Metodi genetici : sistema a doppio ibrido

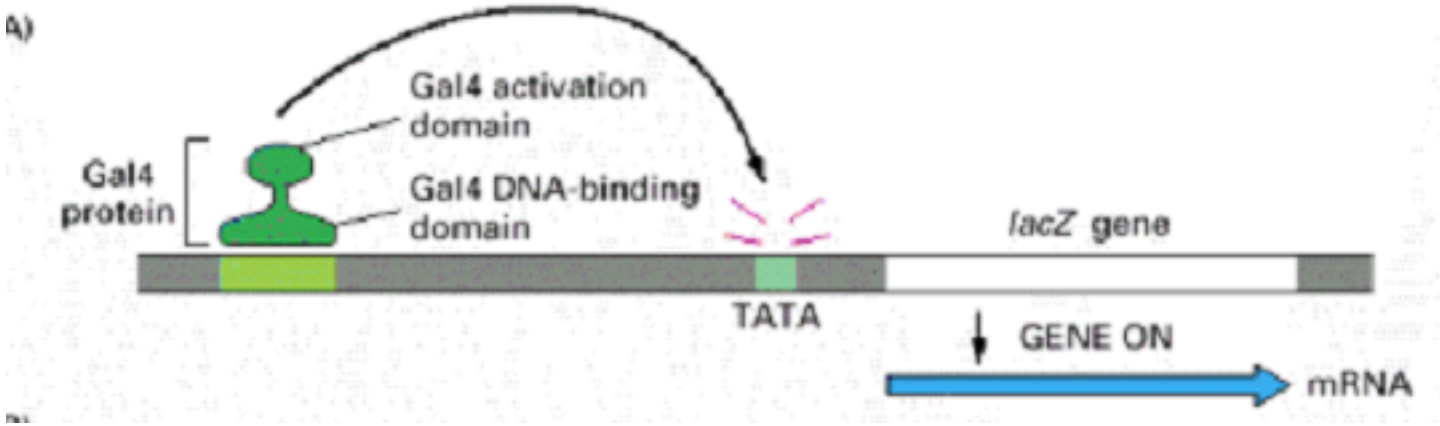
Sistema a doppio ibrido

1989 by Fields and Song

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

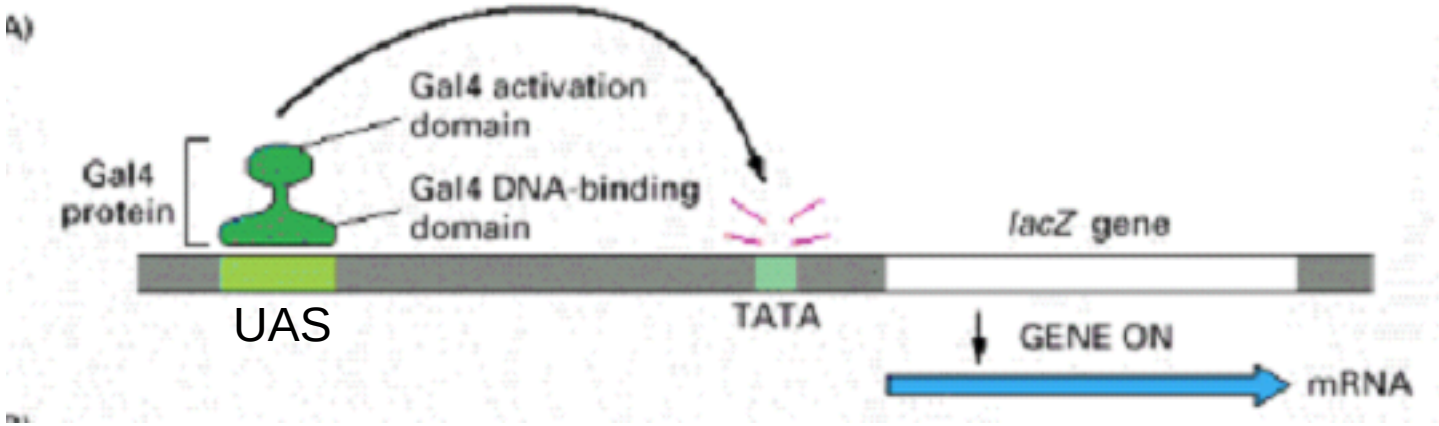
- **Gli attivatori di trascrizione sono delle proteine caratterizzate da un dominio che lega il DNA BD e un dominio che attiva la trascrizione AD**

- **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 ammino terminali codificano per il DNA binding domain (DB) o "bait"
114(residui 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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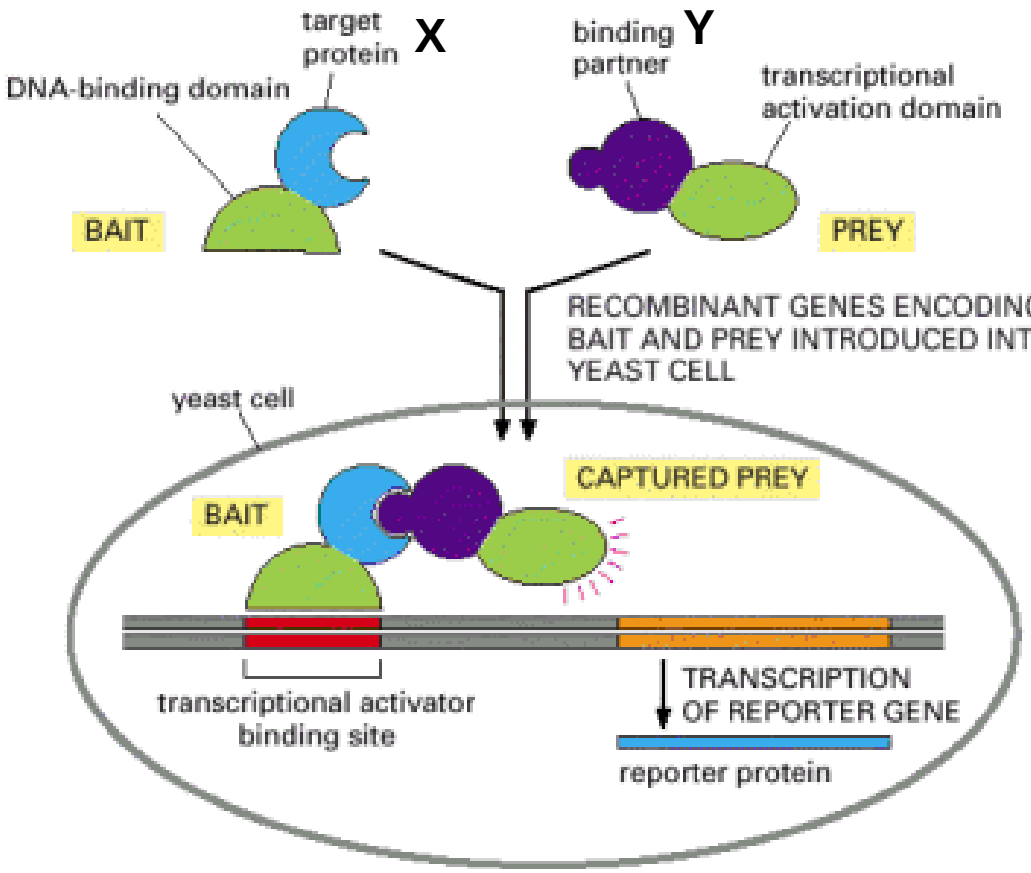
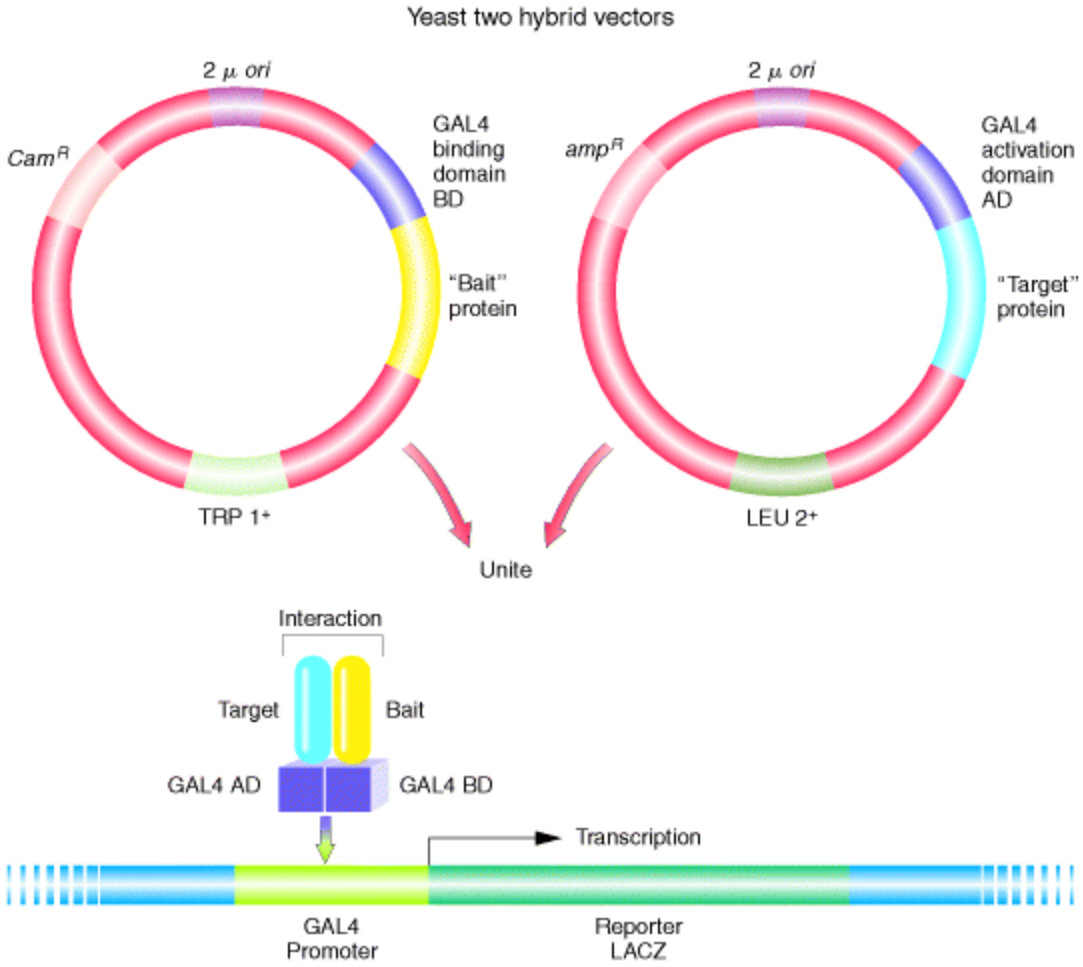


Se X ed Y interagiscono si ha la ricostituzione funzionale dell'attivatore trascrizionale che attiva l'espressione del gene reporter

I due vettori che contengono GAL4binding 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 d'interesse.

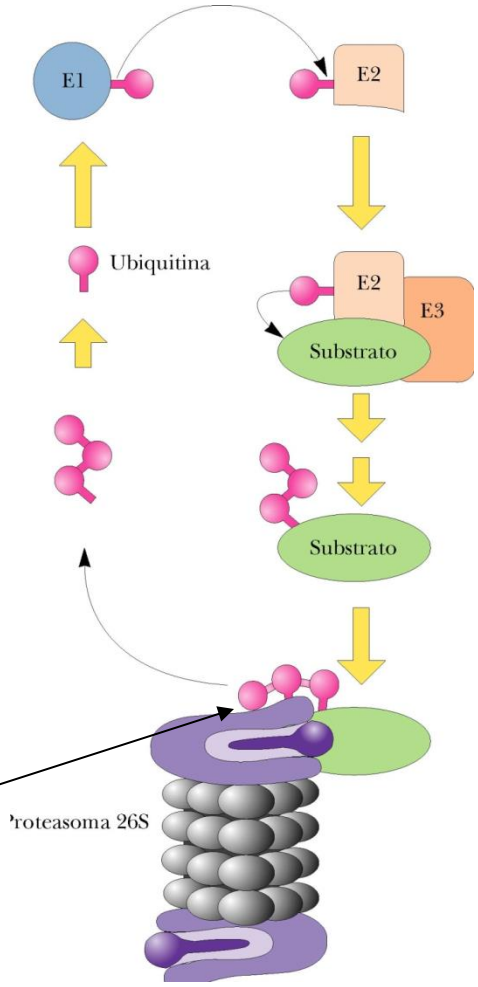
Limiti

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

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



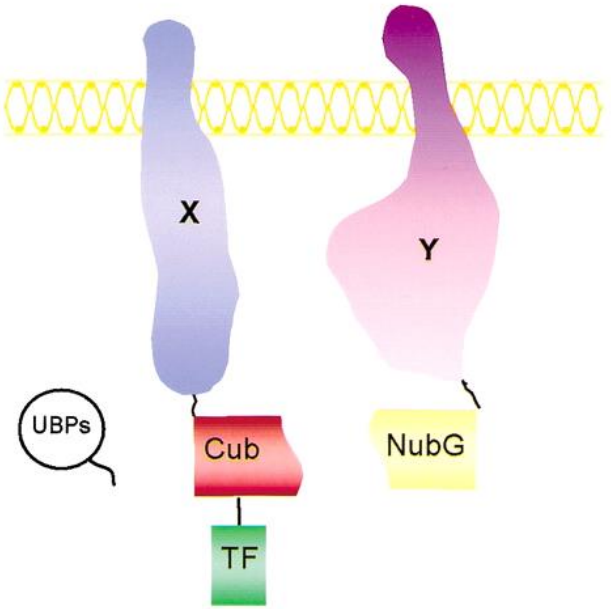
UBPs(ubiquitin- specific- proteases) idrolizzano il legame peptidico tra la proteina “ condannata” e l’ubiquitina

•**L’ubiquitina può essere espressa in due frammenti:**

Nub (N terminal fragment (1-34)

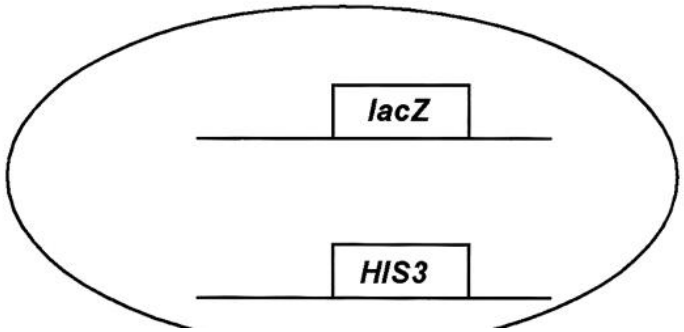
Cub (C-terminal fragment (35-76) che possono riassociarsi a formare l ’ubiquitina

