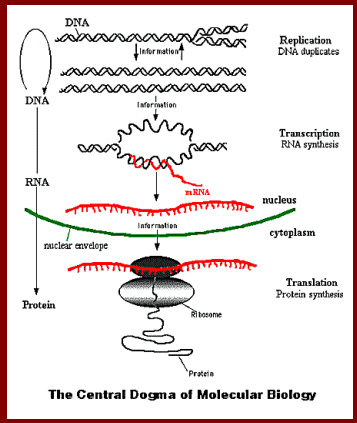
## Directed Evolution of Substrate-Optimized GroEL

Jue Wang

Kim Tipton

Christophe Herman

Carol Gross



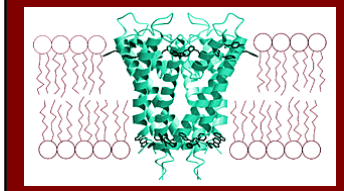

Replication Error Rate $\sim 10^{-8}$

Transcription Error Rate $\sim 10^{-5}$

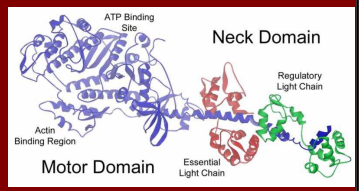
Translation Error Rate $\sim 10^{-3}$-$10^{-4}$

## A Protein's Function is Determined by its Fold

**Potassium Ion Channel**



**Myosin Motor Protein**



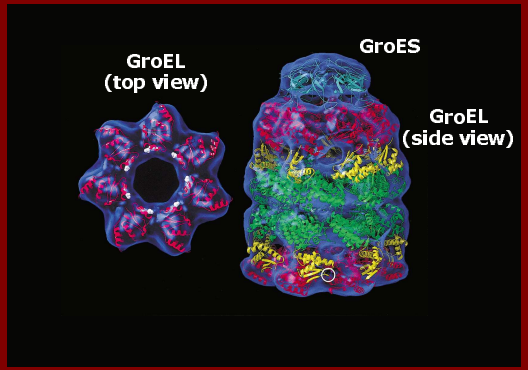

## Protein Folding is the Most Challenging Step in the Reading of Genetic Information

- Folding is an intrinsically complex problem: there is no way of subdividing it into simple steps
- Folding can be very slow: a protein synthesized in minutes may take hours to fold
- Folding is highly error prone: ~33% (up to 95% for some hard cases) of newly made proteins are rapidly degraded
- Many diseases (inherited, spontaneous and even infectious ones) are caused by protein misfolding

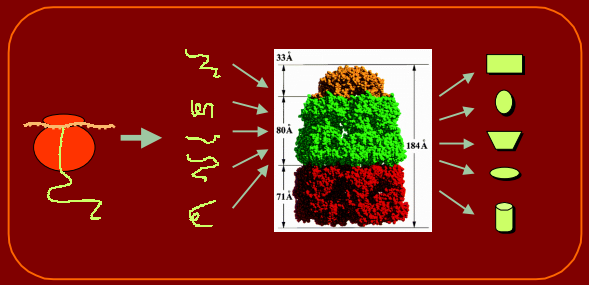
## Protein Folding in the Depends on Helper Proteins Termed Molecular Chaperones

- There are several completely unrelated classes of chaperones
- Chaperones consume energy to assist folding
- The "cocktail" of chaperones determine what proteins can be produced in a given cell type
- Different chaperones systems predominate in different organism (e.g. eukaryotes vs. prokaryotes)

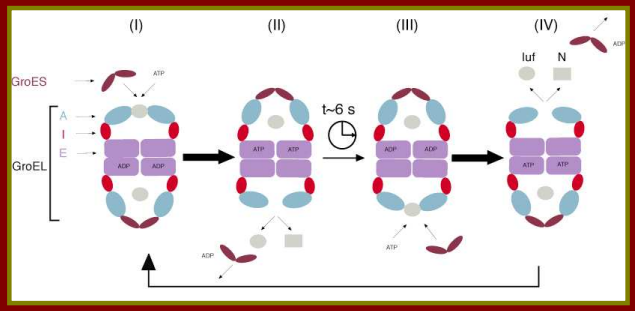
## GroEL/ES: A Conserved and Essential Chaperone Pair Uses ATP Hydrolysis to Enhance Folding



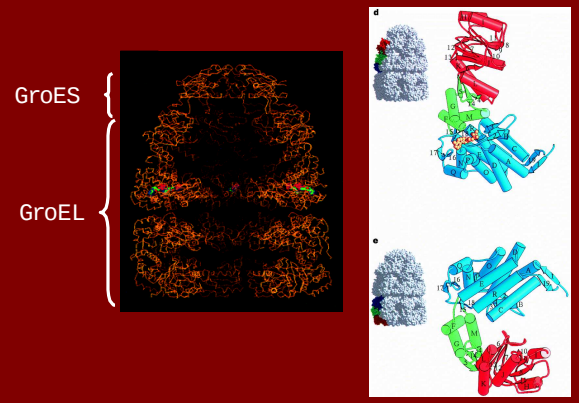

## GroEL Assists Folding of Many Different Proteins




## GroEL Reaction Cycle

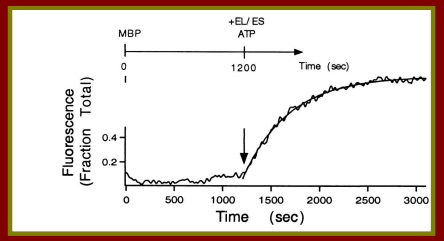


## GroEL-GroES Forms a Protected Folding Cavity

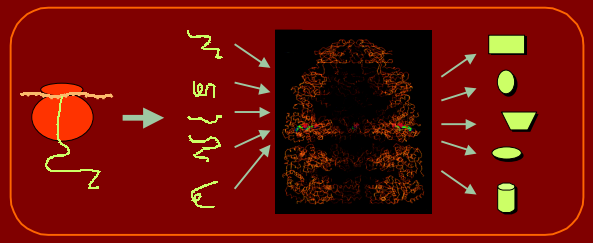



## Beyond the Passive Box: GroEL Catalyzes the Folding of Some Substrates

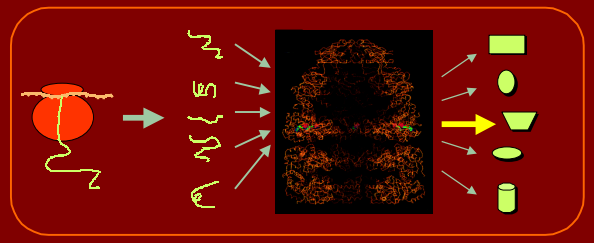
Substrate :
MBP

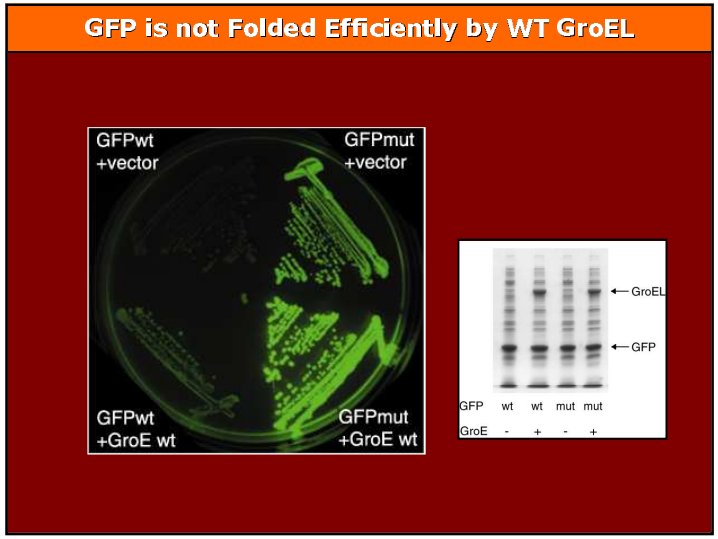


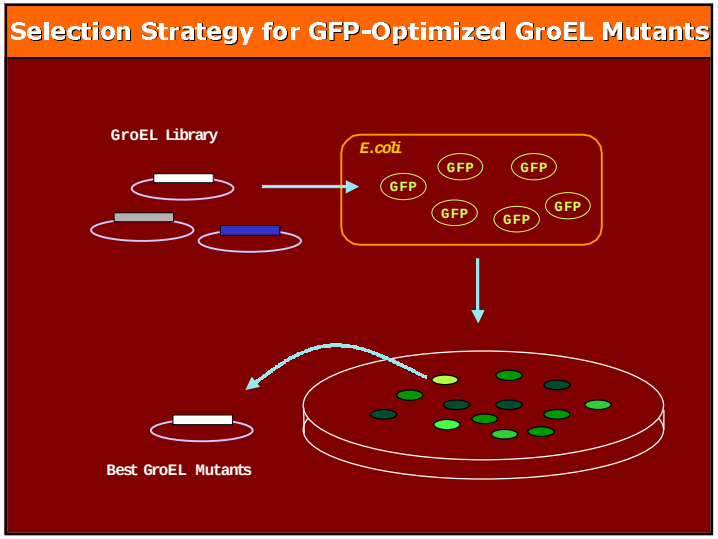

## What are the Limits to GroEL as a Universal Folding Machine?

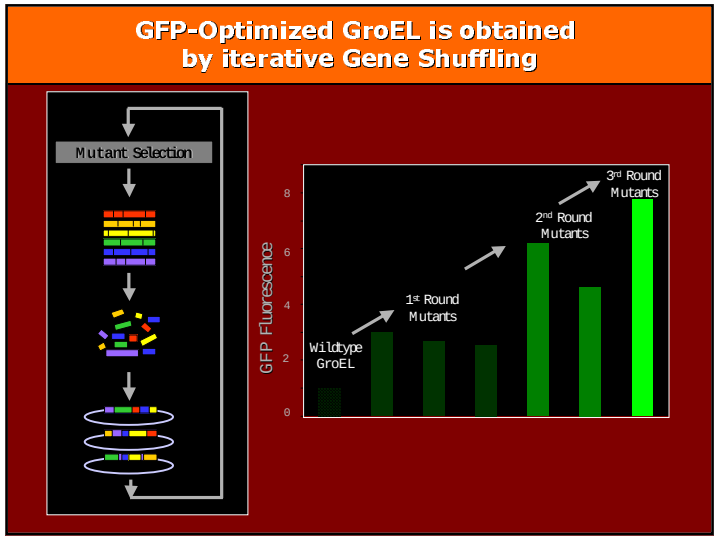


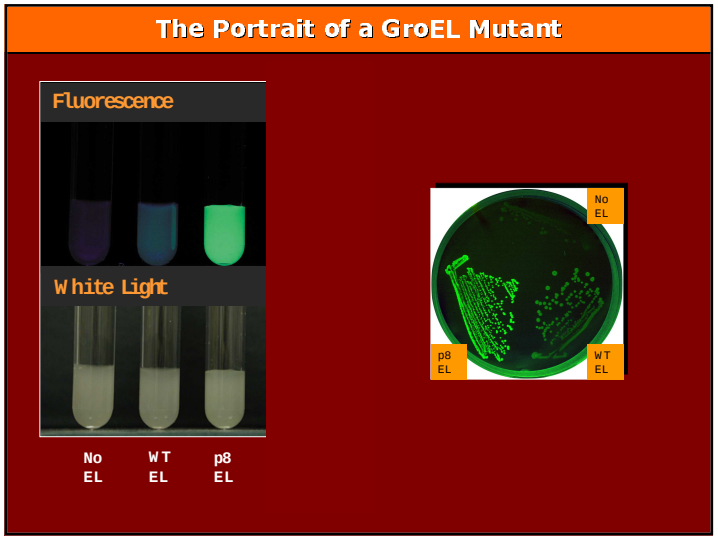
## What are the Limits to GroEL as a Universal Folding Machine?

