

# **Produzione di proteine ricombinanti**

# Sistemi di espressione

## Procariotici

**E. Coli, B. Subtilis**

## Eucariotici:

**Saccharomyces Cerevisiae (lievito)**

**Cellule di insetto (Baculovirus)**

**Cellule di mammifero in coltura (CHO etc.)**

**Animali transgenici**

**Piante transgeniche**

## *Pichia pastoris*

- Lievito metilotrofico
- Crescite ad alta densità
- Glicosilazione più simile a quella di mammifero
- Promotori inducibili **AOX1** (promotore dell'alcol ossidasi indotto da metanolo), **CUP1** (rame)
- Promotori costitutivi **GAPD** (gliceraldeide fosfato deidrogenasi), **YPT1**
- Sistemi di esporto delle proteine più efficienti Limitata secrezione nel mezzo di proteine endogene (quella eterologa arriva a rappresentare il 30% del secreto)
- Possibilità di “umanizzare” il pathway glicosilativo (GlycoFi)

## Cellule di mammifero

- Linee cellulari derivate da tumori CHO (Chinese Hamster Ovary), BHK (Baby Hamster Kidney) di criceto e le HEK293 (Human Embryonic Kidney) Hela umane
- Sistemi molto complessi e costosi
- Crescita lenta (ca 24 ore/raddoppio)
- **Plasmidi episomali o integrativi** con promotori forti inducibili (fitoestrogeni, TetOn/TetOff) o costitutivi (citomegalovirus CMV)
- Trasfezione transiente o stabile (lipocomplessi, elettroporazione)
- Selezione mediante resistenza ad antibiotici o vantaggio selettivo (diidrofolato reductasi, glutamina sintasi)
- Adatte alla produzione di proteine terapeutiche folding e modifiche post-sintetiche generalmente corrette

## Vettori di espressione eucarioti

- **Origine di replicazione**

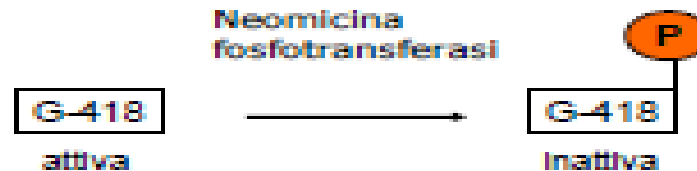
- Generalmente derivata da virus animali (SV40 Simian virus 40)

- **Promotori/sequenze di arresto**

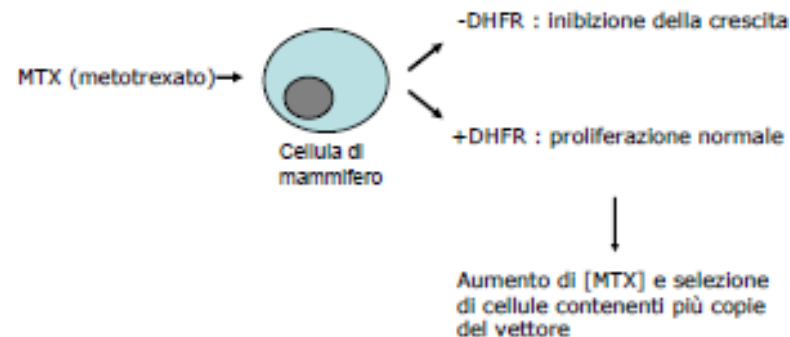
- Generalmente derivati da virus animali o da geni di mammifero altamente espressi SV40, cytomegalovirus (CMV), herpes simplex virus (HSV) actina  $\beta$ , timidina chinasi, ormone della crescita bovino

- **Marcatori selezionabili**

### Neo



- **DHFR:** deidrofolato reduttasi utilizzabile in ceppi DHFR-



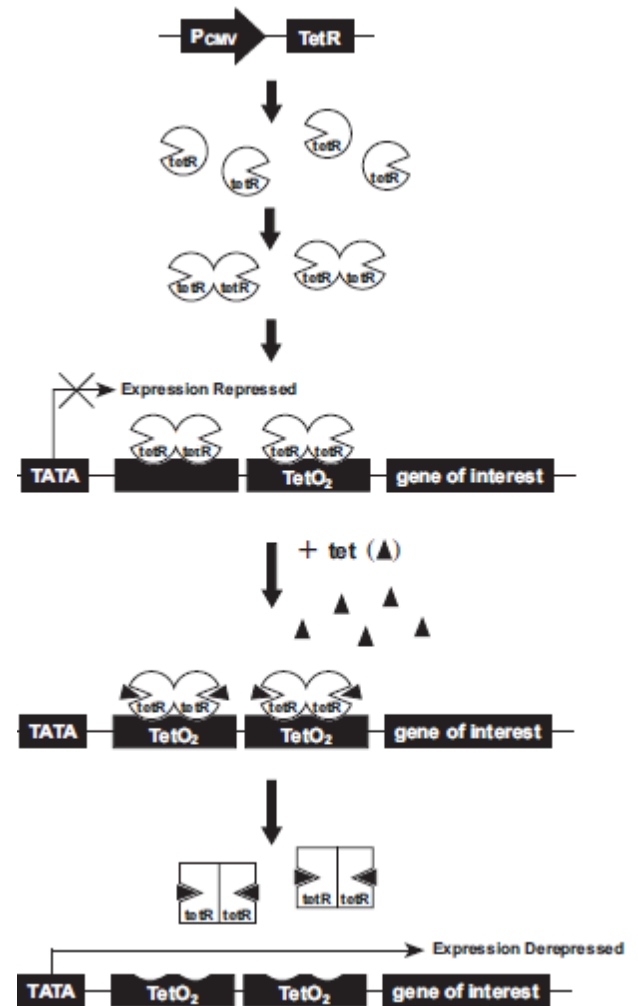
## Induzione dell'espressione indotta da tetraciclina in cellule di mammifero

### Sistema TetOn per l'espressione regolata da tetraciclina

Il repressore batterico TetR lega sequenze specifiche di DNA in assenza di tetraciclina

Il gene di interesse è posto sotto il controllo di un promotore ibrido tetO-CMV e viene inserito in una linea cellulare che esprime costitutivamente TetR

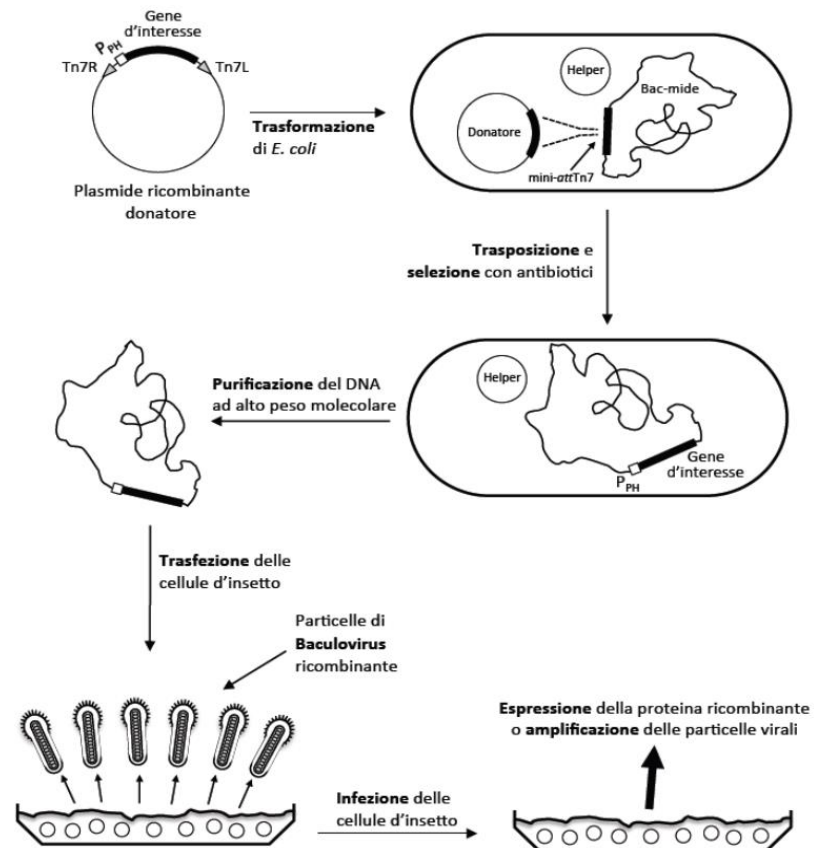
In presenza di tetraciclina TetR non si lega al DNA e permette la trascrizione del gene di interesse



## Cellule di insetto/baculovirus

Cellule *Spodoptera frugiperda* Sf9 infettate con baculovirus ricombinante

- Promotore forte della poliedrina
- Crescita lenta (ca 24 ore/raddoppio)
- Sistemi molto complessi e costosi
- Folding e modifiche post-sintetiche generalmente corrette
- Interferone



## Miglioramento dei livelli di espressione di una proteina ricombinante

- **Miglioramento della traduzione**

- Sequenze di legame per i ribosomi
- Stabilità dell'mRNA
- Codon usage

- **Miglioramento del folding**

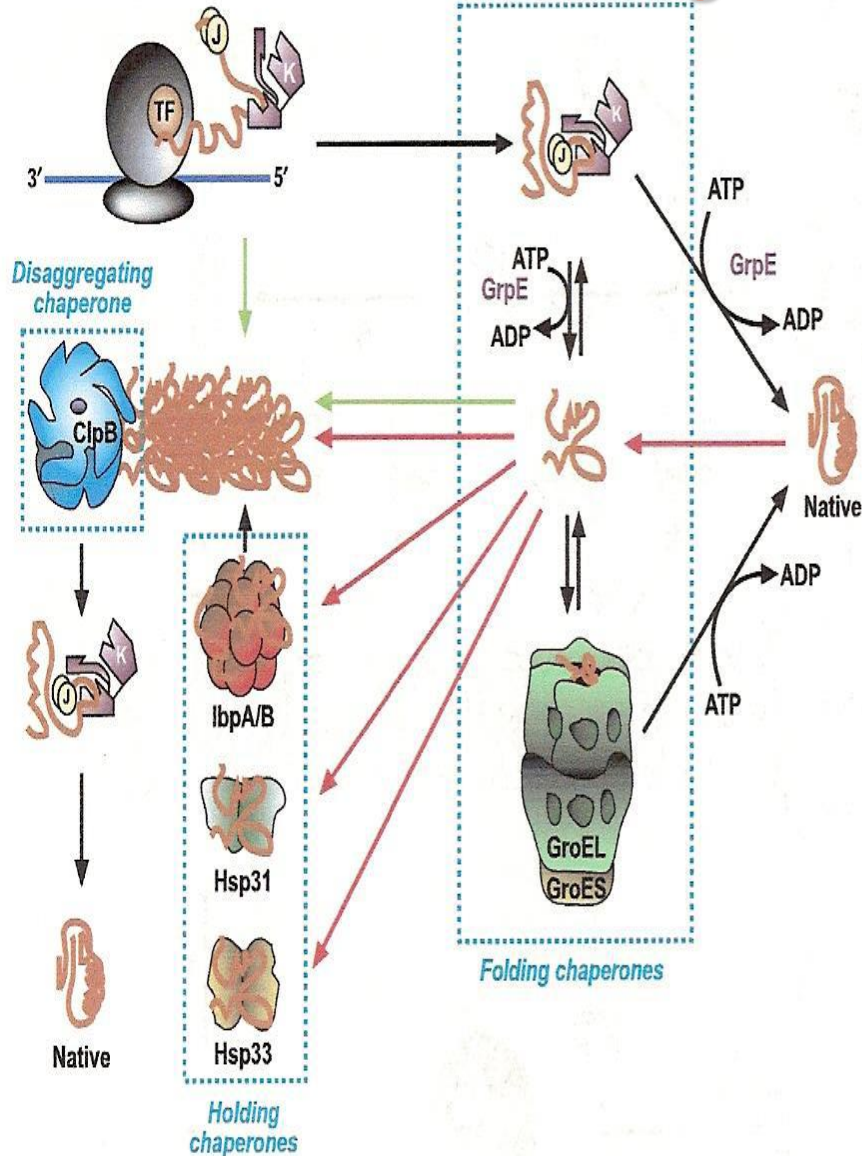
- Variazione delle condizioni di crescita (temperatura, composizione del terreno)
- Coespressione del macchinario di folding (chaperon molecolari)
- Proteine di fusione

