Mechanisms of Cell Proliferation

Nongenotoxic carcinogens

Normal cells

DNA damage

Initiated cell

DNA replication

Pre-neoplastic focus

Proliferation

Tumour

DNA damaged cells deleted

Cells in developing focus deleted

Tumour cells deleted

Apoptosis

Apoptosis

Apoptosis

Nongenotoxic carcinogens
• Multi-cellular organisms depend on cell division/proliferation;
  
• Each organism has a developmental plan that determines its behavior and properties;
  
• Differentiation gives rise to populations of cells which specialize in specific functions;
  
• Almost every cell population in the adult multi-cellular organism is specified by its lineage and environment;
  
• Within the mature organism, cells refrain from exerting their intrinsic potential to grow and divide beyond territories and patterns laid down in the overall developmental plan.

Normal:

• **Growth factor dependence**: proliferation depends on availability of tissue-type specific **growth factors**, which are **signals, not nutrients**. In many cases, factor withdrawal leads to apoptosis.
  
• **Anchorage dependence**: proliferation requires interaction of transmembrane proteins called **integrins** with components of the extracellular matrix (**ECM**) components. Specific integrins recognize specific ECM molecules.
  
• **Contact inhibition**: contact with **like** cell types inhibits **cell movement** and **proliferation**. **Contact inhibition of growth** limits division in culture when cells form a contiguous monolayer. **Contact inhibition of movement** affects the cytoskeletal organization and motility of cells in a monolayer. Contact with **unlike** cells allows motility and hence spontaneous cell sorting.
  
• **Limited proliferation capacity**: vertebrate somatic cells divide a limited number of times (ca. 50-70 divisions for human cells) before the cells enter a senescent state that maintains metabolic activity but stops all further division.
Disruption of normal cell proliferation:
- due to -- mutant alleles inherited from parents,
  -- somatic mutations arising in the organism,
  -- epigenetic changes which alter expression levels of key genes

- **Immortalization and aneuploidy**: diploid cells grown to the point of
  senescence sometimes give rise to clonal lines that survive and grow
  continuously beyond normal limits;

- **Partial or complete loss of growth factor dependence**: transformed
  cells may gain the ability to grow on less rich serum, or at lower initial
  cell density;

- **Loss of contact inhibition of growth**: transformed cells may
  overgrow monolayers and pile up onto each other (foci);

- **Loss of anchorage dependence**: Cells may grow on soft agar or in
  suspension rather attached to a substrate;

- **Loss of contact inhibition of movement**: Transformed cells maintain
  a motile phenotype, which may be a consequence of failure to respond
  properly to cell-cell adhesion signals.

For human fibroblasts, after 50-70 divisions, cells enter a state of
replicative senescence in which cells
are metabolically active, but cease to proliferate. The immediate cause is a strong block to cell cycle
progression and entry to S phase, mediated by cyclin kinase inhibitors (CKI) such as p16\(^{INK4A}\) and p21\(^{CIP1}\).
Cells can be forced to bypass senescence by suppression of the pRB and p53 replication regulators, e.g.
by the action of viral oncogenes such as SV40 large T or adenovirus E1A. Cells thus forced to continue to
divide reach a second proliferative block known as replicative crisis, characterized by drastic
chromosomal instability, leading almost invariably to cell death.
The limit of some 50-70 division cycles for human diploid fibroblasts is mediated by telomere length. The
telomere is an extension of DNA at chromosome ends, generated by the telomerase reverse
transcriptase, (TERT), which uses an internally bound RNA loop as a template. The replication process
terminates before the end of the lagging strand, and telomeres thus shorten with each division unless
maintained by telomerase. Telomerase is active in germline cells, but inactive in somatic cells. Telomere
length correlates with age of cells in culture, and with age in the organism.
Cells reach senescence when the short telomeres trigger the protective mechanisms of p53, which
stimulates the CKIs to halt further cell cycle progress. Cells reach crisis when telomeres are lost,
exposing chromosome ends, and provoking the double strand repair mechanism to make inappropriate
attempts at recombination and ligation
In some cases, immortalized cells maintain telomeres by reactivating telomerase, and maintain relatively
stable chromosomes. However, a significant proportion of immortalized cells are viable in the absence of
telomerase, and use a less well characterized process alternative maintenance of telomeres (ALT).
CDKs and their role in cell-cycle:
During G1, the levels of G1 cyclins rise, and these cyclins associate with cyclin-dependent kinases (CDKs). Activity of G1 CDKs promotes the passage of cells through START (budding yeast), also known as the restriction point (R) in fission yeast and higher eukaryotes. After passing through this point, a cell is committed to continue through the cell cycle.
Three-layer regulation of the cell cycle.

