Methods for Studying the Cell Cycle

cell fusion
live and fixed imaging

genetics
biochemistry
in vitro systems

inhibitors of cellular processes
(transcription, replication, microtubules)
Genetic Screens: Yeast ‘Cell Division Cycle’ (CDC) Mutants

‘Dominos’
sequential, dependent events

‘Oscillator’
central controller

can individual protein mutations block steps or whole process?
Lee Hartwell (cerevisiae); Paul Nurse (pombe)

Goal: find mutants unable to transit the cell cycle

Why yeast?
- Cell shape --> cell cycle stage
- Grow as haploids (easier to find mutants), or diploids (can do genetics)

Problem:
- the screen is for cells that can't grow

Solution:
- temperature sensitive mutants
- Replica plating
**In vitro Dissection of the Cell Cycle - Xenopus Egg Extracts**

**A. Tightly packed eggs**
- Xenopus eggs
- Mineral oil
- Centrifuge hard

**B. Eggs crushed by centrifugation**
- Oil and lipid
- Extract
- Pellet

**C. Added sperm nucleus with membrane removed**

**D. Reassembly of nucleus**

**E. DNA replication**

**F. Mitosis**
- Add Ca^{2+} to trigger exit from mitosis

use to isolate proteins present at particular stages
manipulate proteins-deplete and observe changes to cell cycle
Lecture 2
Introduction to the Cytoskeleton

Outline:
Composition of the cytoskeleton
Polymer Dynamics in theory
Polymer Dynamics in cells

Paper: Identification of pathways regulating cell size and cell-cycle progression by RNAi

Paul Nurse “Controlling the Cell Cycle” !!!
Thu 4 PM, 100 GPBB
Roles of the Cytoskeleton

**Structural scaffold** - cell shape, spatial organization

**Dynamic assemblies** - movement and force production:
- cell migration
- cell division
- intracellular traffic
- contraction

Cytoskeletal functions often involve motor proteins
3 major elements of the cytoskeleton

microtubules

α/β tubulin dimers

25 nm diameter

relatively stiff –
hollow, 13 protofilaments
3 major elements of the cytoskeleton

microfilaments = actin filaments

actin monomers
7 nm diameter
more flexible – 2 helicities
3 major elements of the cytoskeleton

**Intermediate filaments**

- 10 nm diameter
- Fibrous – resistant to shear forces
- Structural – prominent in cells
- Subject to mechanical stress
- Vimentin, nuclear lamins
Introduction to polymer dynamics: 3 cases

1) simple equilibrium polymers

2) polar polymers: asymmetric subunits undergo conformational change during assembly

3) complex polymers: non-equilibrium subunit nucleotide hydrolysis (energy input)

actin and microtubules
assembles & disassembles by addition & loss of subunits at ends

rates = $K_{on}$ and $K_{off}$

$K_{on}$ depends on concentration of subunit, units of $M^{-1}sec^{-1}$

$K_{off}$ does not (unimolecular), units of $sec^{-1}$
Polymer assembly timecourse

1) lag due to kinetic barrier to nucleation
2) growth
3) equilibrium

Polymer grows as time proceeds
subunit concentration drops until $K_{on}[C] = K_{off}$

$[C] = \text{critical concentration } C_c \ (M^{-1}\text{sec}^{-1}[M] = \text{sec}^{-1})$
$K_{on}[C] > K_{off}$

$K_{on}[C] = K_{off}$

$K_{on}[C] < K_{off}$
Critical Concentration

Equilibrium constant $K_{eq}$ determined by change in free energy between free subunits and polymer

$$K_{eq} = \frac{K_{on}}{K_{off}} = \frac{1}{C_c}$$

Concentration of free subunits at which rate of subunit addition = rate of loss

Above $C_c$ net growth, below net shrinkage
Polar Polymer

Two ends polymerize and depolymerize at different rates BECAUSE subunit conformation changes as it incorporates into the polymer.
Plus and minus ends

different $K_{on}$ and $K_{off}$

BUT!

$K_{off} / K_{on}$ ratio or Cc must be the same for both ends:

> the same interactions are broken when a subunit dissociates from either end

> the final state of the subunit is identical

If the plus end grows 3 times faster it must also shrink 3 times faster.

above Cc both ends grow, below Cc, both shrink
Complex Polymer (non-equilibrium): microtubules, actin filaments

due to nucleotide hydrolysis upon assembly of subunit into polymer

D = nucleotide diphosphate
T = nucleotide triphosphate

nucleotide hydrolysis reduces binding affinity
complex polymer properties

internal subunits have different dynamic properties than the ends

T form binds, D form dissociates

\[ K_{\text{Ton}} >> K_{\text{Don}} \quad \text{and} \quad K_{\text{Doff}} >> K_{\text{Toff}} \]

