Conformational Variability – Experience with Ribosomes

Exploration of reconstruction strategy "High-resolution project"

Use small dataset (50,000) to optimize processing, with the idea to switch to larger dataset (130,000)

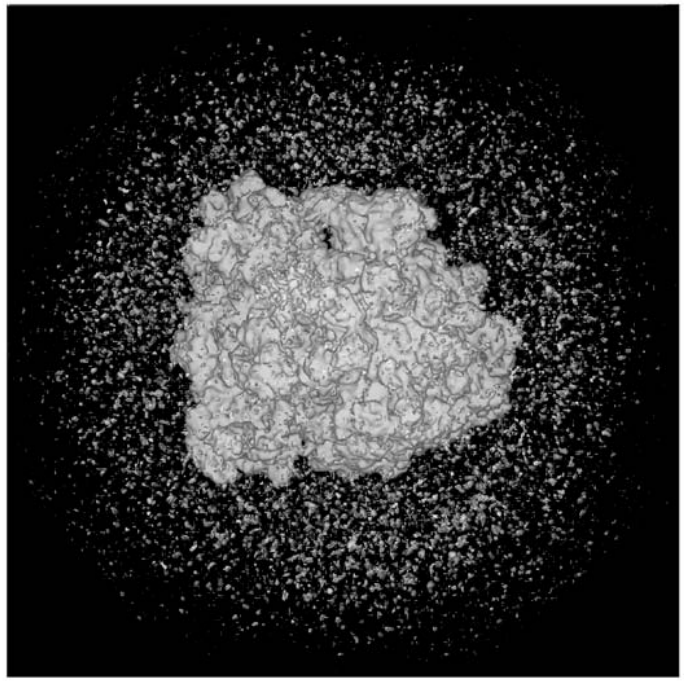
Parameters of image processing:

- Sampling (switch from coarse to fine)
- Window size (to avoid CTF effects)
- Angular spacing
- Amplitude correction in each step of refinement vs. at the very end

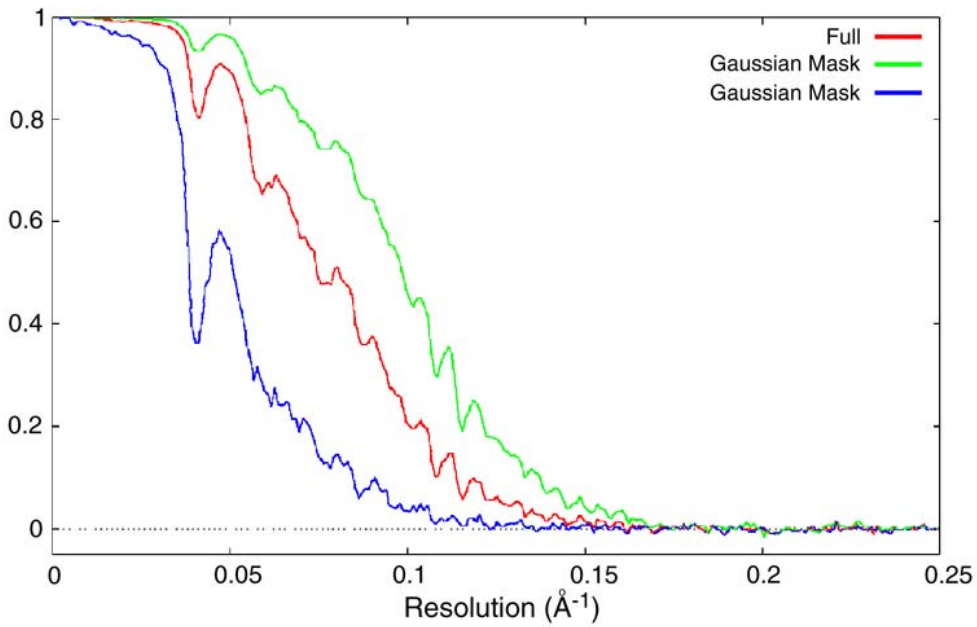
Final parameters:
angular step 0.5 degrees,
angular search range 2 degrees
7 iterations of refinement: 920 hours on a 48-node cluster
Regular window size OK
Sampling (decimation) can be switched mid-way from coarse to fine

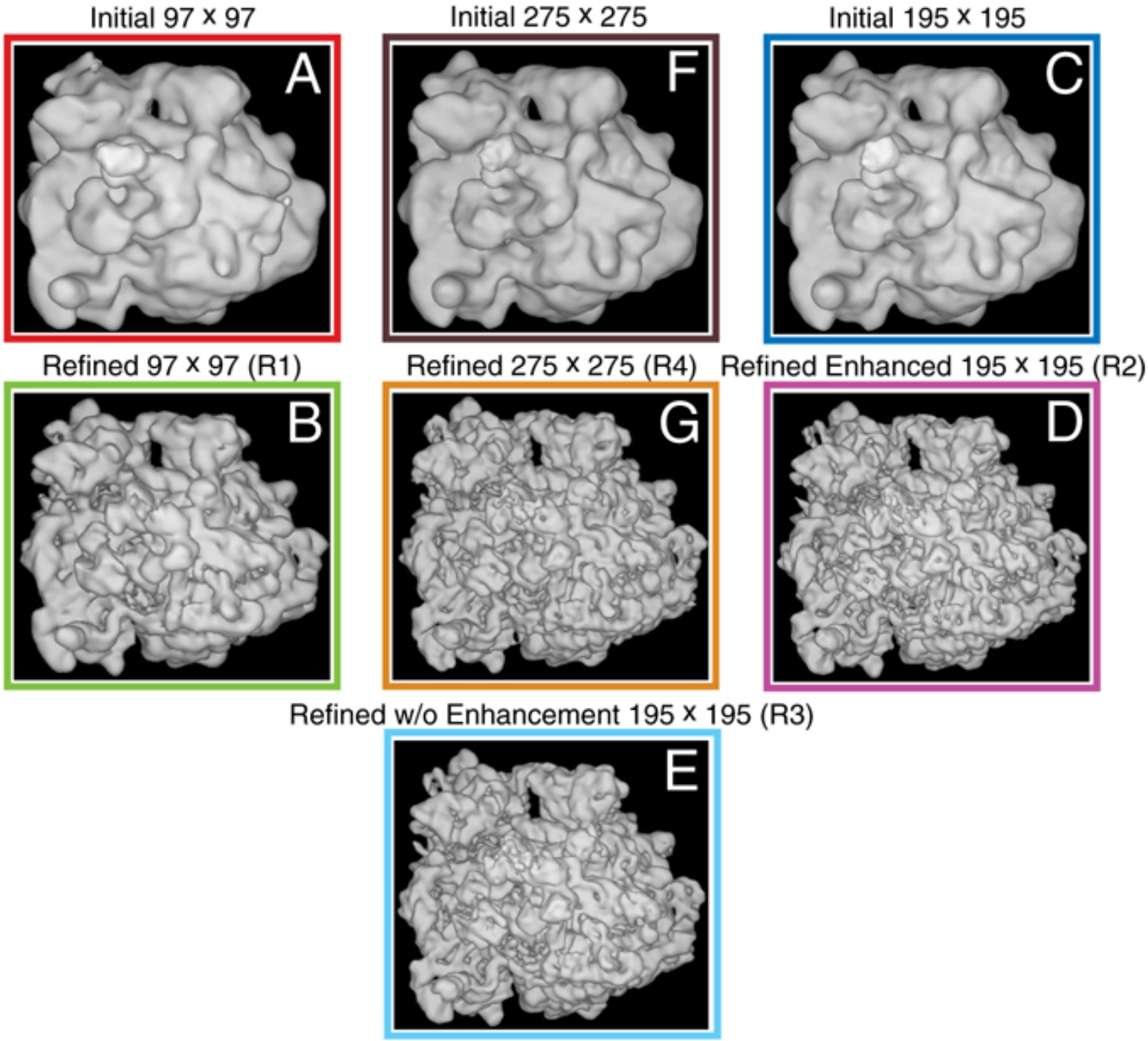
Resolution measurement issues

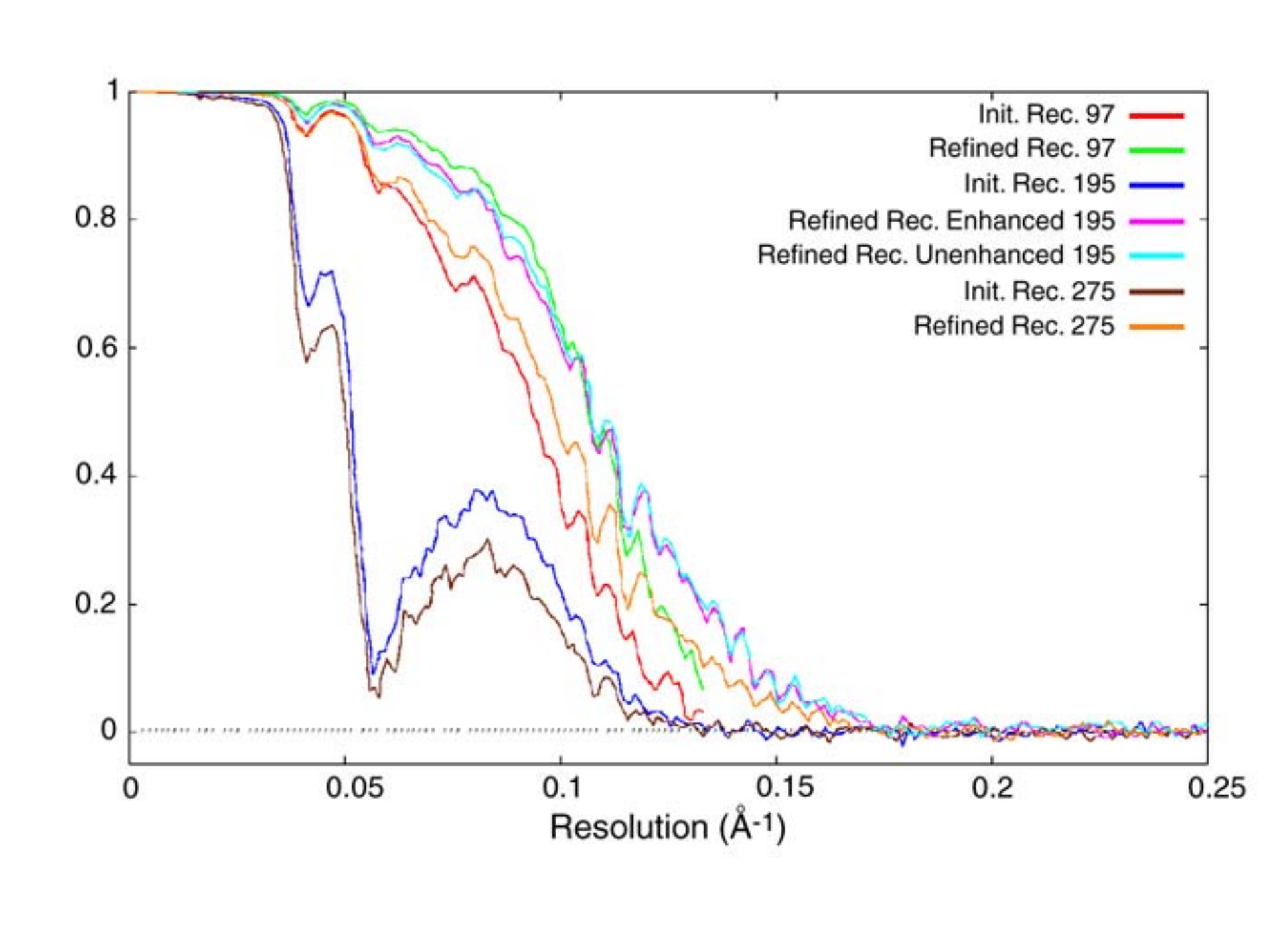
- Apply soft mask to reconstruction to get true resolution!
- Evidence for dependence of resolution *R vs. log(N)*
- Is lin-log dependence general?
- Is it allowed to extrapolate from half to full dataset?

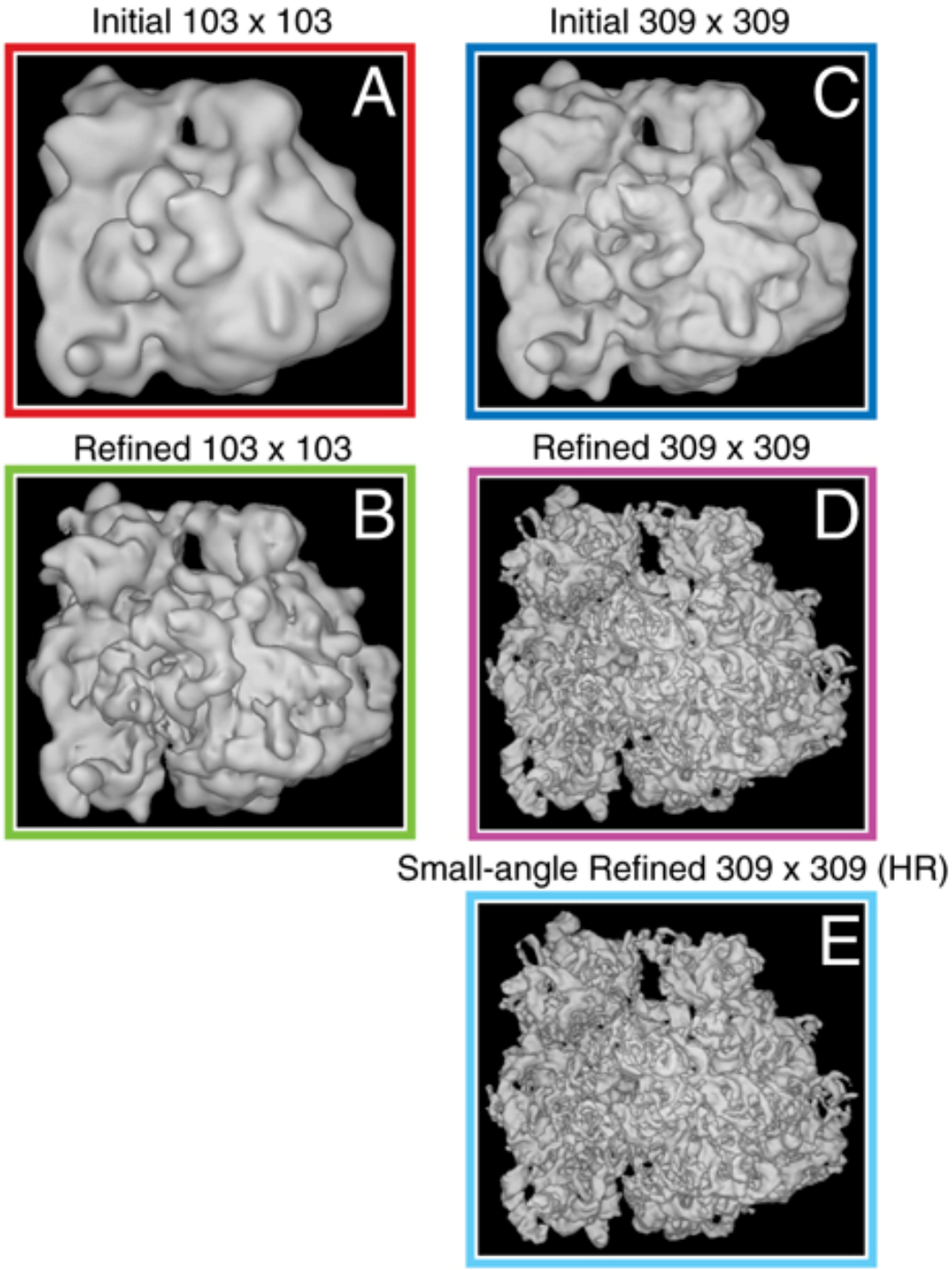


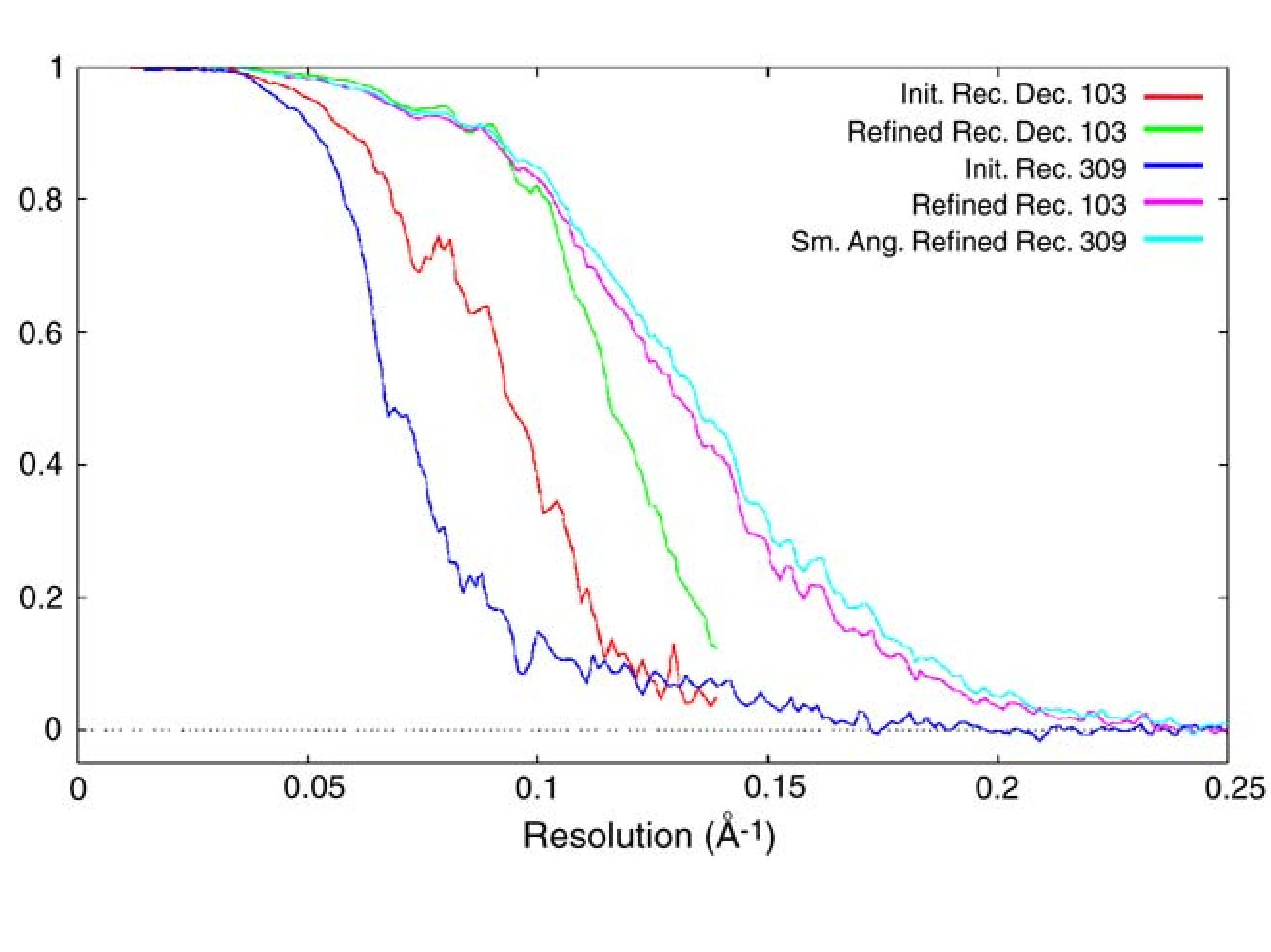
"Clutter"

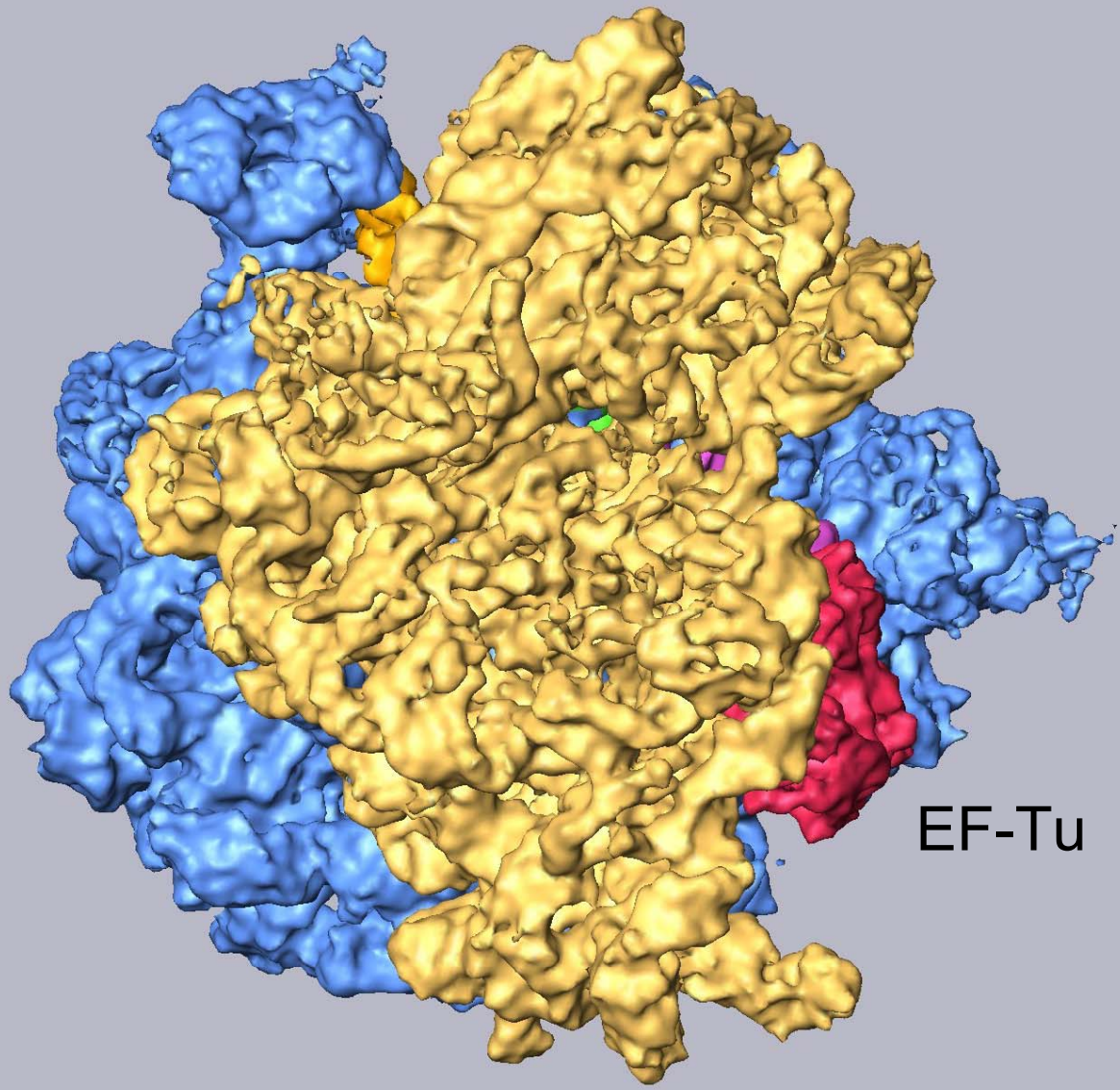

Full
Gaussian Mask
Gaussian Mask
1
0.8
0.6
0.4
0.2
0
0
0.05
0.1
0.15
0.2
0.25
Resolution (Å^{-1})