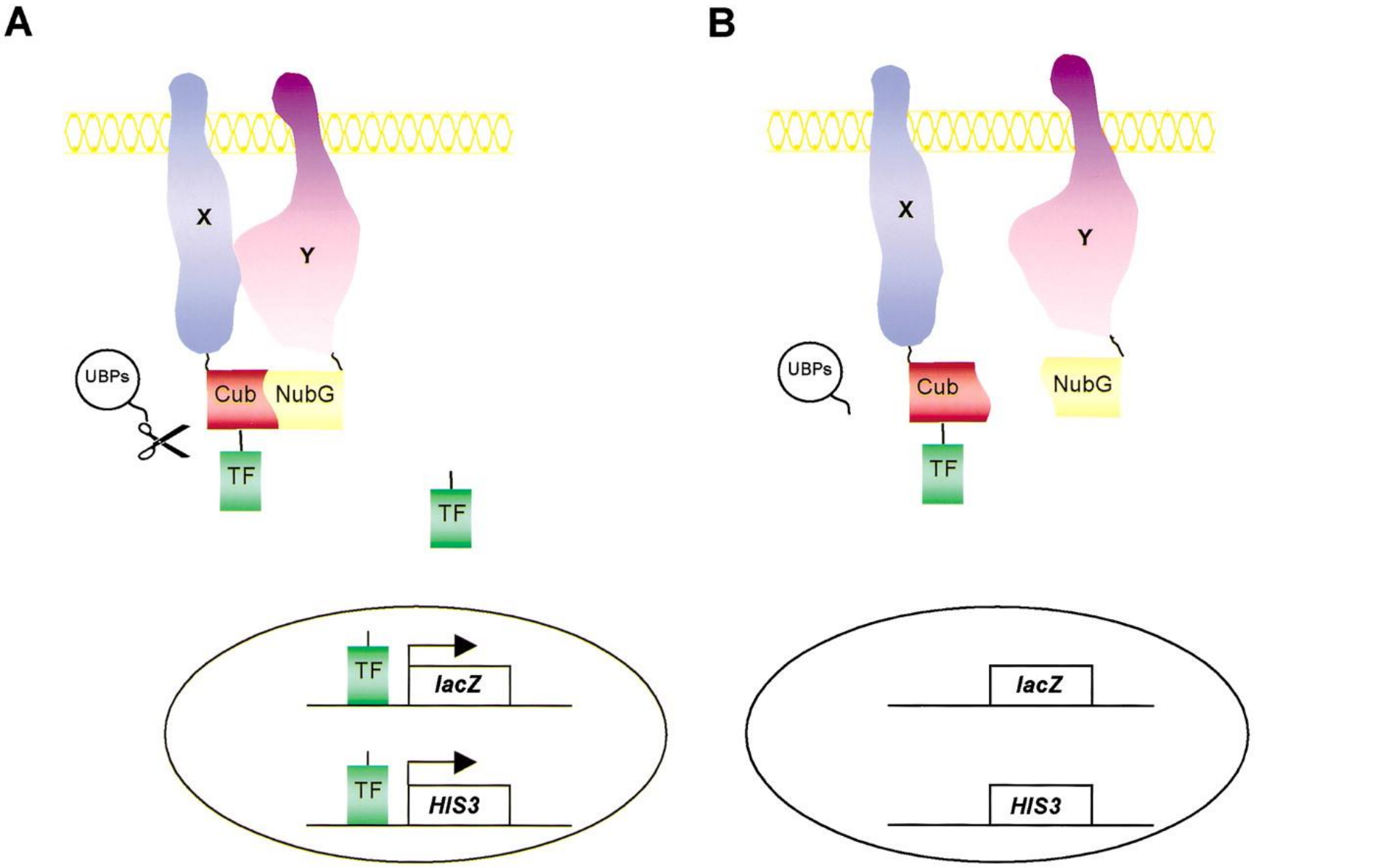
Costruzione dei due ibridi



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**

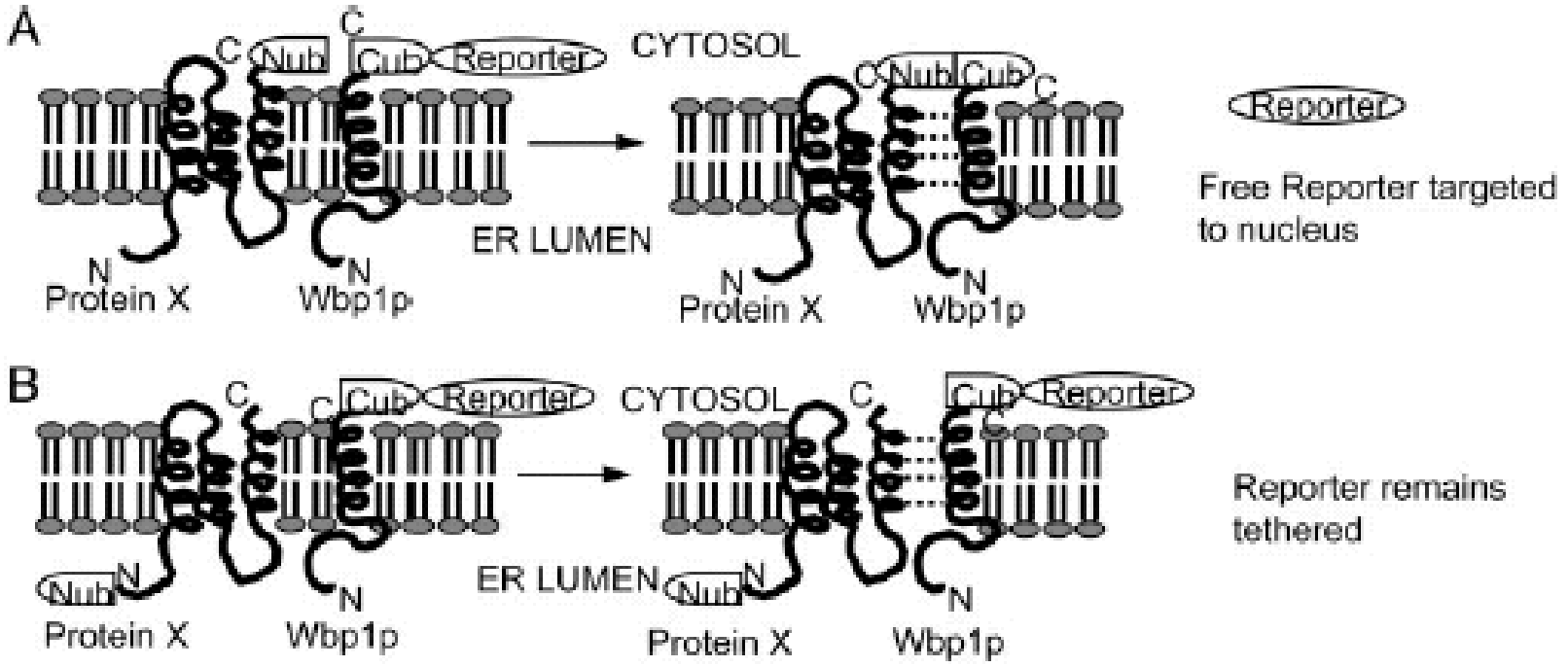



A
X
Y
UBPs
Cub
NubG
TF
TF
TF
lacZ
TF
HIS3
B
X
Y
UBPs
Cub
NubG
TF
lacZ
HIS3

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



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

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Contributed by William J. Lennarz, March 31, 2005

Oligosaccharyl transferase (OT) catalyzes the cotranslational N-

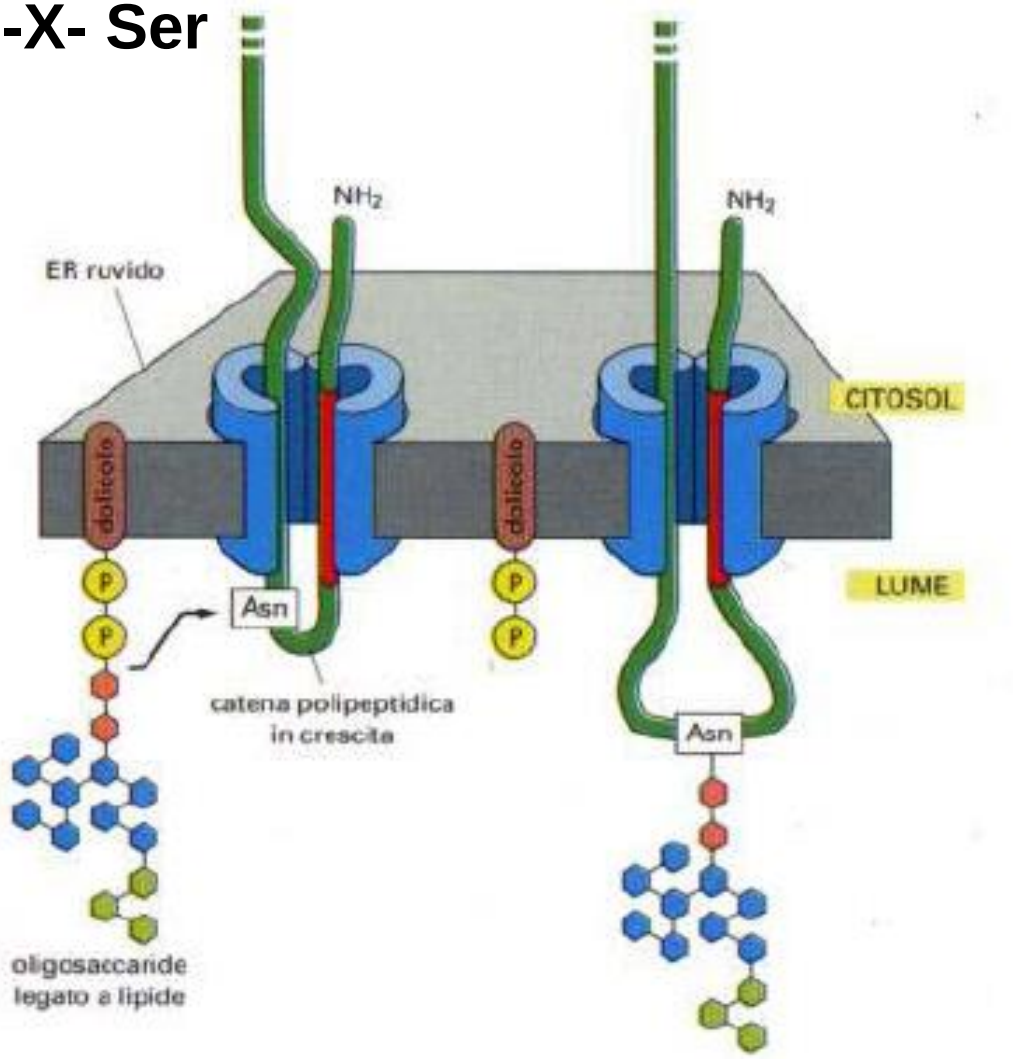
specifically applies to membrane proteins, the split-ubiquitin

L’oligosaccaride trasferasi catalizza il trasferimento di un oligosaccaride complesso(legato da un pirofosfato ad una molecola lipidica chiamata Dolicolo) al gruppo amminico di una asparagina di una sequenza consenso

Asn-X-Thr / Asn-X- Ser

D

b



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

.

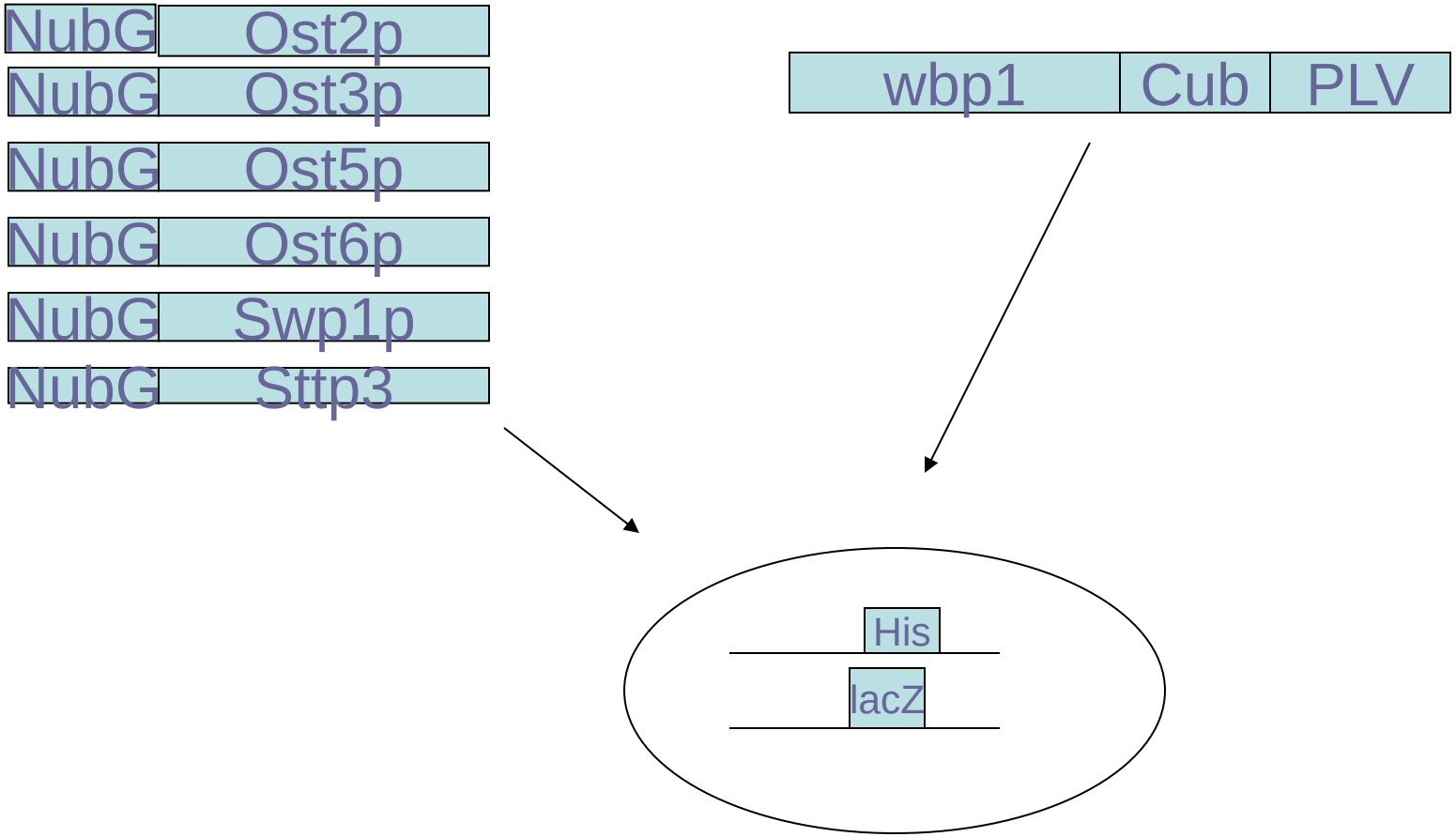
Nel lievito sono stati identificati nove geni che codificano le subunità di questo complesso proteico di membrana OST1,OST2, OST3, OST4, OST5, OST6,WBP1, SWP1, STT3

In questo lavoro mediante "split-ubiquitin system" sono stati chiariti due aspetti:

l'orientamento in membrana di tutti i componenti

il modo in cui le diverse subunità interagiscono *in vivo*.

I Wbp1p è stato usato come esca



Wbp1 interagisce con
Ost2p Ost6p Stt3p

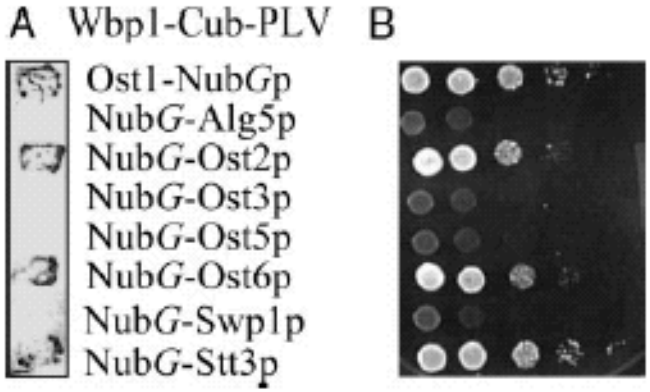
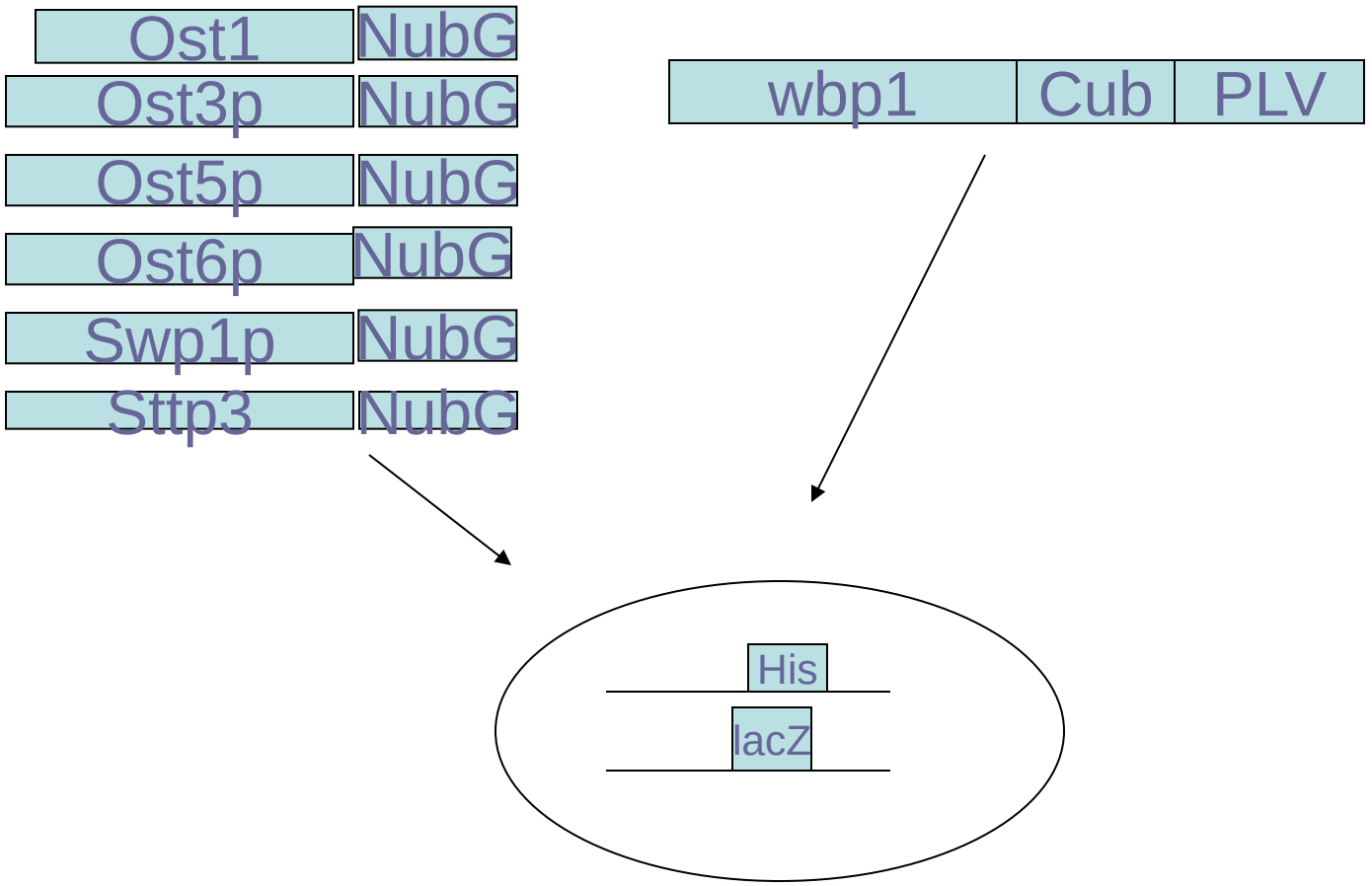