\( C_{\text{ss}} = \) “steady state” concentration

\[ K_{\text{Ton}}[C] = K_{\text{Doff}} \]

\[ C_{\text{ss}} = \frac{K_{\text{Doff}}}{K_{\text{Ton}}} \]
no longer true equilibrium, rather **steady state**
because
ATP or GTP subunits must be replenished

\[ D = \text{diphosphate} \]

\[ T = \text{triphosphate} \]
GTP-tubulin dimer

GTP exchangeable GTP

straight protofilament

GTP Hydrolysis changes subunit conformation and weakens bond in the polymer

curved protofilament

Depolymerization

GDP-tubulin dimer

GDP-GTP exchange
Consequences for polymer dynamics

treadmilling (actin and microtubules)

two different reactions at each end of the polymer

critical concentration different

$\text{Cc}(- \text{end}) > \text{Cc}(+ \text{end})$
Treadmilling

both ends exposed

**Steady state** occurs at concentration between $C_c$ (- end) and $C_c$ (+ end)

**net assembly at the plus end**

**net disassembly at the minus end**

subunits “flux” through the polymer
Treadmilling
Dynamic instability (microtubules)

subunit addition is faster than nucleotide hydrolysis

\[ \text{CAP of GTP-tubulin on polymer ends} \]
\[ K_{\text{Doff}} >> K_{\text{Toff}} : \text{GTP CAP favors growth} \]

GTP CAP present: growth
GTP CAP lost: rapid disassembly

stochastic (unpredictable) transitions

frequency correlates with tubulin concentration
rapid growth with GTP-capped end

accidental loss of GTP cap  CATASTROPHE

rapid shrinkage

regain of GTP cap  RESCUE

rapid growth with GTP-capped end

e tc.
Classic experiments by Mitchison and Kirschner 1984

Microtubules nucleated from seeds - no lag

1) Determine steady state concentration ($C_{ss}$) = 14 µM
2) dilution experiment:
grow microtubule seeds
dilute into tubulin solution above or below \( C_{ss} \)
wait 10 minutes
measure Mt number-concentration, Mt length
(spun onto EM grids)

<table>
<thead>
<tr>
<th>Concentration: ( x10^8/ml )</th>
<th>before dilution</th>
<th>15 uM</th>
<th>7.5 uM</th>
</tr>
</thead>
<tbody>
<tr>
<td>#concentration:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>before dilution</td>
<td>32</td>
<td>32</td>
<td>15</td>
</tr>
<tr>
<td>average length: (( \mu M ))</td>
<td>18</td>
<td>40</td>
<td>22</td>
</tr>
</tbody>
</table>

size is dependent on the concentration of tubulin
Dynamic instability in vitro
Microtubules are really tubes, not simple polymers.
Breakage in middle breaks one bond.

Removal from one end breaks one bond.

Single protofilament: thermally unstable.

Multiple protofilaments: thermally stable.
Summary

**simple equilibrium:**
exchange only at the ends
turnover only with dramatic changes in subunit concentration

**non-equilibrium:**
Dynamic instability
Treadmilling
complete and rapid polymer turnover at steady state
energy required
Polymer properties regulated in cells

1) nucleation
2) polarity
3) dynamics

1) **Nucleation**: kinetic barrier - slow step

- monomer
- dimer
- trimer = nucleation site

*trimer for actin*
*more complex for microtubules*
The graph illustrates the mass of filaments over time. It is divided into three stages:

1. **Nucleation**: The mass of filaments increases rapidly, indicating the formation of nuclei.
2. **Elongation**: The mass continues to increase, but at a slower rate, as the filaments elongate.
3. **Steady state**: The mass levels off as the system reaches a steady state.

Two curves are shown:

- **+ nuclei**: This curve represents the mass of filaments with nuclei.
- **− nuclei**: This curve represents the mass of filaments without nuclei.
Nucleating factors in cells

**Microtubules:** centrosomes

**Actin:** protein complexes (Arp2/3)
2) Polarity: due to asymmetry of subunits

structural polarity of polymer lattice

visualized by decoration of actin filaments and microtubules

allows cell to generate asymmetric structures and shapes
basis of motility
Actin lattice polarity revealed

Actin decorated with myosin subfragment 1
actin structural and dynamic polarity revealed
Microtubule lattice polarity revealed by hook direction

Microtubules decorated and viewed in cross section
microtubule dynamic polarity revealed
Motor proteins recognize polymer asymmetry

**Mechanochemical enzymes**
- hydrolyze ATP to move along filament
- produce force or carry cargo

**Polarity generates directionality**

**More on motors in later lectures ...**
3) dynamics

regulated by many cellular factors

end-capping
subunit sequestering
polymer binding proteins

regulators also regulated
MT dynamics in *pombe*

role in nuclear positioning and deformation

- GFP-tubulin
- nup107-GFP
- 5-s intervals
- 8 min total time
- 60 X realtime.

movement of kinesin motors on growing and shrinking MTs
MT dynamics in vertebrate cells

impact of destabilization of actin
Dynamic Instability

growing

shrinking

Catastrophe

Rescue