Cell-cycle control can be described as a 3-layer process. The immediate phenomena of the cell cycle, including DNA synthesis and chromosome separation (layer a), are qualitatively controlled by phosphorylation. Movement through the cycle (layer b) depends on the activity of cyclin-dependent kinases (CDKs), which are promoted by accelerators — cyclins — and antagonized by brakes — CDK inhibitors (CKIs). The protein levels of cyclins, CKIs and many other cell-cycle-related regulators are quantitatively controlled by ubiquitylating enzymes (layer c).

Nakayama & Nakayama, Nat Rev Cancer 2006

### Major Cell Cycle Regulatory Proteins

**Protein Kinases and Protein Phosphatases that modify CDKs:**
- Cdk-activating kinase (CAK): phosphorylates an activating site in Cdkks
- Wee1 kinase: phosphorylates inhibitory sites in Cdkks, primarily involved in controlling entry into mitosis
- Cdc25 phosphatase: removes inhibitory phosphates from Cdkks, three family members (Cdc25A, B, C) in mammals. Cdc25C is the activator of Cdkks at the onset of mitosis

**CDK Inhibitory Proteins (CKIs):**
- Sic1 (yeast): suppresses Cdk activity in G1, phosphorylation by Cdk1 triggers its ubiquitination
- p27 (mammals): suppresses G0/S Cdk and S-Cdk activities in G1, helps cells to withdraw from cell cycle when they terminally differentiate; phosphorylation by Cdk2 triggers its ubiquitination by SCF
- p21 (mammals): suppresses G0/S Cdk and S-Cdk activities following DNA damage in G1, transcriptionally activated by p53
- p16 (mammals): suppresses G0/Cdk activity in G1, frequently inactivated in cancer

**Ubiquitin Ligases and their Activators:**
- SCF: catalyzes ubiquitylation of regulatory proteins involved in G1 control, including CKIs (Sic1 in budding yeast, p27 in mammals); phosphorylation of target protein usually required for this activity
- APC: catalyzes ubiquitylation of regulatory proteins involved primarily in exit from mitosis, including Securin and M-cyclins; regulated by association with mitotic substrates
- Cdc20: APC-activating subunit in all cells; triggers initial activation of APC at metaphase-to-anaphase transition; stimulated by M-Cdk activity
- Hct1: maintains APC activity after anaphase and throughout G1, inhibited by Cdk activity

**Gene Expression Regulatory Proteins:**
- E2F: promotes transcription of genes required for G1/S progression, including genes encoding G1/S cyclins, S-cyclins, and proteins required for DNA synthesis; stimulated when G0-Cdk phosphorylates Rb in response to extracellular mitogens
- p53: promotes transcription of genes that induce cell cycle arrest (especially p21) or apoptosis in response to DNA damage or other cell stress; regulated by association with Mdm2, which promotes p53 degradation
Evolution of DNA damage during the cell cycle

CELL CYCLE CHECKPOINTS

ENSURE THE COMPLETION OF DEPENDENT EVENTS

PROVIDE MORE TIME FOR REPAIR BEFORE REPLICATION AND MITOSIS
**Cell Cycle Checkpoints**

- **G₁ Delay**
  - UV → DNA Damage
  - IR → DNA Damage
  - ATM → p53 → CHK2 → p21 → Cyclin / CDK's
  - ATR → p53

- **G₂ Delay**
  - IR → DNA Damage
  - ATM/ATR → p53
  - Plk-1 → p21/14-3-3 → Crm1 → CDC25C → Cyclin B1 / CDC2

**DNA Damage and Cell Cycle Checkpoints**

- **G₁ Arrest**
  - Protects Against DNA Replication Errors
  - (e.g. Mutations, Replicative Gaps)

- **G₂ Delay**
  - Protects Against Mitotic Errors:
  - (e.g. Chromosomal Aberrations)

- **Replicon Initiation Inhibition**
  - Protects Against DNA Replication Errors
  - (e.g. Mutations, Replicative Gaps)
Function of p53 in response to DNA Damage

ATR is an essential checkpoint kinase

Ataxia telangiectasia- and rad3-related

ATR-null embryos die at day 3 with severe chromosomal damage

Expression of kinase-inactive ATR overrides checkpoint responses to IR and UVC
Levels of cell cycle regulation: oncogenic factors

