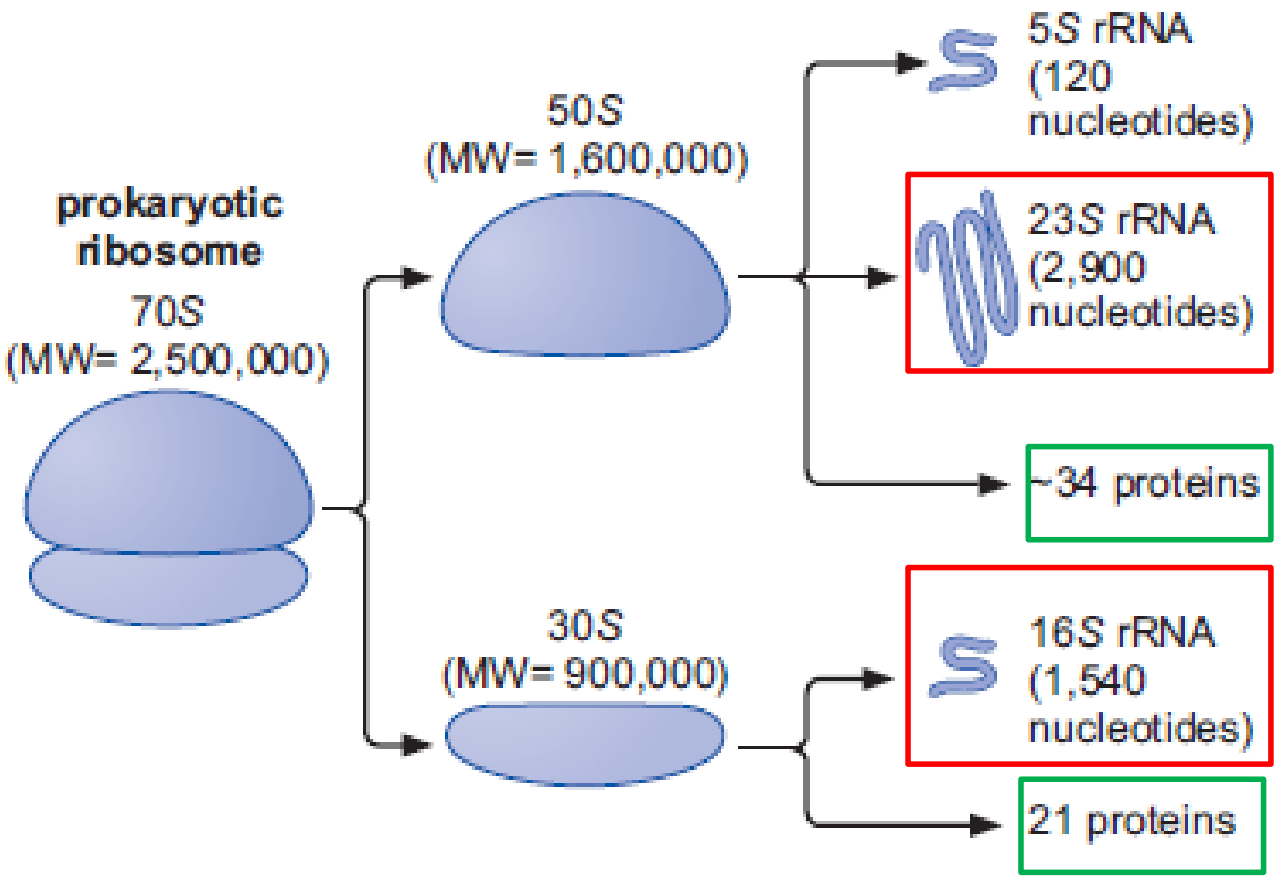
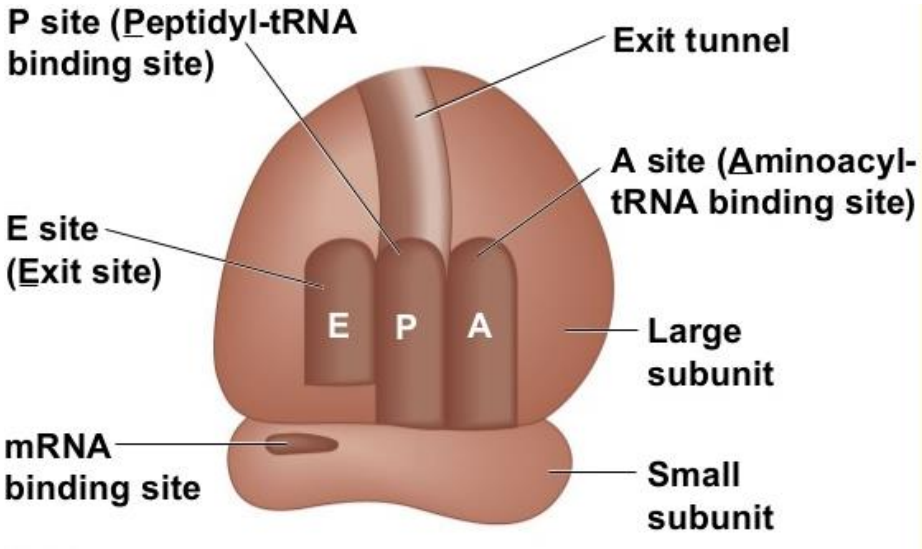
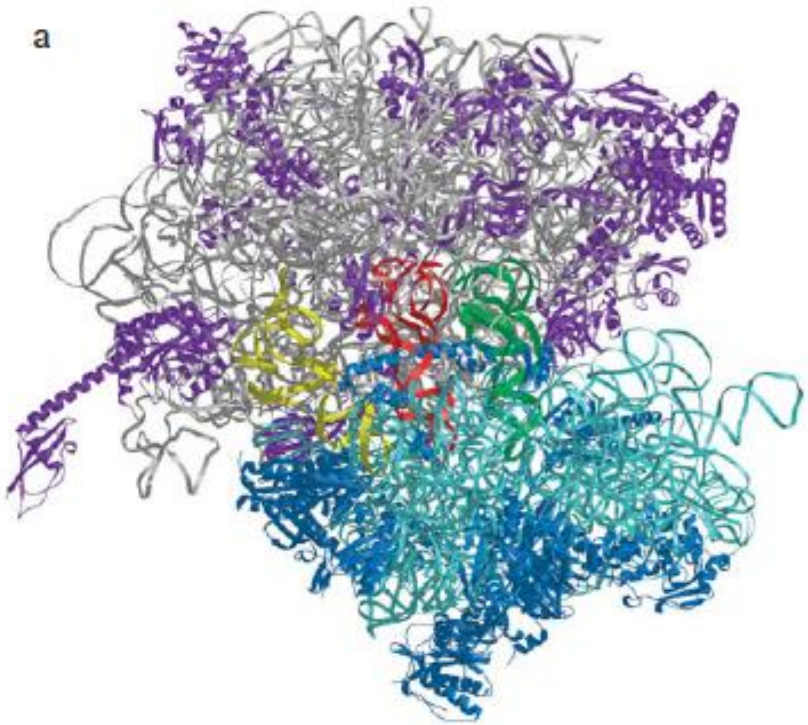
The protagonist of our story: the prokaryotic ribosome



