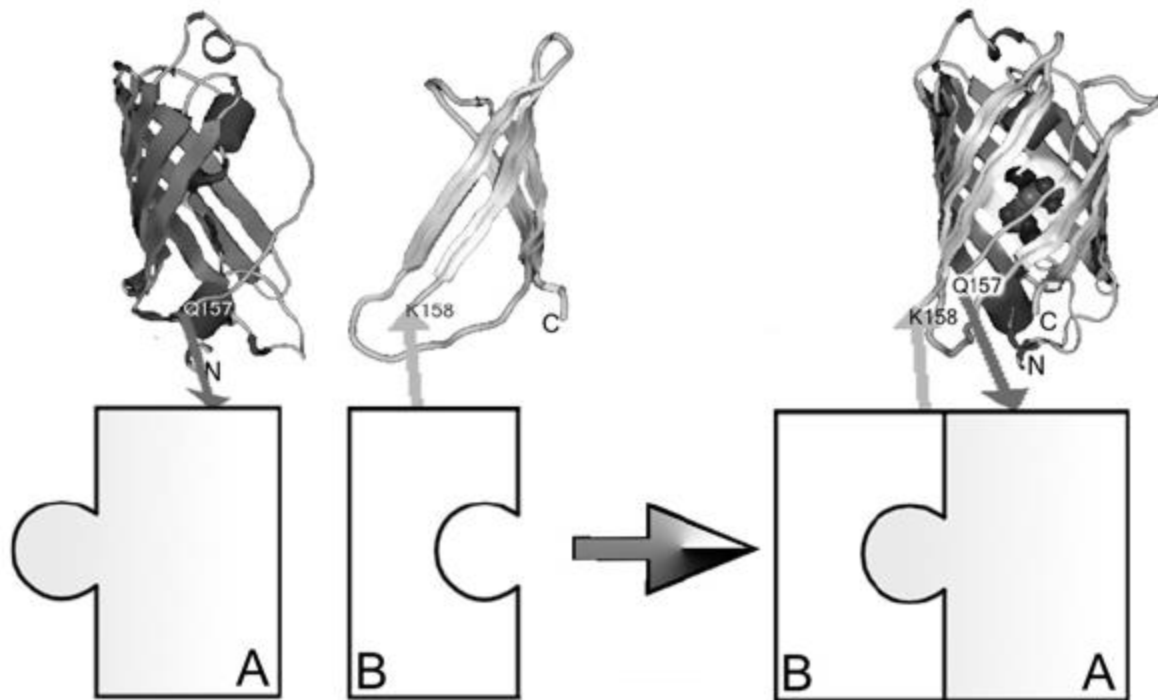
C_α-deprotonation followed by N-deprotonation



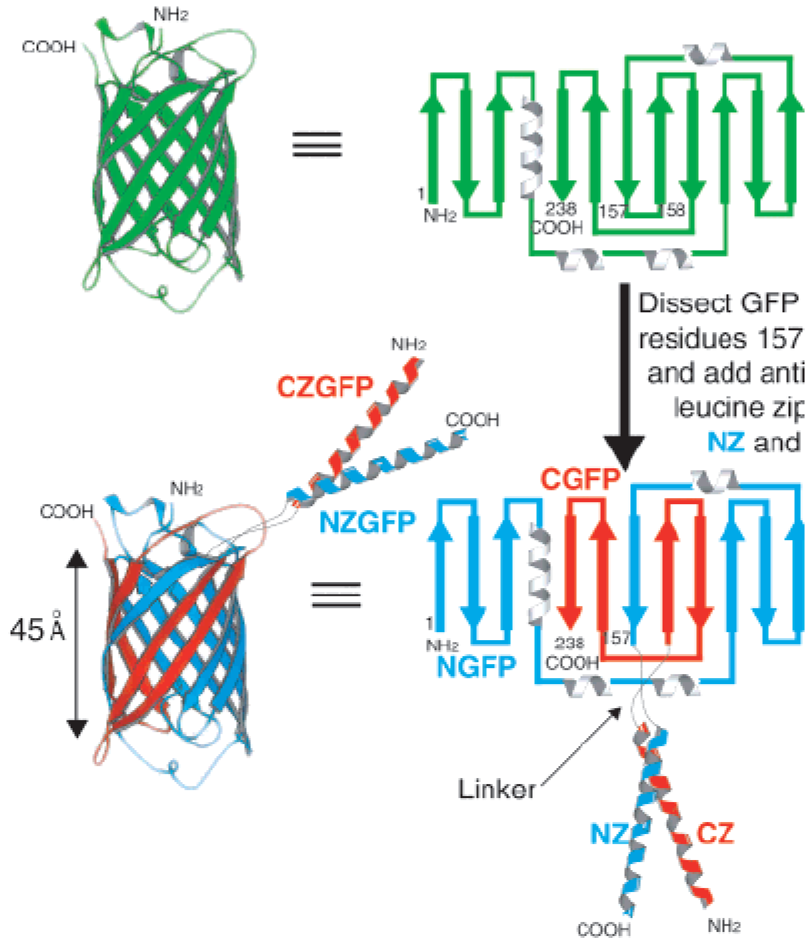




GFP è separata tra i residui 157 and 158 per produrre due frammenti non fluorescenti NGFP e CGFP.



Antiparallel Leucine Zipper-Directed Protein Reassembly: Application to the Green Fluorescent Protein



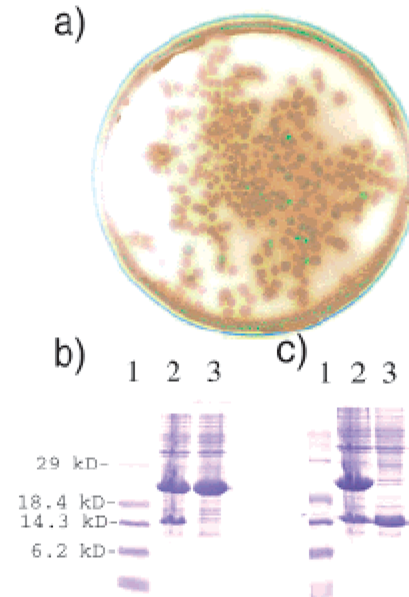
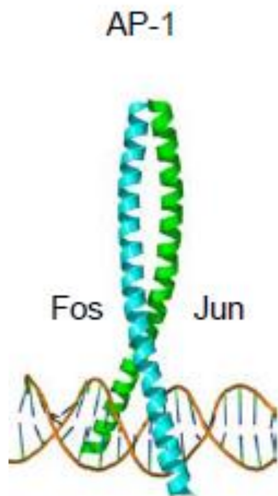
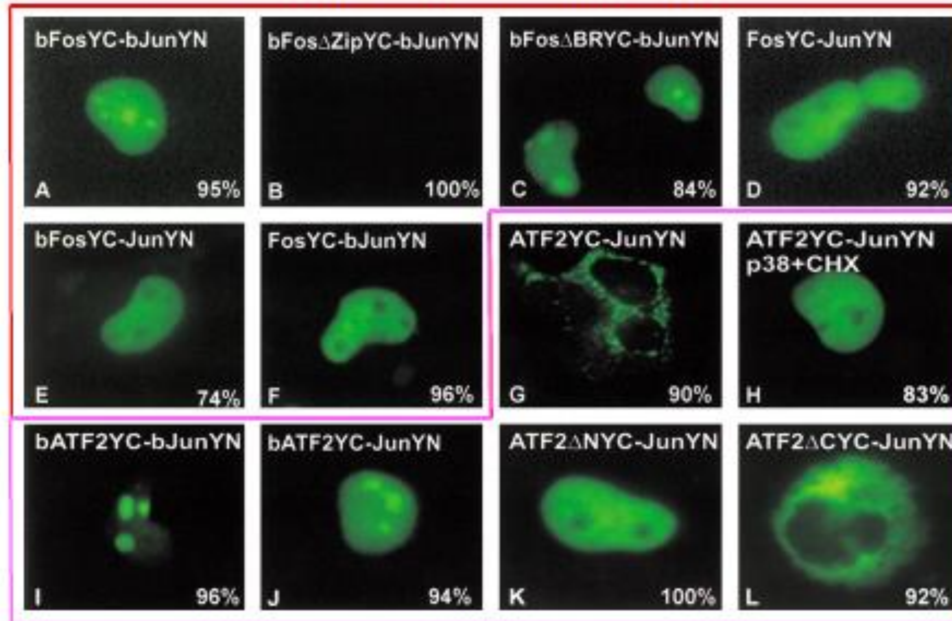


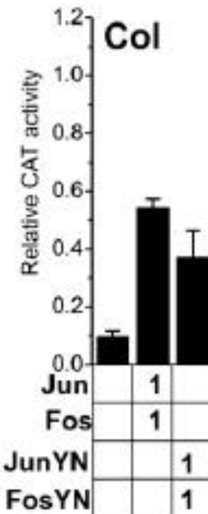
Figure 3. In vitro reconstitution of GFP demonstrated by (a) green fluorescent BL21(DE3) cells and the corresponding SDS gels of (b) lane 1: MW markers; lane 2: protein from cotransformed green colony; and lane 3: protein from colony containing only NZGFP plasmid and (c) lane 1: MW markers; protein from cotransformed green colony; and lane 3: protein from colony containing only CZGFP plasmid.