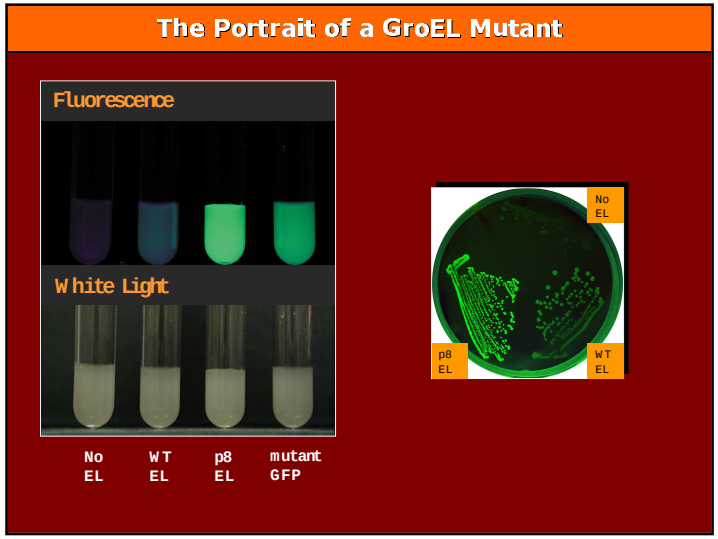


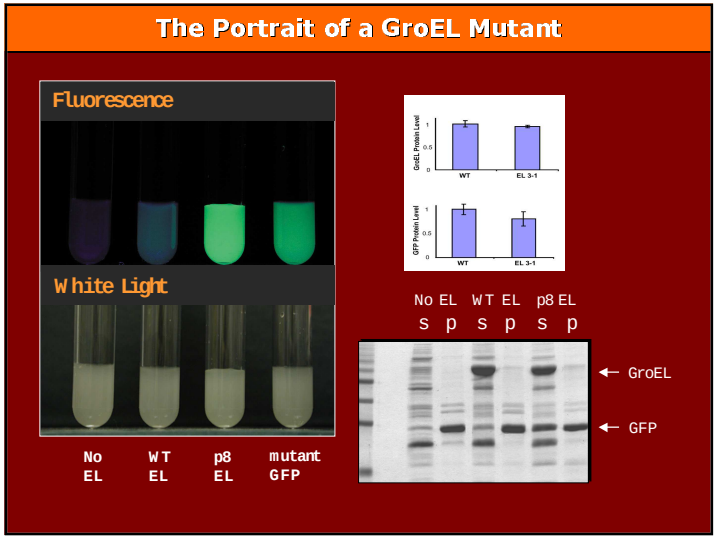

GFP is not Folded Efficiently by WT GroEL
GFPwt +vector
GFPmut +vector
GFPwt +GroE wt
GFPmut +GroE wt
GroEL
GFP
GFP wt wt mut mut
GroE - + - +

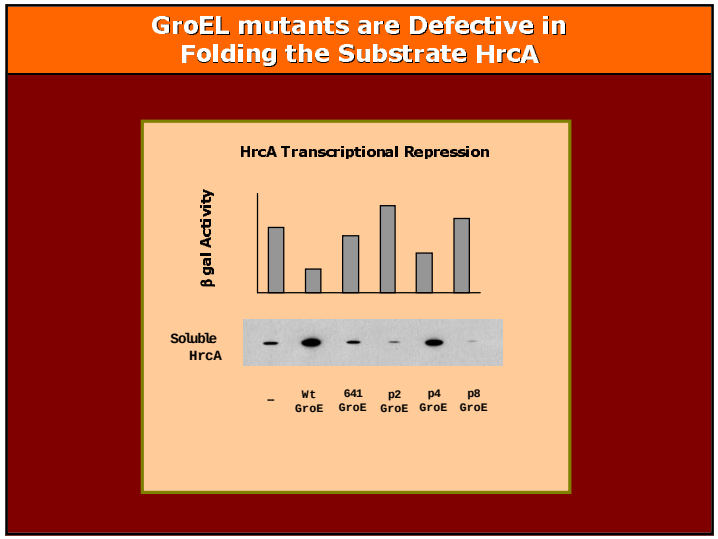

Selection Strategy for GFP-Optimized GroEL Mutants
GroEL Library
E.coli
GFP
Best GroEL Mutants

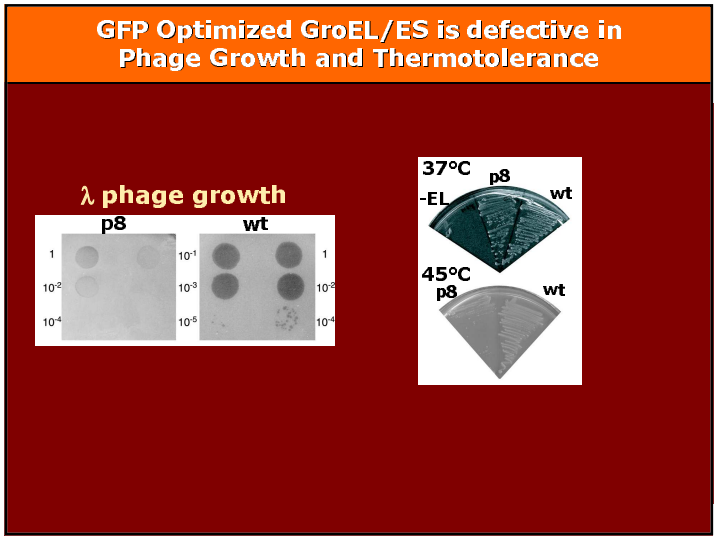

GFP-Optimized GroEL is obtained by iterative Gene Shuffling
Mutant Selection
GFP Fluorescence
Wildtype GroEL
1st Round Mutants
2nd Round Mutants
3rd Round Mutants

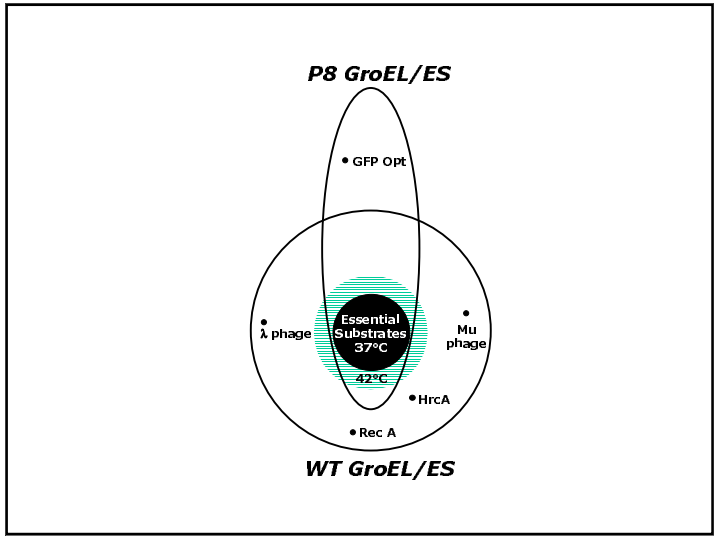

The Portrait of a GroEL Mutant
Fluorescence
White Light
No EL
WT EL
p8 EL


The Portrait of a GroEL Mutant
Fluorescence
White Light
No EL
WT EL
p8 EL
mutant GFP
No EL
p8 EL
WT EL


The Portrait of a GroEL Mutant
Fluorescence
White Light
No EL
WT EL
p8 EL
mutant GFP
No EL WT EL p8 EL
s p s p s p
GroEL
GFP


GroEL mutants are Defective in Folding the Substrate HrcA
HrcA Transcriptional Repression
β gal Activity
Soluble HrcA
–
Wt GroE
641 GroE
p2 GroE
p4 GroE
p8 GroE


GFP Optimized GroEL/ES is defective in Phage Growth and Thermotolerance
λ phage growth
p8
wt
37°C
-EL
p8
wt
45°C
p8
wt

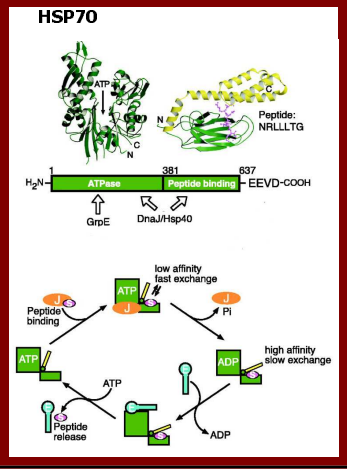


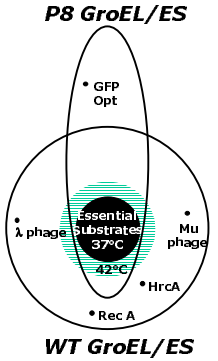

## Conclusions

- There is a remarkable *plasticity* to GroEL which makes it possible to dramatically increase its ability to fold specific substrates
- There is in an intrinsic *conflict* between the requirements of different substrates so that GroEL variants optimized for the folding of one protein lose the ability to fold others

*Why haven't cells evolved many different GroEL Subtypes?*

## The HSP70-HSP40(J) Chaperone system



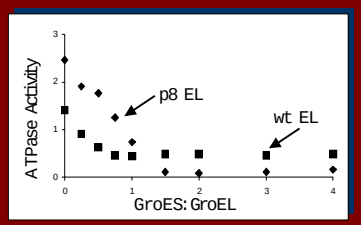

*Hypothesis*: as the proteome expanded, no single chaperone sufficed. The ring nature of GroEL made it relatively difficult to evolve new independent chaperones compared to Hsp70/40.

## Exceptions that "prove" the rule: the use of adaptors to expand the repertoire of a ring complex

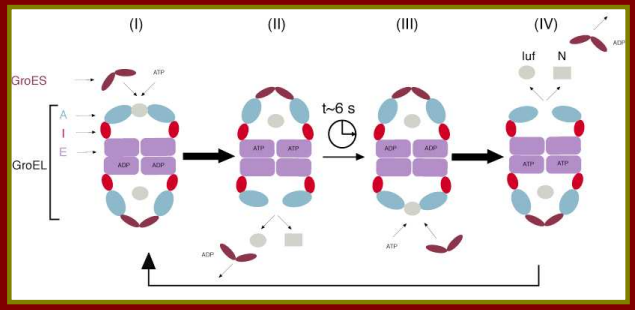
- Nuclear Pores: Importin
- Porteasome: ubiquitin ligases

## Mutations of GroEL are Near ATP Binding Site

## Altered ATPase Activities in the GroEL Mutants




## GroEL Reaction Cycle



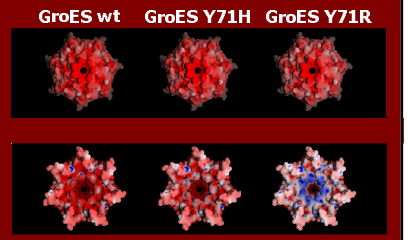
## GroES Residue Y71 is a Hotspot for Mutations

Y71:

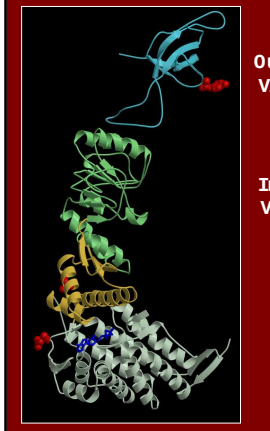
-- Extends into the Central Cavity

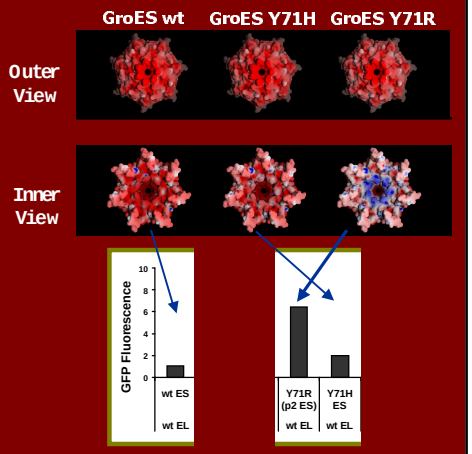
-- Multiple mutations are selected from the screen: Y71H, Y71R, Y71C