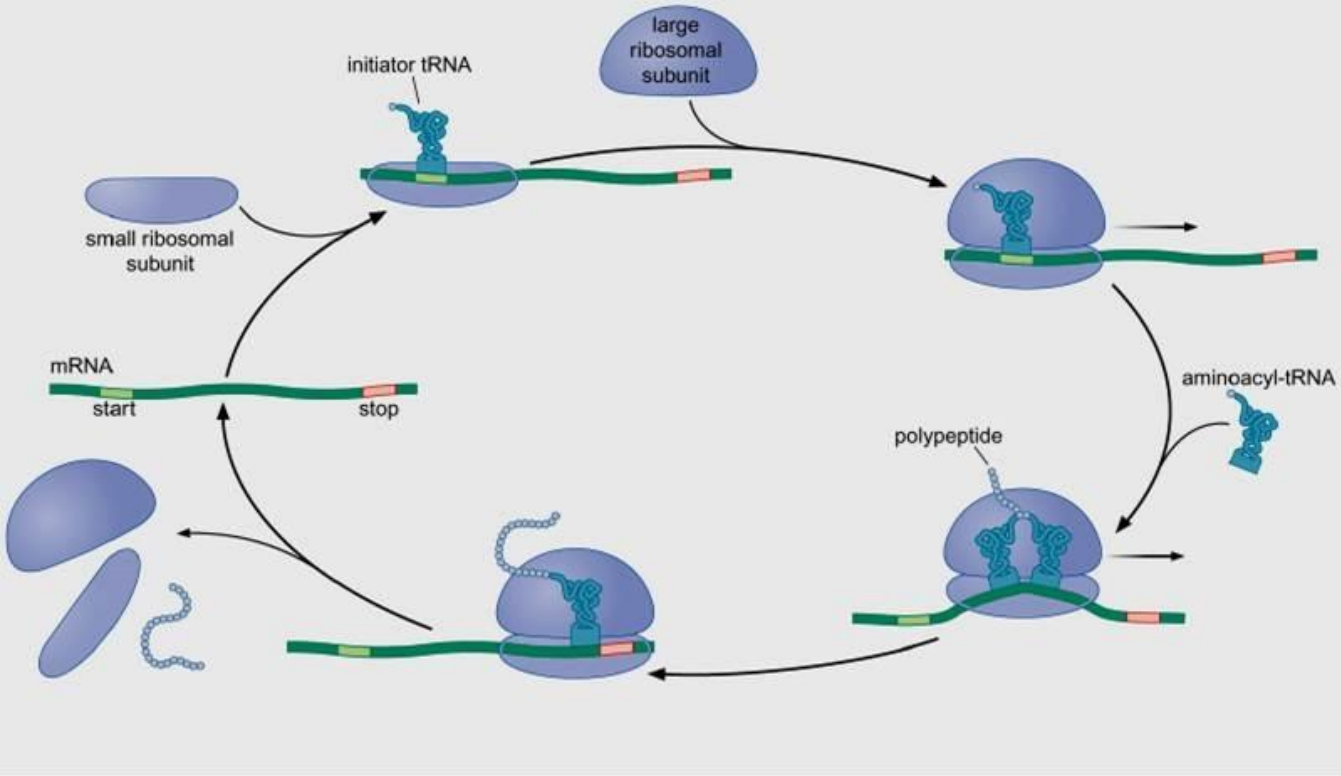




The **core functional domains** of the ribosome (the peptidyl trasferase center and the decoding center) are composed either entirely or mostly from **RNA**

KEY CONCEPT OF MOLECULAR BIOLOGY

Successful expression of the genome requires reversible association and dissociation of the ribosome into individual subunits



Ribosome Cycle

Molecular Biology of the gene, 3° edition

LETTER

doi:10.1038/nature14862

Protein synthesis by ribosomes with tethered subunits

Cédric Orelle[1]†*, Erik D. Carlson[2,3]*, Teresa Szal[1], Tanja Florin[1], Michael C. Jewett[2,3] & Alexander S. Mankin[1]

Nature, August 2015

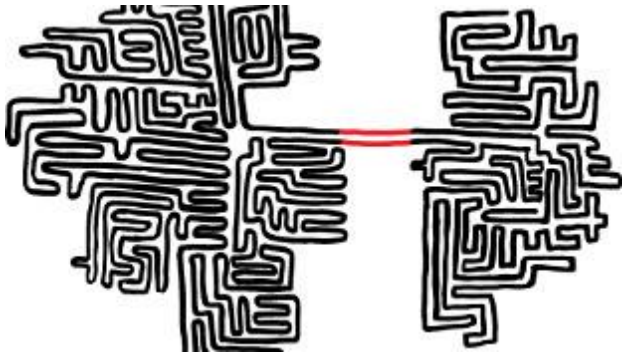
How is it possible to create a ribosome with indissociable subunits?

Dr. Michael J. Hewett

How is it possible to create a ribosome with indissociable subunits?

Dr. Michael J. Hewett

Linkage of 16S and 23S rRNAs in a chimaeric molecule, by the use of short RNA linkers!



Characteristics of a successful chimaeric 16S-23S construct:

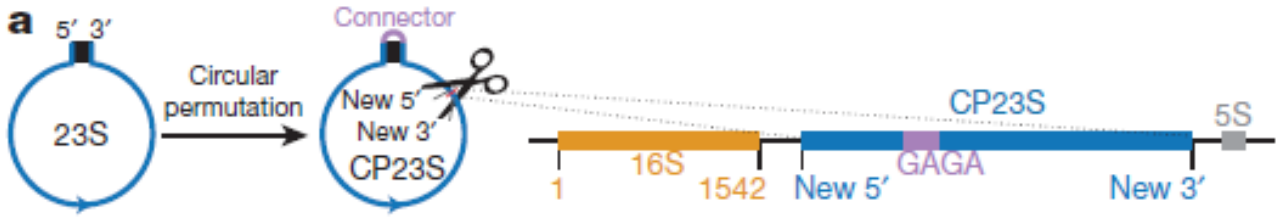
I) Proper interaction with ribosomal proteins and biogenesis factors

II) Able to avoid RNase degradation

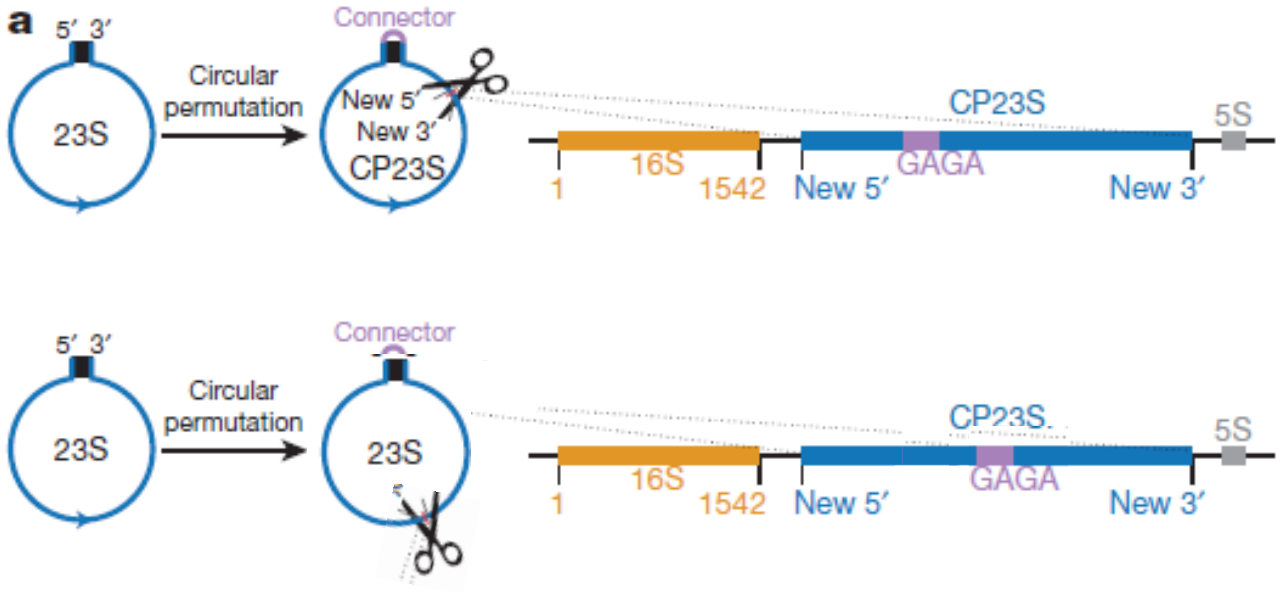
III) Linker sufficiently short to ensure subunit cis-association, yet long enough for minimal interference with subunit movement

Problem! In the native ribosome, the ends of 16S and 23S rRNAs are too far apart to be connected with a nuclease-resistant RNA linker…..