Interactions between the bZIP domains in cells

HU CD., Chichenov Y. and Kerppola T, *Molecular cell*, 2002







Interaction between bZIP domains of Fos and Jun detected by BiFC of bFosYC and bJunYN (A).
No fluorescence complementation upon deletion in the leucine zipper that prevents Fos-Jun dimerization (B).
Mutation of the basic regions of bFos and bJun had no effect on bFos-bJun heterodimer localization (C).
Cells coexpressing either full length FosYC or JunYN or both exhibited predominantly nuclear fluorescence that was excluded from the nucleoli (D, E and F).

Full-length ATF2YC and JunYN dimerization localizes in the perinuclear region (G).
Dimers formed by the bZIP domain of ATF2 with full lenght Jun or bJun display nucleolar localization (I and J).
Deletion of the N terminus in ATF2 results in nucleoplasmic localization of the ATF2ΔNYC-JunYN (K) while ATF2ΔCYC-JunYN is excluded from the nucleus.

Altre proteine split

A

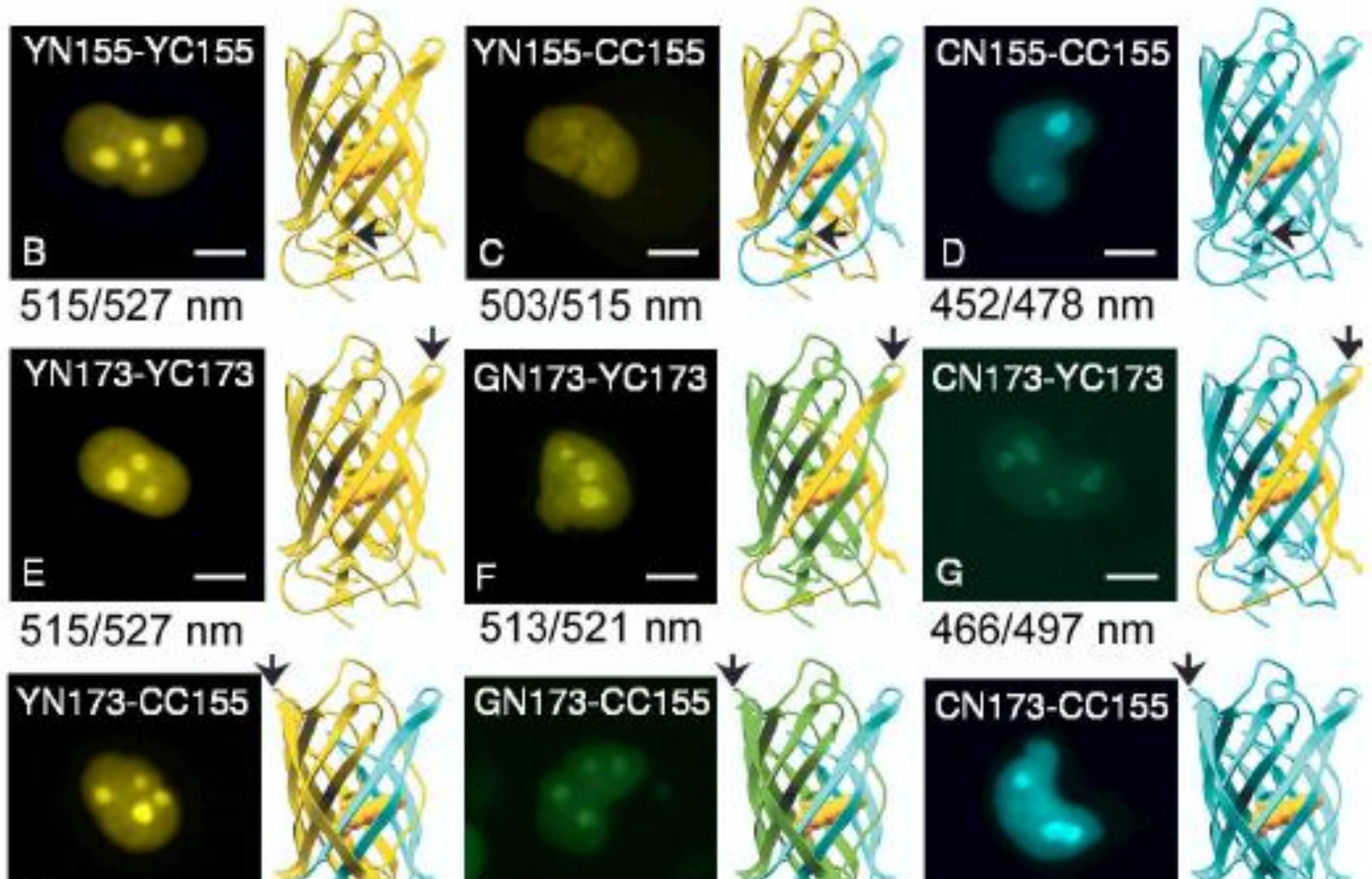
GFP

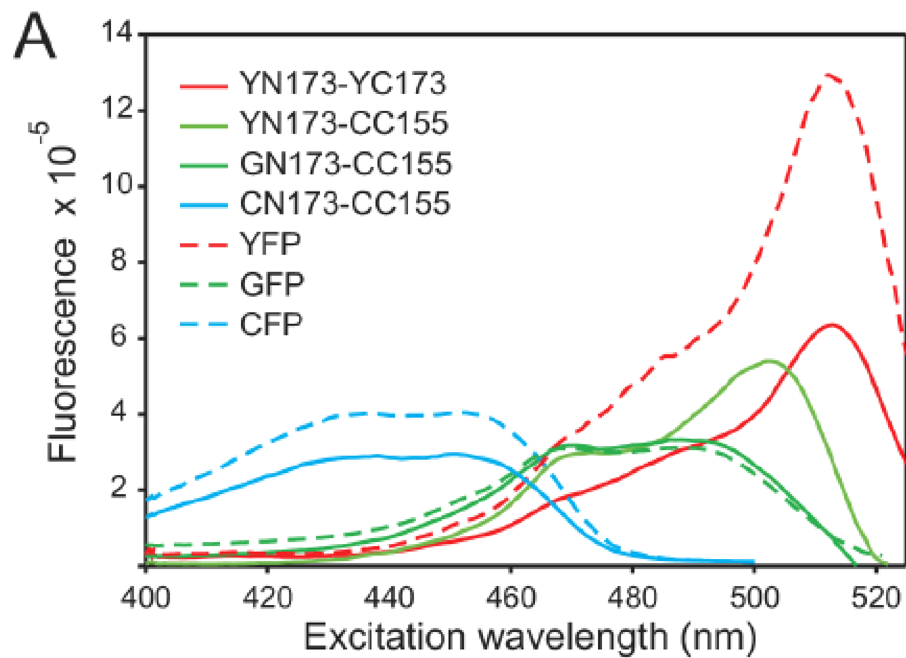
YFP

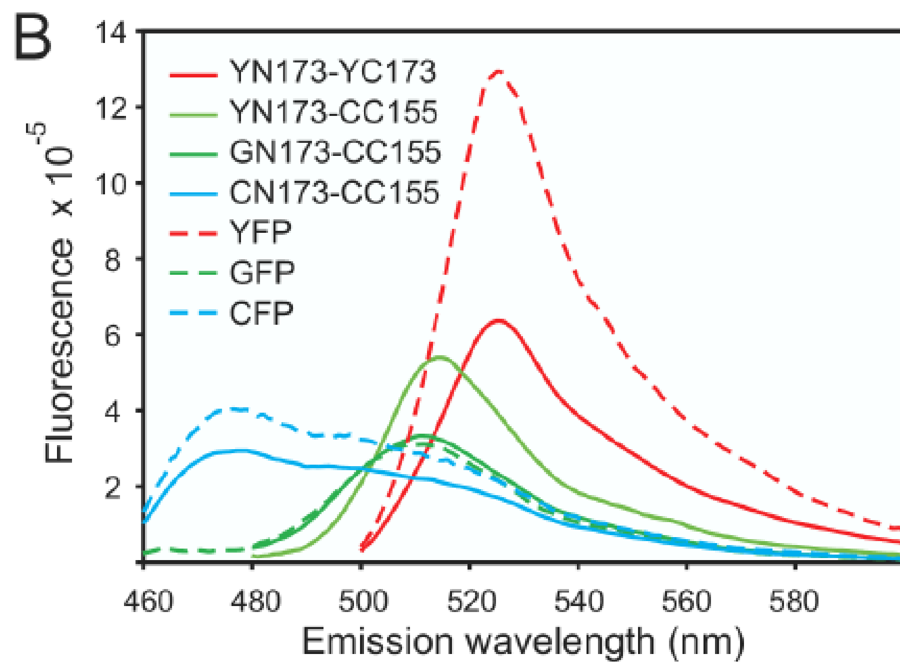
CFP

BFP

isualizzazione della complementazione tra frammenti di
iverse proteine fluorescenti fuse in cui il frammento N-
erminale è fuso a bFos e quello C terminale a bJun




