

## Module 9

# Cell Cycle and Proliferation

## Synopsis

Cell proliferation is the process whereby cells reproduce themselves by growing and then dividing into two equal copies. Growth factors employ a range of growth factor signalling pathways to activate cells to enter the cell cycle. In most cases, proliferation is mediated by growth factors operating within a highly localized environment so that only those cells in the immediate vicinity are instructed to grow. To understand how growth factors control cell proliferation, we have to consider both the nature of the signalling mechanisms and how they impinge upon the cell cycle machinery that regulates cell growth and cell division. One of the most active areas of cell signalling concerns this growth factor signalling/cell cycle interface.

An analysis of the information flow during proliferative signalling reveals that there is a complex interplay between different signalling mechanisms operating throughout the early phase of the cell cycle. In order to activate cell proliferation, growth factors have to do two things. They have to induce cells to enter the cell cycle and they also function in cell growth control by increasing cell mass through an increase in macromolecular biosynthesis. The integrity of the genome is maintained by a process of checkpoint signalling that operates to arrest the cell cycle in response to DNA damage.

Proliferation is most evident during development, when the single-celled zygote begins many rounds of growth and division to make up the cell mass of the developing organism. As the adult organism is formed, many cells stop growing when they differentiate to perform their specialized functions. In the case of neurons and muscle, this growth arrest is permanent, but, for many other cells, this arrest is temporary in that they retain the option of growing again to replace cells that are lost either through normal processes of wear and tear or through damage. There are many examples of such proliferation of specific cell types.

Interest in the signalling pathways that regulate cell proliferation increased when it became apparent that these were altered in many forms of cancer. These signalling pathways also control tumour angiogenesis. Very often, key components of the signalling pathways are proto-oncogenes that become constitutively active to function as oncogenes. Many of the tumour suppressors function as negative regulators of growth factor-mediated signalling pathways. Changes in these proliferative control mechanisms are also relevant to other diseased states, such as atherosclerosis, psoriasis, hypertension and polycystic kidney disease.

## Cell cycle

Cells enter the cell cycle through two quite distinct processes (Module 8: Figure cellular life history). One is the unique process of fertilization, and the other is cell proliferation activated by growth factors. Here the primary focus will be on the cell cycle events that occur when  $G_0$  cells are stimulated to proliferate by growth factors. There is a complex cell cycle network of signalling pathways that interact with each other to control whether or not cells will grow and divide. There is an extensive cell cycle toolkit that

contains both the signalling molecules and the large number of targets that are engaged as the cell passes through the cell cycle. Growth factors act early in  $G_1$  through a growth factor signalling/cell cycle interface to engage the cell cycle signalling system that then takes over and presides over the orderly sequence of events that culminates in cell division to give two daughter cells.

Defects in the cell cycle have been linked to various diseases, such as microcephaly.

## Cell cycle events

The sequence of events that occur when a cell is stimulated to grow and divide constitutes the cell cycle. Resting cells, which have zero growth ( $G_0$ ), have to be stimulated by growth factors in order to enter the cell cycle, which

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begins with the first period of growth ( $G_1$ ) during which it prepares for a period of DNA synthesis (S) (Module 9: Figure cell cycle). Towards the end of  $G_1$ , there is a restriction point (R), which marks the point where the cell becomes irreversibly committed to traverse the rest of the cell cycle. In effect, the control of subsequent events is handed over from the growth factor-mediated signalling pathways to the internal cell cycle signalling system. The latter then controls events during the S phase and the final events of  $G_2$  and mitosis, when the cell divides. Once division is complete, control swings back to the growth factor-mediated signalling pathways. To understand how proliferation is controlled it is therefore necessary to follow the sequence of events during  $G_1$  that leads up to R. Once R is breached, cells become independent of growth factors in that further progression through the cell cycle is now in the hands of the cell cycle control system (blue arrows in Module 9: Figure cell cycle).

Once it has duplicated its chromosomes, the cell enters a second period of growth ( $G_2$ ) when it prepares to divide into two daughter cells during the period of mitosis (M). This M phase is separated into a series of discrete steps that begin with prophase and then pass through prometaphase, metaphase, anaphase and finally the process of cytokinesis, which divides the cell into two equal halves (Module 9: Figure mitotic events).

### Prophase

During prophase, the paired centrosomes begin to separate. The paired chromosomes that were replicated during the S phase begin to condense to form the sister chromatids. Multisubunit condensing complexes and histone phosphorylation are used to condense chromatin to form the two sister chromatids that are held together by cohesin molecules located at the centromeres. The nuclear membrane begins to break down. This initiation of mitosis is driven by the cyclin B/cyclin-dependent kinase 1 (CDK1) complex working in conjunction with the Polo-like kinases (Plks) (Module 9: Figure mitotic entry).

### Prometaphase

In prometaphase, the nuclear membrane disappears and the spindle begins to assemble. There are three types of spindle microtubules: the astral microtubules, the polar microtubules, which overlap each other in the middle to push the poles apart, and the kinetochore spindles that attach to the kinetochore (yellow dots) on the centromeres of the sister chromatids (Module 9: Figure mitotic events).

### Metaphase

In metaphase, the chromatids align in one plane at the metaphase plate. The sister chromatids are attached via the kinetochore microtubule to opposite poles of the spindle. Considerable tension develops between the two chromatids because their microtubules are trying to pull them apart. Separation is prevented by the spindle checkpoint that delays separation until all the chromatids are in place.

### Anaphase

When the spindle checkpoint control is satisfied that all the chromatids are properly aligned, a specific signal triggers the separation process that is driven by the anaphase-promoting complex (APC) that cleaves the cohesin molecules that hold the chromatids together. There are indications that anaphase is triggered by a pulse of  $Ca^{2+}$  (Module 9: Figure chromosome separation).

### Telophase

As the chromosomes separate and move towards the spindle poles, the cell begins the final process of cell cleavage: telophase. Cell division is driven by a process of cytokinesis, which depends upon a contractile ring of actomyosin that gradually contracts to form a constriction that cleaves the cell into two daughter cells (Module 9: Figure cytokinesis).

These events of the cell cycle are controlled by an extensive cell cycle toolkit (Module 9: Table cell cycle toolkit).

## Cell cycle toolkit

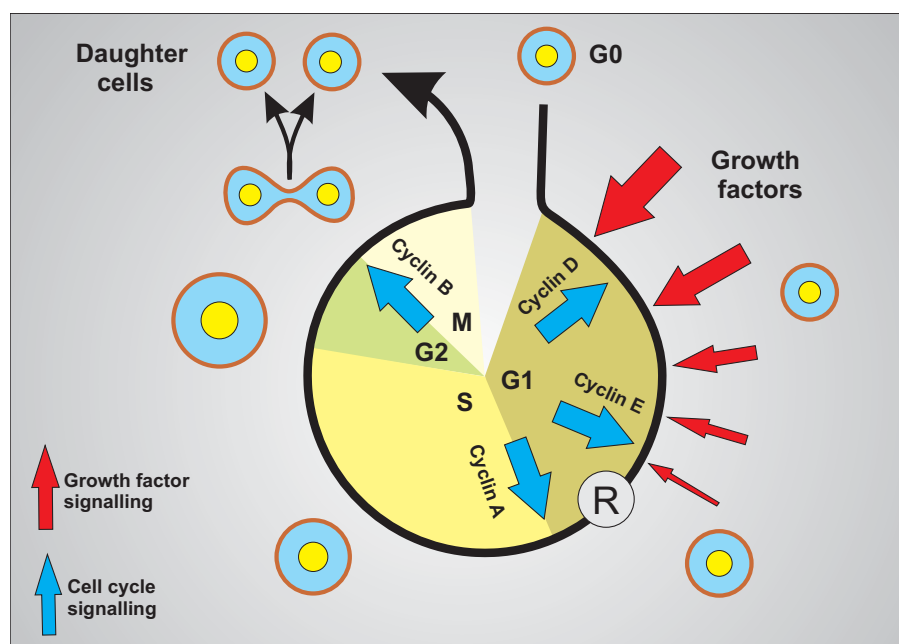
The main components of the cell cycle machinery are a family of proteins known as cyclins and their associated cyclin-dependent kinases (CDKs) (Module 9: Table cell cycle toolkit). Various combinations of these two components regulate the orderly progression through the cell cycle. In addition, there are a number of CDK inhibitors (Cip/Kip and p16 families) and a number of other molecules that either regulate or carry out the downstream events, such as the pocket proteins (Rb, p107, p130), the E2F family of transcription factors and the mitotic components such as the Polo-like kinases (Plks), checkpoint kinases, regulatory phosphatases, centrosome and spindle components, chromosome separation proteins and the spindle midzone complex. The challenge is to understand how all these components come together to control the orderly sequence of events that constitutes the cell cycle. Some of these cell cycle control elements operate early in  $G_1$  and represent the targets for the proliferative signalling pathways, which are part of the cell cycle network.

### Cyclin-dependent kinases (CDKs)

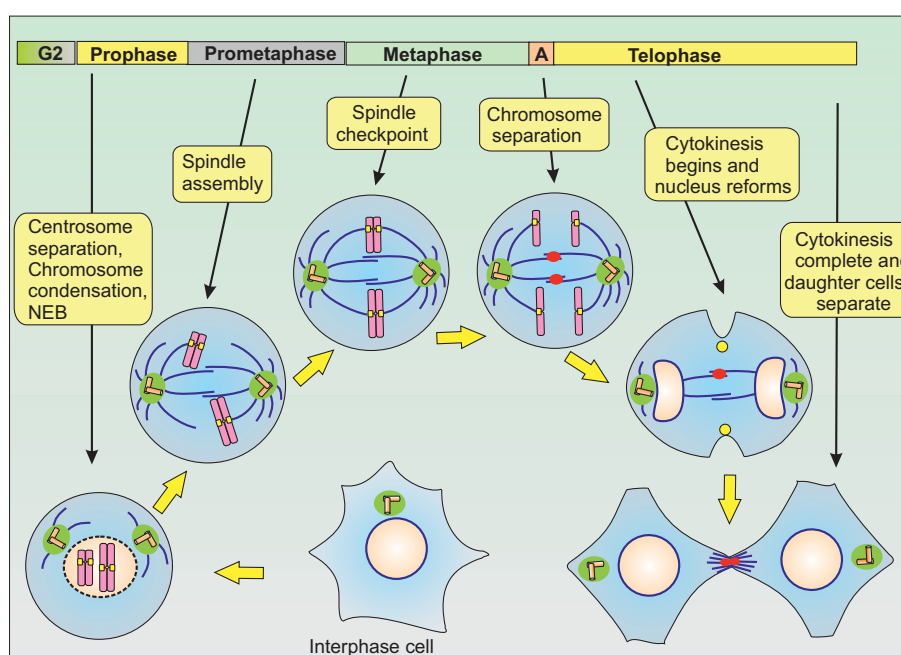
The cyclin-dependent kinases (CDKs) are responsible for regulating the cyclins. Each cyclin binds to a specific CDK (Module 9: Table cell cycle toolkit). The activity of the CDKs depends upon this association with the cyclins to form the cyclin-CDK complexes that control cell cycle progression (Module 9: Figure cell cycle signalling mechanisms). In addition to activating the CDKs, some of the cyclins, such as cyclin B, also determine the cellular location of the cyclin-CDK complex.

Since the cyclins lack enzymatic activity, their action is mediated by binding to cyclin-dependent kinases (CDKs) to form cyclin-CDK complexes. The cyclin-CDK complexes have multiple functions in cell cycle signalling:

- Cyclin A control of DNA synthesis
- Cyclin B/cyclin-dependent kinase 1 (CDK1) control of mitosis
- Cyclin D controls  $G_1$  progression
- Cyclin E controls  $G_1$  progression and DNA synthesis

**Module 9:** | Figure cell cycle**Operation of the growth factor and cell cycle signalling system during different phases of the cell cycle.**

Growth factor signalling (red arrows) operates primarily during G<sub>1</sub> to drive the cell towards the restriction (R) point, after which control moves over towards the internal cell cycle signalling system (blue arrows). The different cyclin proteins, which are a central feature of the cell cycle signalling system, operate at different points during the cell cycle (Module 9: Figure cell cycle dynamics). Note how the cell grows in size over the course of the cell cycle.

**Module 9:** | Figure mitotic events**Summary of the morphological changes that occur during the mitotic phase.**

The final phase of the cell cycle begins when cells leave the G<sub>2</sub> phase and enter the mitotic (M) phase. The events that occur at mitosis are the morphological manifestation of cell division. As cells pass through the G<sub>2</sub>/M boundary, they begin to divide into two equal daughter cells as they pass through a series of steps (see the text for details). NEB, nuclear envelope breakdown.

**Cell cycle network**

There is a complex cell cycle network that has a number of signalling systems that control whether or not a cell

enters the cell cycle. These signalling systems determine the choices that are available when cells exit from the cell cycle (Module 9: Figure cell cycle network). They can follow

**Module 9: | Table cell cycle toolkit**

Molecular components of the cell cycle signalling system.

Cell cycle component	Comments
<b>Cyclins</b>	
Cyclin A1	Associates with CDK2
Cyclin A2	Associates with CDK2
Cyclin B1	Associates with CDK1
Cyclin B2	Associates with CDK1
Cyclin D1	Widely expressed, interacts with CDK4 and CDK6
Cyclin D2	
Cyclin D3	
Cyclin E1	Associates with CDK2
Cyclin E2	Associates with CDK2
Cyclin H	Associates with CDK7 to form the cyclin-dependent kinase (CDK)-activating kinase (CAK)
<b>Cyclin/CDK-associated proteins</b>	
Mat1	Stabilizes the cyclin H/CDK7 complex that forms the cyclin-dependent kinase (CDK)-activating kinase (CAK) (Module 9: Figure cyclin D/CDK action)
<b>Cyclin-dependent kinases (CDKs)</b>	
CDK1	
CDK2	
CDK4	
CDK6	
CDK7	A component of the cyclin-dependent kinase (CDK)-activating kinase (CAK) (Module 9: Figure cyclin D/CDK action)
<b>CDK inhibitors</b>	
Cip/Kip family	
p21	Expression strongly increased by p53 (Module 4: Figure Myc as a gene repressor)
p27	
p57	
p16 family	
p16 <sup>INK4a</sup>	This CDK4 inhibitor shares the same gene locus as the tumour suppressor alternative reading frame (ARF)
p15 <sup>INK4b</sup>	Expression increased by transforming growth factor $\beta$ (TGF- $\beta$ )
p18 <sup>INK4c</sup>	
p19 <sup>INK4d</sup>	
<b>Pocket proteins</b>	
Rb/p105	
p107	
p130/Rb12	
<b>E2F family of transcription factors</b>	
E2F1	E2 promoter-binding factor 1
E2F2	
E2F3	
E2F4	
E2F5	
E2F6	
E2F7	
<b>E2F-binding partners</b>	
DP1	
DP2	
<b>Regulatory kinases</b>	
Polo-like kinases (Plks)	
Plk1	Plays a major role in regulating mitosis (Module 9: Figure mitotic entry)
Plk2	
Plk3	
Plk4	
Wee1	Phosphorylates Tyr-15 on CDK1 (Module 9: Figure mitotic entry)
Myt1	Phosphorylates Thr-14 and Tyr-15 on CDK1 (Module 9: Figure mitotic entry)
Nek2	A kinase that may play a priming role in phosphorylating Plk substrates

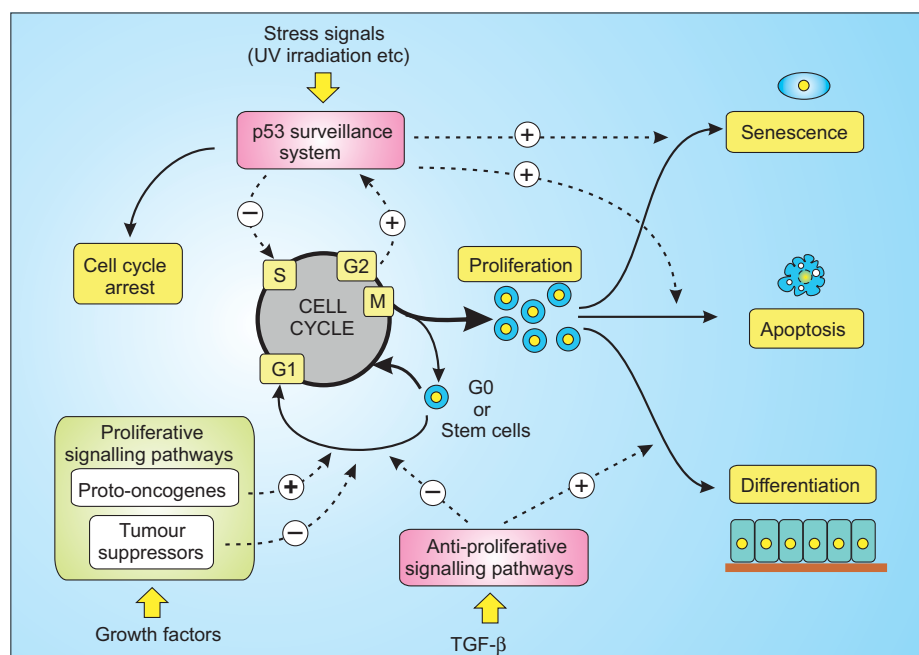
**Module 9: | Table continued**

Cell cycle component	Comments
Citron kinase	Phosphorylates myosin light chain (MLC) during cytokinesis (Module 9: Figure cytokinesis)
<b>Checkpoint kinases</b>	
ATM	Ataxia telangiectasia mutated
ATR	ATM- and Rad3-related
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
Aurora B	Phosphorylates MgRac-GAP and MYPT1 during cytokinesis (Module 9: Figure cytokinesis)
<b>Regulatory phosphatases</b>	
Cdc25	
Cdc25A	Dephosphorylates CDK2 in late G <sub>1</sub> (Module 9: Figure cell cycle signalling mechanisms)
Cdc25B	Dephosphorylates CDK1 at G <sub>2</sub> /M (Module 9: Figure mitotic entry)
Cdc25C	Dephosphorylates CDK1 at G <sub>2</sub> /M (Module 9: Figure mitotic entry)
Cdc14a	Dephosphorylates components of the spindle midzone complex during cytokinesis (Module 9: Figure cytokinesis)
<b>Centrosome and spindle components</b>	
Nlp	Ninein-like protein
Katanin	A microtubule-severing protein
TCTP	A microtubule-stabilizing protein
Stathmin	A microtubule-destabilizing protein
ASPM	Abnormal spindle protein-like, microcephaly-associated
<b>Chromosome separation</b>	
Cohesin	A protein that “glues” chromatids together during spindle assembly
Securin	A scaffolding protein that inactivates separase (Module 9: Figure chromosome separation)
Separase	A cysteine protease that cleaves cohesin (Module 9: Figure chromosome separation)
<b>Spindle midzone complex</b>	
MKLP1	Mitotic kinesin-like protein 1
MKLP2	Mitotic kinesin-like protein 2
Plk1	Translocates MKLP2 to the spindle
MgRac-GAP	A Rho-GEF
ECT2	A microtubule-associated protein (MAP)
PRC1	Protein regulator of cytokinesis 1
KIF1	A kinesin-4 family member that interacts with PRC1
INCENP	Inner centromere protein
Survivin	See Module 9: Figure cytokinesis

four possible pathways, resulting in markedly different cell fates:

- Senescence
- Apoptosis
- Differentiation
- Cell proliferation

It is this last option of cell proliferation that is controlled by the proliferative signalling pathways that induce resting (G<sub>0</sub>) or stem cells to enter the cell cycle. These proliferation signalling pathways are highly dynamic systems, with both positive and negative elements. Many of the positive elements are proto-oncogenes, which often are mutated to become constitutively active to form the oncogenes found in many tumour cells. Conversely, there are negative elements, and many of these are tumour suppressors that are inactivated in many tumour cells. There also are

**Module 9:** | Figure cell cycle network

The cell cycle network of signalling systems that control cell proliferation and related cellular processes.

The cell cycle is at the centre of a number of major cellular signalling systems that not only control cell proliferation, but also determine the subsequent fate of the daughter cells by controlling the processes of differentiation, apoptosis or senescence. Some of the major signalling components associated with this network are shown in more detail in Module 9: Figure proliferation signalling network.

anti-proliferative signalling pathways that inhibit cells from entering the cell cycle. An important example is the Smad signalling pathway controlled by the transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily, which not only prevents cells from entering the cell cycle, but also drives cells to differentiate.

Presiding over the operation of the cell cycle is the p53 surveillance system, which is constantly checking on the performance of all the cell cycle processes, and particularly those concerned with DNA synthesis. The p53 system is also responsive to cell stress from external sources such as irradiation, which often results in causing damage to DNA (Module 4: Figure p53 function). All such cell defects are relayed through p53, which then takes appropriate action, depending on their severity. If the damage is not too severe, p53-induced cell cycle arrest occurs while the DNA damage is repaired (Module 9: Figure cell cycle network). However, if damage is more severe, the cell is driven towards senescence or p53-induced apoptosis.

With regard to cell proliferation, the main question to focus on is how the information flowing into the cell from the proliferative signalling pathways activates the endogenous cell cycle signalling system responsible for guiding the cell through the rest of the cell cycle. This growth factor signalling/cell cycle interface is of central importance because it deals with how the growth factor signalling systems instruct the cell cycle signalling system to proceed with another cycle of cell division.

Such a holistic view of the cell cycle network is critical for understanding the causes of cancer, because tumorigenesis develops from multiple alterations in components

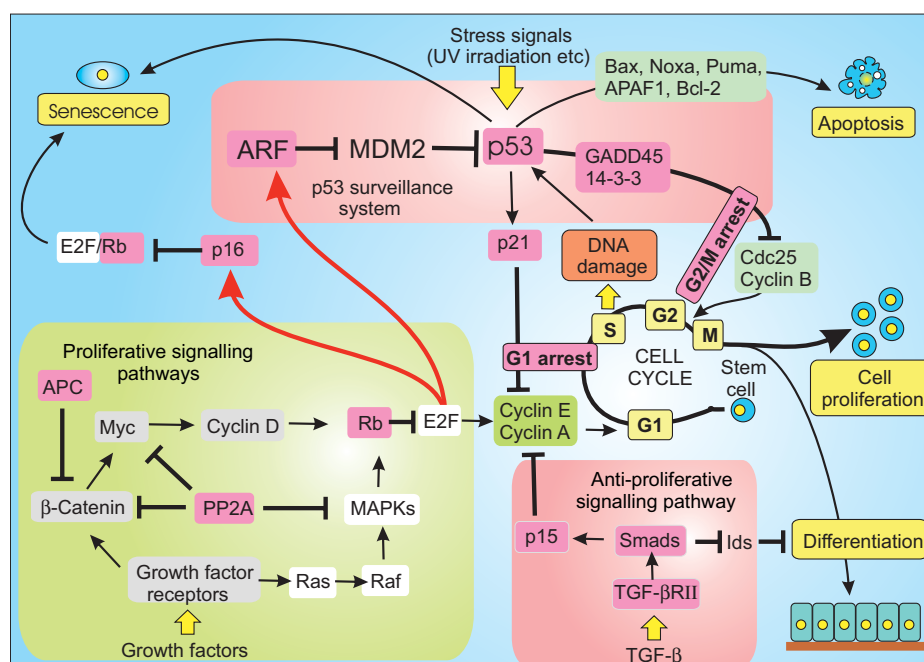
distributed throughout the signalling network that regulates the cell cycle (Module 12: Figure cell cycle network and cancer).

### Growth factor signalling/cell cycle interface

The interface between the growth factor signalling pathways and the cell cycle signalling system is a critical event in the control of cell proliferation. It is during this process that information flowing in from the cell surface sets in motion the sequence of events that culminates in cell division. The cell cycle network illustrates how the cell cycle is controlled by a number of signalling systems (Module 9: Figure cell cycle network). An overview of how the proliferative signalling pathways engage the cell cycle signalling pathway is shown in Module 9: Figure G<sub>1</sub> proliferative signalling. A key event is the activation of cyclin D control of G<sub>1</sub> progression, which then activates the E2F transcriptional system, which is critical for initiating the events that lead to DNA synthesis. The nature of the molecular interface between these signalling systems and the cell cycle signalling system is shown in more detail in Module 9: Figure proliferation signalling network.

The cyclins are the primary targets of the signals coming into the cell from growth factor receptors on the cell surface. Proliferative signalling pathways drive cells into the cell cycle by activating events early in G<sub>1</sub>. One of these pathways depends on Ras and the mitogen-activated protein kinase (MAPK) signalling pathway, and another is the Wnt signalling pathway that acts through  $\beta$ -catenin

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**Module 9:** | Figure proliferation signalling network

Control of cell proliferation by a network of signalling mechanisms.

The hub of this network is the cell cycle, during which proliferative competent cells, such as stem cells, traverse the cell cycle from which they emerge as two daughter cells that then face a number of possible fates. They can re-enter the cell cycle or they can differentiate. Various stresses acting on the p53 system can direct cells towards senescence or apoptosis. Cell proliferation is controlled by a number of signalling systems that act to regulate different components of the cell cycle. This figure illustrates how the various signalling systems outlined in Module 9: Figure cell cycle network interface with the cell cycle by targeting the different cyclin molecules that drive the cell cycle.

to increase the level of the transcription factor Myc. The latter then increases the transcription of cyclin D, which is an early component of the cell cycle signalling machinery (Module 9: Figure cell cycle dynamics). The cyclin D then acts on retinoblastoma protein (Rb) to remove its inhibitory effect on E2F, which then activates the formation of cyclin E and cyclin A, which are responsible for driving the cell into the cell cycle (Module 9: Figure proliferation signalling network). Myc also increases the expression of the inhibitor of DNA binding (Id) proteins that play a key role in the proliferation-differentiation switch (Module 8: Figure proliferation-differentiation switch).

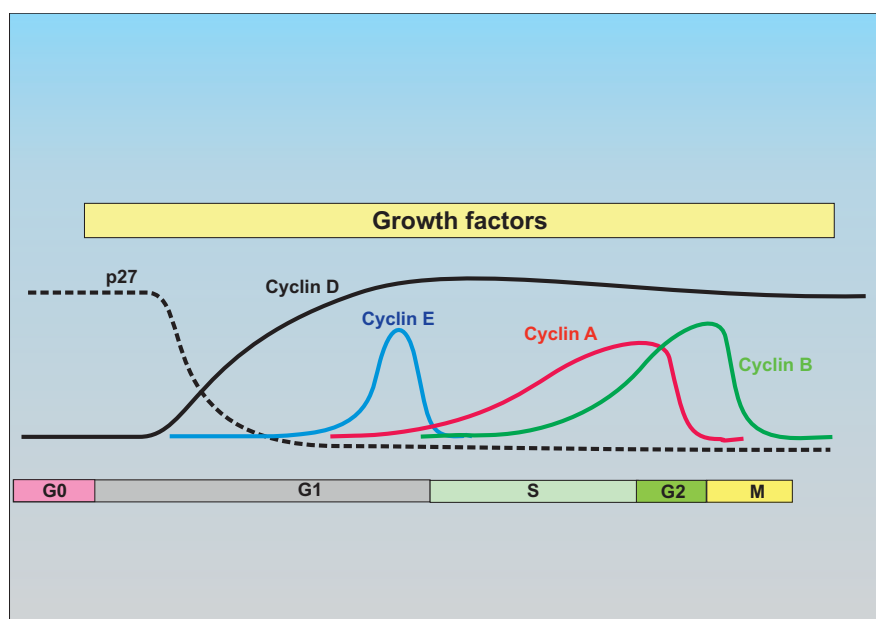
In contrast with the positive effects that certain growth factors have in initiating the cell cycle, other growth factors such as transforming growth factor- $\beta$  (TGF- $\beta$ ) act by inhibiting the cell cycle signalling pathway by interfering with cyclin activation. The transforming growth factor- $\beta$  inhibition of cell proliferation depends upon the up-regulation of the cyclin-dependent kinase (CDK) inhibitor p15 that inhibits cyclin E and cyclin A. The Smads also act to prevent the formation of the transcription factor Myc, which is a key regulator of cell proliferation (Module 4: Figure Myc as a gene activator).