- **Miglioramento della stabilità**

- Secrezione (è necessario un segnale di secrezione all'N-terminale)
- Proteine di fusione
- Modifiche post-traduzionali

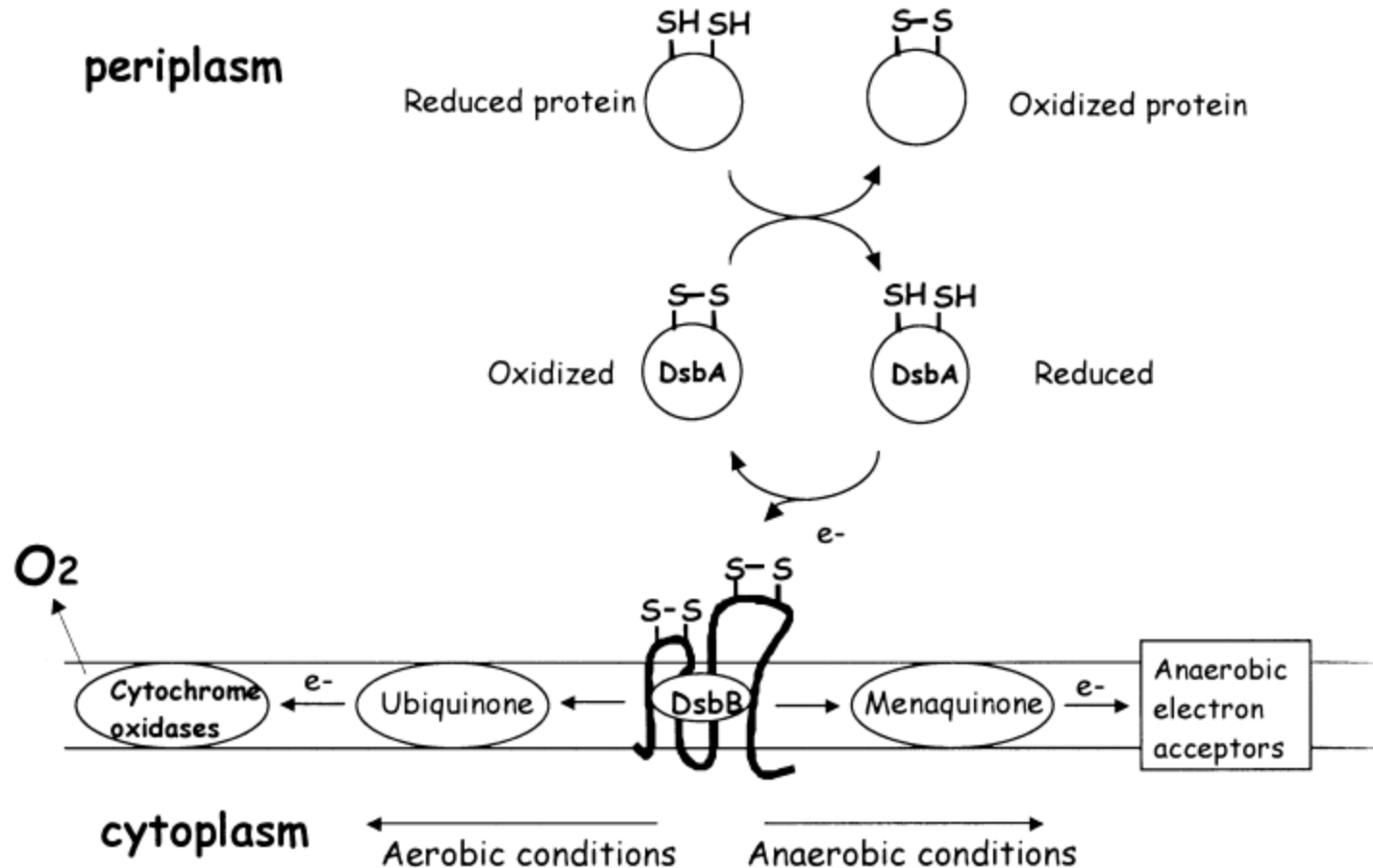


# Folding delle Proteine in E.Coli



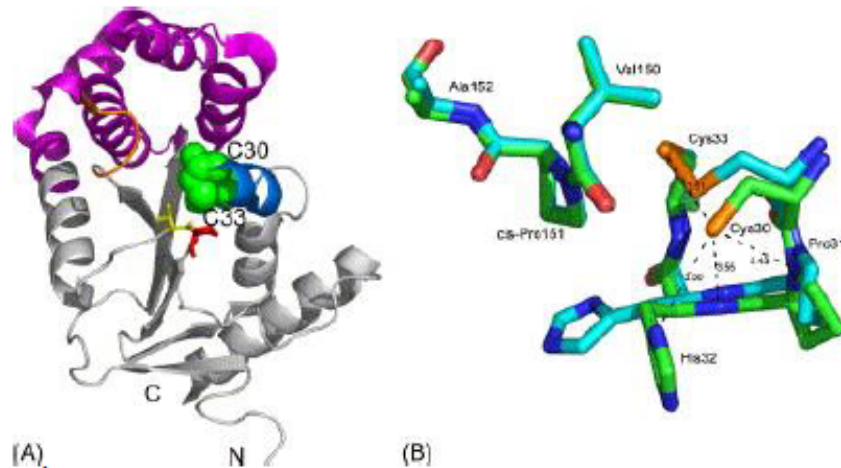
**Figure 1** Chaperone-assisted protein folding in the cytoplasm of *E. coli*. Nascent polypeptides requiring the assistance of molecular chaperones first encounter TF or DnaK-DnaJ. Both chaperones engage solvent-exposed stretches of hydrophobic amino acids, shielding them from the solvent and each other. After undocking from TF- or GrpE-mediated release from DnaK, folding intermediate may reach a native conformation, cycle back to DnaK-DnaJ or be transferred to the central chamber of GroEL for folding at infinite dilution upon GroES capping. In times of stress (red arrows), thermolabile proteins unfold and aggregate. IbpB binds partially folded proteins on its surface to serve as a reservoir of unfolded intermediates until folding chaperones become available and intercalates within large aggregates. The holding chaperones Hsp33 and Hsp31 become important under oxidative and severe thermal stress, respectively. ClpB promotes the shearing and disaggregation of thermally unfolded host proteins and cooperates with DnaK-DnaJ-GrpE to reactivate them once stress has abated. Recombinant proteins that miss an early interaction with TF or DnaK/DnaJ, that undergo multiple cycles of abortive interactions with folding chaperones or titrate them out, accumulate in inclusion bodies (green arrows).

# Formazione ponti disolfuro in E.coli



# Meccanismo d'azione di DsbA

Sito attivo Cys<sub>30</sub>Pro His Cys<sub>33</sub>



Maggiore stabilità nello stato ridotto

Alta reattività nello stato ossidato

Stabilizzazione stato ridotto che determina Cys 30 con basso pKa, nello stato ridotto formazione:

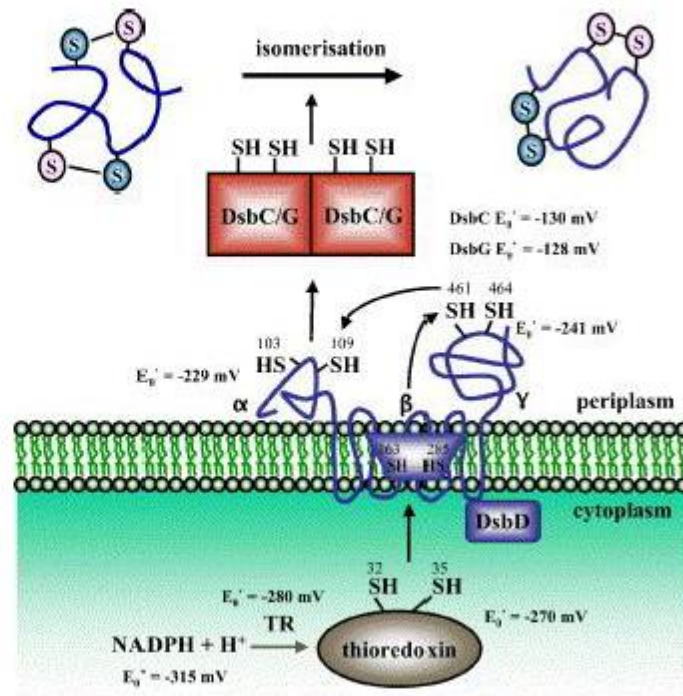
- legame elettrostatico estremità positiva di un'  $\alpha$ -elica
- legame idrogeno con His 32.

DsbA ossidata ha una struttura flessibile che permette il legame di un gran numero di substrati

DsbA ridotta ha una struttura rigida che permette il legame solo di DsbB

# Isomerasi: DsbC / DsbG

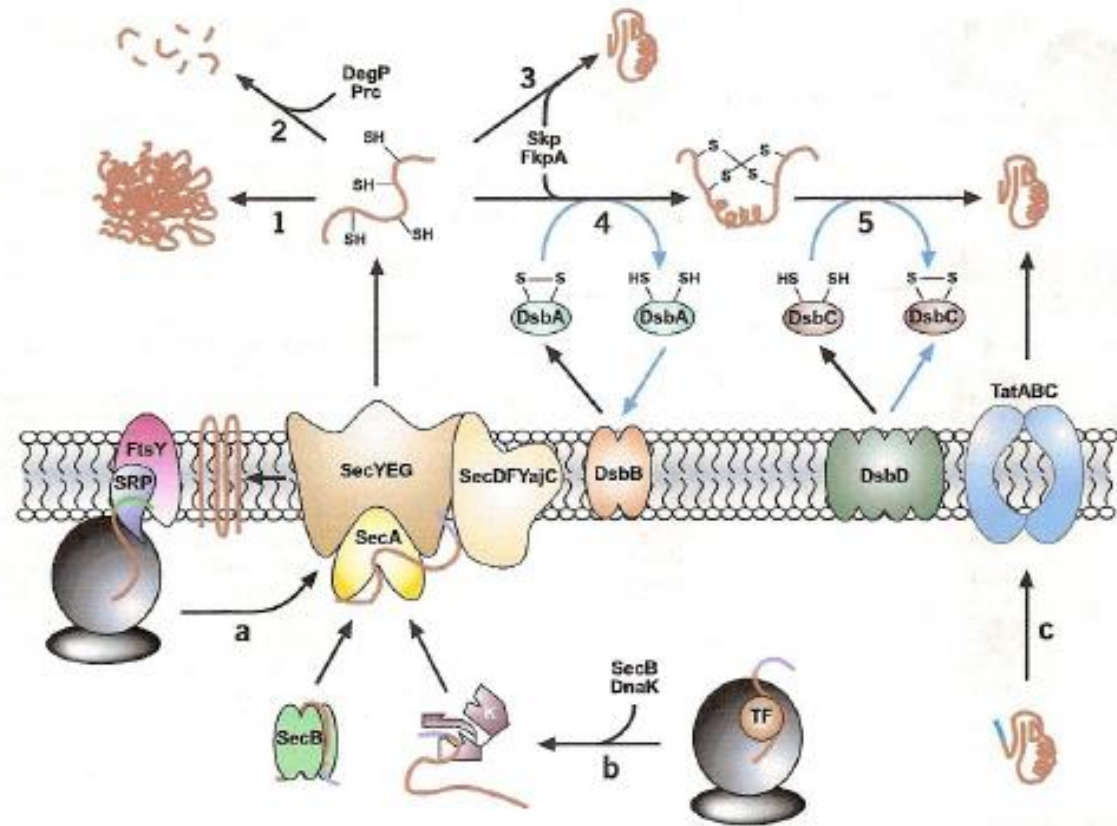
NADPH  
 Tioredossina reduttasi  
 Tioredossina  
 DsbD $\alpha$ -DsbD $\beta$ - DsbD $\gamma$   
 DsbC/DsbG  
 Rottura ponti disolfuro non  
 corretti