A
YN173-YC173
YN173-CC155
GN173-CC155
CN173-CC155
YFP
GFP
CFP
Fluorescence x 10-5
Excitation wavelength (nm)
0
2
4
6
8
10
12
14
400
420
440
460
480
500
520


B
YN173-YC173
YN173-CC155
GN173-CC155
CN173-CC155
YFP
GFP
CFP
Fluorescence x 10-5
Emission wavelength (nm)
2
4
6
8
10
12
14
460
480
500
520
540
560
580

Monitoraggio dei cambiamenti conformazionali della MBP in seguito al legame con il maltosio

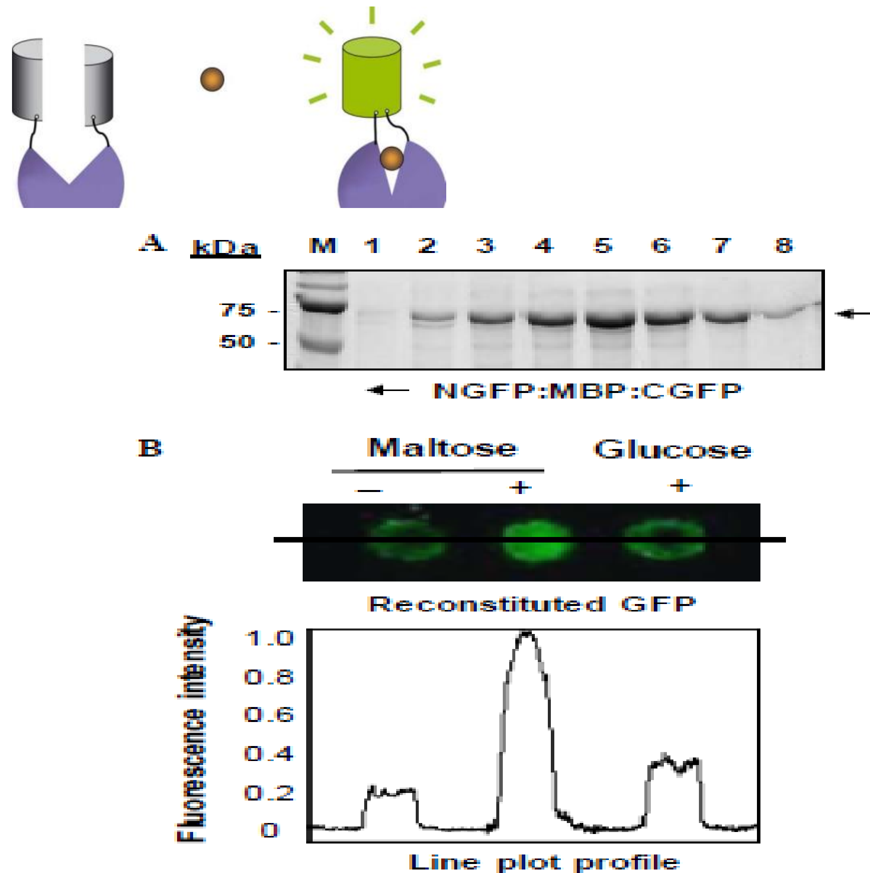


Fig. 2. In vitro visualization of conformational changes in MBP. (Purification of recombinant NGFP:MBP:CGFP protein using immo lized metal ion affinity chromatography (IMAC). Purified NGFP:MI CGFP was analyzed on 10% SDS–PAGE gel, and the arrow indicates NGFP:MBP:CGFP-containing fractions. The procedure is described detail in Materials and methods. (B) Fluorescence complementation as for the monitoring of structural changes in MBP upon maltose bindi After 1 h of sample incubation, the fluorescence images of NGFP:MBP:CGFP proteins treated with maltose (or glucose as a contr were obtained with a GenePix 4200A laser scanner.

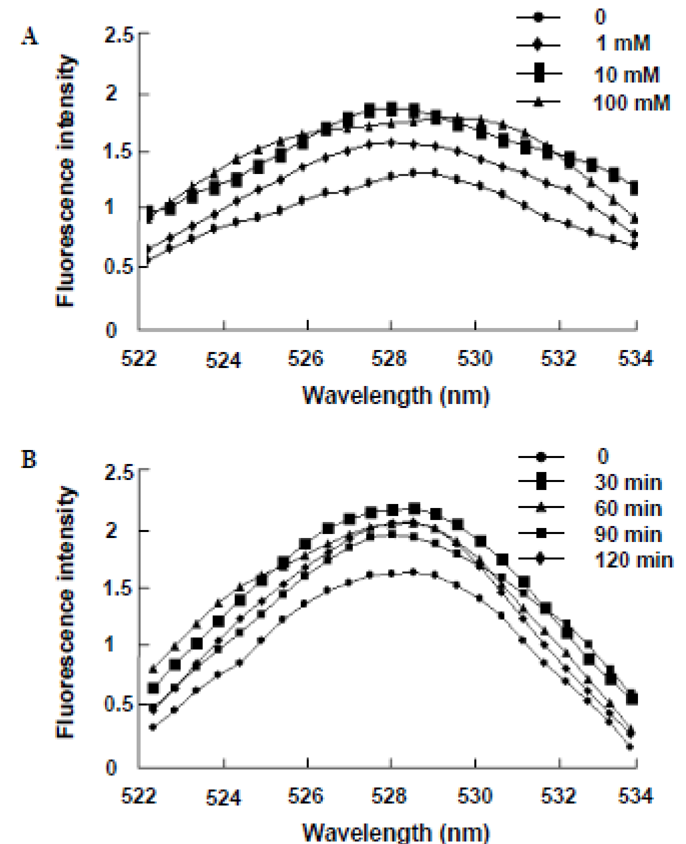
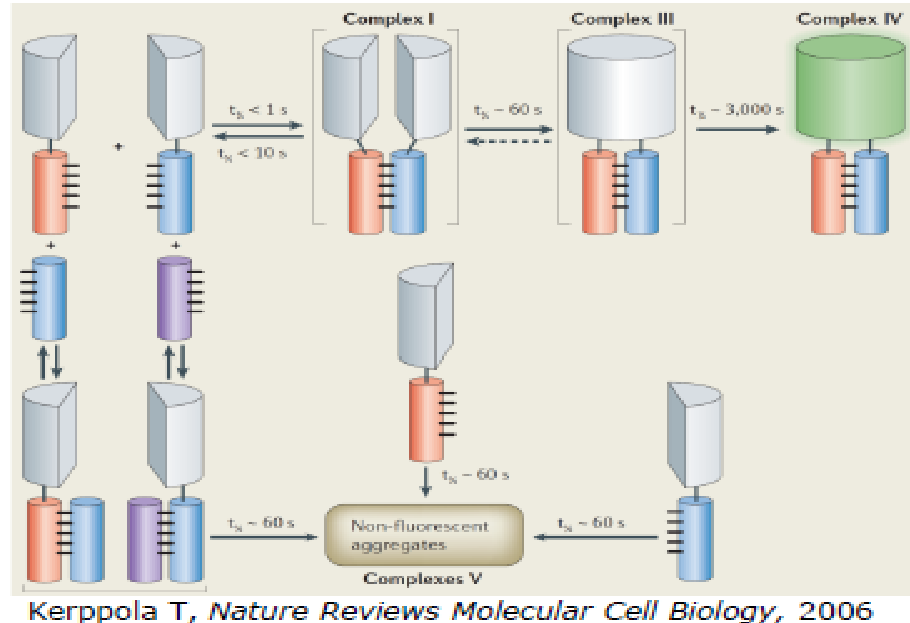


Fig. 3. Analysis of the fluorescence intensity of the purified recombinant NGFP:MBP:CGFP proteins. Maltose-induced conformational changes in MBP were assessed via analyses of the fluorescence emission spectra of NGFP:MBP:CGFP proteins at differing (A) concentrations and (B) incubation times of maltose as a substrate. Fluorescence measurements were conducted using an LS45 Luminescence Spectrometer (Perkin-Elmer Instruments), with an excitation wavelength of 488 nm and emission measured at 525 nm.