Initial 97 x 97
A
Initial 275 x 275
F
Initial 195 x 195
C
Refined 97 x 97 (R1)
B
Refined 275 x 275 (R4)
G
Refined Enhanced 195 x 195 (R2)
D
Refined w/o Enhancement 195 x 195 (R3)
E


Init. Rec. 97
Refined Rec. 97
Init. Rec. 195
Refined Rec. Enhanced 195
Refined Rec. Unenhanced 195
Init. Rec. 275
Refined Rec. 275
1
0.8
0.6
0.4
0.2
0
0
0.05
0.1
0.15
0.2
0.25
Resolution (Å-1)

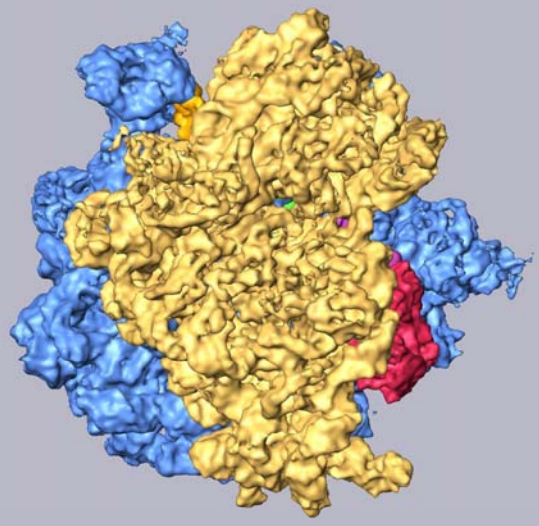

Initial 103 x 103
A
Initial 309 x 309
C
Refined 103 x 103
B
Refined 309 x 309
D
Small-angle Refined 309 x 309 (HR)
E

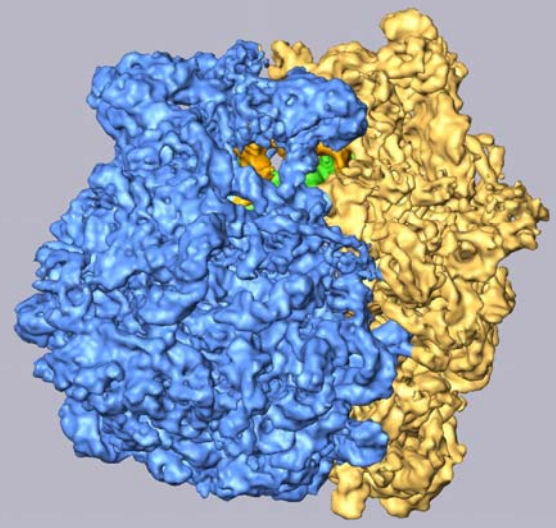

Init. Rec. Dec. 103
Refined Rec. Dec. 103
Init. Rec. 309
Refined Rec. 103
Sm. Ang. Refined Rec. 309
1
0.8
0.6
0.4
0.2
0
0
0.05
0.1
0.15
0.2
0.25
Resolution (Å-1)

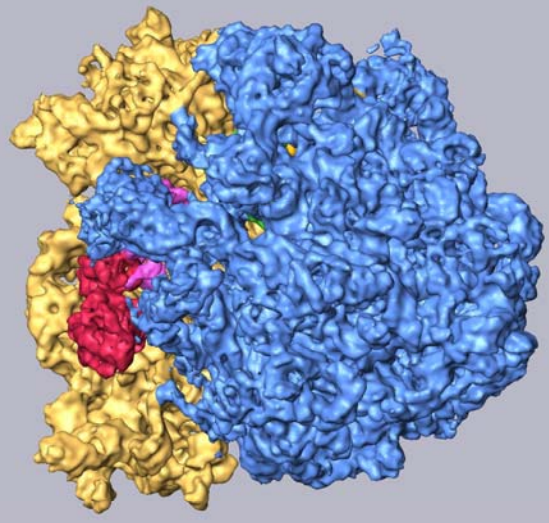


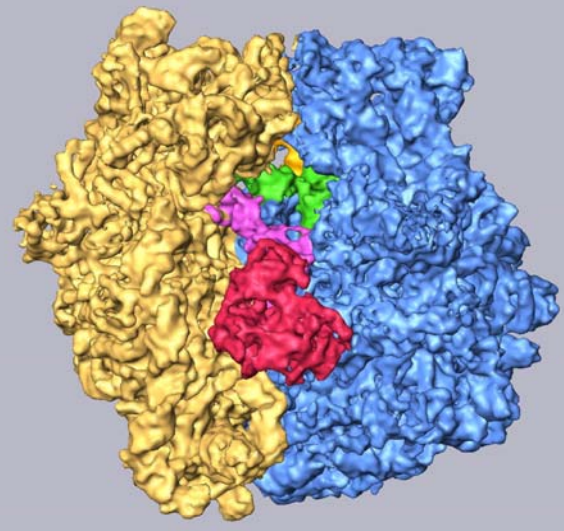
E. coli 70S•aa-tRNA•EF-Tu•GDP•kir at 7.5 Å

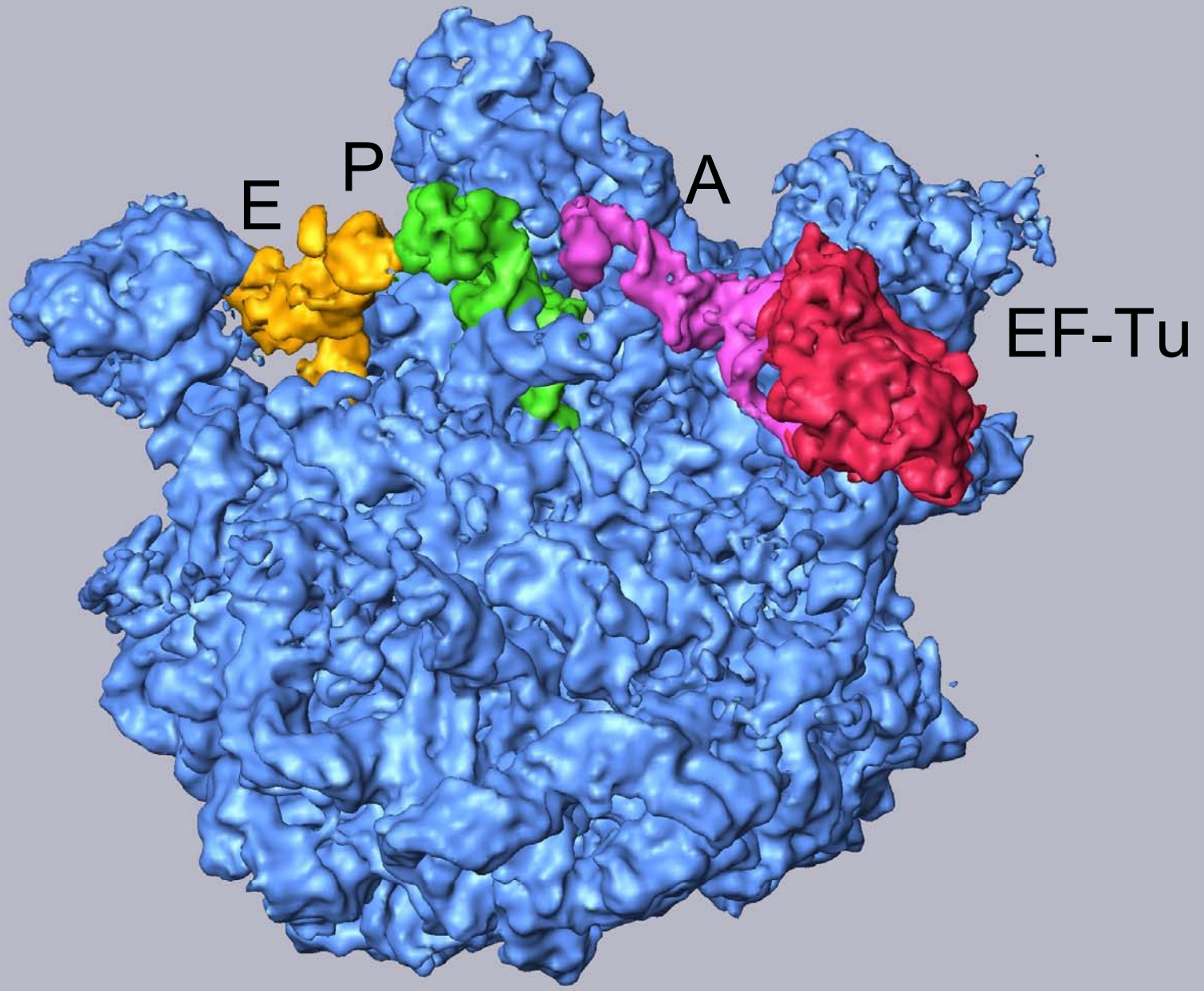
130,000 particles 7.5 Å (FSC=0.5)