# Meccanismi di folding e secrezione in *E. coli*

- **Peptide segnale** fortemente idrofobico viene riconosciuto da SRP il complesso SRP ribosoma lega il recettore FtsY e poi trasferito al traslocone SecYEG e SecDFYajC
- **Peptide segnale** mediamente idrofobico viene intercettato SecB che lega la catena distesa e poi SecA e SecYEG
- **Peptide segnale** contenente 2 arginine viene traslocato attraverso il sistema TatABC dopo che ha raggiunto la forma nativa

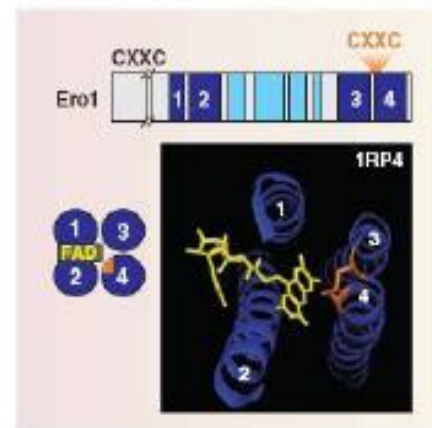
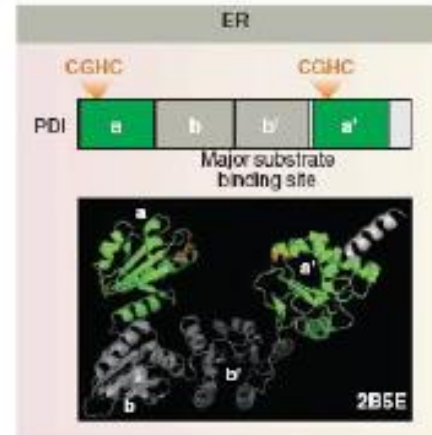
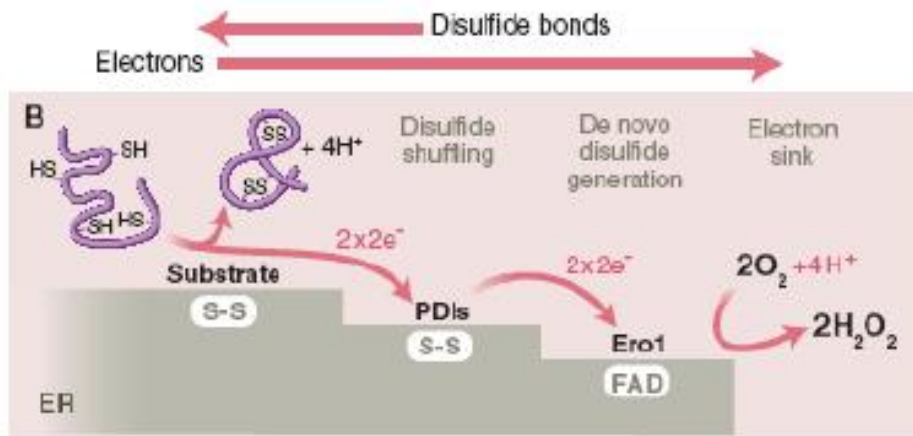


**Figure 2** Export and periplasmic folding pathways. Proteins destined for export can be translocated across the inner membrane in three different fashions. (a) Preproteins with highly nonpolar signal sequences (green) or transmembrane segments of inner membrane proteins are recognized by SRP which, along with TF, scans nascent chains. SRP-dependent export involves delivery of the ribosome-nascent chain complex to FtsY and subsequent translocation through the SecYEG-SecDFYajC translocon. (b) The vast majority of preproteins have less hydrophobic signal sequence (lavender) and undergo Sec-dependent export. TF associates with the nascent polypeptide, halting cotranslational folding. As the chain grows, TF dissociates and the polypeptide is transferred to SecB or DnaK that maintain it in an extended conformation. Delivery to SecA and ATP-dependent translocation through SecYEG completes the process. (c) Preproteins with signal sequences containing the twin-arginine motif (cyan) are exported via the Tat-dependent pathway in a folded form. After cleavage of the signal sequence, partially folded periplasmic proteins may aggregate (1), undergo proteolysis (2) or reach a native conformation, possibly with the assistance of folding modulators (3). Cysteine pairs in proteins containing disulfide bonds are oxidized by DsbA (4) whereas incorrect disulfides are isomerized by DsbC (5). These oxidoreductases are reactivated by DsbB and DsbD, respectively. Black arrows show products obtained after each step, whereas blue arrows represent electron flow.

# Meccanismi di folding e secrezione in cellule eucariotiche

- Reticolo endoplasmatico
  - Rimozione peptide segnale
  - Inizio glicosilazione
  - Formazione ponti disolfuro e folding
- Apparato di Golgi
  - Maturazione oligosaccaridi
- Vescicole di secrezione
  - Trasporto alla membrana plasmatica e secrezione

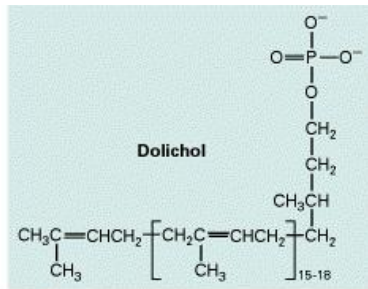
Formazione dei ponti disolfuro nel reticolo endoplasmatico  
 PDI: proteina disolfuro isomerasi  
 Ero1: sulfidril-ossidasi



# Glicosilazione delle proteine. Ruolo della glicocomponente

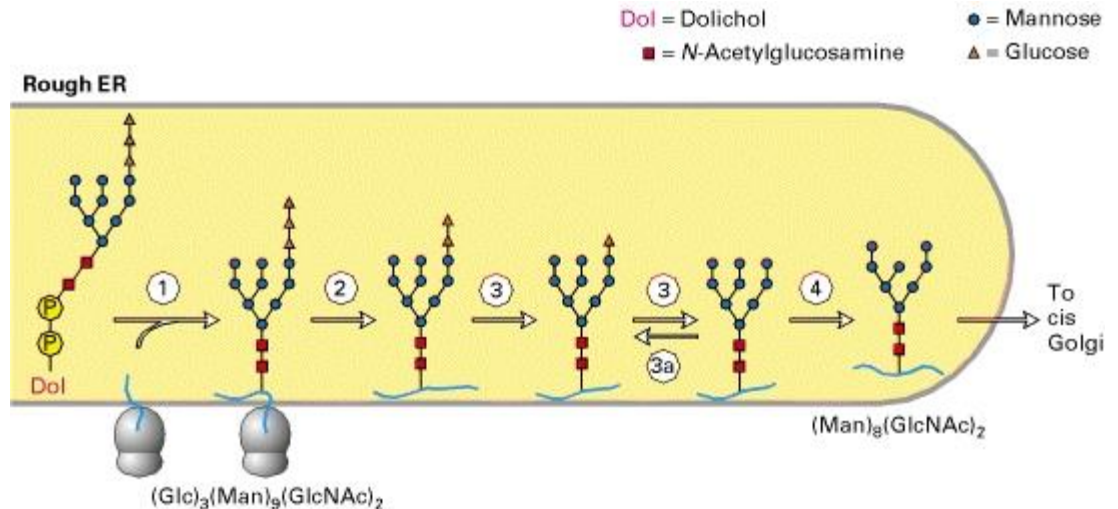
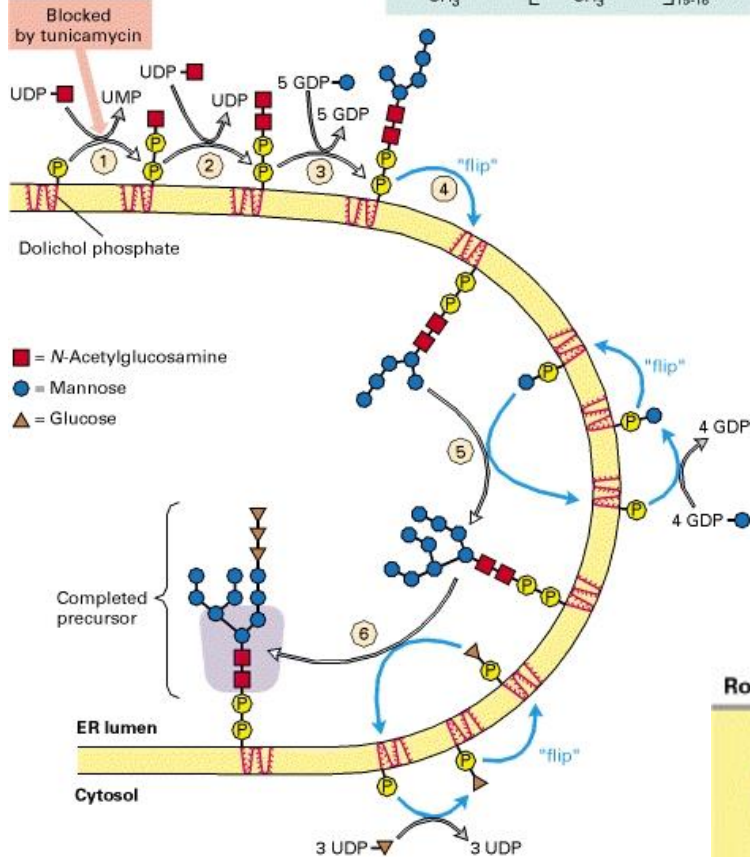
- La glicosilazione può assistere il **folding** della catena polipeptidica attraverso effetti sulla struttura secondaria
- La glicosilazione può indirizzare una proteina alla sua destinazione finale (**trafficking/sorting**)
- Le catene oligosaccaridiche possono contribuire alla **stabilità** di una proteina aumentandone la solubilità, schermando regioni idrofobiche, proteggendo dalle proteasi o favorendo interazioni intra-catena
- Le catene oligosaccaridiche possono partecipare al **riconoscimento/legame** con ligandi o recettori o all' **attività biologica** di una proteina
- L'acido sialico che si trova all'estremità delle catene oligosaccaridiche aumenta l' **emivita** delle proteine plasmatiche
- Le catene oligosaccaridiche possono contribuire all' **immunogenicità** di proteine eterologhe