**Table 22-4** Some Oncogenes and Their Original Sources

<table>
<thead>
<tr>
<th>Oncogene</th>
<th>Original Cancer Type</th>
<th>Original Source</th>
<th>Activity or Product</th>
<th>Cellular Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abelson leukemia</td>
<td>Mouse</td>
<td>Ty protein kinase</td>
<td>Cytoplasm</td>
<td></td>
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<tr>
<td>sisA</td>
<td>Endometriosis</td>
<td>Chicken</td>
<td>Thyroid hormone receptor</td>
<td>Nucleus</td>
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<tr>
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<td>Endometriosis</td>
<td>Chicken</td>
<td>EGF receptor</td>
<td>Plasma membrane</td>
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<tr>
<td>sisC</td>
<td>Myeloblastosis</td>
<td>Chicken</td>
<td>Regulatory protein</td>
<td>Nucleus</td>
</tr>
<tr>
<td>sisD</td>
<td>Feline sarcoma</td>
<td>Cat</td>
<td>Ty protein kinase</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>sisE</td>
<td>Feline sarcoma</td>
<td>Cat</td>
<td>Ty protein kinase</td>
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<td>sisF</td>
<td>Osteosarcoma</td>
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<td>sisH</td>
<td>Sarcoma</td>
<td>Chicken</td>
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<td>sisI</td>
<td>Sarcoma</td>
<td>Cat</td>
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<td>Cytoplasm</td>
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<td>sisJ</td>
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<td>Ty protein kinase</td>
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<td>sisK</td>
<td>Sarcoma</td>
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<tr>
<td>sisN</td>
<td>Osteosarcoma</td>
<td>Nuc</td>
<td>EGF receptor</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>sisO</td>
<td>Many cancers</td>
<td>Human</td>
<td>Tumor suppressor gene</td>
<td>Nucleus</td>
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<tr>
<td>sisP</td>
<td>Sarcoma</td>
<td>Mouse</td>
<td>Ser/Thr protein kinase</td>
<td>Cytoplasm</td>
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<td>sisQ</td>
<td>Sarcoma</td>
<td>Rat</td>
<td>G protein</td>
<td>Plasma membrane</td>
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<tr>
<td>sisR</td>
<td>Retinoblastoma</td>
<td>Human</td>
<td>Tumor suppressor gene</td>
<td>Nucleus</td>
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<td>sisS</td>
<td>Retinoblastoma</td>
<td>Turkey</td>
<td>Regulatory protein</td>
<td>Nucleus</td>
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<tr>
<td>sisT</td>
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<td>Ty protein kinase</td>
<td>Cytoplasm</td>
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<td>sisU</td>
<td>Sarcoma</td>
<td>Monkey</td>
<td>PDGF</td>
<td>Pigmented</td>
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<td>sisV</td>
<td>Carcinoma</td>
<td>Chicken</td>
<td>Regulatory protein</td>
<td>Nucleus</td>
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<td>Ty protein kinase</td>
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<td>sisX</td>
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<td>sisY</td>
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<td>Ty protein kinase</td>
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<tr>
<td>sisZ</td>
<td>Sarcoma</td>
<td>Human</td>
<td>Ty protein kinase</td>
<td>Cytoplasm</td>
</tr>
</tbody>
</table>

**Activation of extracellular receptors**

Growth factors bind to extracellular domains of transmembrane receptors, linked to cytoplasmic domains, many of which have been found to be protein tyrosine kinases. Examples of oncogenic transformation at this level include v-sis, which causes cells to express their own PDGF, and erb-B, which expresses a truncated EGF receptor lacking an extracellular domain, and which is active without a ligand.

Cells may start to secrete their own growth factors, the autocrine effect, instead of depending on an external source. In other cases, cell may lose dependence on growth factors by developing factor-independent receptors with constitutively active kinase modules.

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Ligand induced dimerization of receptors stimulates the kinase

Initially it was assumed that these tyrosine kinases would act through phosphorylation cascades much like Ser/Thr kinases. However, in many cases the best substrate turned out to be the receptor itself. Autophosphorylation, or more correctly, mutual phosphorylation, is mediated by dimerization induced by ligand binding, by subunit orientation modulated by ligand binding, or by other ligand-induced conformational changes.

Autophosphorylation generates key phosphotyrosine sites which are receptive to binding by signaling adapter proteins. These proteins are known as grbs, or growth-factor receptor bound. The factor grb2 consists of a single SH2 domain flanked by two SH3 domains. SH3 domains act as binding sites for proline rich domains in effector molecules acting downstream in the signaling pathway. In addition to the primary phosphorylation sites in receptors such as EGFR, there are secondary sites that bind other SH2 domain proteins with different effector domains; these include additional tyrosine kinases, tyrosine phosphatases, and a variety of other signaling molecules.