## Altered Electrostatic Potential in the GroES Mutants

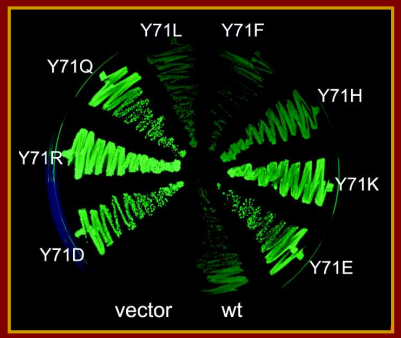



## Altered Central Cavity Might Improve GFP Folding






## Hydrophilic Cavity Improves GFP Folding

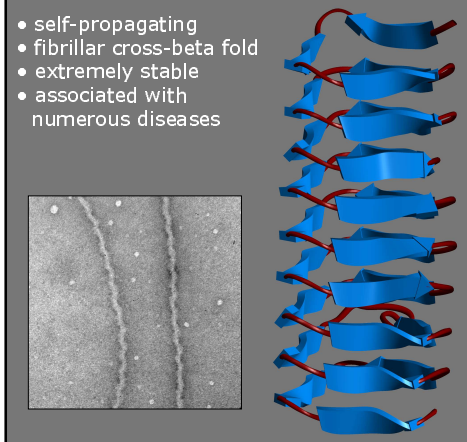


## Summary of in vitro Studies

- The period of ATP-driven capture-release cycle is altered perhaps to fit GFP folding kinetics better
- The folding environment : increased polarity of the central cavity lid enhances GFP folding
- *Mutations were not found in peptide binding domain despite targeted mutagenesis of these regions*

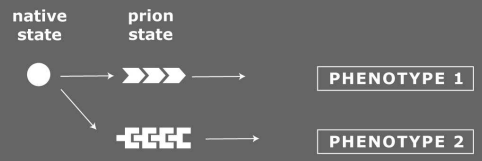
## Amyloids Are Ordered Protein Aggregates

- self-propagating
- fibrillar cross-beta fold
- extremely stable
- associated with numerous diseases



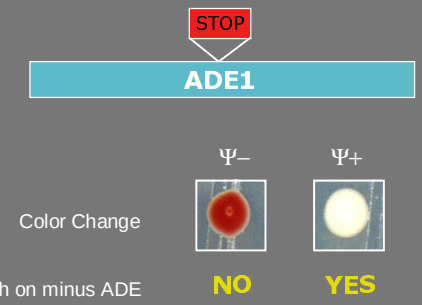

from Serpell and Blake, Adv. Prot. Chem., V.50

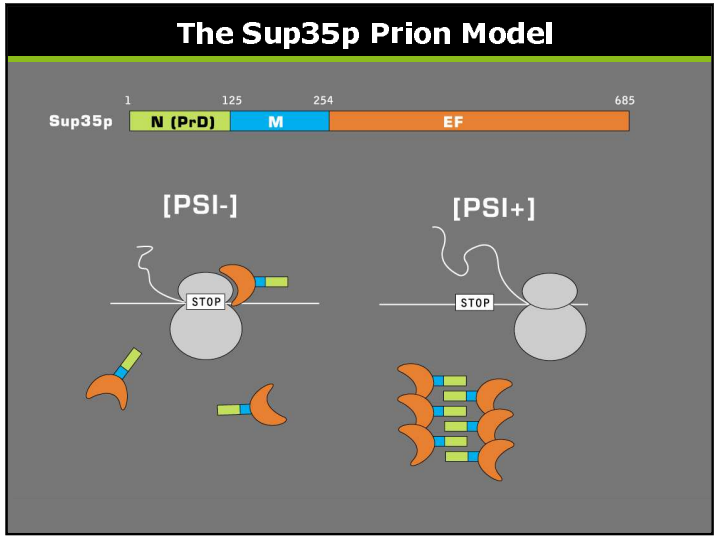
## Requirements of the Prion Hypothesis

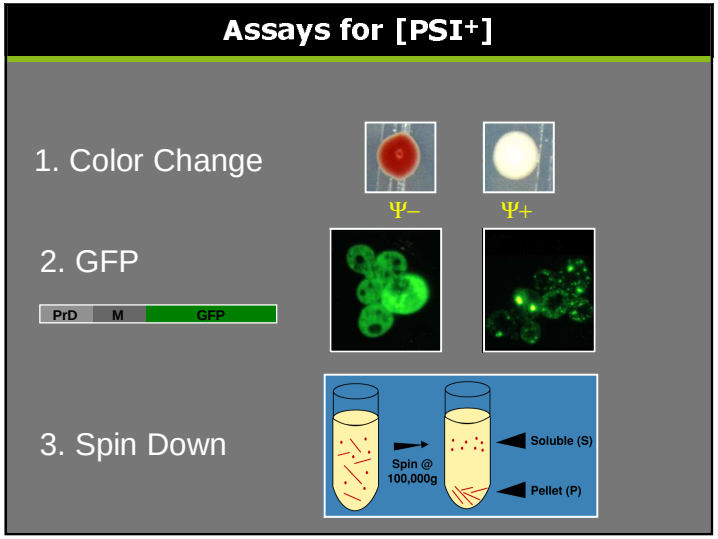


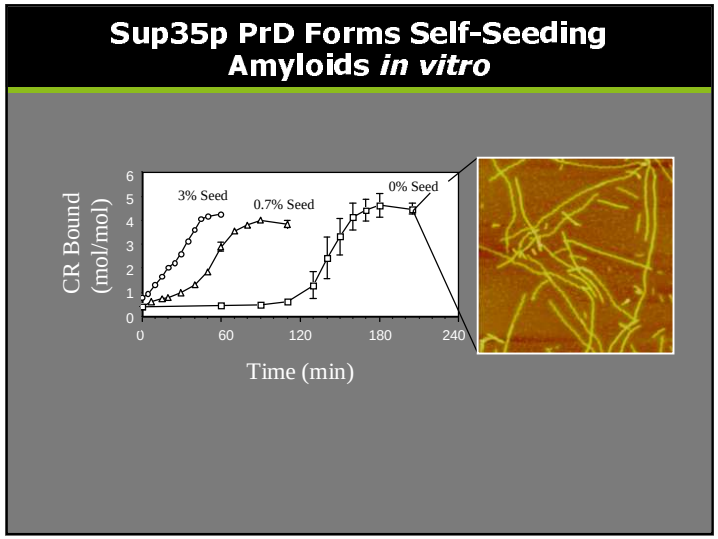

- INFECTION: self-propagating structure
- SPECIES BARRIER: sequence specificity
- STRAINS: structural diversity

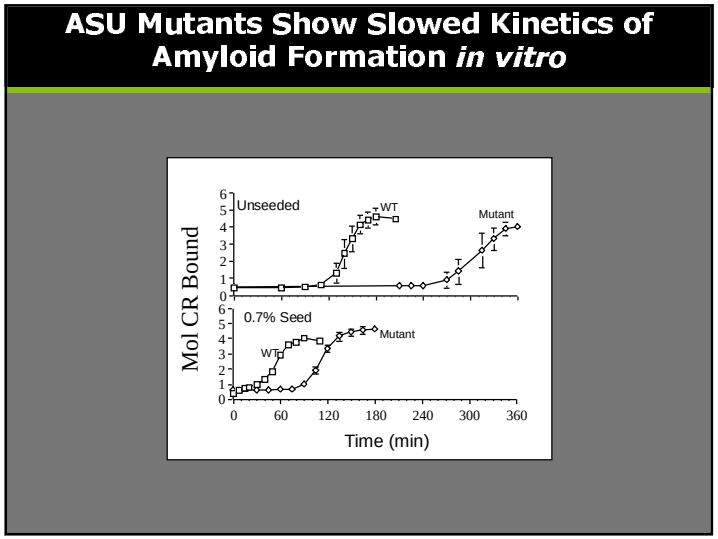
## [PSI+] Causes Suppression of Nonsense Mutations



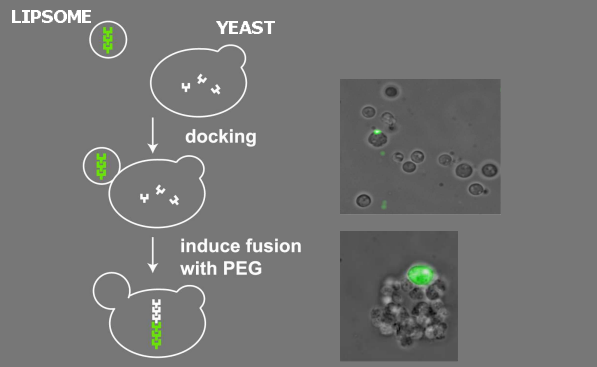

The Sup35p Prion Model
1
125
254
685
Sup35p
N (PrD)
M
EF
[PSI-]
[PSI+]
STOP
STOP


Assays for [PSI+]
1. Color Change
Ψ–
Ψ+
2. GFP
PrD
M
GFP
3. Spin Down
Spin @ 100,000g
Soluble (S)
Pellet (P)


Sup35p PrD Forms Self-Seeding Amyloids *in vitro*
CR Bound (mol/mol)
3% Seed
0.7% Seed
0% Seed
Time (min)

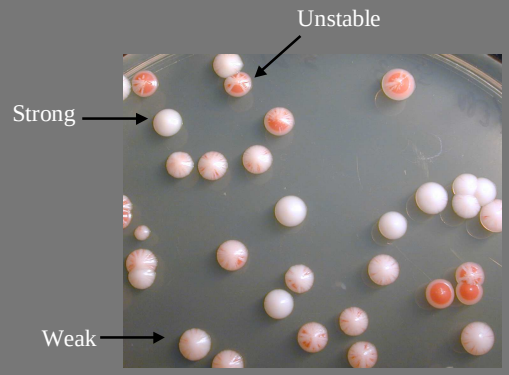

ASU Mutants Show Slowed Kinetics of Amyloid Formation *in vitro*
Mol CR Bound
Unseeded
WT
Mutant
0.7% Seed
WT
Mutant
Time (min)

## Transformation by Liposome Fusion



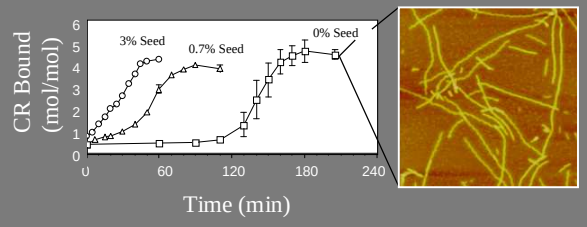

Prion strains: One protein can cause multiple different diseases.