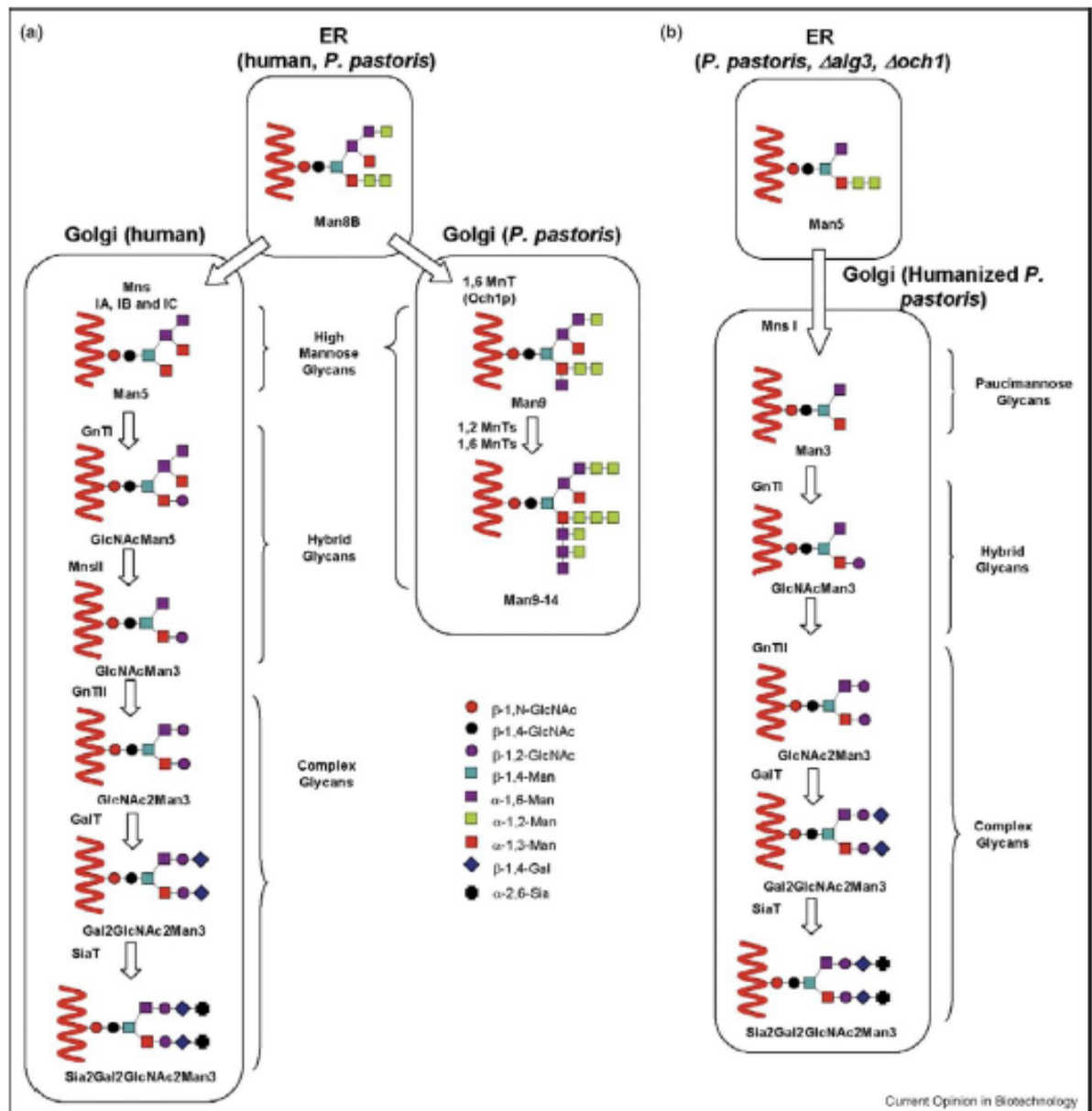
## N-glicosilazione delle proteine

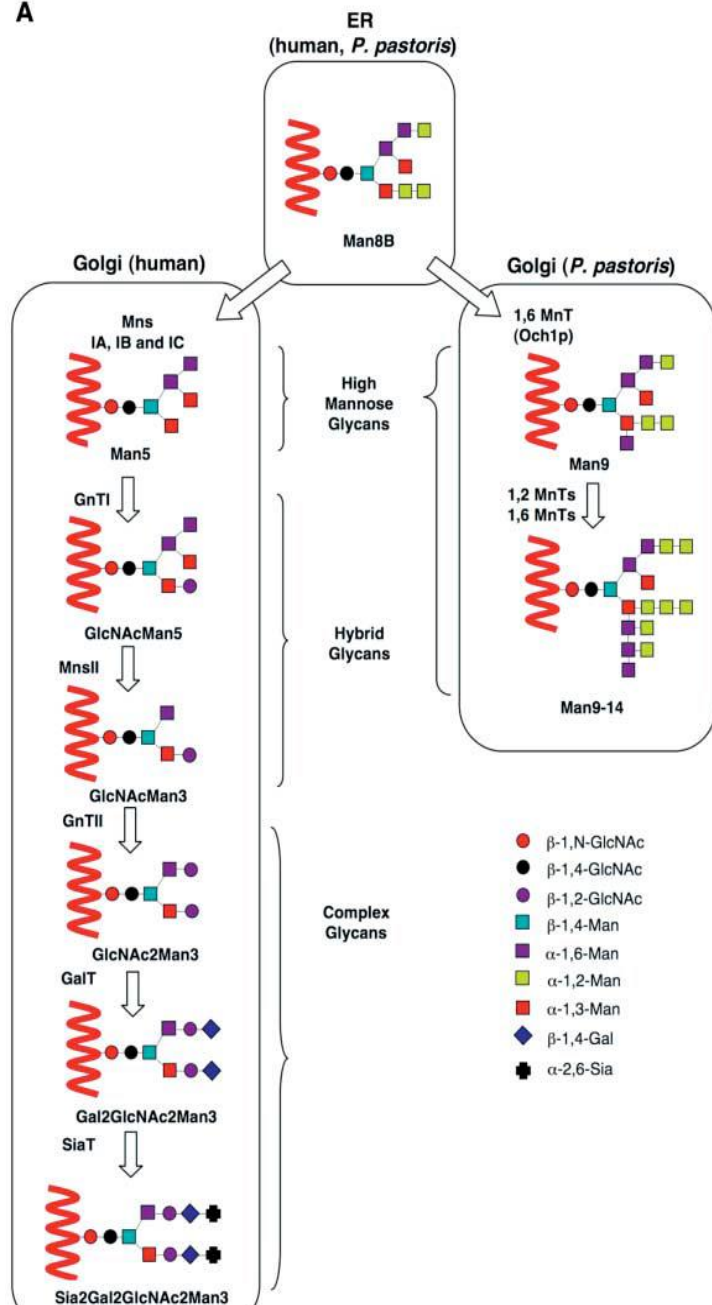
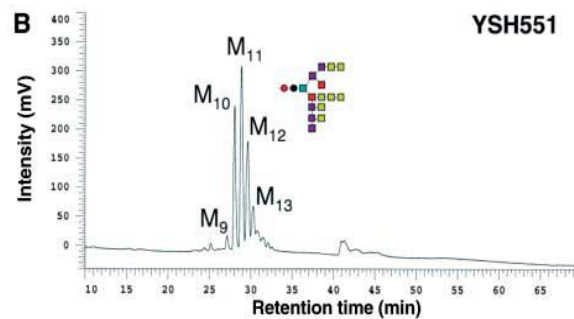
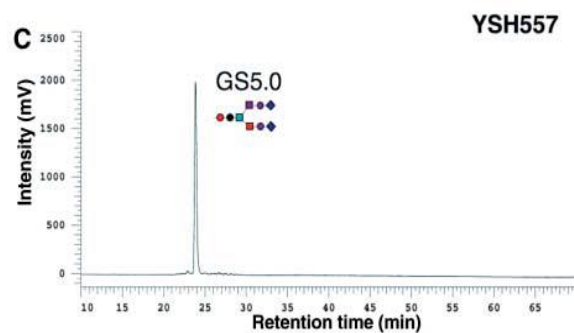
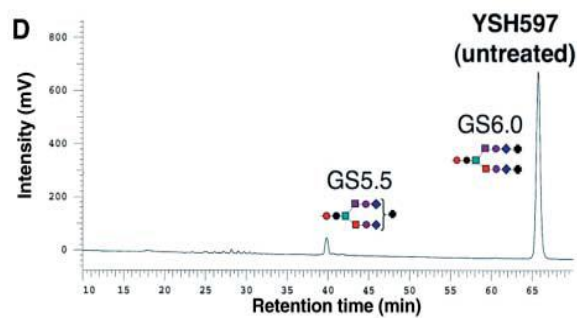
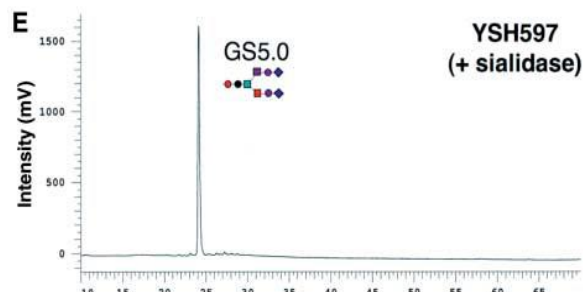
- Formazione del nucleo oligosaccaridico per successive aggiunte di monosaccaridi sul dolicolo fosfato
- Traslocazione nel lumen del reticolo
- Trasferimento mediato dall'oligosaccariltrasferasi sulla sequenza consenso N-X-T/S



# Umanizzazione della glicosilazione del lievito *Pichia pastoris*

N-linked glycosylation pathways in humans and yeast. Representative N-linked glycosylation pathways in humans and *P. pastoris* (a). An alternative humanized N-linked glycosylation pathway in *P. pastoris* (b). Mns;  $\alpha$  1,2-mannosidase, MnsII; mannosidase II, GnTI;  $\beta$  1,2-N-acetylglucosaminyltransferase I, GnTII;  $\beta$  1,2-N-acetylglucosaminyltransferase II, GnTIII;  $\beta$  1,4-galactosyltransferase, GalT;  $\alpha$  2,6-sialyltransferase, SiaT; mannosyltransferase. For simplicity the two GlcNAc residues present at the reducing end of all glycans have been omitted.