Fig. 2. Split-ubiquitin analysis between Wbp1-Cub-PLVp and NubG-Xp (X represents Ost2p, Ost3p, Ost5p, Ost6p, Stt3p, or Swp1p). (*A*) β-Gal activity of the transformants expressing Wbp1-Cub-PLVp together with the NubG-Xp fusion proteins. Cells were grown on plates lacking tryptophan and leucine, transferred 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’



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

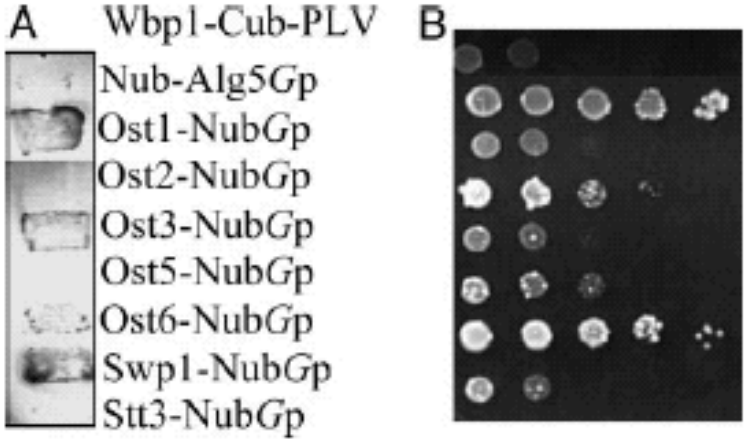


Fig. 3. Split-ubiquitin analysis between Wbp1-Cub-PLVp and X-NubGp (X represents Ost2p, Ost3p, Ost5p, Ost6p, Stt3p, or Swp1p). Shown are β-Gal activity (*A*) and growth of cells coexpressing Wbp1-Cub-PLVp together with X-NubGp fusion proteins on agar plates without histidine (*B*). The procedures, as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

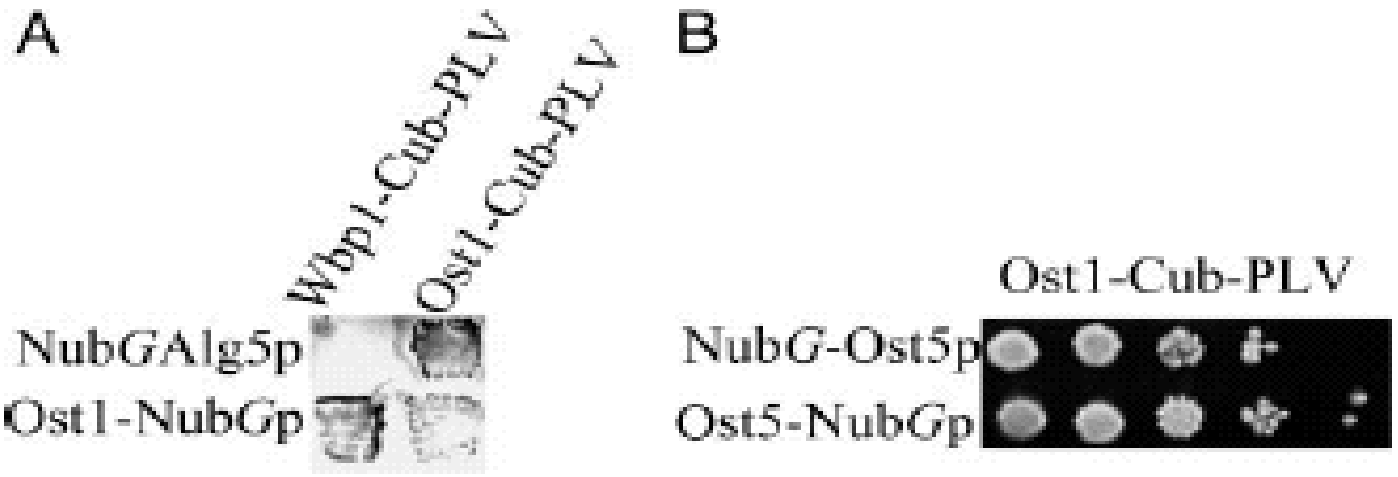
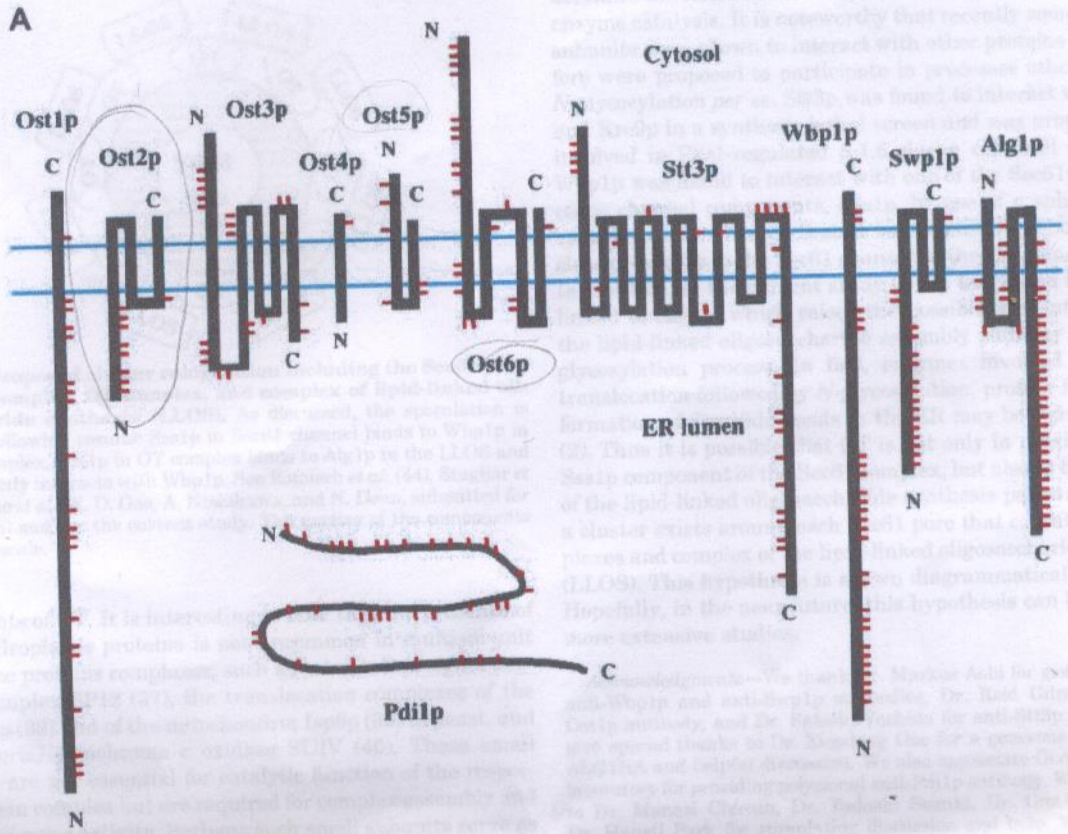


Fig. 4. Split-ubiquitin analysis between Ost1-Cub-PLVp and NubG-Ost5p or Ost5-NubGp. (*A*) β-Gal activity of cells coexpressing Ost1-Cub-PLVp together with NubG-Ost5p or Ost5-NubGp fusion proteins, respectively. (*B*) Growth of the cells coexpressing Ost1-Cub-PLVp with NubG-Ost5p or Ost5-NubGp fusion 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
Ost1p
Ost2p
Ost3p
Ost4p
Ost5p
Ost6p
Stt3p
Wbp1p
Swp1p
Alg1p
ER lumen
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

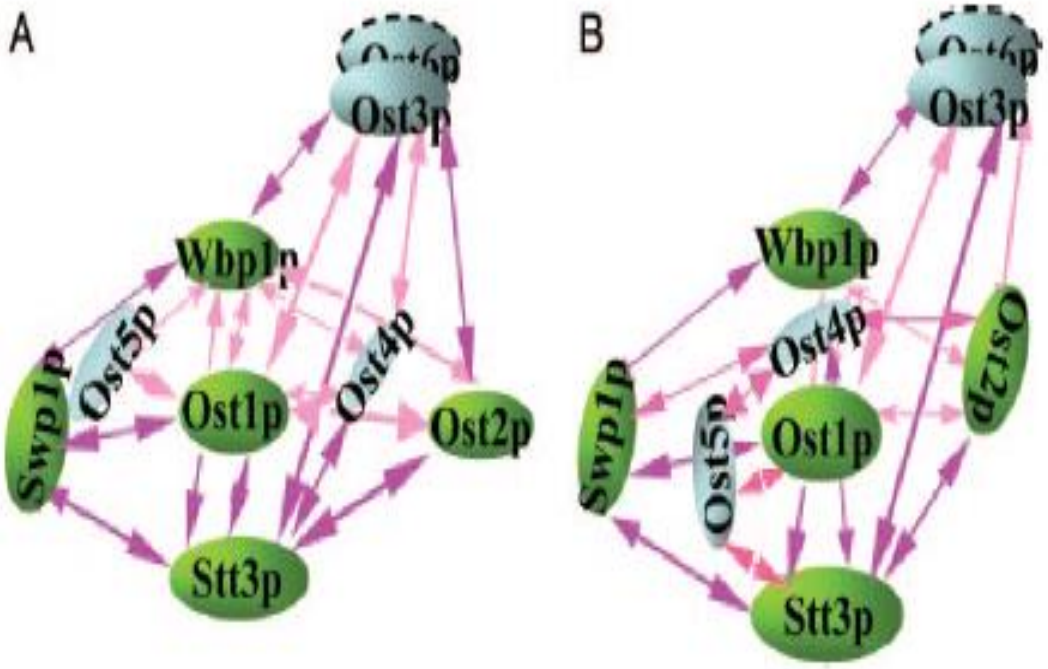
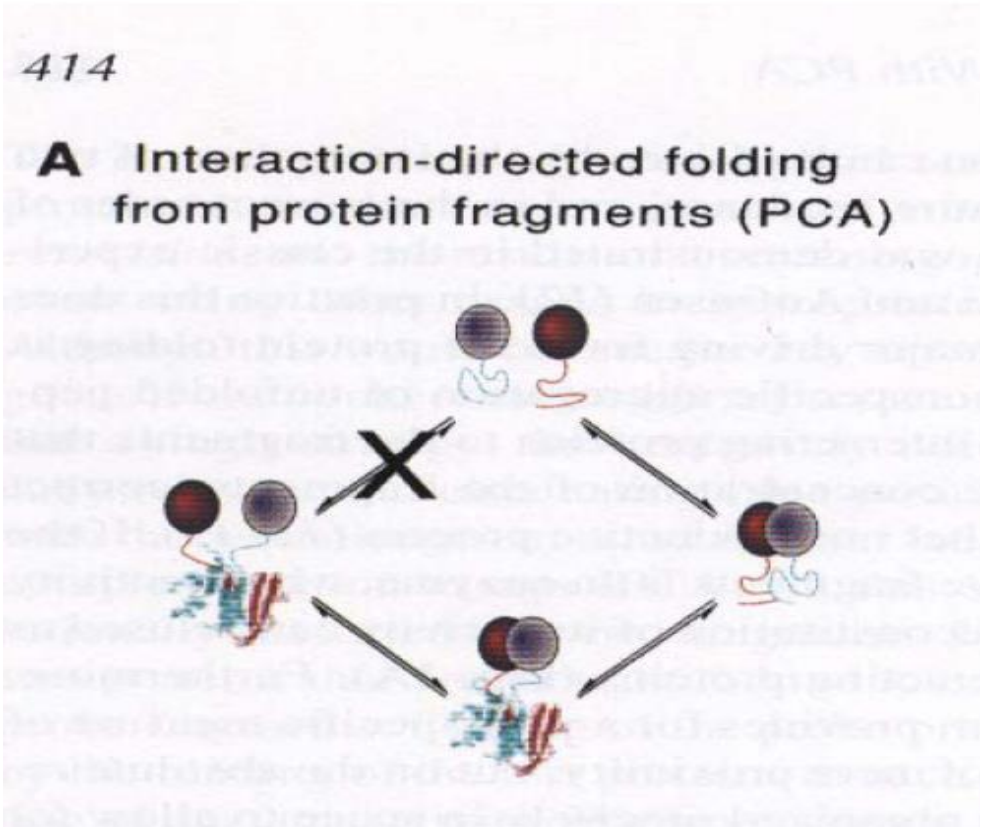
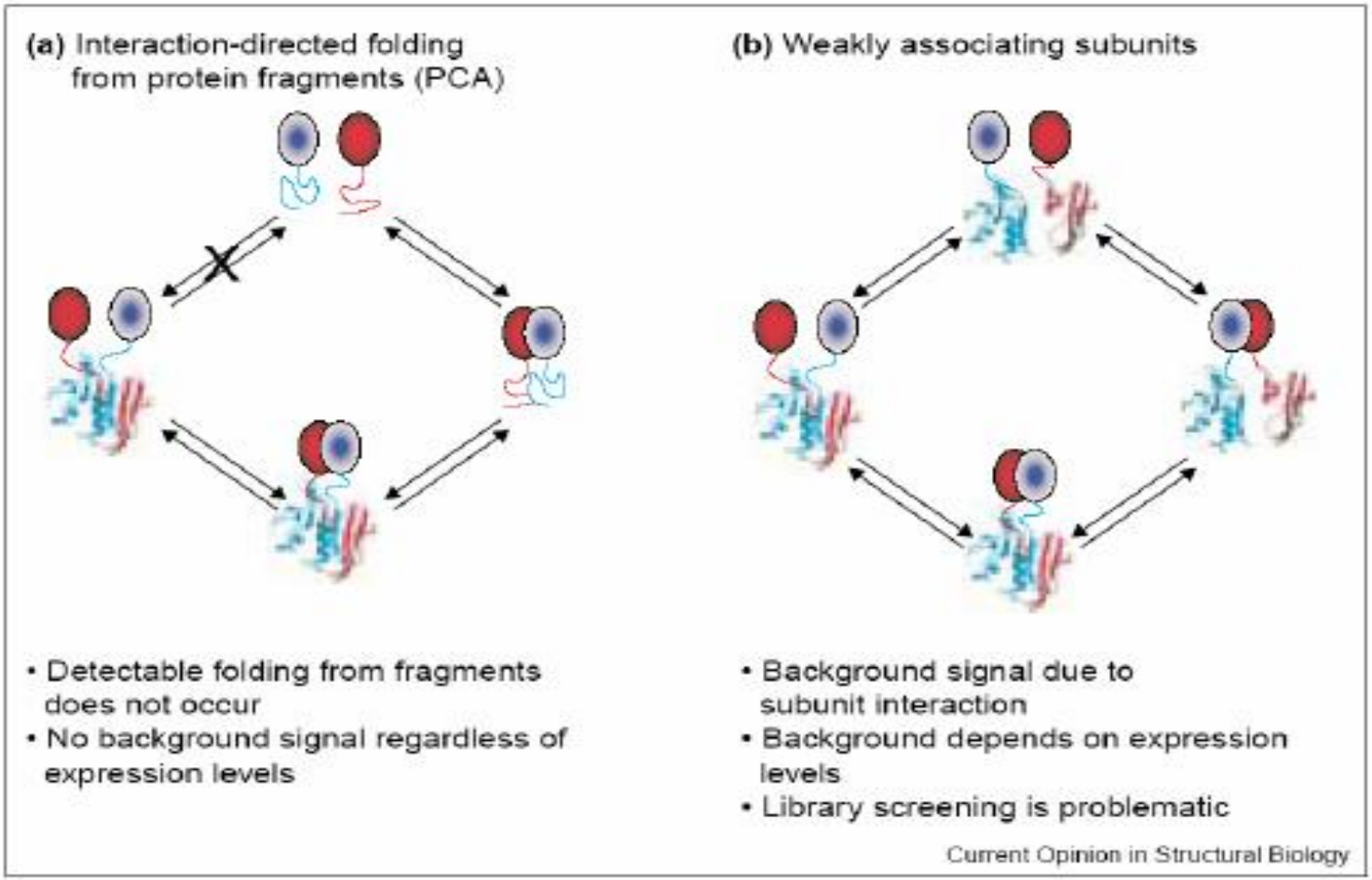


Fig. 5. Comparison of the structural organization of OT complex in a nonworking state (*in vitro*) (*A*) and a working state (*in vivo*) (*B*). The nonworking model was developed based on previous cross-linking studies in isolated microsomal membranes, and the working model was developed based on the *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.