The p53 surveillance system, which has such an important role in checking that the cell cycle proceeds normally with no errors, also exerts its inhibitory effects on the cell cycle by interfering with the cyclins to induce either G<sub>1</sub> or G<sub>2</sub>/M arrest (Module 9: Figure proliferation signalling

network). p53 produces p21, growth-arrest and DNA-damage-inducible protein 45 (GADD45) and the 14-3-3 protein. The p21 inhibits cyclin E to bring about G<sub>1</sub> arrest, whereas 14-3-3 protein and GADD45 are responsible for G<sub>2</sub>/M arrest. The 14-3-3 protein binds to Cdc25, and thus prevents it from activating cyclin B, whereas GADD45 also inactivates cyclin B to prevent it from initiating the onset of mitosis (Module 9: Figure cell cycle signalling mechanisms). In addition, p53 promotes the transcription of apoptotic factors that initiate apoptosis.

The PtdIns 3-kinase signalling pathway contributes to the build-up of cyclin D by increasing the stability and the expression of the cyclin D mRNA. Another important function of this PtdIns 3-kinase pathway is to control some of the key regulators of the cell cycle. For example, the downstream effector of this pathway, protein kinase B (PKB), phosphorylates the forkhead box O (FOXO) transcription factor to reduce its ability to activate transcription of the CDK inhibitor p27. This leads to a reduction in the level of p27, which remains low throughout the rest of the cell cycle (Module 9: Figure cell cycle dynamics), and thus facilitates the operation of the cell cycle signalling events.

The downstream elements of the proliferative and anti-proliferative signalling pathways thus interface with the cell cycle by controlling the activities of the cyclin/CDK complexes, which are central components of the cell cycle signalling system.

**Module 9:** | Figure cell cycle dynamics

Changes in the level of different cyclin isoforms and p27 during the course of the cell cycle.

Following the addition of growth factors, cyclin D appears first and remains at a high level during the cell cycle. The rise in cyclin D is followed later during G<sub>1</sub> by the appearance of cyclin E, which rapidly disappears towards the beginning of DNA synthesis (S phase). Cyclin A begins to appear towards the end of G<sub>1</sub>, continues to rise during the S phase and then rapidly declines as the cell progresses through G<sub>2</sub>. Finally, cyclin B begins to appear during the S phase, and this rise continues into the G<sub>2</sub> phase before rapidly falling during the mitotic (M) phase. In contrast with these periodic fluctuations in the cyclins, the level of the cyclin-dependent kinase (CDK) inhibitor p27 drops rapidly early in G<sub>1</sub> and remains at a low level throughout the cell cycle.

## Cell cycle signalling

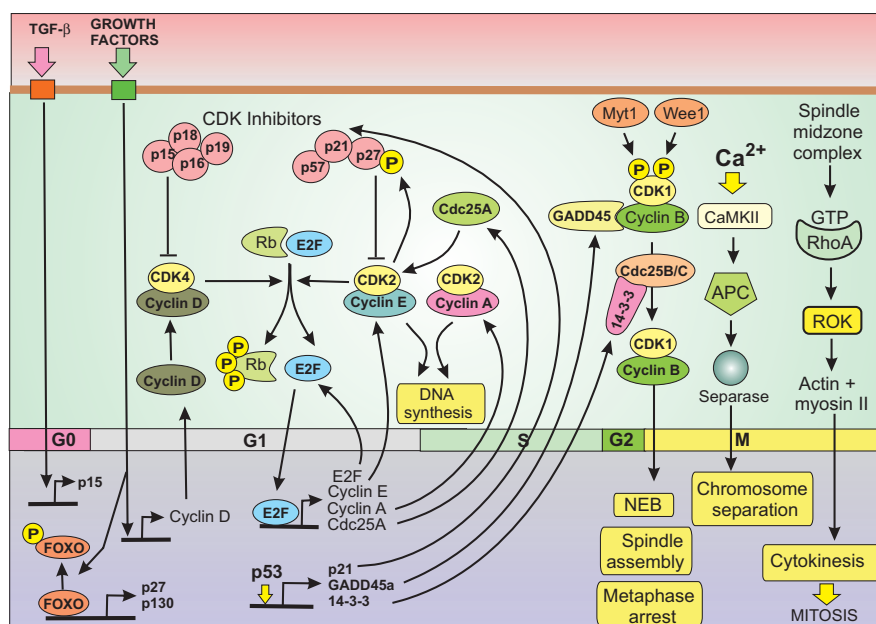
Cell cycle signalling is responsible for controlling the orderly sequence of events that occur when a cell is stimulated to grow and divide. Central features of the signalling system are the waves of cyclin expression that occur during the course of the cell cycle (Module 9: Figure cell cycle dynamics). Since the cyclins lack enzymatic activity, their action is mediated by binding to cyclin-dependent kinases (CDKs) to form cyclin/CDK complexes. Each cyclin binds to a specific CDK (Module 9: Table cell cycle toolkit). The activity of the CDKs depends upon this association with the cyclins to form the cyclin/CDK complexes that control cell cycle progression (Module 9: Figure cell cycle signalling mechanisms). In addition to activating the CDKs, some of the cyclins, such as cyclin B, also determine the cellular location of the cyclin/CDK complex.

Before the CDKs can begin to function as serine/threonine kinases, they have to be activated by the cyclin-dependent kinase (CDK)-activating kinase (CAK). The different complexes can also be regulated by more specific phosphorylation/dephosphorylation reactions that are particularly important in the cyclin B/cyclin-dependent kinase 1 (CDK1) control of mitosis. In addition, the cyclin/CDK complexes can be regulated by a variety of cyclin/CDK-associated proteins. For example, Mat1 stabilizes the cyclin H/CDK7 complex, which is a component of CAK. The cyclin-dependent kinase (CDK) inhibitors are particularly important in regulating the cyclin/CDK complexes that operate early in the G<sub>1</sub> phase. The

sequential activation of different cyclin/CDK regulatory complexes is responsible for the orderly progression of the cell cycle, as illustrated in Module 9: Figure cell cycle signalling mechanisms.

### Cyclin B/cyclin-dependent kinase 1 (CDK1) activation

Once S phase is complete, the cell enters the second growth phase (G<sub>2</sub>) when it prepares to begin mitosis. This entry into mitosis depends upon the activation of the cyclin B/cyclin-dependent kinase 1 (CDK1) complex, which has been kept inactive by phosphorylation of CDK1 by the Wee1 and Myt1 kinases. These inhibitory phosphates are removed by the Cdc25B/C phosphatase, and the active cyclin B/CDK1 complex enters the nucleus, where it activates nuclear envelope breakdown (NEB) and spindle assembly. It also contributes to metaphase arrest, which ensures that anaphase is prevented until all the chromatids are assembled on the spindle. When this is achieved, the final phase of chromosome separation and cytokinesis is triggered by the activation of the anaphase-activating complex (APC). An increase in Ca<sup>2+</sup>, acting through Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaMKII), triggers this process. Once activated, the APC degrades cyclin B, thus cancelling the inhibitory effect of the cyclin B/CDK1 complex on anaphase, and it also hydrolyses securin to release the enzyme separase that is responsible for hydrolysing the cohesin that sticks the chromatids together. Once they are separated, the chromatids once again become functional chromosomes as they move away

**Module 9:** | Figure cell cycle signalling mechanisms**Summary of the major cell cycle control pathways.**

This figure is a roadmap that charts the signalling sequence of events that occurs when growth factors activate their receptors (top left) to bring about mitosis (bottom right). The orderly sequence of events is orchestrated by the different cyclin/cyclin-dependent kinase (CDK) complexes that act at different times during the course of the cell cycle, as described in the text.

from each other towards the opposite poles. Cytokinesis is triggered by the formation of a spindle midzone complex that activates the RhoA signalling mechanisms responsible for activating contraction of actin and myosin to complete the processes of mitosis.

The sequence of events just described in Module 9: Figure cell cycle signalling mechanisms provides a signalling roadmap composed of a number of events that are described in more detail in subsequent sections. The first event is the cyclin D control of  $G_1$  progression. The level of cyclin D, which is low in the resting cell ( $G_0$ ), increases rapidly following growth factor stimulation. As the level of cyclin D rises, it binds to CDK4 or CDK6 and the cyclin D/CDK4/6 complex enters the nucleus, where it drives the transcription of a number of cell cycle components that control the next phases of the cycle. For example, cyclin E controls  $G_1$  progression and DNA synthesis and is followed by cyclin A control of DNA synthesis. Cyclin E/CDK2 acts together with cyclin A/CDK2 to initiate DNA synthesis. Once DNA synthesis has been switched on, most attention is focused on this process of replication at the expense of other cellular processes such as gene transcription, which are switched off. Little else happens until DNA synthesis is complete, whereupon the cell leaves the S phase and enters the  $G_2$ /M phases, where cyclin B/cyclin-dependent kinase 1 (CDK1) controls mitosis. During this final M phase, there are three main events:

- Nuclear envelope breakdown and spindle assembly controlled by the degradation of cyclin B.

- Chromosome separation at anaphase is controlled by a  $Ca^{2+}$ -sensitive process.
- Cytokinesis is controlled by formation of the spindle midzone complex.

**Cyclin D controls  $G_1$  progression**

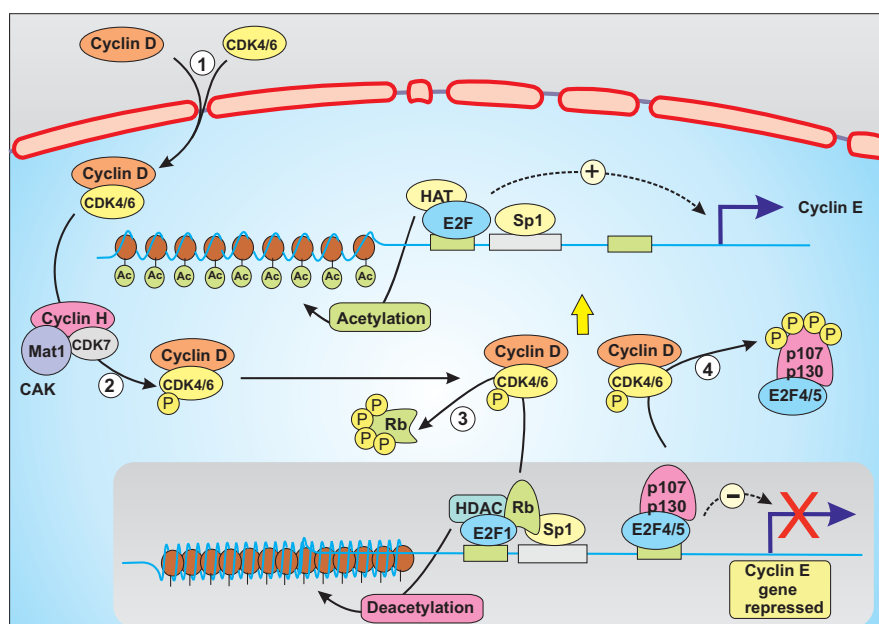
The cell cycle is driven by the waves of cyclin formation that begin when growth factors activate the transcription of cyclin D.

The way in which cyclin D controls  $G_1$  progression depends upon the transcriptional activation and expression of cell cycle signalling molecules.

Cyclin D is a key component of the growth factor signalling/cell cycle interface, because it is the main target of growth factor signalling pathways (Module 9: Figure cell cycle signalling mechanisms). Of the three cyclin D isoforms, cyclin D<sub>1</sub> is the most widely expressed in cells. Their partners cyclin-dependent kinase 4 (CDK4) and 6 (CDK6) are constitutively expressed, but remain inactive until the level of cyclin D rises early in  $G_1$  and begins to activate transcription through the sequence of events shown in Module 9: Figure cyclin D/CDK action:

1. Cyclin D in the cytoplasm interacts with the cyclin-dependent kinase 4 or 6 (CDK4/6) to form a complex that moves into the nucleus.
2. The cyclin D/CDK4/6 complex then interacts with the cyclin-dependent kinase (CDK)-activating kinase (CAK), which is a complex made up of cyclin H, CDK7 and Mat1 that phosphorylates the CDK4/6 to activate its kinase activity. The activated cyclin D/CDK4/6 complex then has two actions:

## Module 9: | Figure cyclin D/CDK action



## Cyclin D/cyclin-dependent kinase 4/6 (CDK4/6) activation of cyclin E transcription.

The grey box at the bottom represents the repressed state of the cyclin E gene as occurs in G<sub>0</sub> when cell proliferation is arrested. There are two promoter sites where the pocket proteins [retinoblastoma protein (Rb), p107 and p130] and the E2F proteins (Module 9: Figure Rb–E2F interactions) interact to repress gene transcription. At one site, the transcriptional activator E2F is repressed by the Rb, which also contributes to repression by binding the histone deacetylase (HDAC). At the other site, the pocket proteins p107 and p130 facilitate the binding of the E2F4/5 transcriptional repressors. When cells are stimulated to grow, there is an increase in the level of cyclin D that sets off a cascade of events that lead to activation of the cyclin E gene, as described in the text.

- It begins to phosphorylate retinoblastoma (Rb), which then moves away from E2F1, allowing it to activate its transcription targets including E2F itself, cyclin E, cyclin A and Cdc25A. Since E2F increases both E2F and cyclin E, it sets up a positive-feedback loop, which serves to increase the phosphorylation of Rb to provide further transcription of the components necessary to drive the cycle on. The Cdc25A phosphatase acts to dephosphorylate CDK2, which then activates cyclin E–CDK2, a complex that co-operates with cyclin A–CDK2 to activate DNA synthesis at the G<sub>1</sub>/S interface (Module 9: Figure cell cycle signalling mechanisms).

In addition to controlling the activity of the E2F transcription factors, the Rb family also regulates transcription by remodelling chromatin through its ability to pull in the histone deacetylases (HDACs). The Rb-associated protein RbAp48 contributes to the complex between Rb and HDAC. The phosphorylation of Rb results in the removal of the HDACs, which are replaced by the histone acetyltransferases (HATs) that open up chromatin so that transcription of cell cycle components can occur.

- The activated cyclin D/CDK4/6 also phosphorylates p107/130, which is then unable to facilitate the repressor activity of E2F4/5. The complex moves away from the promoter site, and E2F1 is now able to activate the transcription of cyclin E.

In addition to controlling the expression of cyclin E, the Rb/E2F signalling systems also control a number of

other genes, such as cyclin A, and thus set up the next waves of cyclin formation to propel the cell cycle forward. One of the processes to occur is that cyclin E controls G<sub>1</sub> progression and DNA synthesis.

As is apparent in Module 9: Figure cell cycle dynamics, the cyclin D level remains high throughout the cell cycle, which means that the daughter cells are able to immediately enter another cell cycle once they complete mitosis, assuming that growth factors are present. However, if growth factors are withdrawn during the cell cycle, the level of cyclin D will fall, and the daughter cells enter a G<sub>0</sub> state.

Cyclin E controls G<sub>1</sub> progression and DNA synthesis

Cyclin E is a nuclear protein that pairs up with cyclin-dependent kinase 2 (CDK2) to form the cyclin E/CDK2 complex that has two main functions (Module 9: Figure cell cycle signalling mechanisms). Firstly, it contributes to G<sub>1</sub> progression by phosphorylating the retinoblastoma (Rb) family of proteins. This process sets up a positive-feedback loop, because two of the genes activated by Rb phosphorylation are cyclin E itself and the E2F1–E2F3 transcription factors. The cyclin-dependent kinase (CDK) inhibitors also play an important role in cyclin E/CDK2 activation. In particular, cyclin E/CDK2 can remove p27, which is one of the major inhibitors that limit its activity. It phosphorylates p27, which is then degraded by the SCF<sup>Skp2</sup> ubiquitin ligase.

Its second action is to contribute to the onset of DNA synthesis. One of its substrates is the transcription factor nuclear protein mapped to the ataxia telangiectasia locus (NPAT), which regulates the transcription of histone that will be required during the S phase. It also phosphorylates cyclic AMP response element-binding protein (CREB)-binding protein (CBP)/p300 to enhance its histone acetyltransferase (HAT) activity, which is essential for the transcription of many of the genes that are required for driving the cell into DNA synthesis. Finally, it can initiate the process of centrosome duplication by phosphorylating nucleophosmin to cause it to leave the centrosome.

The activity of the cyclin E/CDK2 complex is regulated by Cdc25A, which is one of the dual-specificity Cdc25 phosphatases that act by removing inhibitory phosphates on CDK2.

Cyclin E is somewhat unstable, and its level in the cell is determined by the balance between synthesis and proteolysis. The latter is carried out by two separate ubiquitination pathways. Monomeric cyclin E is degraded by the pathway that depends on the Cul-3 protein. On the other hand, the cyclin E/CDK2 complex is degraded by the SCF<sup>Fbw7</sup> ubiquitin ligases. As the cell cycle proceeds, these proteolytic mechanisms gain the upper hand and the level of cyclin E falls, and its role is then taken over by the cyclin A control of DNA synthesis.

Roscovitine, which is a potent inhibitor of cyclinE/CDK2, is being tested as an anticancer drug. It may also prove useful for treating polycystic kidney disease.

### Cyclin A control of DNA synthesis

Cyclin A is a nuclear protein of which there are two isoforms. The cyclin A1 is normally restricted to germ cells, whereas the cyclin A2 functions in most cells to control both the G<sub>1</sub>/S and G<sub>2</sub>/M transitions. In effect, it provides a link between the early events in G<sub>1</sub> and the final events that occur during the G<sub>2</sub>/M phase, when cyclin B/cyclin-dependent kinase 1 (CDK1) controls mitosis.

### Cyclin B/cyclin-dependent kinase 1 (CDK1) controls mitosis

The cyclin B/cyclin-dependent kinase 1 (CDK1) complex controls the final events of the cell cycle, during which the cell divides into two daughter cells (Module 9: Figure cell cycle signalling mechanisms). This mitotic phase can be divided into three main phases. The first phase is nuclear envelope breakdown and spindle assembly. The second is chromosome separation at anaphase, and, finally, there is the process of cytokinesis, during which the cell divides into the two daughter cells.

#### Nuclear envelope breakdown and spindle assembly

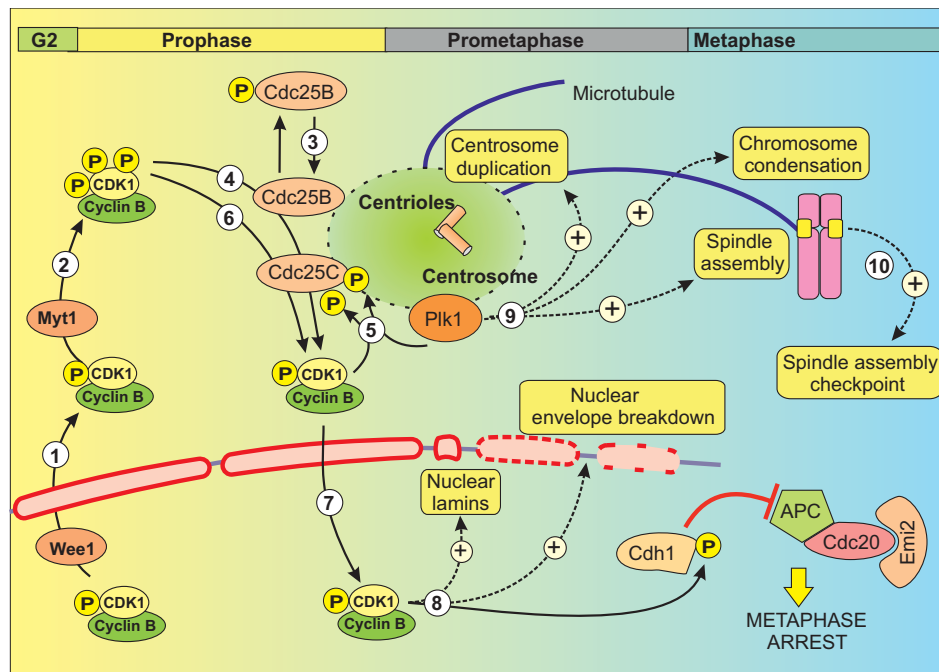
The entry into mitosis at the G<sub>2</sub>/M interface depends upon the activation of the cyclin B/cyclin-dependent kinase 1 (CDK1) complex. The main events that control passage through the G<sub>2</sub>/M boundary are illustrated in Module 9: Figure mitotic entry:

1. The cyclin B/CDK1 complex is kept quiescent during interphase, during which it tends to shuttle in and

out of the nucleus. This nuclear shuttling is controlled by a nuclear localization signal (NLS) that targets the complex in an importin- $\beta$ -dependent manner. In addition, there is a cytoplasmic retention signal (CRS) that has a nuclear export signal (NES) responsible for Crm1-dependent export. The nuclear export system predominates, and the inactive cyclin B/CDK1 complex is located mainly in the cytoplasm during G<sub>2</sub>. In addition, there are two inhibitory phosphorylation sites (Thr-14 and Tyr-15) that are phosphorylated by the kinases Wee1 and Myt1. Wee1 phosphorylates Tyr-15 in the nucleus.

2. Myt1 phosphorylates both Thr-14 and Tyr-15 in the cytoplasm, and these two phosphorylations serve to inactivate cyclin B/CDK1 and to retain it in the cytoplasm.
3. The critical event for the activation of cyclin B/CDK1 is the removal of these inhibitory phosphates by the Cdc25 phosphatases, with the Cdc25B acting together with Cdc25C. Cdc25B, which seems to initiate the process, is activated first following its dephosphorylation.
4. Cdc25B begins to dephosphorylate cyclin B/CDK1.
5. Activation of Cdc25C is enhanced by phosphorylation through both CDK1 and the Polo-like kinases (Plks). This is an example of how these two kinases co-operate with each other. The CDK1 acts as a priming kinase by adding a phosphate to the Cdc25C, which then acts as a binding site for Plk1 that attaches itself to add another phosphate. This phosphorylation of Cdc25C provides a positive-feedback loop that functions to sharpen and enhance the activation of the cyclin B/CDK1 complex.
6. The activated Cdc25C is then able to contribute to the dephosphorylation and activation of cyclin B/CDK1.
7. The dephosphorylated cyclin B/CDK1 enters the nucleus, and acts together with Plk1 to begin the mitotic events.
8. Cyclin B/CDK1 phosphorylates nuclear lamins and triggers nuclear envelope breakdown. One of the important actions of cyclin B/CDK1 is to inhibit the anaphase-promoting complex (APC), which is responsible for metaphase arrest. One of the activators of APC is Cdh1, which is inactivated following its phosphorylation by cyclin B/CDK1. One component of this metaphase arrest is cyclin B/CDK1, which phosphorylates and thus inactivates Cdh1, which is an activator of APC. The APC is also inactivated by the spindle-assembly checkpoint signalling pathway (see 10 below).
9. Plk1 controls the maturation of the centrosome by recruiting  $\gamma$ -tubulin and by phosphorylating some of the centrosomal proteins, such as ninein-like protein (Nlp), katanin (a microtubule-severing protein), translationally controlled tumour protein (TCTP), stathmin (a microtubule-destabilizing protein) and abnormal spindle protein-like, microcephaly-associated (ASPM) protein. Once Nlp is phosphorylated, it leaves the centrosome

## Module 9: | Figure mitotic entry



## Entry into the final mitotic phase of the cell cycle.

Passage through the G<sub>2</sub>/M boundary depends upon the cyclin B/cyclin-dependent kinase 1 (CDK1) complex that is inactivated during G<sub>2</sub>, but is activated by a series of steps as the cells enter prophase (see the text for further details). The operation of the spindle-assembly checkpoint that prevents premature separation of the chromosomes is described in Module 9: Figure spindle assembly checkpoint.

and makes way for the other proteins to form the microtubule organizing centre (MTOC) that orchestrates the polymerization of tubulin to assemble the spindle. Mutation of ASPM has been linked to microcephaly.

10. It is important that the next process of chromosome separation is delayed until all of the chromosomes are located correctly on the spindle. The kinetochores, which are the sites on the centrosome region of the chromosome where the microtubules attach, play an important role in the spindle-assembly checkpoint, which is a surveillance system that ensures that chromosome separation at anaphase is delayed until metaphase is complete. Those kinetochores, which are not attached to microtubules, activate a spindle-assembly checkpoint signalling pathway that ensures that anaphase is delayed until all the chromatids are lined up on the spindle equator (Module 9: Figure spindle assembly checkpoint).

**Microtubule organizing centre (MTOC)**

As its name implies, the microtubule organizing centre (MTOC) functions to organize the polymerization of microtubules into networks that control various cellular processes:

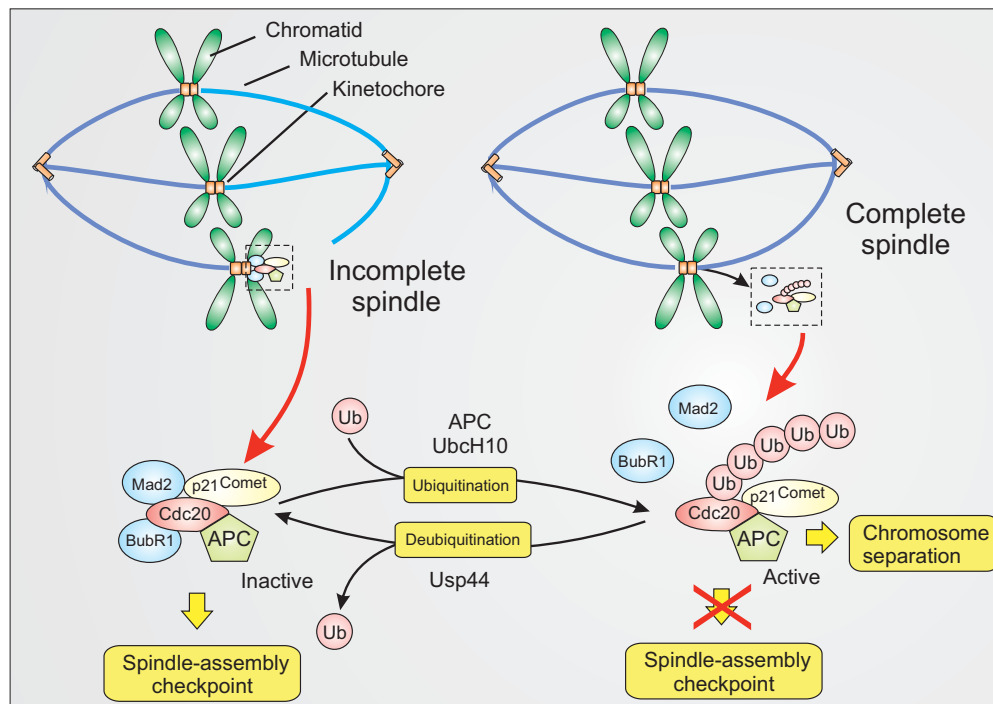
- The MTOC forms the basal bodies that organize flagella and cilia.
- The MTOC is part of the centrosome, which consists of a pair of centrioles, directs spindle assembly (see step 9 in Module 9: Figure mitotic entry). Key components of

the centrosomes are pericentrin,  $\gamma$ -tubulin and ninein-like protein (Nlp).