**A****B****C****D****E**

# Purificazione delle proteine ricombinanti

- Metodi convenzionali: frazionamento con solfato d' ammonio, cromatografia a scambio ionico, gel-filtrazione o cromatografia d' interazione idrofobica
- Cromatografia di affinità sfruttando **tag** proteici o peptidici fusi alla proteina (N- o C-terminali). Immobilizzazione di anticorpi contro i tag peptidici o di substrati dei tag proteici

Tag peptidici: 6xHis, flag, c-myc

Tag proteici: GST (glutathione-S-trasferasi), MBP (maltose binding protein), TRX (tioredossina)

Rimozione tag (inserimento siti di taglio per proteasi in una sequenza linker tra la proteina e il tag)

# Tag peptidici e proteici

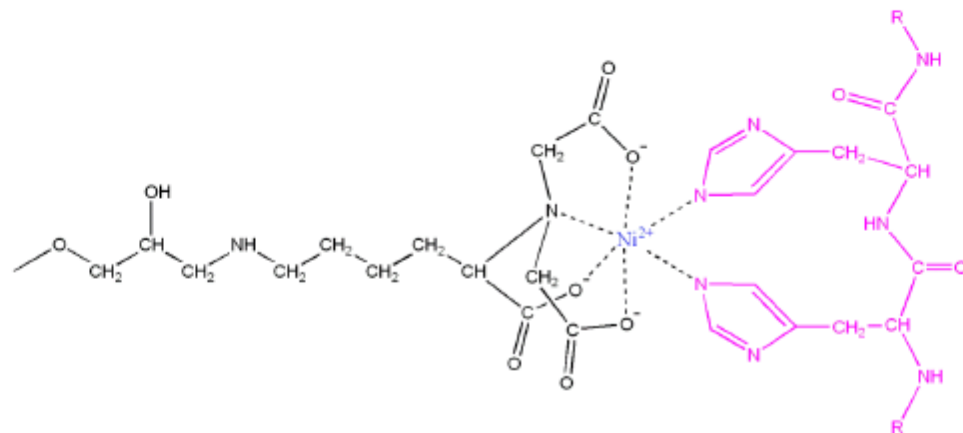
Tag	Residues	Sequence	Size (kDa)
Poly-Arg	5-6 (usually 5)	RRRRR	0.80
Poly-His	2-10 (usually 6)	HHHHHH	0.84
FLAG	8	DYKDDDDK	1.01
Strep-tag II	8	WSHPQFEK	1.06
c-myc	11	EQKLISEEDL	1.20
S-	15	KETAAAKFERQHMDS	1.75
HAT-	19	KDHLIHNVHKEFHAAHANK	2.31
3x FLAG	22	DYKDHDGDYKDHDIDYKDDDDK	2.73
Calmodulin-binding peptide	26	KRRWKNFLAVSAANRFKKISSGAL	2.96
Cellulose-binding domains	27-189	Domains	3.00-20.00
SBP	38	MDEKTTGWRGGHVVEGLAGELEQLRARLEHHPQGQREP	4.03
Chitin-binding domain	51	TNPGVSAWQVNTAYTAGQLVTYNGKTYKCLQPHTSLAGWEPSNVPALWQLQ	5.59
Glutathione S-transferase	211	Protein	26.00
Maltose-binding protein	396	Protein	40.00

Tag **peptidici** facilitano purificazione e rilevazione  
 Tag **proteici** aumentano livelli di espressione e solubilità,  
 facilitano purificazione e rilevazione



# Purificazione delle proteine ricombinanti con tag

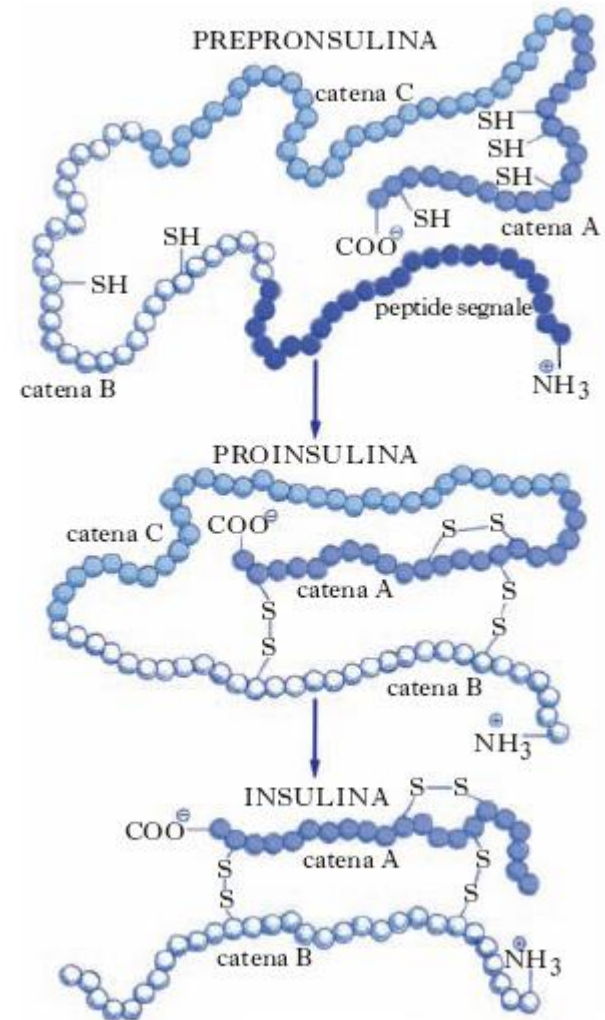
Affinity tag	Matrix	Elution condition
Poly-Arg	Cation-exchange resin	NaCl linear gradient from 0 to 400 mM at alkaline pH>8.0
Poly-His	Ni <sup>2+</sup> -NTA, Co <sup>2+</sup> -CMA (Talon)	Imidazole 20–250 mM or low pH
FLAG	Anti-FLAG monoclonal antibody	pH 3.0 or 2–5 mM EDTA
Strep-tag II	Strep-Tactin (modified streptavidin)	2.5 mM desthiobiotin
c-myc	Monoclonal antibody	Low pH
S	S-fragment of RNaseA	3 M guanidine thiocyanate, 0.2 M citrate pH 2, 3 M magnesium chloride
HAT (natural histidine affinity tag)	Co <sup>2+</sup> -CMA (Talon)	150 mM imidazole or low pH
Calmodulin-binding peptide	Calmodulin	EGTA or EGTA with 1 M NaCl
Cellulose-binding domain	Cellulose	Family I: guanidine HCl or urea>4 M Family II/III: ethylene glycol
SBP	Streptavidin	2 mM Biotin
Chitin-binding domain	Chitin	Fused with intein: 30–50 mM dithiothreitol, $\beta$ -mercaptoethanol or cysteine
Glutathione S-transferase	Glutathione	5–10 mM reduced glutathione
Maltose-binding protein	Cross-linked amylose	10 mM maltose



# Insulina sintetizzata nelle cellule beta del pancreas come precursore inattivo

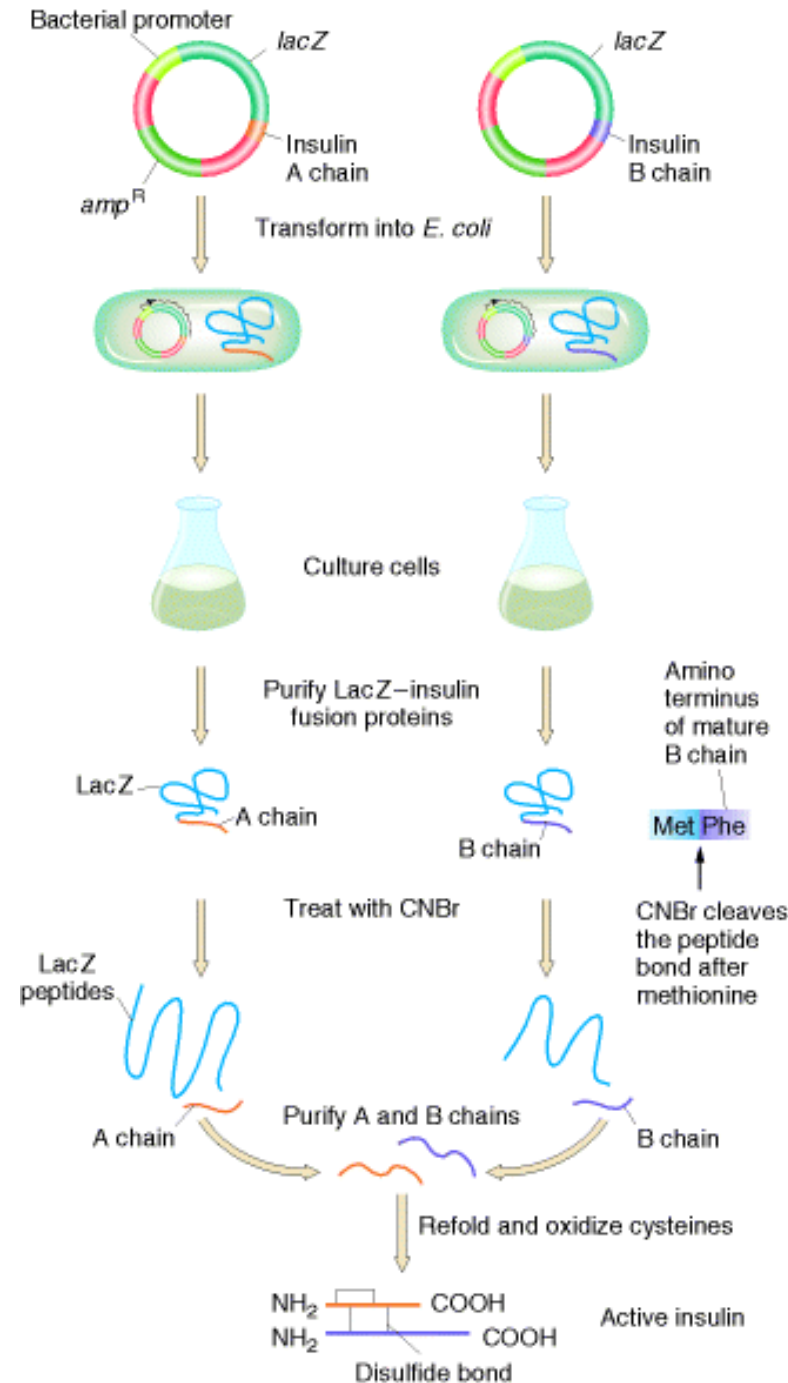
Catena A 21 aa  
Catena B 30 aa  
2 ponti S-S

Nel Golgi viene rimossa la catena C



# PRODUZIONE DI INSULINA RICOMBINANTE IN BATTERI

- Plasmidi separati codificano per Catena A e B
- Le catene sono espresse sotto il controllo di lac come proteine di fusione alla regione Nterm di LacZ
- le sequenze di LacZ sono eliminate per trattamento con bromuro di cianogeno (che taglia dopo una metionina)
- catene purificate, mescolate assieme, in opportune condizioni formano i legami S-S



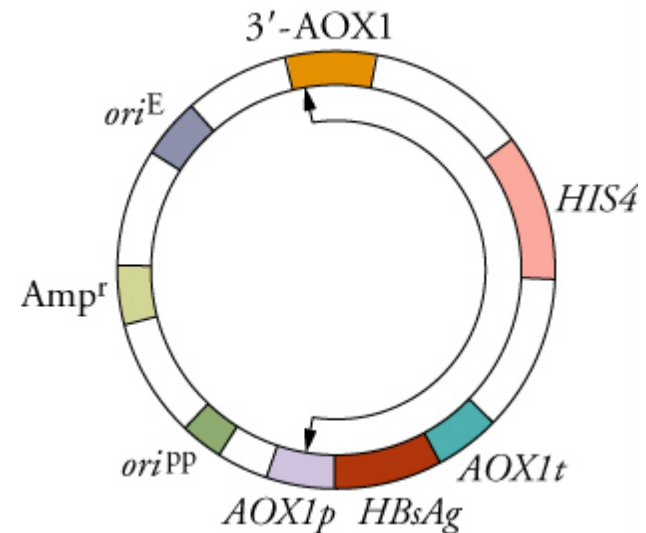


## Produzione del vaccino contro l'epatite C in lievito

- vaccini attenuati sono virus alterati in modo che non possano più riprodursi nell'organismo in cui vengono inoculati
- questi vaccini sono potenzialmente pericolosi: possono essere contaminati con virus infettivi
- i tentativi di produrre antigene di superficie del virus dell'epatite B (HBsAg ) in *E. coli* fallirono
- il gene codificante HBsAg e' stato clonato in un vettore di espressione di lievito.
- il lievito trasformato con questo vettore produce elevate quantità di HBsAg
- utilizzando fermentatori e' possibile ottenere 50-100 mg di proteina per litro di coltura