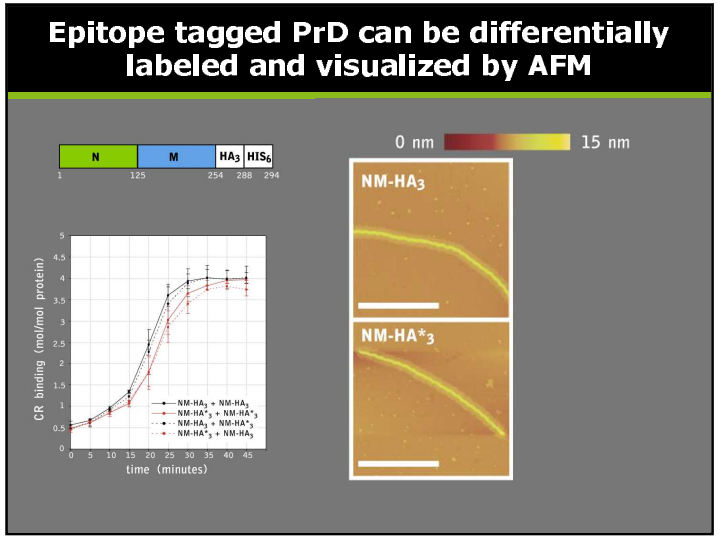
## Yeast Prions also Come in Different Strains

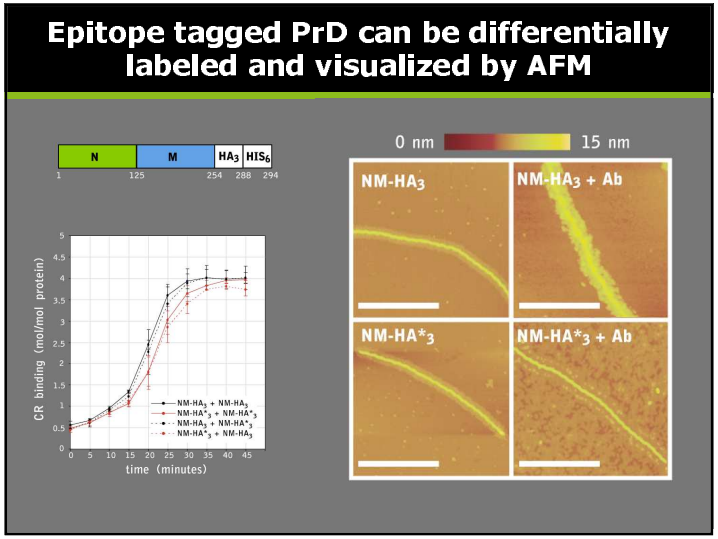


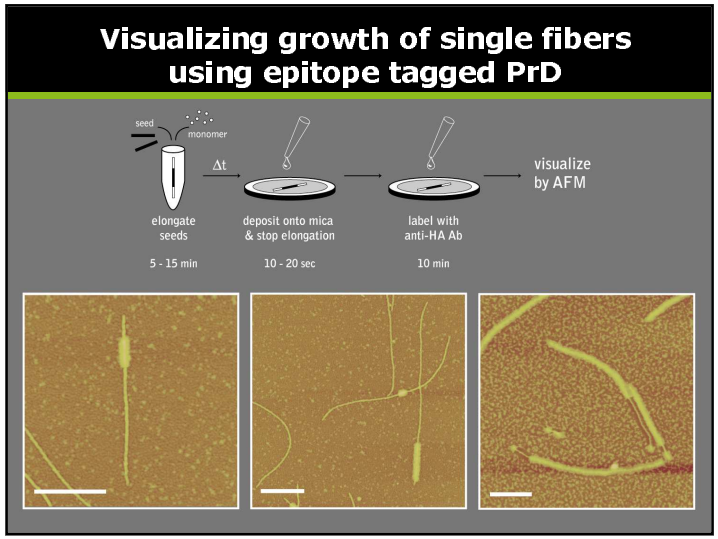

## Do individual Sup35p fibers vary in properties that could account for the variation in strain phenotype?

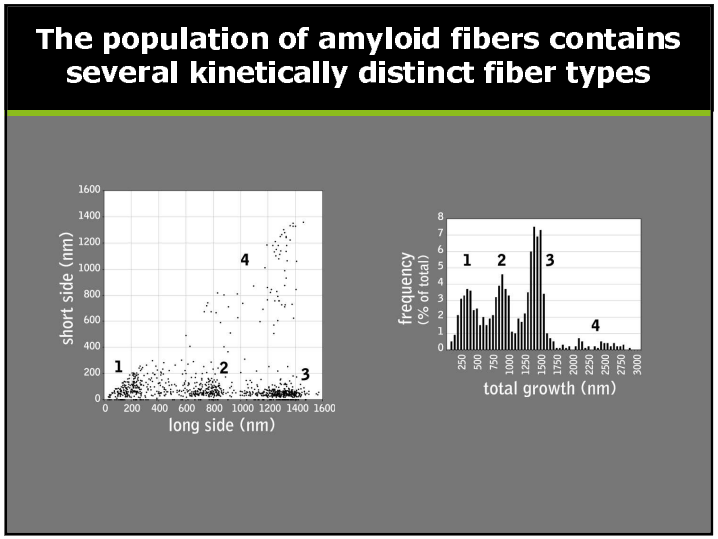
| Phenotype | *In vitro* correlate |
|---|---|
| Strength of nonsense suppression phenotype (amt of soluble Sup35p) | Polymerization rate |



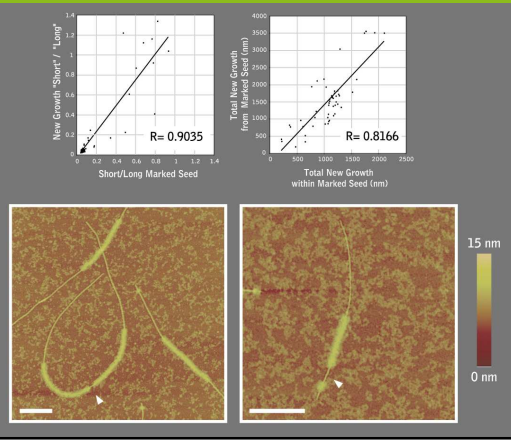

Epitope tagged PrD can be differentially labeled and visualized by AFM
N
M
HA3
HIS6
1
125
254
288
294
0 nm
15 nm
NM-HA3
NM-HA*3
CR binding (mol/mol protein)
time (minutes)


Epitope tagged PrD can be differentially labeled and visualized by AFM
N
M
HA3
HIS6
1
125
254
288
294
0 nm
15 nm
NM-HA3
NM-HA3 + Ab
NM-HA*3
NM-HA*3 + Ab
CR binding (mol/mol protein)
time (minutes)


Visualizing growth of single fibers using epitope tagged PrD
seed
monomer
Δt
visualize by AFM
elongate seeds
5 - 15 min
deposit onto mica & stop elongation
10 - 20 sec
label with anti-HA Ab
10 min

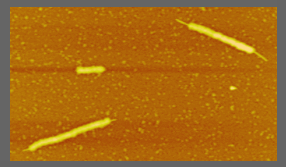

The population of amyloid fibers contains several kinetically distinct fiber types
short side (nm)
long side (nm)
1
2
3
4
frequency (% of total)
total growth (nm)

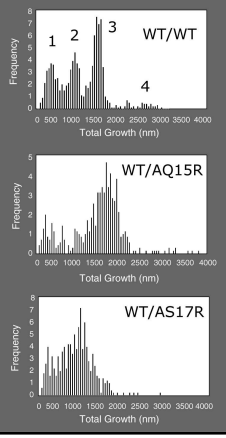
## Individual fibers faithfully propagate polarity and elongation rate



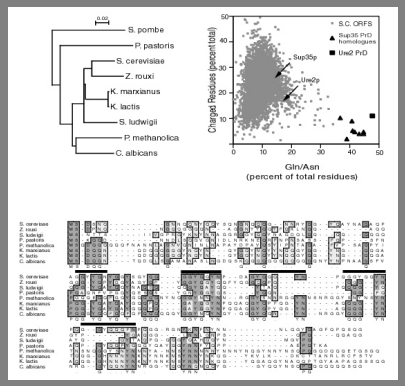

## Effects of Sup35 mutations on fiber strains types

WT growth from mutant seed

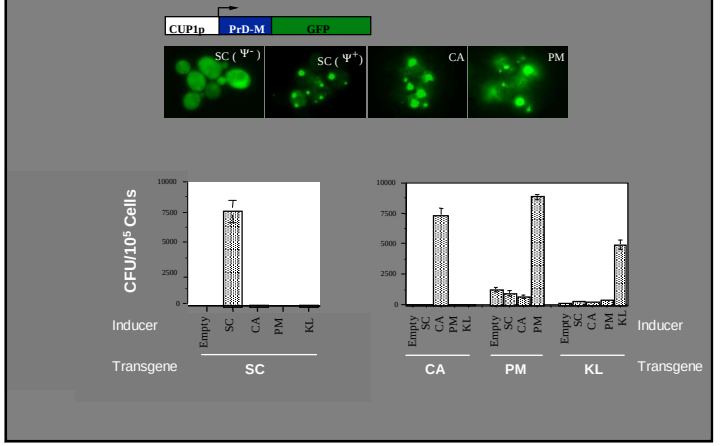





## Sup35 PrD is Conserved in Budding Yeast Evolution

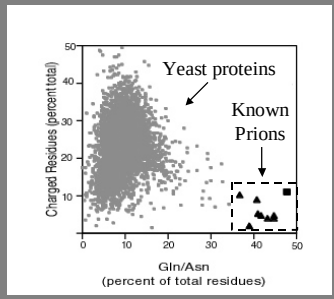



## A Species Barrier to Sup35p Prion Induction



## How Many Prions Are There?

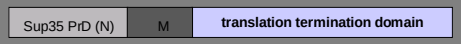
## Searching for Novel Prions



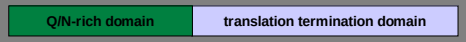

## Making an artificial prion

New1p, a good prion candidate:

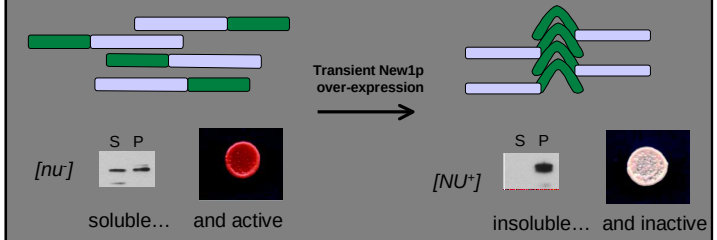

Sup35p, a selectable reporter: X



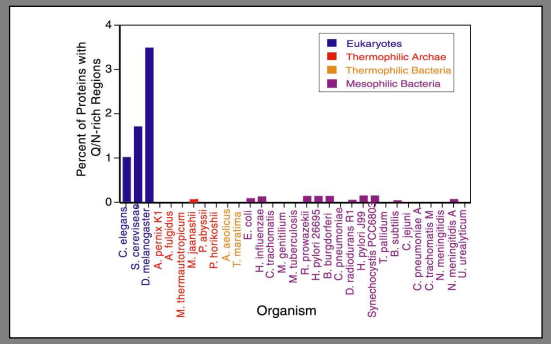

A selectable New1p prion reporter:




## New1p Q/N domain forms a prion, [*NU*⁺]



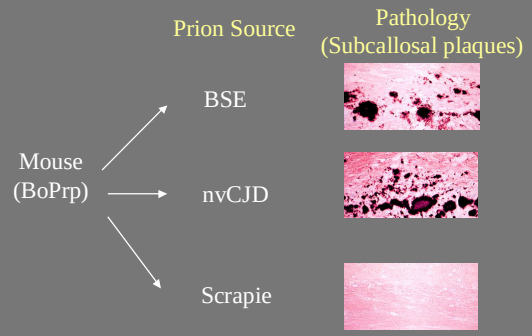
## There are Many Potential Prion-Domains in Eukaryotes




## Acknowledgments

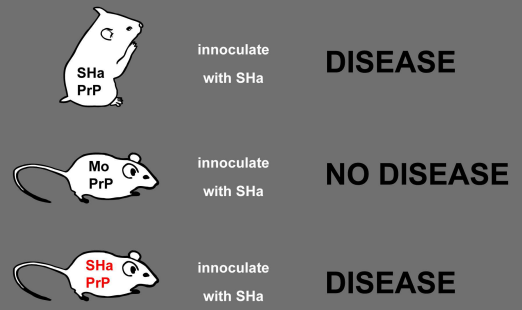
- **Prion Strains:** Peter Chien
- **Prion Mutants Screen:** Angela DePace and Alex Santoso
- **Single Fiber Assay:** Angela DePace
- **Species Barrier:** Alex Santoso and Peter Chien
- **Protein Infection:** Helmut Sparrer
- **New Prions:** Lev Osherovich and Melissa Michelitsch