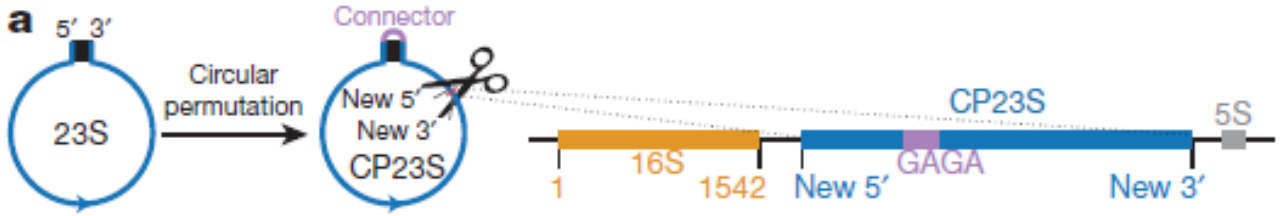
1) Creation of new 23S rRNA ends

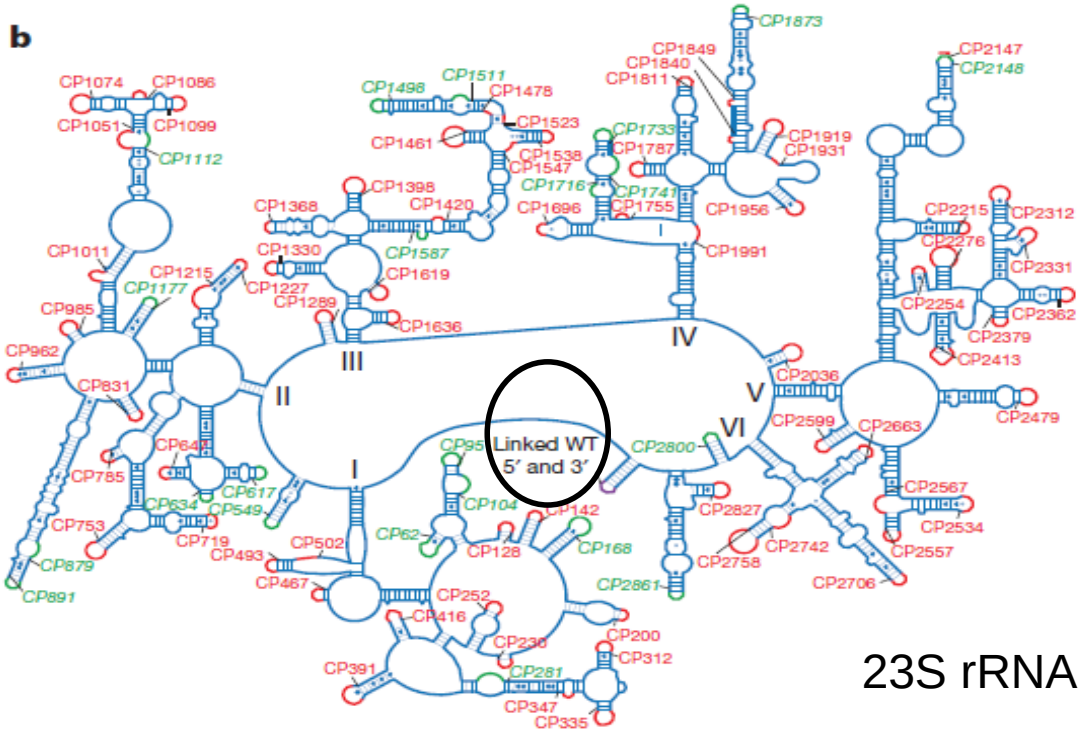


1) Creation of new 23S rRNA ends



1) Creation of new 23S rRNA ends





23S rRNA

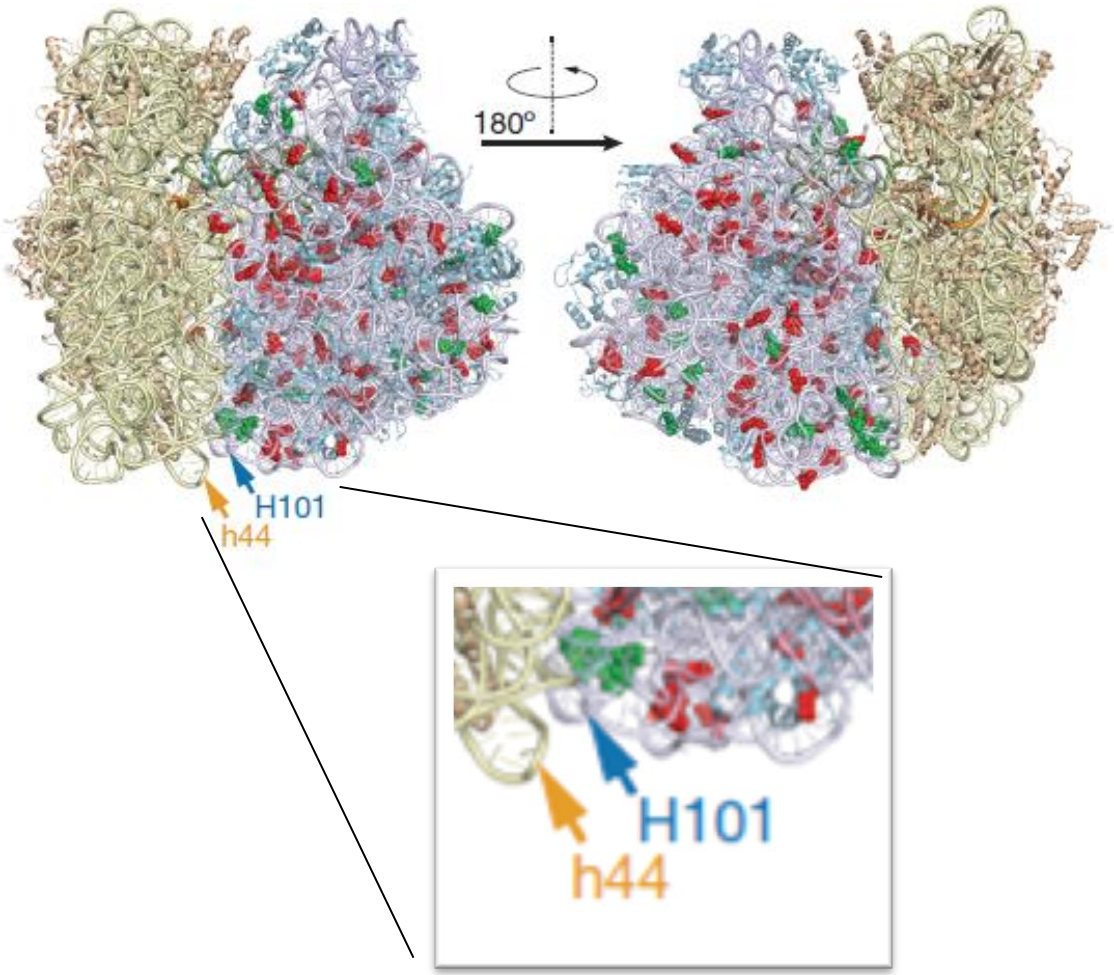
Collection of 91 circularly permutated 23S (CP23S) rRNA mutants with new ends placed at nearly every hairpin

Introduction of the CP23S constructs in a particular strain of ***E.Coli***, which lack chromosomal rRNA alleles but have a plasmid carrying the wild-type rRNA operon

How many of the 91 mutants were able to replace the resident plasmid and support *E.Coli* proliferation?