Protein Complementation Assay

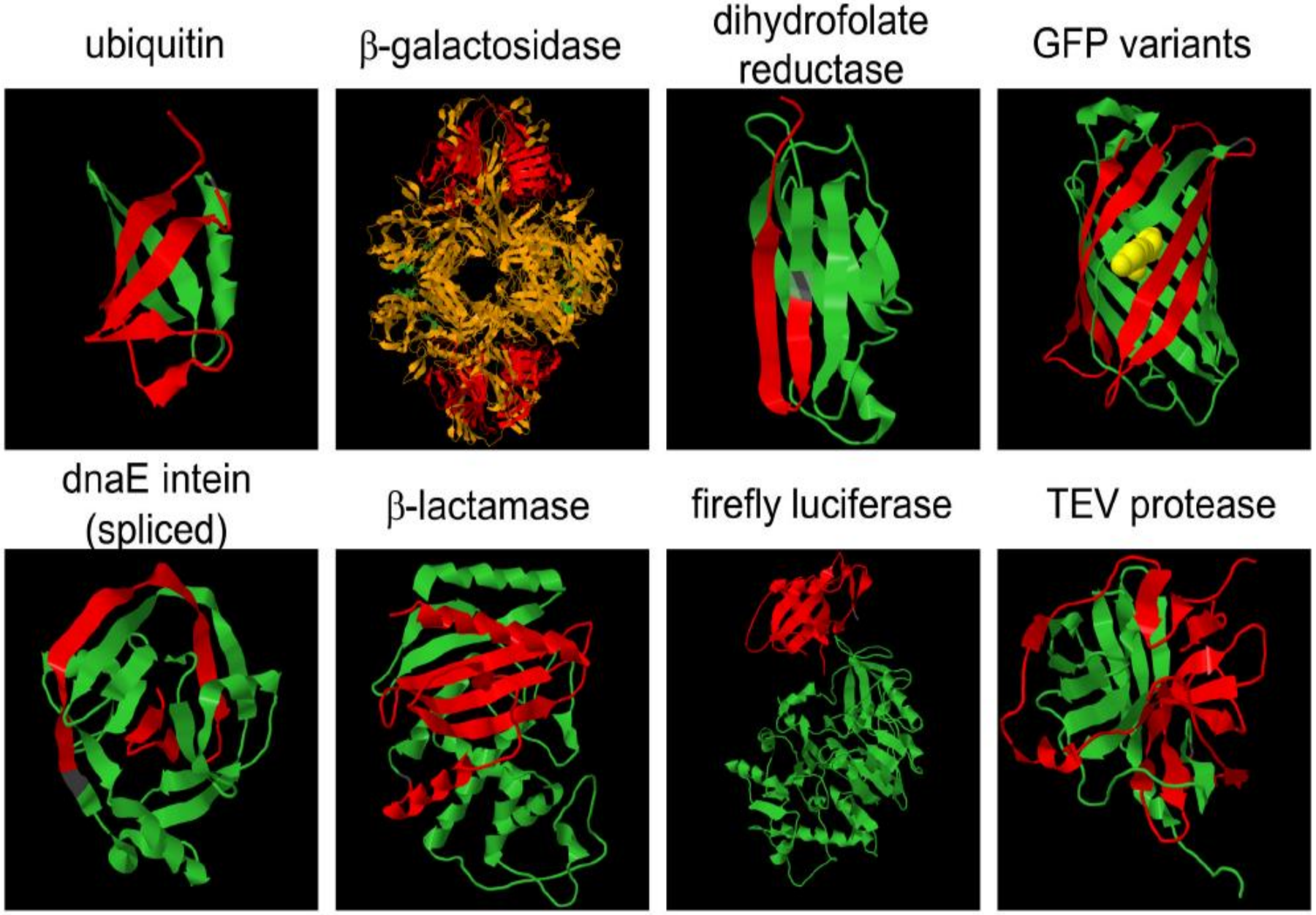
- proteina reporter è separata in due frammenti
- i frammenti sono fusi geneticamente ai potenziali partners dell'interazione
- Il folding della proteina reporter è mediato dall'interazione delle proteine partners





Dihydrofulat reductase
Lactamase
Split YFP

Ubiquitin
Luciferase


ubiquitin
β-galactosidase
dihydrofolate reductase
GFP variants
dnaE intein (spliced)
β-lactamase
firefly luciferase
TEV protease

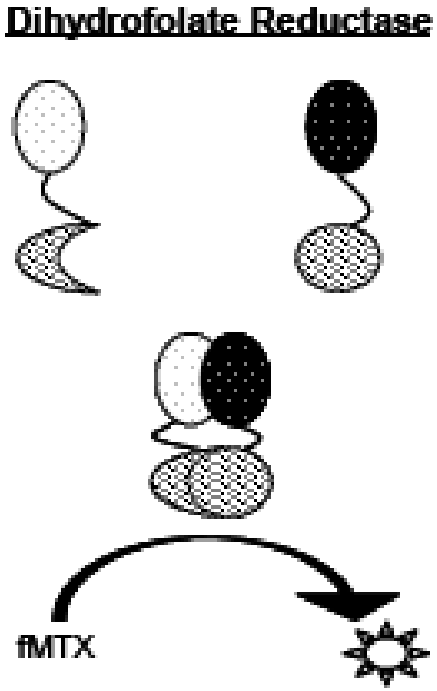
Enzimi
Il folding indotto dall'interazione delle proteine partners porta al ripristino dell'attività enzimatica

Beta galattosidasi , deidrofolato reduttasi (DHFR) o beta lattamasi

Disponibilità di substrati permeabili cromogenici

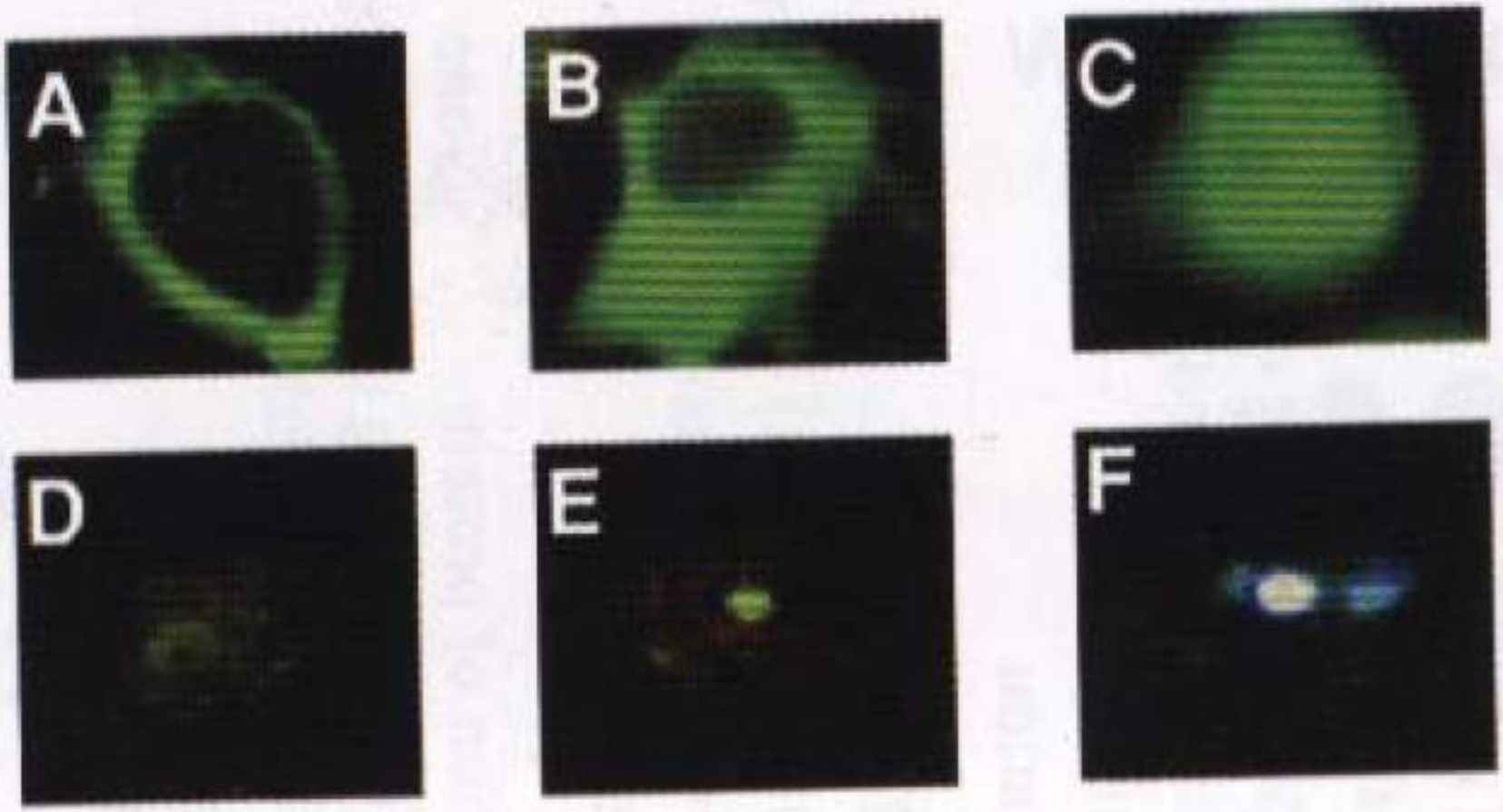
La didrofolatoreduttasi catalizza la riduzione dell'idrofolato in tetraidrofolato importante per la biosintesi dei nucleotidi

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)



f MTX lega con elevata affinità DHFR
Kd = 540 pM

Dihyrofolate reductase (DHFR) is a small



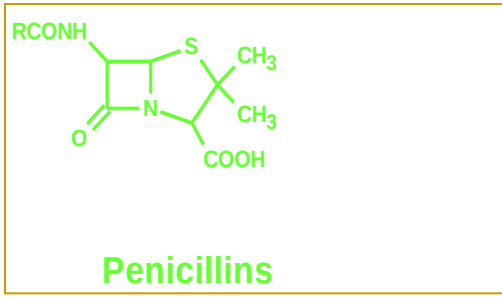
A B C CELLULE COS
D E F localizzazione di NPR1-DHFR E TGA2-DHFR trattate con acido salicilico (E ed F)

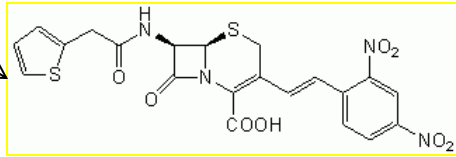
Beta lattamasi enzima monomerico di 29kD

Può essere scisso in due frammenti 24~215 e 216~286 che possono ricostituirsi

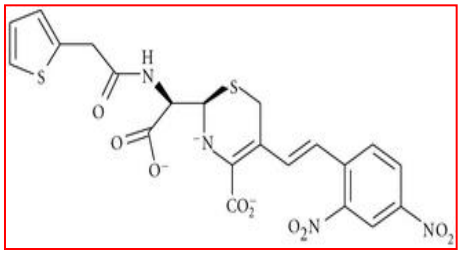
L'interazione tra le proteine partners può essere evidenziata

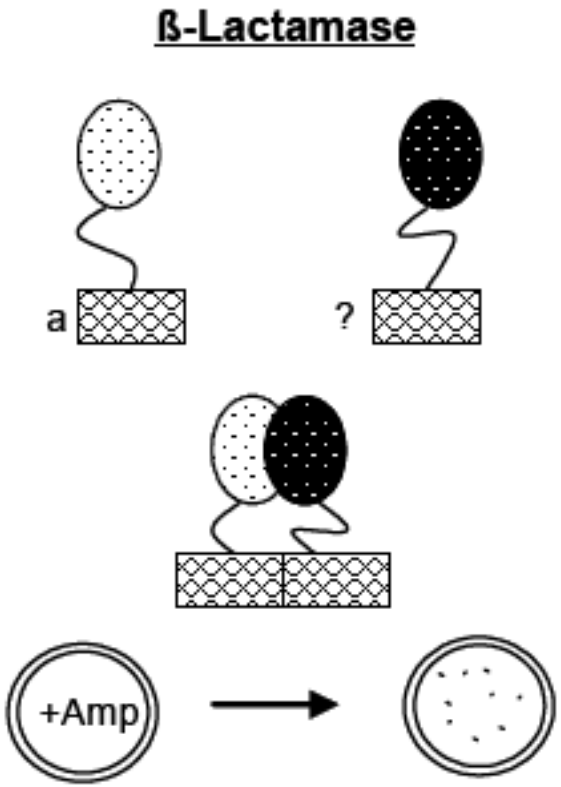
- misurando la resistenza all'ampicillina
- Saggio colorimetrico con nitrocefina





Beta lattamasi





Proteine fluorescenti (Bimolecular Fluorescent Complementation BiFC)

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

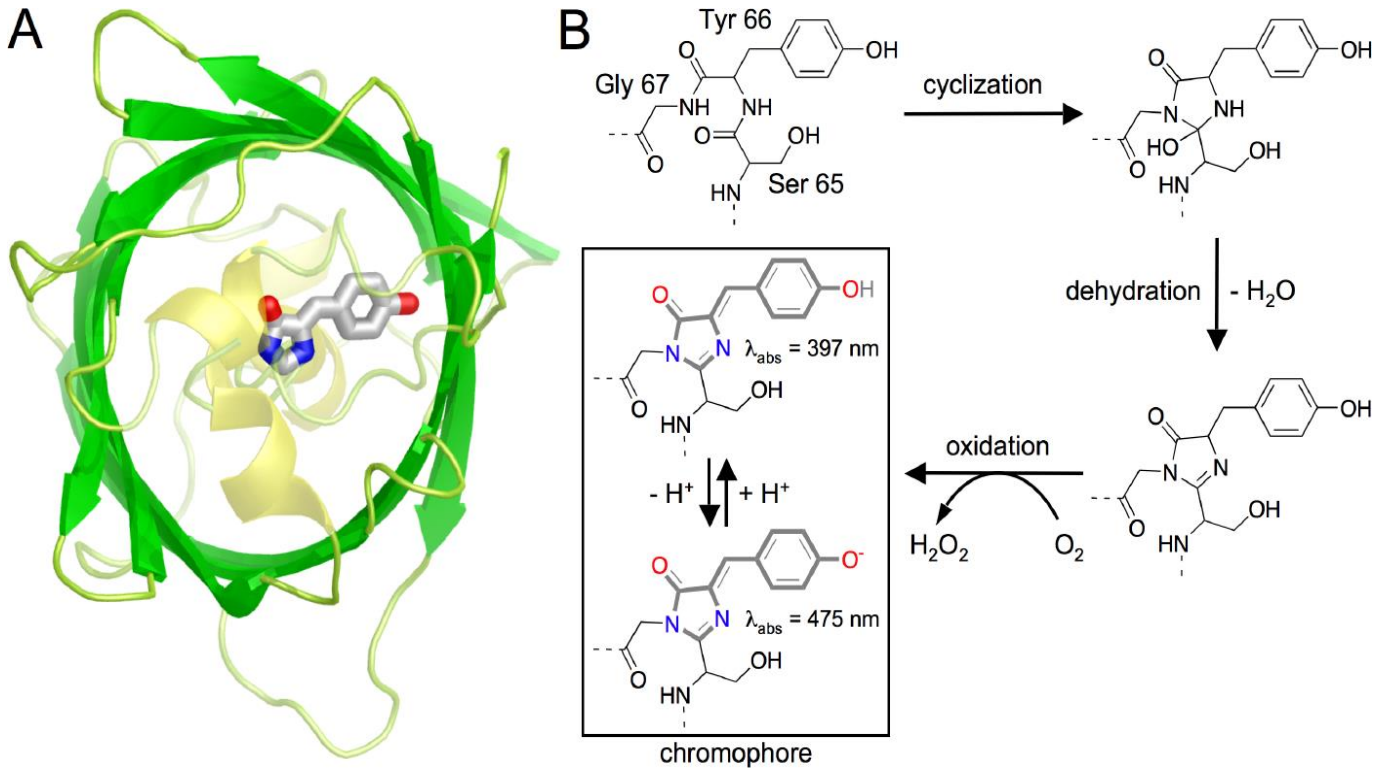
GFP è una proteina di 238 aminoacidi formata da 11 foglietti beta (Prasher *et al*.,1992) isolata dall' *Aequorea victoria*