- During neutrophil chemotaxis, the MTOC functions to align the microtubules in the direction of movement and helps to stabilize cell polarity. It also directs vesicles towards the leading edge (Module 11: Figure neutrophil chemotaxis).
- In natural killer (NK) cells and cytotoxic T lymphocytes (CTLs), the MTOC is orientated between the nucleus and the target cell or antigen-presenting cell respectively. This orientation enables the cells to deliver vesicles to the region of the immunological synapse.
- During Fc $\gamma$ R-mediated phagocytosis in macrophages, the MTOC and Golgi are orientated towards the phagosome and this may facilitate the antigen processing events.

**Spindle-assembly checkpoint signalling**

Errors in chromosome segregation can lead to aneuploidy (abnormal chromosome numbers), which is a feature of many aggressive tumours. Such errors are prevented by a dynamic surveillance mechanism known as the spindle-assembly checkpoint, which ensures that segregation does not begin until all the chromatid pairs are correctly aligned on the mitotic spindle (Module 9: Figure spindle assembly checkpoint). This checkpoint operates by controlling the activity of the anaphase-promoting complex (APC) responsible for initiating chromosome separation at anaphase segregation (Module 9: Figure chromosome separation).

**Module 9:** | Figure spindle-assembly checkpoint**Spindle-assembly checkpoint signalling pathway.**

During assembly of the spindle, the free kinetochores, which have not bound a microtubule, assemble a molecular complex (see box) that inhibits the Cdc20 protein which is responsible for activating the anaphase-promoting complex (APC) that initiates chromatid separation. This checkpoint is removed when the spindle is fully assembled. The inhibitory protein complex dissociates and Cdc20 is able to activate APC and anaphase begins as described in (Module 9: Figure chromosome separation). The hypothesis presented in this figure was developed by Stegmeier et al. 2007.

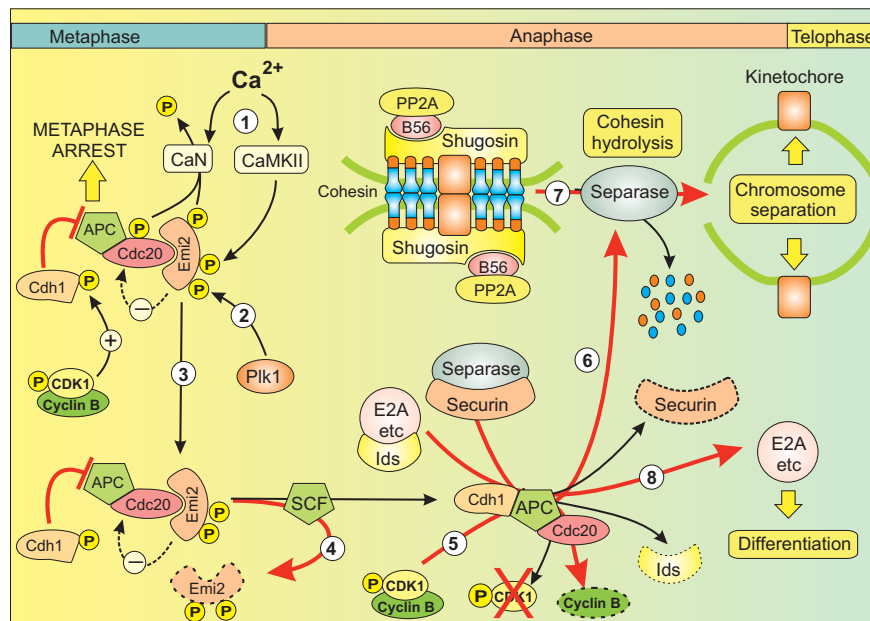
The spindle-assembly checkpoint signalling pathway, which is still being worked out, has to detect the existence of free kinetochores and relay this information to the APC complex to inhibit its activity. The following hypothesis has been proposed to explain how this signalling pathway might operate. The proteins Mad2 and BubR1 appear to be the sensors that detect the free kinetochores on the incomplete spindle (Module 9: Figure spindle assembly checkpoint). These proteins are also able to bind the Cdc20 to prevent it from activating APC. Before Cdc20 can be activated, it must be released from this inhibitory action of Mad2/BubR1 and must also be polyubiquitinated by a combination of APC and E2 ubiquitin ligase UbcH10. The p21<sup>Comet</sup> facilitates the binding of UbcH10 to Cdc20. Even though these two ligases are constitutively active, they are not able to ubiquitinate Cdc20 because any added ubiquitin groups are immediately removed by an ubiquitin-specific protease 44 (Usp44). There appears to be a futile cycle of ubiquitination and deubiquitination, which is biased in favour of the latter, thus ensuring that the Cdc20 is kept inactive.

As soon as the spindle is complete, the complexes located on the kinetochores dissociate to liberate the Cdc20. The latter is then rapidly ubiquitinated and this enables it to stimulate APC to initiate the process of chromosome separation at anaphase (Module 9: Figure chromosome separation).

**Chromosome separation at anaphase**

The spindle-assembly checkpoint, which ensures that anaphase is delayed until all the chromatids are lined up on the spindle equator (metaphase plate), is switched off once all the chromatids are assembled on the spindle (Module 9: Figure spindle assembly checkpoint). Chromosome separation can now begin through a process that appears to be triggered by an increase in  $\text{Ca}^{2+}$  that initiates a signalling sequence that activates the anaphase-promoting complex (APC) through the sequence of steps shown in Module 9: Figure chromosome separation:

1.  $\text{Ca}^{2+}$  activates  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II (CaMKII) to phosphorylate Emi2, which is an inhibitor of the APC.
2. The phosphate on Emi2 provides a binding site for the Polo-like kinase 1 (Plk1).
3. Plk1 then phosphorylates an additional site on Emi2, which creates the phosphodegron [a phosphorylated residue(s)] that makes this inhibitor susceptible to degradation.
4. The ubiquitination complex SCF (Skp/cullin/F-box) then degrades the phosphorylated Emi2 to activate the APC. The APC is inactivated by components of the checkpoint control systems. One of these is exerted by Emi2, which inactivates Cdc20, which is one of the APC activators.

**Module 9:** | Figure chromosome separation**Control of chromosome separation at anaphase.**

The spindle checkpoint ensures that the cell cycle is arrested at metaphase until all the chromatids are lined up on the metaphase plate. The onset of chromosome separation, which marks the beginning of anaphase, is triggered by a specific stimulus. There are indications that a surge in the level of Ca<sup>2+</sup> may be responsible for activating the anaphase-promoting complex (APC) through the sequence of events described in the text.

- One of the functions of the active APC is to degrade cyclin B to inactivate cyclin-dependent kinase 1 (CDK1), which has the effect of allowing the Cdh1 to further activate APC.
- The APC also degrades securin to release the enzyme separase that is responsible for hydrolysing cohesin molecules.
- Sister chromatids (green lines) are held together by cohesin molecules that are part of the kinetochore complex at the centromere. These cohesins are composed of two subunits; Rec8 and stromal antigen 2 (SA2). This SA2 subunit can be phosphorylated by Plk1 and would lead to dissociation of the complex except that stability is maintained by a protein called shugosin that has a binding site for the B56 regulatory subunit of protein phosphatase 2A (PP2A), which ensures that SA2 remains unphosphorylated. Once separase is activated, cohesin is hydrolysed and the two chromatids now separate to become the chromosomes of the two daughter cells. Now the kinetochore microtubules can begin to pull the chromosomes apart. This separation of the chromosomes marks the beginning of cytokinesis.
- APC plays an important role in the proliferation-differentiation switch by hydrolysing the inhibitor of DNA binding (Id) proteins to liberate transcription factors such as E2A that promote differentiation (Module 8: Figure proliferation-differentiation switch).

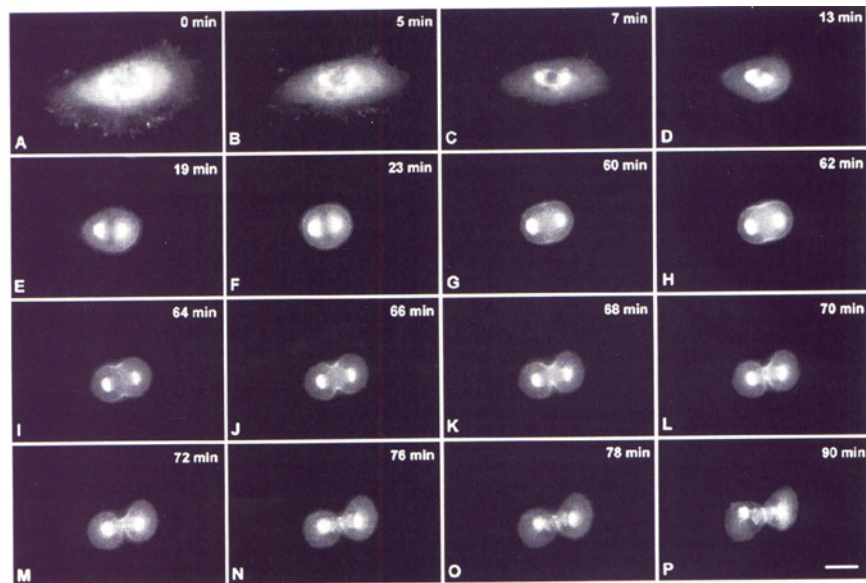
equal daughter cells. As the chromosomes move towards the poles during anaphase, the spindle remains in the form of the overlapping polar microtubules. Proteins that are concentrated at this midzone of the spindle then determine the division plane during cytokinesis. The exact process and how it is controlled is still being worked out, but it appears to depend upon four clearly defined processes that begin with assembly and activation of a spindle midzone complex, which results in the activation of RhoA that then controls assembly of the actomyosin contractile ring and the activation of contraction. Formation of the cleavage furrow depends upon the trafficking and insertion of membrane vesicles.

**Assembly and activation of a spindle midzone complex**

The polar microtubules that are left behind after the chromosomes move towards the poles function to assemble the spindle midzone complex that determines the division plane (Module 9: Figure cytokinesis). This midzone complex is made up of three main groups. There is the Aurora B/inner centromere protein (INCENP)/survivin/Csc1 group, the protein-regulating cytokinesis 1 (PRC1)/KIF1 group and the mitotic kinesin-like protein 1 (MKLP1)/MgcRac GTPase-activating protein (MgRacGAP) group. Some of these components, such as PRC1, MKLP1 and INCENP, are phosphorylated by the cyclin B/cyclin-dependent kinase 1 (CDK1) complex and lie inactive in the cytoplasm. In the case of MKLP1, its phosphorylation inhibits its motor activity, which thus prevents it from binding to the spindle. When the

**Cytokinesis**

The final process of mitosis is cytokinesis, which is responsible for cell division when the cell cleaves into two

**Module 9: | Figure CaM redistribution at mitosis**

**Redistribution of calmodulin during the 90 min time period of mitosis in a HeLa cell.**

At the S/G<sub>2</sub> interface, calmodulin is concentrated in the nucleus (A), and then begins to segregate into two sites where the centrosomes are located (B and C). As the spindle begins to form, it becomes highly concentrated in the polar regions (D and E), where it remains during metaphase (F), anaphase (G and H) and during cytokinesis (I–P). Towards the end of anaphase, calmodulin begins to concentrate in the cortex immediately below the plasma membrane at the site where the cleavage furrow will form (G). The density in this region builds up progressively at the site where the cleavage processes cut the cell into two. Reproduced from Li, C.J., Heim, R., Lu, P., Tsien, R.Y. and Chang, D.C. (1999) Dynamic redistribution of calmodulin in HeLa cells during cell division as revealed by a GFP–calmodulin fusion protein technique. *J. Cell Sci.* 112:1567–1577, with permission from The Company of Biologists; see Li et al. 1999.

cyclin B/CDK1 complex is degraded at anaphase (Module 9: Figure chromosome separation), these phosphorylated proteins are dephosphorylated by the phosphatase Cdc14a and are then incorporated into the spindle midzone complex (Module 9: Figure cytokinesis). The site of insertion of the MKLP1/MgcRacGAP complex may depend upon the Aurora B complex. Aurora B may play a role in stimulating MgcRacGAP, which is a component of the signalling system that controls the assembly of the actomyosin contractile ring.

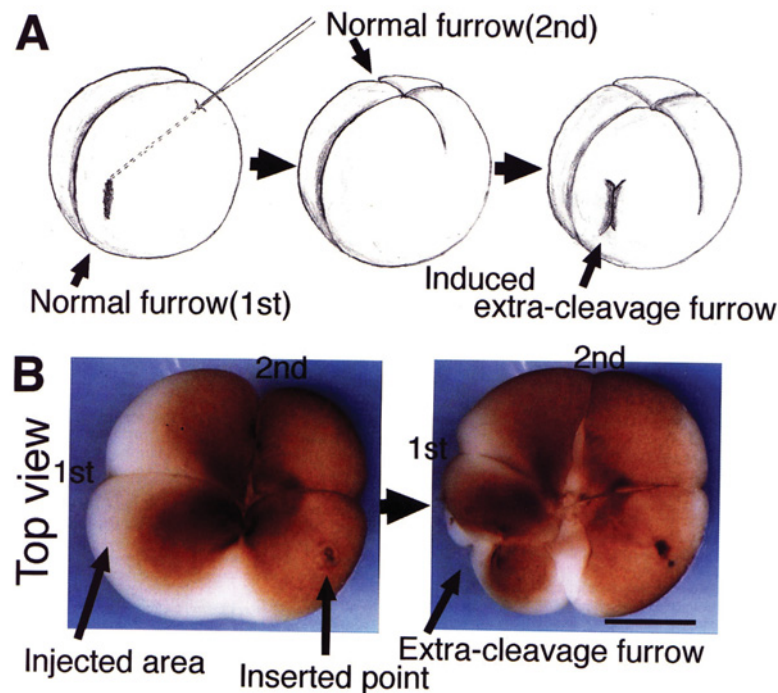
### Assembly of the actomyosin contractile ring

The spindle midzone complex determines the position where the contractile ring is formed, and it also helps to control the activation of RhoA, which is responsible for initiating the assembly of actomyosin fibres (Module 9: Figure cytokinesis). Actin is assembled through an interaction between the proteins formin and profilin. The formin normally functions to cap the barbed ends of the actin filaments to prevent them from growing. However, the activated RhoA-GTP binds to the N-terminal region of formin, its autoinhibition is removed, and the filament begins to grow by nucleating the actin monomers carried in by profilin. As the activation of contraction proceeds and the contractile ring contracts down, there is an overabundance of actin, and this is then reduced by cofilin, which acts to destabilize and sever the redundant actin filaments.

### Activation of contraction

Contraction of the contractile ring is controlled by phosphorylation of the myosin light chains (MLCs) on Ser-19 and Thr-18, which is carried out by a number of mechanisms (Module 9: Figure cytokinesis). This phosphorylation of MLC enables the head of the non-muscle myosin II motor to interact with actin to bring about contraction of the ring. One of the phosphorylation pathways is carried out by the RhoA signalling mechanism, which results in stimulation of Rho kinase (ROCK) that activates contraction by stimulating MLC or by inhibiting myosin phosphatase targeting subunit 1 (MYPT1). The latter is a targeting protein that positions the protein phosphatase 1c (PP1c) at its site of action (Module 5: Table PP1 regulatory, targeting and inhibitory subunits and proteins), which, in this case, is on the myosin molecule, where it can remove the activating phosphates on MLC. The MYPT1 is inhibited following its phosphorylation by both Rho kinase and by Aurora B. Citron kinase, which is another Rho-effector kinase, has also been implicated in the control of myosin activity.

MLC can also be phosphorylated by myosin light chain kinase (MLCK), which is stimulated by Ca<sup>2+</sup> acting through calmodulin (CaM). There certainly are remarkable changes in the distribution of CaM during mitosis (Module 9: Figure CaM redistribution at mitosis). The high concentration at the spindle poles suggests some role in the process of anaphase. The appearance of CaM at the site of the cleavage furrow is consistent with a role for Ca<sup>2+</sup> in controlling cytokinesis (Module 9: Figure cytokinesis).

**Module 9:** | Figure  $\text{InsP}_3$  receptors and cell cleavage

Induction of an extra-cleavage furrow by injecting microsomes rich in inositol 1,4,5-trisphosphate ( $\text{InsP}_3$ ) receptors close to the membrane.

The microsomes were injected into one cell of a *Xenopus* embryo after the first cleavage (A). By the time of the next cleavage, an extra furrow appeared at the site where the inositol 1,4,5-trisphosphate ( $\text{InsP}_3$ )-rich microsomes had been deposited. Reproduced from *Dev. Biol.*, Vol. 214, Mitsuyama, F., Sawai, T., Carafoli, E., Furuichi, T. and Mikoshiba, K., Microinjection of  $\text{Ca}^{2+}$  store-enriched microsome fractions to dividing newt eggs induces extra-cleavage furrows via inositol 1,4,5-trisphosphate-induced  $\text{Ca}^{2+}$  release, pp. 160–167. Copyright (1999), with permission from Elsevier; see Mitsuyama et al. 1999.

Imaging experiments have observed localized  $\text{Ca}^{2+}$  signals in the vicinity of the cleavage furrow. This localized  $\text{Ca}^{2+}$  signal may be released from inositol 1,4,5-trisphosphate ( $\text{InsP}_3$ ) receptors because an extra cleavage furrow was induced by injecting microsomes rich in these receptors near the surface membrane of a *Xenopus* embryo (Module 9: Figure  $\text{InsP}_3$  receptors and cell cleavage).

### Trafficking and insertion of membrane vesicles

The monomeric G protein Rab11, operating through the Rab11-family interacting protein 2 (FIP2), plays a role in controlling the trafficking and insertion of new vesicles to form the cleavage furrow (Module 9: Figure cytokinesis). Annexin A11 also seems to play a role in the trafficking of vesicles to the plasma membrane, and this would suggest that it is a  $\text{Ca}^{2+}$ -mediated process.

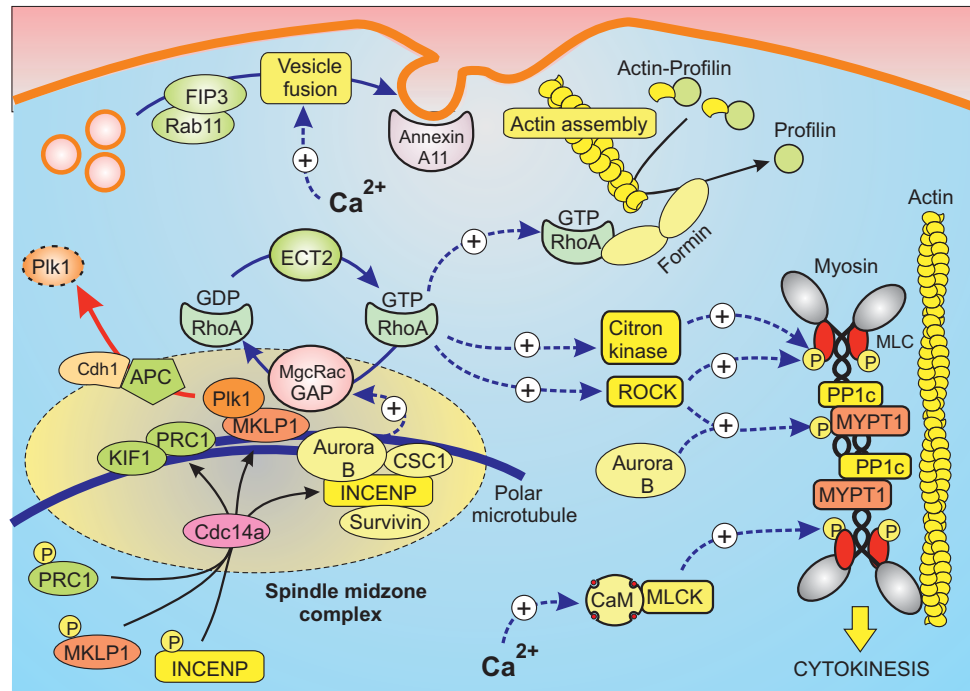
### Cyclin-dependent kinase (CDK)-activating kinase (CAK)

The cyclin-dependent kinase (CDK)-activating kinase (CAK) is a complex composed of cyclin H, CDK7 and Mat1, which is a RING finger protein that stabilizes the complex. CAK functions to activate the cyclin/CDK complexes by phosphorylating a threonine residue located on the T-loop region of the CDKs. This T-loop region inhibits the CDKs by covering up their ATP-binding sites. CAK moves this T-loop out of the way to activate the

CDKs. CAK plays a critical role in activating the CDKs. For example, it activates cyclin D/CDK4/6 once it enters the nucleus (Module 9: Figure cyclin D/CDK action).

### Polo-like kinases (Plks)

The Polo-like kinases (Plks) (Module 9: Table cell cycle toolkit), which are expressed from G<sub>2</sub> onwards and peak at the time of mitosis, are serine/threonine kinases that play a critical role during the M phase by controlling many aspects of mitosis, including nuclear envelope breakdown and spindle assembly, chromosome separation at anaphase and the process of cytokinesis. They have an N-terminal kinase domain and two C-terminal Polo-box (PB) domains (PB1 and PB2) responsible for binding to their substrates. These PB domains form a phosphopeptide-binding motif. In its resting state, these PB domains bend around to interact with the kinase catalytic region to keep it inactive. Activation of the Plks depends upon the phosphorylation of Thr-210 in the T-loop of the kinase domain. They also have a D-box motif, which controls their destruction by the anaphase-promoting complex (APC). The Plks, particularly Plk1, play an important role in controlling various events during the M phase, such as entry into mitosis (Module 9: Figure mitotic entry) and the separation of the chromatids at anaphase (Module 9: Figure chromosome separation).

**Module 9:** | Figure cytokinesis**Control of cytokinesis.**

Cytokinesis begins at telophase, and is driven by formation of an actomyosin contractile ring and by a process of vesicle fusion. Contraction of the ring squeezes the cell such that it finally cleaves in two. Formation of the contractile ring is controlled by the spindle midzone complex that forms in the region where the polar microtubules intersect. One of the components of this complex is MgcRacGAP, which is a GTPase protein that controls the activity of RhoA. The latter has a central role in regulating both the formation of the contractile ring and in the activation of the ring during cytokinesis (see the text for further details).

An interaction between p21, the transcription factor NF-Y and the Polo-like kinases 1 (Plk1) may function to maintain the checkpoint function of p53 to prevent mitotic cell death.

### Cyclin-dependent kinase (CDK) inhibitors

There are two quite distinct families of cyclin-dependent kinase (CDK) inhibitors (Module 9: Table cell cycle toolkit) that play a critical role in regulating the cell cycle: the INK4 family and the Cip/Kip family.

The INK4 family functions to inhibit cyclin D/CDK4/6 complexes (Module 9: Figure cell cycle signalling mechanisms). There is considerable interest in this family because p15 functions in the transforming growth factor  $\beta$  (TGF- $\beta$ ) inhibition of cell proliferation. There also is interest in p16<sup>INK4a</sup>, which is a specific inhibitor of CDK4, because it is a tumour suppressor that plays a role in the development of some cancers. p16<sup>INK4a</sup> has also been implicated in the signalling pathways of compensatory hypertrophy.

The activity of cyclin E/CDK2 is controlled by the Cip/Kip family, which is older than the INK4 family in evolutionary terms, consists of p21, p27 (KIP1) and p57 (KIP2). The p53-induced cell cycle arrest depends upon the transcriptional activation of various cell cycle components, such as p21 (Module 4: Figure p53 function). Similarly,

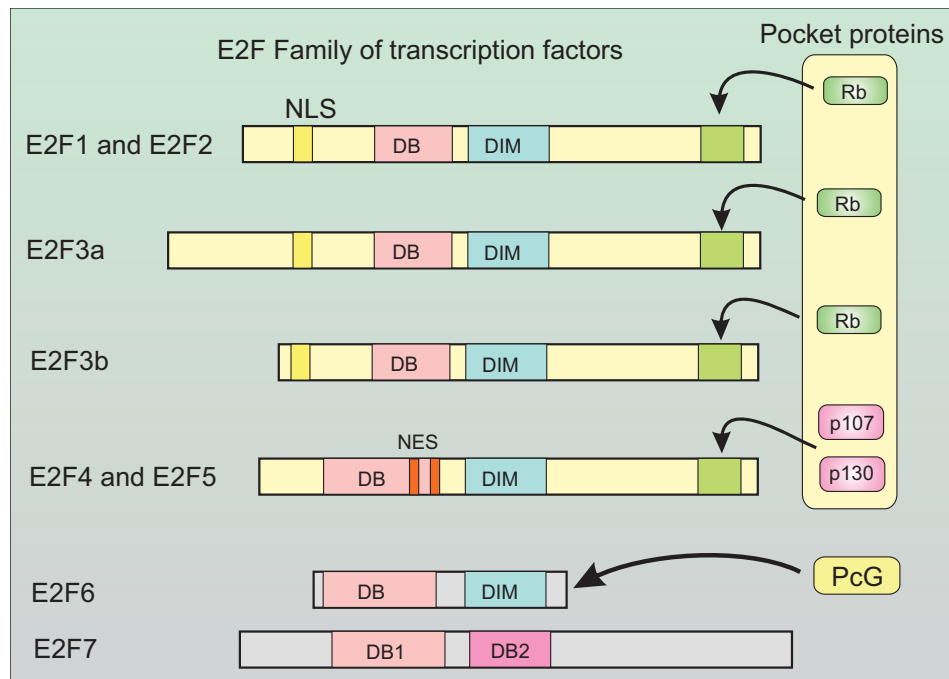
the hormone 1,25-dihydroxyvitamin D<sub>3</sub> [1,25(OH)<sub>2</sub>D<sub>3</sub>] inhibits parathyroid gland hyperplasia by increasing the expression of p21 (Module 7: Figure PTH secretion).

Mutations of p57 have been linked to Beckwith-Wiedemann syndrome.

The p27 also plays an important role in the activation of the cyclin E/CDK2 complex (Module 9: Figure cell cycle signalling mechanisms). The onset of cell proliferation depends upon a rapid fall in the level of p27 early in G<sub>1</sub> (Module 9: Figure cell cycle dynamics).

