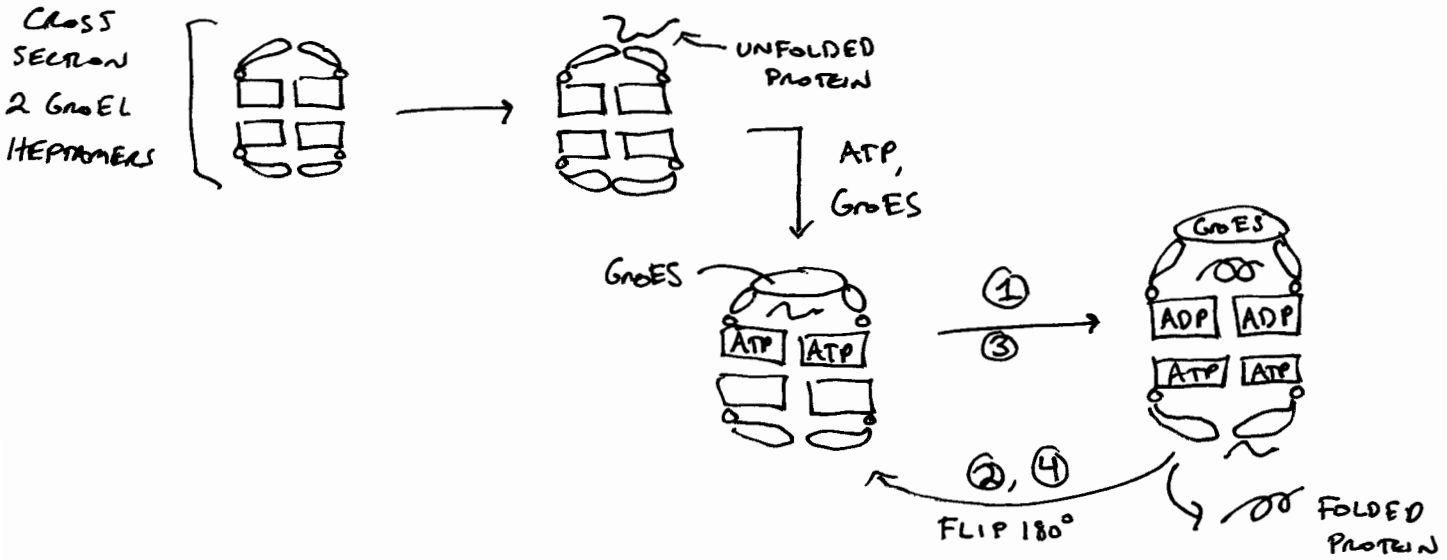
TD 11
Identification of *in vivo* substrates of GroEL
Nature 1999, 402, 147, Hartl et al
Techniques: immunoprecipitation, pulse-chase labeling, 2D gels, mass-spec

I. Questions to be addressed:
-What proteins use GroEL to fold *in vivo*?
(*in vitro*, nearly everything appears to interact with GroEL)
-What comon properties do these proteins have?
-What is the timescale and nature of their interaction with GroEL? (rapid, slow? single? repeated?)

II. Background (*in vitro* working model)
Diagrams are cross-sections showing 2 GroEL heptamers (each monomer is 57kDa)
First, weak, hydrophobic interactions cause association of GroEL and unfolded protein
Then, ATP binds (weakens protein association), and GroES traps protein
1) ATP hydrolysis, then protein and ATP bind to the lower half
2) GroES, ADP and folded protein dissociate from top half, then GroES caps lower half
3) ATP hydrolysis, then protein and ATP bind to the top half
4) GroES, ADP and folded protein dissociate from bottom, then GroES caps top




-Probably hydrophobic interactions mediate initial docking of unfolded protein onto ATp-less GroEL
-The protein has about ~10s to fold while trapped inside the GroEL/GroES chamber, before ATP hydrolysis triggers its release (dependent on rate of ATP hydrolysis)
-The chamber can accommodate proteins up to ~60kDa
-A single protein may need to go in and out of the chamber many times before getting to final correctly folded state
-the reaction is dependent on ATP and GroES

III. Overall Approach

-Treat E.coli (bacteria) w/ $^{35}$S-methionine for 15 sec to label only newly synthesized protein (synthesis rate 10-20 amino acids per second -> can label 150-300 amino acid segments)

-Lyse bacteria with **non-denaturing** detergent (key)

-Add anti-GroEL antibody to immunoprecipitate associated proteins

-Run 2D gel to look at charge and molecular weight distribution ($^{35}$S-visualize)