BiFC: limiti



Kerppola T, *Nature Reviews Molecular Cell Biology*, 2006

L'interazione non è visualizzata in tempo reale
Il complesso è irreversibile(pro e contro)
Formazione di aggregati
Uso di proteine di fusione
Dipendenza dalla temperatura

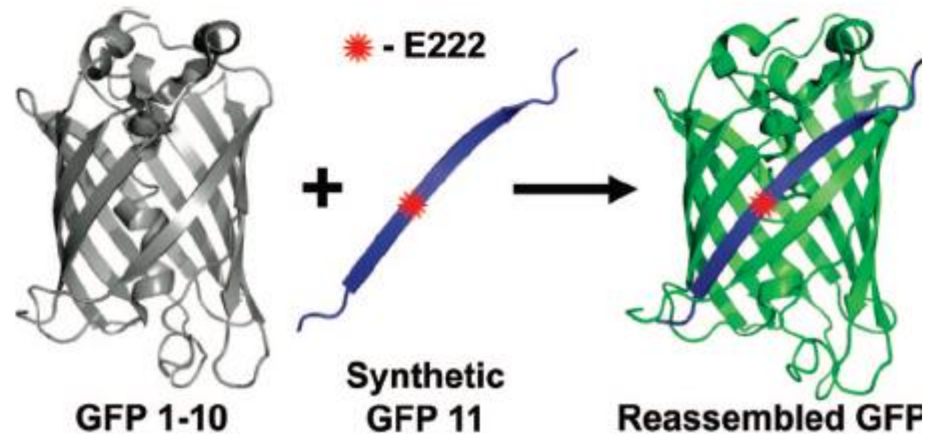
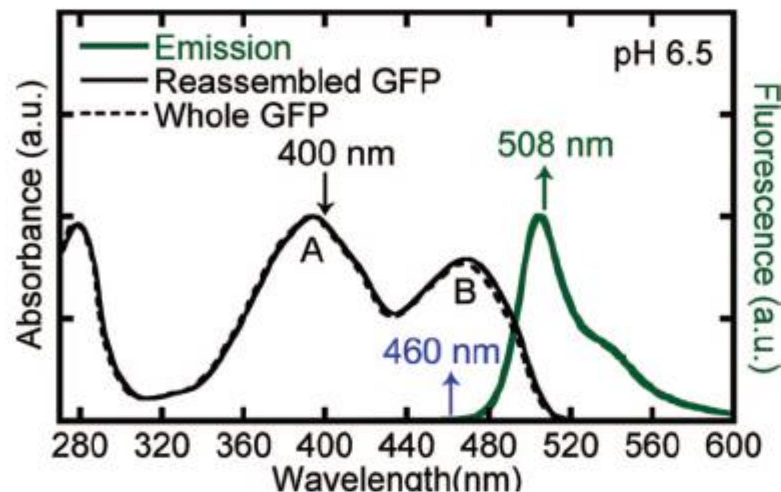
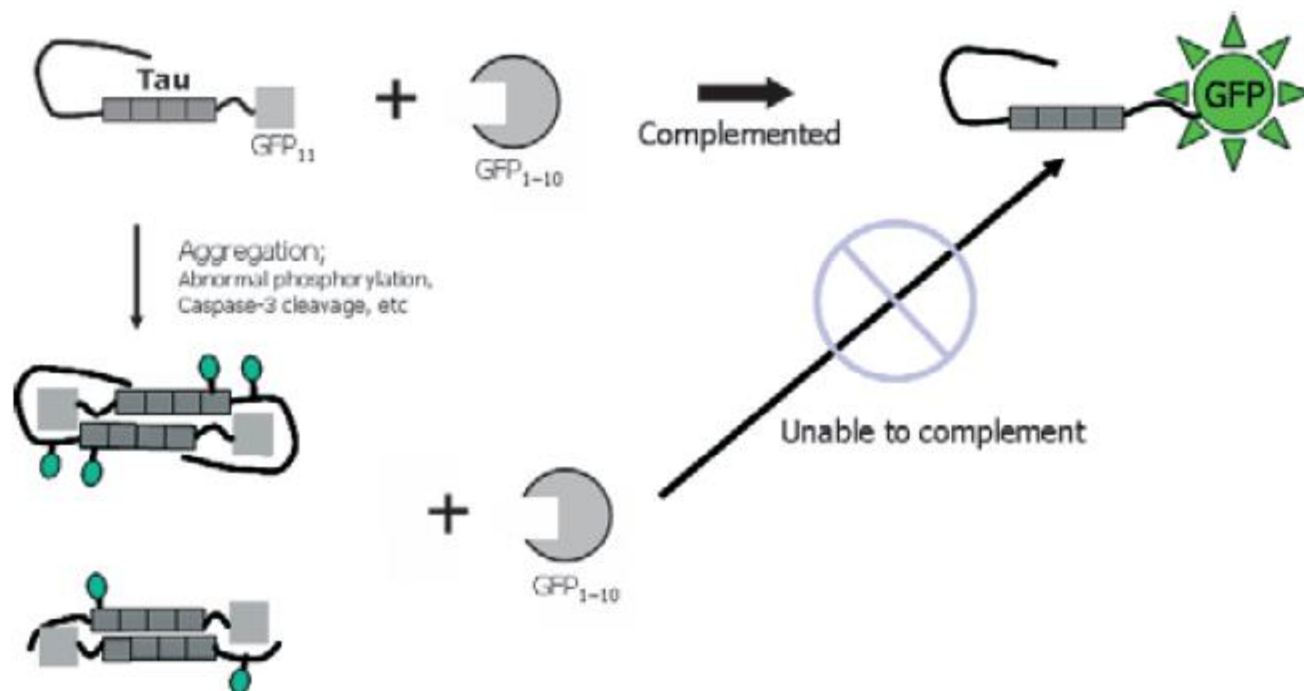


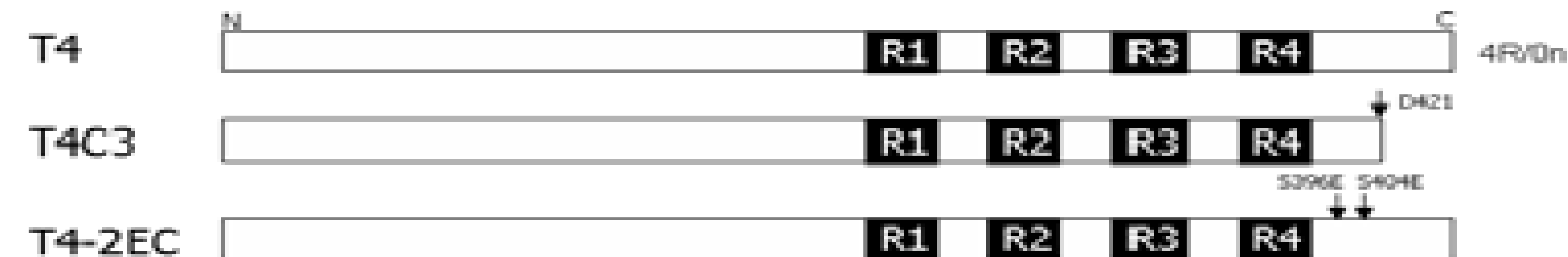
Figure 1. Schematic diagram illustrating reassembly of the eleven-strand
β-barrel of GFP from GFP 1−10 (the first 214 amino acids of GF
including residues 65−67 that become the chromophore) and a synthet
16 amino acid 11th strand, GFP 11. The red star marks residue E222
GFP 11, which is known to be an excited-state proton acceptor in seve
GFP variants.[24,25] The chromophore is not formed in GFP 1−10, but dc
mature upon addition of synthetic GFP 11. The topology of GFP 1−10
solution is not yet well characterized and is drawn based on the structu
of whole GFP minus the 11th strand (see text).