### Pocket proteins

The pocket proteins, represented by the retinoblastoma protein (Rb) family (Module 9: Table cell cycle toolkit) contains three proteins: Rb/p105, p107 and p130/Rbl2. Rb play a central role in regulating the cell cycle through their control of the E2F family of transcription factors that regulate expression of a number of cell cycle components, such as cyclin E, cyclin A, Cdc25A and E2F itself (Module 9: Figure cell cycle signalling mechanisms). The Rb family and the E2F family bind to each other (Module 9: Figure Rb-E2F interactions) to set up a complex web of interactions that function to both activate and repress transcription of a large number of genes. The Rb family acts by regulating the activity of the E2F family, with Rb acting as an activator, whereas p107 and p130/Rbl2 are repressors.

**Module 9:** | Figure Rb–E2F interactions

**The domain structure of the E2F family of transcription factors and their Rb family-binding partners.**

All the E2F transcription factors have a DNA-binding domain (DB), and most of them also have a dimerization domain (DIM). The Rb family-binding domain (green) located in the C-terminal region of the transcriptional activators E2F1–E2F3 is responsible for binding retinoblastoma protein (Rb), whereas p107 and p130 bind to a similar domain on the repressors E2F4 and E2F5. E2F6 and E2F7 do not bind pocket proteins, but E2F6 does bind to the Polycomb group of proteins (PcG). The E2F1–E2F3 isoforms have a nuclear localization signal (NLS), whereas E2F4 and E2F5 have two nuclear export signals (NESs).

The pocket proteins have an important role in maintaining the cell in a  $G_0$  state by repressing the E2F genes (Module 9: Figure cyclin D/CDK action). Most attention has focused on Rb, which is a nuclear protein whose activity is regulated through its phosphorylation state. During the resting  $G_0$  phase, hypophosphorylated Rb binds to E2F to repress its transcriptional activity. However, when cyclin D/cyclin-dependent kinase 4/6 (CDK4/6) begins to phosphorylate Rb, the latter leaves the promoter site to allow E2F1–E2F3 to transcribe a variety of genes that function in the cell cycle (Module 9: Figure cell cycle signalling mechanisms). In the case where Rb family members act to control repressors such as E2F4 and E2F5, the phosphorylation of p107 and p130/Rb12 removes these repressors. Therefore, there are two main mechanisms whereby the Rb family regulates transcription. Rb allows the E2F1–E2F3 isoforms to exert their activator role, whereas p107 and p130 remove the repressor activity of E2F4 and E2F5 (Module 9: Figure cyclin D/CDK action).

### E2F family of transcription factors

The family of E2F transcription factors (Module 9: Table cell cycle toolkit) plays a critical role in cell proliferation by controlling the transcription of many of the key components of the cell cycle. The E2Fs function together with their binding partners DP1 and DP2. The E2F family can be divided into those that are strong transcriptional activators

(E2F1, E2F2 and E2F3a) and those that act as repressors (E2F4–E2F7) (Module 9: Figure Rb–E2F interactions). Both the activators and the repressors are controlled by the family of pocket proteins. Retinoblastoma protein (Rb) acts to regulate E2F1–E2F3, whereas the repressors E2F4 and E2F5 are regulated by the pocket proteins p107 and p130 (Module 9: Figure cyclin D/CDK action). E2F6 and E2F7 act independently of the pocket proteins.

The Rb/E2F families set up a network that not only controls cell proliferation, but also participates in checkpoint controls, differentiation and apoptosis.

### DNA damage and checkpoint signalling

DNA damage occurs frequently during the life of a cell and can lead to mutations and the development of cancer. There are different types of damage that give rise to different types of DNA lesions that are then dealt with by a sophisticated set of repair mechanisms often referred to as the DNA damage response (DDR) pathway. Since these repair processes can take some time to complete, a serious problem arises in cells that are actively proliferating. It would be disastrous if cells began DNA synthesis or mitosis while repairing their DNA. Consequently there are DNA damage checkpoint signalling systems that operate to co-ordinate the processes of DNA repair with the different phases of the cell cycle.

## DNA damage

DNA damage results in genotoxic stress caused by both exogenous and endogenous agents resulting in different types of lesion. These lesions are then recognized by components of the different repair mechanisms. This DNA damage is caused by two main groups of agents. Firstly, there are exogenous agents such as chemicals, ultraviolet (UV) and ionizing radiation (IR). Secondly, there are endogenous agents such as reactive oxygen species (ROS) that induce DNA breaks, oxidation of bases, mismatches caused by replication defects and strand breaks caused by collapse of the replication fork. Double strand breaks (DSBs), which are among the deadliest of lesions, are caused by multiple factors including replication fork collapse induced by blocking lesions caused by ROS, normal genomic rearrangements that occur during meiosis or V(D)J recombination or by the physical stress exerted on dicentric chromosomes as they are pulled apart at mitosis. When such DSBs occur during the S phase, the high levels of CDKs will induce resection of the DSBs resulting in the appearance of single-stranded DNA (ssDNA). Stalling of the DNA polymerase can also result in ssDNA tracts when the replicative helicase is uncoupled from the advancing fork. The checkpoint signalling pathways are triggered by either the DSBs or the ssDNA. The Fanconi anaemia/BRCA pathway also seems to play a central role in both DNA repair and cell cycle control.

Genotoxic stress activation of NF- $\kappa$ B signalling provides another mechanism for protecting cells following DNA damage.

Defects in the DDR signalling pathways have been linked to a number of disorders such as Ataxia telangiectasia (AT) syndrome where mutations in ataxia telangiectasia mutated (ATM) results in defects in the repair of double strand breaks (DSBs).

## Checkpoint signalling

DNA damage activates a checkpoint signalling system that has components similar to those found in many other cell signalling pathways. Components of the checkpoint signalling toolkit (Module 9: Table checkpoint signalling toolkit) are organized into signalling pathways that relay information from the sites of DNA damage to various downstream effectors that can temporarily arrest the cell cycle. The DNA lesions, such as the double-strand breaks (DSBs) or single-stranded DNA (ssDNA), provide the stimuli that are recognized by 'receptors'. The latter are then coupled through transducers/amplifiers to activate the checkpoint kinases that function as messengers that relay information to the various effectors, which are components of the cell cycle machinery. The key components of the checkpoint signalling network are the phosphoinositide 3-kinase related kinases (PIKKs) represented by Ataxia-telangiectasia mutated (ATM), ATM and RAD3-related, and DNA-dependent protein kinase. It is these three PIKKs that feature significantly in checkpoint signalling systems that will be illustrated by considering G<sub>1</sub> checkpoint signalling to DNA double-strand breaks (DSBs) and S and G<sub>2</sub>/M checkpoint signalling to single stranded DNA.

**Module 9:** | Table checkpoint signalling toolkit  
Checkpoint signalling toolkit.

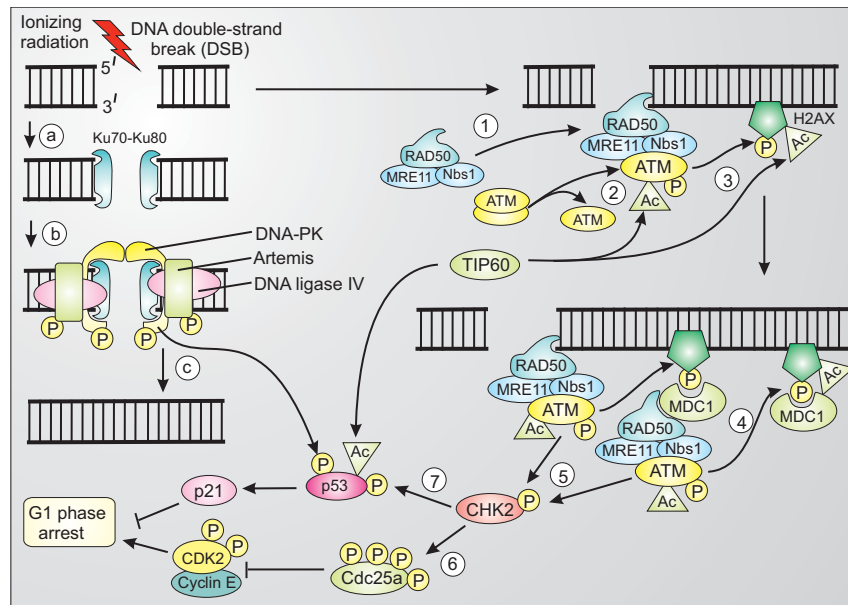
Checkpoint signalling components	Comments
<b>Receptors/sensors</b>	
Ku 70 and Ku80	One of the sensors that recognizes DSBs (Module 9: Figure G <sub>1</sub> checkpoint signalling)
RAD 50	One of the sensors that recognizes DSBs (Module 9: Figure G <sub>1</sub> checkpoint signalling)
RPA	Replication protein A coats single stranded DNA (ssDNA) (Module 9: Figure S/G <sub>2</sub> phase checkpoint signalling)
<b>Transducers/amplifiers</b>	Phosphoinositide 3-kinase related kinases (PIKKs)
ATM	Ataxia-telangiectasia mutated (ATM)
ATR	ATM and RAD3-related
DNA-PK	DNA-dependent protein kinase
TIP60	Protein acetylase
<b>Adaptors/scaffolding proteins</b>	
MRE11	Meiotic recombination protein-11
Nbs1	Nijmegen breakage syndrome protein-1 responsible for recruiting ATM
MDC1	Mediator of DNA damage checkpoint protein-1 is an adaptor that functions in G <sub>1</sub> checkpoint signalling (Module 9: Figure G <sub>1</sub> checkpoint signalling)
ATRIP	ATR-interacting protein is an adaptor that functions in S/G <sub>2</sub> checkpoint signalling (Module 9: Figure S/G <sub>2</sub> phase checkpoint signalling)
RAD17-RFC	RAD17-replication factor C functions as a clamp loader for the 9-1-1 complex
RAD-9	A component of the 9-1-1 complex
RAD1	A component of the 9-1-1 complex
HUS1	A component of the 9-1-1 complex
TOBP1	Topoisomerase-binding protein-1
Claspin	Adaptor protein that connects ATR to CHK1 (Module 9: Figure S/G <sub>2</sub> phase checkpoint signalling)
<b>Checkpoint kinases (CHKs)</b>	
CHK1	Checkpoint kinase 1
CHK2	Checkpoint kinase 2

## G<sub>1</sub> checkpoint signalling to DNA double-strand breaks (DSBs)

DNA double-strand breaks (DSBs) are among the deadliest of lesions and are responsible for triggering the following two G<sub>1</sub> checkpoint signalling cascades (Module 9: Figure G<sub>1</sub> checkpoint signalling):

### *DNA-protein kinase (DNA-PK) signalling pathway*

- The DNA break is recognized by the Ku80 component of the Ku70–Ku80 complex that provides a nucleus to bind other components of the pathway.
- The DNA-PKs, artemis and DNA ligase IV associate with the Ku complex. The DNA-PK is activated through an autophosphorylation reaction.
- The DNA ligase IV then joins the two ends together to complete the repair process. In addition, the DNA ligase can contribute to the process of G<sub>1</sub> arrest by activating p53 as will be described in the following section.

**Module 9:** | Figure G<sub>1</sub> checkpoint signalling**G<sub>1</sub> checkpoint signalling mechanisms.**

Ionizing radiation causes double-strand breaks (DSBs) that induce two major DNA damage signalling pathways that arrests cells in the G<sub>1</sub> phase of the cell cycle. The DNA-protein kinase (DNA-PK) signalling system (a-c). The ATM/checkpoint 2 signalling pathway (1-7).

*The ATM/checkpoint 2 signalling pathway*

- Another way in which the DSB is recognized depends on the binding of a MRN complex that is made up from the meiotic recombination protein-11 (MRE-11), RAD50 and Nijmegen breakage syndrome protein-1 (Nbs1) proteins. It is the RAD50 that is responsible for recognizing the DSB.
- The MRN complex recruits ataxia-telangiectasia mutated (ATM) during which it switches from an inactive dimer to an active phosphorylated monomer. It is the Nbs1 that binds this activated ATM. TIP60 also plays a role by acetylating and phosphorylating ATM.
- One of the first actions of activated ATM is to phosphorylate the C-terminal tails of the histone variant H2AX, which initiates an amplification process. The acetylase TIP60 also plays a role by opening up chromatin by acetylating H2A and H4.
- This amplification process is completed by the phosphorylated H2AX recruiting mediator of DNA damage checkpoint protein-1 (MDC1), which is an adaptor that recruits additional MRN-ATM complexes to build up a local population of activated ATM molecules.
- The activated ATM then phosphorylates checkpoint kinase 2 (CHK2), which is responsible for G<sub>1</sub> phase arrest through two main pathways.
- CHK2 hyperphosphorylates Cdc25A, which is one of the dual-specificity Cdc25 phosphatases that act by removing inhibitory phosphates on CDK2 that controls Cyclin E. The Cyclin E con-

trols G<sub>1</sub> progression and DNA synthesis and the inhibition of this critical step results in G<sub>1</sub> phase arrest (Module 9: Figure cell cycle signalling mechanisms).

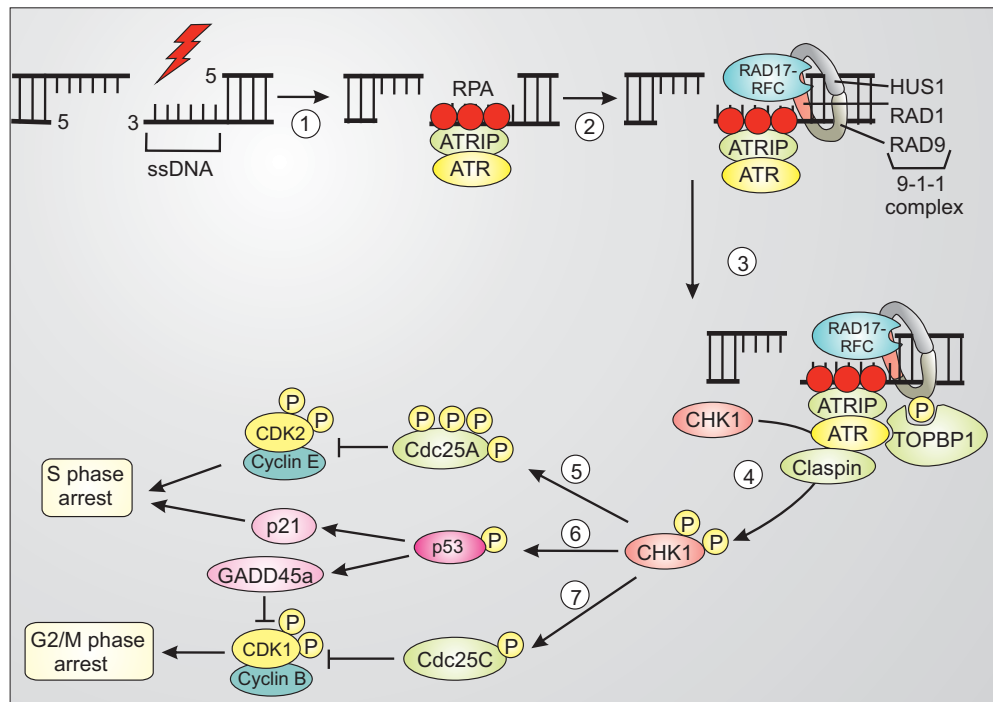
- The activated CHK2 can also arrest cells by phosphorylating and activating the transcription factor p53 (Module 4: Figure p53 function). One of the main mechanisms used by p53 is to activate G<sub>1</sub> arrest by activating p21, which is a potent cyclin-dependent kinase (CDK) inhibitor (Module 9: Figure proliferation signalling network). p21 inhibits the CDK2 that activates transcription of the E2F-regulated genes that are required for the onset of DNA replication (Module 9: Figure cell cycle signalling mechanisms). A number of other p53-dependent mechanisms can inhibit the cell cycle as described in the section on p53-induced cell cycle arrest.

The protein acetylase TIP60 also plays a role in growth arrest by acetylating p53 on lysine-120.

**S and G<sub>2</sub>/M checkpoint signalling to single-stranded DNA**

An ATR/checkpoint 1 signalling pathway, which occurs mainly during the S and G<sub>2</sub> phases of the cell cycle, operates during the process of homologous recombination. The primary trigger that initiates this checkpoint signalling response is the existence of single-stranded DNA (ssDNA) as outlined in the following sequence of events (Module 9: Figure S/G<sub>2</sub> phase checkpoint signalling):

- The first step in the signalling pathway is the coating of the single-stranded regions with replication protein A

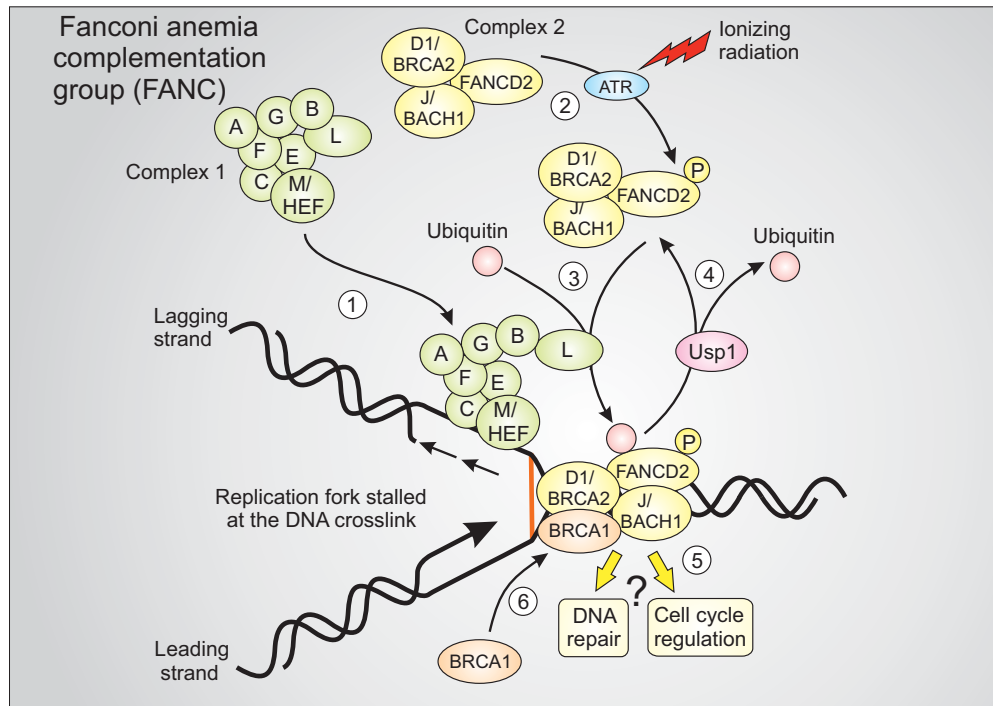
**Module 9:** | Figure S/G<sub>2</sub> phase checkpoint signalling**S and G<sub>2</sub> phase checkpoint signalling.**

An ATR/checkpoint 1 signalling pathway responds to single-stranded DNA (ssDNA) by initiating a signalling cascade that results in either S or G<sub>2</sub> arrest depending on the phase of the cell cycle. See text for details of the numbered sequence of events

- (RPA). The latter then functions as a receptor to recruit ATR-interacting protein (ATRIP), which functions as an adaptor that brings ATM and RAD3-related (ATR) protein kinase into the site of DNA damage. This RPA-ATRIP-ATR complex is not enough to induce ATR activation, which requires the recruitment of additional components.
- Activation of ATR depends on the 9-1-1 complex, which is a ring-shaped molecule made of RAD9, RAD1 and HUS1. The loading of 9-1-1 at the primer-template junction is facilitated by the clamp loader RAD17-replication factor C (RAD17-RFC).
- Once the 9-1-1 complex is in place, the C-terminal tail of the RAD9 component of this complex is phosphorylated and this provides a binding site that recruits the topoisomerase-binding protein-1 (TOPBP1). The TOPBP1 is positioned close to ATR and is able to activate the latter through its ATR activation domain.
- Once TOPBP1 has activated ATR, the latter can begin to phosphorylate and activate checkpoint kinase 1 (CHK1), which relays information to the cell cycle components that bring about cell cycle arrest. The interaction between CHK1 and ATR is facilitated by the protein claspin.
- CHK2 hyperphosphorylates Cdc25A, which is one of the dual-specificity Cdc25 phosphatases that act by removing inhibitory phosphates on CDK2 that controls Cyclin E. The Cyclin E controls G<sub>1</sub> progression and DNA synthesis and the inhibition of this critical step results in G<sub>1</sub> phase arrest (Module 9: Figure cell cycle signalling mechanisms).
- The activated CHK2 can arrest the cell cycle by phosphorylating and activating the transcription factor p53 (Module 4: Figure p53 function). p53-induced cell cycle arrest depends on a number of mechanisms (Module 9: Figure proliferation signalling network). One of the main mechanisms used by p53 is to activate p21, which is a potent cyclin-dependent kinase (CDK) inhibitor. p21 inhibits the activity of CDK2 that controls transcription of the E2F-regulated genes that are required for DNA replication (Module 9: Figure cell cycle signalling mechanisms). Another protein called GADD45a, which is up-regulated by p53, acts together with the Wilms' tumour suppressor (WT1) to dissociate the cyclin B/CDK1 complex by binding to CDK1 (Module 9: Figure proliferation signalling network).
- CHK1 also phosphorylates Cdc25C, which removes inhibitory phosphates on CDK1 that controls the cyclin B that is responsible for the final events of the cell cycle and thus contributes to the G<sub>2</sub>/M phase arrest (Module 9: Figure cell cycle signalling mechanisms).

**Fanconi anaemia/BRCA pathway**

The Fanconi anaemia/BRCA pathway operates during DNA damage that is induced by DNA cross-linking and other forms of genotoxic stress. The components of this pathway came to light during the analysis of Fanconi anaemia, which is a genetically heterogeneous disease that results in chromosome instability. There is a Fanconi anaemia complementation group (FANC) that has at least 12 components (A, B, C, D1, D2, E, F, G, I, J, L and M). These FANC proteins, which seem to function as two

**Module 9:** | Figure Fanconi anaemia pathway**Fanconi anaemia pathway in DNA repair and cell cycle control.**

The Fanconi anaemia pathway functions in DNA repair, particularly in response to DNA crosslinks (vertical red line). The Fanconi anaemia complementation group (FANC) proteins operate as part of two macromolecular complexes that are drawn into such regions when DNA replication is stalled at the crosslinks. Complex 1 (green proteins) has the ubiquitin ligase (L) that ubiquitinates FANCD2, which is part of complex 2 (yellow proteins). Monoubiquitination of the FANCD2 is particularly important in the activation of this pathway. The information used to construct this figure was taken from Kennedy and D'Andrea (2009).

separate complexes (the green and yellow groups of proteins shown in Module 9: Figure Fanconi anaemia pathway), co-operate with each other in the operation of a signalling system that responds to various defects in DNA. The pathway is particularly evident during DNA synthesis when the replication fork stalls due to defects such as a DNA cross-link. Just how all these proteins function is still being worked out and the following hypothesis illustrates some of the key features of the pathway (Module 9: Figure Fanconi anaemia pathway):

1. The A, B, C, E, F, G, L, M and possibly I (coloured green) form complex 1 that binds to the stalled lagging strand. The M protein has been identified as helicase-associated endonuclease for forked structured DNA (Hef).
2. The other group of FANC proteins form complex 2 that have three members (coloured yellow): D1 [(also known as breast cancer 2 (BRCA2)], D2 (referred to here as FANCD2) and the J protein that has been identified as BRCA1-interacting protein C-terminal helicase (BRIP1), which is also known as BACH1. Both phosphorylation and ubiquitination reactions have been implicated in the activation and attachment of these proteins to DNA. The Ataxia telangiectasia mutated and Rad3-related (ATR) kinase, which is activated during ionizing radiation (Module 9: Figure S/G<sub>2</sub> phase check-

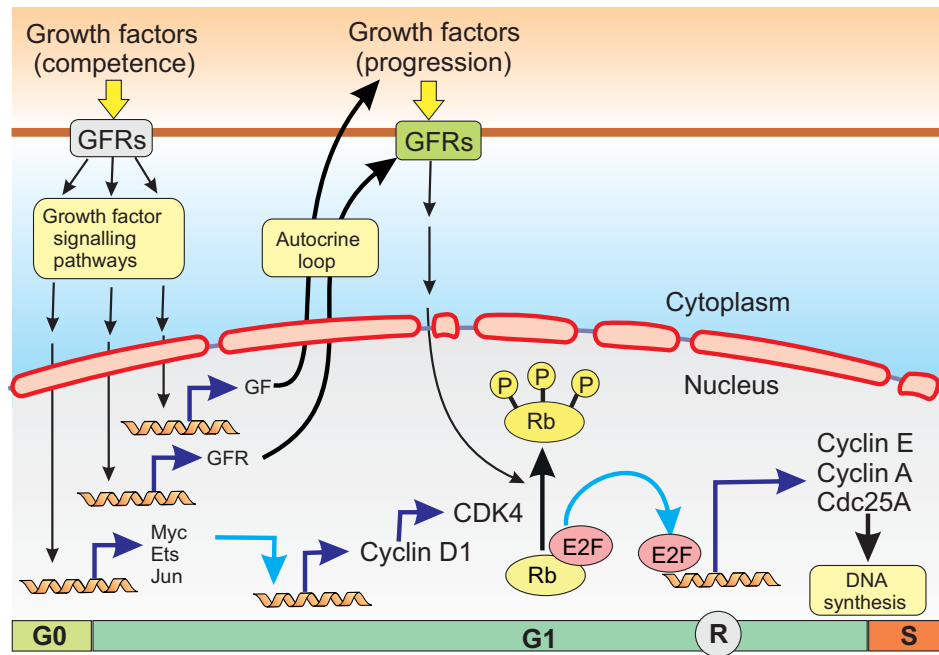
point signalling), is one of the kinases that can phosphorylate FANCD2.

3. The L subunit of complex 1 is an ubiquitin ligase that ubiquitinates and activates FANCD2 to facilitate the attachment of complex 2 to the replication fork.
4. The ubiquitin-specific protease 1 (Usp1) reverses this activation process by deubiquitinating FANCD2.
5. The precise function of complex 2 is still not clear, but it appears to contribute to both DNA repair and cell cycle regulation. For example, FANCD2 may participate in DNA repair by interacting with various recombination enzymes.
6. Breast cancer 1 (BRCA1) is also drawn into the complex and has a role in carrying out the processes responsible for DNA repair

Mutations in components of the Fanconi anaemia/BRCA pathway are responsible for the genetically heterogeneous disease Fanconi anaemia.