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

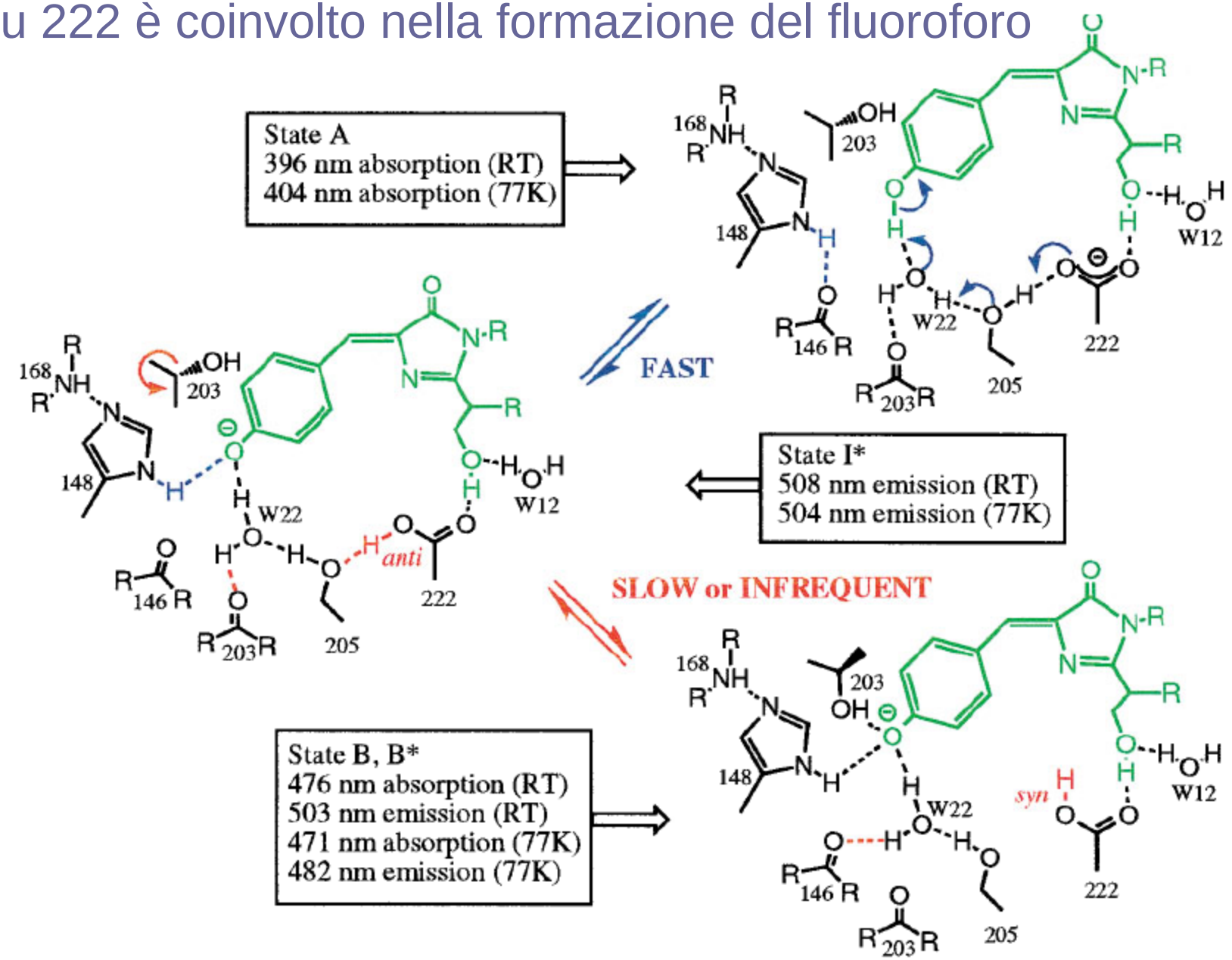
S65T produce il fluoroforo 4 volte più velocemente del wt

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

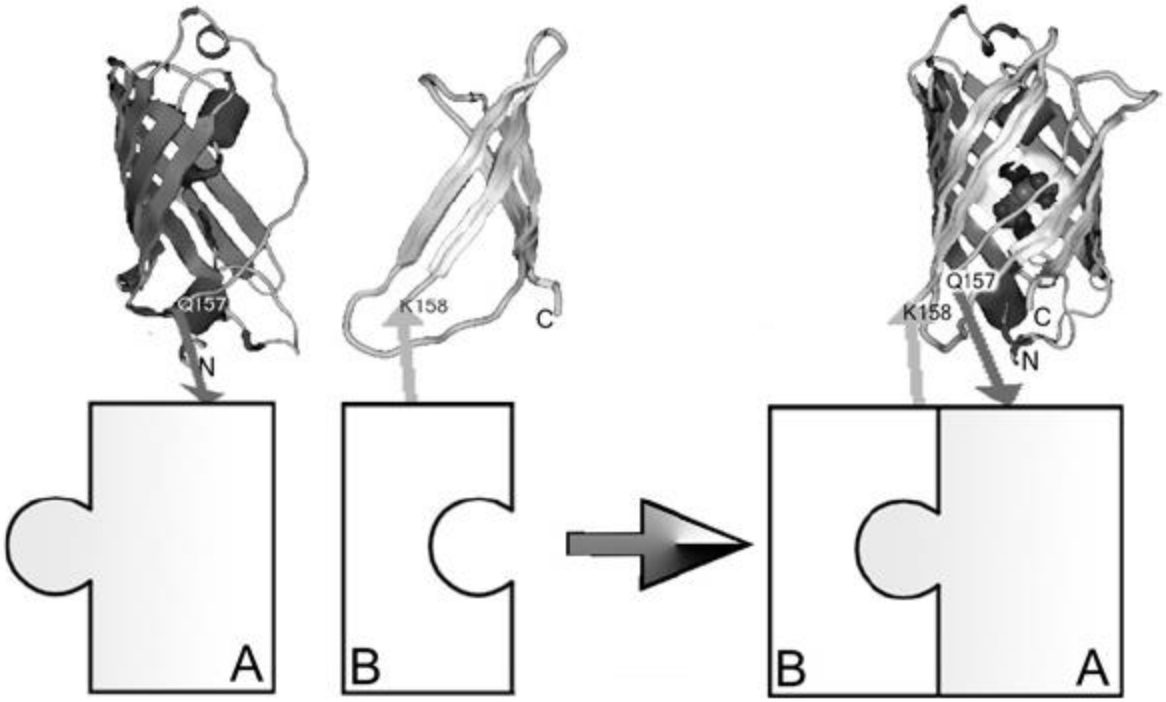
Formazione del cromoforo

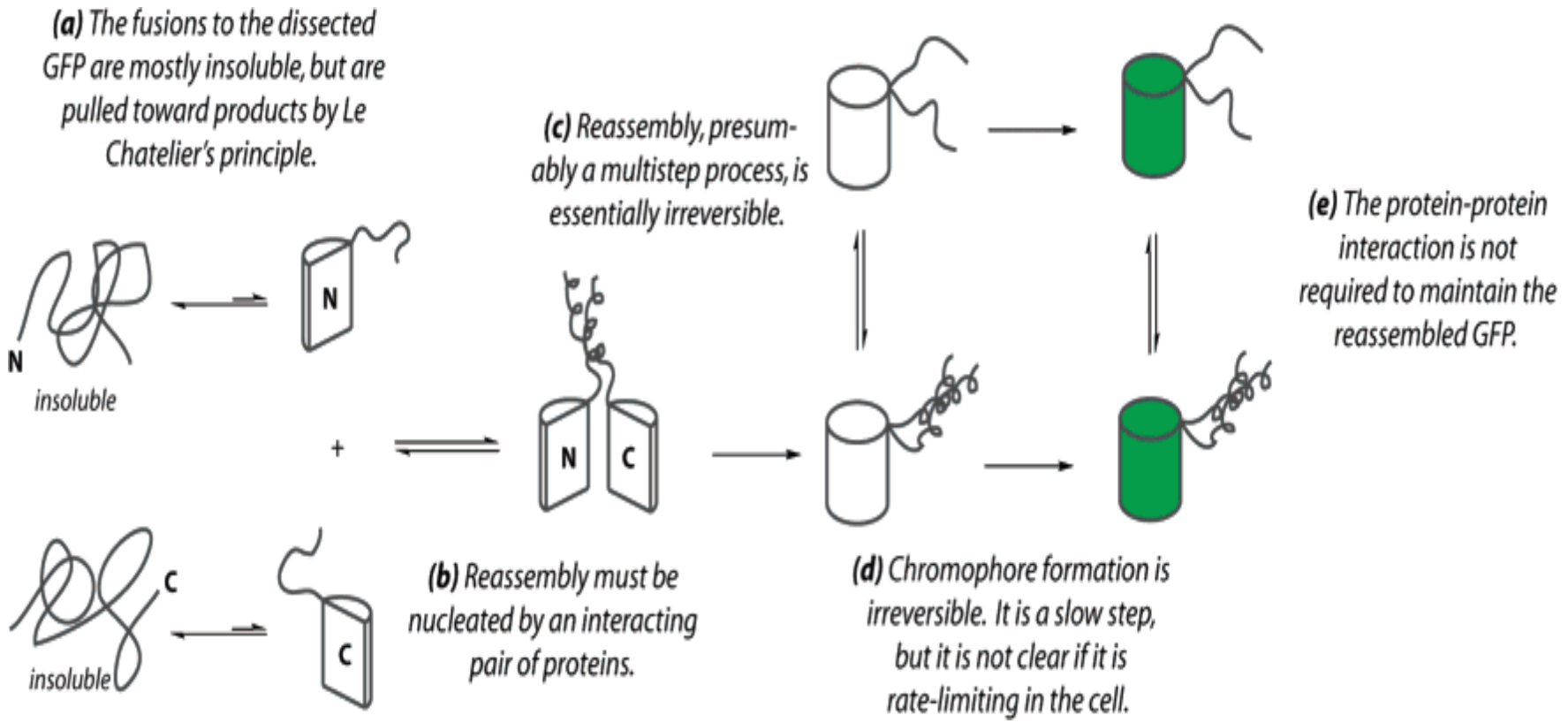


Glu 222 è coinvolto nella formazione del fluoroforo

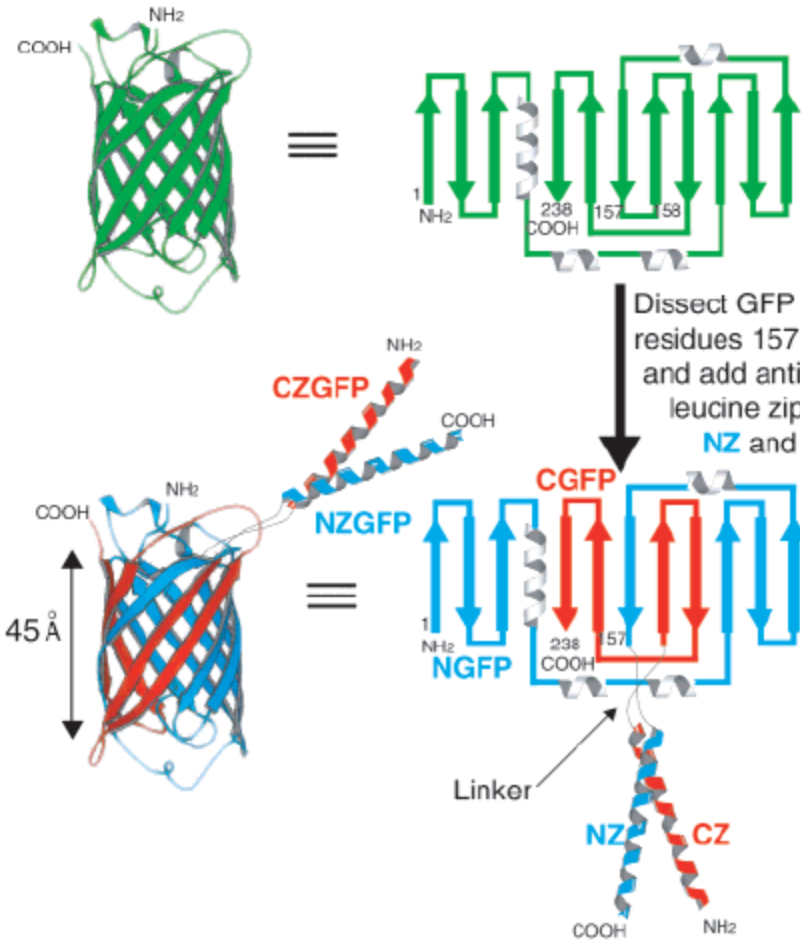


GFP è separata tra i residui 157 and 158 per produrre due frammenti non fluorescenti NGFP e CGFP.




(a) The fusions to the dissected GFP are mostly insoluble, but are pulled toward products by Le Chatelier's principle.
N
insoluble
N
+
C
insoluble
C
(b) Reassembly must be nucleated by an interacting pair of proteins.
N
C
(c) Reassembly, presumably a multistep process, is essentially irreversible.
(d) Chromophore formation is irreversible. It is a slow step, but it is not clear if it is rate-limiting in the cell.
(e) The protein-protein interaction is not required to maintain the reassembled GFP.