Split GFP complementation assay: a novel approach to quantitatively measure aggregation of tau *in situ*: effects of GSK3β activation and caspase 3 cleavage



T4 = (tau wt)
T4C3 (tau cleaved)
T4-2EC pseudofosforilata S396/E, S404E



K18 wild type

ΔK mutant ΔLys 280

Proline mutant (K18/ΔK280/2P)

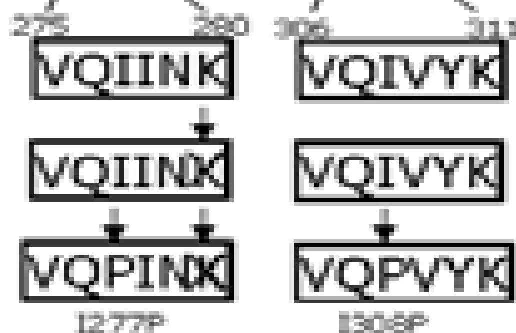
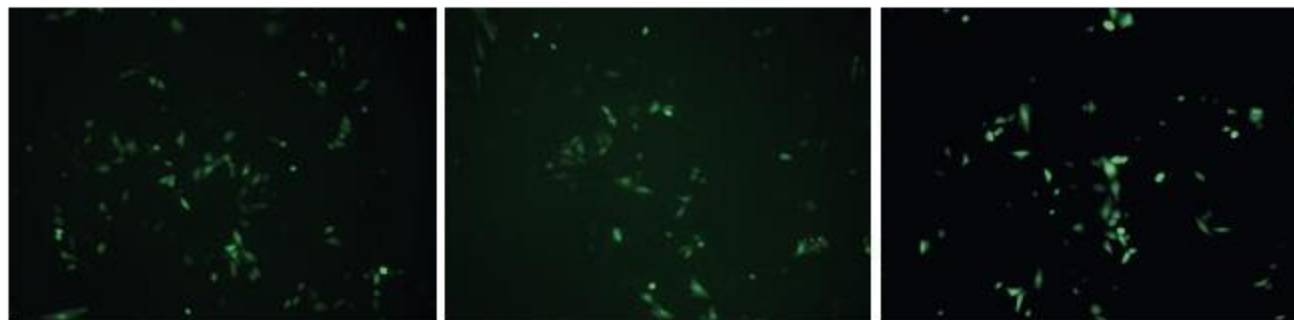
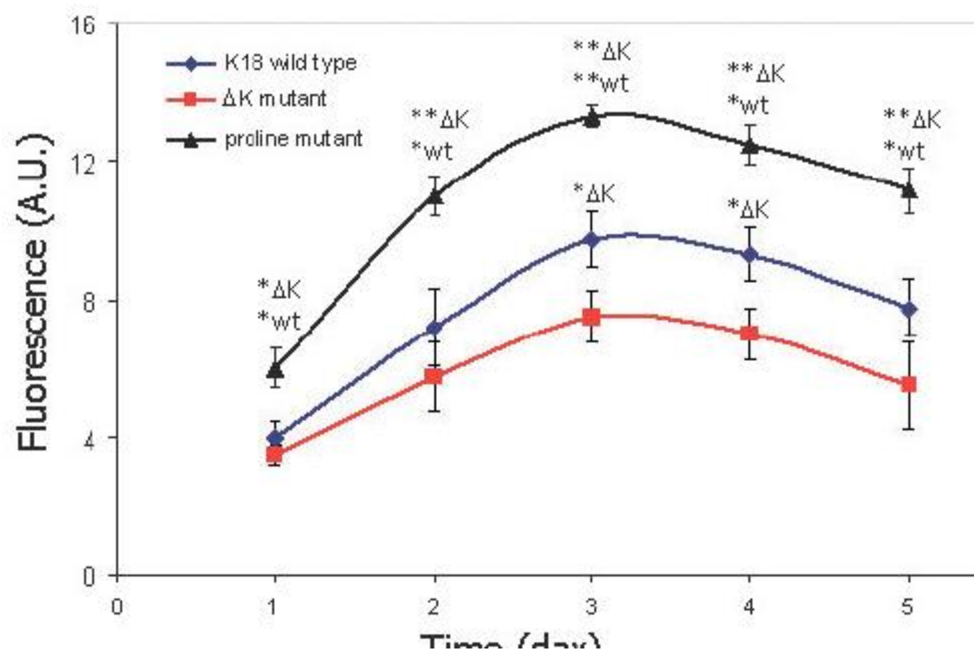


Fig. 2 Diagram of tau and tau_{MBD} constructs used for the split green


K18 wild type
ΔK mutant
Proline mutant


K18 wild type
ΔK mutant
proline mutant
Fluorescence (A.U.)
Time (day)
16
12
8
4
0
0
1
2
3
4
5
*ΔK
*wt
**ΔK
*wt
**ΔK
**wt
*ΔK
**ΔK
*wt
*ΔK
**ΔK
*wt

La complementazione di GFP è concentrazione dipendente

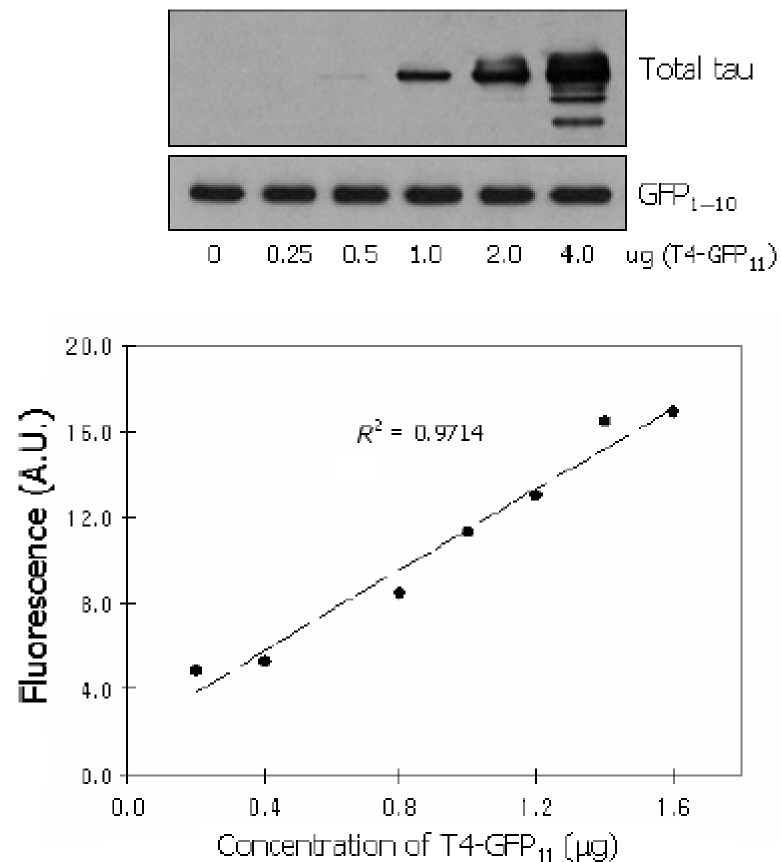
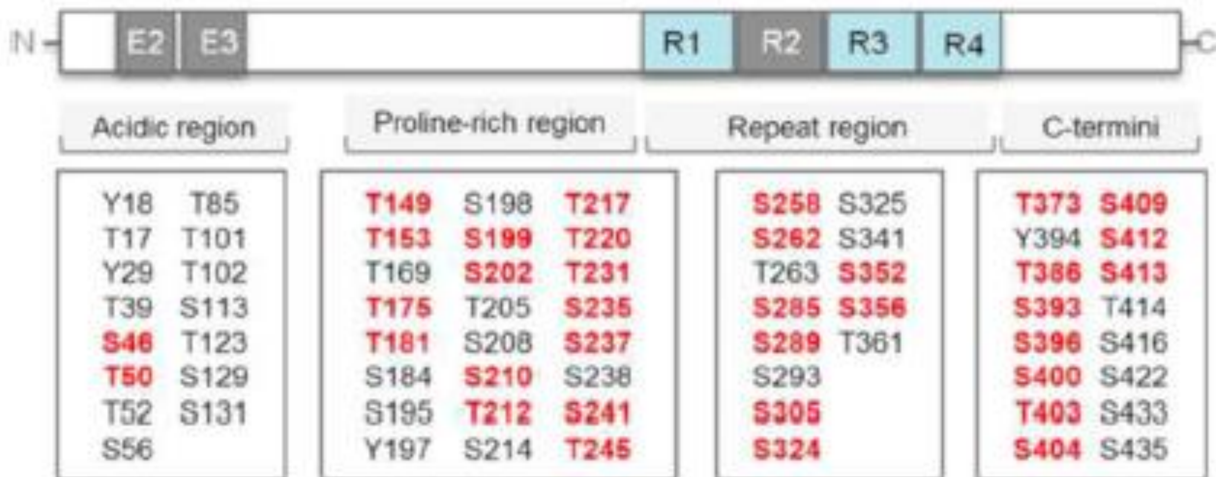