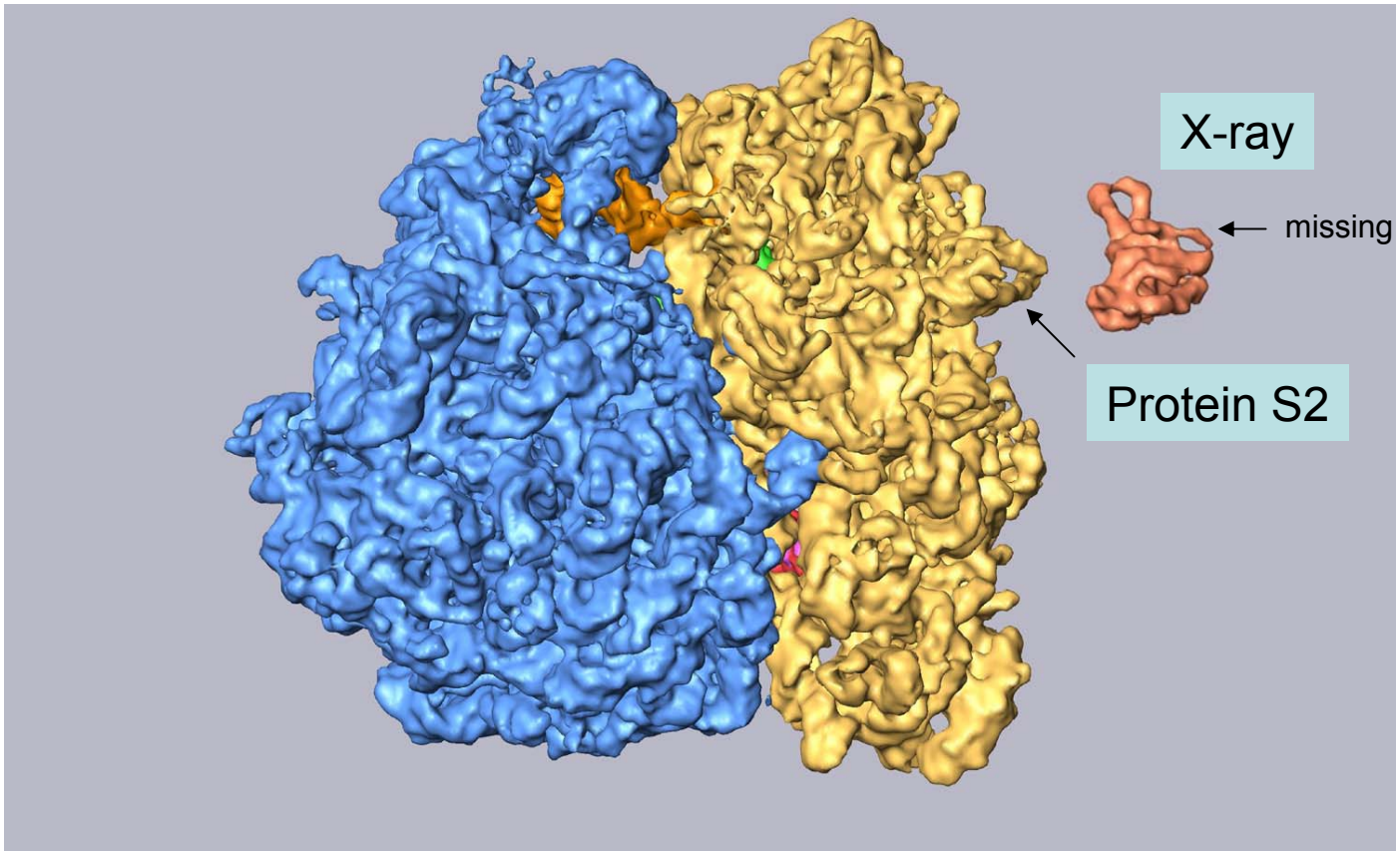




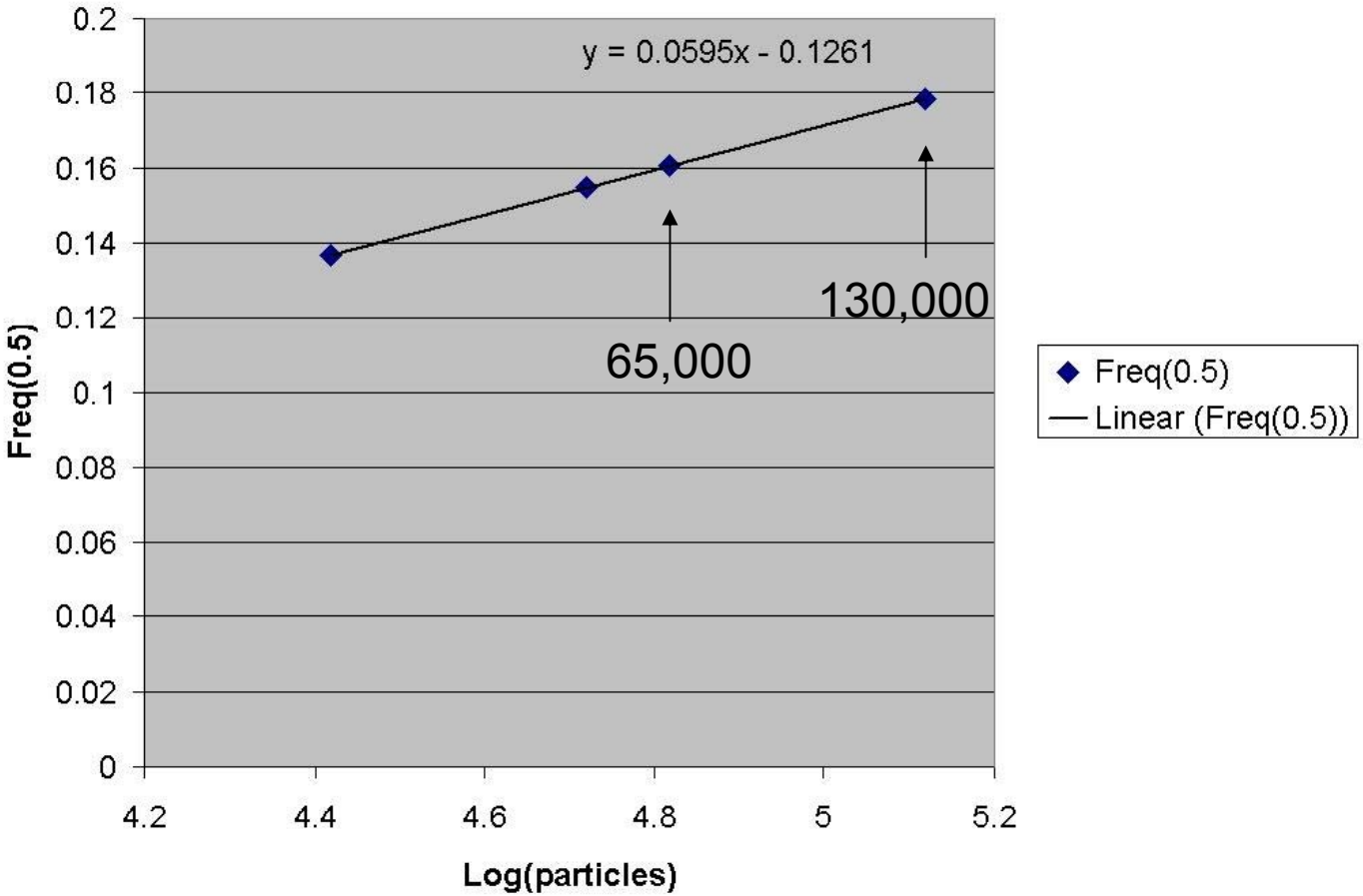


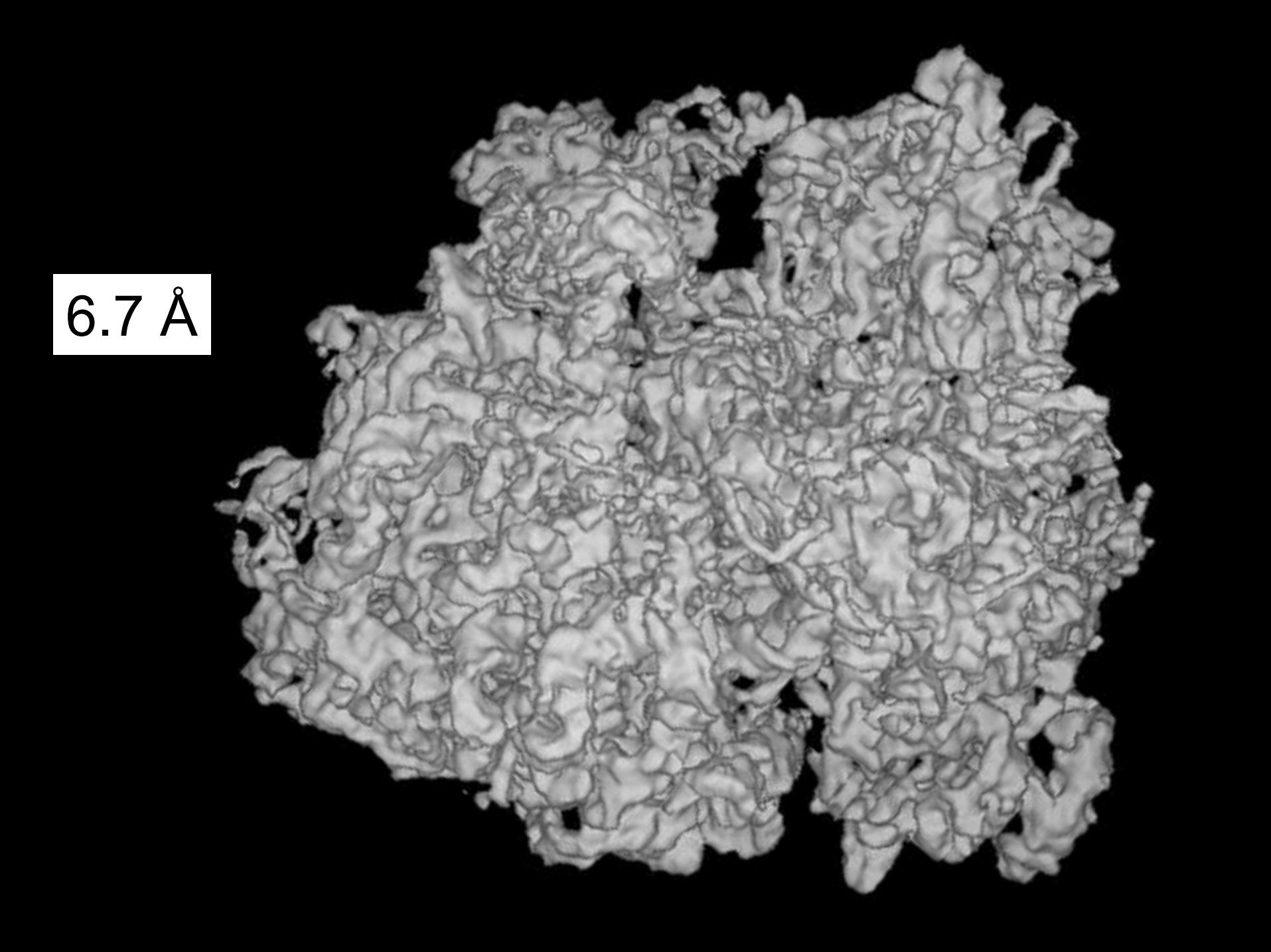


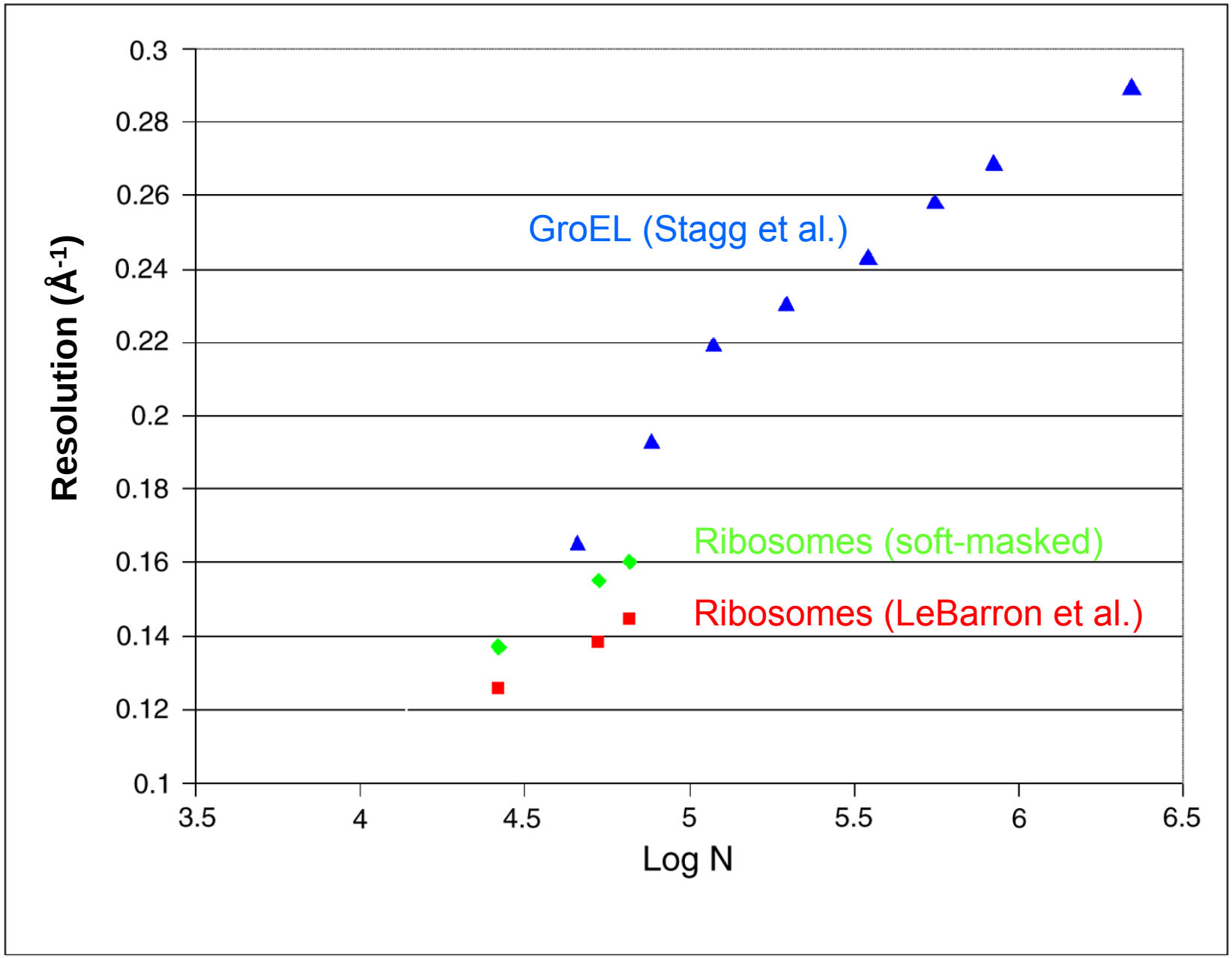

E
P
A
EF-Tu

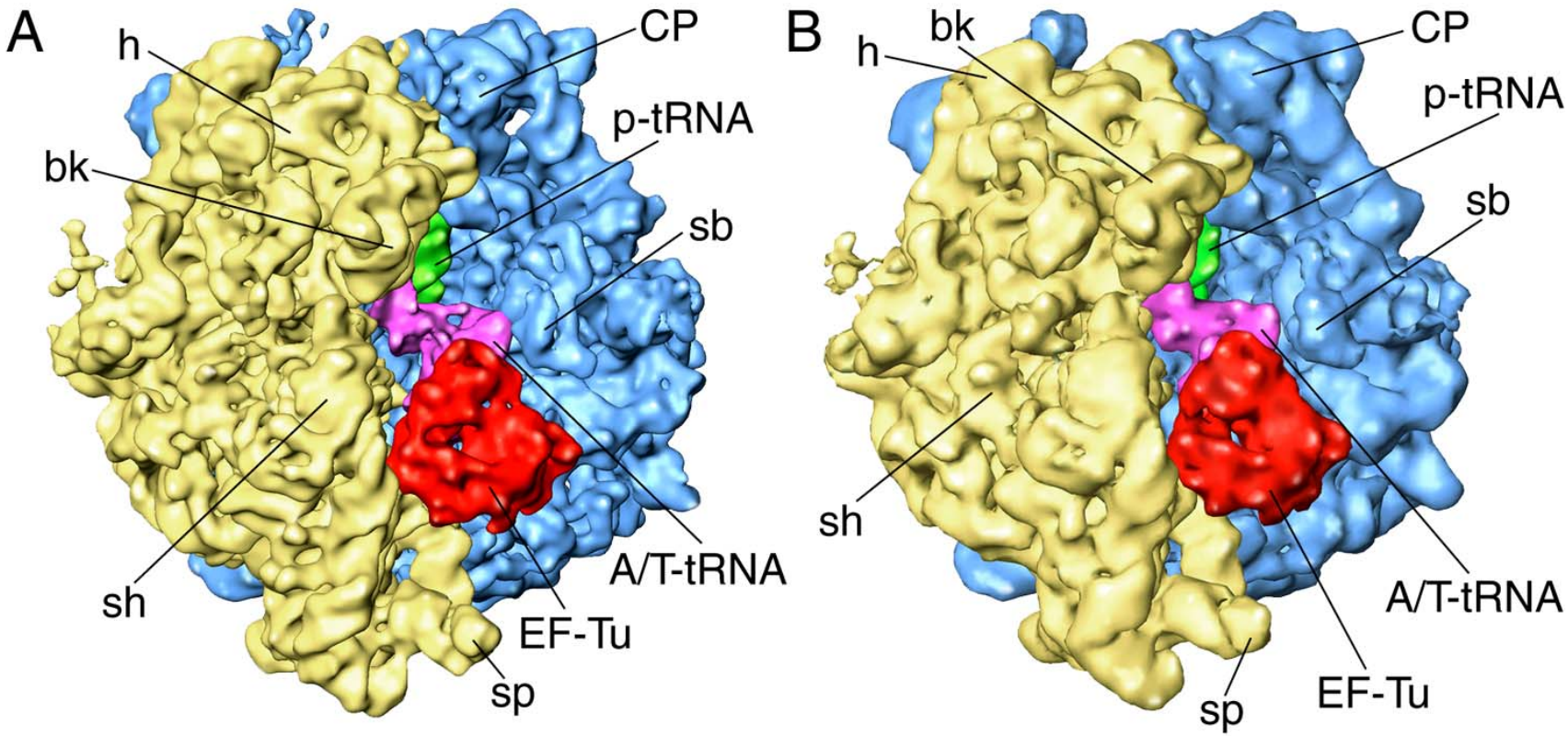

X-ray
missing helix
Protein S2

Extrapolation of FSC resolution to full set



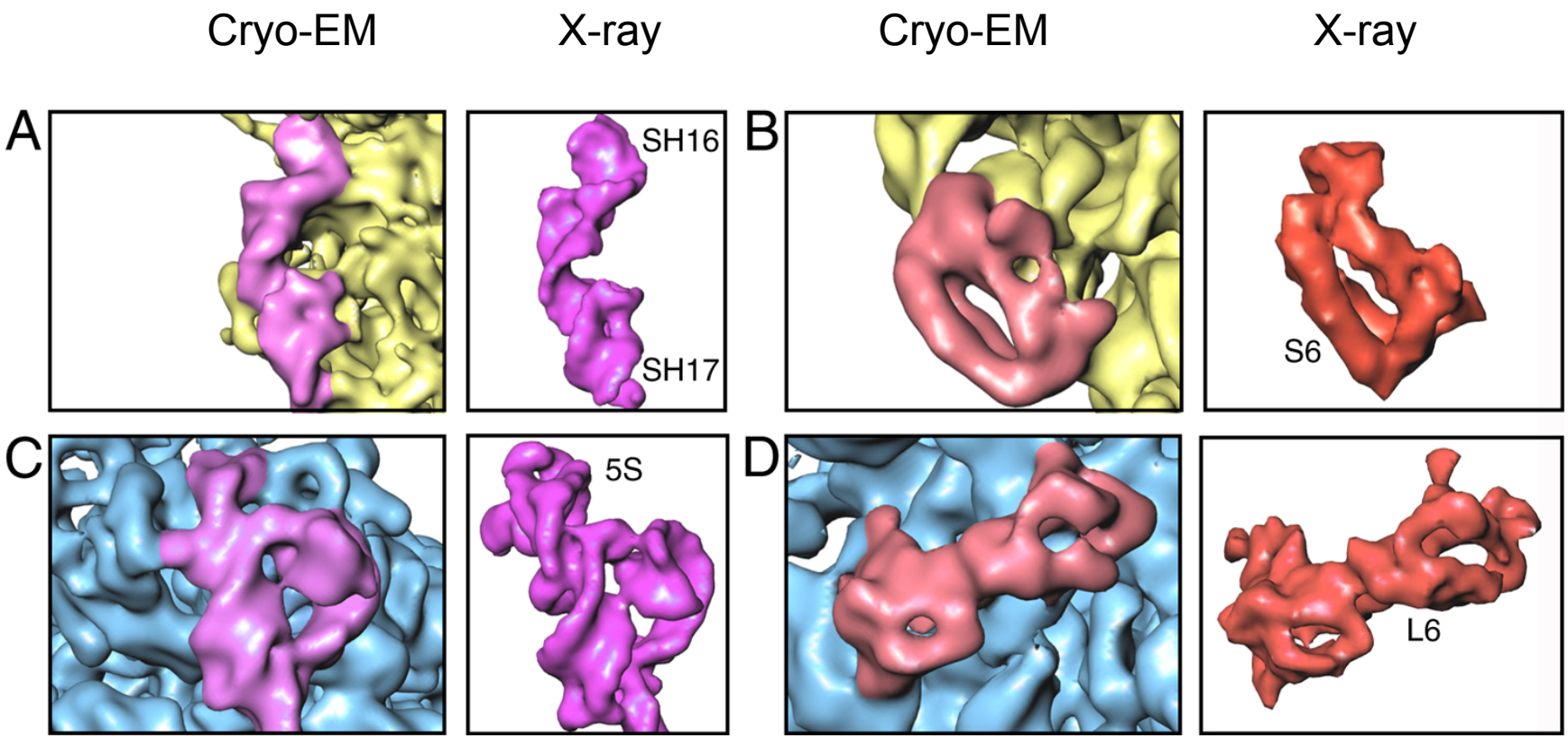

6.7 Å

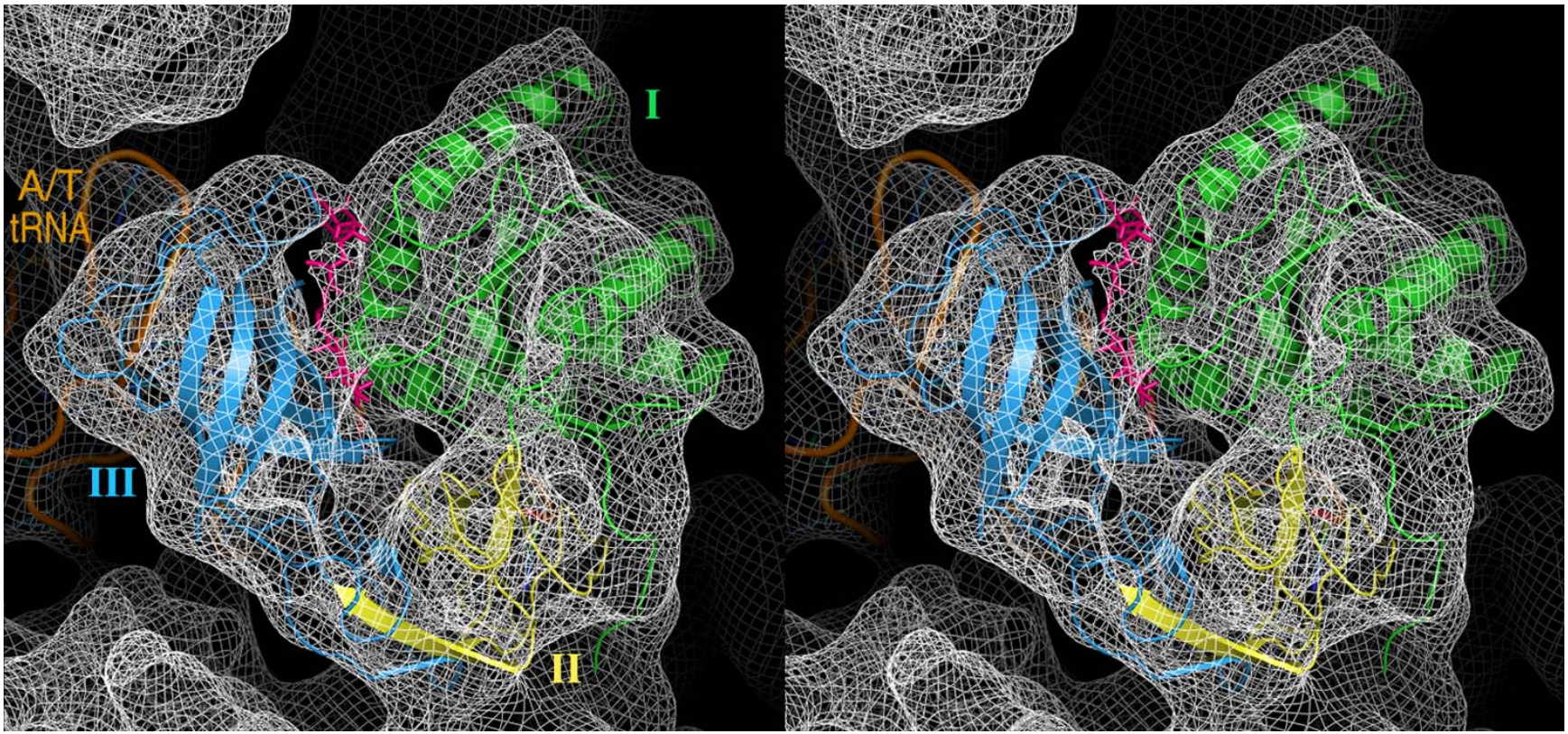

Resolution (Å-1)
0.3
0.28
0.26
0.24
0.22
0.2
0.18
0.16
0.14
0.12
0.1
3.5
4
4.5
5
5.5
6
6.5
Log N
GroEL (Stagg et al.)
Ribosomes (soft-masked)
Ribosomes (LeBarron et al.)



6.7 Å (LeBarron et al., in prep.)

10 Å (Valle et al., NSB 2003)


Cryo-EM
X-ray
Cryo-EM
X-ray
A
SH16
SH17
B
S6
C
5S
D
L6



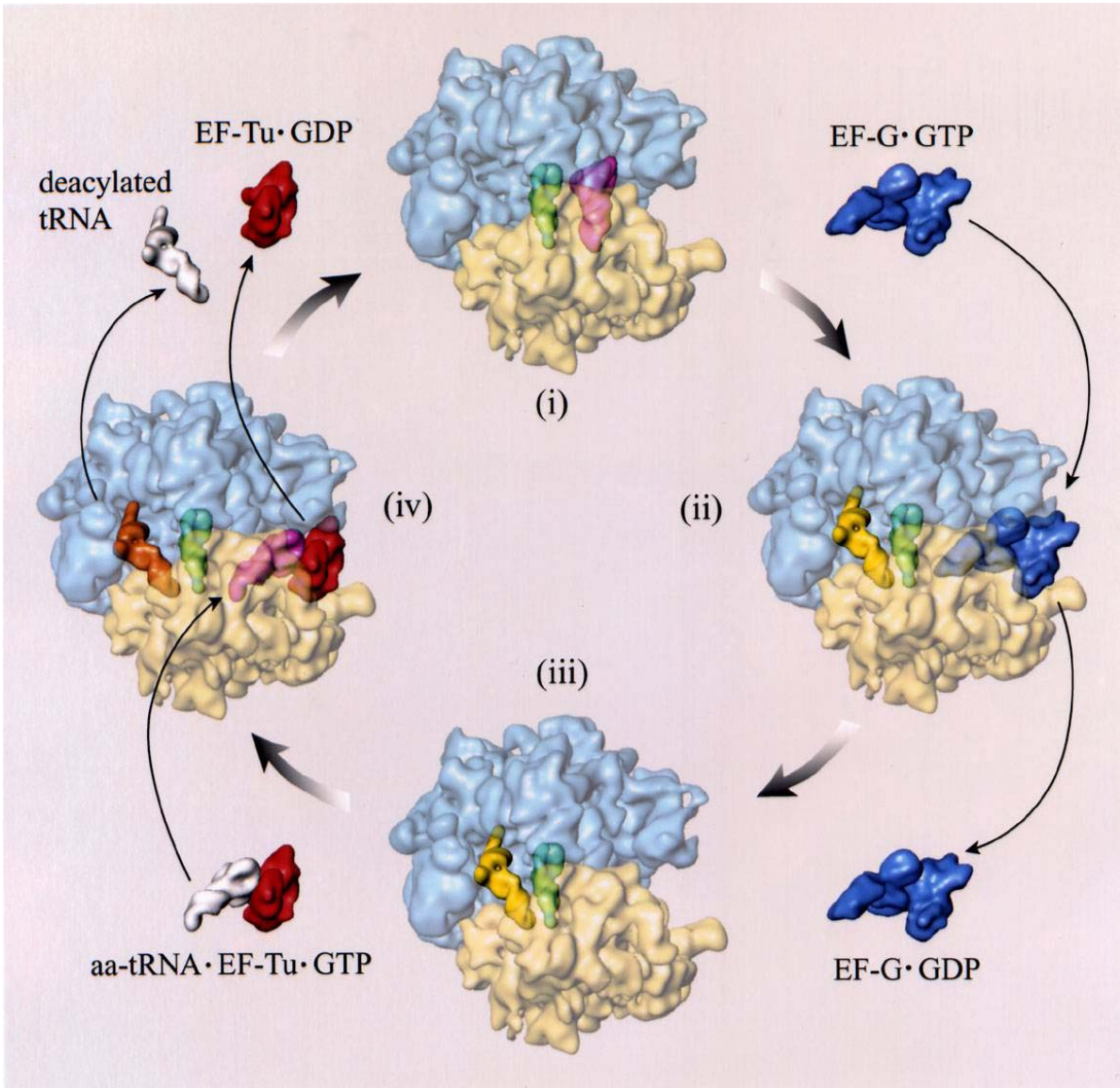
Definition of EF-Tu domains

Elongation Cycle

Animation

decoding

kirromycin
GDPNP



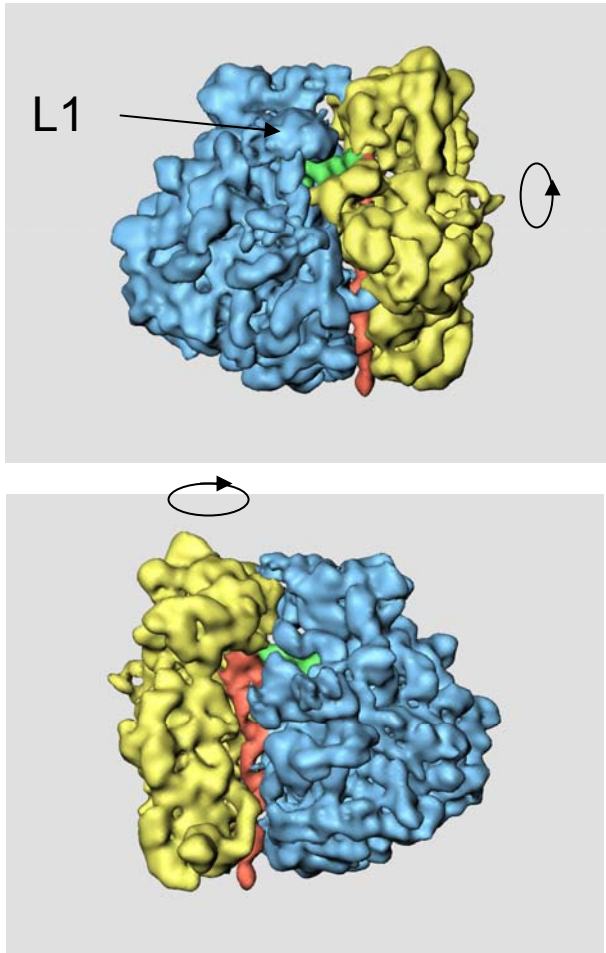
translocation

fusidic acid
thiostrepton
GDPNP

Dynamics of Translation

- We draw inferences about movements by comparing EM maps in different states.
- To what extent are such inferences supported by other data?

- L1 stalk move → X-ray
- Small subunit head rotation → X-ray
- Ratchet motion in translocation → smFRET
- tRNA selection → smFRET



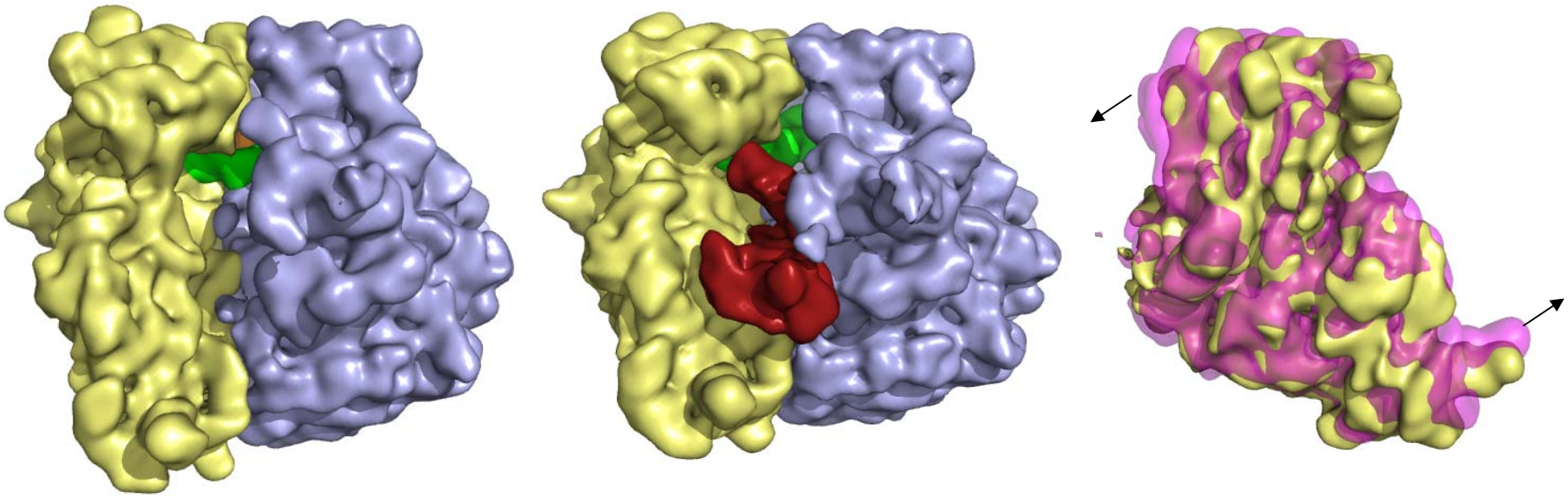
Ratchet motion induced by EF-G binding

- Cryo-EM: (1) differences between conformations in two different states

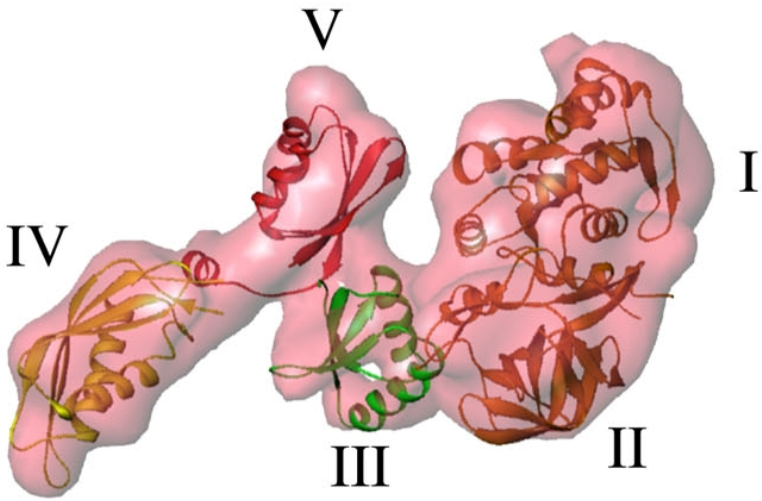
  (2) evidence of conformational variability -- coexistence of different conformations in the specimen (blurring, 3D variance)
- Hydroxyl radical probing: changes of Pb^{2+} – induced rRNA cleavage pattern along elongation cycle (Polacek et al., 2000)
- Bulk FRET (Ermolenko et al., 2006)
- Single-molecule FRET (Cornish et al., 2007)