-excise spots, trypsin digest and Maldi mass spec to ID the proteins

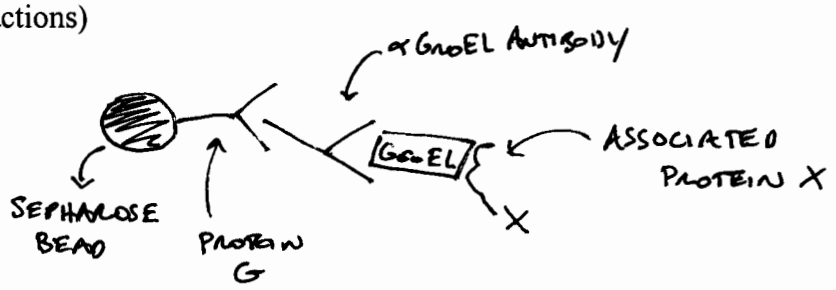
IV. Technique 1: Immunoprecipitation (IP)

- A way to detect stable protein-protein interactions within complex mixtures

Strong interactions survive mild cell lysis conditions

* Use EDTA in lysate to chelate $Mg^{2+}$; prevents ATP binding to GroEL and release of substrate proteins

1) Add anti-GroEL anitbody (will stick to GroEL, GroEL is stuck to substrate protein)
2) Add protein G sepharose beads; incubate (protein G binds the constant region of GroEL antibody)
3) spin down beads; rinse to get rid of all other protein
4) elute GroEL and associated proteins off beads with 8M urea and detergent (break all interactions)




V. Technique 2: Pulse chase labeling

* To determine how long GroEL substrate proteins are remaining bound to GroEL

Protein synthesis in *E.coli* is completely unsynchronized, bit if you add $^{35}$S-methionine (radiolabeled amino acid building block) for 15 sec, you can label all proteins that are synthesized during only that 15 sec interval

[$^{35}$S]-methionine (15 sec) -> lyse cells (+EDTA) -> IP with anti-GroEL
This will detect only newly synthesized proteins that interact with GroEL

How to determine if protein substrates interact for short or long time with GroEL? Use a chase with unlabeled methionine:

[$^{35}$S]-methionine (15 sec) -> wash out -> methionine (10 minutes, unlabeled) -> lyse cells (+EDTA) -> IP with anti-GroEL

This will detect only newly synthesized proteins that are still bound to GroEL, 10 minutes after synthesis

Examples:
Protein shows up w/ pulse but disappears after 5 minute chase = This is a GroEL substrate, must interact < 5 min

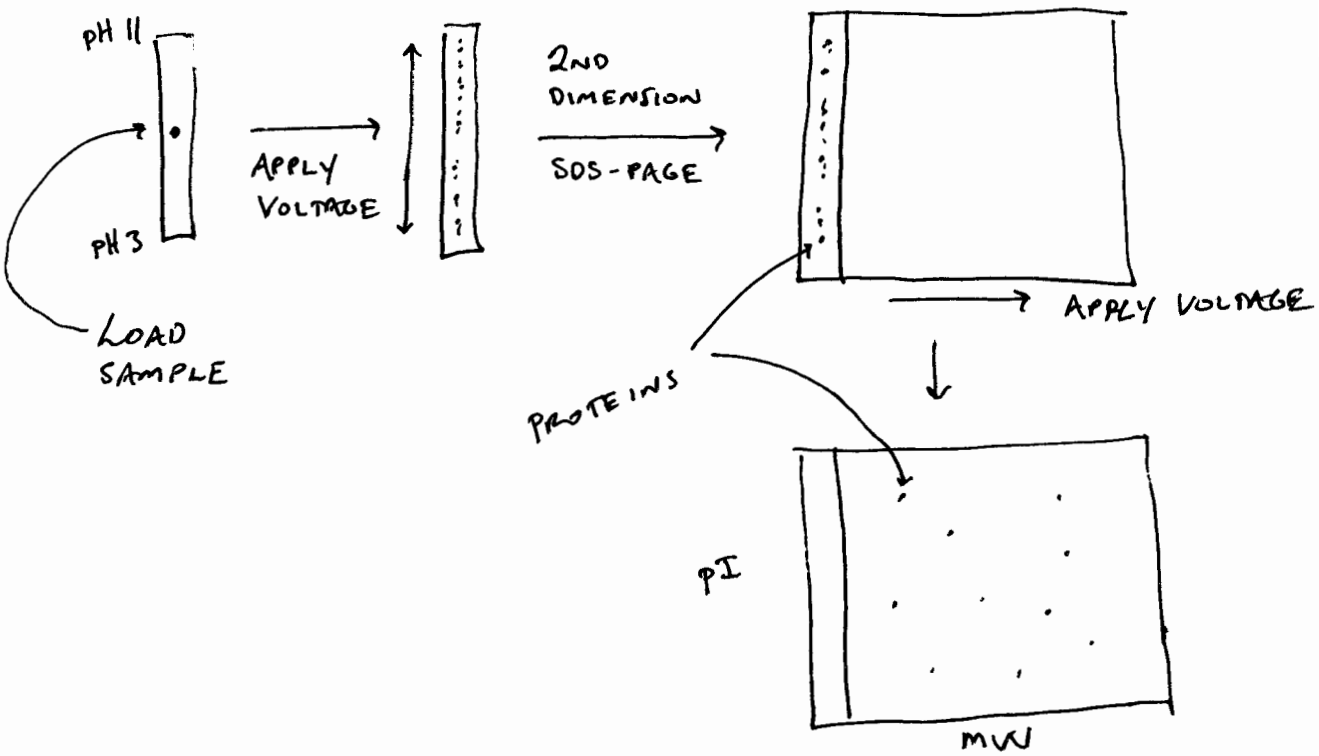
Protein shows up & still remains after 10min chase= This is a GroEL substrate, stays bound to GroEL 10 min or longer after synthesis