22

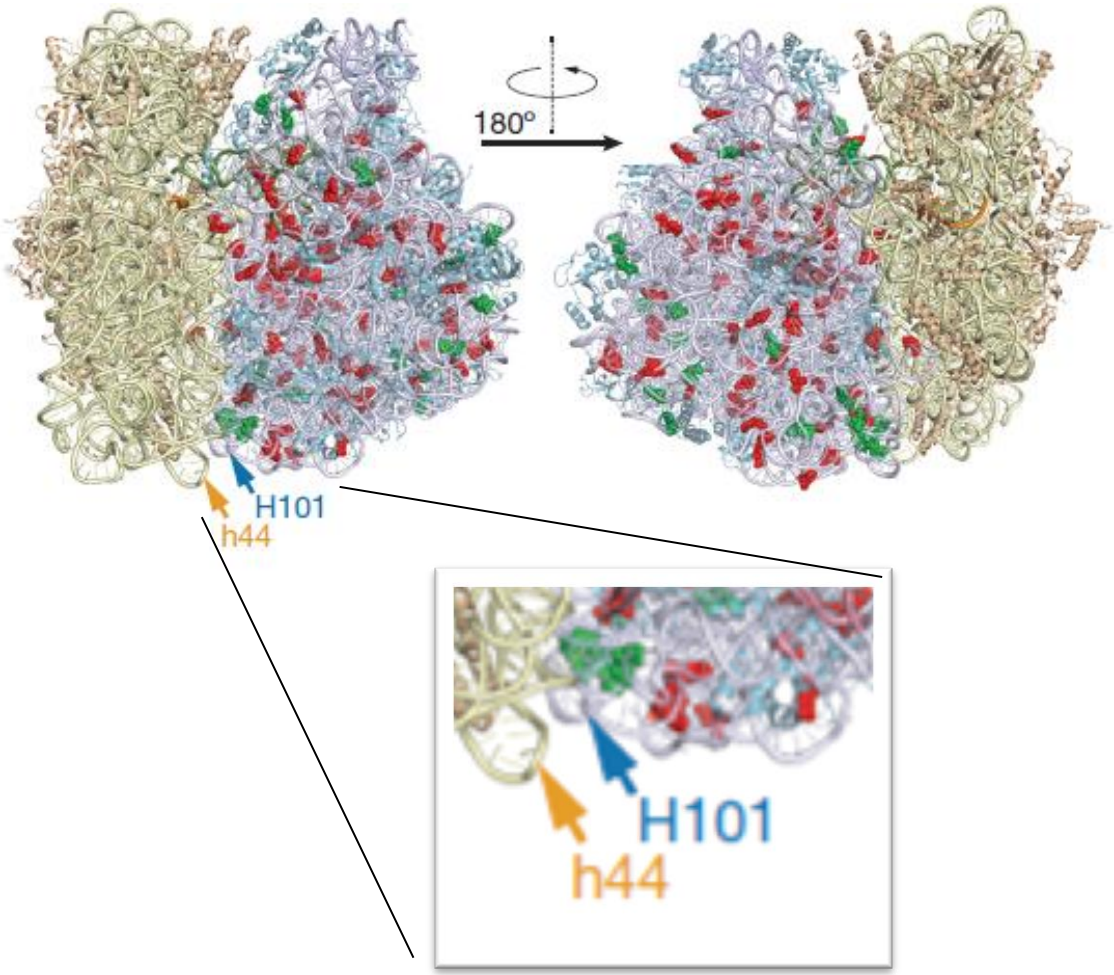
2) Linkage of 23S and 16S rRNAs by short RNA linkers



One of the 22 viable mutants had a peculiar characteristic:

h44 loop is not conserved and can tolerate mutations

2) Linkage of 23S and 16S rRNAs by short RNA linkers

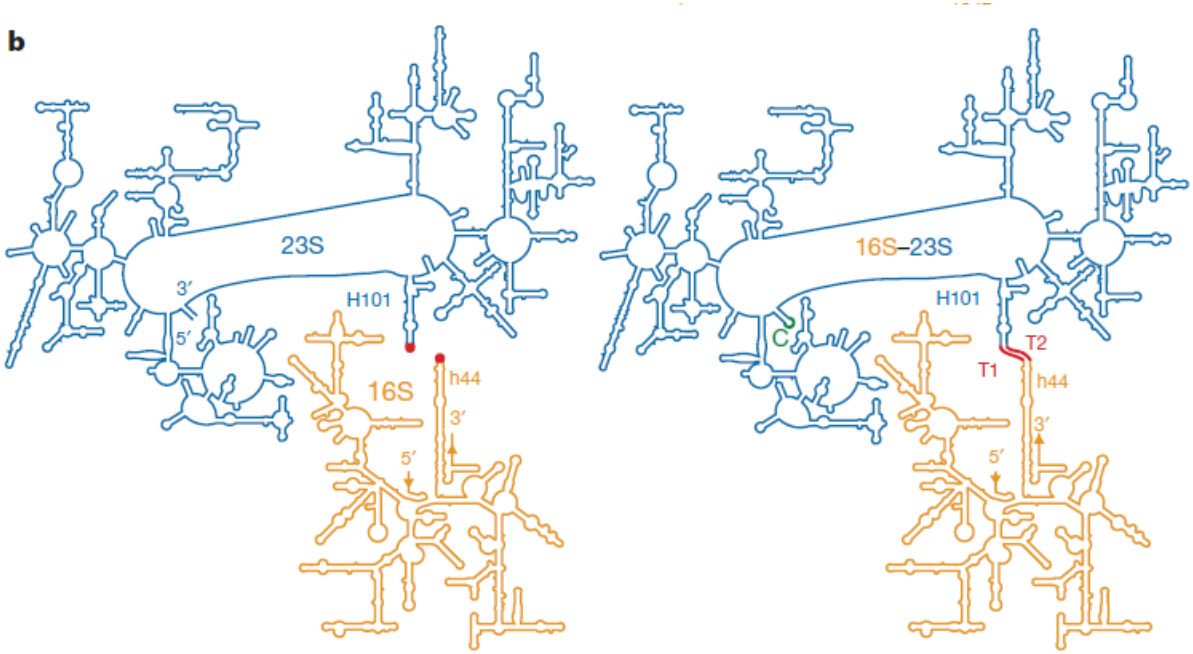


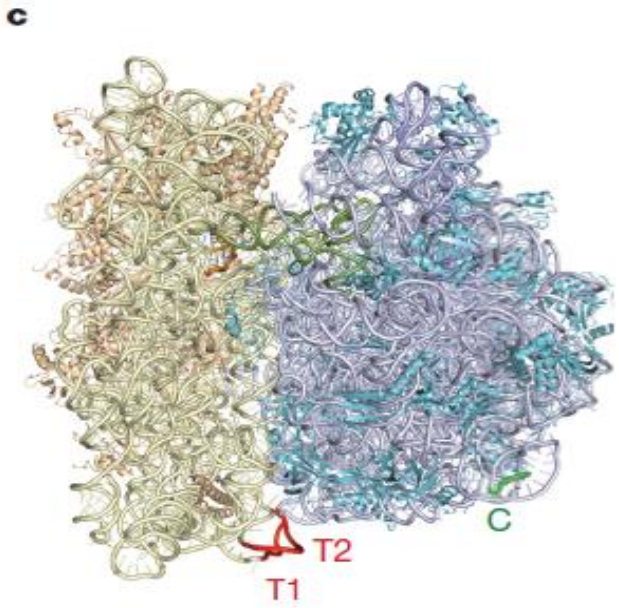
One of the 22 viable mutants had a peculiar characteristic:

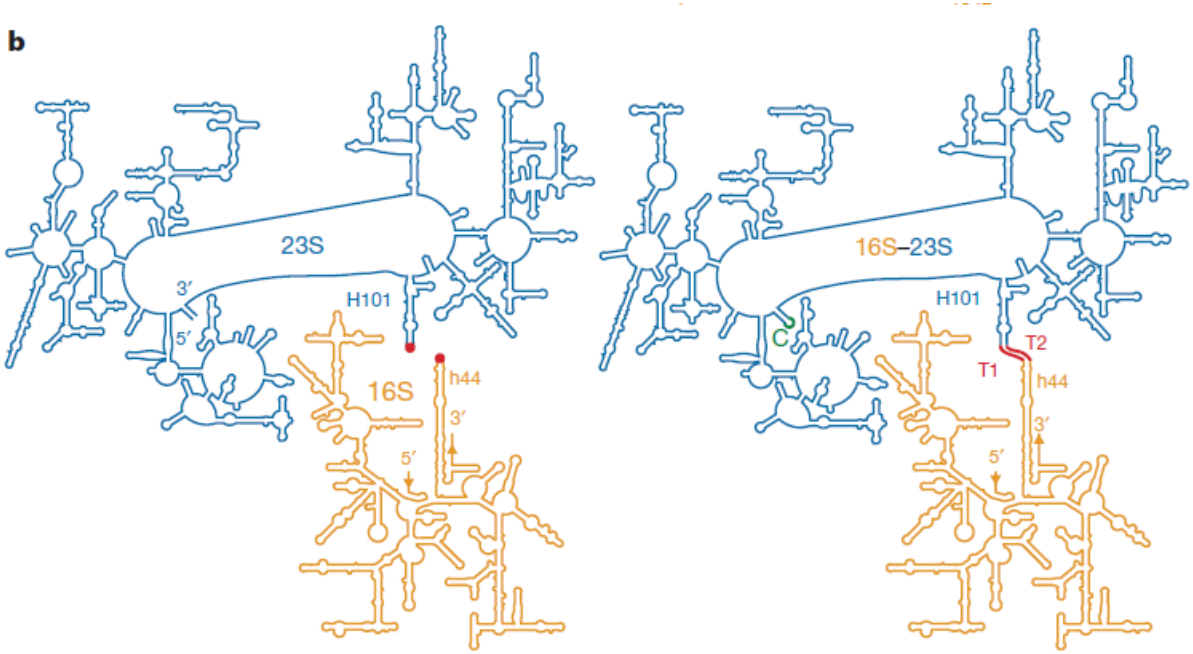
h44 loop is not conserved and can tolerate mutations

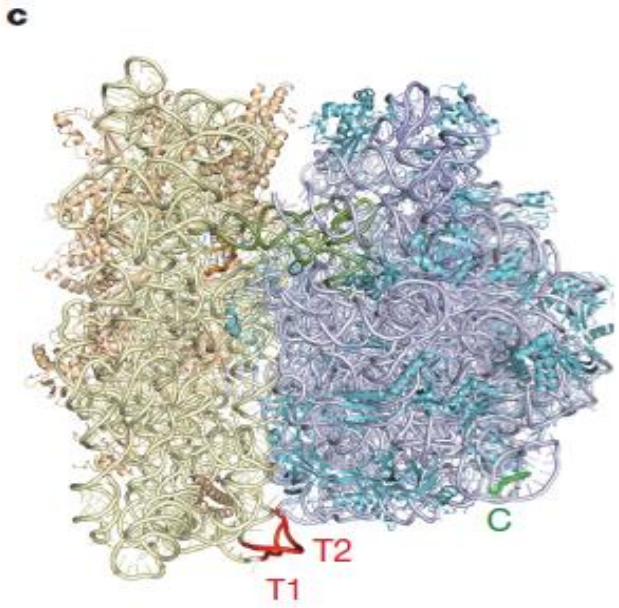
….we found a good site for the linkers!

2) Linkage of 23S and 16S rRNAs by short RNA linkers









Dr. Mankin describes this as “two different people holding hands.” He explains, “We have created ribosomes that can’t let go of their hands.”

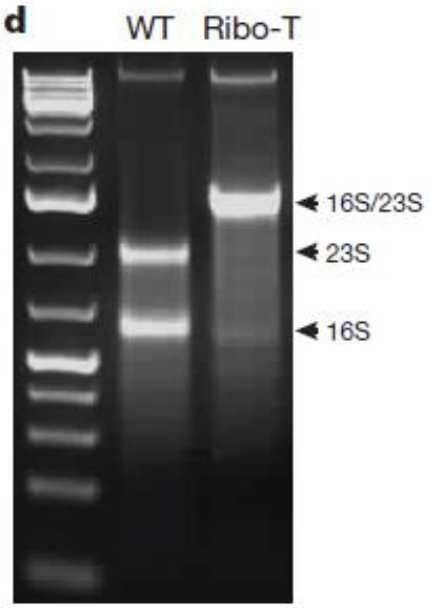
Introduction of the **contructs with T1-T2 linkers** in *E.Coli*, as before…..

Introduction of the **contructs with T1-T2 linkers** in *E.Coli*, as before…..

VIABLE CLONES!!

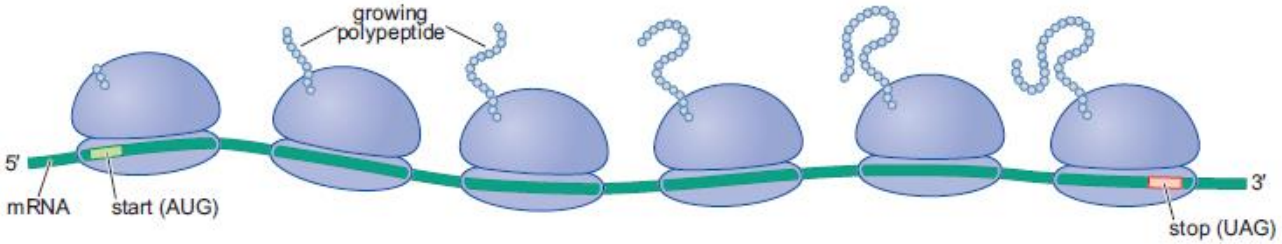
3) Characterization of RiboT *in vivo*

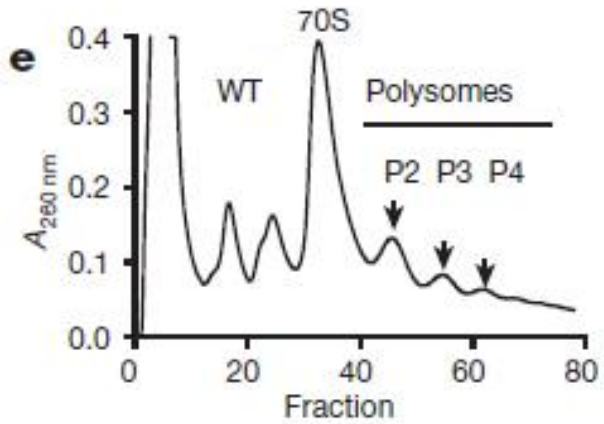
Analysis of <u>RNA</u> extracted from WT or RiboT clones

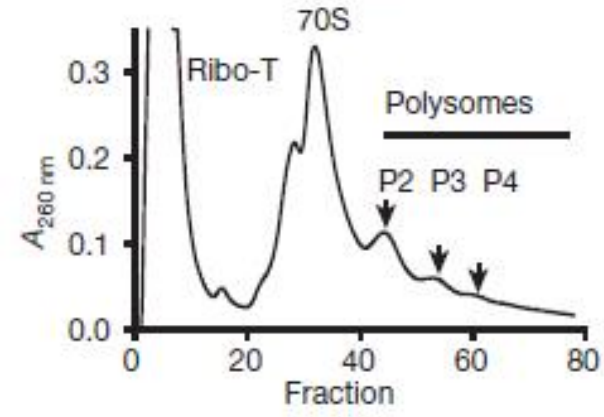


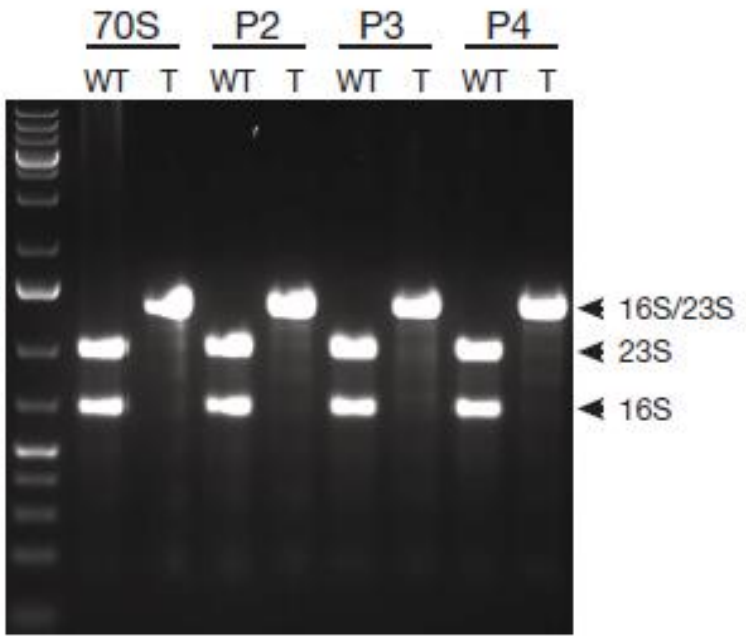
First evidence that the bipartite nature of ribosomes is <u>dispensable</u> for succesful protein synthesis and CELL VIABILITY