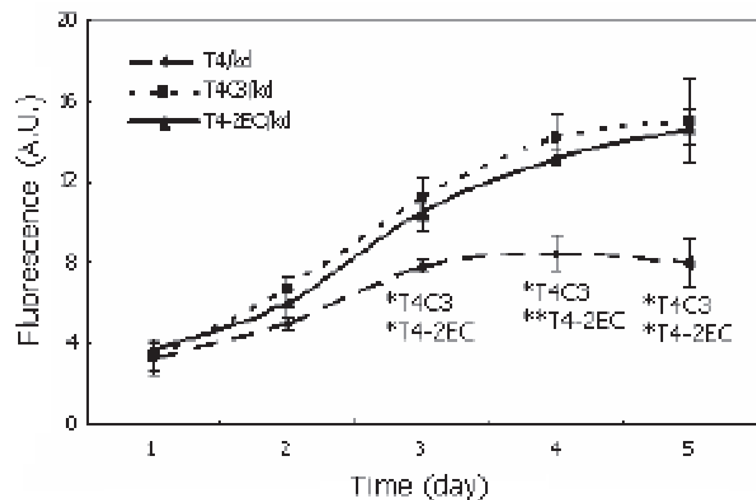
Fig. 4 Quantification of *in situ* split green fluorescence protein (GFP)

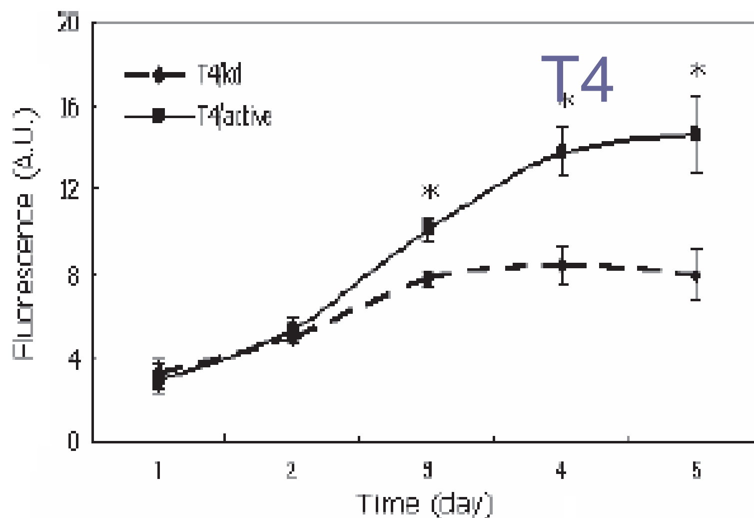
a
Ser/Thr/Tyr
Phosphorylation
GSK3β
Tau
HO-P=O
Ser/Thr/Tyr
Tau-P
Neurotoxic aggregates

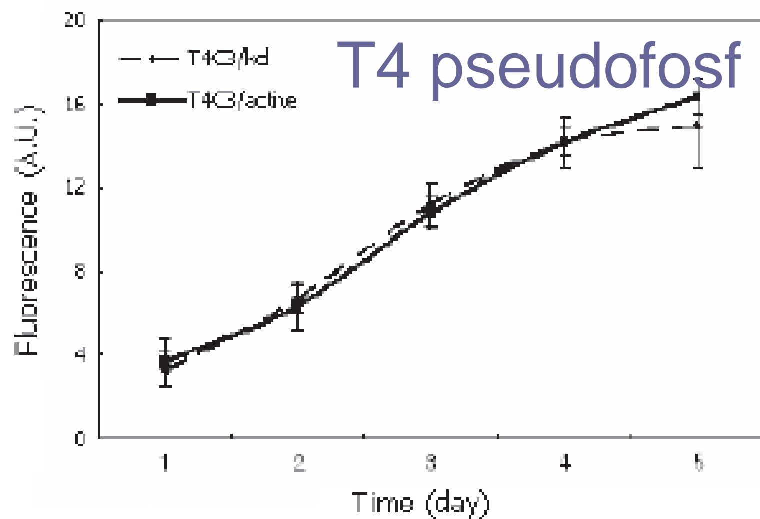


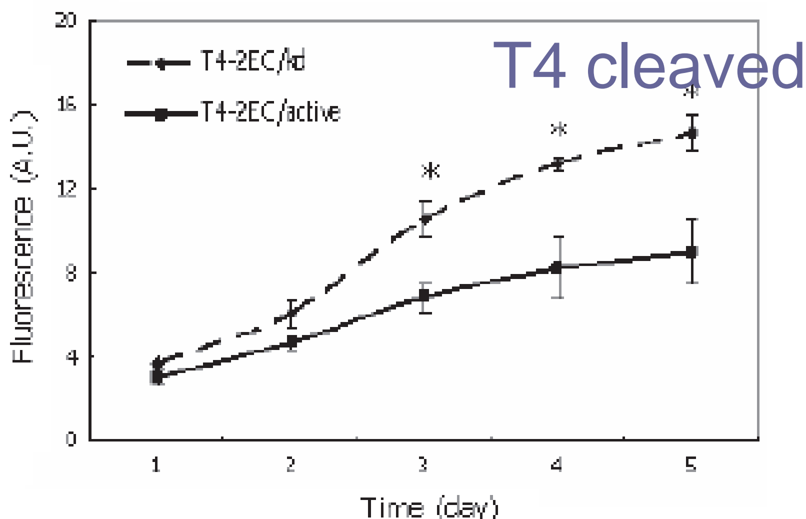
b
N
E2
E3
R1
R2
R3
R4
C
Acidic region
Proline-rich region
Repeat region
C-termini
Y18 T85
T17 T101
Y29 T102
T39 S113
S46 T123
T50 S129
T52 S131
S56
T149 S198 T217
T153 S199 T220
T169 S202 T231
T175 T205 S235
T181 S208 S237
S184 S210 S238
S195 T212 S241
Y197 S214 T245
S258 S325
S262 S341
T263 S352
S285 S356
S289 T361
S293
S305
S324
T373 S409
Y394 S412
T386 S413
S393 T414
S396 S416
S400 S422
T403 S433
S404 S435

Effetto della glicogeno sintasi sull'aggregazione Complementazione effettuata con glicogeno sintasi GSK3β attiva e denaturata (kd)