EF-G/eEF2 binding induces ratcheting of the small subunit

70S-EF-G

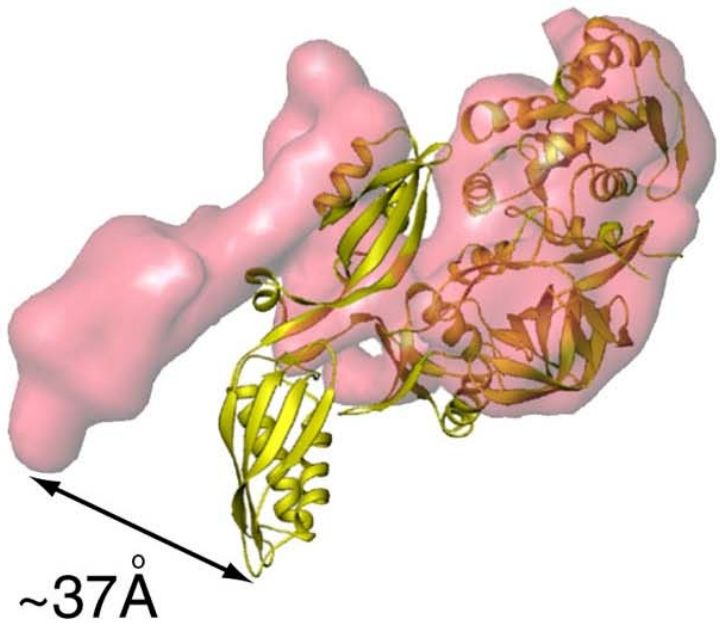


Agrawal *et al*. (1999) *Nat. Str. Biol.* **6**:643-7 and Valle *et al.* (2003) *Cell* **114**: 123-134



X-ray structure of EF-G•GDP, domains III, IV, V rotated

"Induced fit" – both ribosome and EF-G undergo structural changes, such that a match of binding sites is achieved



X-ray structure of EF-G•GDP

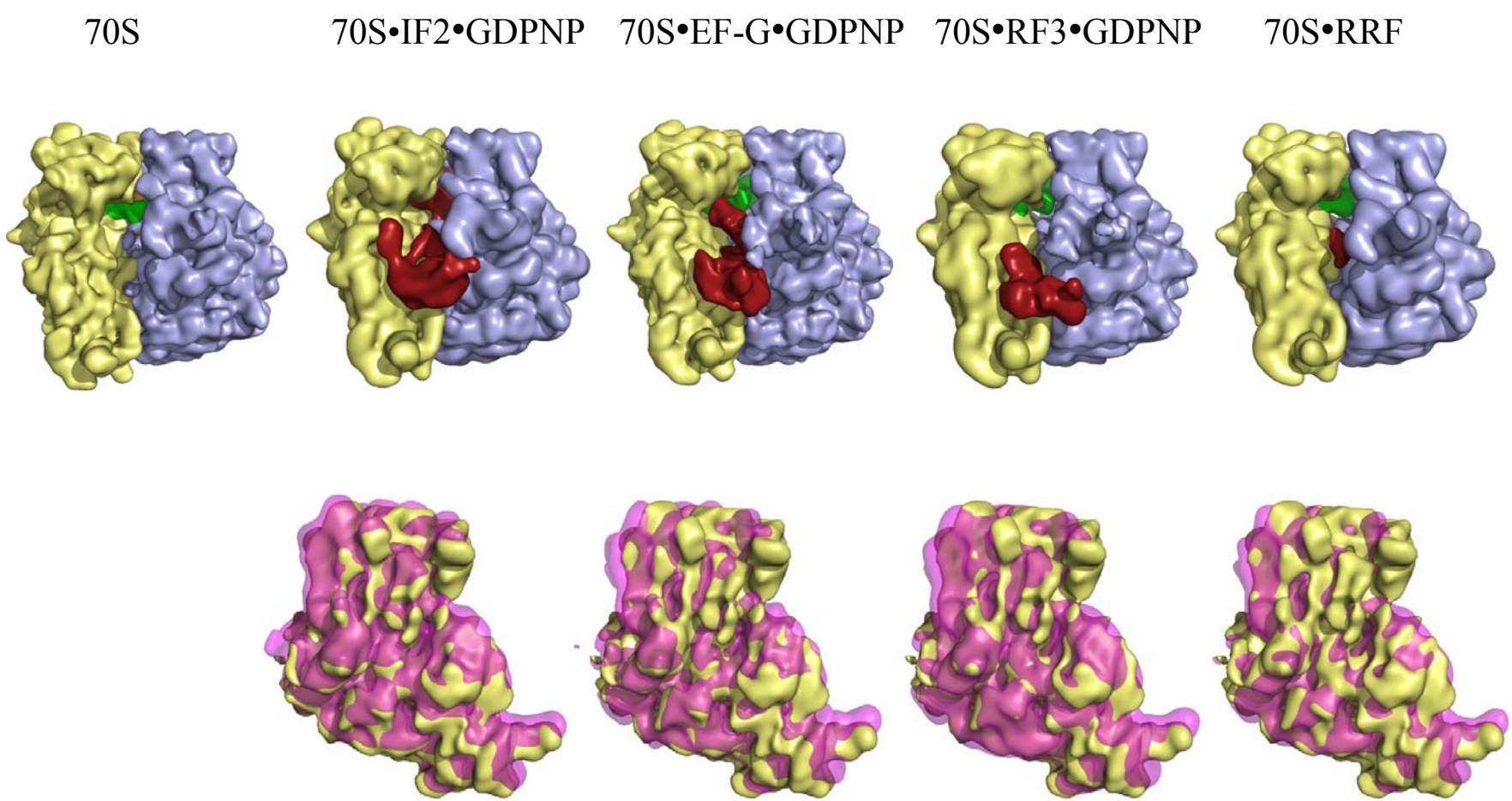
What is the Purpose of the Ratchet Motion in mRNA-tRNA Translocation?

Mechanism of **mRNA translocation** on the small subunit, in two parts

Translocation, Step I:
mRNA moves along with 30S, relative to 50S (lock is closed)

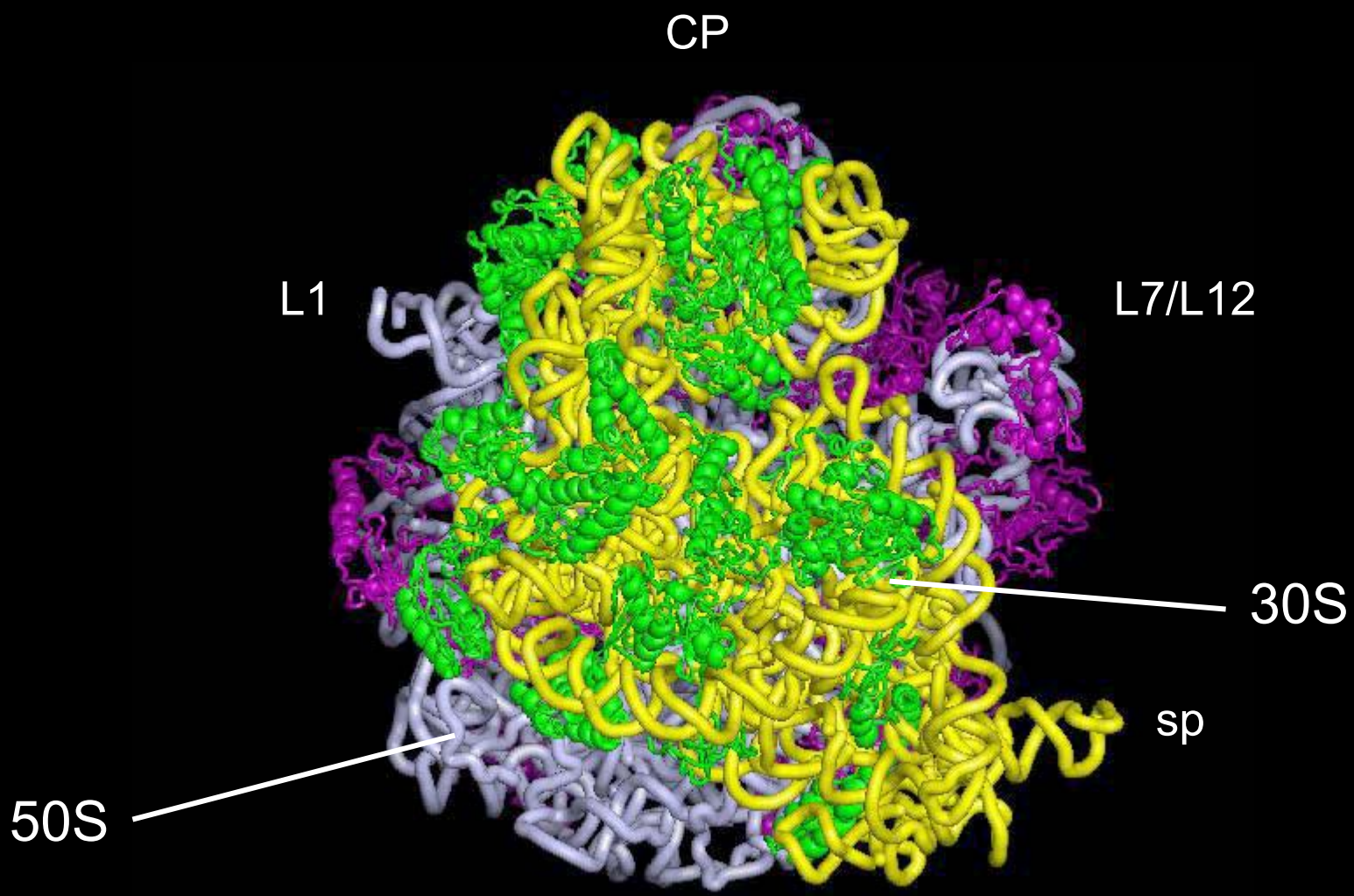
Translocation, Step II:
30S moves back, relative to mRNA and 50S (lock is open)

Modularity of the Machine: Macro-state II is trapped by several factors in entirely different functional contexts. Common mechanism for activating GTPase mechanism?



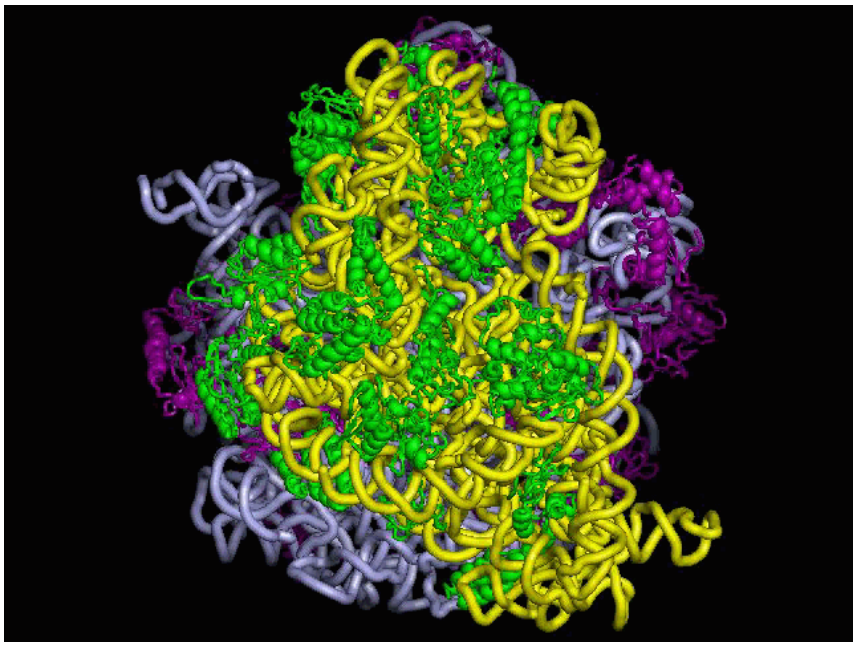
Gabashvili et al., 2000 Allen et al., 2005 Valle et al., 2003 H. Gao et al., subm. N. Gao et al., 2005

Frank & Agrawal, 2000

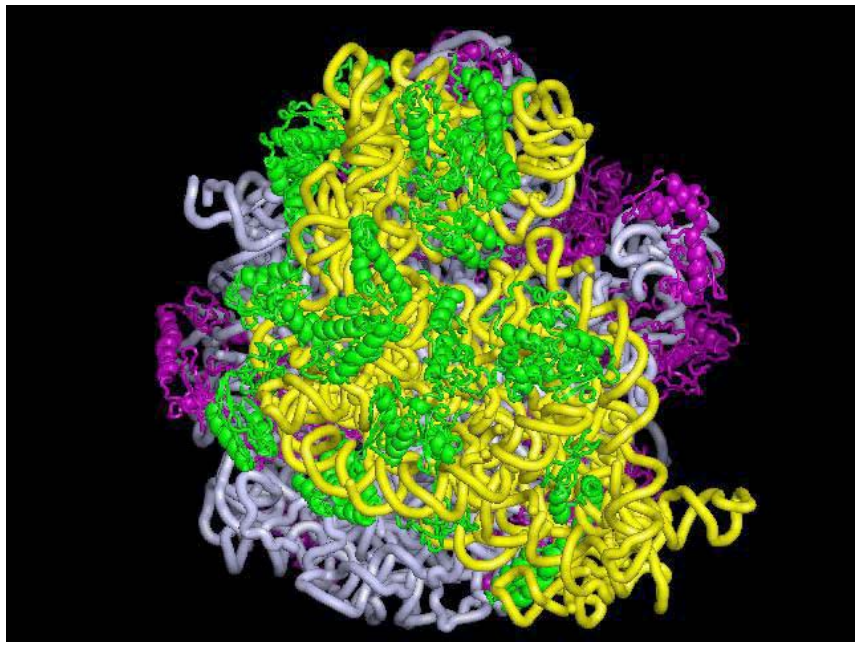


Atomic models of the ratcheting ribosome, upon binding of EF-G (Valle et al. Cell 2003), obtained by real-space refinement (Gao et al., unpublished).

Ratchet motions triggered by EF-G and RF3 are virtually indistinguishable



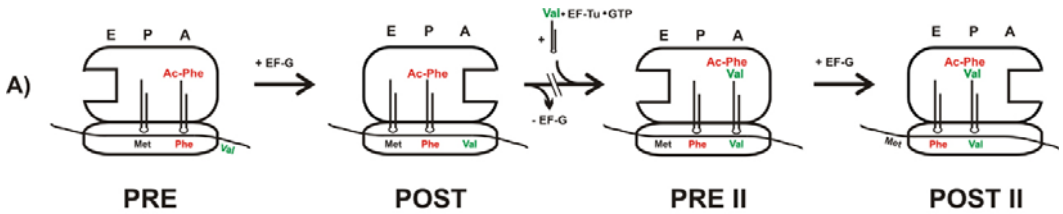
EF-G

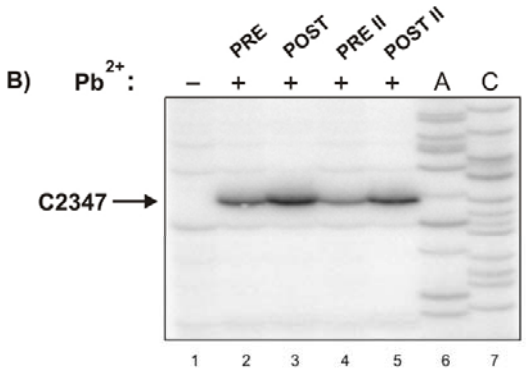


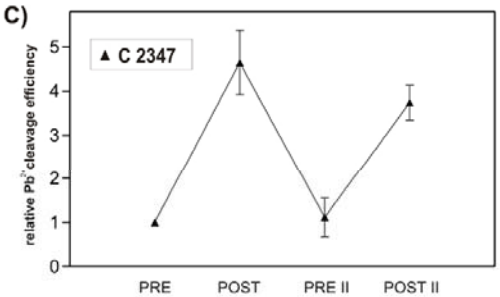
RF3

Evidence for Conformational Changes:

Pb^{2+} induced rRNA cleavage pattern near the peptidyl-transferase center undergoes periodic changes during the elongation cycle



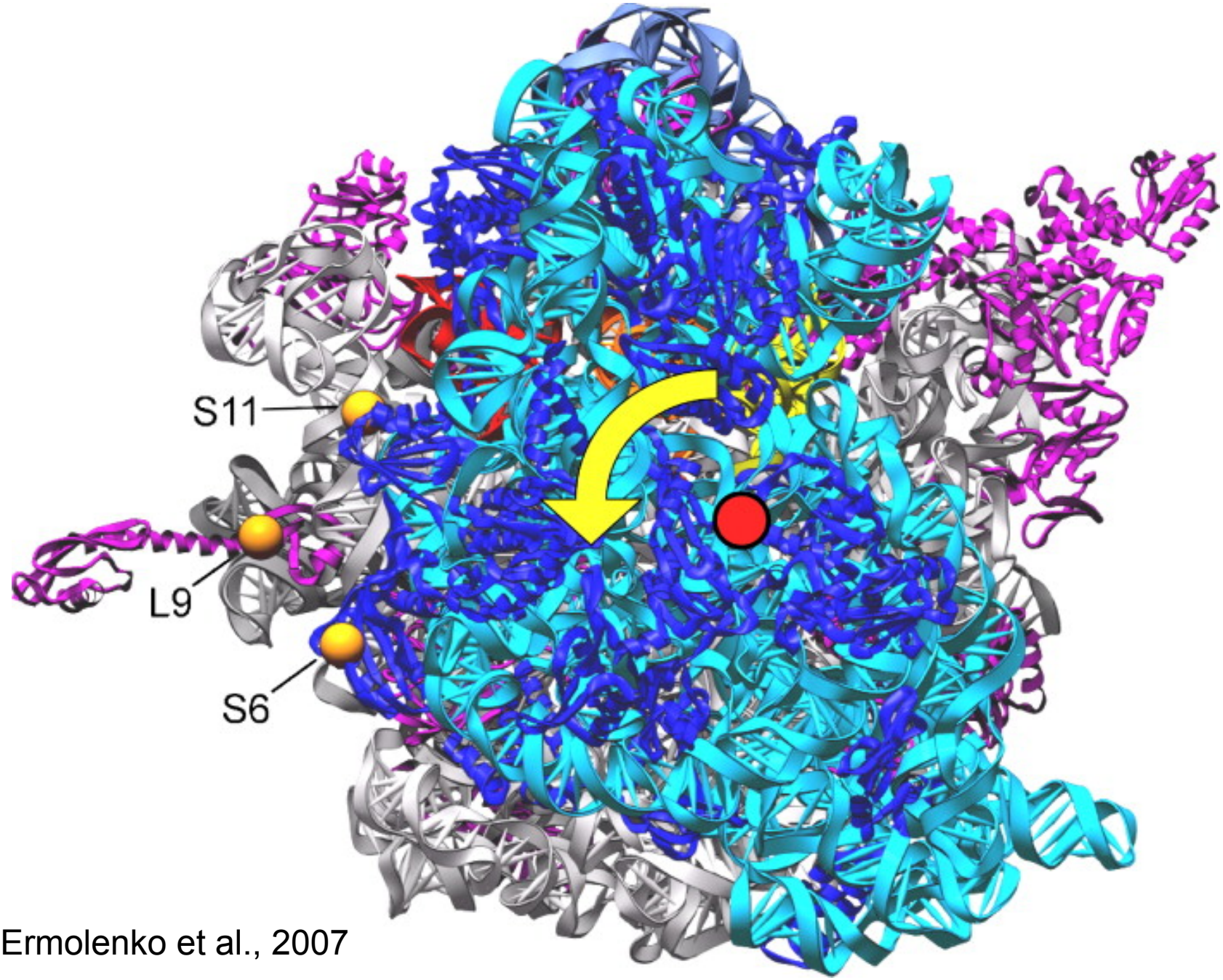




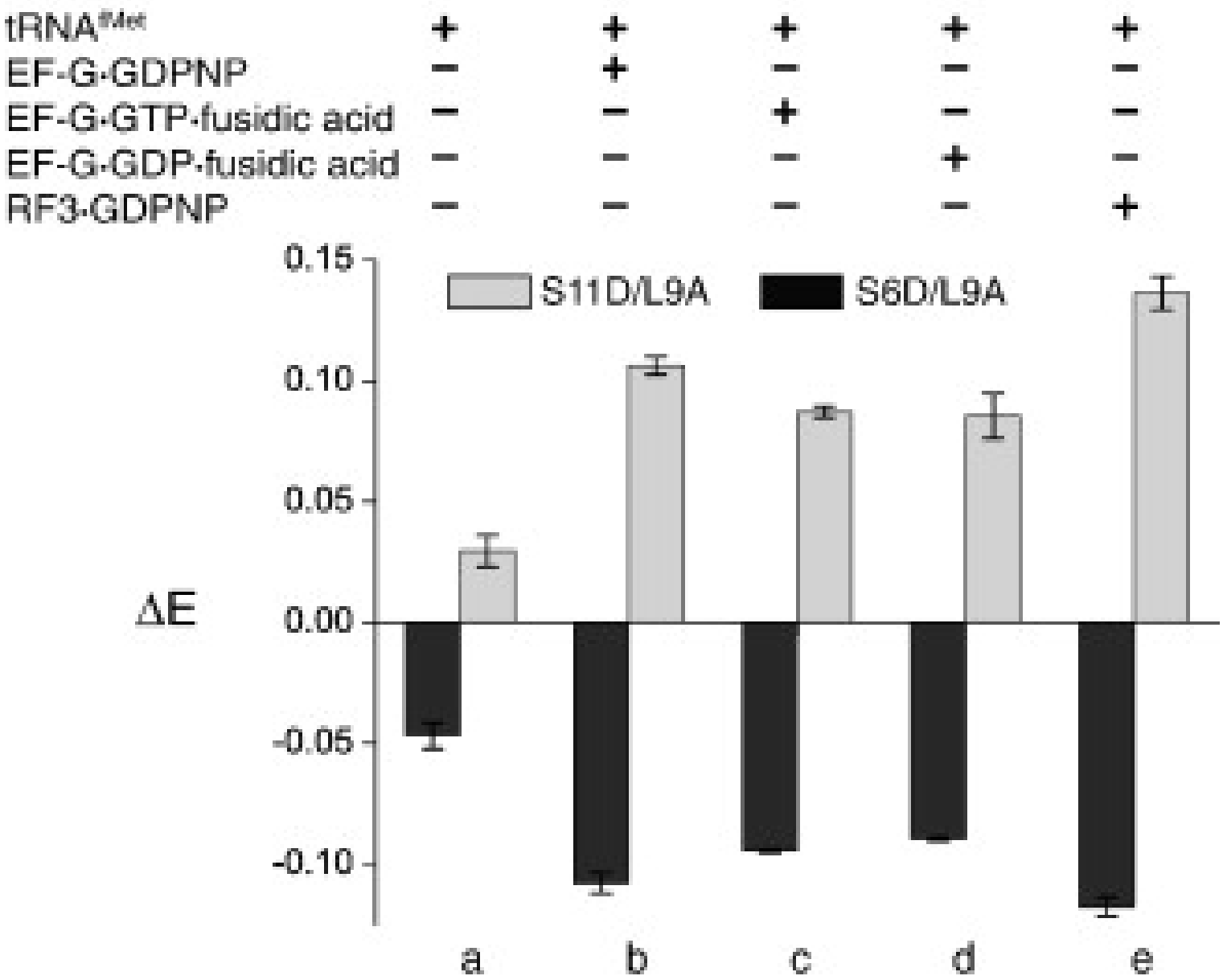
D)