**Genotoxic stress activation of NF- $\kappa$ B signalling**

Genotoxic stress resulting from DNA damage caused by a variety of insults, such as ionizing radiation and anticancer drugs, can induce a protective pathway based on activation of the NF- $\kappa$ B signalling pathway (see Step 10 in Module 2: Figure NF- $\kappa$ B activation). The activity of the ubiquitin E3 ligase Parkin may be particularly relevant with regard to cell survival because one of the genes activated by NF- $\kappa$ B is

**Module 9:** | Figure G<sub>1</sub> proliferative signalling**Spatiotemporal aspect of proliferative signalling.**

Competence growth factors initiate a number of signalling pathways that carry information into the nucleus to activate immediate early genes. Some of these early genes encode information for the expression of progression growth factors (GFs) and their receptors (GFRs) to set up an autocrine loop to drive later events. Other early genes encode transcription factors such as E twenty-six (ETS), Jun and Myc to initiate transcription of later genes, such as cyclin D, which combines with the cyclin-dependent kinases (CDKs) to phosphorylate the retinoblastoma protein (Rb): one of the key gate-keepers guarding passage through the restriction point (R). A key event at the restriction point (R) is the phosphorylation of Rb, which removes its inhibition of E2F and enables this transcription factor to increase the expression of the cell cycle components (e.g. cyclin E, cyclin A and Cdc25A) responsible for initiating DNA synthesis. Further details of this activation sequence are provided in Module 9: Figure cell cycle signalling mechanisms.

optic atrophy 1 (OPA1), which is critical for maintaining the cristae in mitochondria and thereby prevents apoptosis (Module 5: Figure OPA1 and mitochondrial cristae remodelling).

**Cell proliferation**

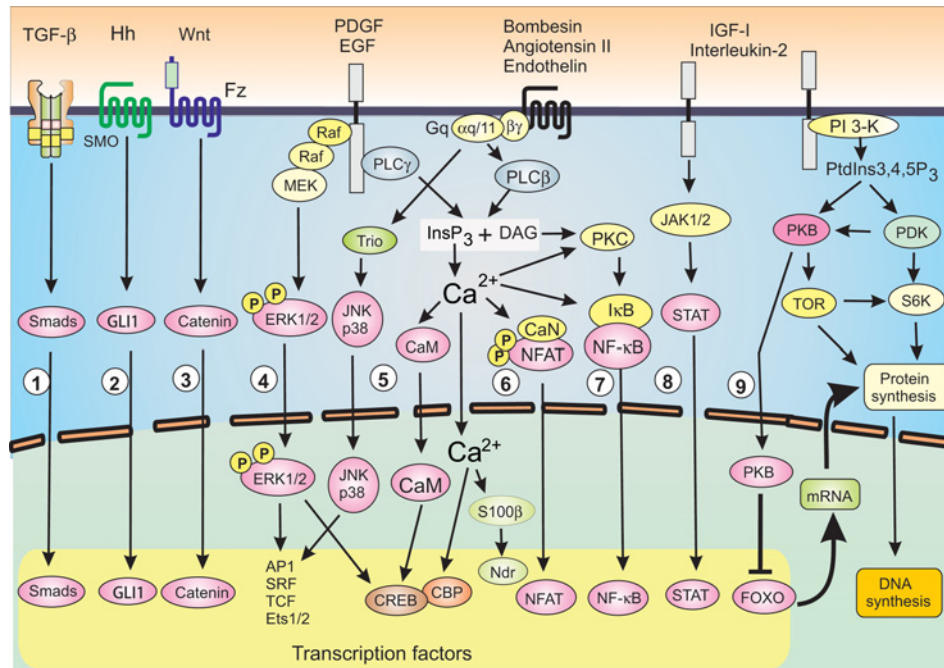
Resting cells in a zero growth phase (G<sub>0</sub>) are induced to re-enter the cell cycle by growth factors that function by stimulating a process of cell proliferation (Module 9: Figure cell cycle network).

Spatial and temporal aspects feature significantly in the activation of cell proliferation by growth factor signalling pathways (Module 9: Figure G<sub>1</sub> proliferative signalling). The spatial problem concerns the fact that growth factors acting at the cell surface have to transfer information to internal targets, many of which are found in the nucleus. With regard to the temporal problem, the signalling pathways have to operate over long periods in G<sub>1</sub>. This question of the time it takes to stimulate cells to enter the cell cycle is also apparent during the completion of meiosis at fertilization, where Ca<sup>2+</sup> spikes have to persist for 2 h before the zygote is committed to begin its growth and development. Another example is Ca<sup>2+</sup> signalling in lymphocytes, where a similar long period of stimulation is required to drive cells into the cell cycle. Therefore the information flow during proliferative signalling is adapted

to transmit information from growth factor receptors to the nucleus over long periods of time.

**Information flow during proliferative signalling**

Most of the signalling pathways used to transmit information into the cell are made up of sequential elements, some of which are sufficiently stable and diffusible to disperse information deep into the cell, thus solving the spatial problem. These early signalling events are driven by the so-called competence growth factors in that they set the stage for the action of the later progression growth factors (Module 9: Figure G<sub>1</sub> proliferative signalling). Solution of the temporal problem has two elements. Firstly, the signalling mechanisms show little desensitization, thus enabling them to operate over prolonged periods. Secondly, the signalling pathways trigger a cascade of gene transcriptional events that serve to maintain the flow of information during the critical G<sub>1</sub> period. One way of doing this is to induce the expression of additional signalling pathways that operate later in G<sub>1</sub>. It is during this transcriptional cascade that the progression growth factors and their receptors are put in place to provide additional signalling pathways that carry information in from the outside to drive some of the later cell cycle signalling elements responsible for initiating DNA synthesis.

**Module 9:** | Figure growth factor signalling

Growth factors use a large number of signalling pathways to activate cell proliferation.

A number of signalling pathways co-operate with each other to mediate the action of growth factors. Those components coloured in red are responsible for carrying information from the cell surface into the nucleus. Some of these components are transcription factors, whereas others are signalling components such as Ca<sup>2+</sup> and protein kinases such as extracellular-signal-regulated protein kinase 1/2 (ERK1/2) and protein kinase B (PKB) that target transcription factors resident within the nucleus. All of these transcription factors control the expression of the cell cycle components that are required to induce DNA synthesis. Further details of these signalling pathways are given in the text.

In summary, growth factors use a number of signalling mechanisms to initiate the process of cell proliferation. Early in G<sub>1</sub>, these signalling pathways transmit information into the nucleus to trigger a transcriptional cascade that culminates in the phosphorylation of retinoblastoma protein (Rb) at the restriction point. As the role of the growth factor-dependent signalling pathways decline during G<sub>1</sub>, the onus for driving the cell cycle to completion is gradually handed over on to the endogenous cell cycle signalling mechanisms based on the cyclin cascade (blue arrows in Module 9: Figure cell cycle). These cell cycle events are set in motion by a number of growth factor signalling pathways.

### Growth factor signalling pathways

Growth factors use a variety of signalling pathways to transmit information into the nucleus. In describing these pathways, the emphasis will be placed on the way in which information travels from the cell surface to the cytosolic and nuclear targets that co-ordinate the onset of DNA synthesis, as outlined in Steps 1–10 in Module 9: Figure growth factor signalling:

1. The Smad signalling pathway mediates the transforming growth factor β (TGF-β) inhibition of cell proliferation. Stimulation of the TGF-β receptor results in the activation of the Smad transcription factors (Module 2: Figure TGF-βR activation),

which then translocate into the nucleus (Module 2: Figure Smad signalling). One of the functions of the Smads is to increase the expression of the cyclin-dependent kinase (CDK) inhibitor p15 (Module 4: Figure Myc as a gene repressor), which acts to inhibit cell proliferation.

2. The Hedgehog (Hh) signalling pathway activates the zinc-finger transcriptional activator GLI1, which translocates into the nucleus to activate a number of genes, some of which are components of other signalling systems (Module 2: Figure Hedgehog signalling pathway).
3. The canonical Wnt/β-catenin pathway activates the transcription factor β-catenin, which translocates into the nucleus to activate a number of the genes that function in cell proliferation (Module 2: Figure Wnt canonical pathway).
4. The mitogen-activated protein kinase (MAPK) signalling pathway plays a central role in cell proliferation. Activation of tyrosine kinase-linked receptors results in the phosphorylation of extracellular-signal-regulated kinases 1/2 (ERK1/2), which then translocate into the nucleus (Module 2: Figure ERK signalling). One of the main targets of ERK1/2 in the nucleus is the transcription factor cyclic AMP response element-binding protein (CREB) (Module 4: Figure CREB activation). In addition, ERK1/2 activates a number of other transcription factors (Module 9: Figure growth factor signalling).

5.  $\text{Ca}^{2+}$  signalling plays a key role in controlling the process of cell proliferation in many primary cells. The importance of  $\text{Ca}^{2+}$  as a proliferative signal may also explain the connection between  $\text{K}^+$  channels and cell proliferation. The opening of  $\text{K}^+$  channels is critical for keeping the membrane hyperpolarized to maintain the driving force for  $\text{Ca}^{2+}$  entry.

The G protein-coupled receptors (GPCRs) that function as growth factor receptors are linked to different signalling systems. Most of the GPCRs that stimulate proliferation are coupled to the heterotrimeric G protein Gq that dissociates into  $\text{G}_{\beta\gamma}$  and  $\text{G}_{\alpha q}$ . The latter has two actions. Firstly it can activate Trio, which is a guanine nucleotide-exchange factor (GEF) that acts through Rho and Rac to engage the c-Jun N-terminal kinase (JNK) pathway and the p38 pathway to activate a number of transcription factors. The  $\text{G}_{\alpha q}$  can also stimulate the formation of inositol 1,4,5-trisphosphate ( $\text{InsP}_3$ ) and diacylglycerol (DAG) (Module 2: Figure  $\text{InsP}_3$  and DAG formation). The DAG released following hydrolysis of  $\text{PtdIns}4,5\text{P}_2$  by phospholipase C (PLC) has an important role in regulating cell proliferation. One function is to activate protein kinase C (PKC), which then phosphorylates inhibitor of nuclear factor  $\kappa\text{B}$  (I $\kappa\text{B}$ ), causing it to be hydrolysed, and the released nuclear factor  $\kappa\text{B}$  (NF- $\kappa\text{B}$ ) is then free to diffuse into the nucleus (Module 9: Figure growth factor signalling).

The  $\text{InsP}_3$  releases  $\text{Ca}^{2+}$ , which can stimulate cell proliferation in a number of ways. Some of the cytoplasmic targets of  $\text{Ca}^{2+}$  are the transcription factors nuclear factor of activated T cells (NFAT) (see Step 6) and NF- $\kappa\text{B}$  (Step 7). In addition to acting in the cytoplasm,  $\text{Ca}^{2+}$  and calmodulin (CaM) can invade the nucleus, where they activate various effectors such as  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase IV (CaMKIV), the CREB-binding protein (CBP) or the S100B-dependent nuclear protein kinase (Ndr). One of the functions of CaMKIV is to activate CREB (Module 4: Figure CREB activation).

The  $\text{Ca}^{2+}$  sensor CaM undergoes a profound redistribution during the course of the cell cycle. During  $\text{G}_1$ , much of the calmodulin is in the cytoplasm, but becomes concentrated in the nucleus during the S phase and is localized at the spindles and at the site of cell cleavage during the process of mitosis (Module 9: Figure CaM redistribution at mitosis).

One reason the importance of  $\text{Ca}^{2+}$  in regulating cell proliferation has not been fully appreciated is that much of the experimental work has been carried out on immortalized cell lines, which may have a signalling phenotype very different from that of primary cells. In particular, it is possible that many of these cells may have  $\text{Ca}^{2+}$  signalling pathways that are constitutively active, and thus appear to be independent of  $\text{Ca}^{2+}$ . Some evidence for this is that a large number of transformed cells can continue to proliferate at low levels of external  $\text{Ca}^{2+}$  that completely inhibit the growth of normal cells.

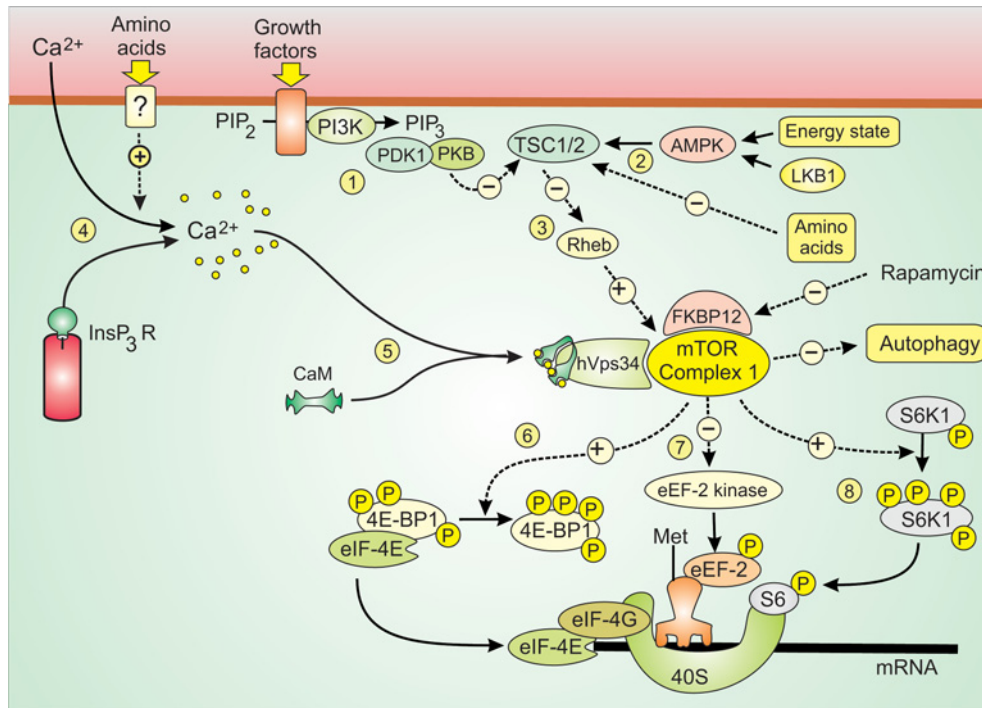
6. One of the functions of  $\text{Ca}^{2+}$  is to activate the NFAT (Module 4: Figure NFAT activation). In its inactive state, it is phosphorylated and located in the cytoplasm. When  $\text{Ca}^{2+}$  rises, it activates the enzyme calcineurin (CaN) to remove the phosphate to release the active form of NFAT, which diffuses into the nucleus to initiate gene transcription (Module 9: Figure growth factor signalling).
7.  $\text{Ca}^{2+}$  is one of the messengers responsible for activating the nuclear translocation of NF- $\kappa\text{B}$ . The latter is retained in the cytoplasm through its attachment to I $\kappa\text{B}$ , which is hydrolysed following its phosphorylation through various signalling pathways, including the  $\text{Ca}^{2+}$  and the DAG/PKC pathways.
8. The Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signalling pathway functions by activating the STATs (Module 2: Figure JAK/STAT function), which then translocate into the nucleus to activate gene transcription.
9. The  $\text{PtdIns}3$ -kinase has a number of functions in regulating cell proliferation (Module 9: Figure growth factor signalling). The lipid second messenger  $\text{PtdIns}3,4,5\text{P}_3$  activates both phosphoinositide-dependent kinase 1 (PDK1) and protein kinase B (PKB). The latter translocates into the nucleus, where it inactivates the forkhead box O (FOXO) transcription factors (Module 4: Figure FOXO control mechanisms). FOXO is responsible for driving the expression of various inhibitors of proliferation, such as p27 and Rb. Inhibition of this transcriptional activity accounts for the rapid decline in the level of p27 that occurs when cells are stimulated to enter the cell cycle (Module 9: Figure cell cycle dynamics). PDK1/2 acts through ribosomal S6 protein kinase (S6K) to stimulate protein synthesis. PKB also contributes to the target of rapamycin (TOR) signalling pathway that functions in the control of protein synthesis (Module 9: Figure target of rapamycin signalling). Through this control of protein synthesis, the  $\text{PtdIns}3$ -kinase signalling pathway plays a key role in cell growth control.

The signalling mechanisms summarized in Module 9: Figure growth factor signalling represent a proliferative signalling toolkit from which specific systems are assembled to control the proliferation of specific cell types.

### **$\text{K}^+$ channels and cell proliferation**

There are many examples of  $\text{K}^+$  channels playing a role in controlling cell proliferation. The primary candidate for carrying out this role is the intermediate-conductance (IK) channel  $\text{K}_{\text{Ca}3.1}$  (Module 3: Table properties of  $\text{Ca}^{2+}$ -sensitive  $\text{K}^+$  channels), which is one of the  $\text{Ca}^{2+}$ -sensitive  $\text{K}^+$  channels. The primary action of  $\text{K}_{\text{Ca}3.1}$  is to hyperpolarize the membrane and thereby maintain the driving force for the entry of external  $\text{Ca}^{2+}$ , as has been demonstrated for lymphocytes (Module 9: Figure T cell  $\text{Ca}^{2+}$  signalling).

Proliferation is reduced by toxins (charybdotoxin and iberiotoxin) or inhibitors that block these  $\text{K}^+$  channels.

**Module 9:** | Figure target of rapamycin signalling**Integration of growth factor and nutrient signalling by the target of rapamycin (TOR).**

The target of rapamycin (TOR) is a major integrative serine/threonine protein kinase that responds to information coming in from growth factor receptors and from metabolic signalling pathways that assess the nutritional status of the cell. All this information is integrated by the tuberous sclerosis complex 1 and 2 (TSC1 and TSC2), which then relay signals to TOR through the Ras homologue enriched in brain (Rheb) protein. TOR acts through a number of targets to control the rate of protein synthesis. TOR also has an important role to inhibit autophagy (Module 11: Figure autophagy).

Overexpression of these channels has been found in many tumours.

**Cell growth control**

The growth factors that control cell proliferation have two major actions. They must exert both cell cycle control and cell growth control. The latter is concerned with the control of the macromolecular synthetic events that are necessary for cells to double their size prior to mitosis. This control of growth is complex, because not only is it stimulated by growth factors, but also it is regulated by nutrients such as amino acids and by the energy state of the cell. What is remarkable about these different regulatory inputs is that they all impinge upon a common regulatory system based on the mammalian target of rapamycin (TOR), which then controls the target of rapamycin (TOR), which has a major role in regulating protein synthesis by phosphorylating both ribosomal S6 protein kinase 1 (S6K1) and eukaryotic initiation factor 4E (eIF4E)-binding protein (eIF4E-BP1). TOR is a protein kinase that forms two complexes: TOR complex 1 and TOR complex 2. Most information is available for TOR complex 1, which consists of TOR, regulatory-associated protein of TOR (raptor), G protein-like protein (GβL) and proline-rich PKB/Akt substrate 40 kDa (PRAS40). This TOR complex 1 is regulated through two separate signalling mechanisms (Module 9: Figure target of rapamycin signalling). One pathway is

activated by hormones and growth factors and uses signalling components such as the tumour suppressor proteins tuberous sclerosis complex 1 and 2 (TSC1/2) and the Ras-homologue enriched in brain (Rheb). The other pathway is regulated by and increase in circulating amino acids that activate a Ca<sup>2+</sup> signalling mechanism. The following sequence of events indicates how these two pathways function to activate TOR to regulate protein synthesis (Module 9: Figure target of rapamycin signalling):

1. Growth factors operating through the PtdIns 3-kinase (PI 3-K) signalling cassette generate the lipid messenger PtdIns3,4,5P<sub>3</sub> (PIP<sub>3</sub>) (Module 2: Figure PtdIns 3-kinase signalling), which then functions to activate both protein kinase B (PKB) and phosphoinositide-dependent kinase 1 (PDK1). One of the functions of PKB is to inhibit tuberous sclerosis complex 1 and 2 (TSC1/2). TSC2 is phosphorylated on Ser-969 and Thr-1462.
2. TSC1/2 also plays a critical role in co-ordinating signal inputs relating to energy state and the level of amino acids (Module 9: Figure target of rapamycin signalling). TSC1/2 relays information to TOR concerning the nutritional status of the cell. For example, if the level of amino acids falls, their inhibitory effect on TSC1/2 is removed, enabling the latter to shut off protein synthesis by inhibiting Rheb. Likewise, a decrease in the energy state activates AMP-activated protein kinase (AMPK),

which then phosphorylates TSC2 to enhance its inhibitory action on Rheb.

3. As noted above, TSC1/2 acts by inhibiting Rheb, which is a major activator of TOR. This inhibitory action is mediated through TSC2, which is a GTPase-activating protein capable of stimulating Rheb. Rheb acts by stimulating TOR, which exerts a major influence over protein synthesis by regulation both the initiation and elongation phases.
4. The activity of TOR is also activated by an increase in the concentration of circulating amino acids, which bring about an elevation of intracellular  $\text{Ca}^{2+}$  through a mechanism that depends on both the entry of external  $\text{Ca}^{2+}$  and the release of  $\text{Ca}^{2+}$  from internal stores. The  $\text{Ca}^{2+}$  response often appears as  $\text{Ca}^{2+}$  oscillations with relatively long periodicities (3–5 mins). These amino acid-induced oscillations appear to be driven by the mechanism of  $\text{Ca}^{2+}$  oscillations found in many other cell types.
5. The increase in  $\text{Ca}^{2+}$  then binds to calmodulin (CaM) that then activates the human vacuolar protein sorting 34 (hVps34), which is a Class III PtdIns 3-kinase that functions to generate PtdIns3P (Module 2: Figure PI 3-K family).
6. Control of initiation depends upon the ability of TOR to phosphorylate the eIF4E-BP1. When eIF4E-BP1 is phosphorylated, eIF4E is released to contribute to the formation of the initiation complex, together with other initiation factors such as eIF4G.
7. TOR can regulate the translocation step of elongation by altering the activity of eukaryotic elongation factor 2 (eEF2). This factor is inactivated when it is phosphorylated on Thr-56 by an eEF2 kinase. The activity of this eEF2 kinase is inhibited by TOR.
8. Control of translation depends upon TOR phosphorylating ribosomal S6 protein kinase 1 (S6K1), which then phosphorylates the S6 protein component of the 40S ribosome subunit.

## Tuberous sclerosis complex 1 and 2 (TSC1/2)

The tuberous sclerosis complex 1 and 2 (TSC1/2) plays a critical role in regulating cell growth control because it co-ordinates many of the inputs that regulate the control of protein synthesis by the protein kinase target of rapamycin (TOR) (Module 9: Figure target of rapamycin signalling). TSC is a heterodimer composed of TSC1 (also known as hamartin), which contains a coiled-coil structure, and TSC2 (also known as tuberlin), which is a GTPase-activating protein (GAP). TSC1 and 2 are phosphorylated by protein kinase B (PKB) during the action of growth factors.

TSC1 and TSC2 are considered to be tumour suppressor genes because they act by inhibiting the growth promoting signals from nutrients. The genes encoding TSC1/2 are inactivated in the autosomal dominant disorder tuberous sclerosis.

## Target of rapamycin (TOR)

The target of rapamycin (TOR), which is also referred to as mammalian target of rapamycin (mTOR), derives its name from the fact that it is inhibited by the drug rapamycin that is a potent inhibitor of cell proliferation. Some analogues of rapamycin are being tested as anti-tumour agents. Rapamycin exerts its inhibitory action by first binding to the immunophilin FK506-binding protein (FKBP) of 12 kDa (FKBP12) (Module 9: Figure target of rapamycin signalling).

TOR is a serine/threonine protein kinase, which operates as a nutrient-sensitive cell cycle checkpoint. The highly conserved kinase domain is located at the C-terminus close to an FRB domain that binds the FKBP12. The activity of TOR is switched off, and cell proliferation ceases under conditions when amino acid levels are low or when energy is limiting. This inhibition of TOR is also responsible for the onset of autophagy (Module 11: Figure autophagy).

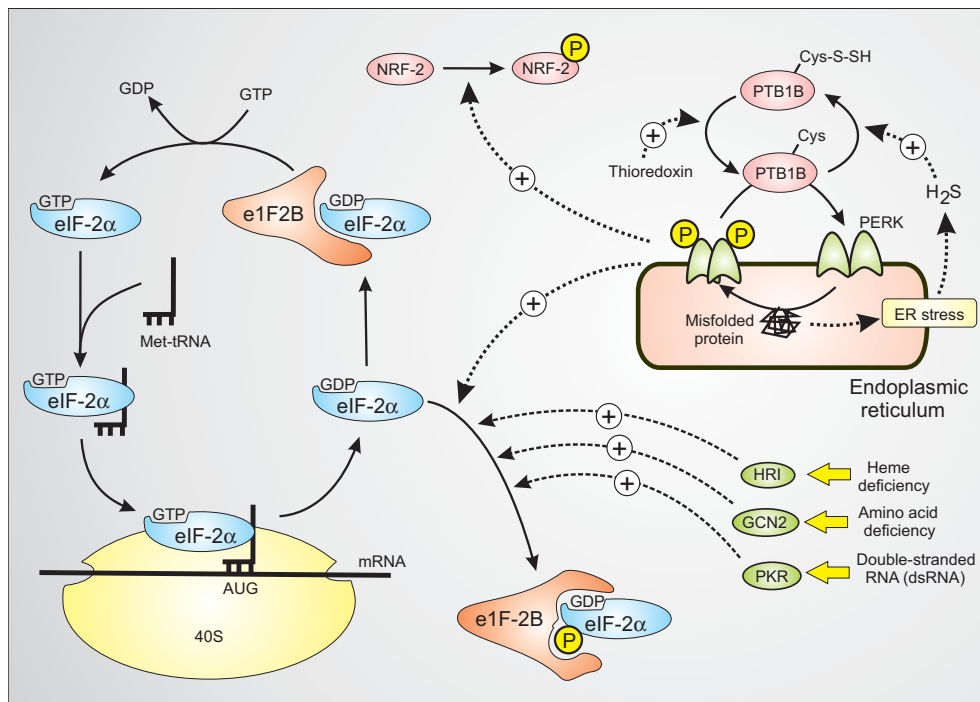
The N-terminal region of TOR contains a large number of HEAT repeats. These helical repeat units fold into an extended superhelical scaffold-like structure that may provide interfaces for interactions with other molecules. TOR is organized into two complexes. In the TOR complex 1, TOR is associated with a regulatory-associated protein of TOR (raptor), G protein-like protein (GβL) and a proline-rich PKB/Akt substrate 40 kDa (PRAS40). Raptor is a scaffolding protein that provides a bridge between TOR and its downstream targets of eIF4E-BP1 and ribosomal S6 protein kinase 1 (S6K1). The TOR complex 2 contains TOR together with GβL, rapamycin-insensitive companion of TOR (rictor), mammalian stress-activated protein kinase (SAPK)-interacting protein 1 (mSin1) and protein observed with rictor (protor).

TOR is also sensitive to the phospholipase D (PLD) signalling pathway (Module 2: Figure PLD signalling).

## Protein synthesis

Protein synthesis is often referred to as translation because it depends on a process of converting the codon sequences of mRNA being translated into an amino acid polypeptide chain. This translation process consists of three discrete mechanisms: initiation, elongation and termination.

1. **Initiation** – Protein synthesis begins when a ribosome attaches to the mRNA and starts to form a protein through an initiation sequence that is orchestrated by one of the eukaryotic initiation factors known as eukaryotic initiation factor 2 (eIF-2), which consists of three subunits (eIF-2α, eIF-2β and eIF-2γ). The eIF-2α subunit is a typical GTP-binding protein that contributes to the pre-initiation complex by binding to the methionine-charged initiator transfer RNA (Met-tRNA), which is then positioned on the AUG start codon (Module 9: Figure regulation of eIF-2α cycling). Once the Met-tRNA is in place, eIF-2α hydrolyses GTP to GDP and leaves the ribosome. The sequence can be repeated when eIF2B, which is a guanine nucleotide-exchange factor (GEF), exchanges GDP for GTP

**Module 9:** | Figure regulation of eIF2- $\alpha$  cycling**Regulation of eIF2- $\alpha$  cycling.**

The loading of methionine transfer RNA (Met-tRNA) on to messenger RNA (mRNA) is driven by the binding of GTP to the eukaryotic initiation factor 2 $\alpha$  (eIF-2 $\alpha$ ). Once the Met-tRNA is positioned on the AUG start sequence, the GTP is hydrolysed to GDP and eIF-2 $\alpha$  is released from the 40S ribosomal subunit. The sequence can be repeated when eIF2B GEF exchanges GDP for GTP.

enabling the activated eIF-2 $\alpha$ /GTP complex to initiate the synthesis of another protein.