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



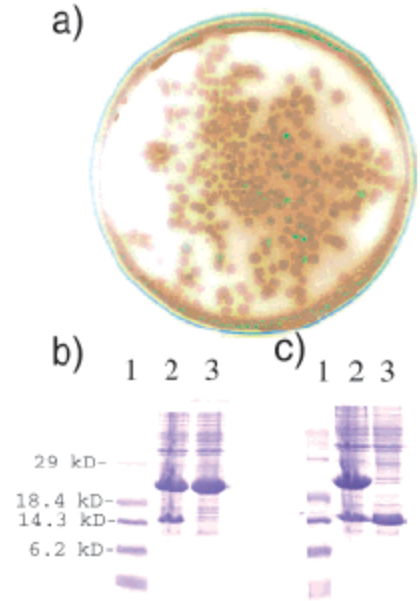


Figure 3. In vitro reconstitution of GFP demonstrated by (a) green fluorescent BL21(DE3) cells and the corresponding SDS gels of (b) lane 1: MW markers; lane 2: protein from cotransformed green colony; and lane 3: protein from colony containing only NZGFP plasmid and (c) lane 1: MW markers; protein from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

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

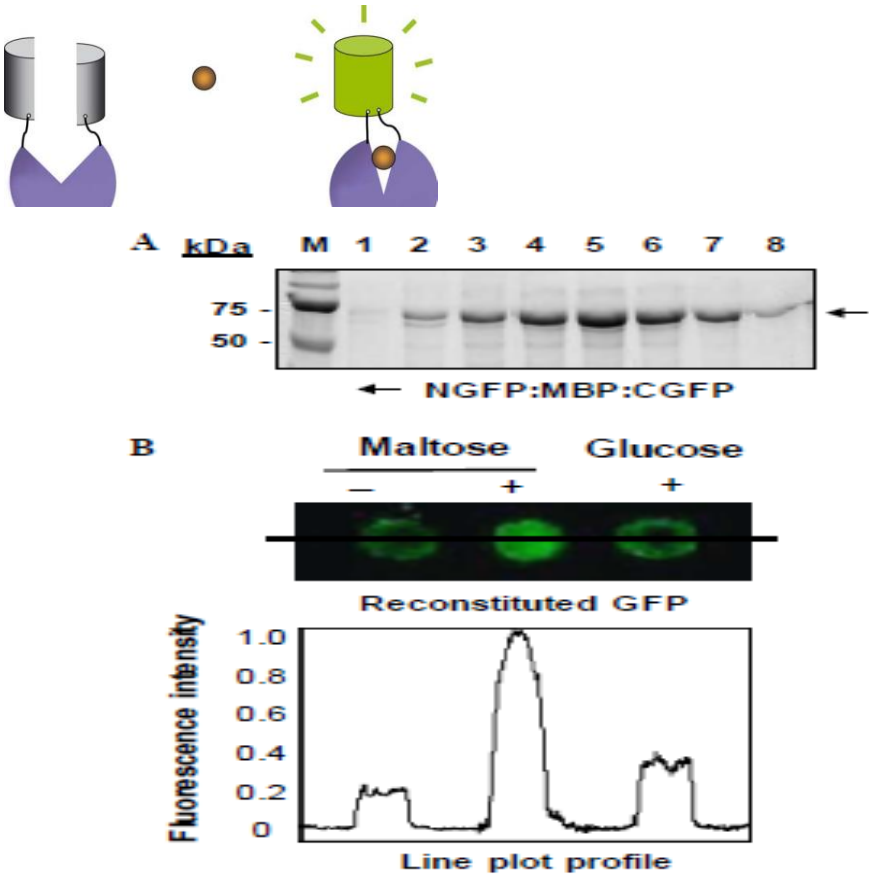


Fig. 2. In vitro visualization of conformational changes in MBP. (Purification of recombinant NGFP:MBP:CGFP protein using immo lized metal ion affinity chromatography (IMAC). Purified NGFP:MI CGFP was analyzed on 10% SDS–PAGE gel, and the arrow indicates NGFP:MBP:CGFP-containing fractions. The procedure 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.

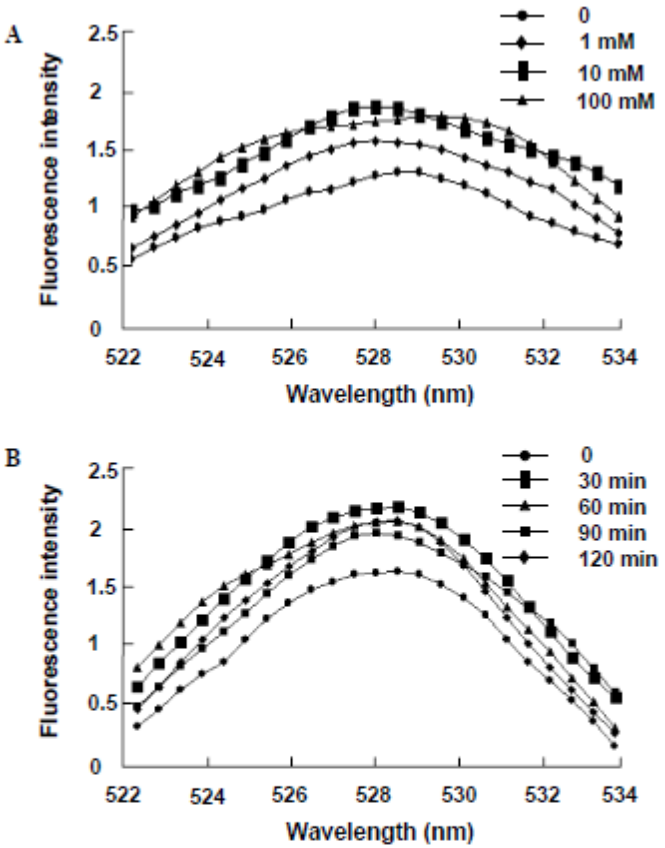
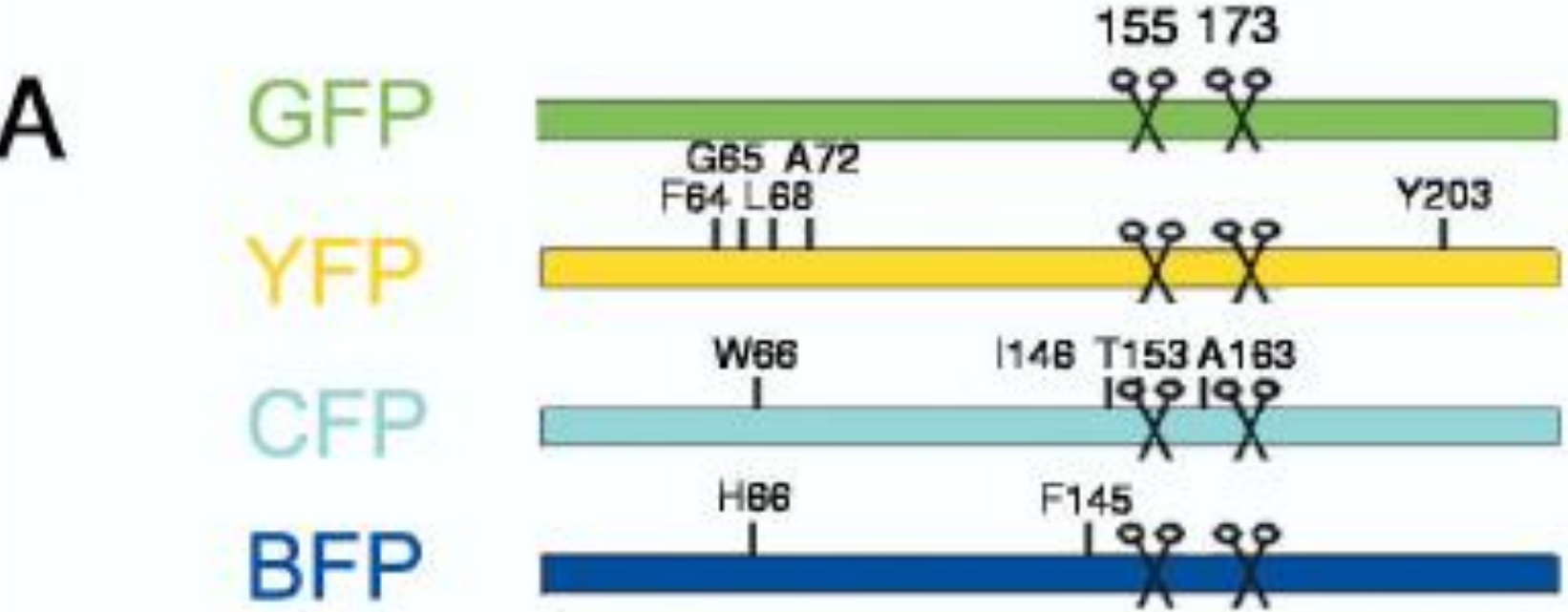
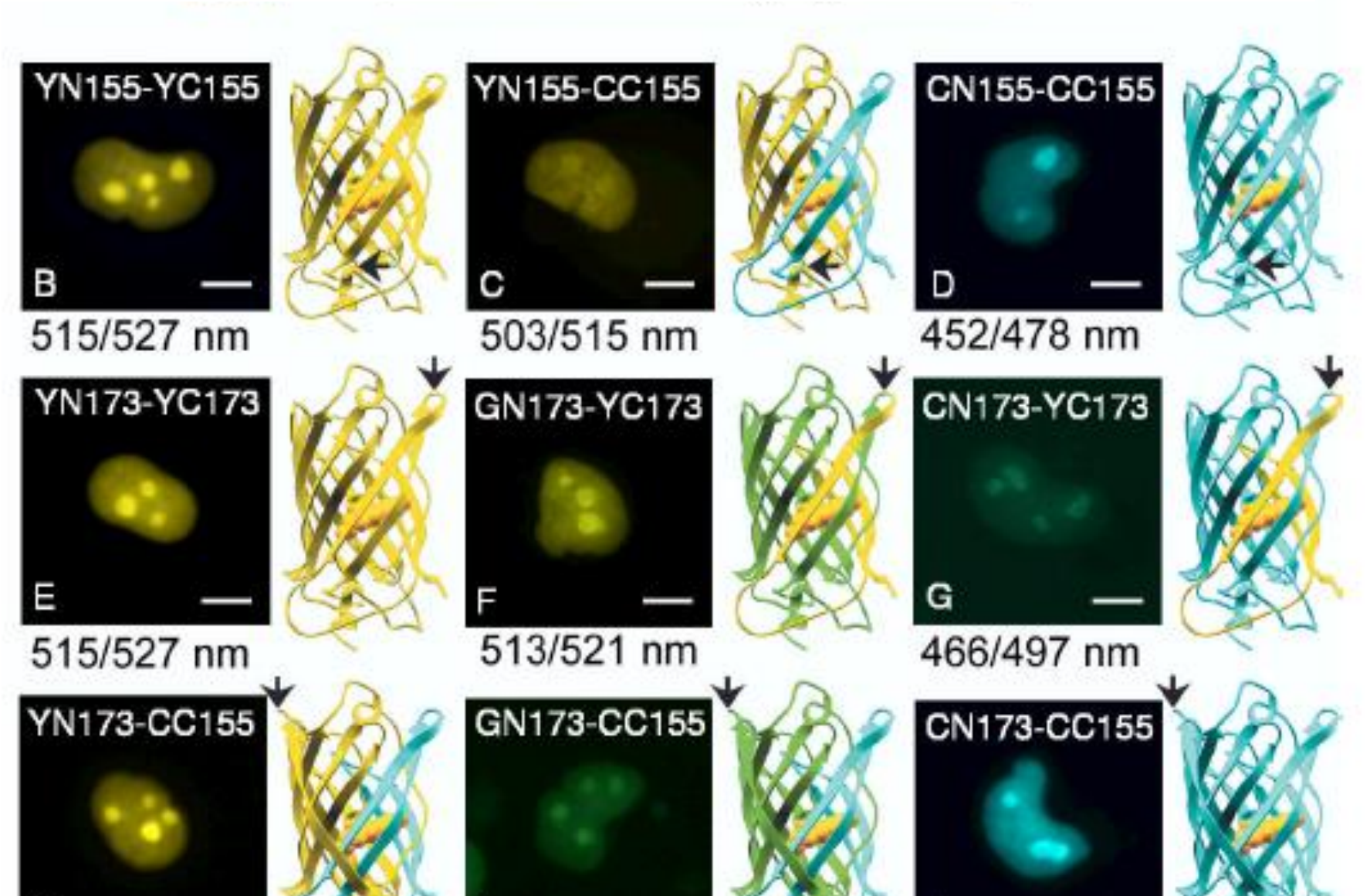


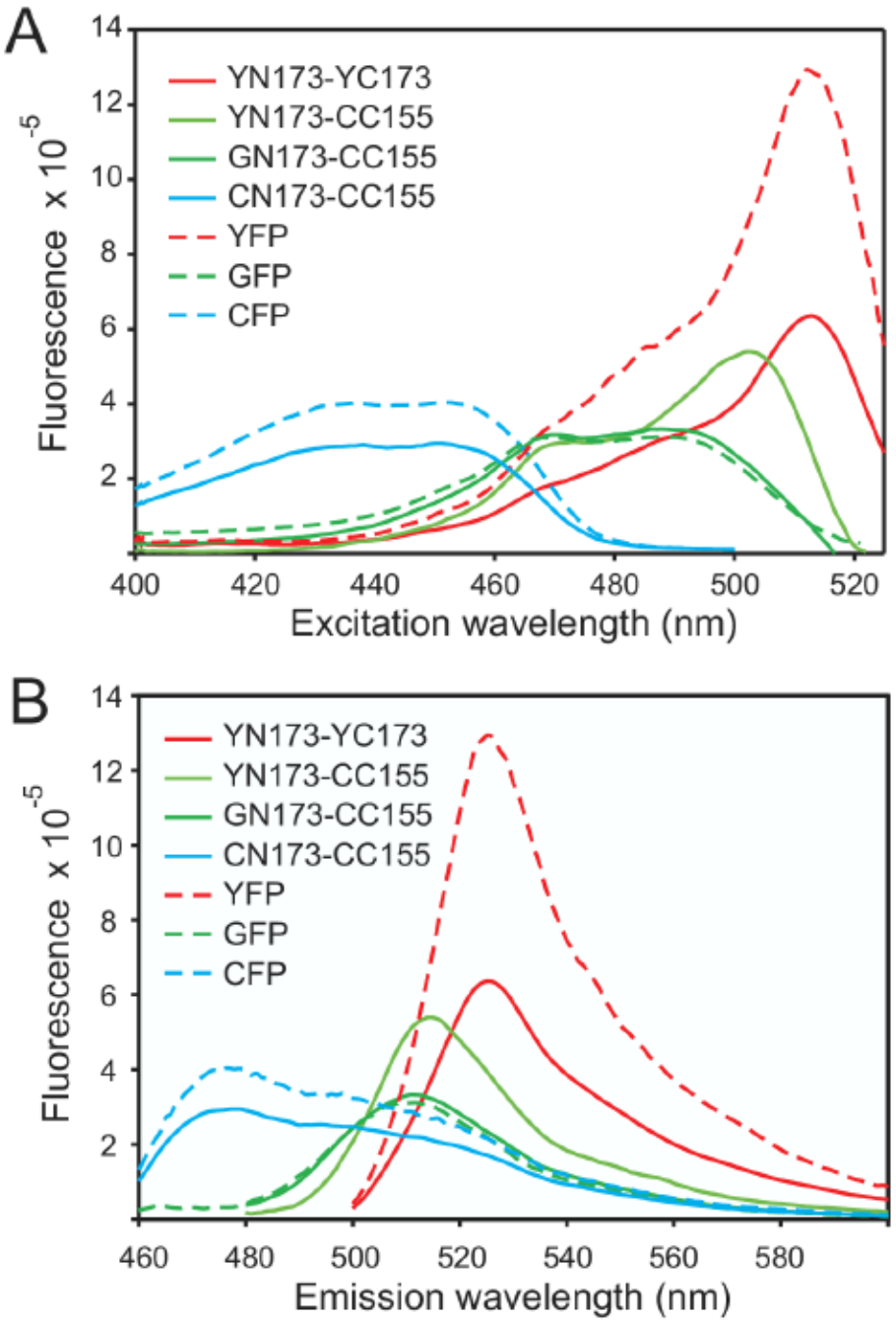
Fig. 3. Analysis of the fluorescence intensity of the purified recombinant NGFP:MBP:CGFP proteins. Maltose-induced conformational changes in MBP were assessed via analyses of the fluorescence emission spectra of NGFP:MBP:CGFP proteins at differing (A) concentrations and (B) incubation times of maltose 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.