## BSE and nvCJD are the Same Strain: Humans are Susceptible to Mad Cow Disease

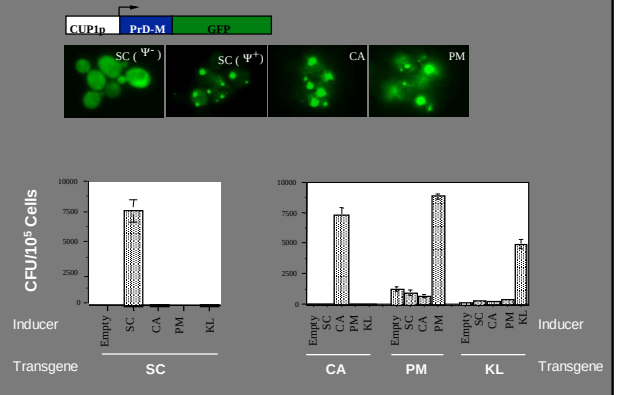



*Scott, Prusiner et al. PNAS 96(1999)

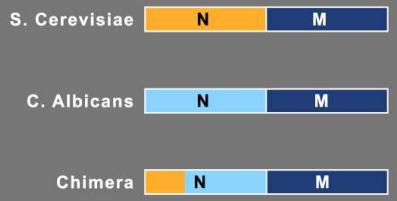
## Prion Protein Sequence Determines Species Barrier



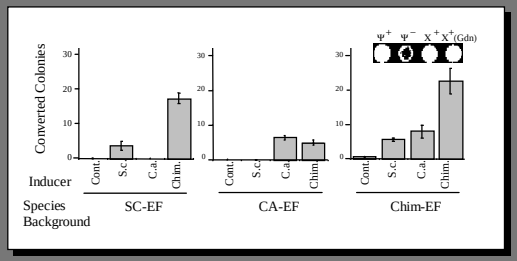
## A Species Barrier to Sup35p Prion Induction



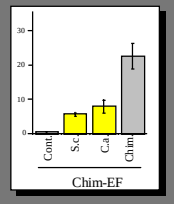

## A Chimeric Prion Domain



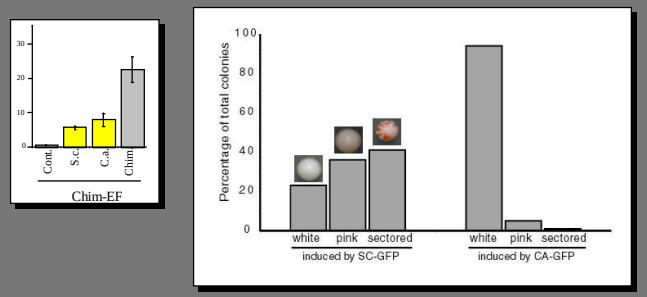

## The Chimera Bridges the Species Barrier



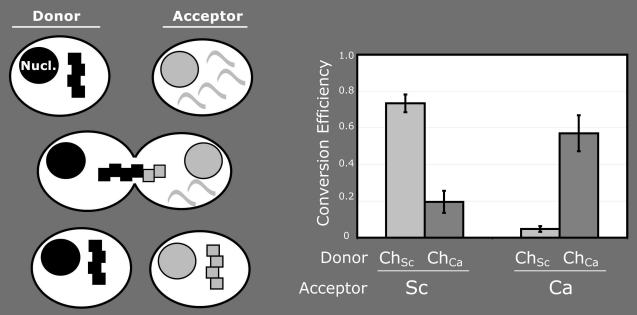

## Efficiency of Chim Prion Conversion is the Same for Both Pure Parent Species. But...



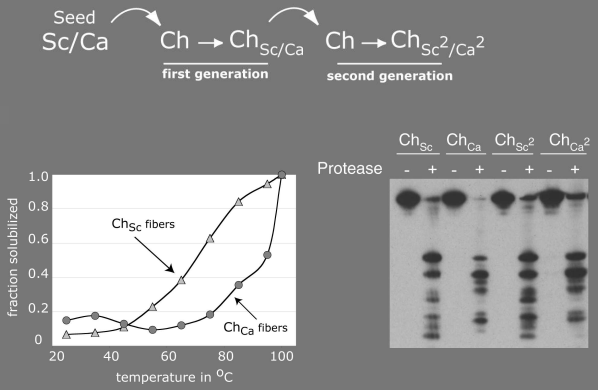
## ...“Infecting” Species Determines Strain Properties of Chim Prion



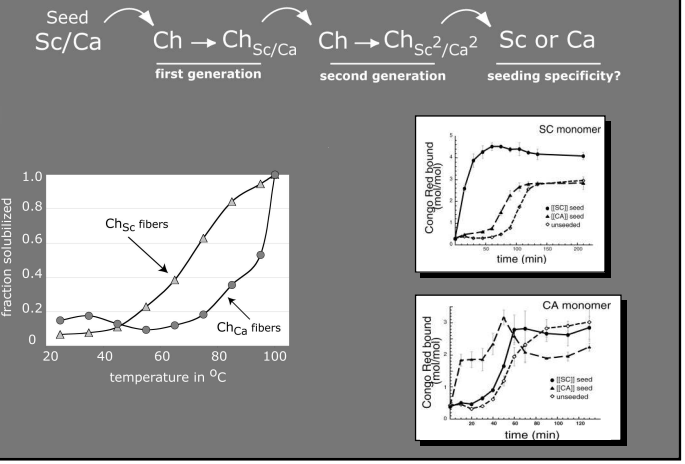

## “Infecting” Species Determines Species Preference of Chim Prion

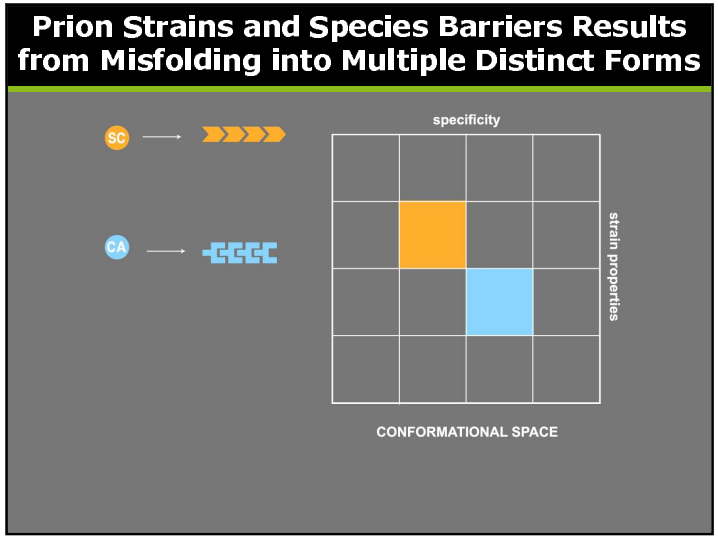


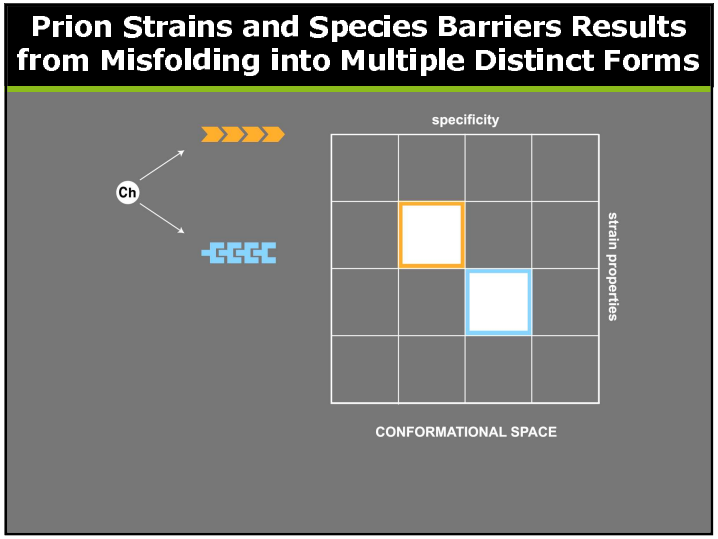

## Prion strains in vitro

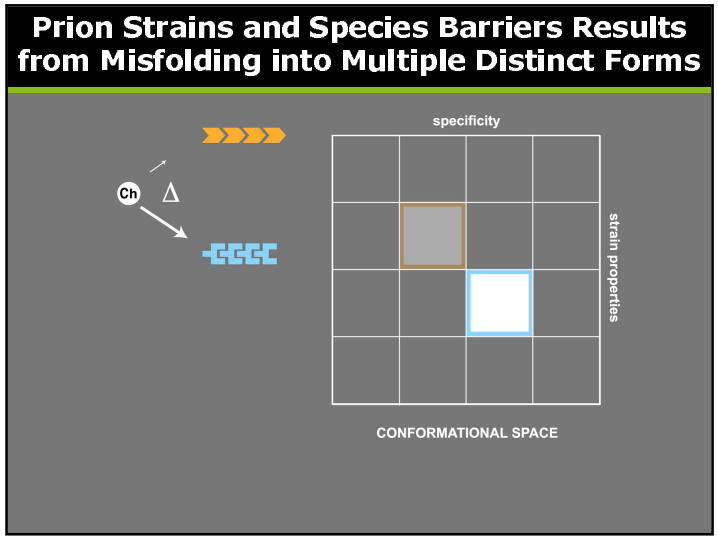


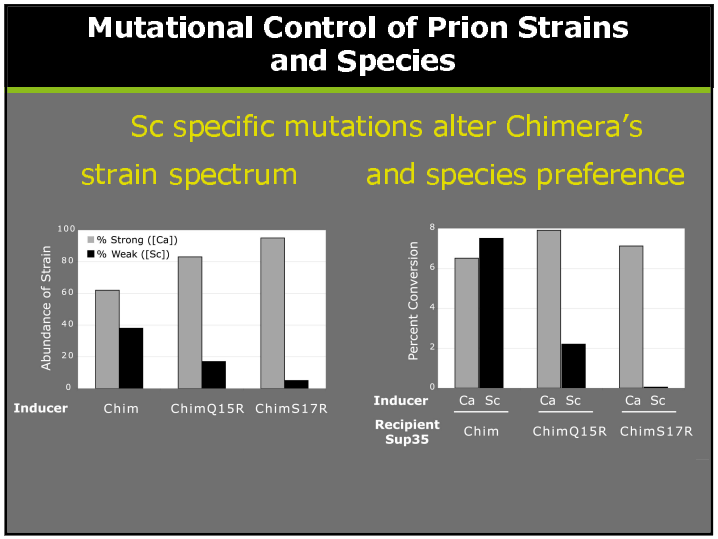

## Prion strains determine species preference

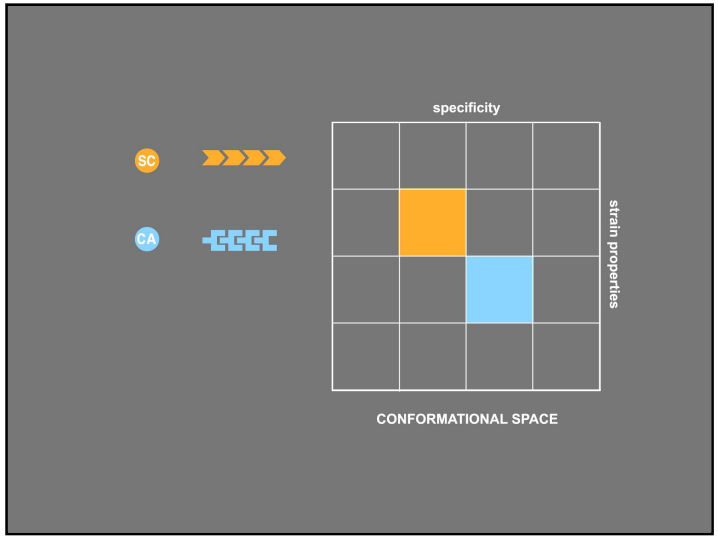


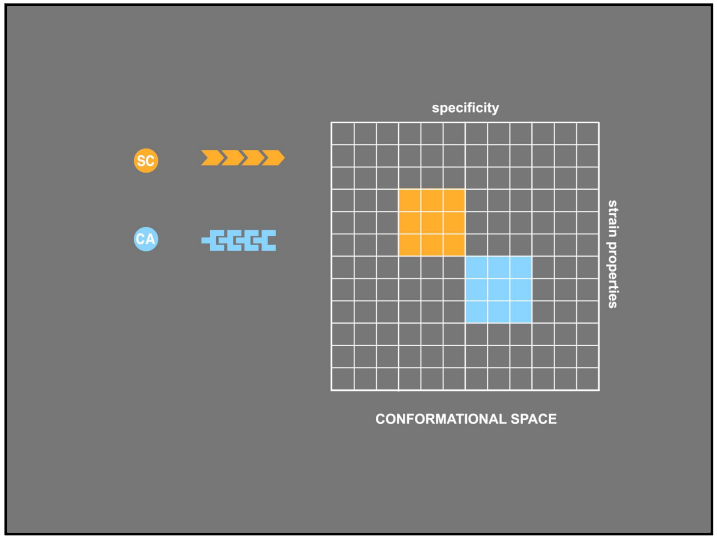

Prion Strains and Species Barriers Results from Misfolding into Multiple Distinct Forms
SC
CA
specificity
strain properties
CONFORMATIONAL SPACE