The activity of eIF-2 $\alpha$  is a key control point for eukaryotic protein synthesis and is altered in response to various stresses such as viral infection, nutrient deprivation and the endoplasmic reticulum (ER) stress signalling response (Module 2: Figure ER stress signalling). Many of these cellular stresses inhibit protein synthesis by activating different members of the eIF2 protein kinase family such as Haem-regulated inhibitor (HRI), double-stranded (ds) RNA activated protein kinase (PKR), general control non-repressible-2 (GCN2) and protein kinase RNA-like endoplasmic reticulum kinase (PERK). In the case of PERK, which is a dual-specificity protein kinase, misfolded proteins within the lumen of the ER induce dimerization during which there is a tyrosine autophosphorylation reaction (Module 9: Figure regulation of eIF-2 $\alpha$  cycling). Once it is phosphorylated, PERK can then phosphorylate eIF-2 $\alpha$  that then binds to eIF2B and prevents it from carrying out its role of exchanging GDP for GTP and thus effectively represses translation.

PERK is inactivated by protein tyrosine phosphatase 1B (PTP1B), which is located on the surface of the endoplasmic reticulum. An interesting aspect of this dephosphorylation reaction is that it can be inhibited by a sulfhydration of the hyper-sensitive cysteine-215 by the neurotransmitter hydrogen sulfide (H<sub>2</sub>S).

- 2. Elongation** – after the Met-tRNA is in place the other tRNAs bring in the amino acids that correspond to the subsequent codons and in so doing the ribosome progresses down the mRNA strand.
- 3. Termination** – Once the final mRNA codon (i.e. the STOP codon) is reached, the synthesis ceases and the completed peptide chain is released.

**Eukaryotic initiation factor 2 (eIF-2)**

The eukaryotic initiation factor 2 (eIF-2), which consists of three subunits (eIF-2 $\alpha$ , eIF-2 $\beta$  and eIF-2 $\gamma$ ), has a critical role in protein synthesis in that it facilitates attachment of mRNA to the ribosome (Module 9: Figure regulation of eIF-2 $\alpha$  cycling). The activity of eIF-2 is inhibited by phosphorylation of serine-51 on the  $\alpha$ -subunit and this markedly reduces protein synthesis. Four kinases have been identified that are capable of reducing protein synthesis by phosphorylating eIF-2 $\alpha$ . While all four kinases share a conserved kinase domain, they have divergent regulatory regions that enable them to respond to different types of cell stress (Module 9: Figure regulation of eIF-2 $\alpha$  cycling):

- Haem-regulated inhibitor (HRI), which is activated by deficiency of haem.
- Double-stranded (ds) RNA activated protein kinase (PKR). As its name implies, PKR is stimulated by double-stranded RNA (dsRNA).

- General control non-derepressible-2 (GCN2) is sensitive to UV irradiation and is also activated by deficiency of amino acids.
- Protein kinase RNA-like endoplasmic reticulum kinase (PERK), which is stimulated by misfolded proteins in the ER as a result of endoplasmic reticulum (ER) stress signalling (Module 2: Figure ER stress signalling).

## Proliferation of specific cell types

Cell proliferation is evident in many different cell types. Stem cells are specialized for cell proliferation. For example, in the intestine, there are nests of undifferentiated stem cells that are constantly proliferating to replace absorptive cells that are sloughed off from the ends of the villi. In the immune system, lymphocyte activation plays a vital role during clonal expansion of unique lymphocytes programmed to fight off specific infections. There also are many differentiated primary cells that perform their specific functions while retaining the option of returning to the cell cycle (Module 8: Figure cellular life history). In all of these examples, it is clear that cell proliferation is carefully orchestrated to maintain cellular homeostasis.

The following are some examples of *in vivo* cell proliferation events that can be induced with the appropriate stimuli:

- Lymphocyte activation
- Smooth muscle cell proliferation
- Insulin-secreting  $\beta$ -cell proliferation
- Astrocyte proliferation
- Intestinal stem cell proliferation (Module 12: Figure colon cancer)
- Neuronal stem cell proliferation during neurogenesis (Module 10: Figure granule cell neurogenesis)
- Endothelial cell angiogenesis (Module 9: Figure angiogenesis)
- Parathyroid gland hyperplasia
- Mesangial cell proliferation (Module 7: Figure mesangial cell). Excessive mesangial cell proliferation is one of the major causes of diabetic nephropathy.
- Hepatic stellate cell stimulation is induced during liver injury (Module 7: Figure hepatic stellate cell).

## Lymphocyte activation

The immune system employs a highly sophisticated chemical surveillance system to recognize and combat foreign substances. The foot soldiers of this defence force are the lymphocytes, which are separated into thymus-derived cells (T cells) and bone marrow-derived cells (B cells). The T cells act against intracellular pathogens (cell-mediated immunity), whereas the B cells deal with extracellular pathogens (humoral immunity). There are two main types of T-cell: the cytotoxic cells, which can kill infected cells, and the helper cells, which, as their name implies, help B cells to fight infections by providing specific cytokines. From a signalling point of view, T cell activation and B cell activation are of interest because they are examples of how cells are stimulated to proliferate in response to a specific

stimulus, in this case antigen. In addition, these cells can also respond to antigen either by dying through apoptosis or by entering an unresponsive state known as anergy. Exactly which course they take depends on the nature of the signalling information they receive through a variety of receptor pathways.

### T cell activation

T cell activation depends upon a sequential series of events that begin when the circulating T cell responds to the cytokines emanating from the source of an infection. Through a process of T cell chemotaxis, the T cell homes in on the infected area, where it makes intimate contact with the antigen-presenting cell to form an immunological synapse. The chemotactic response and the formation of the synapse are very dependent upon T cell cytoskeletal reorganization. As the two cells come into contact, the major histocompatibility complex II (MHCII) molecules on the antigen-presenting cell present the antigenic peptides to the T cell receptors to initiate the T cell receptor (TCR) signalling responsible for T cell activation. Activation through the TCR is facilitated by the CD28 co-stimulatory pathway.

### T cell chemotaxis

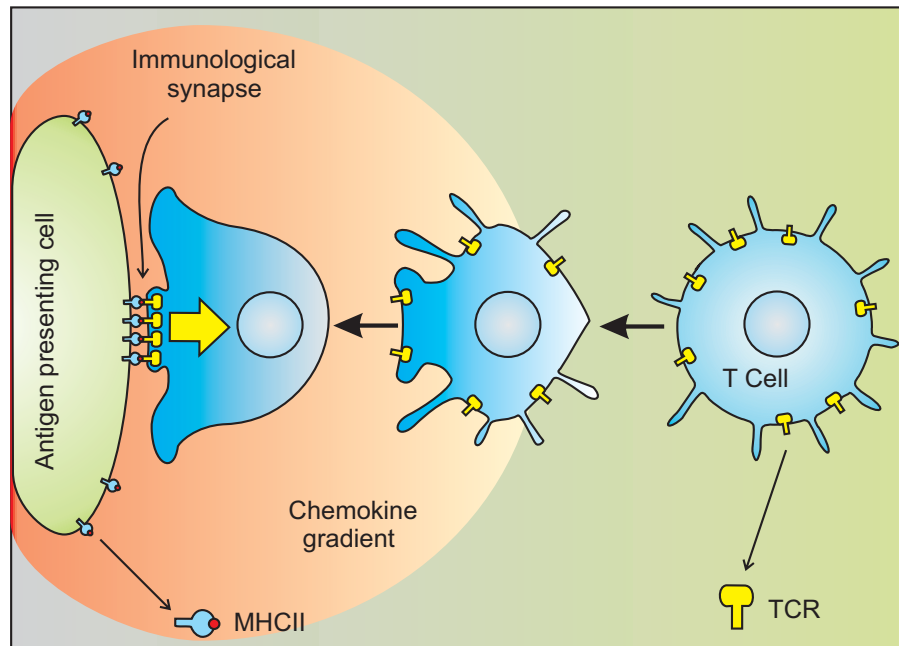
Circulating T cells detect and move towards sites of infection by a process of chemotaxis. This chemotactic response is usually driven by a gradient of chemokines (Module 9: Figure T cell chemotaxis). Resting T cells have a round shape with numerous small microvilli. This uniform shape, which is maintained by a cortical web of microfilaments, undergoes a rapid change when the T cell begins to detect chemokines. This T cell cytoskeletal reorganization induces a clearly defined polarity that contributes to the process of chemotaxis.

### T cell cytoskeletal reorganization

Remodelling of the actin cytoskeleton plays a key role in T cell activation. It is responsible for the dramatic changes in cell shape that occur during T cell chemotaxis, and it comes into play at a later stage to help assemble the immunological synapse. These cytoskeletal rearrangements are controlled by an interplay between the PtdIns 3-kinase signalling pathway and the PtdIns4,5P<sub>2</sub> signalling cassette. The reorganization of the actin cytoskeleton that occurs during T cell chemotaxis is very similar to that seen in neutrophil chemotaxis.

As chemotaxis proceeds and the two cells begin to make contact with each other, there is another cytoskeletal reorganization that forms the immunological synapse (Module 9: Figure T cell actin scaffold).

When the T cell makes contact with potential antigen-presenting cells, it carries out an initial surveillance that causes a change in shape (see Sequence a–d in Module 9: Figure T cell shape change). If this target is a naïve cell, there is no further response, and the T cell moves away. However, if the T cell recognizes an antigen-presenting cell, there is a dramatic change in shape as it throws out pseudopodia, almost totally engulfing its target (see Sequence e–h in Module 9: Figure T cell shape change). This

**Module 9:** | Figure T cell chemotaxis

T cell migration towards antigen-presenting cells through a process of chemotaxis.

Antigen-presenting cells release cytokines that attract T cells. When they reach the antigen-presenting cell, they form the immunological synapse where the T cell receptor (TCR) on the T cell binds to the major histocompatibility complex II (MHCII) molecules on the antigen-presenting cell. The antigenic peptide (shown in red) is held on the surface of the MHCII molecules. Details of the synapse are shown in Module 9: Figure immunological synapse.

rapid change in shape is accompanied by a large  $\text{Ca}^{2+}$  transient, which is thought to stabilize the interaction with the antigen-presenting cell.

### Immunological synapse

The antigenic peptides responsible for activating the T cell are not free in solution, but are held by the major histocompatibility complex II (MHCII) molecules embedded on the surface of the antigen-presenting cell (Module 9: Figure T cell chemotaxis). As a result of the process of chemotaxis, the two cells come together to form an immunological synapse made up of highly organized adhesive and signalling components (Module 9: Figure immunological synapse structure). These adhesive components, such as lymphocyte function-associated antigen (LFA-1), form a protective outer ring surrounding an inner core containing the signalling components. Within this synapse, therefore, molecules appear to be segregated into 'supramolecular activation clusters' (SMACs). The peripheral SMAC (pSMAC) contains the adhesion molecules, whereas the central SMAC (cSMAC) contains the signalling components.

An important aspect of the immunological synapse is the polymerization of actin to provide an 'actin-scaffold signalling highway' that helps in the relay of information from the cSMAC into the cell. The organization of the actin cytoskeleton depends upon the recruitment of the Wiskott-Aldrich syndrome protein (WASP) and the WASP-interacting protein (WIP) into the immunological synapse. This translocation may be assisted by a Crk-like (CrkL) adaptor protein. The ability of WIP to inhibit the

activity of WASP may be alleviated by phosphorylation of the former by protein kinase  $\text{C}\theta$  ( $\text{PKC}\theta$ ), which is known to play a role in T cell activation. This actin scaffold may also act to reorientate the microtubule organizing centre (MTOC) so that it faces towards the antigen-presenting cell. This actin remodelling was also found to be necessary for the activation of *Oria1*, which is essential for lymphocyte activation (Module 3: Figure STIM-induced  $\text{Ca}^{2+}$  entry).

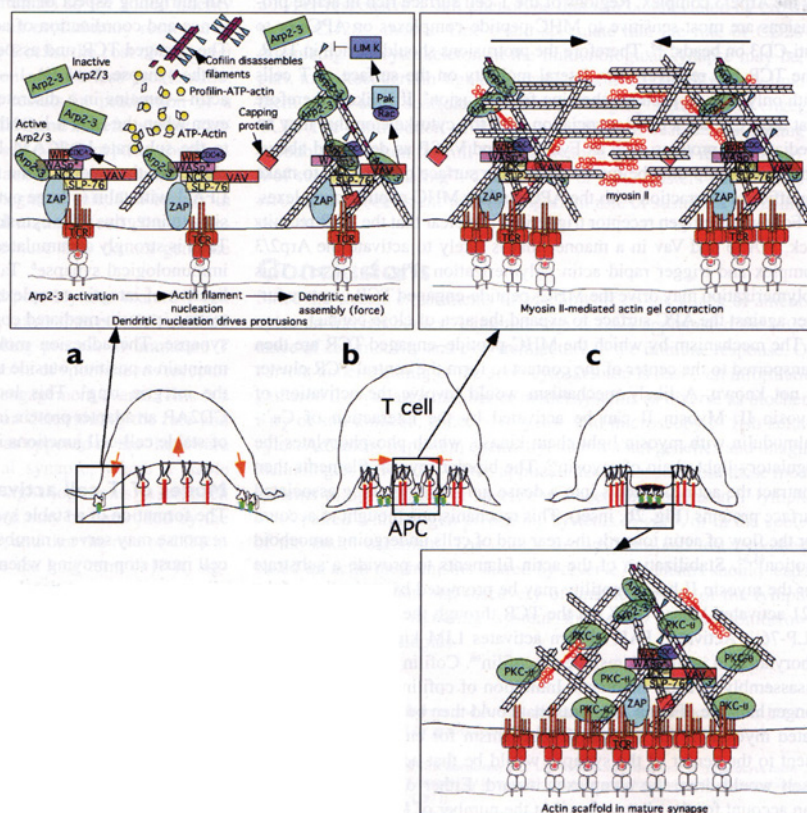
The important principle is that the efficient functioning of the signalling system depends upon the construction of an elaborate scaffold made up of both the cytoskeleton and a range of scaffolding proteins that contributes to the molecular organization of a functional T cell receptor (TCR) (Module 9: Figure TCR signalling). One of the important signalling events regulated by the cytoskeleton is  $\text{Ca}^{2+}$  entry, which seems to be controlled by actin remodelling carried out by a signalling pathway that includes Vav, Rac and WASP verprolin homologous 2 (WAVE2) (Module 3: Figure STIM-induced  $\text{Ca}^{2+}$  entry).

There is a disruption in lymphocyte signalling in Wiskott-Aldrich syndrome, which results from mutations of WASP that result in a disruption in the formation of the immunological synapse.

### T cell receptor (TCR) signalling

The T cell receptor (TCR) signalling processes responsible for the activation of proliferation contain a bewildering array of components. The main components of the T cell signalling toolkit are collected together into functional groups (Module 9: Table T cell signalling toolkit). Many

## Module 9: | Figure T cell actin scaffold



## Formation of an actin scaffold at an immunological synapse.

At stage a, the T cell has made contact with the antigen-presenting cells, and the T cell receptor has begun to assemble the signalling components [e.g. Src homology 2 domain-containing leukocyte protein of 76 kDa (SLP-76), Nck, Vav, Wiskott–Aldrich syndrome protein (WASP) and actin-related protein 2/3 (Arp2/3) complex] necessary to form actin filaments, as shown in b. In c, myosin II begins a contraction process that pulls together the individual actin scaffolds associated with each receptor complex to produce the mature synapse shown in the panel at the bottom. The signalling components of the T cell receptor complex are shown in Module 9: Figure TCR signalling. Reproduced by permission from Macmillan Publishers Ltd: *Nat. Immunol.* Dustin, M.L. and Cooper, J.A. (2000) The immunological synapse and the actin cytoskeleton: molecular hardware for T cell signalling. 1:23–29. Copyright (2000); <http://www.nature.com/ni>; see Dustin and Cooper 2000.

of these components are collated together on a spatiotemporal map, which provides a visual framework to illustrate how they interact with each other to drive T cells into DNA synthesis (Module 9: Figure T cell signalling map). In effect, this is a spatiotemporal flow diagram that begins at the top left with the arrival of the antigen and ends with DNA synthesis in the bottom right-hand corner. Using such a map, it is possible to determine approximately where and when different signalling components function during the activation process.

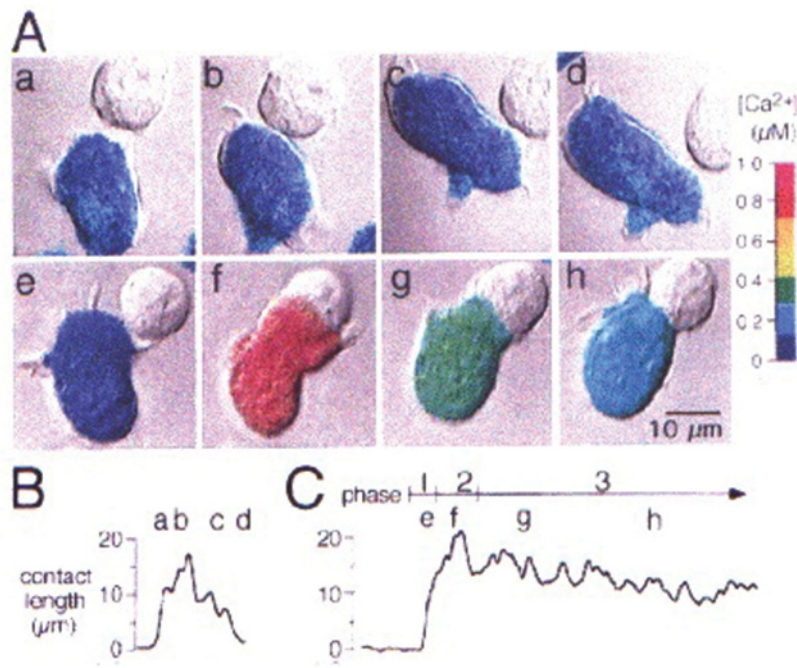
The signalling events proceed through a conventional sequence, as shown in the green panel on the left of the map. The activation process begins with the ligands [e.g. antigen held on the major histocompatibility complex II (MHCII)] that bind to the large TCR complex that is coupled to transducers to create the messengers that relay information through various signalling cassettes to activate the cytosolic and nuclear effectors that initiate the transcriptional cascade that finally results in DNA synthesis. Some of the main components of T cell signalling are outlined below.

## T cell ligands

T cells respond to a number of ligands, most of which are held on the surface of the antigen-presenting cells (Module 9: Figure T cell signalling map). The primary ligand for T cell activation is the antigen itself. The antigen is taken up by the antigen-presenting cell, where it is chopped up into short fragments (8–15 amino acids) that are bound to the major histocompatibility complex II (MHCII). The MHCII molecules then display this antigen fragment on the cell surface, where it is recognized by the T cell receptor (TCR). In effect, this peptide fragment acts as a growth factor to initiate cell proliferation. For a successful response, however, the T cell must be stimulated by additional ligands, such as CD86, that act through the CD28 receptor.

## T cell receptors, precursors and transducers

The T cell receptor (TCR) is a complicated multimolecular complex. It begins with a few fixed components, but as soon as it binds antigen, it draws in a large number of additional transducing and scaffolding components to build

**Module 9:** | Figure T cell shape change

**T cell shape change and  $\text{Ca}^{2+}$  signals triggered by contact with an antigen-presenting cell.**

A. Images of fura-2-loaded T cells approaching either a naïve B cell (a–d) or a B cell coated with an antigen (e–h). In the latter case, there was a dramatic increase in the level of  $\text{Ca}^{2+}$  accompanying the change in shape. B and C. These traces illustrate the time course for the change in shape and intracellular  $\text{Ca}^{2+}$  taken during the responses shown in A. Reproduced from *Immunity*, Vol. 4, Negulescu, P.A., Krasieva, T.B., Khan, A., Kerschbaum, H.H. and Cahalan, M.D., Polarity of T cell shape, motility, and sensitivity to antigen, pp. 421–430. Copyright (1994), with permission from Elsevier; see Negulescu et al. 1996.

a huge macromolecular complex, which has also been referred to as a ‘transducisome’, which functions to relay information into the cell (Module 9: Figure TCR signalling). The TCR consists of transmembrane proteins ( $\alpha$ ,  $\beta$  and  $\zeta$ ) that are associated with the CD3 components (CD3 $\gamma$ ,  $\delta$  and  $\epsilon$ ). This TCR complex is associated with the accessory co-receptor CD4 that binds to the major histocompatibility complex II (MHCII). The TCR $\alpha$  and TCR $\beta$  subunits form an immunoglobulin-like receptor that is responsible for recognizing the antigen fragment held in the jaws of the MHC. Activation of the TCR creates a large macromolecular complex responsible for relaying information through a number of signalling cassettes.

Since the  $\alpha$  and  $\beta$  subunits of the TCR have very short cytoplasmic tails, they are incapable of engaging the signal transducing elements, and this function is carried out by other members of the receptor. The CD3 subunits ( $\gamma$ ,  $\delta$  and  $\epsilon$ ) and the  $\zeta$  subunits have long cytoplasmic chains that contain immunoreceptor tyrosine-based activation motifs (ITAMs) (red bars), which provide the docking sites to assemble the receptor scaffold. The ‘glue’ that directs and holds the scaffold together is provided by the phosphate groups that are added sequentially to the various ITAMs. The assembly of this signalling scaffold follows an orderly sequence of events:

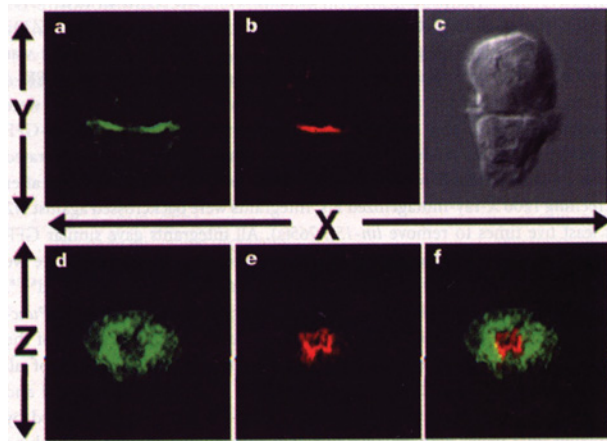
Positioning the tyrosine kinases (Module 9: Figure TCR signalling). Since the TCR has no enzymatic activity, this

is assembled by recruiting various non-receptor protein tyrosine kinases such as Lck and  $\zeta$ -associated protein of 70 kDa (ZAP70). An important early event is the recruitment of Lck, which is drawn in to the TCR through its attachment to CD4, the co-receptor that interacts with MHCII.

Activation of the tyrosine kinases. Lck is kept inactive by phosphorylation of a tyrosine residue in its C-terminal region by a C-terminal Src kinase (CSK). To activate these kinases, this inhibitory phosphate group must be removed by the transmembrane tyrosine phosphatase CD45.

Phosphorylation of the ITAM docking sites. Lck phosphorylates tyrosine residues on the ITAMs located on the CD3 and  $\zeta$  chains.

Recruitment of ZAP70 and scaffolding elements. One of the key components of the developing scaffold is the tyrosine kinase  $\zeta$ -associated protein of 70 kDa (ZAP70) that uses its Src homology 2 (SH2) domains to bind to the phosphorylated ITAMs. Once in place, ZAP70 then phosphorylates scaffolding components such as a linker for activation of T cells (LAT) and an SH2-domain-containing leukocyte protein of 76 kDa (SLP-76), which are drawn into the growing central supra-molecular activation cluster (cSMAC) and function as docking sites to communicate to the different signalling cassettes. An important function of both SLP-76 and LAT is to provide a scaffold, which includes Vav1. This

**Module 9: | Figure immunological synapse structure**

An immunological synapse formed between a T cell and an antigen-presenting cell.

The cells have been stained with an antibody directed against talin (green), which highlights the outer ring of adhesive elements that constitute the peripheral supramolecular activation cluster (pSMAC). An antibody against protein kinase C $\theta$  (PKC $\theta$ ) has stained the inner core of signalling components (red), which make up the central SMAC (cSMAC), as illustrated in Module 9: Figure immunological synapse. The synapse is viewed in two orientations. In the top panels (a and b), it is seen from the side (i.e. the x-y axis), whereas in panels d-f, it is seen from the top (i.e. the x-z axis), which clearly shows the bull's-eye appearance of the synapse. Reproduced by permission from Macmillan Publishers Ltd: *Nature*, Monks, C.R.F., Freiberg, B.A., Kupfer, H., Sciaky, N. and Kupfer, A. (1998) Three-dimensional segregation of supramolecular activation clusters in T-cells. 395:82-86; <http://www.nature.com>; see Monks et al. 1998.

scaffolding complex then assembles the transducers, such as phospholipase C $\gamma$ 1 (PLC $\gamma$ 1), Ras and PtdIns 3-kinase that are responsible for activating the TCR signalling cassettes that relay information into the nucleus.

**T cell receptor (TCR) signalling cassettes**

The T cell receptor (TCR) activates cell proliferation by relaying information to the nucleus using many of the conventional growth factor signalling pathways (Module 9: Figure T cell signalling map):

**T cell receptor (TCR) Ca<sup>2+</sup> signalling**

One of the major signals for activating lymphocyte proliferation is Ca<sup>2+</sup>, which is generated by the inositol 1,4,5-trisphosphate (InsP<sub>3</sub>)/Ca<sup>2+</sup> signalling cassette. The phosphate group of LAT binds to the Src homology 2 (SH2) domain of phospholipase C $\gamma$ 1 (PLC $\gamma$ 1) through the conventional mechanism for activating the  $\gamma$  isoform of PLC (Module 2: Figure PLC structure and function). The PLC $\gamma$ 1 is thus positioned close to the membrane, where it can begin to initiate the following sequence of events (Module 9: Figure T cell Ca<sup>2+</sup> signalling):

1. Antigen initiates the process by binding to the T cell receptor (TCR), which activates phospholipase C $\gamma$ 1 (PLC $\gamma$ 1) to hydrolyse PtdIns4,5P<sub>2</sub> to generate inositol 1,4,5-trisphosphate (InsP<sub>3</sub>). This process is enhanced by PtdIns 3-kinase that forms the lipid messenger PtdIns3,4,5P<sub>3</sub>, which acts through inducible T cell kinase (Itk) to enhance PLC $\gamma$ 1.