	PRE	POST	PRE II	POST II
bound tRNA / 70S				
Ac-[^{14}C]-Phe-tRNA	nd	0.75	nd	0.65
[^{3}H]-Val-tRNA	nd	—	nd	0.47
Puromycin - assay				
Ac-[^{14}C]-Phe-Puro	0.13 pmol	1.55 pmol	—	—
Ac-[^{14}C]-Phe-[^{3}H]-Val-Puro	—	—	0.28 / 0.16 pmol	1.22 / 1.26 pmol
specificity of the complexes	91 %		77 - 87 %	
translocation efficiency	> 85 %		> 78 %	

Polacek et al., Molecular Cell 6 (2000) 159-171

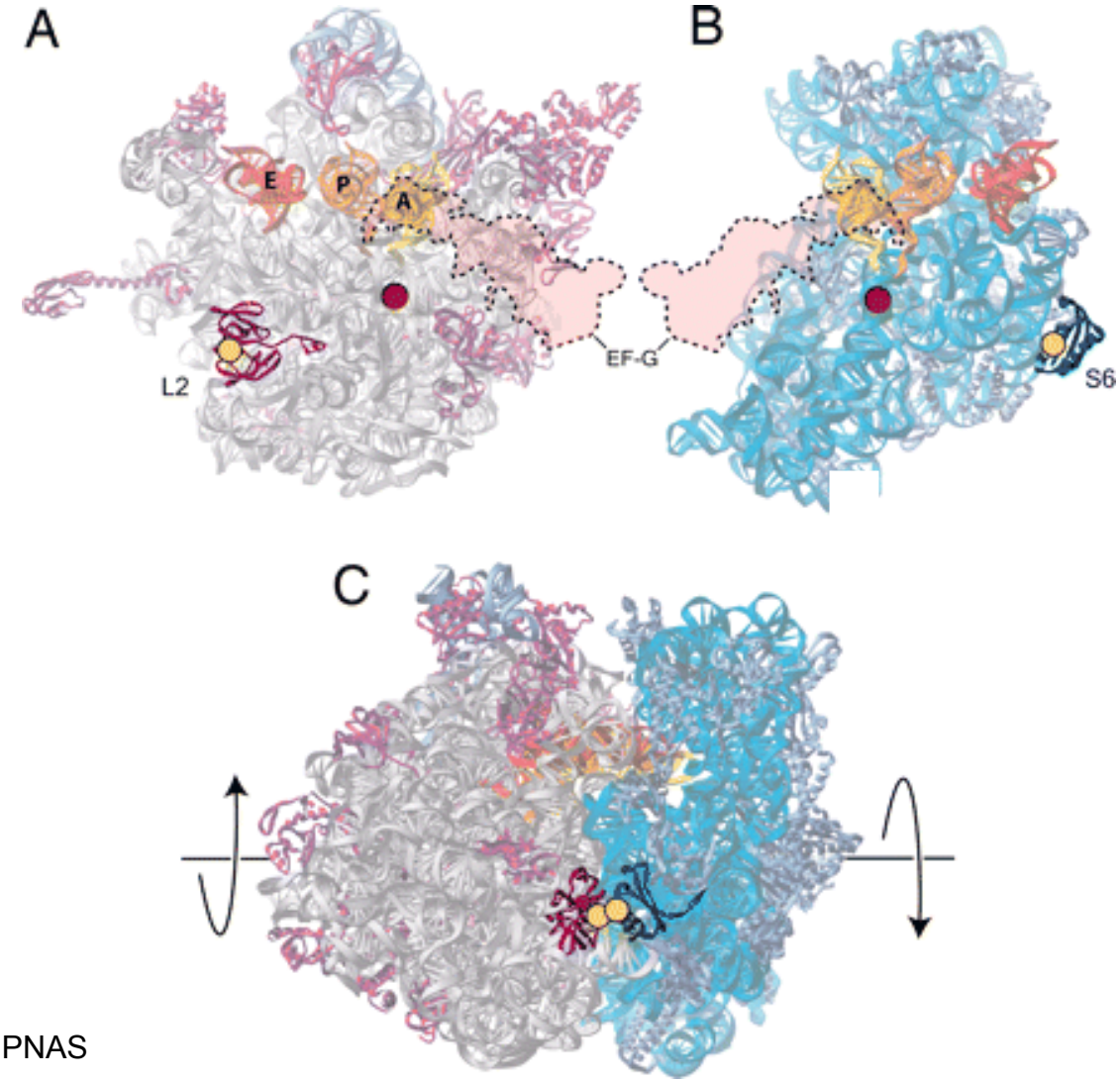


Ermolenko et al., 2007


tRNA^fMet
EF-G·GDPNP
EF-G·GTP·fusidic acid
EF-G·GDP·fusidic acid
RF3·GDPNP
+ + + + +
− + − − −
− − + − −
− − − + −
− − − − +
S11D/L9A
S6D/L9A
ΔE
0.15
0.10
0.05
0.00
-0.05
-0.10
a
b
c
d
e

Ratchet motion is necessary for translocation: experimental findings

L2 – S6 cross-link Inhibits translocation



Horan & Noller (2007), PNAS

“Macro-States” of the Ribosome

- The ribosome possesses two “macro-states” (I and II) with distinct conformations that differ by a change in the angle between the subunits (“ratchet motion”)
- *Along with the change in intersubunit angle, a structural reorganization takes place in both subunits, which affects the properties of several sites on both subunits.*
- Although one of the states is preferred, the two macro-states have similar stability, and they appear to be separated by a very small energy barrier (no GTP hydrolysis required to go from one to the other).
- *This transition is instrumental to translocation (recent Noller results), but it will not take place unless the P-site tRNA is deacylated (Zavialov et al., 2003; Valle et al., 2003)*
- Binding of a variety of factors (at the same ribosomal site) temporarily stabilizes state II: EF-G (translocation), IF2 (initiation), RF3 (termination), RRF (recycling).
- *Spontaneous ratcheting (along with transition to P/E state) has been observed by Harry Noller.*

Ratchet motion: example for heterogeneity (one of the many)

- Two populations co-exist:

  (1) non-ratchet + A,P,E (2) ratchet + P/E + EF-G
- Need for classification
- Supervised classification: need to know what we are looking for
- Unsupervised (preferable): no or minimal prior knowledge

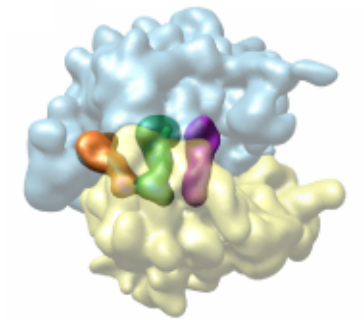
  1) “Maximum likelihood” (S. Scheres et al., 2007)

  2) Cluster tracking (Jie Fu & J. Frank, 2006)

  3) Mirek Kalinowski’s/Gabor Herman’s approach of graph cutting (Kalinowski et al., Ultramicroscopy 2007)

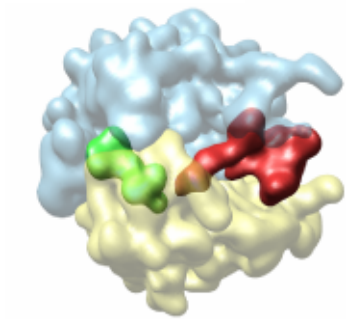
Observation of hybrid state (stabilized by EF-G•GDPNP and ratchet motion) by cryo-EM

Non-ratcheted



E/E P/P A/A

Ratcheted



P/E EF-G

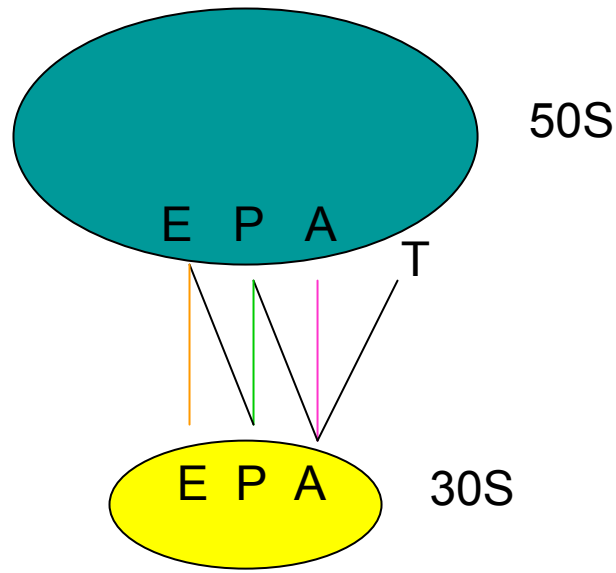
Digression: Passage of tRNA through the ribosome: canonical and hybrid states

tRNA proceeds "one step at the time":

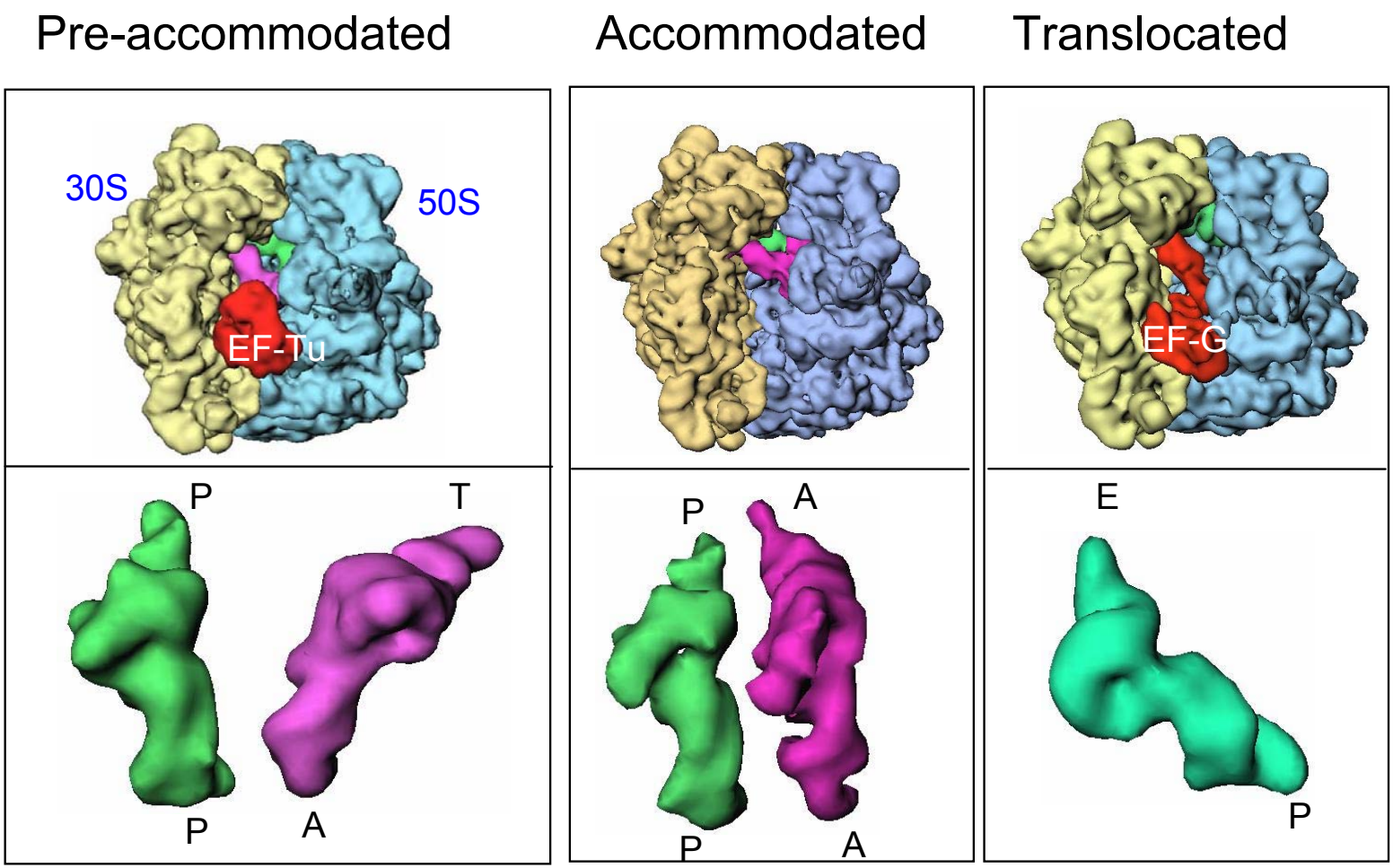
A/T → A/A → A/P → P/P → P/E → E/E

Nomenclature: [position on small subunit] / [position on large subunit]

T bound with EF-Tu
A aminoacyl
P peptidyl
E exit

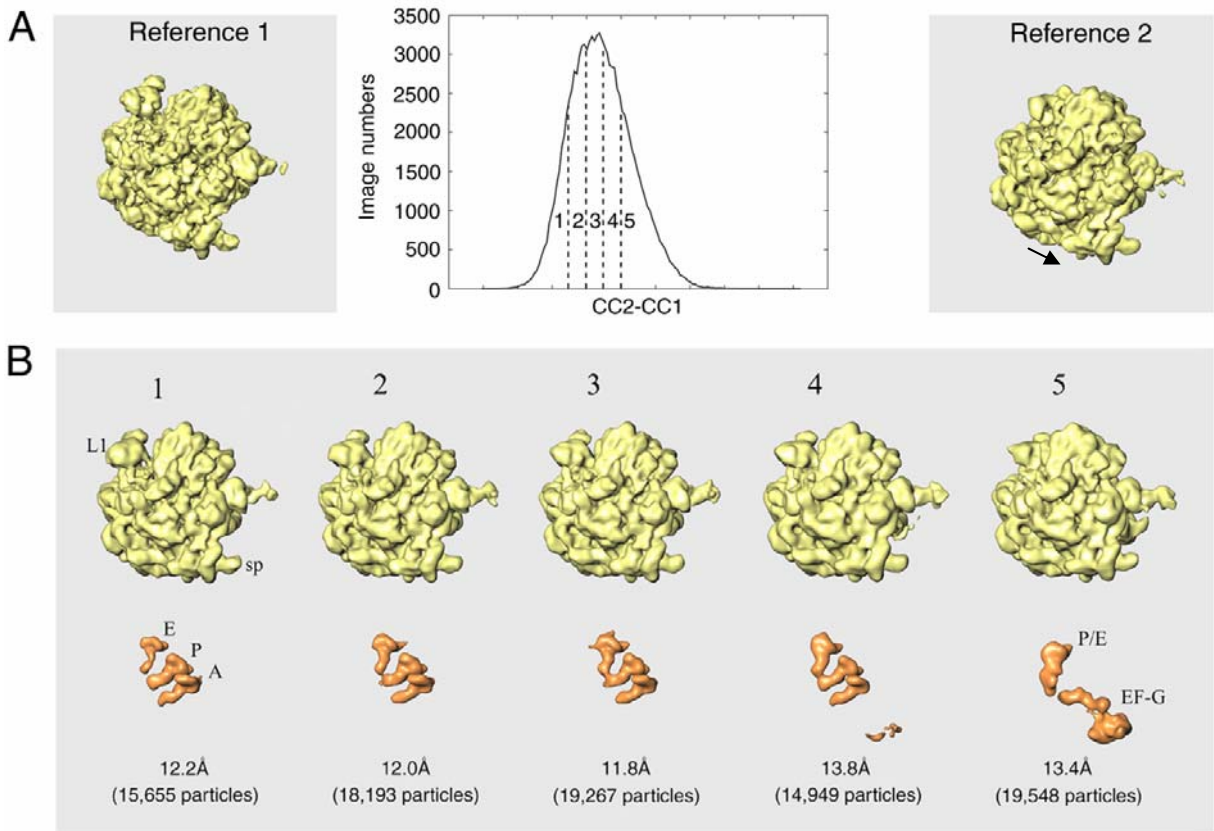


tRNA observed in cryo-EM maps



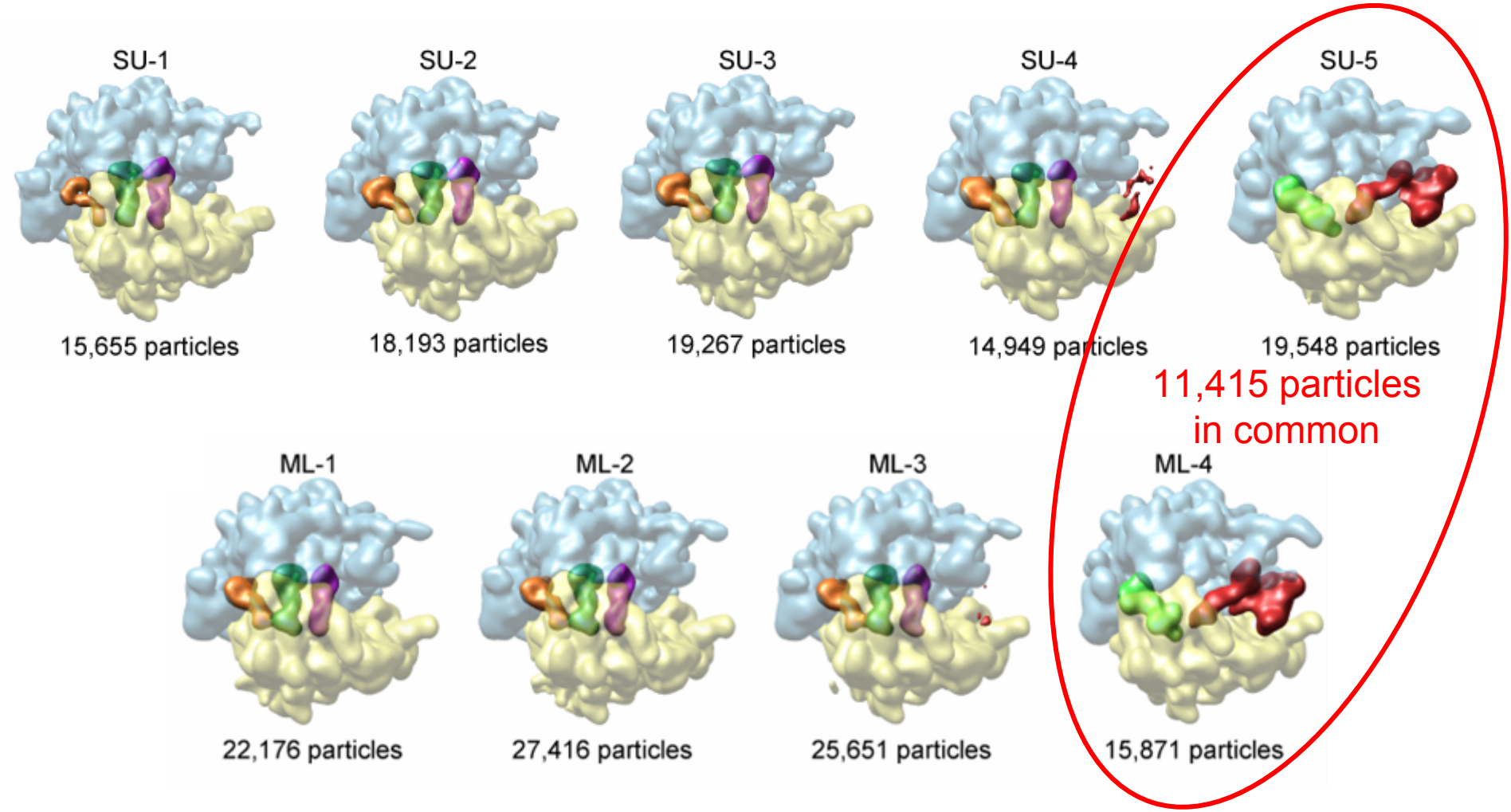
Supervised Classification

- Use ribosome maps in both ratchet states but without ligands:
- Successful classification will show tRNAs and EF-G at the expected locations in the two classes.