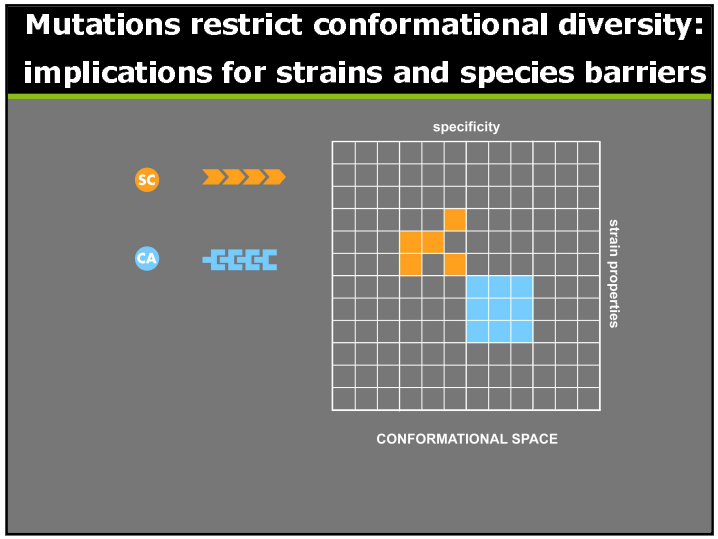

Prion Strains and Species Barriers Results from Misfolding into Multiple Distinct Forms
Ch
specificity
strain properties
CONFORMATIONAL SPACE

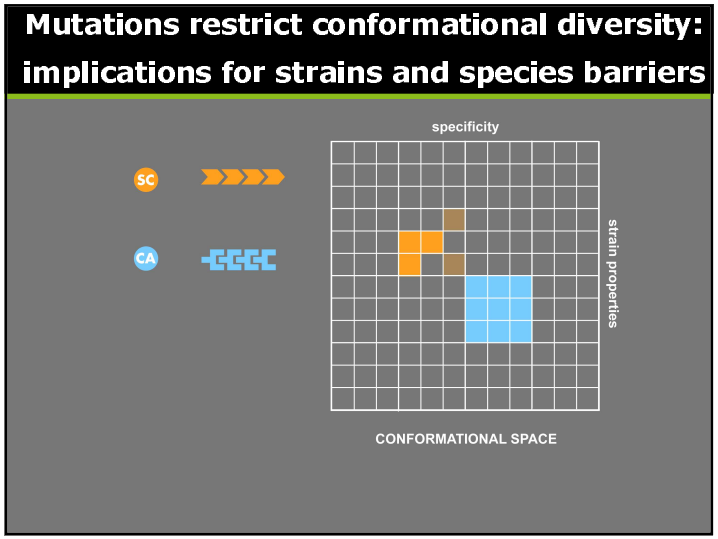

Prion Strains and Species Barriers Results from Misfolding into Multiple Distinct Forms
Ch
Δ
specificity
strain properties
CONFORMATIONAL SPACE

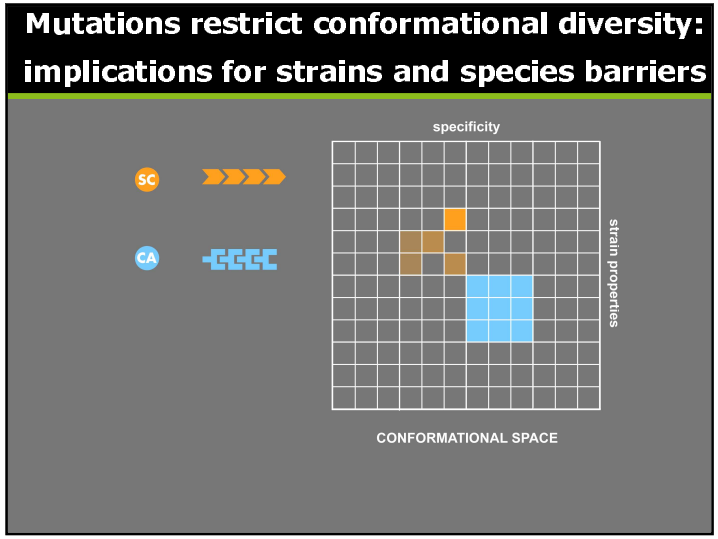

Mutational Control of Prion Strains and Species
Sc specific mutations alter Chimera's
strain spectrum
and species preference
% Strong ([Ca])
% Weak ([Sc])
Abundance of Strain
Inducer
Chim
ChimQ15R
ChimS17R
Percent Conversion
Ca
Sc
Recipient Sup35

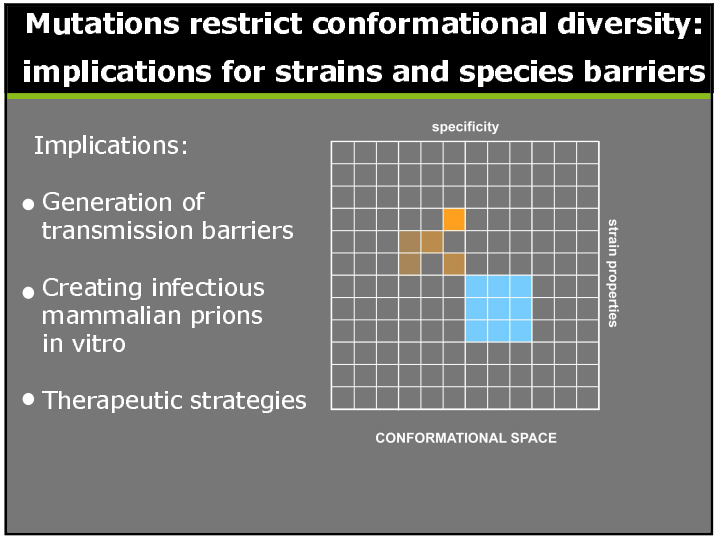

specificity
SC
CA
strain properties
CONFORMATIONAL SPACE

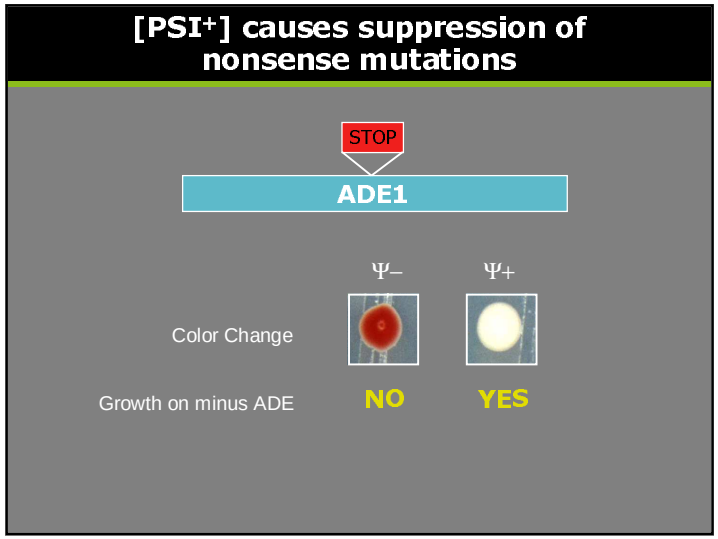

specificity
SC
CA
strain properties
CONFORMATIONAL SPACE

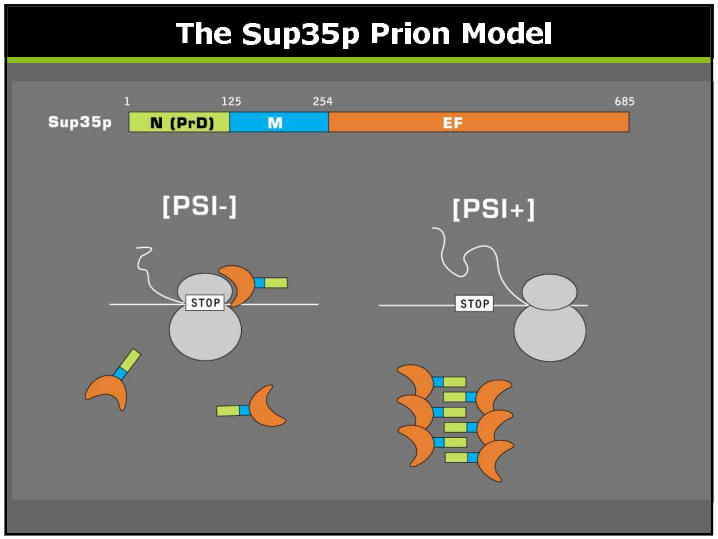

Mutations restrict conformational diversity:
implications for strains and species barriers
specificity
SC
CA
strain properties
CONFORMATIONAL SPACE


Mutations restrict conformational diversity:
implications for strains and species barriers
specificity
SC
CA
strain properties
CONFORMATIONAL SPACE

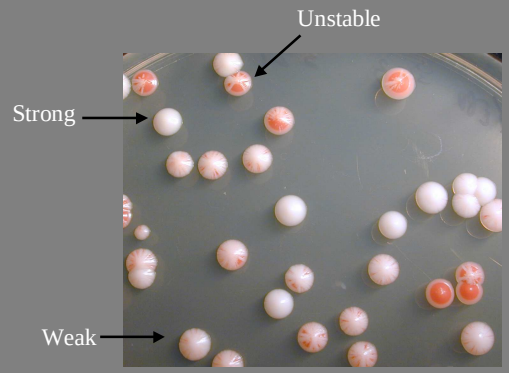

Mutations restrict conformational diversity: implications for strains and species barriers
specificity
SC
CA
strain properties
CONFORMATIONAL SPACE


Mutations restrict conformational diversity: implications for strains and species barriers
Implications:
• Generation of transmission barriers
• Creating infectious mammalian prions in vitro
• Therapeutic strategies
specificity
strain properties
CONFORMATIONAL SPACE

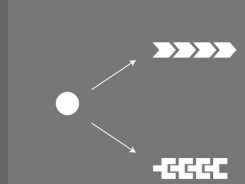

[PSI+] causes suppression of nonsense mutations
STOP
ADE1
Ψ–
Ψ+
Color Change
Growth on minus ADE
NO
YES


The Sup35p Prion Model
1
125
254
685
Sup35p
N (PrD)
M
EF
[PSI-]
[PSI+]
STOP
STOP

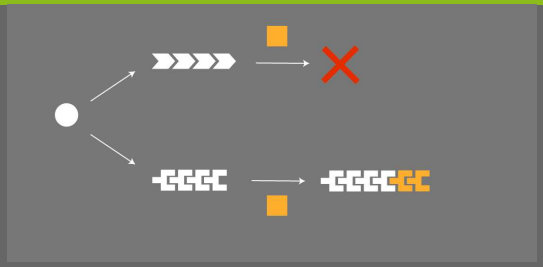
## Yeast Prions also Come in Different Strains