Analysis of polysomes

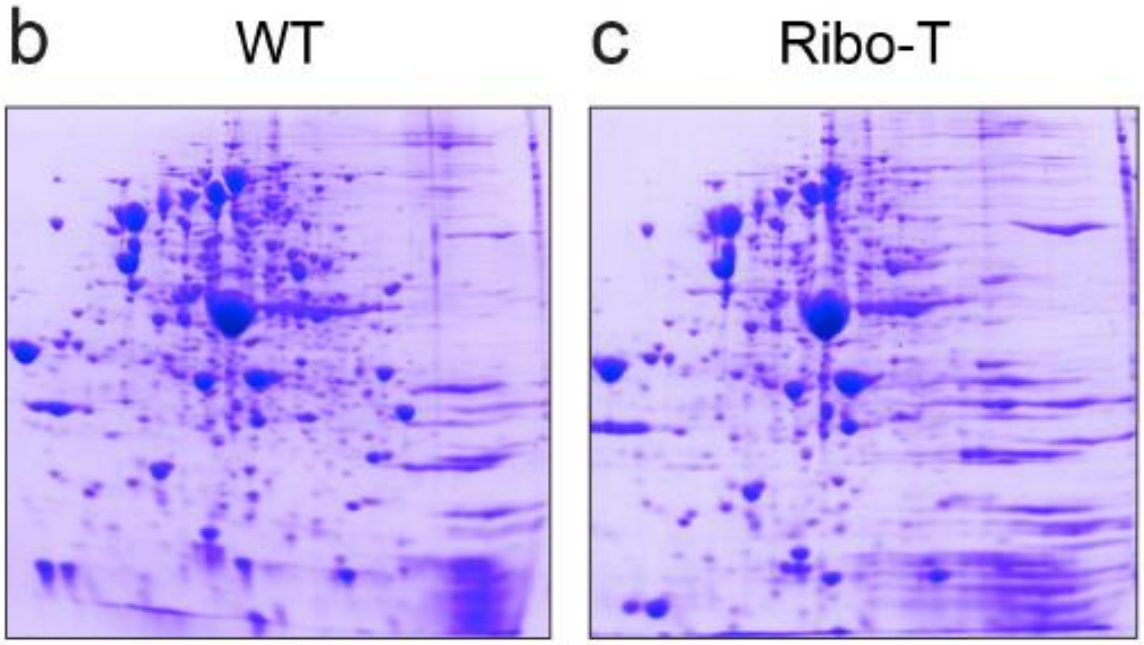








Proteome expression by 2D gel analysis

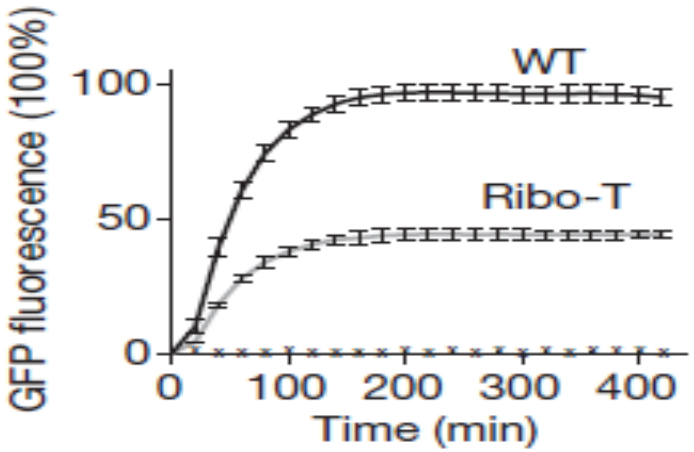


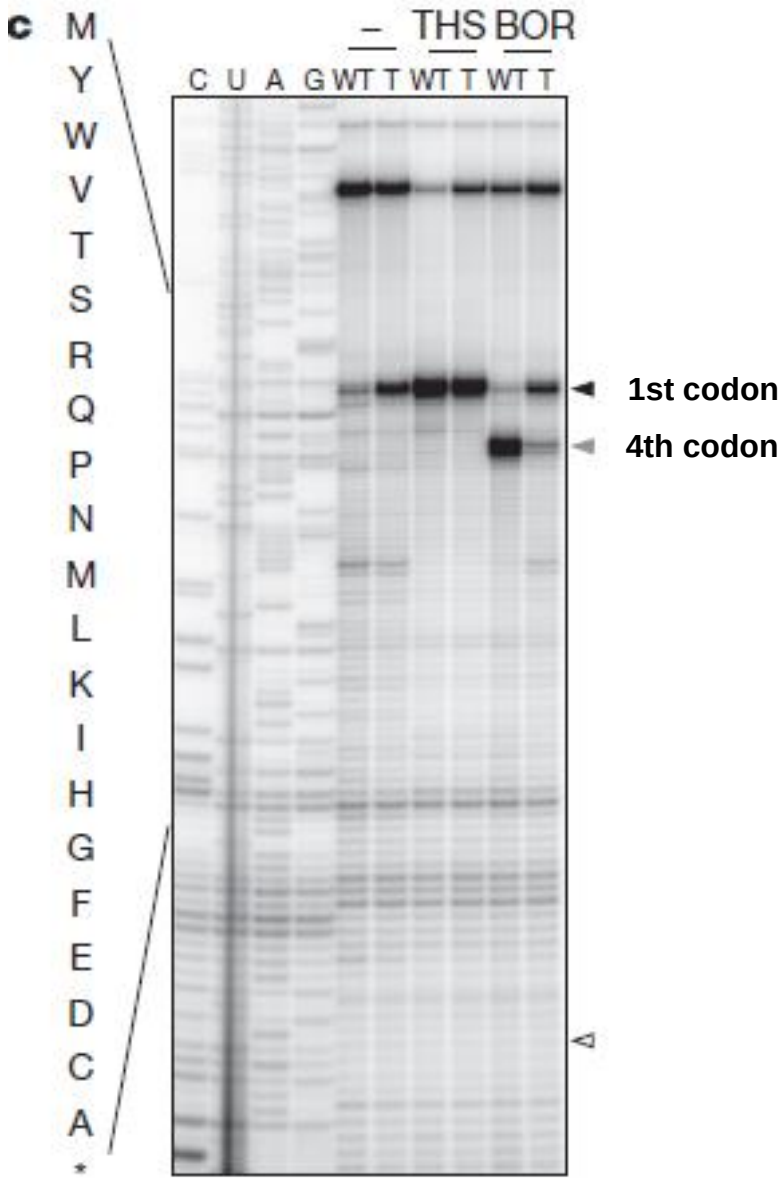
4) Characterization of RiboT *in vitro*

Activity of RiboT tested in the PURExpress translation system lacking native ribosomes

↓

The rate of RiboT protein synthesis reaches approximately **45% of that of the wild-type ribosomes……why?**