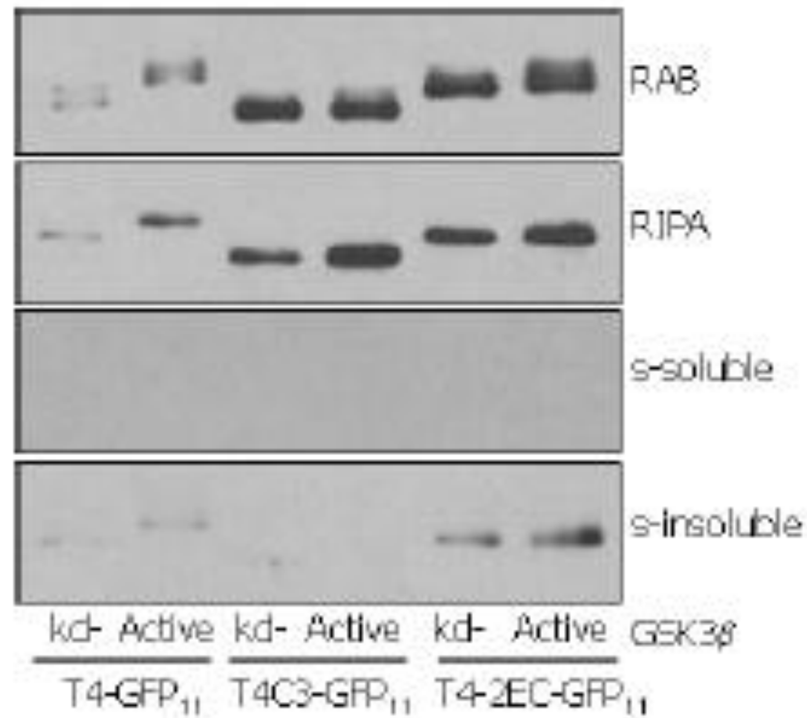


Studi di complementazione tau, tau pseudofosforilata , tau cleaved rivelano che

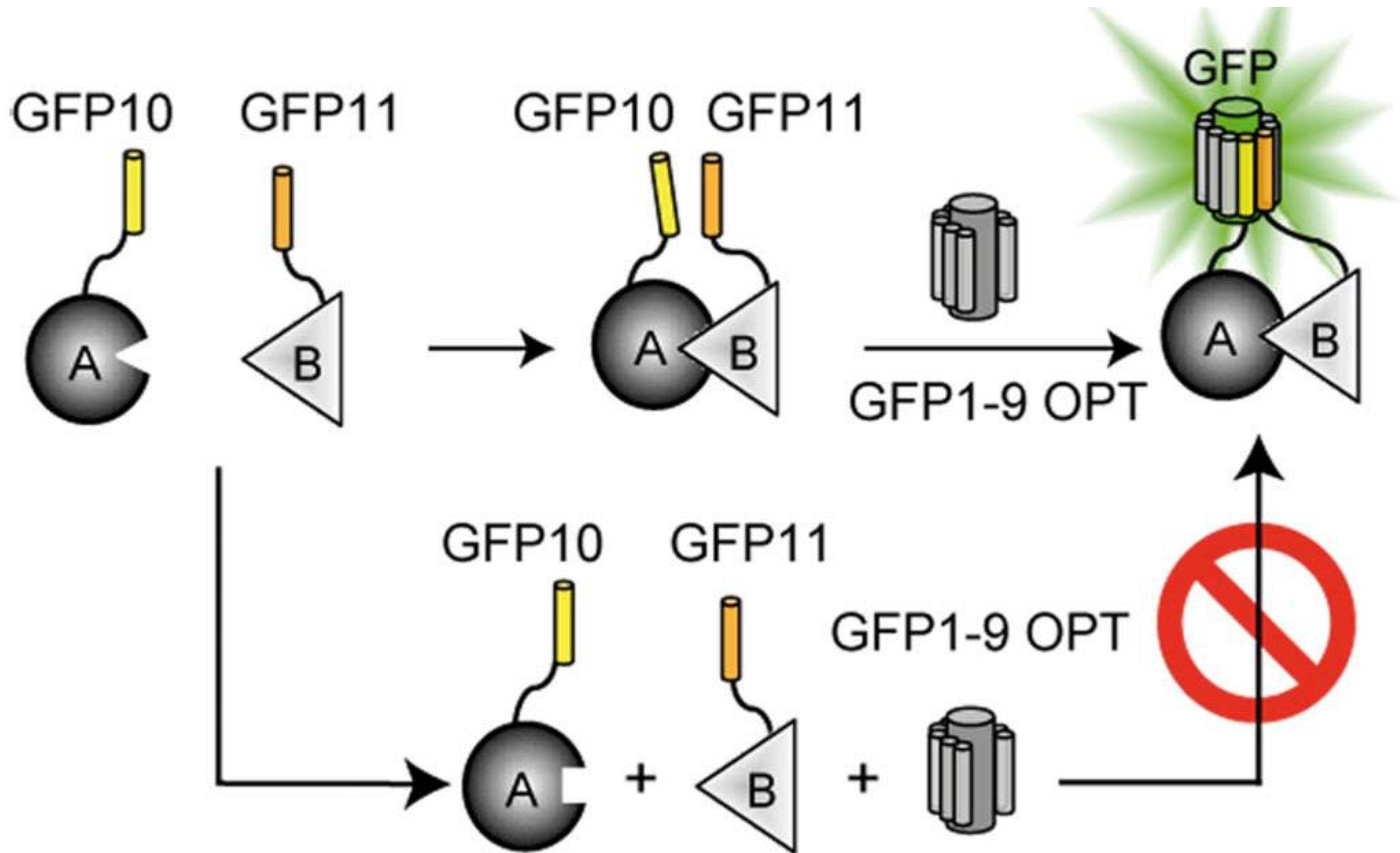
La glicogeno fosforilasi attiva

1. diminuisce l’aggregazione di T4(aumenta la complementazione)
2. non ha effetto sull’aggregazione di T4 pseudofosforilata
3. aumenta l’aggregazione di T4 cleaved

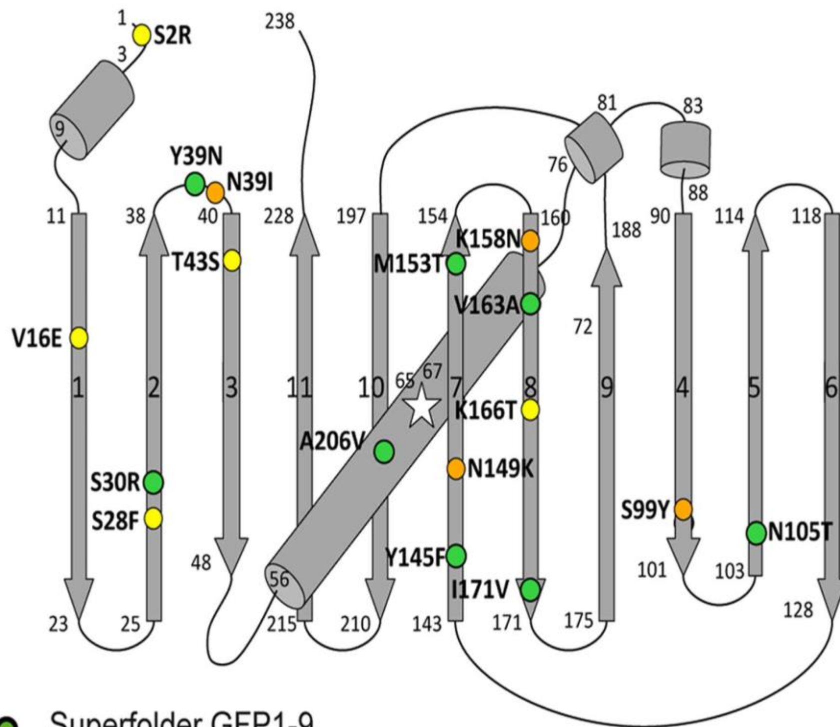
T43EC (pseudofosforilata)sia attiva che inattiva in presenza di GSK3 si ritrova associata ai corpi di inclusione



Tripartite Split-GFP system

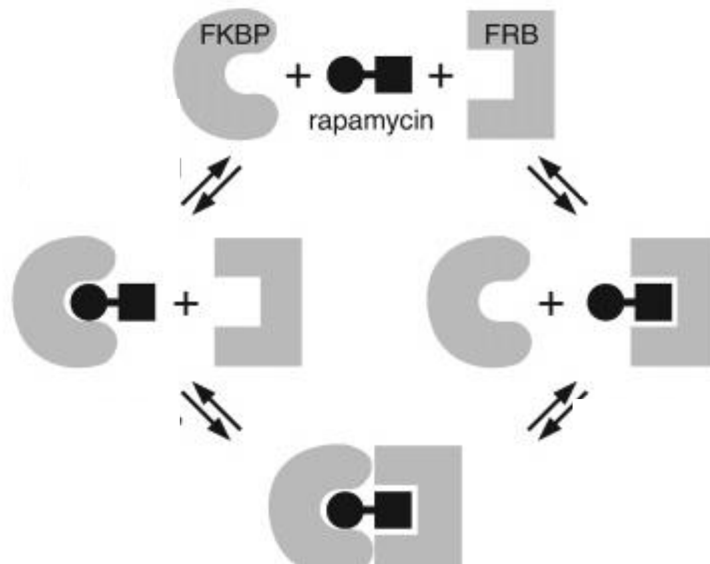


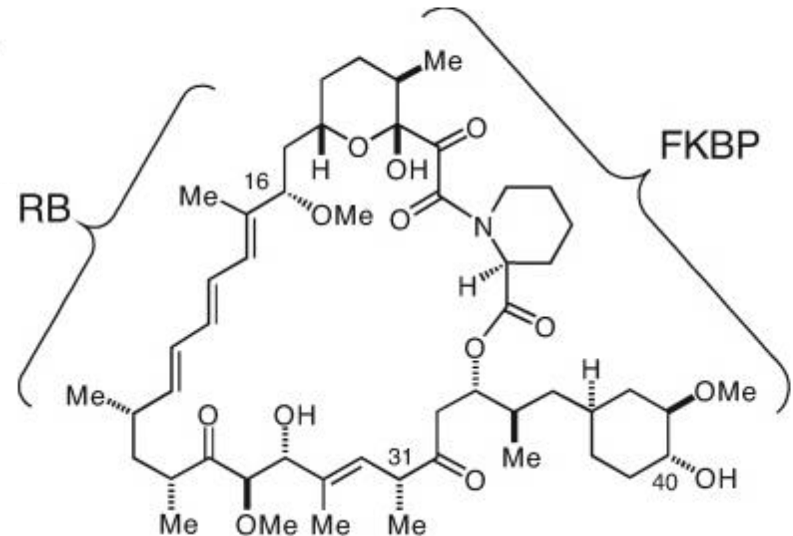
GFP10–11	194–238	Inserito un linker flessibile che include un sito di clonaggio
GFP1–9	1–193	Random PCR per migliorare il folding