## Strains require the existence of multiple pathogenic conformations



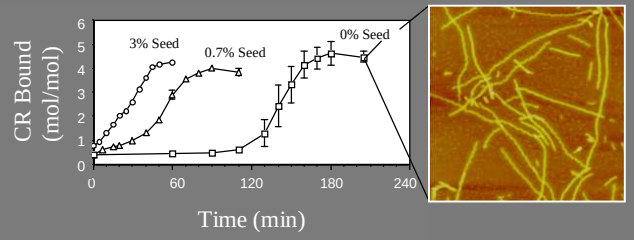
## Strains require the existence of multiple pathogenic conformations

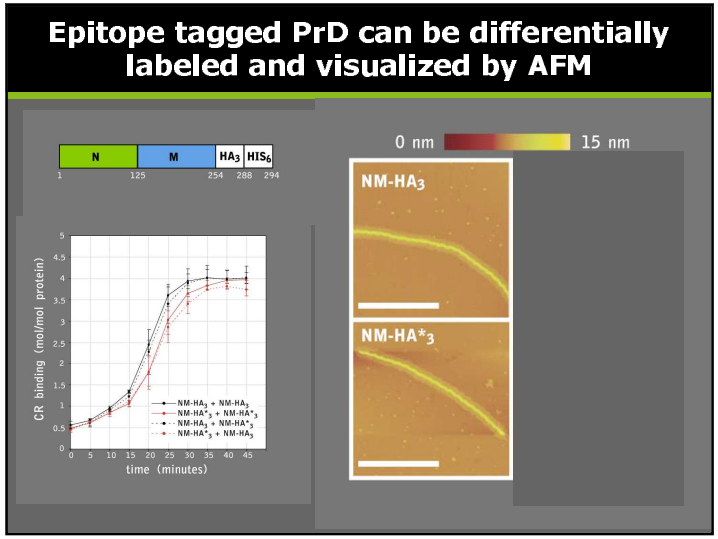


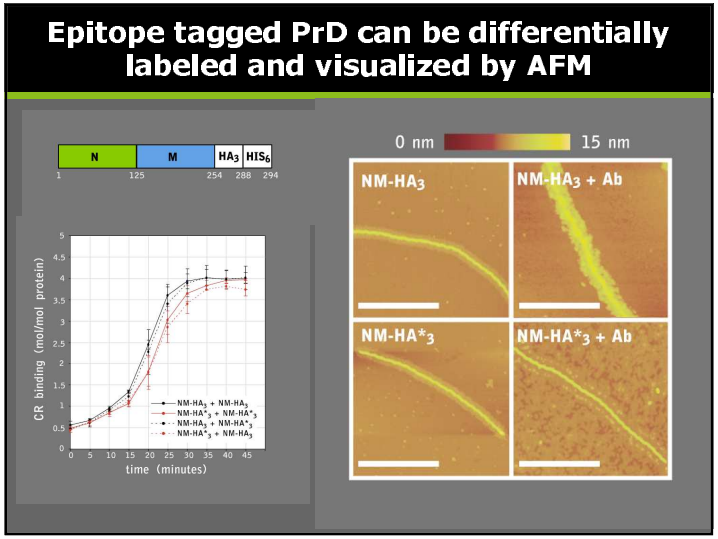
A single protein **can** exist in multiple distinct prion conformations

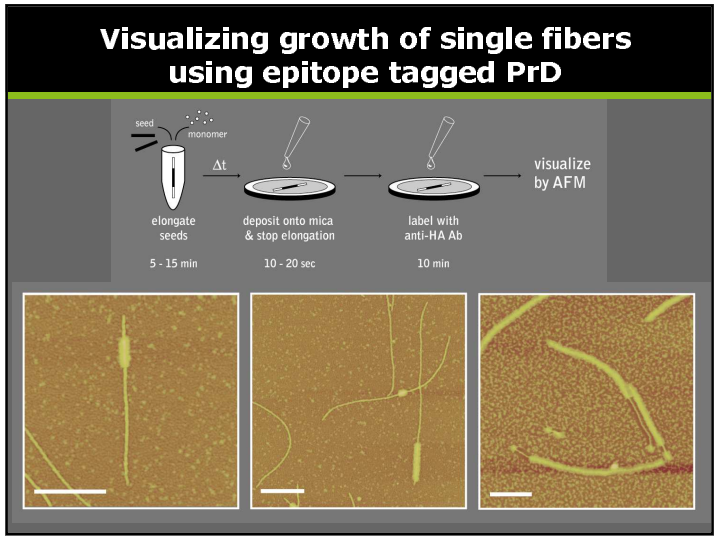
These conformations effect:
- species preference
- strain phenotype

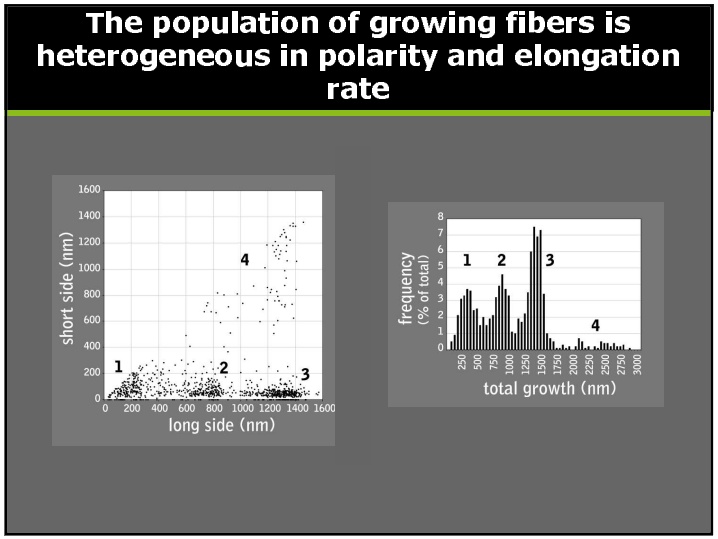
## Sup35p PrD Forms Self-Seeding Amyloids *in vitro*



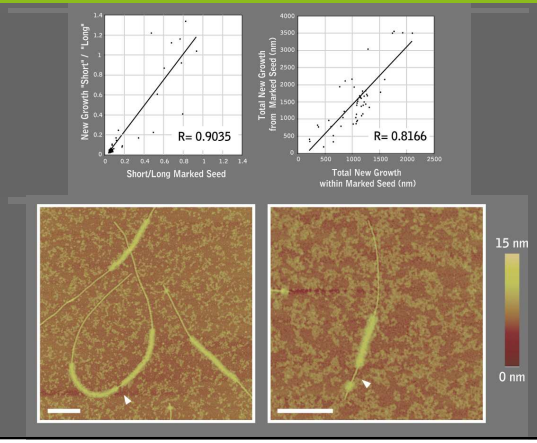

Epitope tagged PrD can be differentially labeled and visualized by AFM
N
M
HA3
HIS6
1
125
254
288
294
0 nm
15 nm
NM-HA3
NM-HA*3
CR binding (mol/mol protein)
time (minutes)


Epitope tagged PrD can be differentially labeled and visualized by AFM
N
M
HA3
HIS6
1
125
254
288
294
0 nm
15 nm
NM-HA3
NM-HA3 + Ab
NM-HA*3
NM-HA*3 + Ab
CR binding (mol/mol protein)
time (minutes)


Visualizing growth of single fibers using epitope tagged PrD
seed
monomer
Δt
visualize by AFM
elongate seeds
5 - 15 min
deposit onto mica & stop elongation
10 - 20 sec
label with anti-HA Ab
10 min

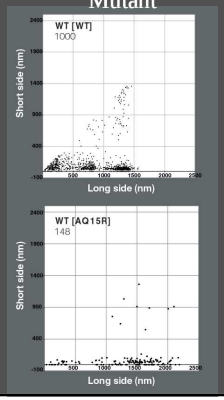

The population of growing fibers is heterogeneous in polarity and elongation rate
short side (nm)
long side (nm)
1
2
3
4
frequency (% of total)
total growth (nm)

## Individual fibers faithfully propagate polarity and elongation rate

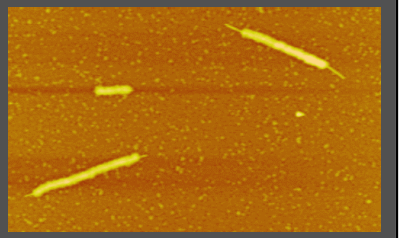



## Effects of Sup35 mutations on fiber strains

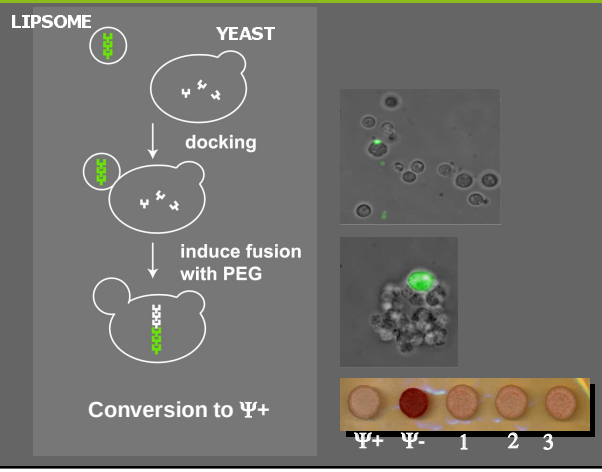
Enhanced Seeding Mutant




Curing Mutant (WT growth from mutant seed)



## Transformation by Liposome Fusion

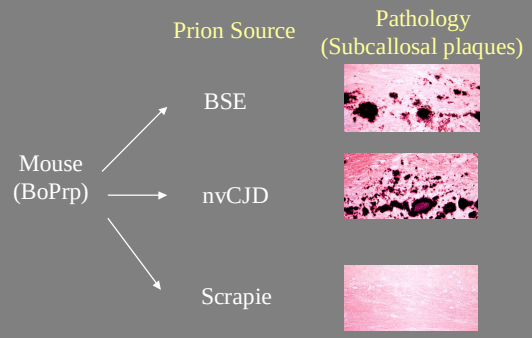






## Molecular Basis of Prion Strains

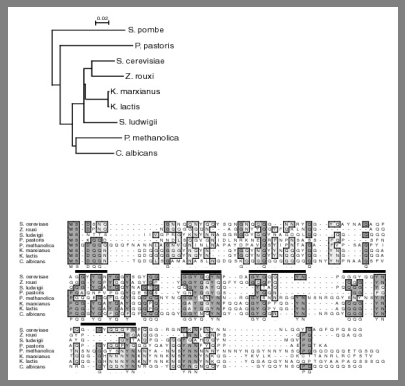
## BSE and nvCJD are the Same Strain: Humans are Susceptible to Mad Cow Disease



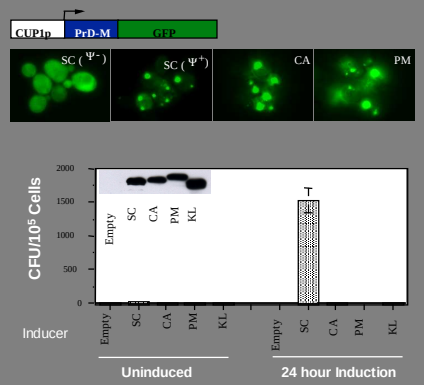

*Scott, Prusiner et al. PNAS 96(1999)