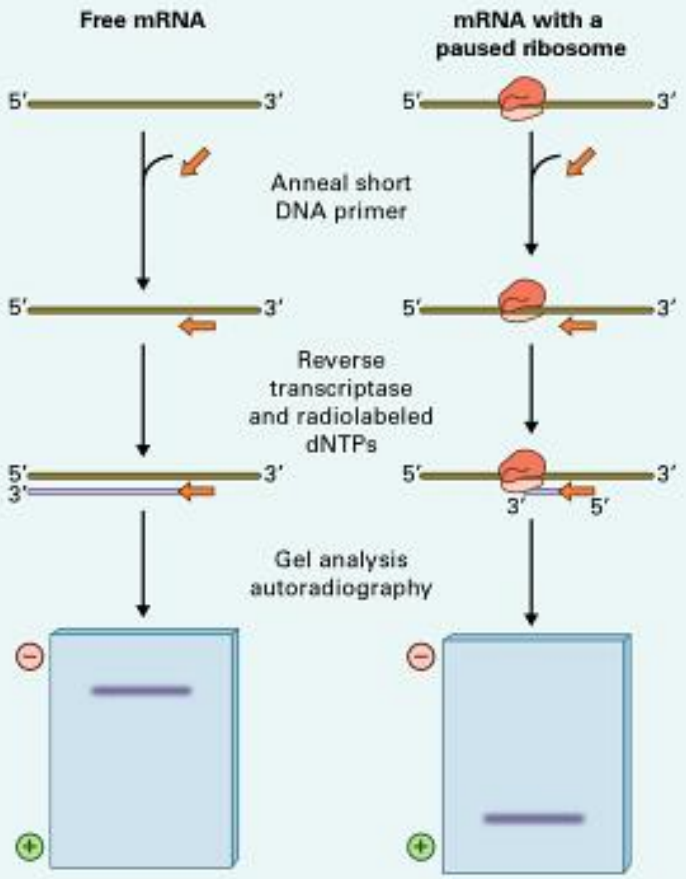


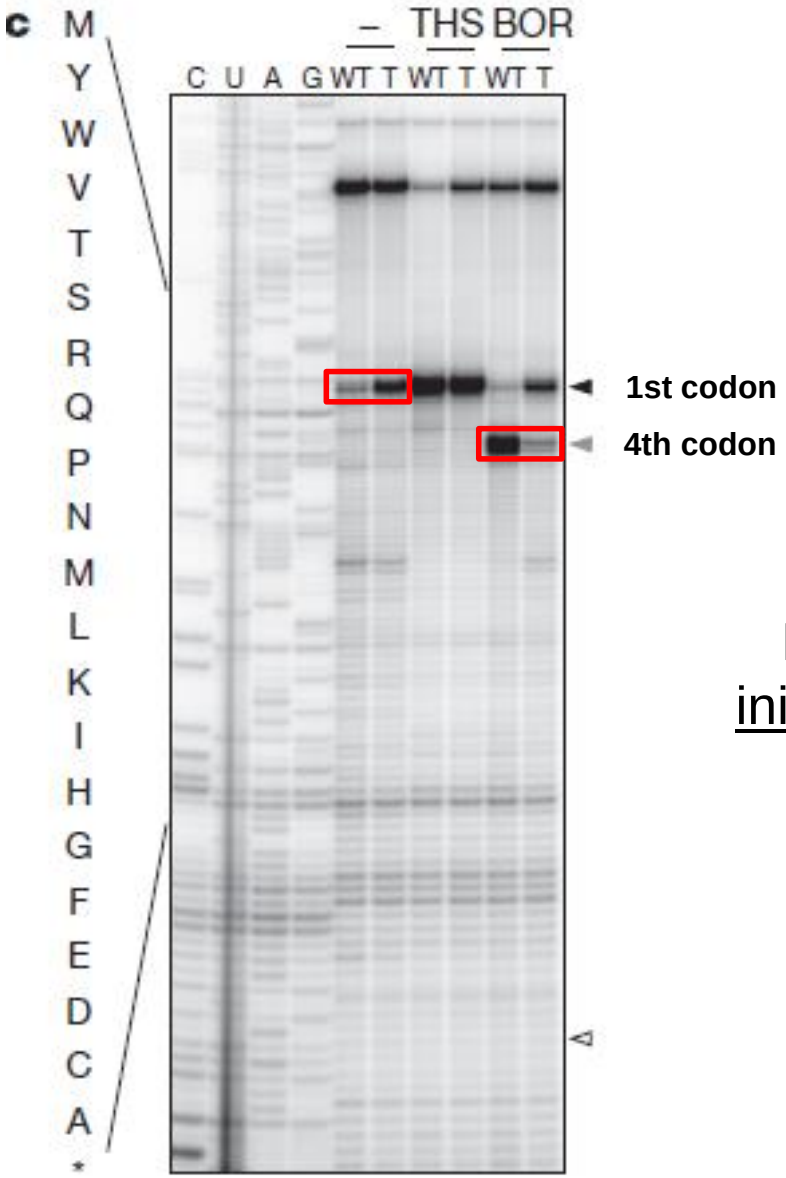
Toeprinting analysis

BOX 10.7

Toeprinting can be used to determine where ribosomes pause during protein synthesis

The positions where ribosomes pause on an mRNA can be determined by mapping the edge of the ribosome on the 3′ side of the region of the mRNA covered by the ribosome. In this procedure, known as **toeprinting**, mRNAs with ribosomes bound at the stalled position are isolated, and a short DNA primer is hybridized to the mRNA downstream from the predicted pause site. Reverse transcriptase is used to synthesize a cDNA from the mRNA/primer complex. When no ribosomes are present on the mRNA, the reverse transcriptase can potentially copy the mRNA all the way to the 5′ end; however, if a ribosome is present between the site where the primer is annealed and the 5′ end of the mRNA, the progress of the reverse transcriptase is blocked. When it reaches the edge of the ribosome, the enzyme stops and dissociates from the mRNA, creating a shorter cDNA that locates the leading edge of the ribosome.





Toeprinting analysis

RiboT is impaired in translation initiation at a step subsequent start codon recognition

Okay, let’s stop for a moment….
RiboT is a fantastic molecule: a ribosome with tethered subunits, that is able to substain the expression of an entire genome!
That’s incredible! So, what now?

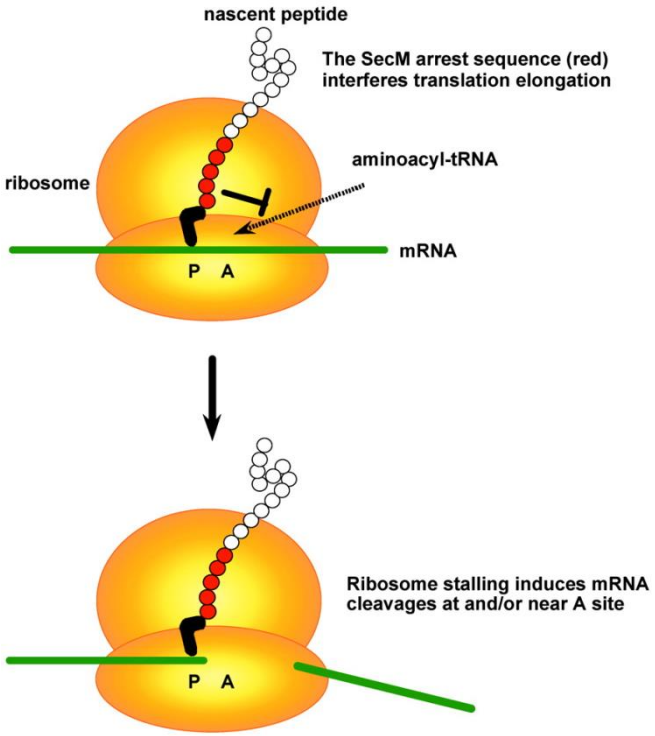
Okay, let’s stop for a moment….
RiboT is a fantastic molecule: a ribosome with tethered subunits, that is able to substain the expression of an entire genome!
That’s incredible! So, what now?