# Espressione dell'antigene di superficie del virus dell'epatite B in *Pichia pastoris* (nel citoplasma)

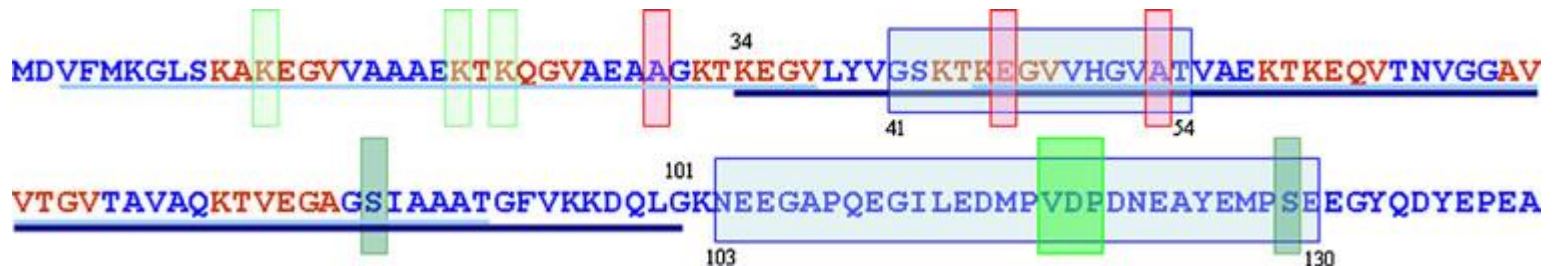
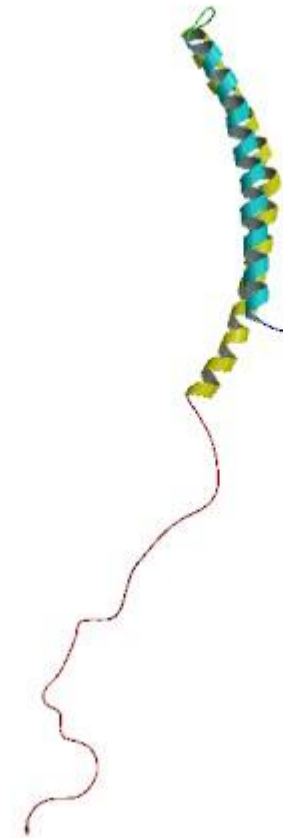
Promotore alcol ossidasi  
Segnali di arresto e poliadenilazione  
Regolato dalla presenza di metanolo  
Seq 3' alcol ossidasi per facilitare integrazione del DNA durante crossing over  
Gene per la biosintesi della istidina

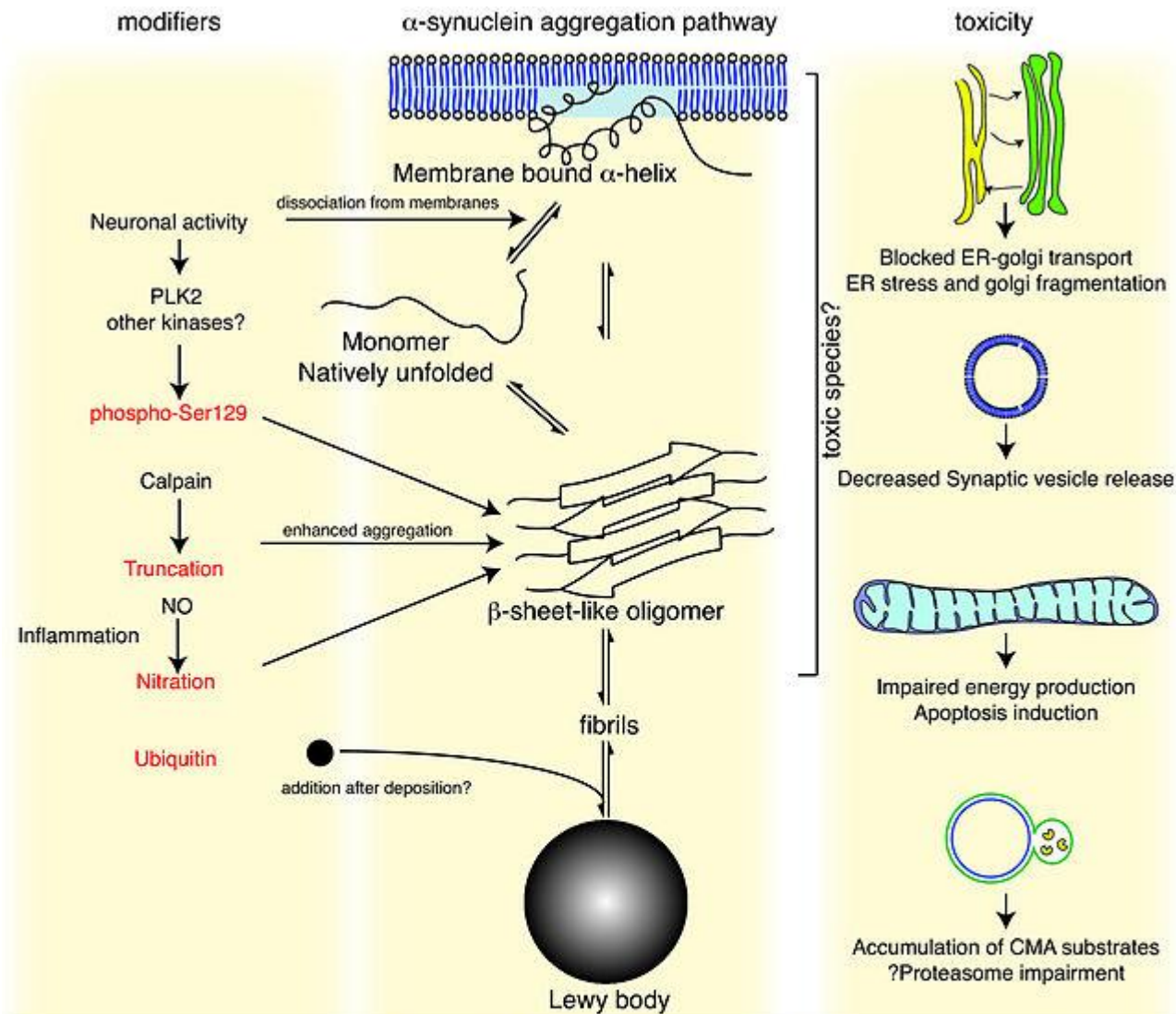


Vettore di espressione  
che si integra in *P.*  
*pastoris*

- $\alpha$  - sinucleina (  $\alpha$  - syn ) , è una proteina di 14kDa associata a malattie neurodegenerative.
- Presente abbondantemente nel cervello (talamo , sostanza nigra, ippocampo etc)
- Espressa maggiormente nei neuroni che nelle cellule gliali
- E' coinvolta nel «trafficking» delle vescicole sinaptiche

regione N-terminale(1–60)contiene 11 residui ripetuti coinvolti nella formazione di alfa elica anfipatica interazione con i lipidi  
 regione centrale coinvolta nell'aggregazione tra (residui 61–95)  
 regione acidica C-terminale ( 101–140) .





Potential therapeutic opportunities

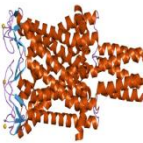
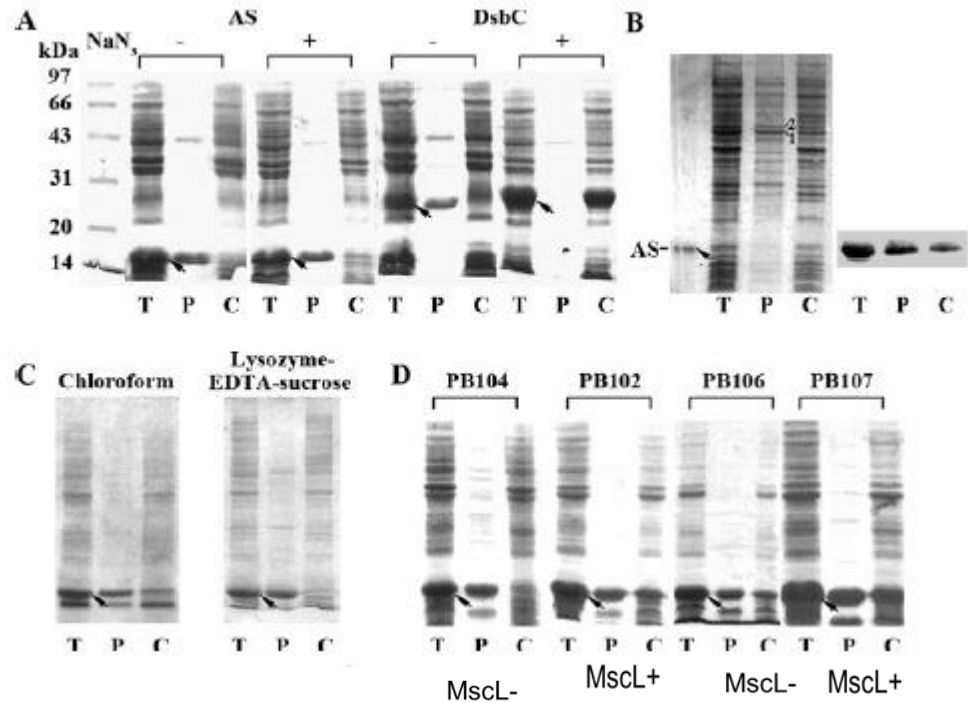
Kinase inhibitors  
Protease inhibitors  
Anti-inflammatory?

Knockdown total  $\alpha$ -synuclein  
Limit formation or remove  
oligomers or other aggregates

Improve organelle function  
Limit cell death

# Espressione in E.coli della alfa -sinucleina

Espressione periplasmatica  
 -Non è coinvolta SecA  
 -Non avviene per shock osmotico  
 -Ne' mediante MscL: Large Mechanosensitive channel



Alfa-syn non può liberamente penetrare nelle LUV( large unilamellar vesicles )

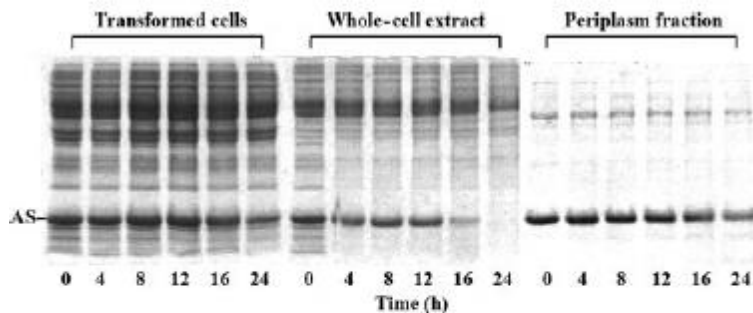


FIG. 2. The periplasm is a safer compartment for the life of AS than is the cytoplasm. AS was expressed in strain BL21(DE3). Transformed cells, the whole-cell extract obtained by sonication, and the periplasm fraction obtained by osmotic shock were incubated at 37°C for different times as indicated and were then analyzed by SDS-PAGE.