## Conservation and speciation in a yeast prion

## Sup35 PrD is Conserved in Budding Yeast Evolution

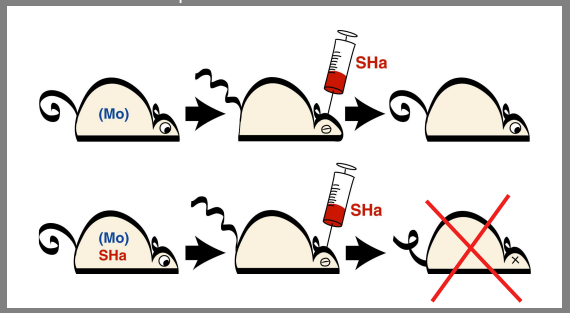



## Foreign Sup35s Aggregate.... But Fail to Convert S. cerevisiae to [PSI+]

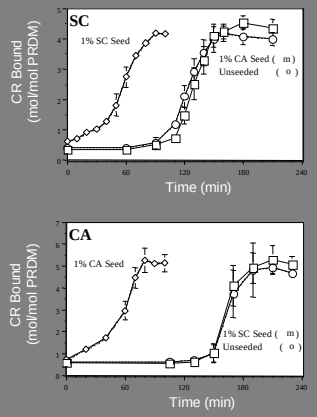


## Prion Protein Sequence Determines Species Barrier

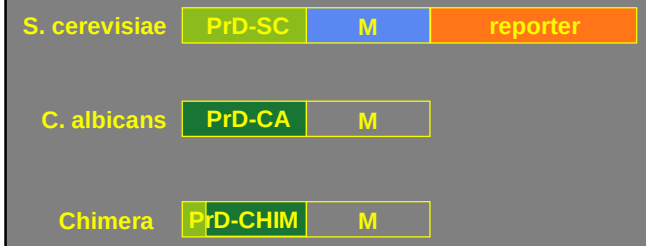
Mouse - Hamster Species Barrier



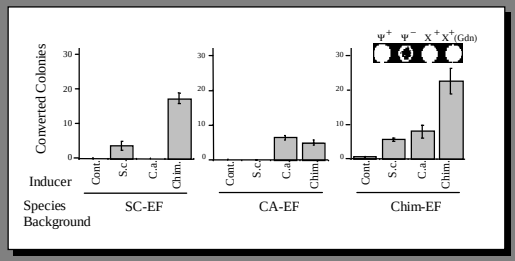

## The Species Barrier Can Be Recapitulated *in vitro* with Pure Protein



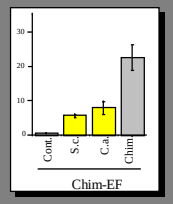

## A Chimeric Prion Domain



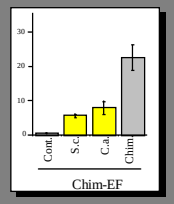

## The Chimera Bridges the Species Barrier

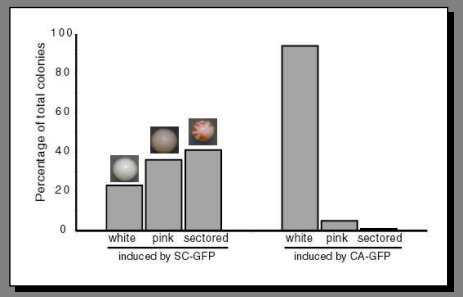


## Efficiency of Chim Prion Conversion is the Same for Both Pure Parent Species. But...

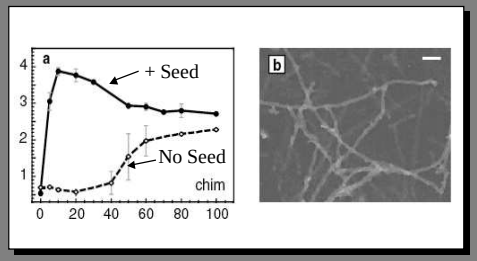



## ..."Infecting" Species Determines Strain Properties of Chim Prion




## Chimera forms self-seeding fibers *in vitro*




## Prion strains *in vitro*

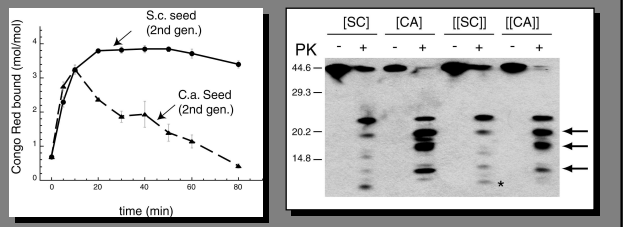
X → chim → chim[X] (first generation) → chim → chim[[X]] (second generation)

X = SC or CA

## Prion strains *in vitro*

X → chim → chim[X] (first generation) → chim → chim[[X]] (second generation)

X = SC or CA





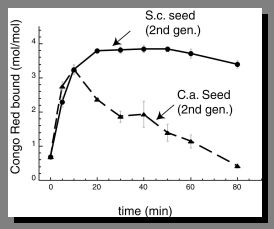


Chimera monomer

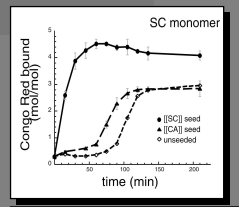
## Prion strains determine species preference

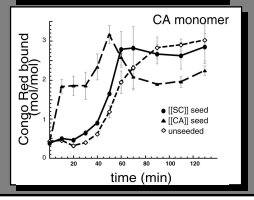
X → chim → chim[X] (first generation) → chim → chim[[X]] (second generation) → SC or CA (strain preference)

X = SC or CA




Chimera monomer



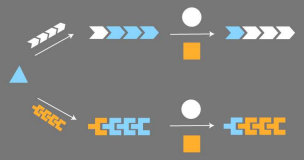

## Prion Strain Conformations Act in Concert with the Primary Structure to Determine Transmission Barrier

INTACT SPECIES BARRIER

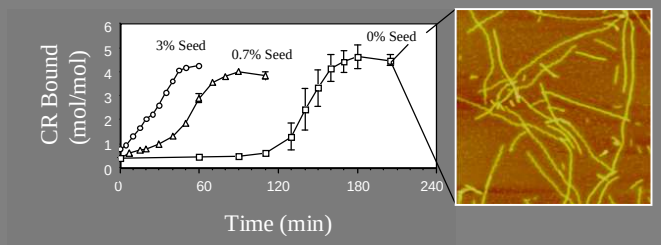

## Prion Strain Conformations Act in Concert with the Primary Structure to Determine Transmission Barrier

INTACT SPECIES BARRIER


BYPASSED SPECIES BARRIER



## Do different prion strain conformations form spontaneously?



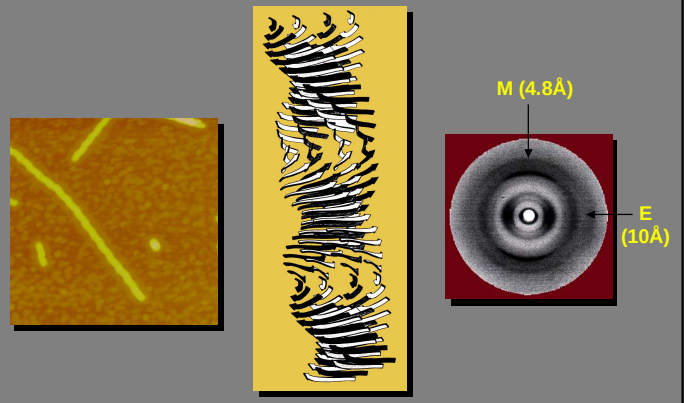

## Summary

- An epigenetic switch: Prions as a novel and conserved mechanism of regulating protein function
- Molecular basis of a yeast prion species barrier
- A prion's conformation (strain) plays a critical role in determining its ability to be transmitted across a species barrier
- Identified a novel yeast prion [$NU^{+}$] which acts as a general regulator of protein aggregation

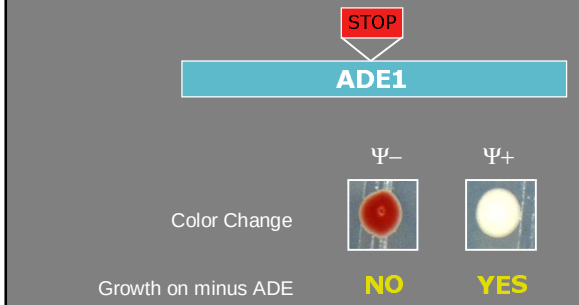
## Acknowledgments


- **Prion Strains:** Peter Chien
- **Prion Mutants Screen:** Angela DePace and Alex Santoso
- **Single Fiber Assay:** Angela DePace
- **Species Barrier:** Alex Santoso and Peter Chien
- **Protein Infection:** Helmut Sparrer
- **New Prions:** Lev Osherovich and Melissa Michelitsch

## Amyloids Are Ordered Protein Aggregates




## [PSI+] causes suppression of nonsense mutations



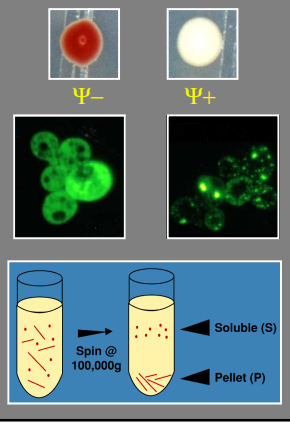

## Assays for [PSI+]

1. Color Change

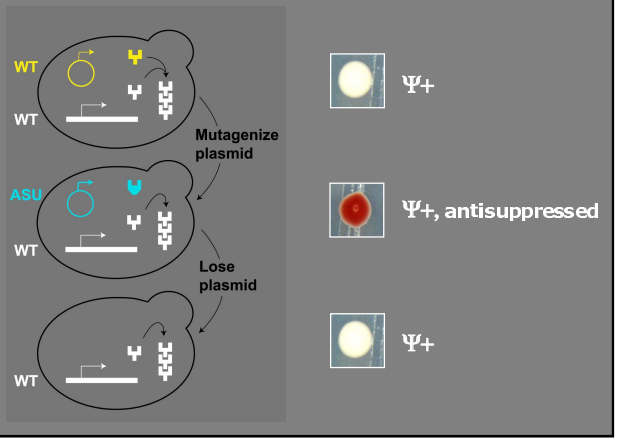
2. GFP

PrD M GFP

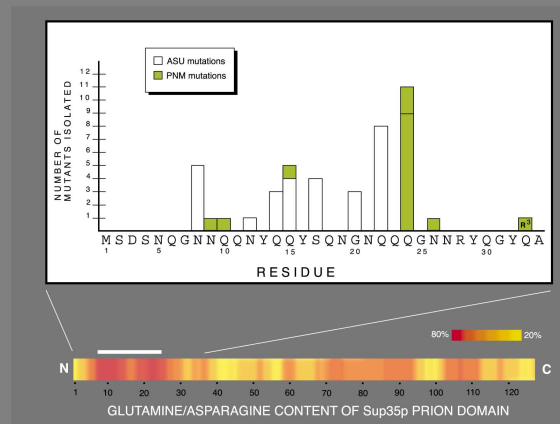
3. Spin Down



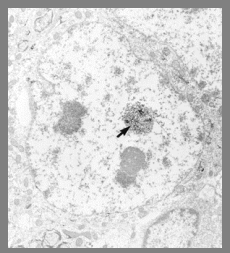

## A Screen for AntiSUppressor (ASU) Mutants



## Sup35 PrD Mutants Cluster to a Gln/Asn Rich Region




## CAG repeats (polyGln) cause protein aggregation and neurodegeneration

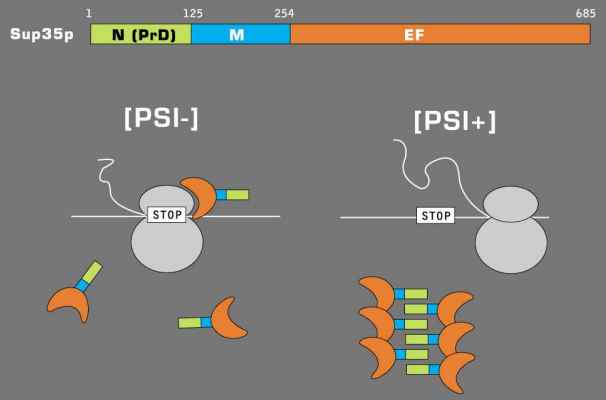


* Immuno-EM Showing Huntingtin Nuclear aggregates

- Nuclear aggregates are a common feature CAG-repeat diseases
- Expression of protein fragments containing Gln repeats leads to nuclear aggregates and Neurodegeneration in Mice Worms, and Drosophila
- Poly Gln-repeats form amyloids in vitro (as predicted by Perutz)
- Poly Gln aggregates in prion-like manner in yeast

*Bates et al. Cell 90, pp537.

## The Sup35p Prion Model




## ASU Mutants Show Slowed Kinetics of Amyloid Formation *in vitro*