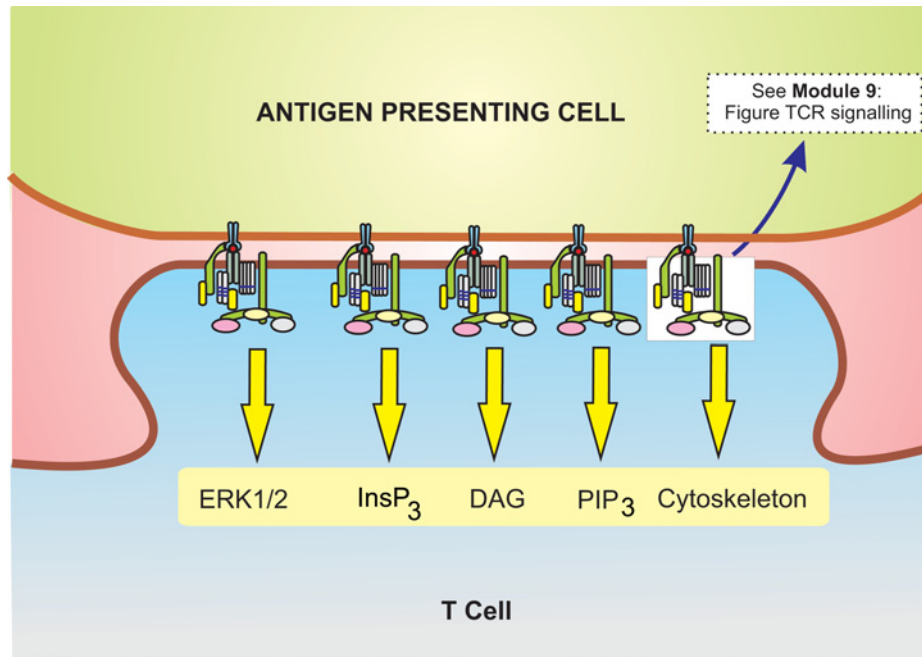
2. The InsP<sub>3</sub> acts through the InsP<sub>3</sub> receptor (InsP<sub>3</sub>R) to release Ca<sup>2+</sup> from the internal store.
3. As the ER empties, it sends a signal to the Ca<sup>2+</sup> release-activated Ca<sup>2+</sup> (CRAC) channel responsible for maintaining the Ca<sup>2+</sup> entry current  $I_{CRAC}$  for the 2 h period required to induce lymphocytes to grow. The Orai1 protein has been identified as the channel responsible for carrying  $I_{CRAC}$ . Mutation of the Orai1 has been linked to severe combined immune deficiency (SCID) (Module 3: Figure Ca<sup>2+</sup> signalling in SCID). The coupling between store emptying and activation of the CRAC channel is still unclear. A stromal interaction molecule (STIM) in the ER may function as the sensor of store emptying (Module 3: Figure STIM-induced Ca<sup>2+</sup> entry). In addition, there is a role for Wiskott-Aldrich syndrome protein (WASP) verprolin homologous 2 (WAVE2), which may function to stabilize the coupling complex responsible for Ca<sup>2+</sup> entry by remodelling the actin cytoskeleton.
4. Mitochondria play an important role in enhancing the entry process by removing Ca<sup>2+</sup> from the vicinity of the  $I_{CRAC}$  channel, thereby negating the inhibitory effect of Ca<sup>2+</sup>. The membrane hyperpolarization, which is necessary to maintain Ca<sup>2+</sup> entry, depends on both a voltage-dependent potassium (K<sub>v</sub>) channel (K<sub>v</sub>1.3) and the intermediate-conductance (IK) channel (K<sub>Ca</sub>3.1).
5. Ca<sup>2+</sup> acts to stimulate the transcription factor nuclear factor of activated T cells (NFAT) (see Module 4: Figure NFAT activation for details).
6. NFAT enters the nucleus where it switches on the genes for the IL-2 signalling pathway. In addition, NFAT activates the expression of Down's syndrome critical region 1 (DSCR1) and carabin, which inhibit calcineurin to set up a negative-feedback loop (Module 9: Figure T cell Ca<sup>2+</sup> signalling).

An important point to stress is that the Ca<sup>2+</sup> signalling pathway has to remain active for at least 2 h in order to stimulate lymphocytes to proliferate. During this activation period, the Ca<sup>2+</sup> signals are presented as prolonged oscillations (Module 9: Figure T cell Ca<sup>2+</sup> oscillations). An increase in Ca<sup>2+</sup> is thus a key regulator of cell proliferation. Lymphocyte activation and smooth muscle cell proliferation provide paradigms for such a role for Ca<sup>2+</sup> in controlling cell proliferation.

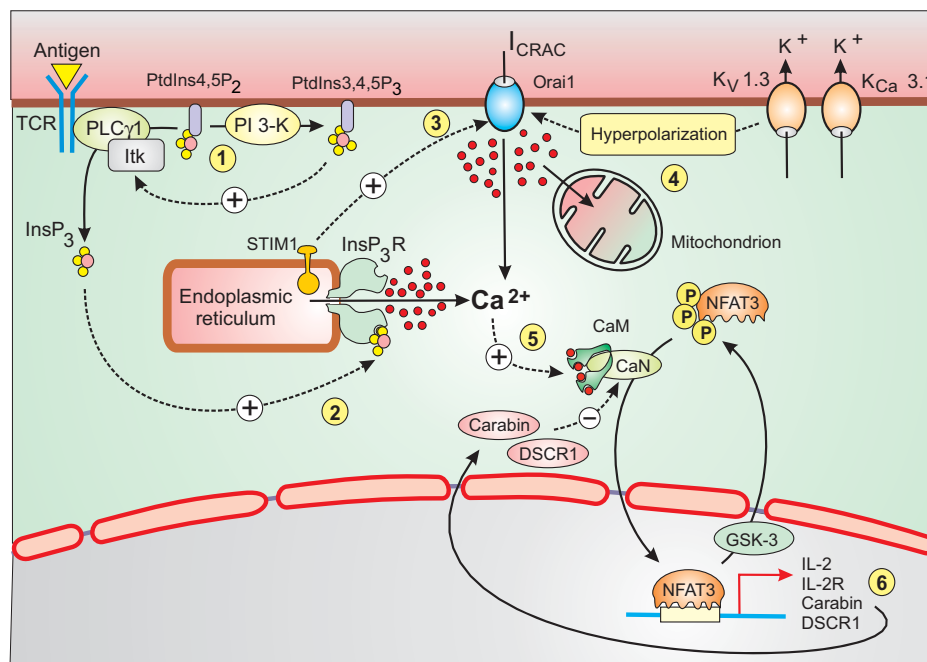
T cell activation provides a good example of the link between K<sup>+</sup> channels and cell proliferation. At least two K<sup>+</sup> channels are responsible for hyperpolarizing the membrane to maintain the electrical gradient necessary for the prolonged influx of external Ca<sup>2+</sup> through the Orai1 channels (Module 9: Figure T cell Ca<sup>2+</sup> signalling). There is K<sub>v</sub>1.3 and the intermediate-conductance (IK) channel (K<sub>Ca</sub>3.1). Hypoxia results in a decrease in the expression of the K<sub>v</sub>1.3 channel and this can lead to a decrease in cell proliferation.

**The Ras/mitogen-activated protein kinase (MAPK) cassette**

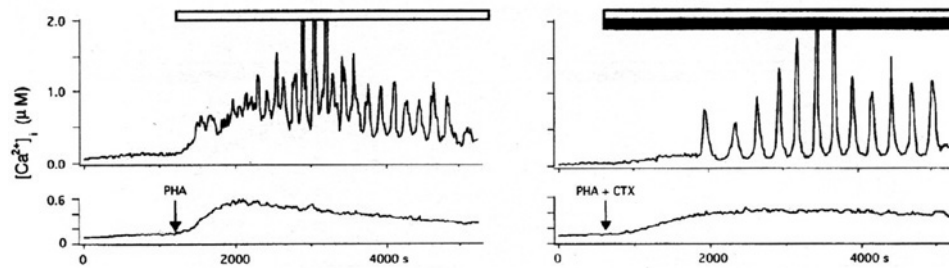
(Module 9: Figure T cell signalling map). The mitogen-activated protein kinase (MAPK) signalling pathway

**Module 9:** | Figure immunological synapse**The immunological synapse.**

When a T cell makes contact with an antigen-presenting cell, the resulting signalling events are localized to an immunological synapse, where T cell receptor complexes are concentrated in a two-dimensional array. The region illustrated here is the central supramolecular activation cluster (cSMAC) shown in Module 9: Figure immunological synapse structure. For sake of clarity, the peripheral SMAC (pSMAC) containing the adhesion molecules and the actin scaffold has not been included. The signalling complexes, which co-operate with each other to generate the signalling information that induces the T cell to proliferate, are shown in more detail in Module 9: Figure T cell Ca<sup>2+</sup> signalling.

**Module 9:** | Figure T cell Ca<sup>2+</sup> signalling**The role of Ca<sup>2+</sup> signalling in lymphocyte proliferation.**

Antigen initiates the proliferative process by binding to the T cell receptor (TCR), which activates phospholipase Cγ1 (PLCγ1) to hydrolyse PtdIns4,5P<sub>2</sub> to generate inositol 1,4,5-trisphosphate (InsP<sub>3</sub>). InsP<sub>3</sub> binds to the InsP<sub>3</sub> receptor (InsP<sub>3</sub>R) to release Ca<sup>2+</sup> from the endoplasmic reticulum (ER) and this initiates the signalling cascade described in the text. The way in which Ca<sup>2+</sup> acts to stimulate the transcription factor nuclear factor of activated T cells (NFAT) is described in Module 4: Figure NFAT activation.

**Module 9:** | Figure T cell  $\text{Ca}^{2+}$  oscillations**Oscillations in activated T cells.**

Response of single human peripheral lymphocytes to phytohemagglutinin (PHA; open bars) or to PHA plus charybdotoxin (PHA + CTX; closed bar). The traces on the bottom represent the average response from 200 cells. Reproduced from *Cell Calcium*, Vol. 21, Verheugen, J.A.H., Le Deist, F., Devignot, V. and Korn, H., Enhancement of calcium signaling and proliferation responses in activated human T lymphocytes: inhibitory effects of  $\text{K}^+$  channel block by charybdotoxin depend on the T cell activation state, pp. 1–17. Copyright (1997), with permission from Elsevier; see Verheugen et al. 1997.

plays an important role in lymphocyte activation. The growth-factor-receptor-bound protein 2 (Grb2) subunit is drawn into the complex where, together with Son-of-sevenless (SoS), it helps to assemble the membrane complex that initiates the extracellular-signal-regulated kinase (ERK) signalling pathway (Module 2: Figure ERK signalling).

**Small GTP-binding proteins and the cytoskeleton**

(Module 9: Figure T cell signalling map) The scaffold protein Src homology 2 domain-containing leukocyte protein of 76 kDa (SLP-76) binds Rac, Vav and Nck, which function to organize the cytoskeleton. For example, Vav is a guanine-nucleotide exchange factor (GEF) for the Rho family of monomeric G proteins such as Rac, which function by organizing the cytoskeleton (Module 2: Figure Rac signalling). Modification of the cytoskeleton may also depend on the ability of Vav to bind talin and vinculin, which function to anchor the cytoskeleton to the plasma membrane. In addition, Vav may influence  $\text{Ca}^{2+}$  signalling by acting through Rho to stimulate the PtdIns4P 5-kinase (PtdIns4P 5-K) to provide the precursor lipid PtdIns4,5P<sub>2</sub> used by the PLC $\gamma$ 1 and PtdIns 3-kinase signalling cassettes (Module 9: Figure TCR signalling).

**T cell PtdIns 3-kinase signalling cassette**

(Module 9: Figure T cell signalling map). The regulatory subunit of the PtdIns 3-kinase has Src homology 3 (SH3) and two Src homology 2 (SH2) domains that enable it to bind to the activated receptor, thus enabling its associated catalytic subunit to begin to phosphorylate PtdIns4,5P<sub>2</sub> to the second messenger PtdIns3,4,5P<sub>3</sub> (PIP<sub>3</sub>) (Module 2: Figure PtdIns 3-kinase signalling). One of the functions of PIP<sub>3</sub> is to facilitate the activation of phospholipase C $\gamma$ 1 (PLC $\gamma$ 1) by binding directly to the enzyme, and also by activating the protein inducible T cell kinase (Itk) (Module 9: Figure TCR signalling).

**CD28 co-stimulatory pathway**

Activation of the T cell receptor (TCR) by itself is not sufficient to induce cell proliferation, even though it is capable of sending information down a number of prom-

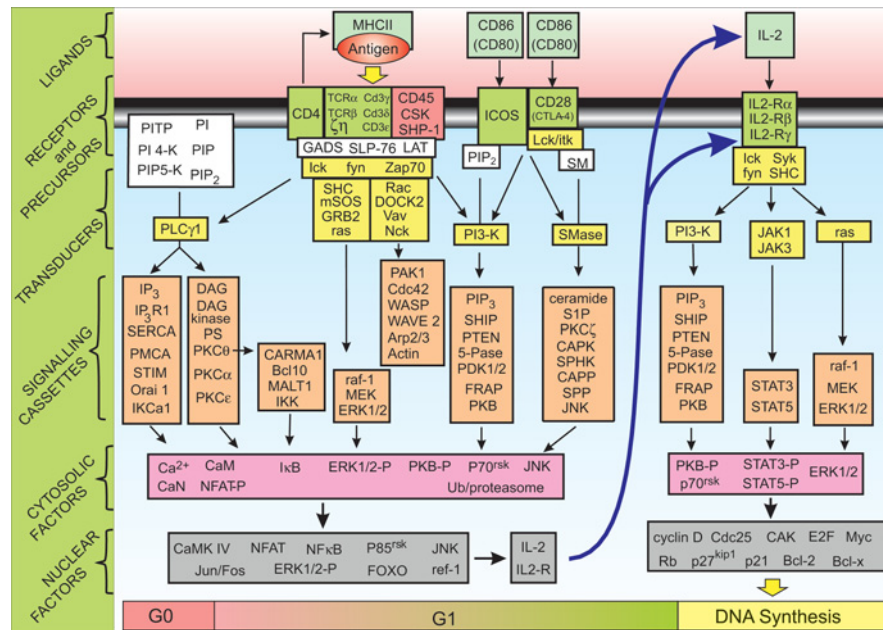
inent signalling cassettes. To complete its role in initiating events early in G<sub>1</sub>, the TCR complex requires assistance from the co-stimulatory pathway (Module 9: Figure T cell signalling map). In addition to facilitating the proliferative signal, the CD28 pathway also prevents the TCR from entering into an unresponsive state that has been referred to as clonal anergy. Just how the CD28 pathway acts to prevent the TCR from becoming unresponsive is still very much a mystery.

CD28 is a transmembrane glycoprotein that functions as a homodimer linked together through disulphide bonds in the extracellular domain. The ligands for CD28 are CD80 and CD86, which are members of an immunoglobulin family located in the membrane of the antigen-presenting cell (Module 9: Figure TCR signalling). The cytoplasmic domain of CD28 lacks enzymatic activity, but it does contain tyrosine residues that become phosphorylated following stimulation, and which may then recruit signalling elements such as PtdIns 3-kinase. There also is an indication that CD28 may activate the sphingomyelin signalling pathway, resulting in the activation of a number of pathways including the JNK pathway.

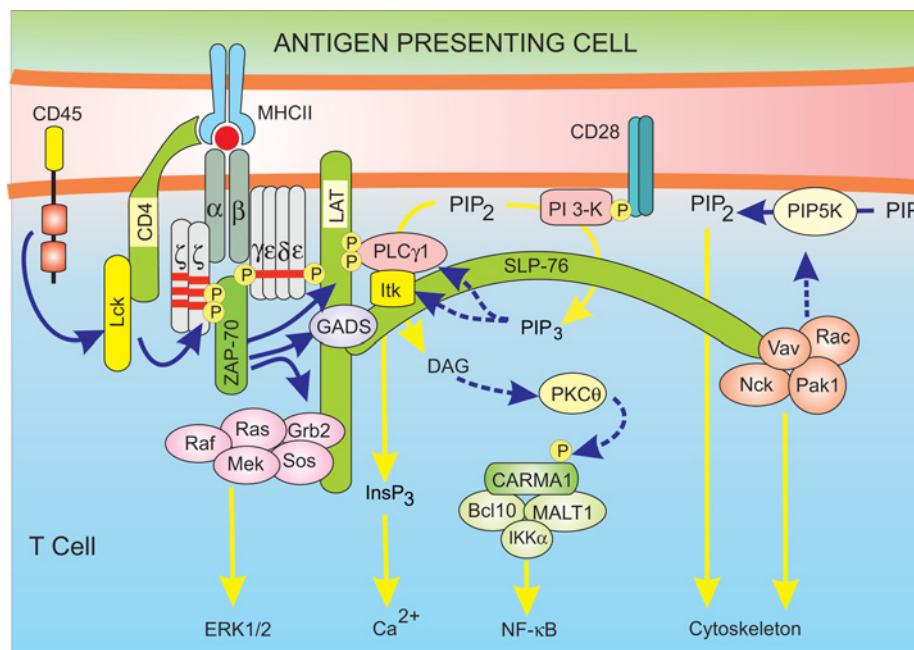
The inducible costimulatory molecule (ICOS), which is also known as CD278, is related to CD28 and also acts by providing positive signals during T cell activation. CD28 and ICOS have unique and overlapping functions and act synergistically to promote cell activation. CD28 is expressed in resting T cells, whereas the expression of ICOS is induced once T cells are activated. For example, ICOS is induced in follicular helper T cells (T<sub>FH</sub> cells) during B cell differentiation in the lymph node (Module 8: Figure B cell maturation signalling). ICOS is activated by the ICOS ligand (ICOSL), also known as B7RP-1, which acts by stimulating the PtdIns 3-kinase signalling pathway to induce the production of effector cytokines such as interleukin 4 (IL-4) and interleukin 21 (IL-21).

**Signalling lymphocyte activation molecule (SLAM)**

There are nine genes in the signalling lymphocyte activation molecule (SLAM) family (SLAMF1–9) that play an

**Module 9:** | Figure T cell signalling map**A spatiotemporal map of T cell activation.**

Signalling begins at the top left with the major histocompatibility complex II (MHCII) presenting the antigen (red) to the T cell receptor (TCR) complex, which then initiates a sequence of events summarized in the green panel on the left of the figure. The sequence begins with the TCR interacting through various transducers to activate signalling cassettes, which relay information into the nucleus through cytosolic messengers to induce gene transcription. Activation of these early gene products sets up an autocrine loop to put in place the interleukin-2 (IL-2) signalling system that activates additional signalling cassettes to complete the signalling cascade by inducing DNA synthesis. The names and abbreviations of all the components of this signalling map are described in Module 9: Table T cell signalling toolkit.

**Module 9:** | Figure TCR signalling**Molecular organization of a functional T cell receptor (TCR).**

Details of the signalling complexes located within the immunological synapse (Module 9: Figure immunological synapse). Signalling begins when the  $\alpha$  and  $\beta$  subunits of the T cell receptor (TCR) detect antigen (red dot) held in the jaws of the major histocompatibility complex II (MHCII) embedded in the surface of the antigen-presenting cell. The sequence begins with CD4 associating with MHCII, thereby bringing Lck into the complex to begin the phosphorylation cascade that also depends upon  $\zeta$ -associated protein of 70 kDa (ZAP70). The phosphates on the scaffolding protein then draw in the signal transducers, such as phospholipase  $\gamma$ 1 (PLC $\gamma$ 1), PtdIns 3-kinase (PI 3-K) and the Ras complex, to activate the various signalling pathways as described in more detail in the text. The activation of  $\text{Ca}^{2+}$  signalling by inositol 1,4,5-trisphosphate (InsP $_3$ ) is described in Module 9: Figure T cell  $\text{Ca}^{2+}$  signalling.

**Module 9 | Table T cell signalling toolkit**

An inventory of the major signalling components used to stimulate T cell proliferation.

Component	Comments
<b>Ligands</b>	
MHC class II	Major histocompatibility complex II
CD86	Transmembrane glycoproteins (on antigen-presenting cells) responsible for stimulating CD28
CD80	Transmembrane glycoproteins (on antigen-presenting cells) responsible for stimulating CTLA4
IL-2	Interleukin-2
<b>Receptors</b>	
TCR $\alpha$	T cell receptor $\alpha$ subunit
TCR $\beta$	T cell receptor $\beta$ subunit
CD3 $\gamma$	The $\gamma$ chain of the CD3 component of the TCR
CD3 $\delta$	The $\delta$ chain of the CD3 component of the TCR
CD3 $\epsilon$	The $\epsilon$ chain of the CD3 component of the TCR
TCR $\zeta$	The $\zeta$ chain of the TCR
TCR $\eta$	The $\eta$ chain of the TCR
CD4	An accessory co-receptor that binds to MHC class II
CD8	An accessory co-receptor that binds to MHC class I
CD28	A co-stimulatory receptor that responds to CD86
ICOS	Inducible co-stimulatory molecule that acts synergistically with CD28
IL-2R $\alpha$	IL-2 receptor $\alpha$ subunit
IL-2R $\beta$	IL-2 receptor $\beta$ subunit
IL-2R $\gamma$	IL-2 receptor $\gamma$ subunit
<b>Precursors</b>	
PIP	Phosphatidylinositol transfer protein
PI	Phosphatidylinositol
PIP <sub>2</sub>	Phosphatidylinositol 4-phosphate
PI 4-K	Phosphatidylinositol 4,5-bisphosphate
PIP 5-K	Phosphatidylinositol 4-kinase
SM	Phosphatidylinositol 4-phosphate 5-kinase
<b>Transducers</b>	
<b>Enzymatic components</b>	
Lck	A non-receptor tyrosine kinase (a member of the Src family)
Fyn	A non-receptor tyrosine kinase (a member of the Src family)
Itk	Inducible T cell kinase, T cell-specific tyrosine kinase
ZAP70	$\zeta$ -Associated protein of 70 kDa, a tyrosine kinase closely related to ZAP70
Syk	Spleen tyrosine kinase closely related to ZAP70
CD45	A transmembrane tyrosine protein phosphatase
CSK	C-terminal Src kinase
PLC $\gamma$ 1	Phospholipase C $\gamma$ 1
SHP	Src homology 2 (SH2) domain-containing protein tyrosine phosphatase
<b>G protein components</b>	
Rac	A monomeric G protein
Cdc42	A monomeric G protein
Vav	A guanine nucleotide exchange factor (GEF) for Rho family GTPases
<b>Scaffolding components</b>	
LAT	Linker for activation of T cells
SLP-76	Src homology 2 (SH2)-domain containing leukocyte protein of 76 kDa
GADS	Grb2-related adaptor protein
CrkL	Crk-like scaffolding protein
Nck	A scaffolding protein
CARMA1	A scaffolding protein that links PKC $\theta$ to the activation of the NF- $\kappa$ B signalling pathway (Module 9: Figure TCR signalling)

**Module 9: | Table continued**

Component	Comments
Bcl10	A scaffolding protein that links CARMA1 to the activation of the NF- $\kappa$ B signalling pathway
MALT1	Mucosa-associated lymphoid tissue protein-1 that functions with CARMA1 and Bcl10 to the activation of the NF- $\kappa$ B signalling pathway (Module 9: Figure TCR signalling)
<b>Signalling cassettes</b>	
<b>InsP<sub>3</sub>/Ca<sup>2+</sup> pathway</b>	
InsP <sub>3</sub>	Inositol 1,4,5-trisphosphate
InsP <sub>3</sub> R1	Inositol 1,4,5-trisphosphate receptor type I
InsP <sub>3</sub> R2	Inositol 1,4,5-trisphosphate receptor type II
InsP <sub>3</sub> R3	Inositol 1,4,5-trisphosphate receptor type III
IP <sub>3</sub> 3-K	InsP <sub>3</sub> 3-kinase
IP <sub>3</sub> 5-Pase	InsP <sub>3</sub> 5-phosphatase
K <sub>Ca</sub> 3.1	Ca <sup>2+</sup> -activated intermediate-conductance (IK) K <sup>+</sup> channel
SERCA	Sarco/endo-plasmic reticulum Ca <sup>2+</sup> -ATPase
PMCA	Plasma membrane Ca <sup>2+</sup> -ATPase
STIM1	Stromal interaction molecule 1 (Module 3: Figure SOC signalling components)
Orai1	The main component of the Ca <sup>2+</sup> release-activated Ca <sup>2+</sup> (CRAC) channel (Module 3: Figure SOC signalling components)
<b>DAG/PKC pathway</b>	
DAG	Diacylglycerol
DAG kinase	Diacylglycerol kinase
PS	Phosphatidylserine
PKC $\alpha$	Protein kinase C $\alpha$
PKC $\epsilon$	Protein kinase C $\epsilon$
PKC $\zeta$	Protein kinase C $\zeta$
PKC $\theta$	Protein kinase C $\theta$
<b>PI 3-kinase pathway</b>	
PIP <sub>3</sub>	Phosphatidylinositol 3,4,5-trisphosphate
PI 3-K	Phosphatidylinositol 3-kinase
PTEN	Phosphatase and tensin homologue deleted on chromosome 10
SHIP	Src homology 2 (SH2) domain-containing inositol polyphosphate 3-phosphatase
PKB	Protein kinase B (also known as Akt)
FRAP	FK506-binding protein 12 (FKBP12) rapamycin-associated protein
p70 <sup>S6K</sup>	70 kDa ribosomal S6 kinase
p85 <sup>S6K</sup>	85 kDa ribosomal S6 kinase
<b>MAPK pathway</b>	
Ras	A small GTP-binding protein
mSoS	Mammalian Son-of-sevenless
Shc	Src homology 2 (SH2)-domain-containing protein (an adaptor protein for Ras activation)
Grb2	Growth factor receptor-bound protein 2
Raf-1	Cellular homologue of the v-Raf oncogene (a serine/threonine protein kinase)
MEK	MAPK/extracellular-signal-regulated kinase (ERK) kinase
MAPK	Mitogen-activated protein kinase
<b>JAK/STAT pathway</b>	
JAK1	Janus kinase 1
JAK3	Janus kinase 3
STAT3	Signal transducer and activator of transcription 3
STAT5	Signal transducer and activator of transcription 5
<b>Sphingomyelin pathway</b>	
S1P	Sphingosine 1-phosphate
Ceramide	A second messenger released from sphingomyelin
SMase	Sphingomyelinase
CAPK	Ceramide-activating protein kinase
CAPP	Ceramide-activated protein phosphatase
SPHK	Sphingosine kinase
SPP	S1P phosphatase
<b>Actin remodelling</b>	
WASP	Wiskott-Aldrich syndrome protein

**Module 9: | Table continued**

Component	Comments
WAVE2	WASP verprolin homologous; related to WASP and regulates actin cytoskeleton
WIP	WASP-interacting protein
Arp2/3	Actin-related proteins 2 and 3
Cytosolic factors	
CaM	Calmodulin
CaN	Calcineurin
CaMKIV	Ca <sup>2+</sup> /calmodulin-dependent protein kinase IV
Ub	Ubiquitin
Proteasome	A multisubunit complex responsible for degrading ubiquitin/protein conjugates
NFAT	Nuclear factor of activated T cells (cytoplasmic component)
I $\kappa$ B	Inhibitor of NF- $\kappa$ B
NF- $\kappa$ B	Nuclear factor- $\kappa$ B
Nuclear factors	
E2F	E2 promotor binding factor
Jun	A nuclear transcription factor (a component of activating protein 1)
Fos	A nuclear transcription factor (a component of activating protein 1)
Ref-1	Redox factor-1
p21	A 21 kDa inhibitor of cyclin-dependent kinases
p27 <sup>kip1</sup>	A 27 kDa inhibitor of cyclin-dependent kinases
Cyclin E	A cell cycle protein that functions late in G <sub>1</sub> to control onset of DNA synthesis
CDK2	Cyclin-dependent kinase 2 (a threonine/serine protein kinase)
CAK	CDK-activating kinase
Rb	Retinoblastoma gene product

The purpose of this T cell signalling toolkit, which is by no means complete, is to highlight the fact that cell proliferation depends upon the co-operation of a large number of signalling components. The description of the abbreviations often helps to describe their different functions. The way in which many of these components contribute to T cell activation to transfer information from the cell surface into the cytoplasm and nucleus is summarized in Module 9: Figure T cell signalling map.

important role in controlling the various interactions that lymphocytes make with different cells. They belong to an immunoglobulin superfamily that is located on the cell surface of haematopoietic cells. Most of the family are type I transmembrane glycoproteins whereas SLAMF2 is attached to the membrane through a glycosylphosphatidylinositol anchor and functions as a receptor for SLAMF4. All of the other SLAMs interact in a homophilic mechanism in that their ectodomains can interact with each other. The cytoplasmic tails of six of these receptors (SLAM1, SLAM3–7) carry unusual intracellular tyrosine-based switch motifs (ITSMs). When the SLAMs interact with each other, these ITSMs are phosphorylated and recruit various SH2-domain-containing adaptor proteins such as the SLAM-associated protein (SAP) and EWS/FLI activated transcript-2 (EAT-2).