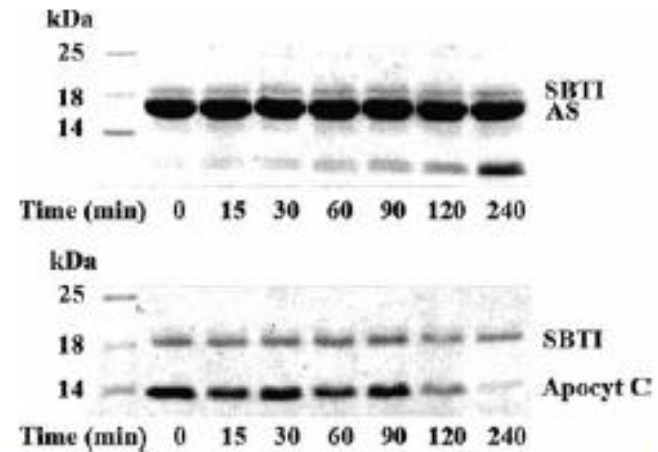


FIG. 3. Translocation of AS into trypsin-entrapped LUV. A 3.6-nmol sample of AS and apocytochrome *c* (Apocyt C) was incubated in 100  $\mu$ l of Tris buffer containing 108 nmol of trypsin-entrapped LUV and 10  $\mu$ g soybean trypsin inhibitor (SBTI) at 37°C, respectively. Reaction mixture samples of 12  $\mu$ l were taken at different times as indicated for SDS-PAGE analysis.

Ed è stabile nel periplasma



# La traslocazione non è mediata da Tat ABC ma da SRP

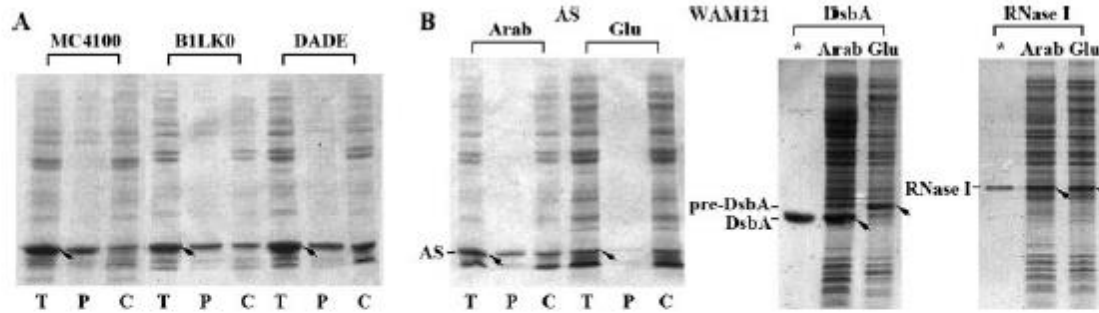


FIG. 4. Subcellular location of AS expressed in Tat-negative strains and strain WAM121. AS was expressed (A) in MC4100 (wild type), B1LK0 ( $\Delta tatC$ ), and DADE ( $\Delta tatABCDE$ ) and (B) in WAM121 in the presence of 0.2% arabinose (Arab) or glucose (Glu). The cell fractions after osmotic shock were examined by SDS-PAGE. T, P, and C mean the same as in Fig. 1. DsbA and RNase I were expressed in WAM121 (B) to verify effectively controlled expression of Ffh. Arrows indicate the positions of expressed AS, DsbA, Pre-DsbA (DsbA precursor), and RNase I. Stars denote the input of the purified proteins.

# La regione carbossi-terminale è necessaria per la traslocazione

2782 REN ET AL.

J. BACTERIOL.

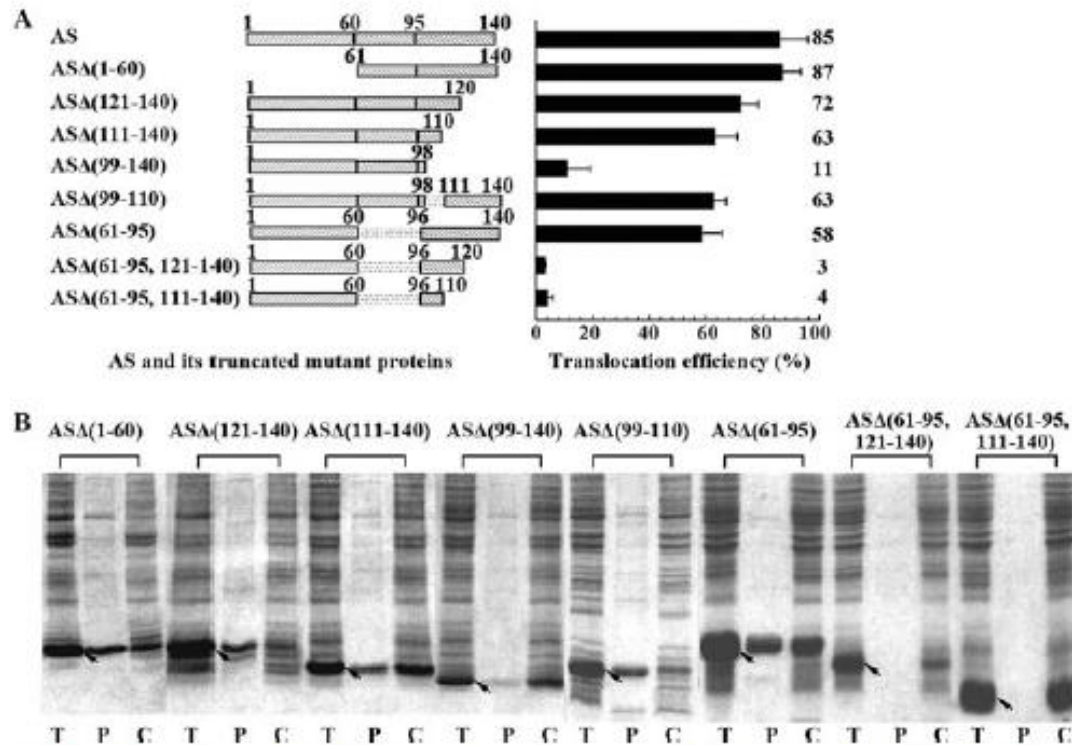


FIG. 5. Subcellular location of truncated AS mutant proteins expressed in M15(pREP4). (A) Schematic representation of intact AS and truncated mutant proteins (left) and their corresponding translocation efficiencies (right, estimated from panel B). Data are expressed as the mean  $\pm$  the standard error ( $n \geq 3$ ). (B) Subcellular location of the AS mutant proteins was examined by SDS-PAGE after osmotic shock. T, P, and C mean the same as in Fig. 1, and arrows indicate the positions of the corresponding mutant proteins.



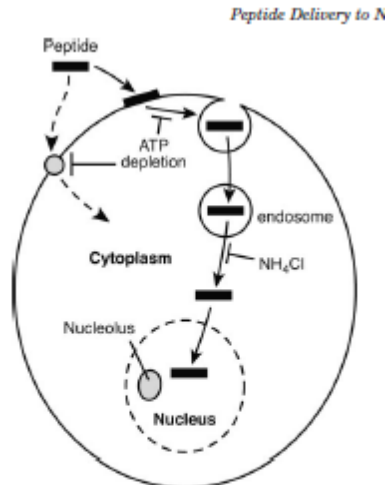
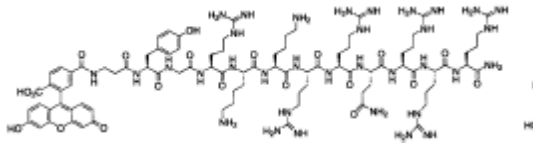
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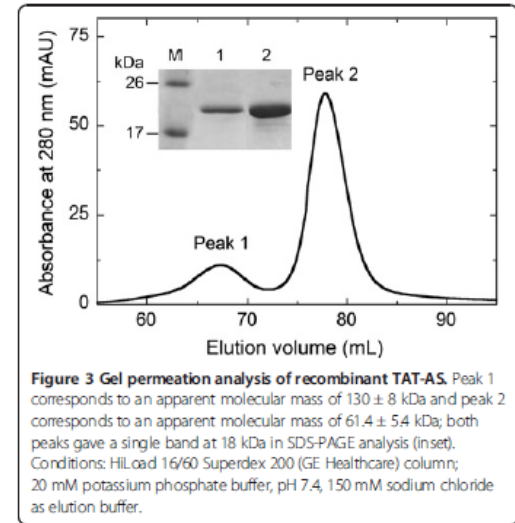
# One single method to produce native and Tat-fused recombinant human $\alpha$ -synuclein in *Escherichia coli*

Laura Caldinelli<sup>1,2</sup>, Diego Alboni<sup>3</sup> and Loredano Pollegioni<sup>1,2\*</sup>

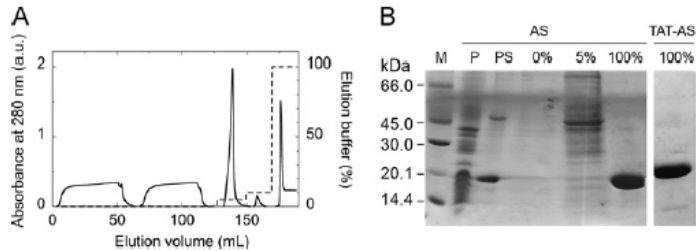
## YGRKKRRQRRR



**Fig. 8 Model for the entry of Fla-TAT and Fla- $\beta$ -VRR<sub>1</sub> into the cytoplasm and nucleus of cells.** The schematic is based on data presented in this paper. Peptides bind to the cell surface and are endocytosed, a process that is blocked in ATP-depleted cells (solid arrow). Escape of peptides from the endosome into the cytoplasm and nucleus occurs by an unknown mechanism that depends on endosomal pH and a threshold concentration of peptide. Escape is blocked by ammonium chloride treatment. An alternative, energy-dependent pathway for direct movement of peptides across the plasma membrane is also shown (dotted arrow).



**Figure 3 Gel permeation analysis of recombinant TAT-AS.** Peak 1 corresponds to an apparent molecular mass of  $130 \pm 8$  kDa and peak 2 corresponds to an apparent molecular mass of  $61.4 \pm 5.4$  kDa; both peaks gave a single band at 18 kDa in SDS-PAGE analysis (inset). Conditions: HiLoad 16/60 Superdex 200 (GE Healthcare) column; 20 mM potassium phosphate buffer, pH 7.4, 150 mM sodium chloride as elution buffer.