Transferring the kinase signal into the cell

Autophosphorylation of the receptor tyrosine kinase allows it to recruit other factors by interaction of SH2 domains with the phosphotyrosine. Grb2 can bind directly or indirectly via Shc; Grb2 recruits Sos. This localizes Sos to the membrane, where it acts as a guanine nucleotide exchange factor (GEF) for Ras GTPase.

Key signalling components such as Ras and c-Src are anchored to the membrane, by polyisoprenyl and myristoyl chains respectively. Recruitment of the signalling adapter proteins close to the membrane increases their local concentration, allowing the signal to pass effectively. Ras in turn activates Raf, a Ser/Thr protein kinase, which is the starting point for the MAP kinase pathway controlling cell proliferation.
What keeps the signal on track is the assembly of the cascade components onto a scaffolding factor such as Ste5, which appears to restrict the signal to lie within the intended mating pathway. Scaffolding proteins MP1 (MEK Partner) and JIP1 (Jun N-terminal kinase interacting protein) have now been found to interact with the components of the ERK growth receptor/proliferation pathway and JNK/SAPK stress pathways.

Scaffolds may serve to insulate signal pathways from interference or undesired cross-talk. They may serve to condition the signal so that variable input produces all-or-none output.

Normal proliferation pathways contain negative feedback or signal relaxation elements, so that the end result is dependent on continuous maintenance of the growth factor signal.

- Activation of Ras by RTK accelerates Ras turnover;
- Intrinsic hydrolysis of GTP in Ras causes it to revert to inactive GDP-Ras;
- MAPK phosphorylation of Sos causes its relocation back into the cytoplasm;
- SH2-domain bearing protein tyrosine phosphatase PTP1
Integrins can adopt inactive and active configurations, which differ by change in relative orientation of the α- and β-subunits. The active orientation has enhanced affinity for both external and cytoplasmic ligands. Binding of ligand on either side promotes the change to active form, so cytoplasmic ligands can promote binding to ECM, and ECM binding can enhance interaction with cytoplasmic ligands or binding partners. Normal cells in a normal ECM environment are quiescent, whereas transformed cells become actively motile. The invasiveness and metastatic potential of tumor cells is strongly dependent on this transition.

**Intracellular integrin targets:**

- Expression and activation of Cdks and cyclins A,B,D,E
- Activity of Cdk inhibitors p21CIP1 and p27KIP
- Progression through G1-S checkpoint and pRb phosphorylation
- The actin cytoskeleton and myosin light chain kinase
- Regulatory GTPases Cdc42, Rac and Rho

Cell-cell interaction, and specifically which results from like-cell contacts, involves extracellular adhesion proteins called cadherins (Ca dependent adhesion proteins) and intracellular effectors called catenins.

Cadherin forms clusters at points of cell-cell contact in the membrane, forming the junction between cells. The local concentration of cadherin at junction helps sequester the cytoplasmic pool of β-catenin, which limits the amount that can enter the nucleus. In the nucleus, β-catenin associates with transcription factors LEF and Tcf, and activates expression of genes such as cyclin D, thus stimulating cell proliferation.

Cytoplasmic β-catenin also binds to APC (Adenomatous Polyposis Coli protein). APC forms a scaffold for phosphorylation of β-catenin by the serine/threonine kinase GSK3. Phosphorylation activates a ubiquitination signal leading to the rapid turnover of free β-catenin.
Liver regeneration and hepatocellular proliferation

Liver histopathology, APAP-protein adduct distribution, and cell proliferation in mice pretreated with saline or APAP and challenged with saline or APAP. Mouse liver sections were stained with (HE) (A, D), immunohistochemical stain for 3-Cys-A (B, E), or PCNA (C, F). C: central vein; P: portal area.

A-C is from a representative control animal pretreated for 8 days with saline and challenged on day 9 with saline. (A) HE stain showing normal liver. (B) stain for 3CysA protein adduct is negative. (C) stain for PCNA showing normal (low) level of hepatocyte proliferation. S, cell in S phase.

D-F is from an animal pretreated for 8 days with APAP 2 hours after saline challenge. (D) HE stain showing moderate CL necrosis. Note substantial inflammatory infiltrate (arrow). (E) stain for 3CysA shows diffuse adduct indicated by deposition of the red-brown diaminobenzidine reaction product in the CL area. Note relative absence of 3CysA in cells immediately adjacent to the central vein. (F) stain for PCNA shows substantial numbers of hepatocytes proliferating, arrow marks inflammatory infiltrate.