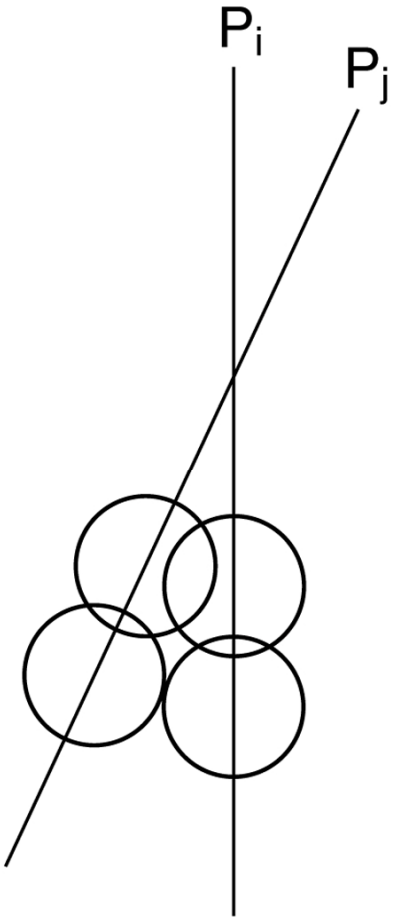
#1500 *Journal of Structural Biology* Fu (2007)

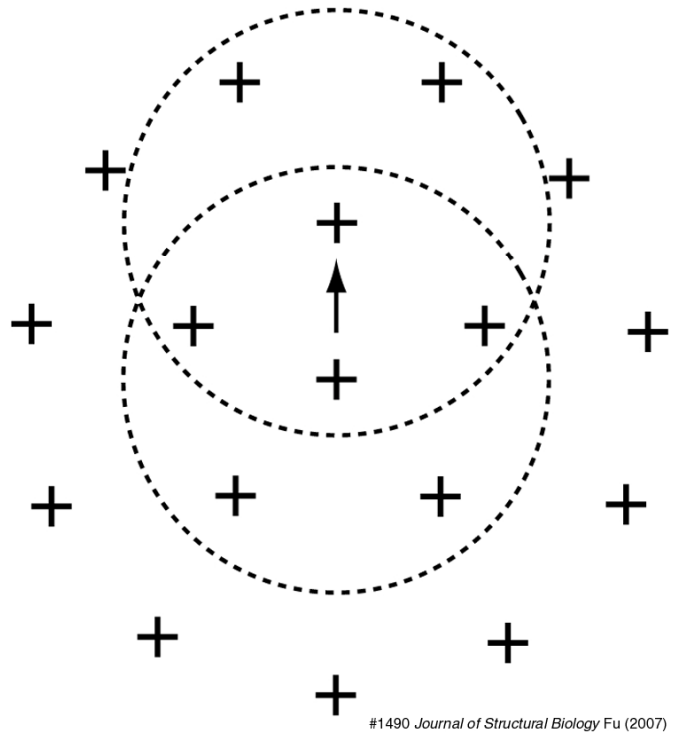
Supervised vs. Unsupervised (Maximum Likelihood) Classification of 90,000 Ribosome Images (+/- EF-G•GDPNP)



Scheres et al., Nature Methods 2007

Cluster tracking method: cluster continuity is a consequence of data overlap in Fourier space





Jie Fu and J. Frank, 2007

Cluster tracking

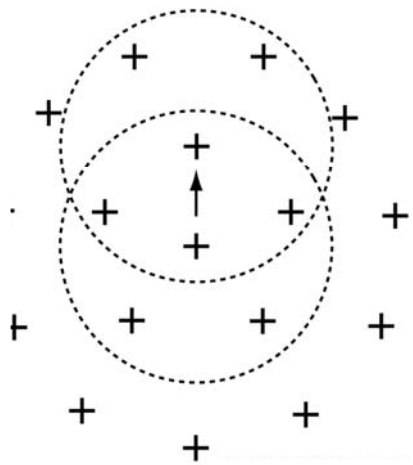
Strategy:

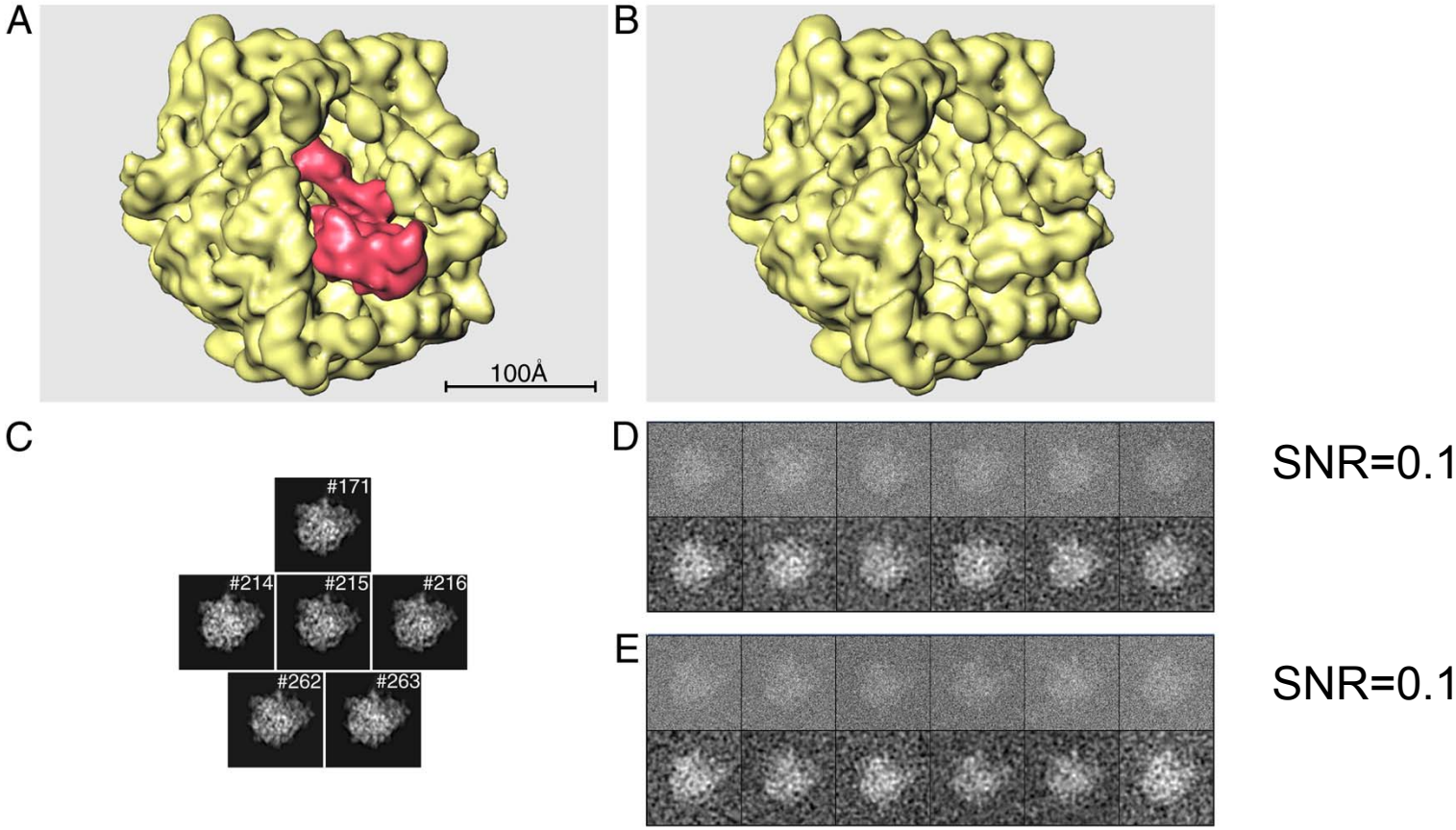
classify data first into orientations on angular grid,

then classify all data falling in narrow angular neighborhoods.

Slide angular neighborhoods along the (half-) globe

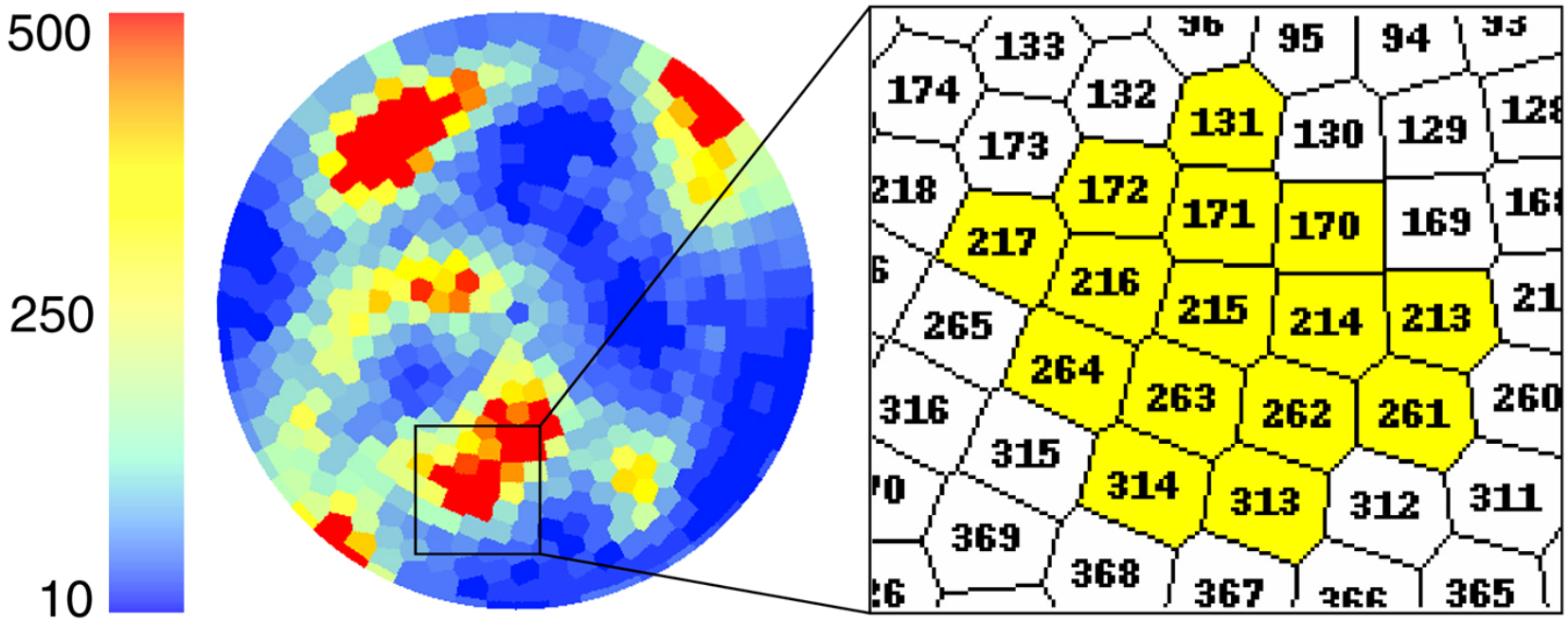
Track clusters as you go along





#1492 *Journal of Structural Biology* Fu (2007)

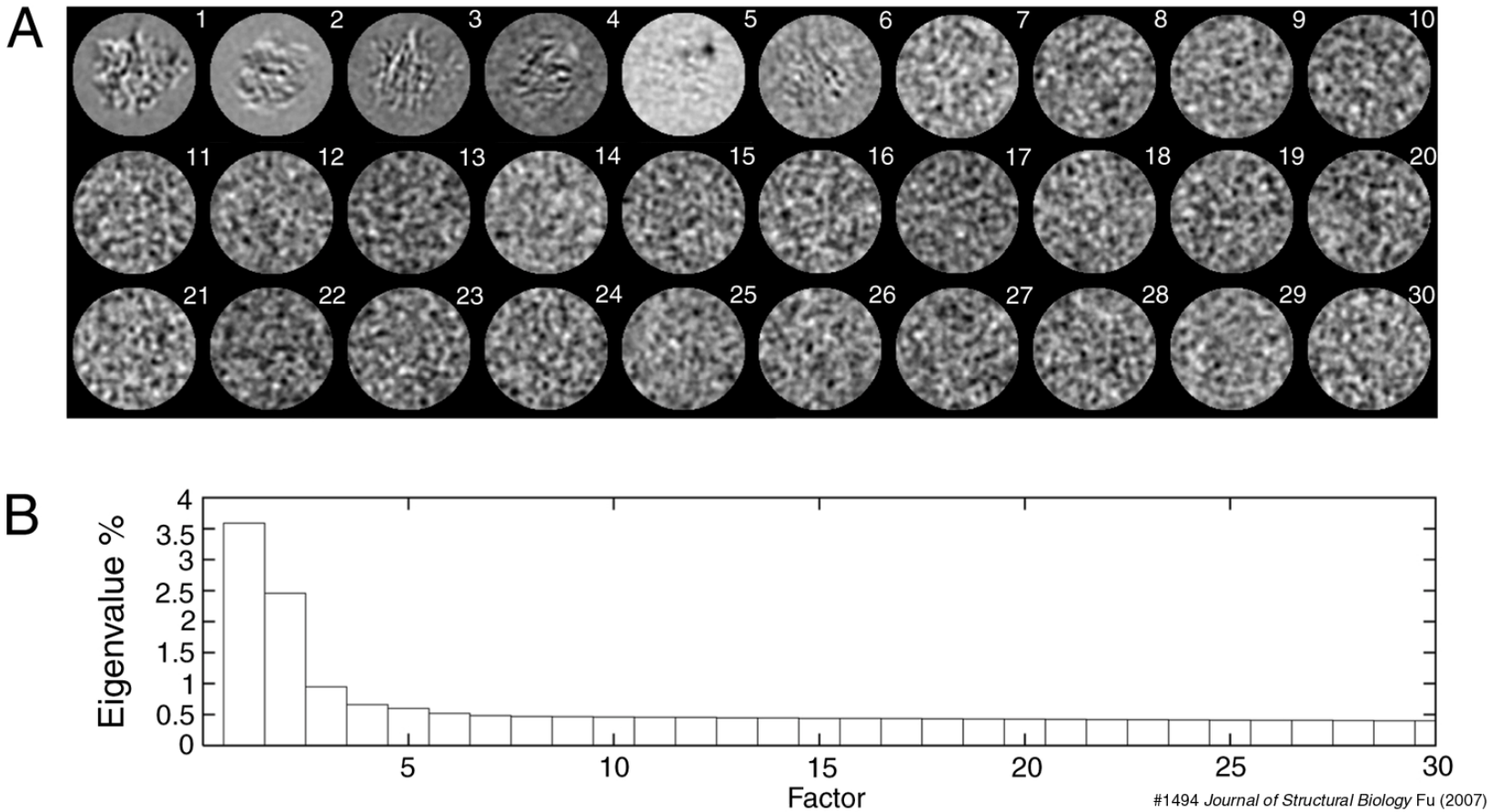
90,000 particles: angular distribution



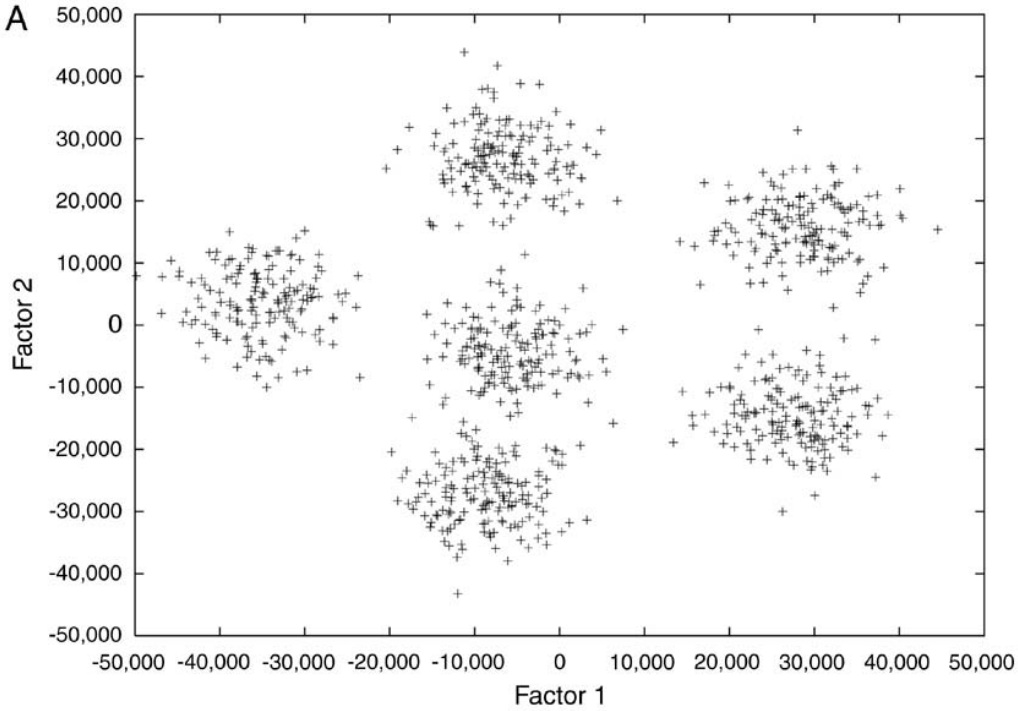
#1493 *Journal of Structural Biology* Fu (2007)

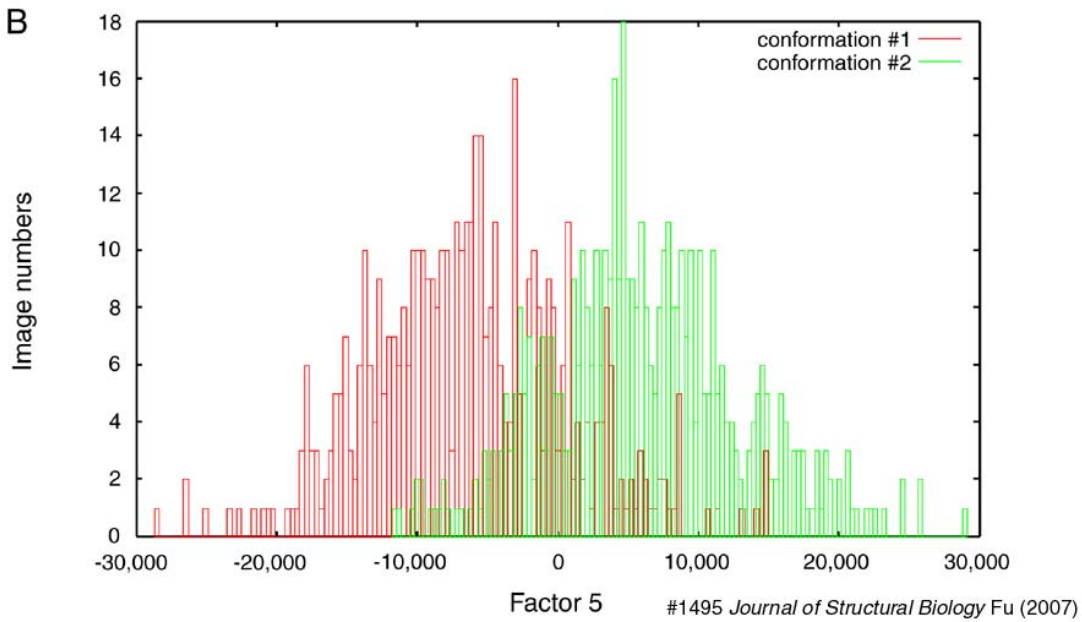
Color code for # of particles per tile

(tile #)



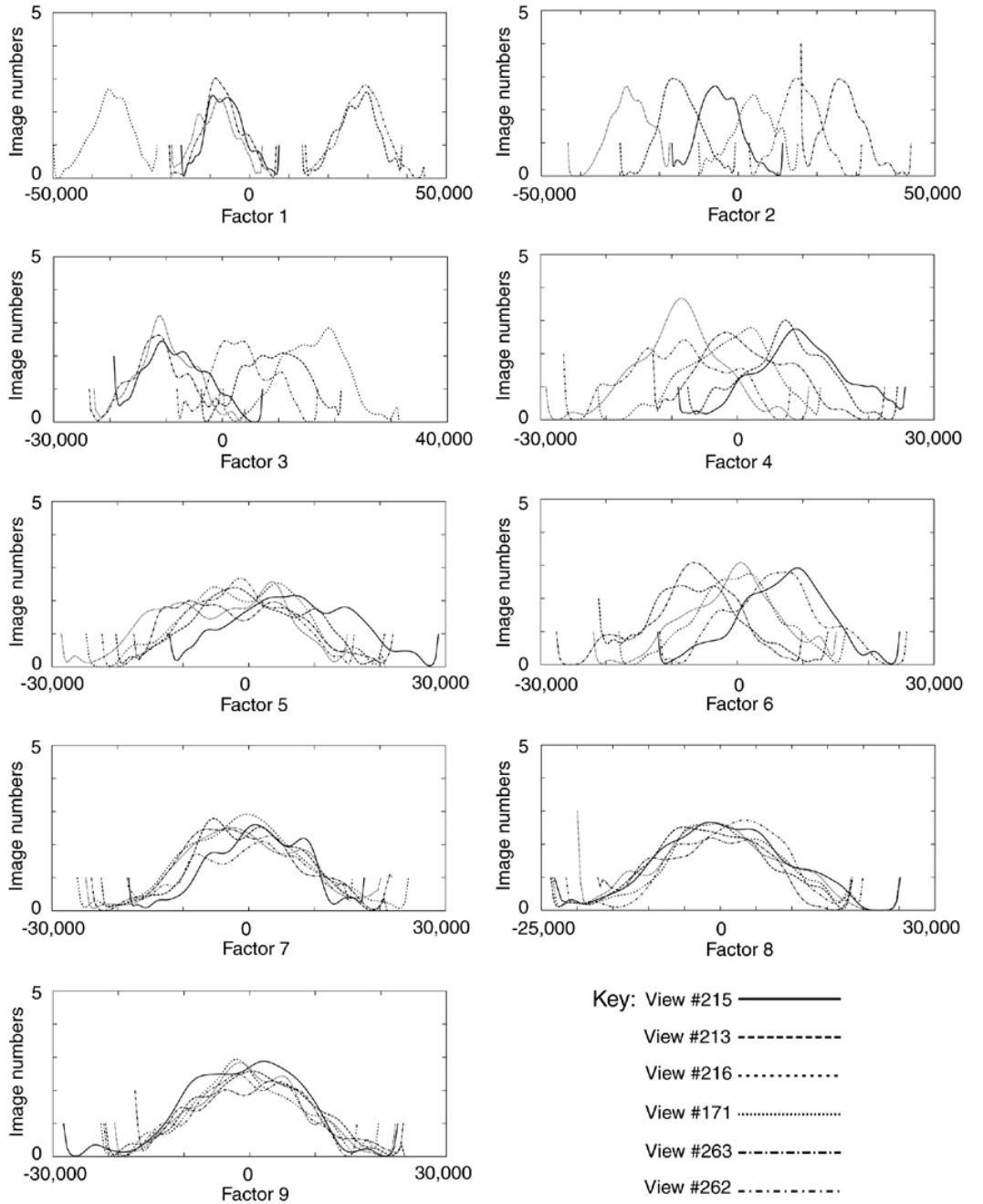
Phantom data – main variation due to orientation is in factors 1 vs 2





#1495 *Journal of Structural Biology* Fu (2007)

Factors should
not be sensitive to
orientation
(successive exclusion)



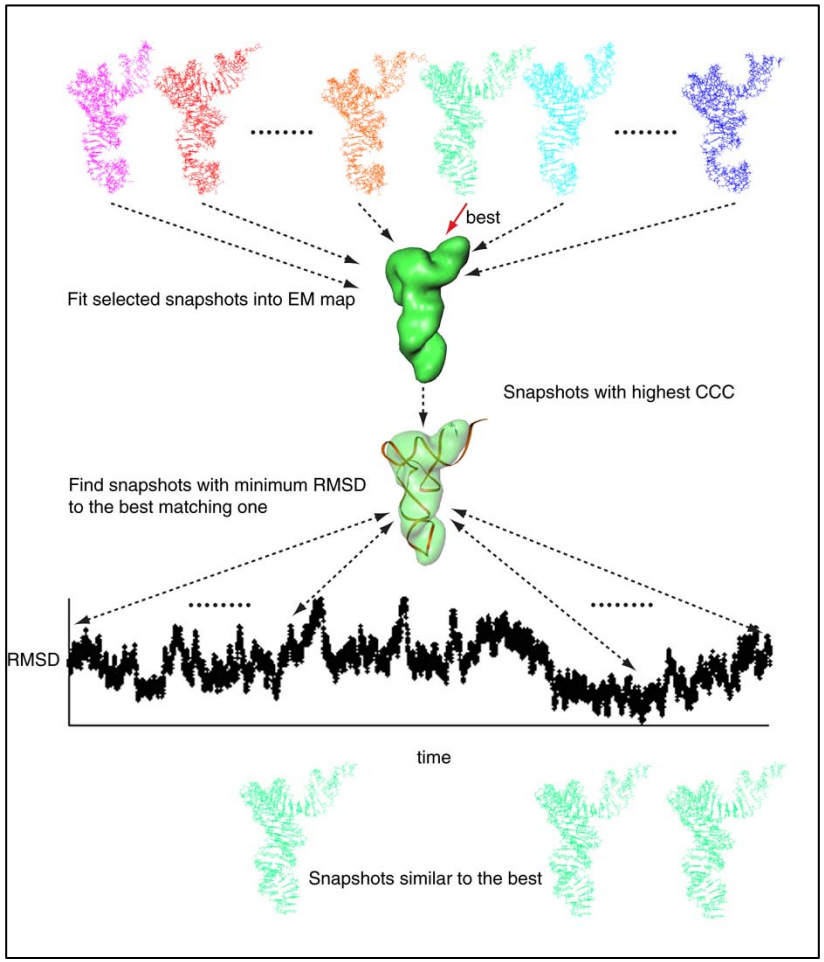
#1496 *Journal of Structural Biology* Fu (2007)

Cluster tracking

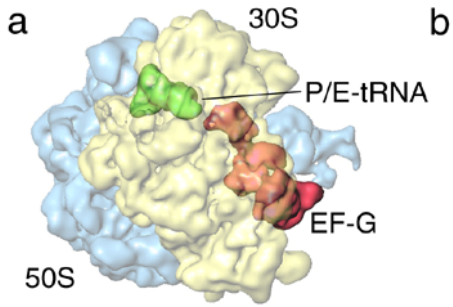
- Problem of discontinuity of angular distribution
- Solution: (a) collect more data

  (b) use CCCL (cross-correlation of common lines) between clusters established on each “island”.