We can do even better than this!!!

Dr. Michael J. Hewett

5) Evolvability of RiboT to identify gain-of-function mutations that facilitate synthesis of problematic protein sequences

The model: SecM polypeptide presents a classic example of an amino acid sequence for which translation is problematic for the ribosome



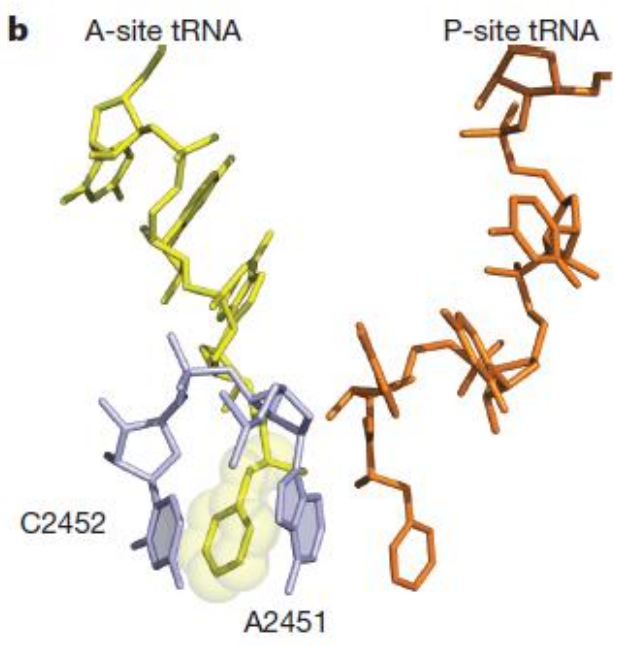
Programmed **ribosome stalling** at the Pro166 codon of *SecM*.

Translation arrest ensues because specific interactions of the SecM nascent chain with the ribosomal exit tunnel impair the PTC (Peptydil-Trasferase Center) function, preventing the transfer of the 165-amino-acid long peptide to the incoming prolyl transfer-RNA (Pro-tRNA).

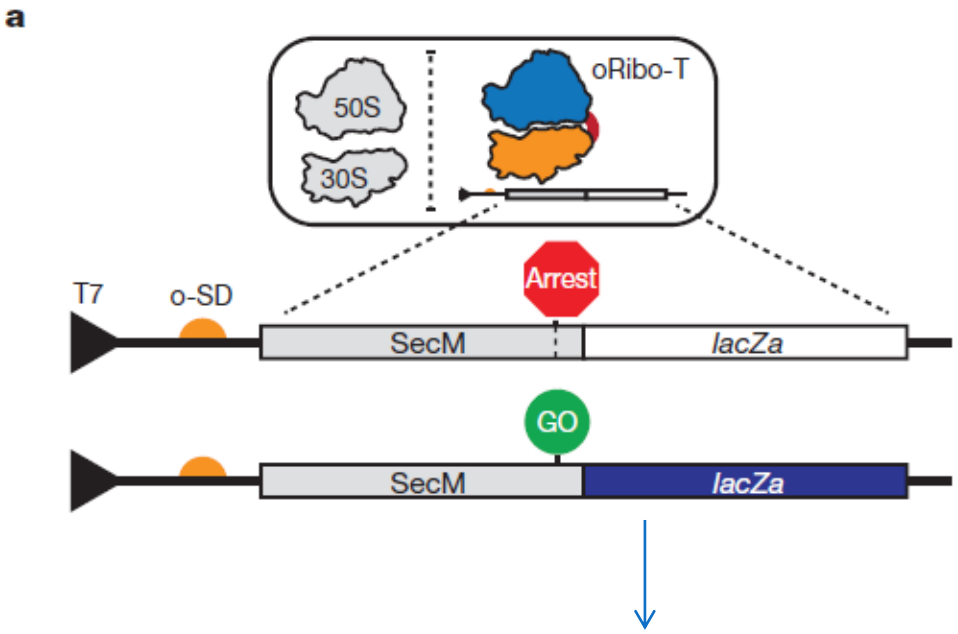
rRNA residues in the PTC-A site have been proposed to **have a key role** in the mechanism of ribosome stalling.

Mutations in the PTC A-site are **dominantly lethal in wild-type ribosomes**.
But what about **Ribo-T**?

Library of mutations at two 23S residues (A2451 and C2452), that form the amino acid binding pocket in the PTC A-site



Reporter gene encoding the SecM arrest sequence fused in frame with *lacZ* gene



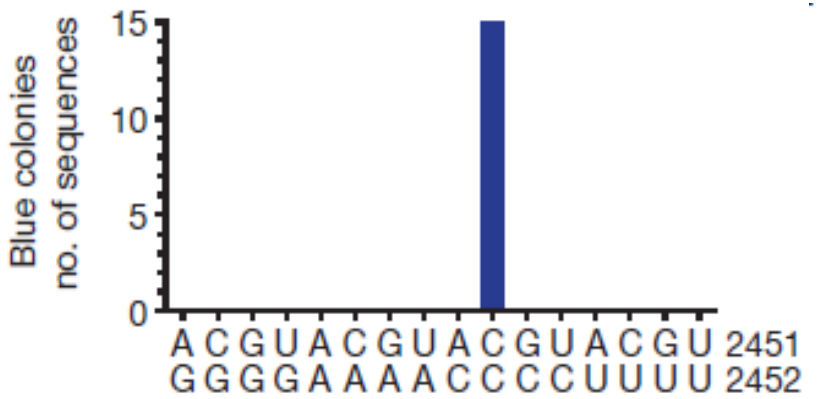
Blue colonies

5) Gain of function mutations in PTC A-site of RiboT

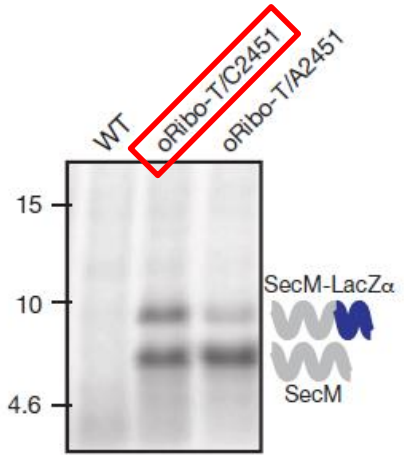
E.Coli transformed with the reporter *SecM* and the RiboT mutant library……

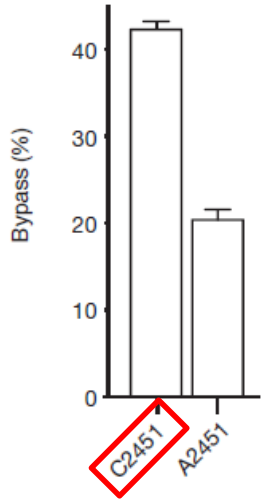
E.Coli transformed with the reporter *SecM* and the RiboT mutant library……

Blue colonies!



Validation *in vitro* of the discovered role of A2451C mutation in the mechanism of SecM translation arrest





RiboT with mutations in the PTC A-site (normally lethal!)

- were functional in cellular protein synthesis
- **gained the ability** to bypass translation arrest caused by the *SecM* sequence

Conclusions

- Revision of one of the key concepts of molecular biology: reversible association and dissociation of ribosomal subunits is not essential in order to succesfully express the entire genome

- Ribosome with inseparable subunits (**RiboT**) are able to substain the **expression of entire bacterial genome**

- RiboT can be used for **studying** in cells **mutations** of functionally crucial rRNA residues that are **dominantly lethal**

- Future prospects of **engineering ribosomes** capable of programmed polymerization of unnatural amino acids and backbone-modified analogues

“A lot of people consider the ribosome to be the chef of translation and so one of the things we’re curious to know now is if you have the ability to make specialized chefs, chefs that make different types of cuisines, what kind of chefs would you make? Put another way, could we evolve the ribosome to perform new types of chemistry?”