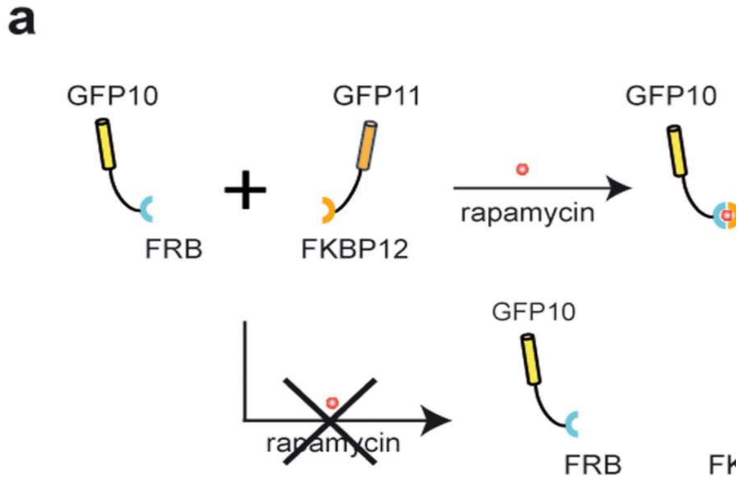
Studio dell’interazione tra rapamicina , FRB, FKBP

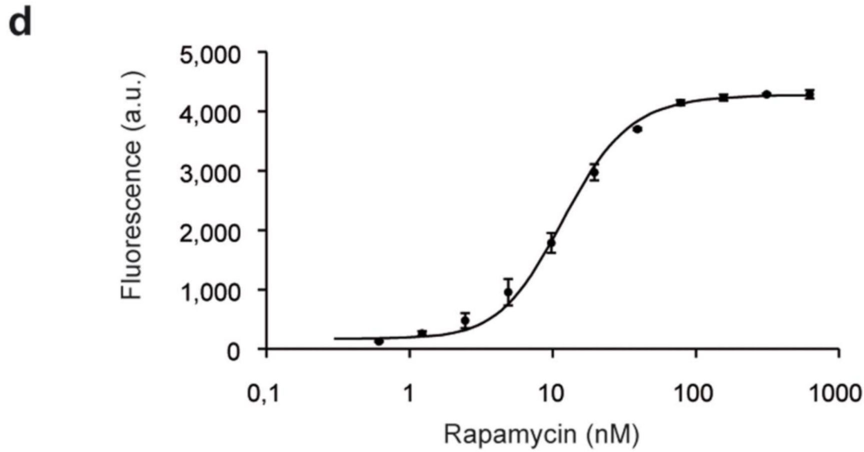
FRB rapamicina binding domain di **mTOR** (acronimo di **mammalian target of rapamycin**, protein-chinasi che fosforila serina e treonina che regola la crescita, la proliferazione, la motilità e la sopravvivenza delle cellule, la sintesi proteica e la trascrizione

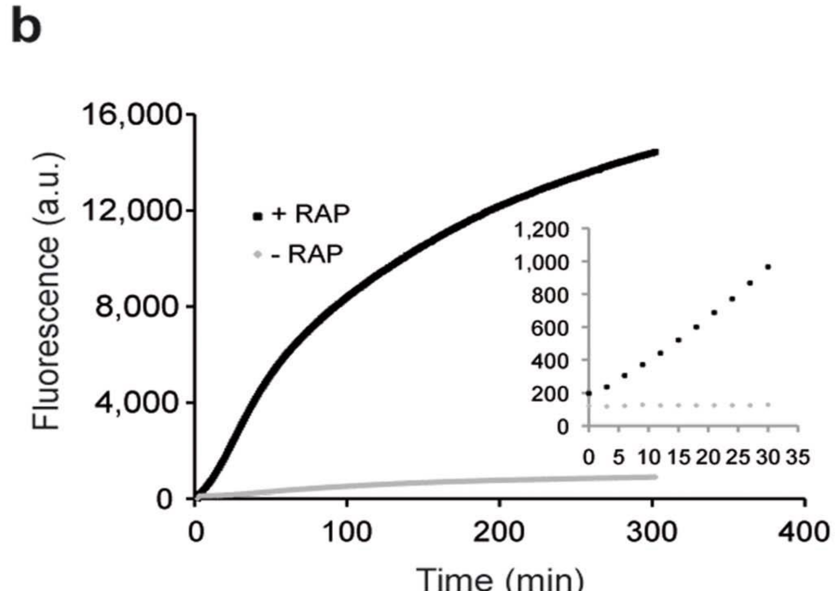


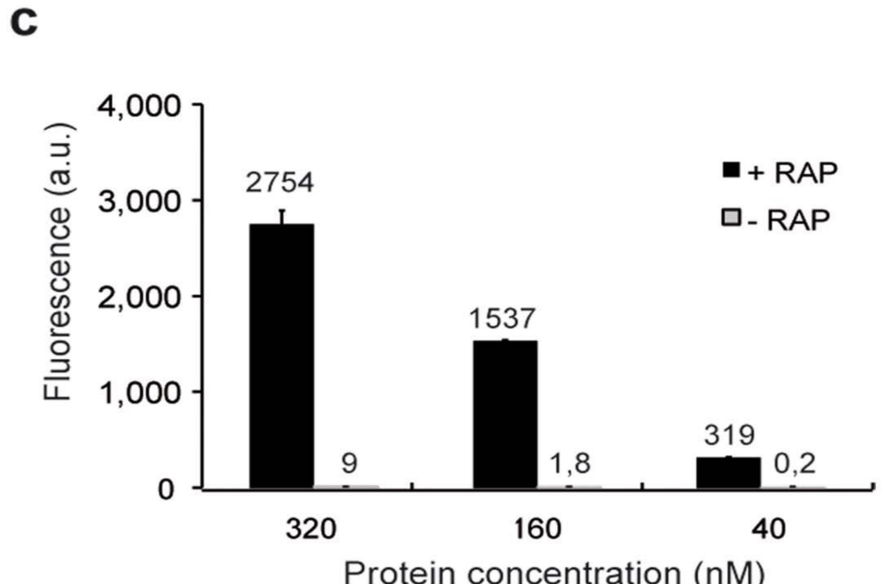


Studio dell'interazione tra rapamicina , FRB, FKBP

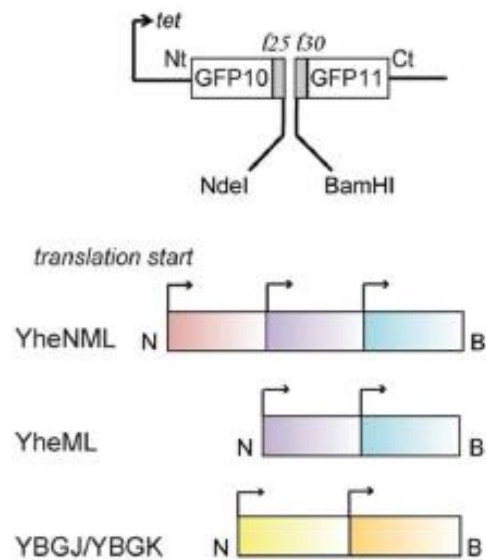




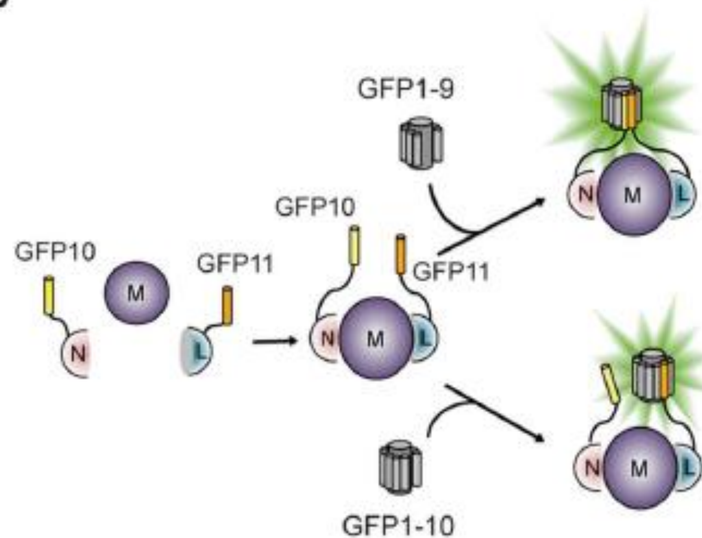




a



b



c