From Shayiq et al, Hepatology 1999.
# Known growth factors/mitogens

<table>
<thead>
<tr>
<th>Factor</th>
<th>Related Family Members</th>
<th>Broad or Narrow Specificity</th>
<th>Representative Actions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet-derived growth factor (PDGF)</td>
<td>- three subtypes</td>
<td>broad</td>
<td>stimulate proliferation of connective tissue cells and some neural cells</td>
</tr>
<tr>
<td>Epidermal growth factor (EGF)</td>
<td>transforming growth factor α (TGF-α); Lin-3 protein (in C. elegans)</td>
<td>broad</td>
<td>stimulate proliferation of many cell types; acts as an inductive signal in embryonic development</td>
</tr>
<tr>
<td>Insulin-like growth factor I (IGF-I)</td>
<td>insulin</td>
<td>broad</td>
<td>promote cell survival; stimulate cell metabolism; collaborate with other factors to stimulate cell proliferation</td>
</tr>
<tr>
<td>Transforming growth factor β (TGF-β)</td>
<td>- multiple subtypes; activins; bone morphogenetic proteins (BMPs); Decapentaplegic protein (in Drosophila, Vg) protein (in Xenopus)</td>
<td>broad</td>
<td>potentiate or inhibit responses of most cells to other factors, depending on cell type; regulate differentiation of some cell types; act as inductive signals in embryonic development</td>
</tr>
<tr>
<td>Fibroblast growth factor (FGF)</td>
<td>- multiple subtypes</td>
<td>broad</td>
<td>stimulate proliferation of many cell types; inhibit differentiation of various types of stem cells; act as inductive signals in embryonic development</td>
</tr>
<tr>
<td>Interleukin-2 (IL-2)</td>
<td></td>
<td>narrow</td>
<td>stimulate proliferation of activated T lymphocytes</td>
</tr>
<tr>
<td>Nerve growth factor (NGF)</td>
<td>brain-derived neurotrophic factor (BDNF); neurotrophin-3 (NT-3); neurotrophin-4 (NT-4)</td>
<td>narrow</td>
<td>promote survival and nerve process outgrowth; specific classes of neurons</td>
</tr>
<tr>
<td>Erythropoietin</td>
<td></td>
<td>narrow</td>
<td>promote proliferation, differentiation, and survival of red blood cell precursors</td>
</tr>
<tr>
<td>Interleukin-3 (IL-3)</td>
<td>hemopoietic colony stimulating factors (CSFs)—multiple types</td>
<td>narrow</td>
<td>stimulate proliferation and survival of various types of blood cell precursors</td>
</tr>
</tbody>
</table>
Repair and regeneration of renal proximal tubular cells (RPTC) following acute sublethal toxicant injury. Sublethally injured RPTC either repair physiological functions and restore normal tubular function or dedifferentiate, migrate, and/or proliferate to replace lost cells, then differentiate and resume normal function. The processes of repair and regeneration work in concert to ensure relining of the damaged nephron and restoration of renal function.


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Interaction of AhR with retinoblastoma tumor suppressor protein (Rb), and its possible consequences for cell cycle regulation and apoptosis. Mitogenic signaling via protein kinases and cell contact-mediated mitoinhibition via protein phosphatases affect the Rb protein, a key G1 restriction checkpoint. The hypophosphorylated Rb has been shown to interact with the AhR. Two mechanisms may be operative in TCDD-mediated growth arrest, coactivation and corepression. While being required for S-phase progression, E2F may be involved in both progression of the cell cycle and the apoptosis machinery of the cell.

From Boch & Kohle Biochem Pharmacol 2005
## Methods to Study Cell Proliferation

<table>
<thead>
<tr>
<th>In vitro (cell culture)</th>
<th>In vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Mitotic figure count</td>
<td>• BrdU incorporation</td>
</tr>
<tr>
<td>• $^3$H-thymidine incorporation</td>
<td>• PCNA staining</td>
</tr>
<tr>
<td>• Colony/cell count</td>
<td>• Transcription Factor activation</td>
</tr>
<tr>
<td>• Flow cytometry</td>
<td>• Rb phosphorylation</td>
</tr>
<tr>
<td></td>
<td>• Cyclins/Cyclin-dependent kinases</td>
</tr>
</tbody>
</table>

![Cell Cycle Diagram](image)
Flow cytometry: DNA content (ploidy)

Propidium Iodide Fluorescence

Cell Counts

- Cells in G1
- Cells in S
- Cells in G2 and M

Propidium Iodide Fluorescence

Relative DNA content

Cell number

0 1 2

0 1 2

0 1 2
Activation/ phosphorylation of cell cycle factors

From Wheeler et al., Am J Physiol 2003