**Figure 1 Representative purification of recombinant native AS on HiTrap chelating column (5 mL).** **A)** An amount of periplasmic sample corresponding to 2 L of fermentation broth was loaded and eluted in 20 mM sodium phosphate, pH 7.4, 1 M NaCl. Following two washing steps at 5 and 10% of elution buffer to remove proteins specifically bound to the column, AS was eluted with 500 mM imidazole in 20 mM sodium phosphate, pH 7.4 (elution buffer). **B)** SDS-PAGE analysis of fractions eluted from HiTrap chelating column. P and PS: cell pellet after osmotic shock and periplasmic space content (both corresponding to 0.2 mL of fermentation broth); 0%, 5% and 100%: fractions eluted at different percentage of elution buffer; M: molecular mass standard proteins (GE Healthcare). TAT-AS: purified TAT-AS recombinant protein eluted at 100% of elution buffer.

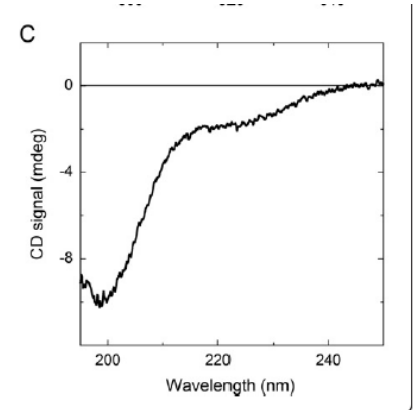
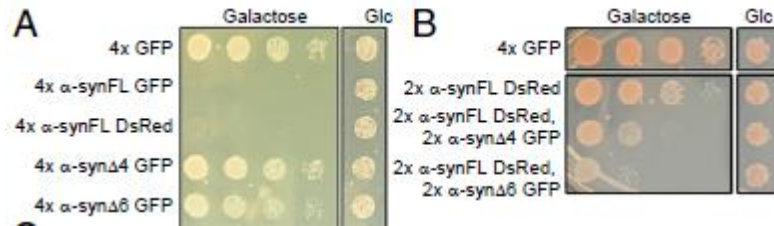


Table 1  
Nanoparticles delivered inside cells by CPPs

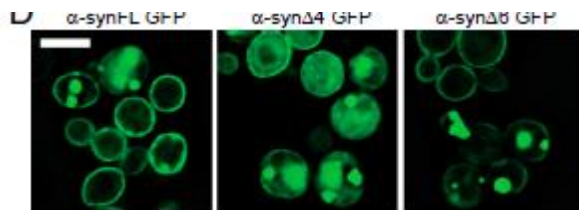
Particle and size, CPP used	Target cell and the aim of the study	References
CLIO (MION) particles, 41 nm, TAT peptide	Mouse lymphocytes, human natural killer, HeLa, human hematopoietic CD34+, mouse neural progenitor C17.2, human lymphocytes CD4+, T cells, B cells, macrophages, immune cells, stem cells For intracellular labeling, MRI, magnetic separation of homed cells, cell imaging	[70–73]
Gold particles, 20 nm, TAT peptide	NIH3T3, HepG2, HeLa, human fibroblast HTERT-BJ1 For intracellular localization studies	[74,85]
Quantum dot-loaded polymeric micelles, 20 nm, TAT peptide coupled to the linker	Mouse endothelial cells, bone marrow-derived progenitor cells For studying tumor pathophysiology under dynamic conditions, useful for concurrent imaging and distinguishing tumor vessels from perivascular cells and matrix	[84]
pH-sensitive polymeric micelles, 20–45 nm, TAT peptide	Various cells For tumor-specific intracellular drug delivery	[93]
PEG–polylactic acid micelles, 20–45 nm, TAT peptide	For drug delivery to acidic solid tumors	[93]
Nanocomplexes of polyethyleneimine and DNA, TAT peptide	SH-SY5Y cells For gene delivery to neurotypic cells	[95]
PEG–PEI conjugates, TAT peptide	For DNA delivery to the lung in mice	[100]
Sterically stabilized liposomes, 200 nm, TAT peptide coupled to the linker	Mouse LLC, human BT20, rat H9C2, LLC tumor in mice To show the potential of TAT–liposome for intracellular gene delivery <i>in vitro</i> and <i>in vivo</i>	[88,89]
Sterically stabilized liposomes, 65–75 nm, TAT peptide and penetratin coupled to the linker	Human bladder carcinoma HTB-9, murine colon carcinoma C26, human epidermoid carcinoma A431, human breast cancer SK-BR-3, MCF7/WT, MCF7/ADR, murine bladder cancer MBT2, dendritic cells For kinetics studies of the internalization of CPP-modified liposomes	[89,90]
Liposomes, TAT peptide, Antennapedia, octaarginine	Airway cells For intracellular drug delivery by inhalation	[79]
Liposomes, lipid-modified TAT peptide	Various cells	[92]
Liposomes, polyarginine	For siRNA delivery into cells and gene silencing	[102]
Dendrimers, various CPPs	For oligonucleotide delivery into various cells	[101]
Boron carbide nanoparticles, TAT peptide	Murine EL4 lymphoma cells, B16F10 melanoma cells For boron neutron capture therapy	[86]



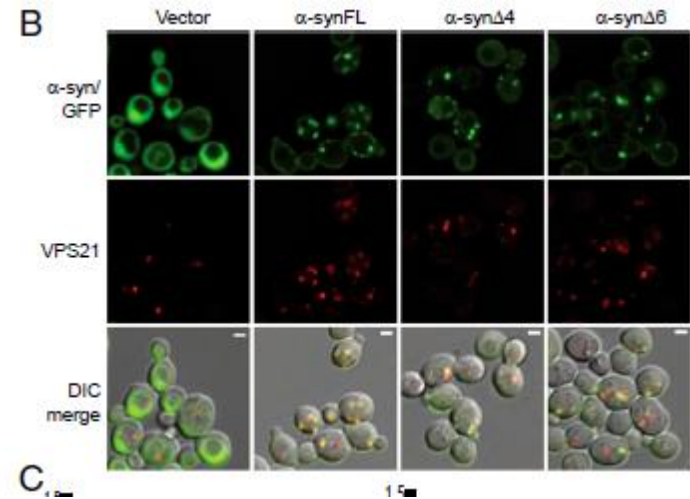
## Splice Isoforms of $\alpha$ -Syn Display Different Toxicity.



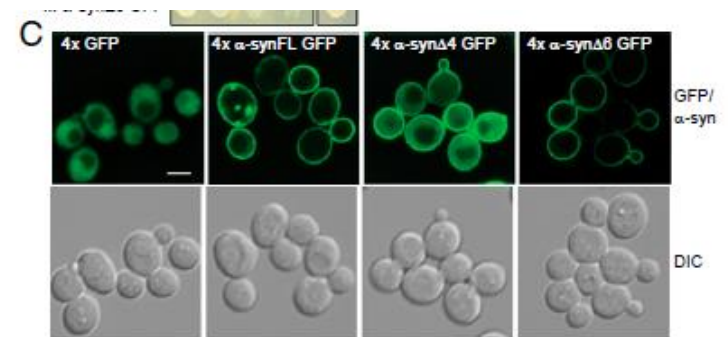
**Fig. 3.** Higher levels of  $\alpha$ -syn splice isoform expression lead to higher toxicity and foci formation. ( )



## Expression of $\alpha$ -Syn Splice Isoforms Blocks Vesicular Trafficking

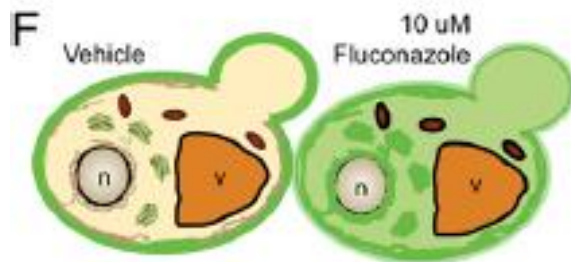
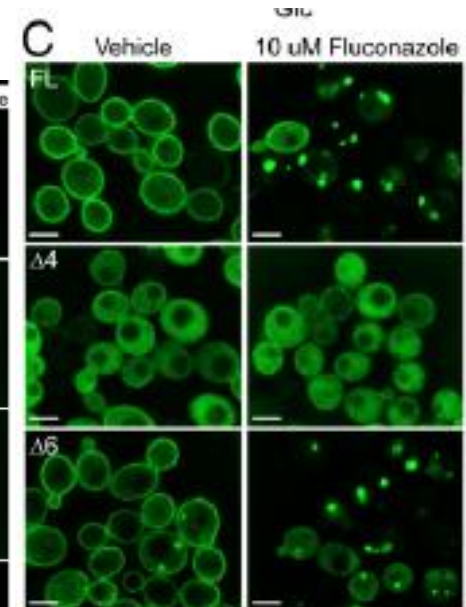
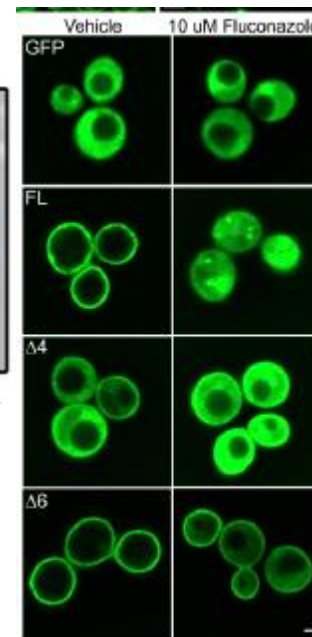
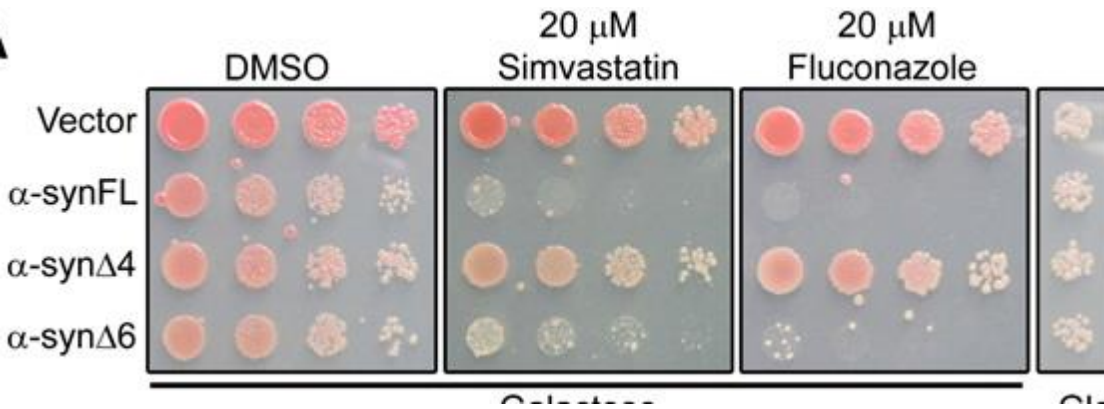


## Splice Isoforms of $\alpha$ -Syn Display Different Localization



Inibitori della sintesi di colesterolo aumentano la tossicità sia di syn FL che di syn  $\Delta 6$

Fluconazolo aumenta la percentuale synucleina associata alle vescicole di secrezione



# Saccharomyces cerevisiae

**Yip** vettore di integrazione, deriva da un vettore plasmidico batterico.

- marker di selezione di lievito
- manca di origine di replicazione

Integrazione per ricombinazione omologa tra un marcatore funzionale di lievito e il corrispondente marcatore difettivo in un lievito mutante. L'integrazione porta alla duplicazione della sequenza bersaglio. Il DNA duplicato fiancheggia il vettore. I plasmidi integrativi sono propagati stabilmente, ma occasionalmente possono essere "spinti fuori" per ricombinazione tra le due sequenze duplicate.