Altre proteine split



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




A
Fluorescence x 10^-5
14
12
10
8
6
4
2
YN173-YC173
YN173-CC155
GN173-CC155
CN173-CC155
YFP
GFP
CFP
400
420
440
460
480
500
520
Excitation wavelength (nm)
B
Fluorescence x 10^-5
14
12
10
8
6
4
2
YN173-YC173
YN173-CC155
GN173-CC155
CN173-CC155
YFP
GFP
CFP
460
480
500
520
540
560
580
Emission wavelength (nm)

- 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

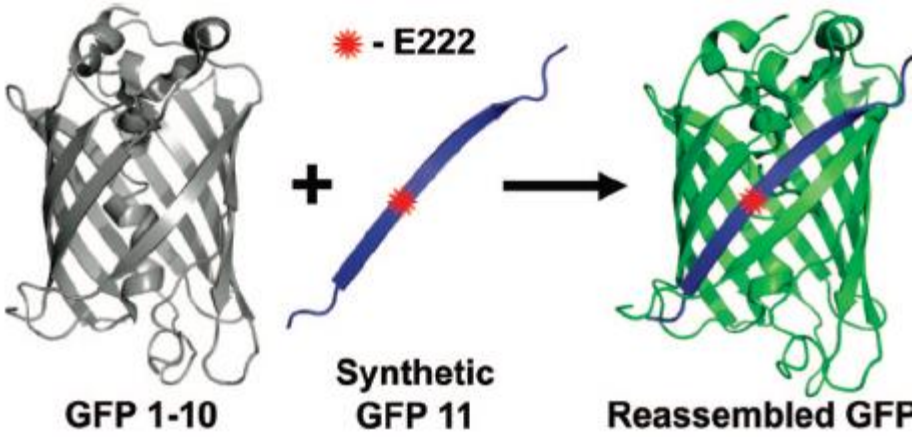
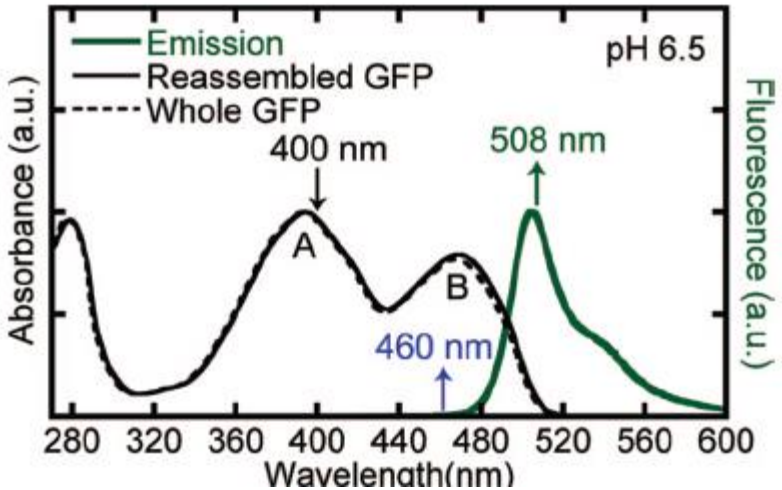
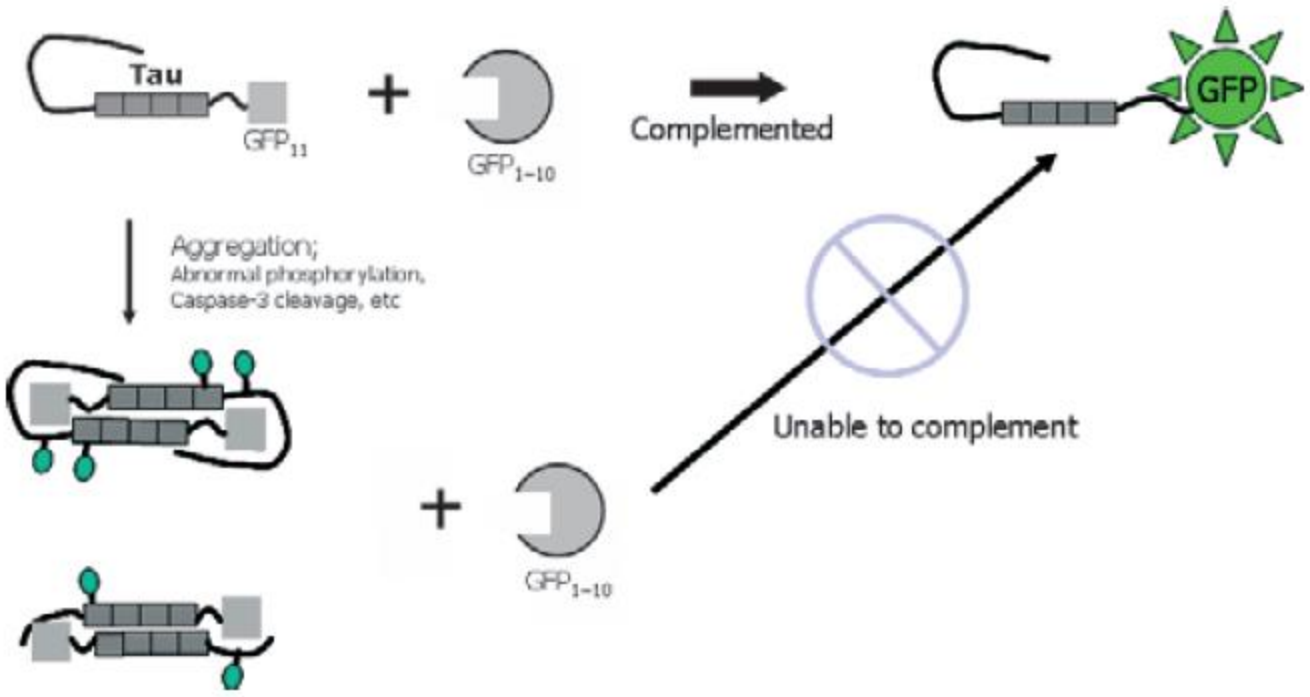


Figure 1. Schematic diagram illustrating reassembly of the eleven-strand β-barrel of GFP from GFP 1−10 (the first 214 amino acids of GF including residues 65−67 that become the chromophore) and a synthet 16 amino acid 11th strand, GFP 11. The red star marks residue E222 GFP 11, which is known to be an 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 pseudofosforilato

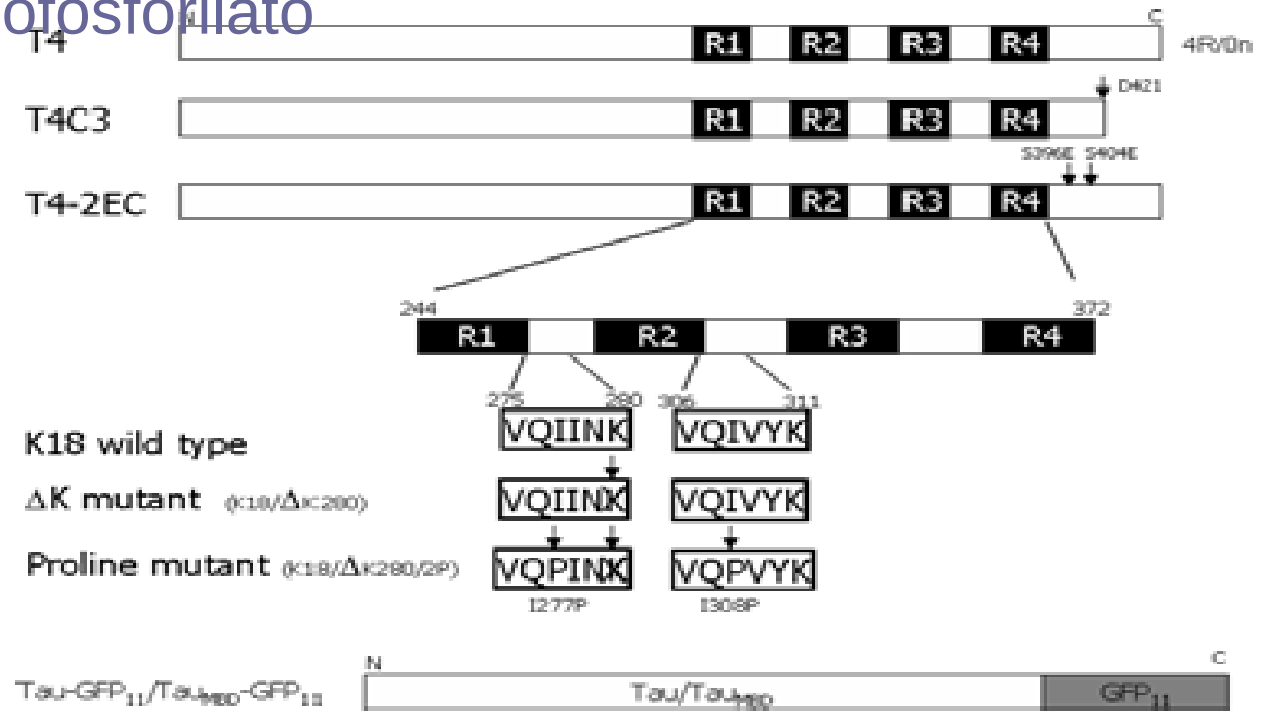
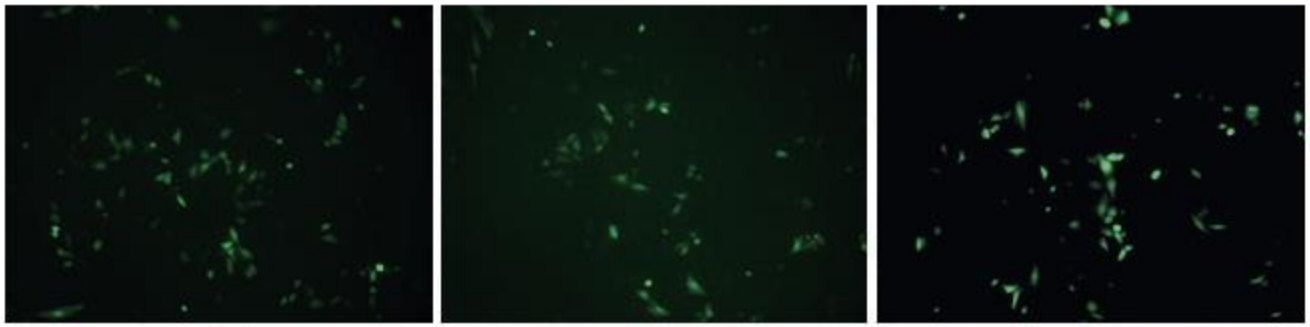
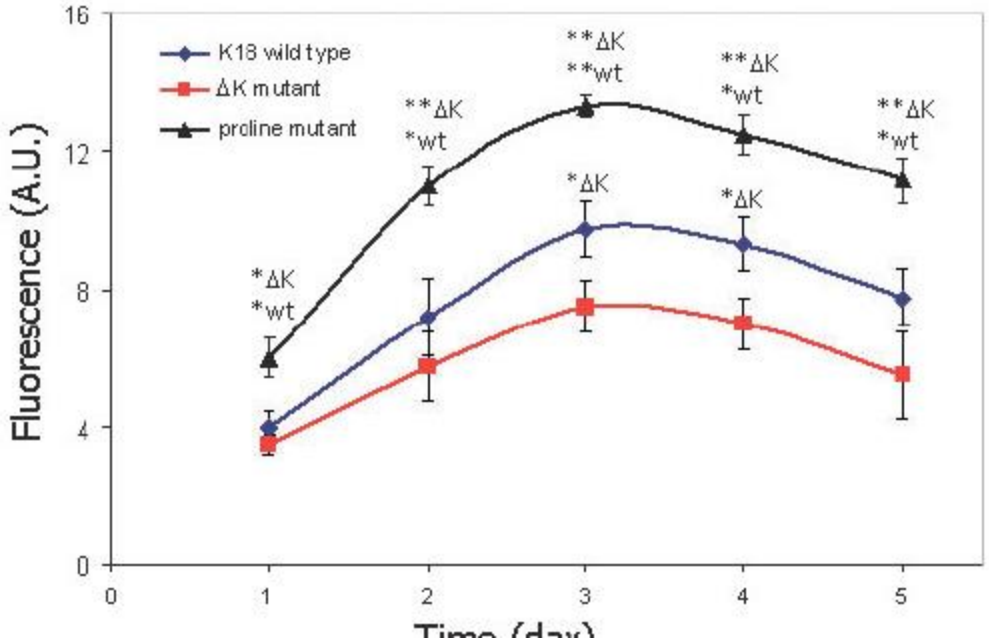


Fig. 2 Diagram of tau and tau$_{MBD}$ constructs used for the split green fluorescence protein (GFP) complementation study. The T4 tau construct is human tau with four microtubule-binding repeats but without exons 2 and 3, and T4C3 is the same as T4 except the last 20 amino acids have been deleted to mimic caspase cleavage of tau at Asp421. T4-2EC is the same as T4 except there are two Ser-to-Glu mutations at Ser396/S404 to mimic phosphorylation of these sites. Three



K18 wild type ΔK mutant Proline mutant



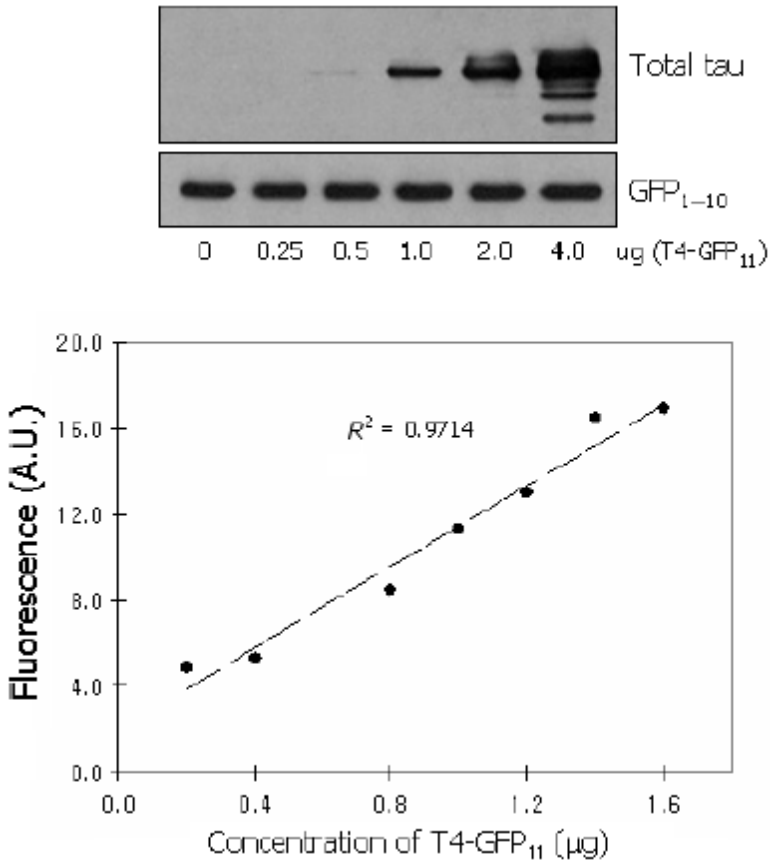


Fig. 4 Quantification of *in situ* split green fluorescence protein (GFP)

(b)