VI. Technique 3: 2D gels

* To resolve protein on the basis of 2 physical properties, usually charge and molecular weight (MW)

Review TD 7 Gel electrophoresis (especially SDS-PAGE gels and IEF gels)
remember SDS-PAGE separates based on MW only
IEF separates based on charge

IEF = 1str dimension
1. Load sample onto middle of strip with pH gradient
2. Apply voltage- separates based on charge
3. place strip at top of SDS-page gel, and apply voltage in 2nd direction
4. proteins are now separated in 2 dimensions based on isoelectric point (charge) and MW



VII. Technique 4: Trypsin digest and MALDI mass spec

*To determine the identity of individual protein spots on 2D gel

1) punch out single hot spot from gel (can increase the amount of protein at spot by co-running w/ cold crude bacterial lysate-> there is more of the same, unlabeled protein at that spot for analysis)
2) extract protein from gel
3) complete digest with trypsin protease (cleaves C-terminal to Lys and Arg)
4) MALDI mass spec on fragments -> match collected mass to sequence and ID protein

MALDI= matrix-assisted laser desorption/ionization
matrix of light absorbing organic molecules
ion flight time gives mass to charge ratio (m/z)
-commonly used for obtaining mass of macromolecules (peptides, DNA, and other polymers)
-soft method; doesn't fragment sample
-compare to ESI (used in Walsh study from TD3)
-better accuracy w/ ESI
-MALDI generally easier for high MW samples
-MALDI requires smaller sample size
-Both are used commonly for proteomics

VIII. Identification of GroEL *in vivo* substrates
See Nature 1999, 402, 147, Hartl et al

Figure 1
Figure 1a shows 35S images of newly synthesized proteins on 2D gel
This is a control of 2,500 labeled cytoplasmic proteins in lysate before IP (4,300 proteins in genome- not all are expressed during 15 min labeling, and some are insoluble)
Figure 1c shows after anti-GroEL IP
now there are only 250-300 spots – 10% compared to control (indicates proteins that interact with GroEL), notice the predominance of larger proteins
Figures 1e and f show how pI (isoelectric point) and MW compare to total proteins
-pI distribution is about the same as control
Most GroEL substrates are greater than 20kDa and most are less than 60kDa
Figures 1 c and d when compared show the duration of interaction with GroEL (fig 1d shows anto-GroEL IP after 10 minute chase)
About ½ of the proteins are gone after 10 min chase (these proteins interact with groEL less than 10 minutes)

Figure 2
Figure 2a is an example of analysis of release kinetics of individual proteins
The y axis shows % bound to GroEL and the x-axis is the time of the chase
Analysis shows that
2/3 of <60kDa proteins are released between 20sec and 2 minutes
~100 proteins <60kDa remain associated for >10 minutes
several proteins >60kDa are released very slowly

Figure 4
Mass spec ID of GroEL substrates
shows 2D gel with interacting proteins circled. These proteins were cut out of gel and identified
Common feature: Most contain 2 or 3 domains with alpha/beta folds with extensive hydrophobic surfaces (expected to fold slowly and be prone to aggregation)
No common role of these proteins within the cell (they are involved in a wide variety of functions/pathways)