P/E tRNA model by MD simulation and CC with cryo-EM

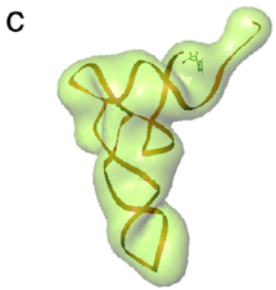


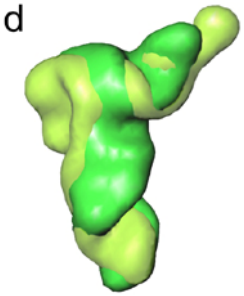
Search for representative structures along MD simulation trajectory for free tRNA



P/E cryo

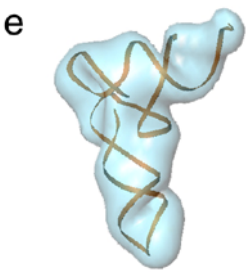
X-ray of P-tRNA

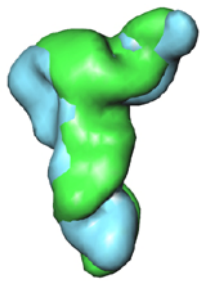




(b + c)

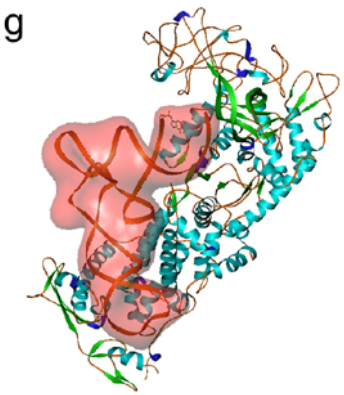
tRNA unbound

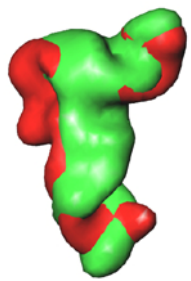




(b + e)

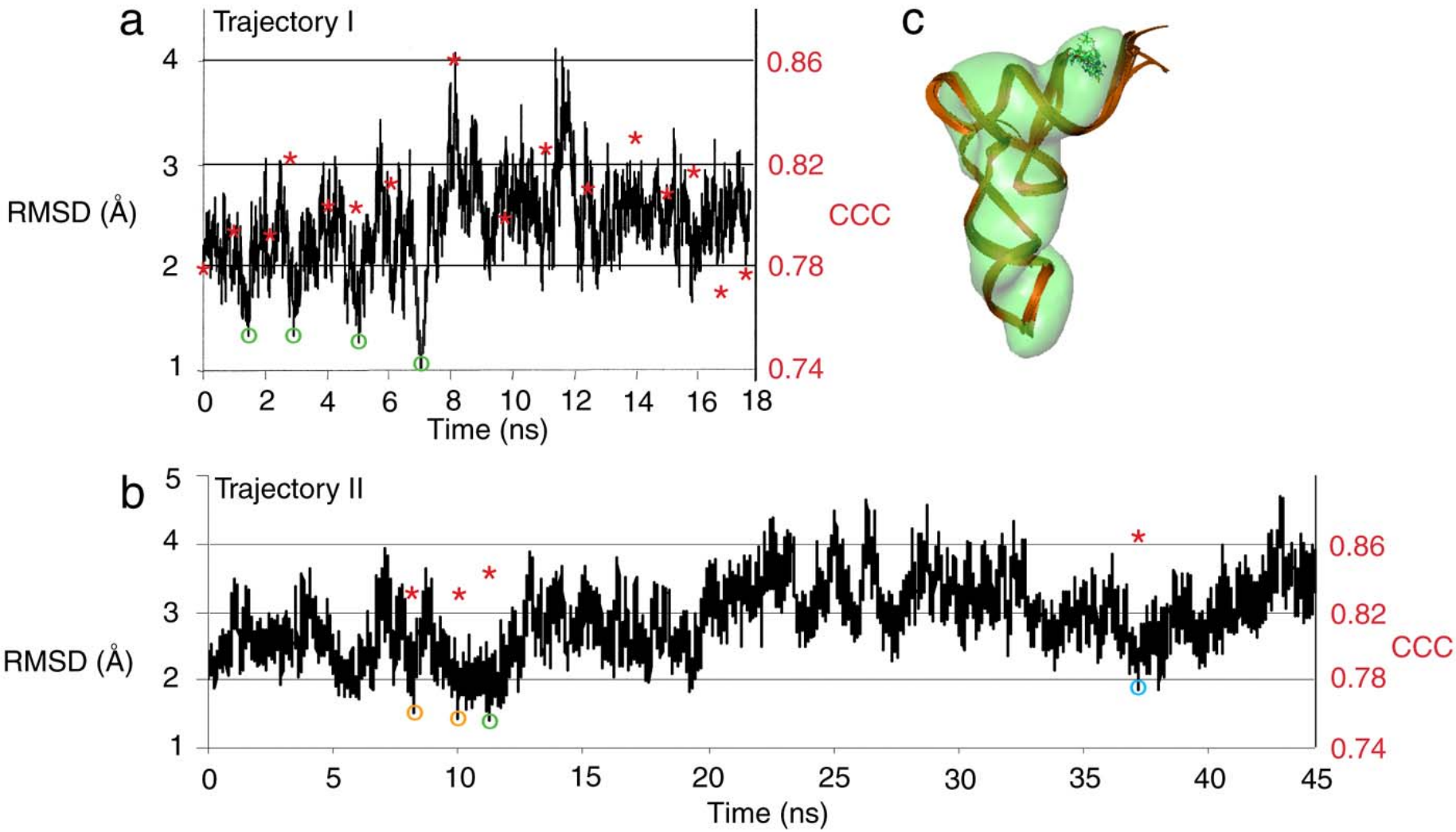
X-ray of $tRNA^{Ile}$
with synthetase





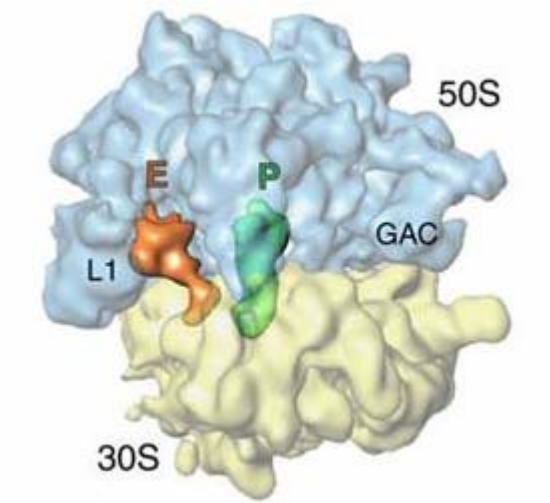
(b + g)

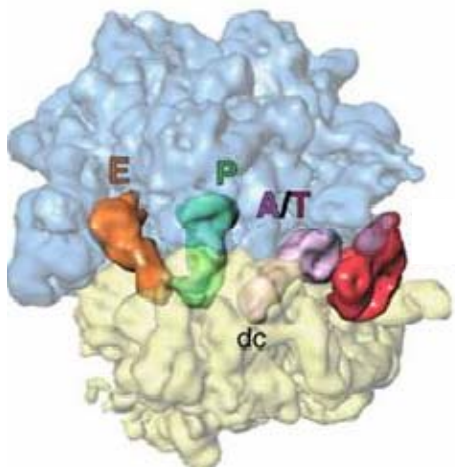
Conformation of observed P/E-tRNA is visited in MD simulations of free tRNA (Wen Li and J. Frank, subm.)

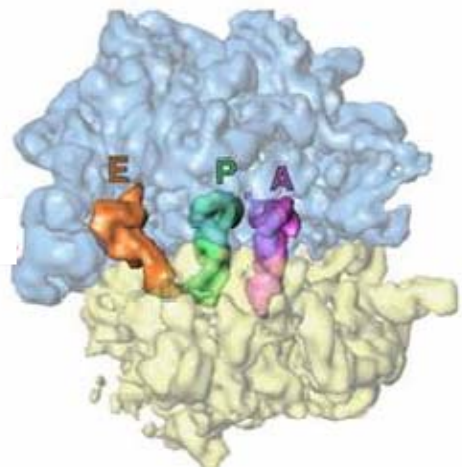


RMSD with respect to candidate structure with high cross-correlation

tRNA Selection and Accommodation: Cryo-EM 3D Snapshots in three States







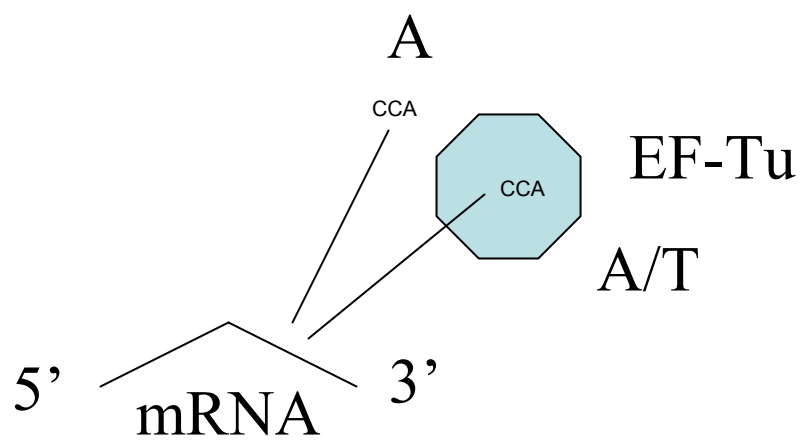
Post-initiation
(post-translocation)

"A/T"
Phe-tRNA^{Phe}•EF-Tu•GDP•kir

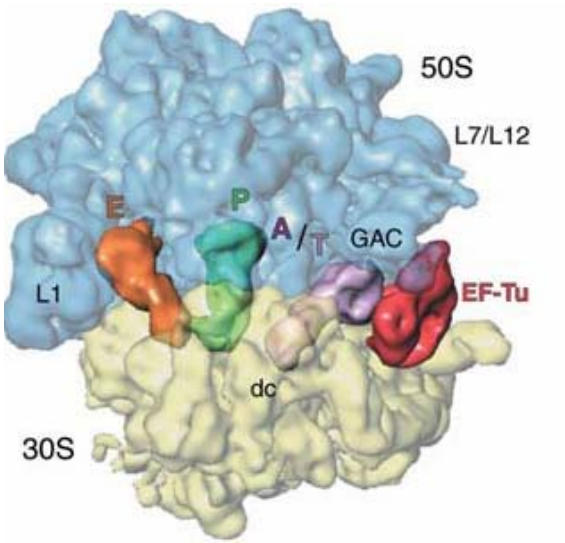
"A"

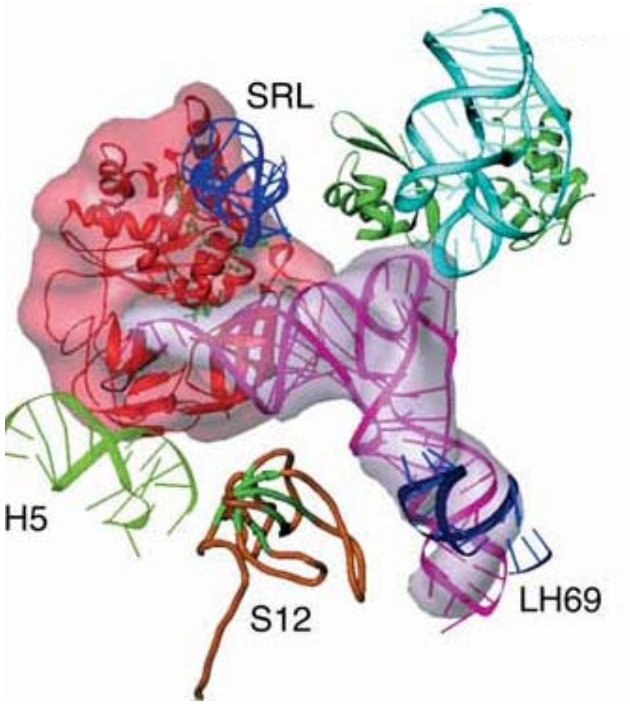
Valle et al., NSMB 10 (2003) 899

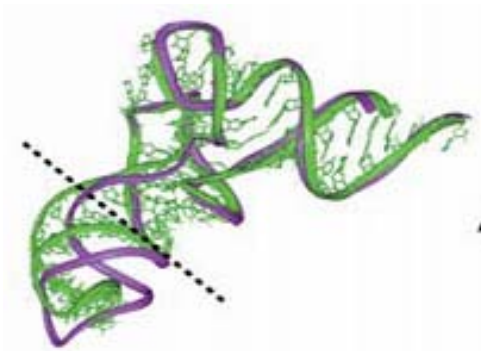
The initial approach of aa-tRNA presents a steric problem



Phe-tRNA^{Phe} in A/T state: interaction with ribosome is accompanied by a distortion in the anticodon stem

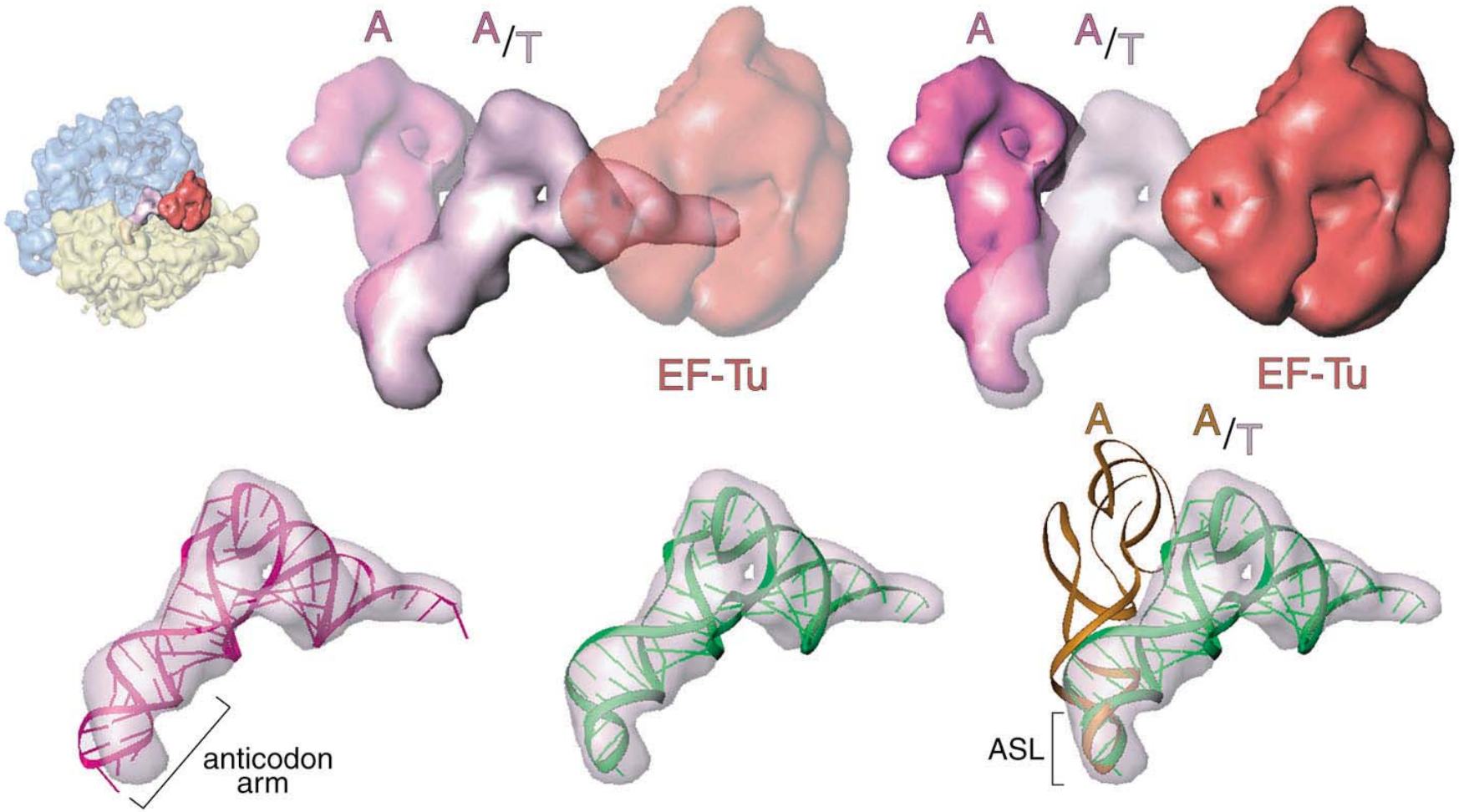






Valle et al., NSB 2003

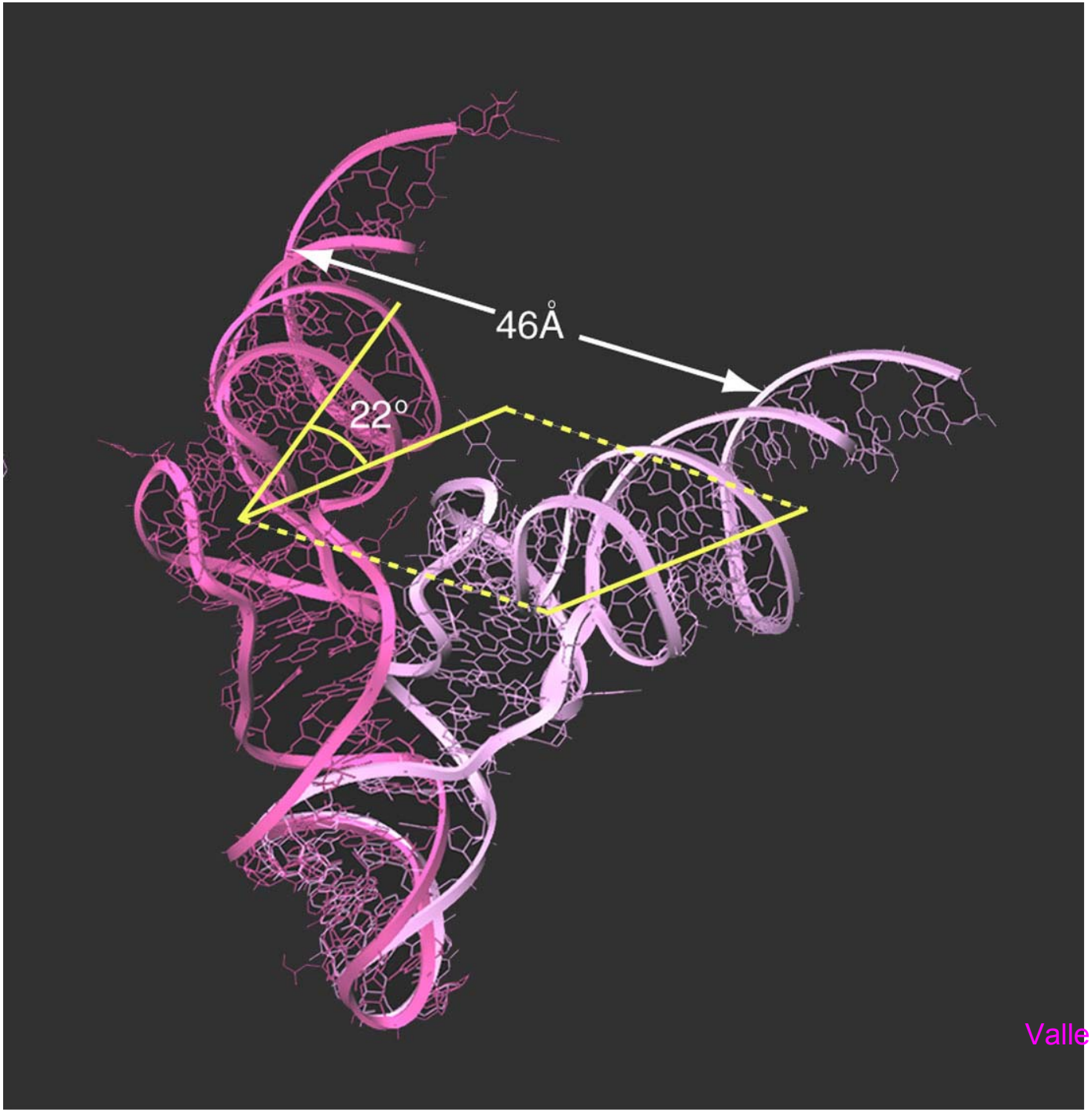
A/T conformation: the tRNA is in a high-energy state.
A/T → A: relaxation of a molecular spring



X-ray

remodeled to fit

Valle et al., NSB 10 (2003) 899

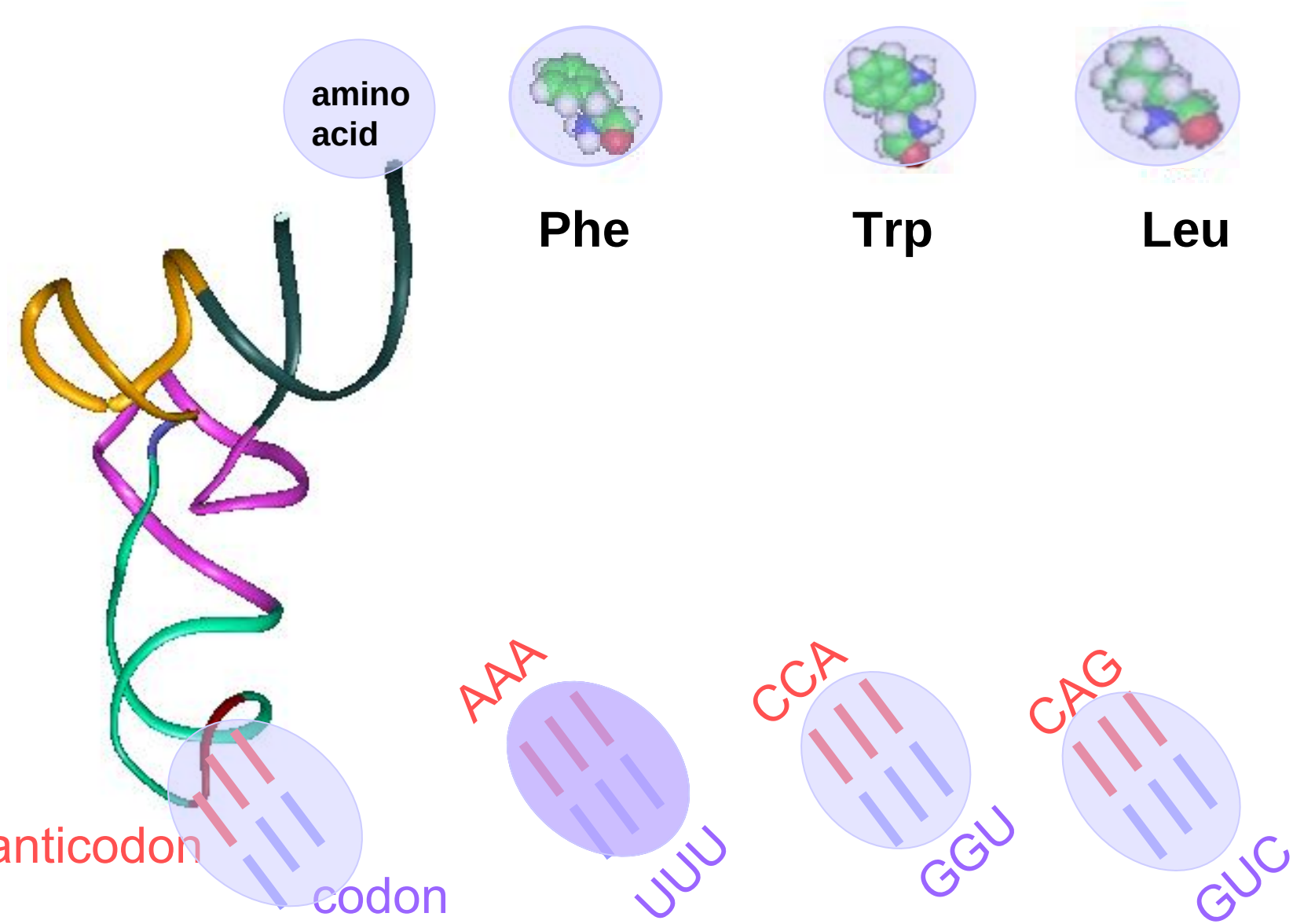


Valle et al., NSB 11 (2003) 899

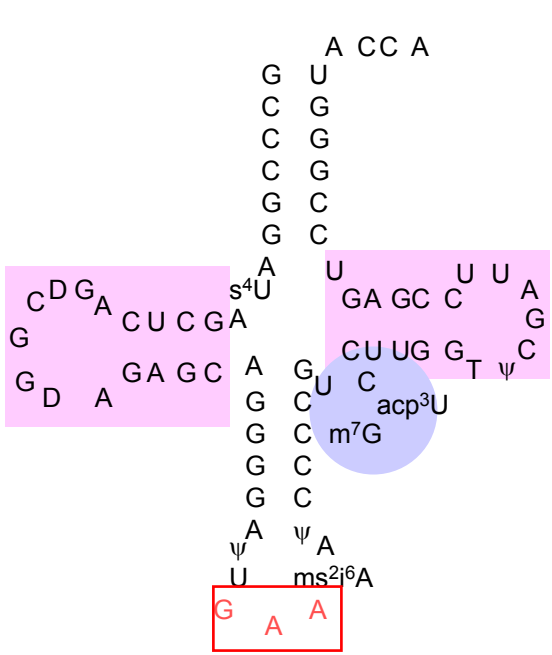
Are the dynamic features of tRNA selection universal?