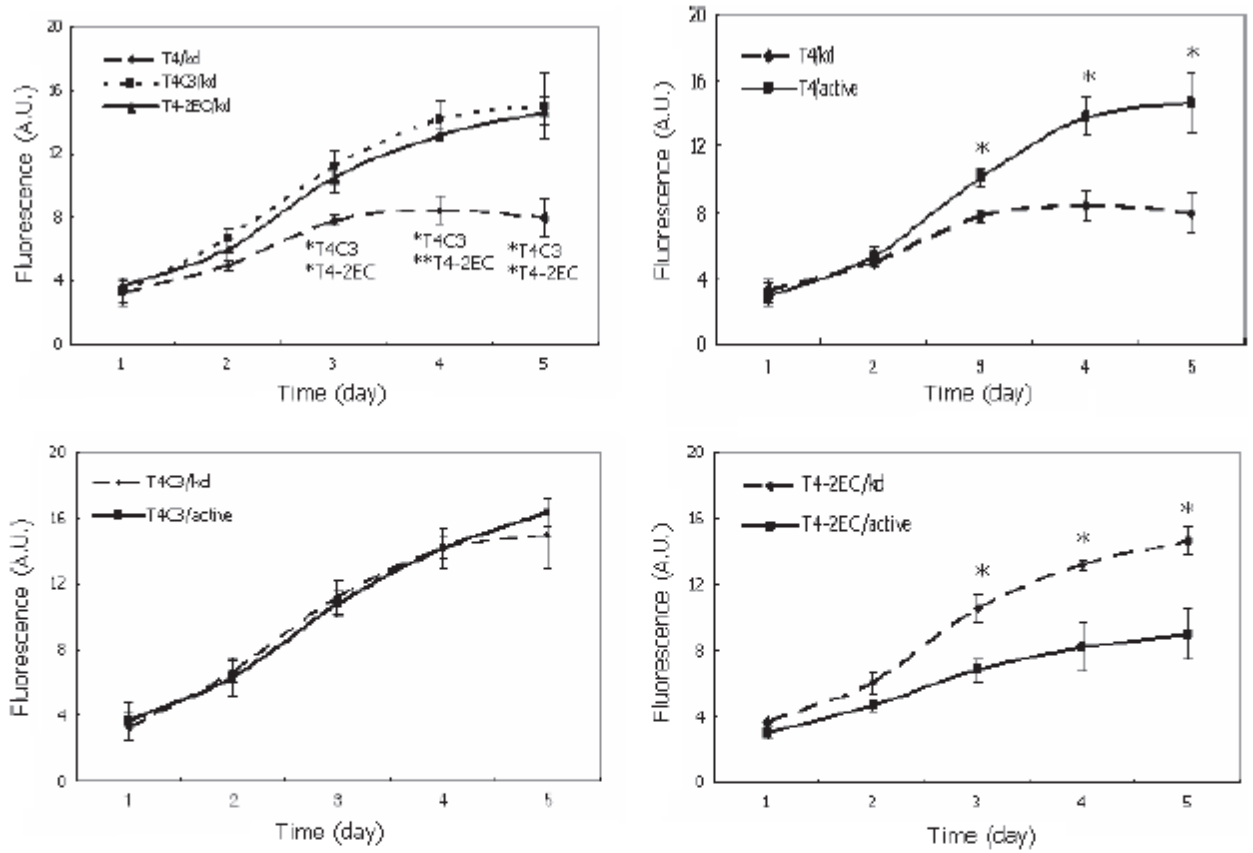
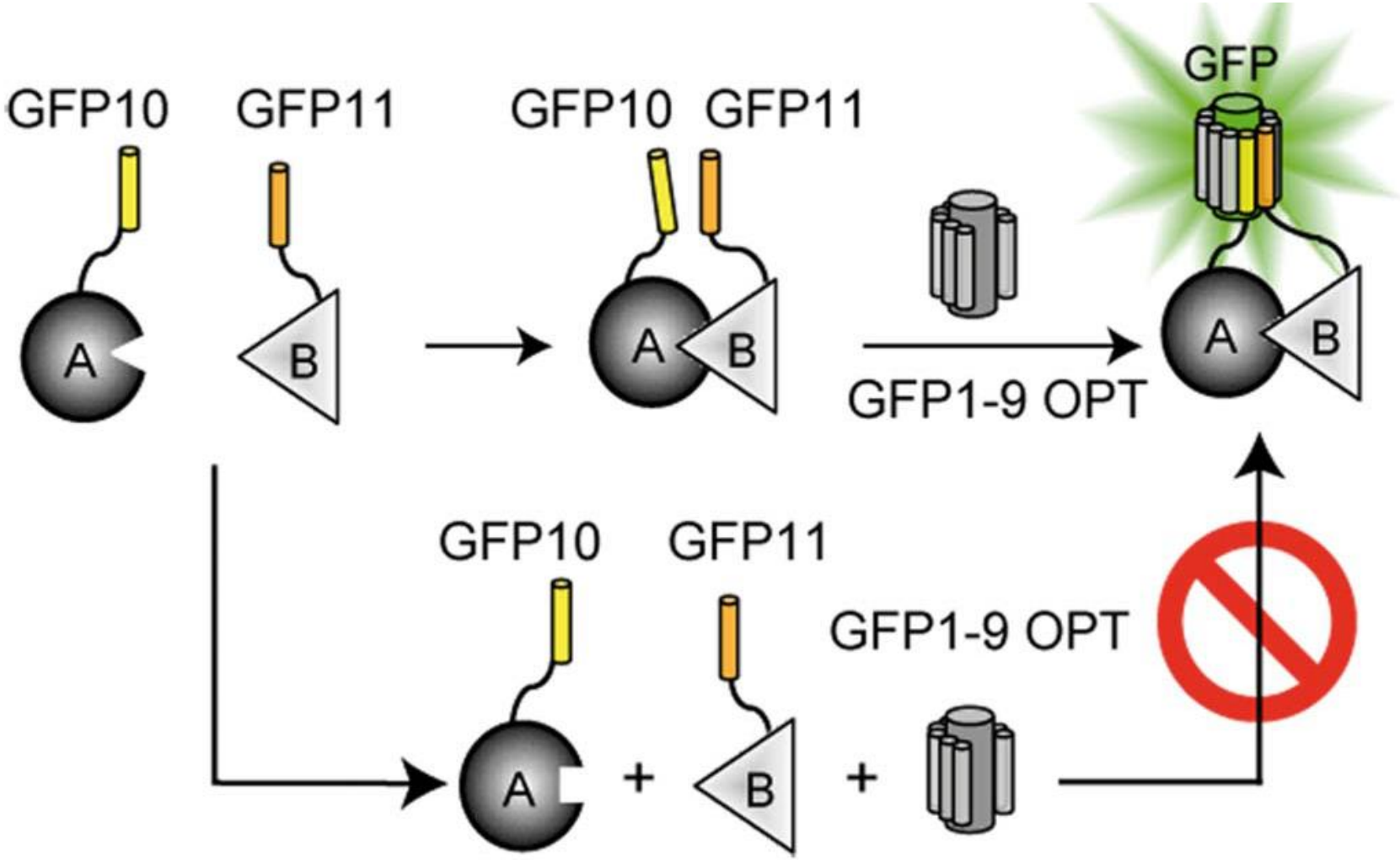


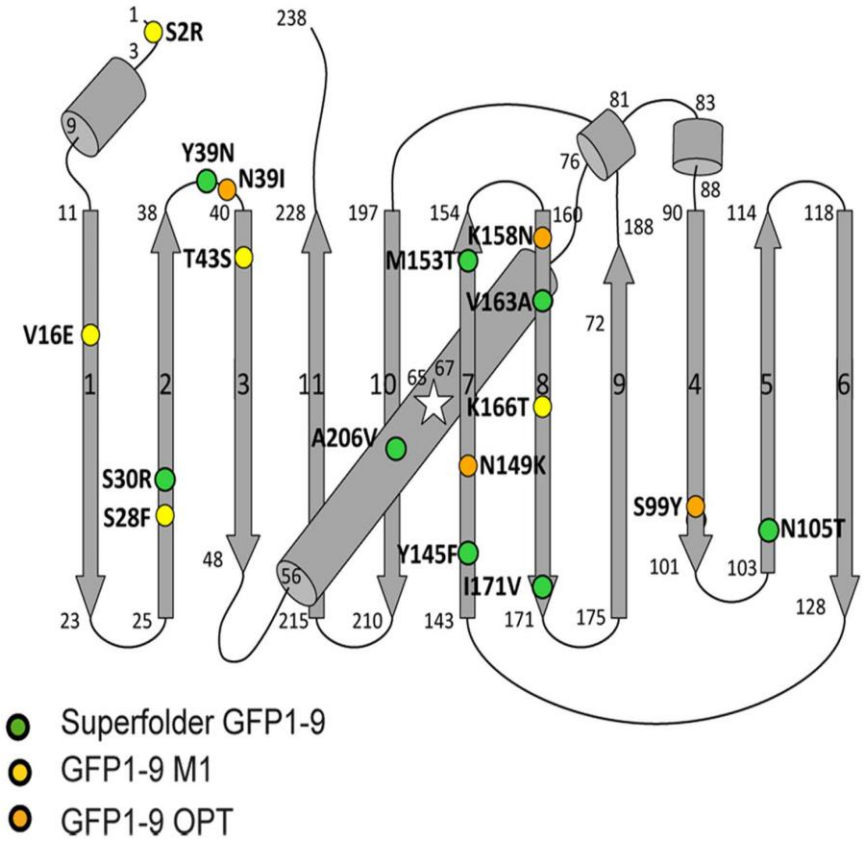
Fig. 6 Effects of glycogen synthase kinase 3β (GSK3β) activation and caspase 3 cleavage on the expression levels of tau and *in situ* split green fluorescence protein (GFP) complementation. Each tau-GFP_{11} (0.8 μg) was transiently co-transfected with $GFP_{1–10}$ (1.6 μg) in human embryonic kidney cells in the presence of kinase-dead (kd)-GSK3β (0.6 μg) or active GSK3β (0.6 μg. (a) Representative immunoblots of expression levels of each tau protein in the presence of active or kd-GSK3β. To compare the expression levels among groups, representative immunoblots were displayed for each day (top) or group (bottom) as indicated. (b) Effects of active GSK3β and caspas cleavage on *in situ* split GFP complementation. Each tau-GFP_{11} wi $GFP_{1–10}$ was co-transfected in the presence of kd- or active-GSK3β human embryonic kidney cells in 96-well microtiter plates. The GF intensity was quantitatively measured every 24 h up to 5 days (λe e = 488 nm/λem = 530 nm) and plotted as mean ± SE from thre independent experiments ($n = 3$). There was no detectable GF emission right after transfection; therefore, day 0 was not included the graph. *$p < 0.05$ and **$p < 0.01$ compared with T4-GFP_{11}.

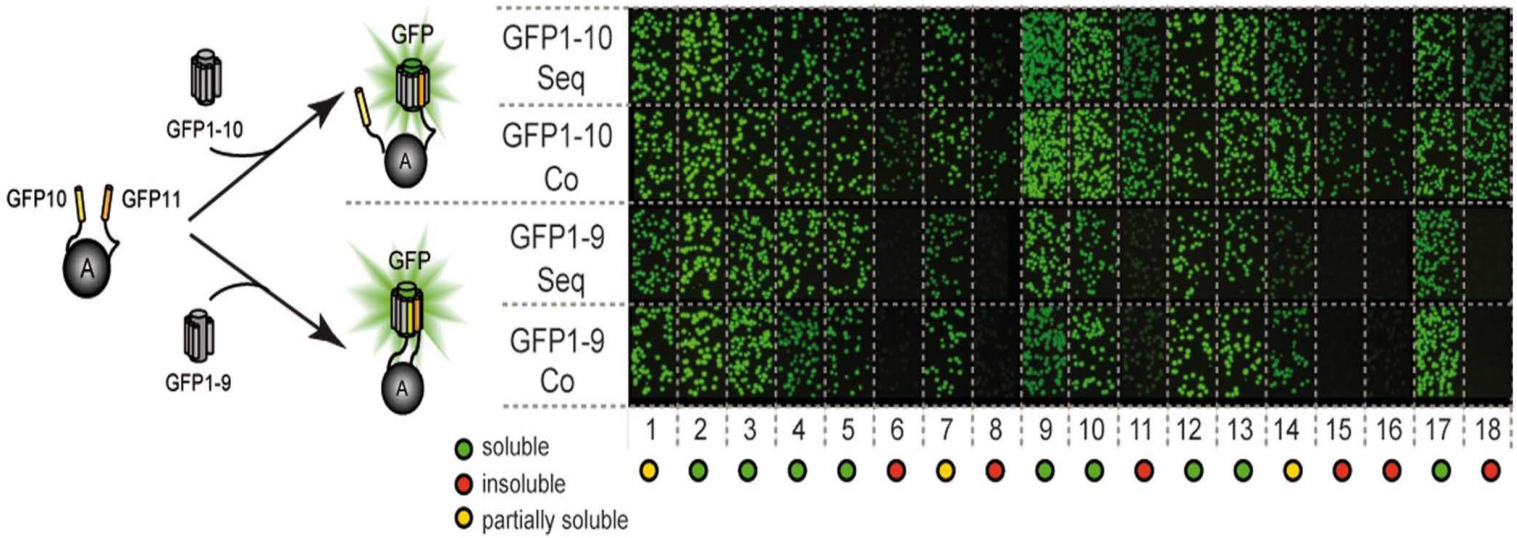
Tripartite Split-GFP system



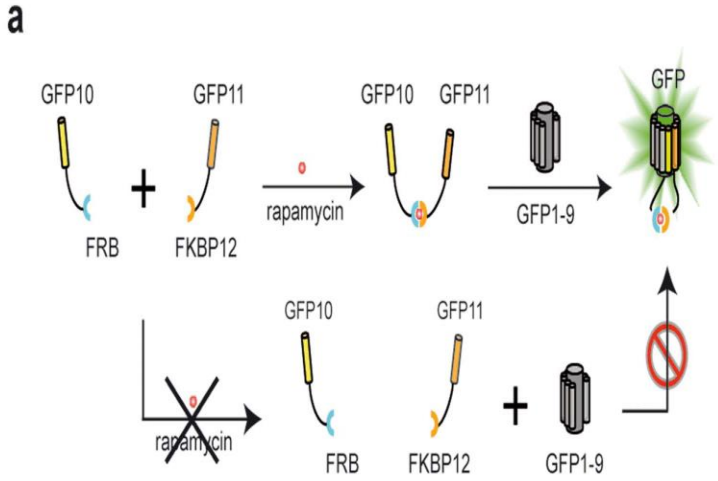
GFP10–11 194–238 Inserito un linker flessibile che include un sito di clonaggio

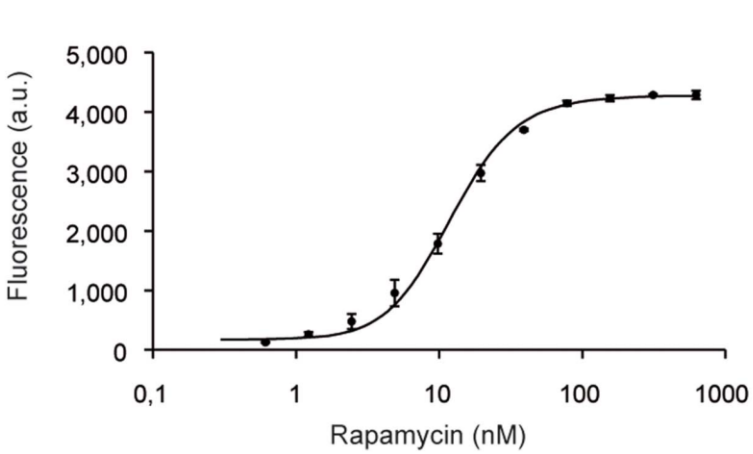
GFP1–9 1–193 Random PCR per migliorare il folding

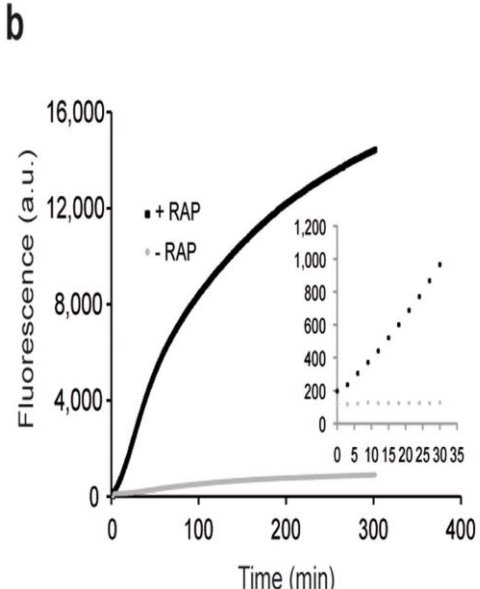


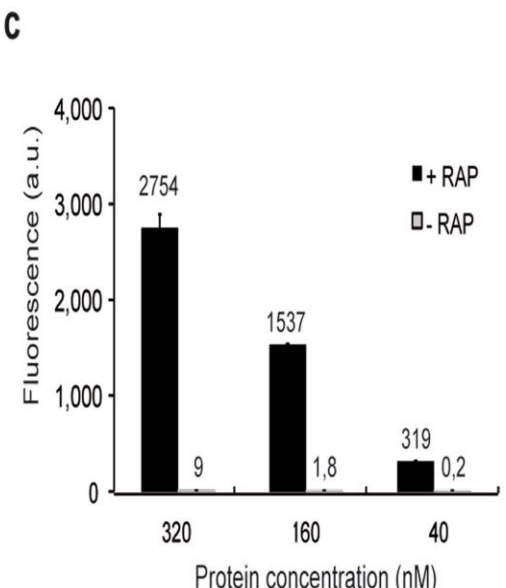

GFP
GFP1-10
GFP10
GFP11
A
GFP1-9
GFP1-10 Seq
GFP1-10 Co
GFP1-9 Seq
GFP1-9 Co
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
soluble
insoluble
partially soluble

Studio dell'interazione tra rapamicina e (FRB FKBP)









Comparison of complementation methods using fragments of different proteins.

Protein	Detection	Spatial resolution[1]	Time resolution[1]	Experimental systems[1]	Reference
Ubiquitin	Ub-protease coupled reporters	Cell population	Day	Yeast	(99)
β-galactosidase	FDG hydrolysis	Cellular	Hours	Cultured cells, *D. melanogaster*	(52)
Dihydrofolate reductase	Fl-MTX binding	Sub-cellular	Minutes	Cultured cells, plants	(89)
GFP variants	Intrinsic fluorescence	Sub-cellular	Minutes-Hours	Cultured cells, plants, fungi	(45)(34)
*Synechocystis*dnaE intein	Reporter ligation	Cell population	Hours	Cultured, implanted cells	(81)
β-lactamase	CCF2/AM hydrolysis	Cellular	Minutes	Cultured cells, primary neurons	(32,106,123)
Firefly luciferase	Luciferin hydrolysis	Cell population	Hours	Cultured, implanted cells	(87)
Renilla luciferase	Coelenterazine luminescence	Cell population	Minutes-Hours	Cultured, implanted cells	(86)
Gaussia luciferase	Coelenterazine luminescence	Cell population	Minutes	Cultured cells	(96)
TEV protease	Coupled reporters	Cellular	Minutes	Cultured cells	(122)