- Phe-tRNA -- existing results: Valle et al. Cell 2003
- Leu-tRNA – Wen Li et al.: collab. with Mans Ehrenberg and Suparna Sanyal
- Trp-tRNA – Xabier Agirrazabala et al.: collab. with Rachel Green (Hirsh suppressor wild-type)

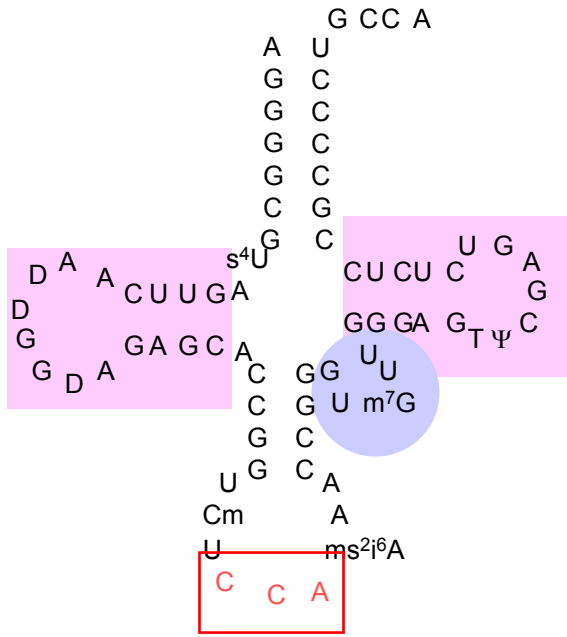
Aminoacyl-tRNA selection



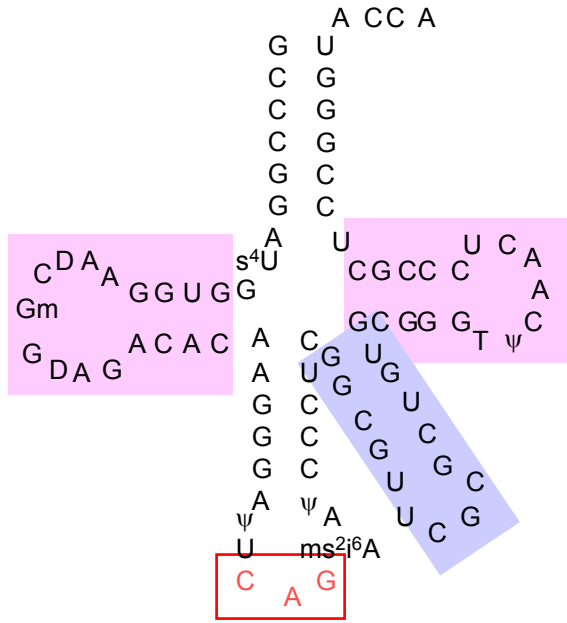
Aminoacyl-tRNA sequences



Phe

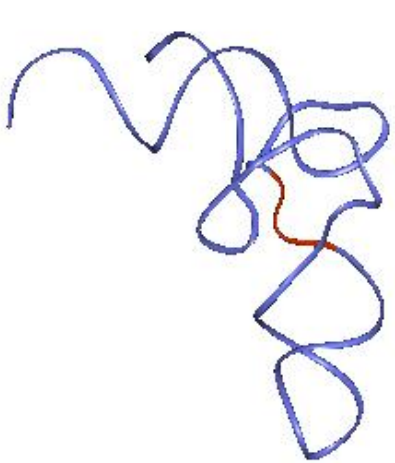


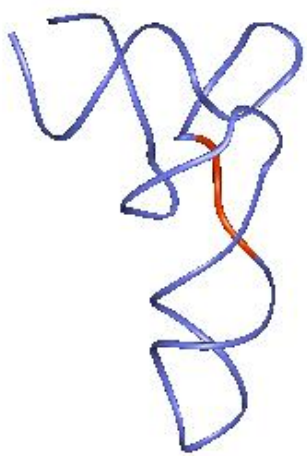
Trp

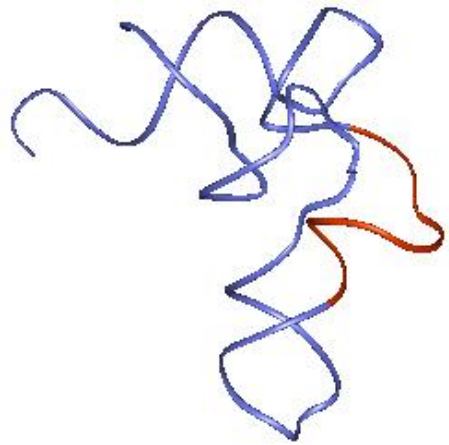


Leu

tRNA







Phe

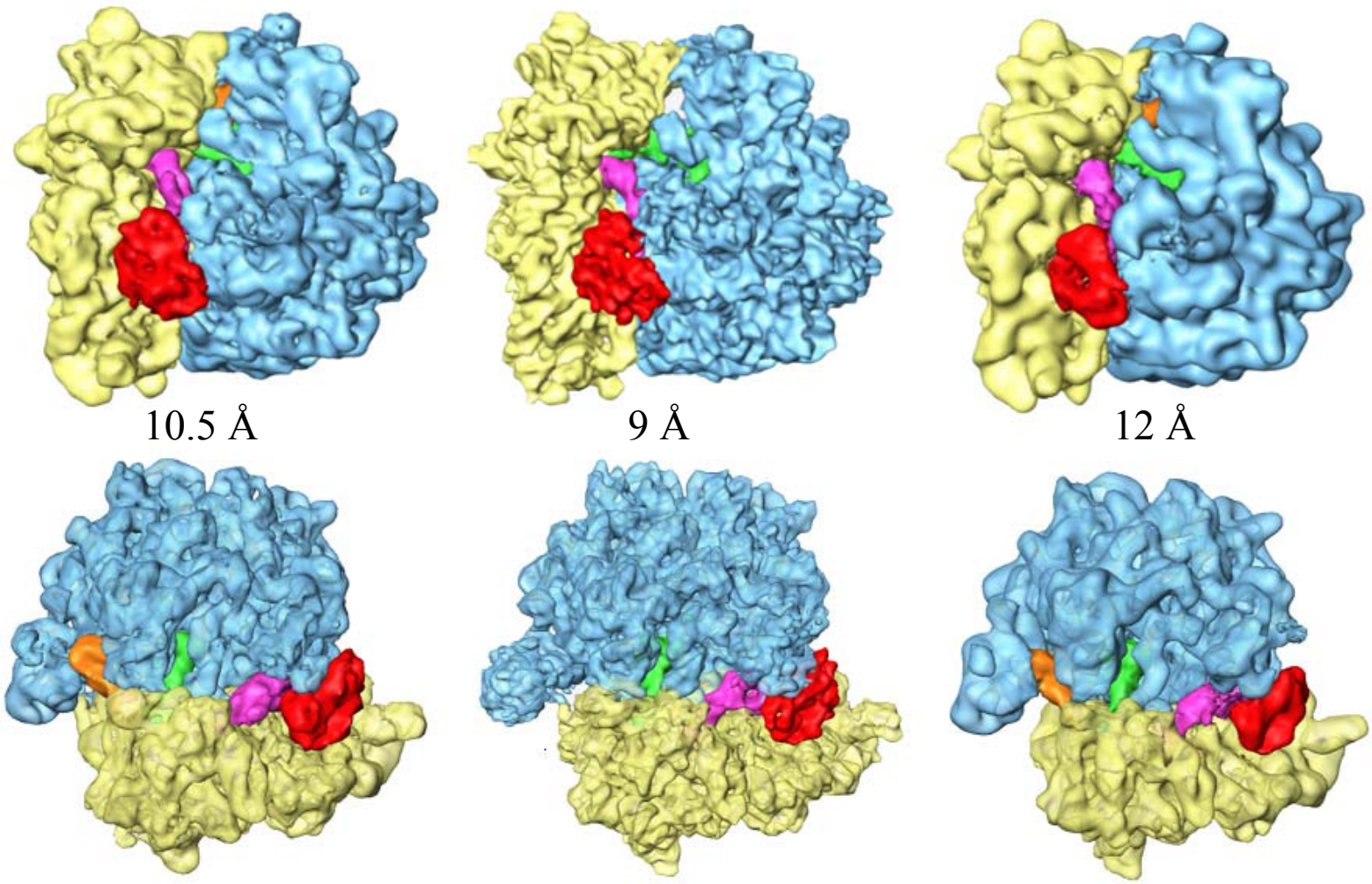
Trp

Leu

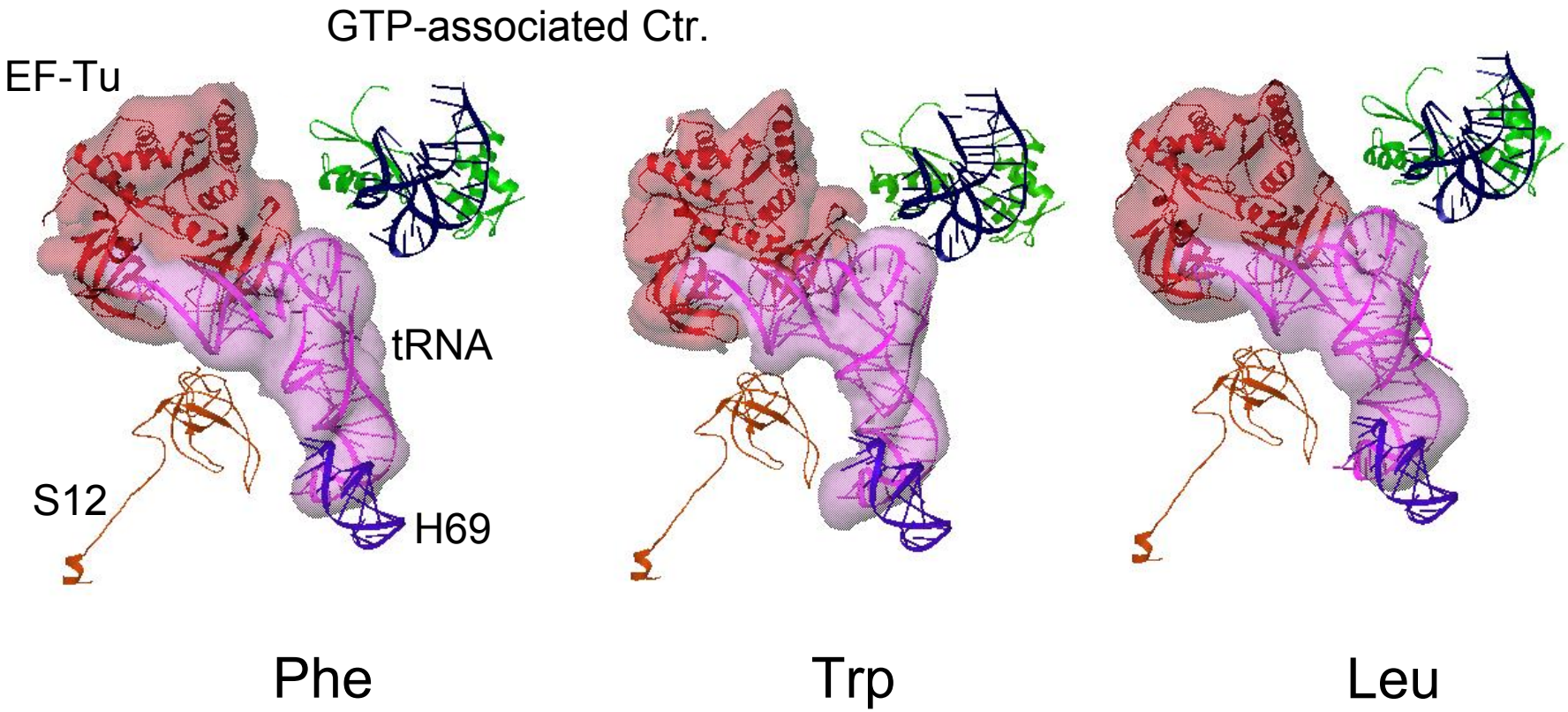
Class I tRNA

Class II tRNA

Three different aminoacyl-tRNAs in pre-accommodated complexes



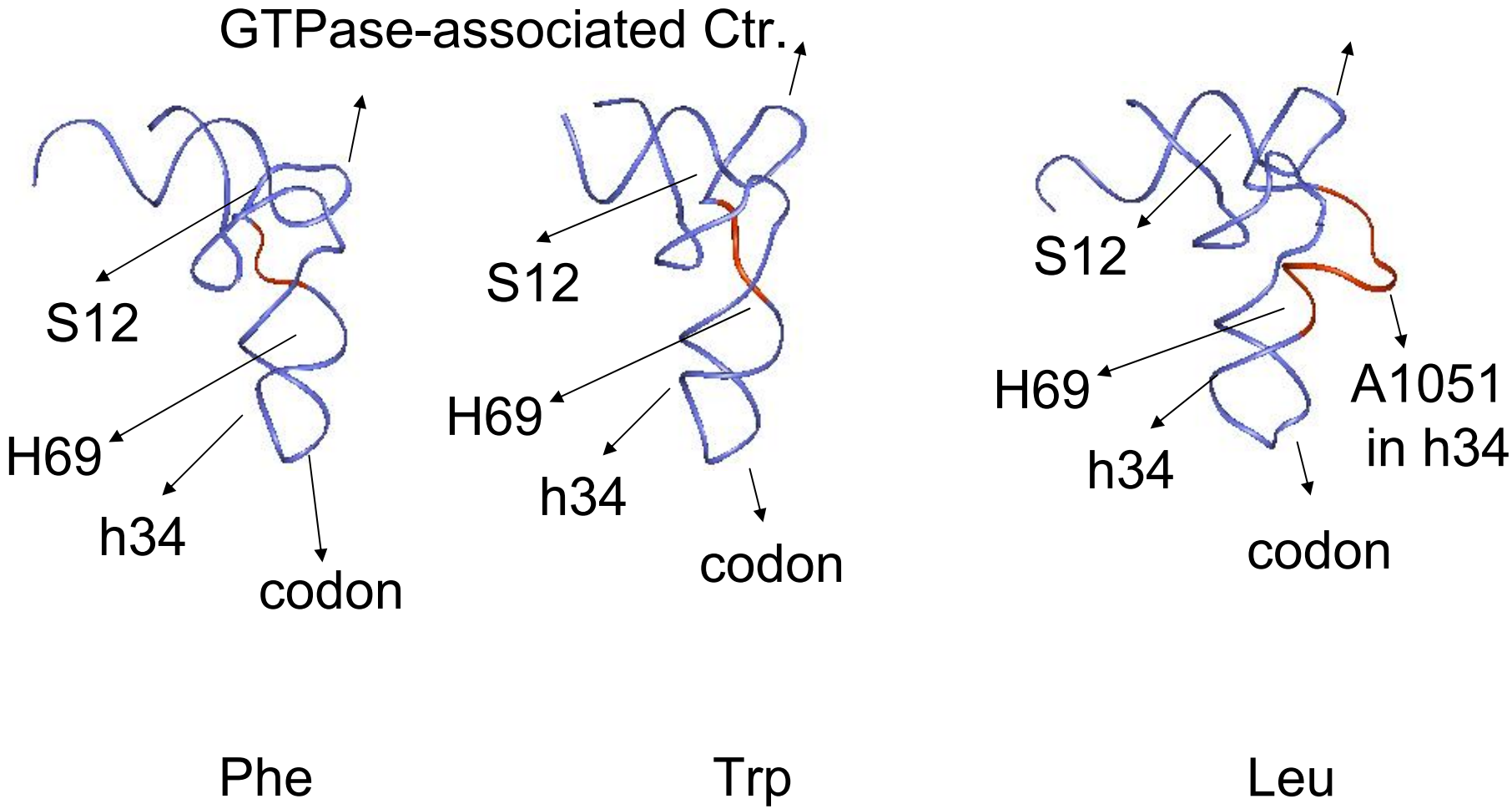
All three aa-tRNAs in A/T state show a distortion (kink and twist) in the selection step



Models from real-space refinement -- 4 rigid pieces for Phe and Trp/ 5 rigid pieces for Leu

In all three aa-tRNA investigated, ribosomal contacts are the same -- selection occurs solely on the basis of codon-anticodon interaction

[contact of variable loop of $tRNA^{leu}$ with h34 is weak]

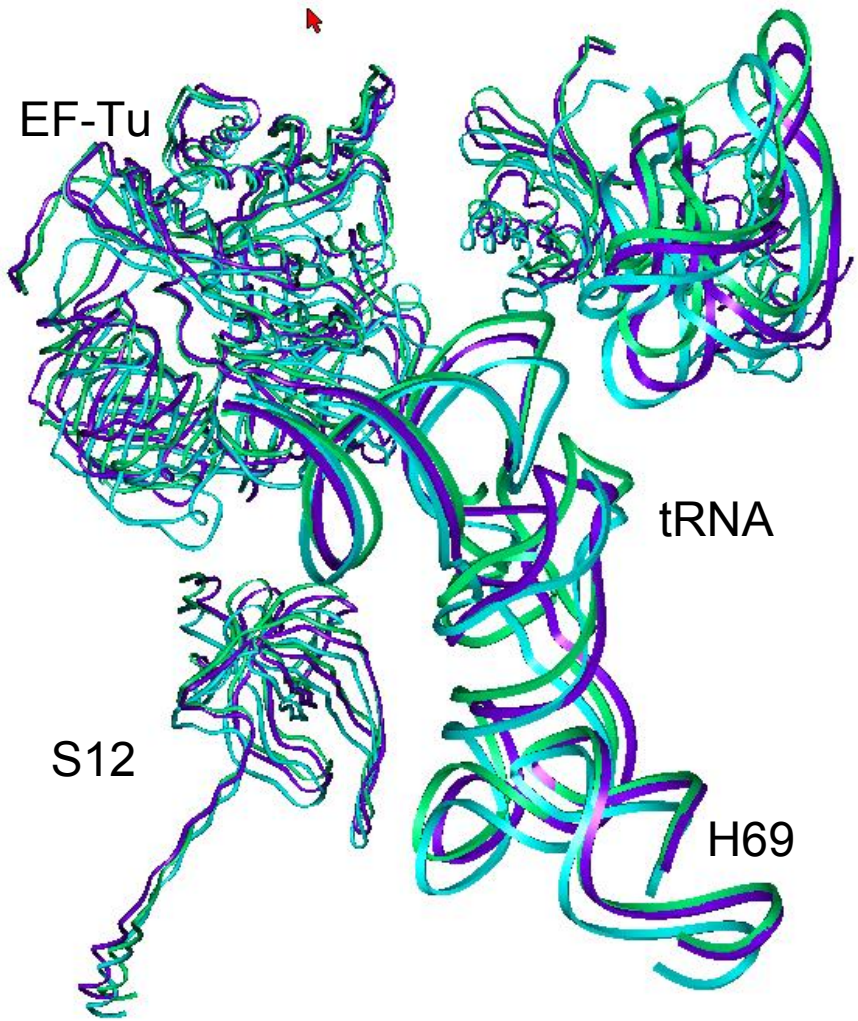


Three aa-tRNA in A/T state -- same ribosome binding sites

Trp

Phe

Leu



Distortion of the anticodon stem loop, apparently instrumental for tRNA selection, kinetic proofreading, and accommodation

- Cryo-EM findings

  [Valle et al., EMBO J. 2002; Stark et al., NSMB 2002; Valle et al., CELL 2003]

- tRNA mutations affecting translation fidelity – “waggle hypothesis”

  [Yarus and Smith, “Transfer RNA” (Eds Soll & RajBhandary) pp. 443-469 (1995)]

- Normal mode analysis of free tRNA produces deformation close to A/T conformation

  [Bahar and Jernigan, J. Mol. Biol. 281 (1998) 871]

- Aaron Klug’s initial predictions of instability in the anticodon arm, based on X-ray structure

  [Robertus et al., Nature 250 (1974) 546; Nucl Acid Res. 1 (1974) 927]

□

Contributors (tRNA A/T)

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Xabier Agirrazabala
Jayati Sengupa (GDPNP complex)
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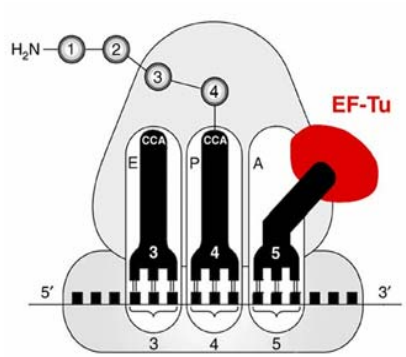

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