The SLAM/SAP signalling pathway functions in a large number of lymphocyte interactions to control processes such as the development of T cell lineages, natural killer T cells (NKT cells) and the affinity maturation of B cells. For example, during B cell differentiation in the lymph node, there is an increase in the expression of SAP and SLAM in the T<sub>FH</sub> cells that are responsible for the B cell selection within the germinal centre (Module 8: Figure B cell maturation signalling).

### SLAM-associated protein (SAP)

SLAM-associated protein (SAP) and the related EWS/FLI activated transcript-2 (EAT-2), which is expressed mainly in antigen-presenting cells, are SH2-domain adaptor proteins that bind to the phosphotyrosine motifs on the cytoplasmic tails of some of the signalling lymphocyte activation molecule (SLAM) family (SLAM1, SLAM3–7). Activated SAP is known to relay information to other signalling components such as Fyn.

Mutations in the gene Src homology 2 (SH2) domain protein 1A (SH2D1A) causes X-linked lymphoproliferative syndrome (XLP).

### T cell gene transcription

The signalling cassettes activated by the T cell receptor (TCR) produce various cytosolic factors (signalling components and activated transcription factors) that enter the nucleus to initiate a programme of early gene transcription (Module 9: Figure T cell signalling map). Some of the key early genes that are activated code for the interleukin-2 (IL-2) signalling pathway, which is a classical autocrine mechanism that is critical for completing the signalling events that end with the onset of DNA synthesis (Module 9: Figure G<sub>1</sub> proliferative signalling). The promoter region of the IL-2 gene is thus an example of one of the early genes. It has an array of response elements that bind different transcription factors activated by the different signalling cassettes. Such promoter regions integrate information relayed to them by a number of different transcription factors:

- **Nuclear factor of activated T cells (NFAT).** One of the key transcription factors is NFAT, which mediates the action of Ca<sup>2+</sup>. It has two components: one is located in the nucleus (NFAT<sub>n</sub>) and is rapidly induced by the diacylglycerol (DAG)/protein kinase C (PKC) signalling cassette; the second component is cytosolic NFAT (NFAT<sub>c</sub>), which resides in the cytoplasm as an inactive phosphorylated intermediate. Ca<sup>2+</sup> acts by stimulating the enzyme calcineurin (CaN), which dephosphorylates NFAT<sub>c</sub>, thus enabling it to enter the nucleus to initiate gene transcription (Module 4: Figure NFAT activation). Once it enters the nucleus, NFAT<sub>c</sub> combines with NFAT<sub>n</sub> to form a dimer that binds to two separate sites. At one of these sites, NFAT co-operates with another factor, activating protein 1 (AP-1) (see below), thus illustrating the combinatorial aspect of gene transcription. One of the genes activated by NFAT codes for carabin, which is an inhibitor of calcineurin, and thus constitutes a negative-feedback loop to limit T cell signalling. This Ca<sup>2+</sup>-dependent gene transcription mediated through NFAT is of particular significance, because it represents the site of action of a potent class of immunosuppressants, such as cyclosporin A (CsA) and FK506. These drugs bind to the immunophilins to block lymphocyte proliferation by inhibiting the calcineurin-dependent dephosphorylation of NFAT<sub>c</sub>. These drugs inhibit calcineurin by binding to cyclophilin A or a FK506-binding protein (FKBP).

- Activating protein 1 (AP-1) (Fos/Jun). The transcription factor activating protein 1 (AP-1) is a heterodimer of c-Fos and c-Jun. This AP-1 (Fos/Jun) dimer also binds to NFAT to form a transcriptional complex. The activation of AP-1 is complicated in that it requires an induction of the genes that code for the two components, and, once they have formed the AP-1 complex, this has to be activated through phosphorylation by kinases linked to different signalling pathways (Module 4: Figure SRF and AP-1).
- Nuclear factor  $\kappa$ B (NF- $\kappa$ B). IL-2 gene transcription also depends upon activation of the nuclear factor  $\kappa$ B (NF- $\kappa$ B) signalling pathway. In the case of T cells, the formation of DAG and its activation of protein kinase C $\theta$  (PKC $\theta$ ) plays a role in stimulating the transcription factor NF- $\kappa$ B (Module 9: Figure TCR signalling). The PKC $\theta$  acts by assembling a scaffolding complex by phosphorylating CARMA1, which is then recruited into the immunological synapse. The CARMA1 then associates with a pre-existing complex that consists of the CARD protein Bcl10 and the mucosa-associated lymphoid tissue protein-1 (MALT1 also known as paracaspase) to form the CARMA–Bcl10–Malt1 scaffolding complex. This complex then brings about the UBC13-dependent ubiquitination of I $\kappa$ B kinase (IKK) resulting in activation of the NF- $\kappa$ B signalling pathway. See Module 2: Figure NF- $\kappa$ B activation for details of how IKK phosphorylates the I $\kappa$ B that is targeted for destruction by the ubiquitin-proteasome system. Once I $\kappa$ B is destroyed, the NF- $\kappa$ B dimer can enter the nucleus, where it helps to switch on the genes for IL-2 and the IL-2 receptor.

As a result of these early transcriptional events, genes such as those for IL-2 and the IL-2 receptor initiate the autocrine loop that results in the activation of the interleukin-2 (IL-2) signalling pathway that functions later in G<sub>1</sub> to send signals to the nucleus to interact with the cell cycle control elements to switch on DNA synthesis (Module 9: Figure T cell signalling map).

### Interleukin-2 (IL-2) signalling pathway

Interleukin-2 (IL-2) is a cytokine that functions through non-enzyme-containing receptors (Module 1: Figure cytokines). IL-2 provides the link between the earlier events initiated by the T cell receptor (TCR) to the cell cycle components necessary to initiate DNA synthesis (Module 9: Figure T cell signalling map). The IL-2 receptor (IL-2R) has three subunits ( $\alpha$ ,  $\beta$  and  $\gamma$ ), which have to come together to form a functional unit. The IL-2 signalling pathway transfers information to the cell cycle components using the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signalling pathway (Module 2: Figure JAK/STAT heterogeneity). In addition, it may also use the PtdIns 3-kinase and mitogen-activated protein kinase (MAPK) signalling pathways.

### B cell activation

B cell activation depends on a number of receptor mechanisms. The primary process is B cell antigen receptor (BCR)

activation, but this response is modulated by information coming from both B cell stimulatory co-receptors (CD19) and B cell inhibitory co-receptors (CD22 and Fc $\gamma$ RIIB).

### B cell antigen receptor (BCR) activation

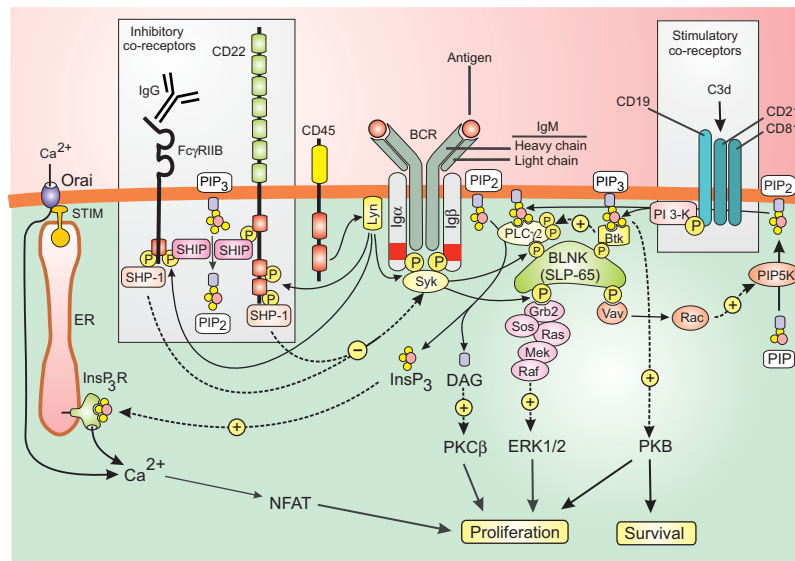
The B cell antigen receptor (BCR) appears early during the differentiation of B cells (Module 8: Figure B cell differentiation). One of the earliest steps in differentiation, which occurs in the pro-B cell stage, is the expression of Ig $\alpha$  (CD79a) and Ig $\beta$  (CD79b) that are key components of the BCR. These two signalling components begin to transmit information to control differentiation and continue to be a major component of the BCR in mature B cells. The BCR is a hetero-oligomeric complex consisting of these two Ig $\alpha$  and Ig $\beta$  signalling proteins associated with the antigen-binding immunoglobulin IgM receptor, which consists of two heavy chains and two light chains (Module 9: Figure B cell activation). When this IgM component detects antigen, a non-receptor tyrosine kinase called Lyn brings about the tyrosine phosphorylation of their immunoreceptor tyrosine-based activation motifs (ITAMs) located on the cytoplasmic regions of Ig $\alpha$  and Ig $\beta$ . The transmembrane protein tyrosine phosphatase CD45 is responsible for activating Lyn to initiate the following cascade of signalling events that emanate from the activated BCR.

The phosphorylated ITAMs on Ig $\alpha$  and Ig $\beta$  recruit and activate the non-receptor spleen tyrosine kinase Syk, which then recruits adaptors and various signalling transducers to form a large macromolecular signalling complex. A key function of Syk is to phosphorylate the B cell linker protein (BLNK) at several sites to provide docking sites to recruit various key signalling components such as Bruton's tyrosine kinase (Btk) and phospholipase C $\gamma$ 2 (PLC $\gamma$ 2). The Btk activates PLC $\gamma$ 2 by phosphorylating Tyr<sup>753</sup> and Tyr<sup>759</sup>. The enzymatic activity of both Btk and PLC $\gamma$ 2 is stimulated by PtdIns3,4,5P<sub>3</sub>, the lipid messenger of the PtdIns 3-kinase signalling pathway. The PtdIns3,4,5P<sub>3</sub> stimulates Btk to phosphorylate PLC $\gamma$ 2 and the latter is also directly activated by this highly charged lipid. The increase in PtdIns3,4,5P<sub>3</sub> formation is described in more detail in the section on the B cell stimulatory co-receptors.

Once PLC $\gamma$ 2 is activated, it hydrolyses PtdIns4,5P<sub>2</sub> to release diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (InsP<sub>3</sub>). The DAG activates protein kinase C $\beta$  (PKC $\beta$ ) to contribute to the onset of proliferation. The InsP<sub>3</sub> plays a primary role in activating proliferation by increasing the intracellular level of Ca<sup>2+</sup> (Module 9: Figure B cell activation). The InsP<sub>3</sub> binds to the InsP<sub>3</sub> receptor to release Ca<sup>2+</sup> from the endoplasmic reticulum (ER) and once this internal store is depleted the store-operated channels (SOCs) are activated to enhance the entry of external Ca<sup>2+</sup>. The mechanism of store-operated channels (SOCs) activation depends on an interaction between STIM proteins in the ER-sensing store depletion to send information to open the Orai channels in the plasma membrane. One of the actions of Ca<sup>2+</sup> is to activate NFAT that leads on to the increase in proliferation.

The phosphorylated BLNK adaptor is also connected to other signalling pathways such as the

## Module 9: | Figure B cell activation

**B cell activation.**

B cells have complex activation processes driven by information from the primary B cell receptor (BCR), which detects antigen, and a number of stimulatory co-receptors (CD19) and inhibitory co-receptors (CD22 and FcγRIIB), which function to modulate the activity of the BCR. See the text for further details.

MAP kinase signalling pathway and the Rac signalling mechanism (Module 9: Figure B cell activation). The adaptor growth factor receptor-bound protein 2 (Grb2) binds to one of the phosphorylated tyrosine residues and then assembles the signalling components of the MAP kinase signalling pathway that results in the activation of ERK1/2 that contributes to the onset of proliferation (Module 2: Figure ERK signalling). Another output signal from the BLNK signalling complex is the Rac signalling mechanism (Module 2: Figure Rac signalling), which is initiated by the guanine nucleotide-exchange factor Vav that then stimulates Rac to promote the membrane localization and activation of PtdIns4P 5-kinase (PIP5K in Module 9: Figure B cell activation). Following activation of PIP5K, the resulting local formation of PtdIns4,5P<sub>2</sub> provides the substrate used by both PLCγ2 and the PtdIns 3-kinase. The latter is a key signalling component of the B cell stimulatory co-receptors.

The BCR is of interest because it appears early during B cell differentiation in the bone marrow (Module 8: Figure B cell differentiation) and plays a critical role in providing the proliferative and survival signals to drive B cells through their programme of differentiation. It is then retained in mature B cells to enable them to function as memory cells to respond to foreign antigens.

**B cell linker protein (BLNK)**

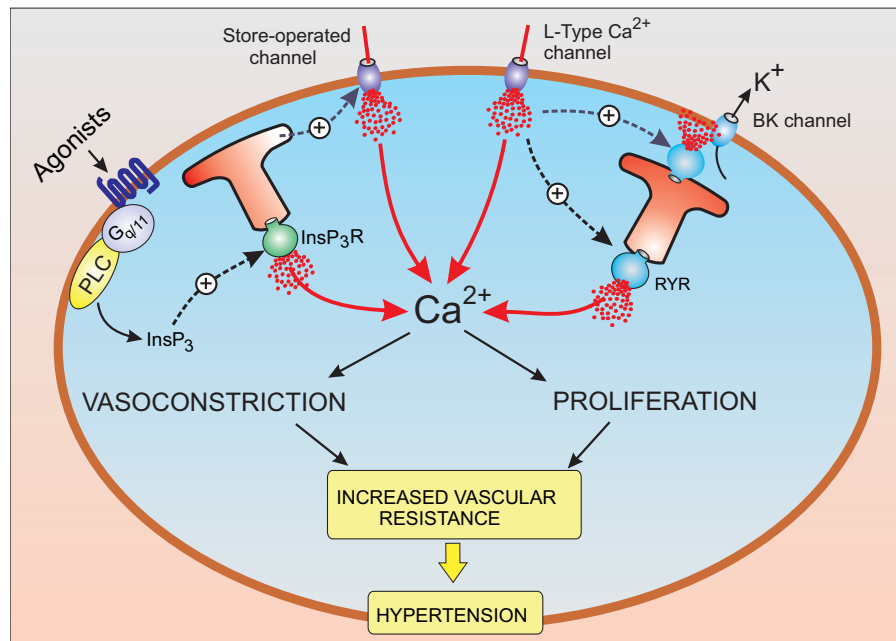
The B cell linker protein (BLNK), which is also known as Src homology 2 (SH2) domain-containing leukocyte protein of 65 kDa (SLP-65), is phosphorylated during B cell antigen receptor (BCR) activation to provide binding sites for both Bruton's tyrosine kinase (Btk) and phospholipase Cγ2 (PLCγ2) (Module 9: Figure B cell activation).

**B cell stimulatory co-receptors**

The B cell stimulatory co-receptors can fine tune the B cell antigen receptor (BCR) response by activating PtdIns 3-kinase signalling to provide the PtdIns3,4,5P<sub>3</sub> to enhance the activity of both Bruton's tyrosine kinase (Btk) and phospholipase Cγ2 (PLCγ2) (Module 9: Figure B cell activation). The trimolecular complex consisting of CD19, CD21 and CD81 is recruited into the vicinity of the BCR complex when CD21 binds to the C3d complement component. CD19, which is a B cell-specific surface protein, first appears in the pre-B cell stage during B cell differentiation in the bone marrow (Module 8: Figure B cell differentiation). The non-receptor tyrosine kinase Lyn, which initiated the BCR activation process, is also responsible for phosphorylating the cytoplasmic domain of CD19 to provide a binding site for the Class 1A PtdIns 3-kinase. The regulatory subunit p85 attaches the catalytic p110 subunits to the phosphorylated tyrosine residue on CD19, thus positioning the enzyme close to the membrane where it can begin to generate the lipid messenger PtdIns3,4,5P<sub>3</sub> (Module 9: Figure B cell activation).

**B cell inhibitory co-receptors**

The B cell inhibitory co-receptors regulate the amplitude of the B cell antigen receptor (BCR) response through a number of inhibitory mechanisms. One such inhibitory process depends on CD22, which is a type I membrane protein that first appears in the pre-B cell stage during B cell differentiation in the bone marrow (Module 8: Figure B cell differentiation). CD22 has seven immunoglobulin (Ig)-like extracellular domains and it is the V-set N-terminal Ig-like domain that binds to its ligand, which is the α2,6-linked sialic acid (2,6Sia) ligand found on the surface of many membrane proteins such as the IgM of

**Module 9:** | Figure SMC proliferation**Role of  $\text{Ca}^{2+}$  in the regulation of smooth muscle cell (SMC) contraction and proliferation.**

Both contraction and proliferation of smooth muscle cells (SMCs) are regulated by  $\text{Ca}^{2+}$ . The role of different ion channels in regulating  $\text{Ca}^{2+}$  signalling is described in Module 7: Figure smooth muscle cell E-C coupling. High levels of such signalling contribute to both vasoconstriction and proliferation.

the BCR or CD45. When it is cross-linked to such proteins, the immunoreceptor tyrosine-based inhibition motifs (ITIMs) on its cytoplasmic tail are phosphorylated to provide docking sites for both Src homology 2 (SH2) domain-containing inositol phosphatase 1 (SHIP1) and Src homology 2 (SH2) domain-containing protein tyrosine phosphatase 1 (SHP1) (Module 9: Figure B cell activation). SHIP-1 hydrolyses  $\text{Ins}1,3,4,5\text{P}_4$  and  $\text{PtdIns}3,4,5\text{P}_3$  and thus reduces the stimulatory effect of this lipid on both Bruton's tyrosine kinase (Btk) and phospholipase  $\text{C}\gamma 2$  ( $\text{PLC}\gamma 2$ ). The primary function of SHP-1, which is a tyrosine phosphatase, is to inhibit the BCR signalling pathways by dephosphorylating the phosphotyrosine residues on components such as Syk, BLNK and CD19.

Another inhibitory co-receptor  $\text{Fc}\gamma\text{RIIB}$ , which also has ITIMs capable of recruiting both SHIP-1 and SHP1, carry out lipid and protein dephosphorylation reactions respectively, similar to those described for CD22.

**Smooth muscle cell proliferation**

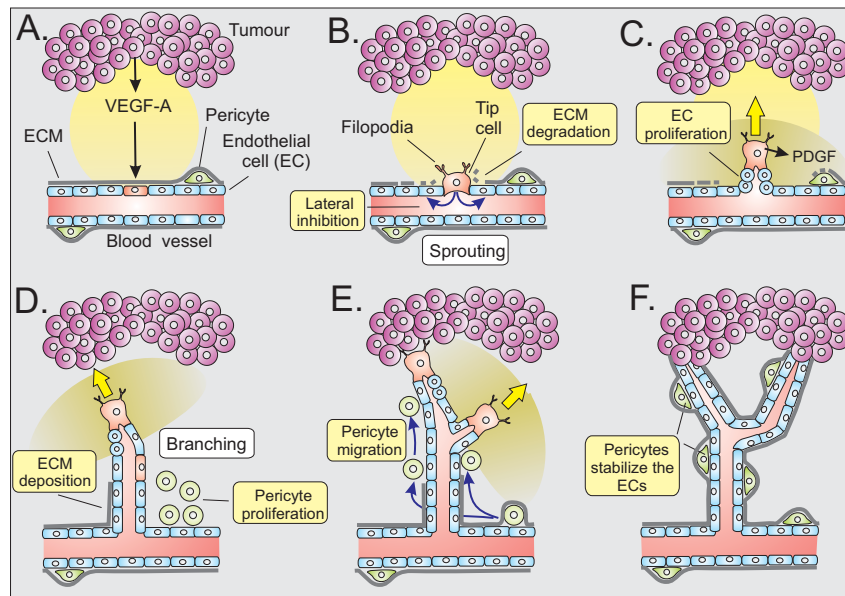
The proliferation of smooth muscle cells plays an important role in wound healing, but this also occurs during vascular remodelling, which can have serious pathophysiological consequences when it leads to pulmonary vasoconstriction and hypertension.

A central role of  $\text{Ca}^{2+}$  in the control of cell proliferation (Module 9: Figure growth factor signalling) is nicely exemplified in the case of smooth muscle cell proliferation. A smooth muscle cell provides a good example of

a proliferation/differentiation switch whereby a fully differentiated cell, which is carrying out its function of contraction, returns to the cell cycle and begins to proliferate. What is so interesting about this dual role of contraction and proliferation is that  $\text{Ca}^{2+}$  plays a role in controlling both processes. Proliferation is also regulated through growth factors acting through transcription factors such as ETS-like transcription factor-1 (Elk-1) and Krüppel-like factor 4 (KLF4) (Module 8: Figure smooth muscle cell differentiation). The microRNAs miR-143 and miR-145 play an important role in converting such proliferating cells back into differentiated smooth muscle cells.

This dual action of  $\text{Ca}^{2+}$  in smooth muscle cells is similar to what occurs in cardiac cells, where  $\text{Ca}^{2+}$  controls both contraction and the transcriptional events responsible for cardiac hypertrophy (Module 12: Figure hypertrophy signalling mechanisms). As for cardiac hypertrophy, it is reasonable to argue that normal levels of  $\text{Ca}^{2+}$  signalling regulate contraction, but when this becomes excessive, cell proliferation is induced. As for other examples of cell proliferation, it is likely that a prolonged period of  $\text{Ca}^{2+}$  signalling might be necessary to stimulate smooth muscle cell proliferation (Module 9: Figure SMC proliferation). In the case of pulmonary artery smooth muscle cells, this prolonged signalling seems to depend upon a constant influx of  $\text{Ca}^{2+}$  through canonical transient receptor potential 1 (TRPC1), i.e. one of the store-operated channels (SOCs) that carry out capacitative  $\text{Ca}^{2+}$  entry, similarly to the  $I_{\text{CRAC}}$  used for lymphocyte activation (Module 9: Figure T cell  $\text{Ca}^{2+}$  signalling).

## Module 9: | Figure angiogenesis

**Angiogenesis.**

This figure depicts the growth of new vessels responding to vascular endothelial growth factor A (VEGF-A) released from a tumour. In the initial response (stages A–C), a single endothelial cell differentiates into a tip cell to initiate the growth of a sprout. As the endothelial cells (ECs) proliferate, this new sprout grows and new tip cells appear (stages D and E) to form a branch. The newly formed vessels then invade the tumour providing a new blood supply. See text for further details.

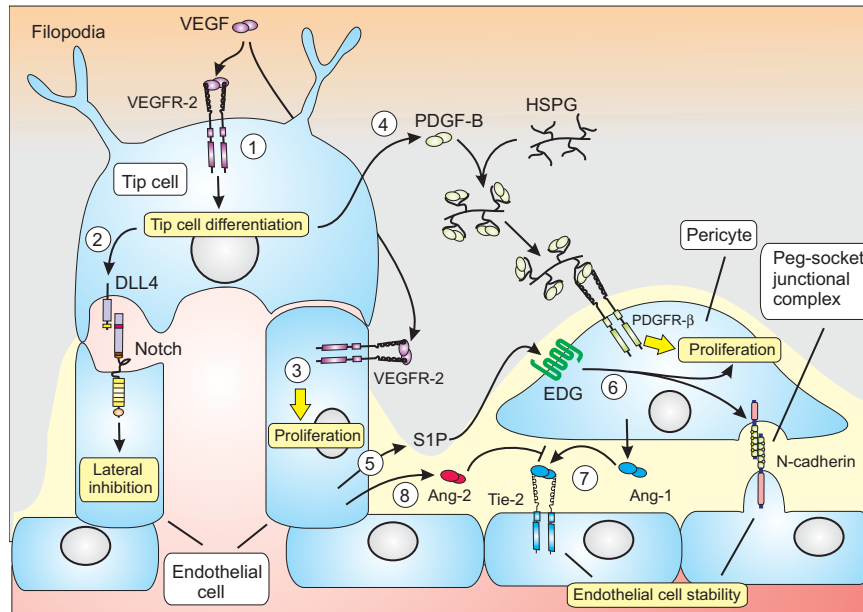
**Angiogenesis**

A process of angiogenesis carries out the growth and repair of blood vessels, during which pre-existing blood vessels give rise to new vessels (Module 9: Figure angiogenesis). Differentiated endothelial cells that line the inner surface of blood vessels are normally in a quiescent state ( $G_0$ ) in that they have a very low turnover, of the order of hundreds of days. During the process of angiogenesis, however, they return to the cell cycle in response to various growth factors. Angiogenesis is usually driven by local tissue demands for oxygen and glucose, or by various mechanical stresses. An important component of this angiogenic response is the activation of hypoxia-inducible factor (HIF), which is a transcription factor that increases the expression of a large number of components such as vascular endothelial growth factor (VEGF) that function to regulate cell proliferation (Module 4: Figure HIF functions). One of the main angiogenic mechanisms is the sprouting and branching of pre-existing vessels, a process triggered by VEGF, which acts on endothelial cells to induce them to proliferate and to migrate to form the new vessels as shown in the following sequence of events (Module 9: Figure angiogenesis):

A. A typical example of angiogenesis is found near tumours, where the growing cell mass begins to run out of oxygen and this triggers the activation of hypoxia-inducible factor (HIF) that switches on the expression and release of vascular endothelial growth factor A (VEGF-A) (Module 4: Figure HIF functions). The VEGF-A (yellow hallow) diffuses out towards neighbouring blood vessels where it acts on the VEGF receptor 2 (VEGFR2) to induce one of the endothelial

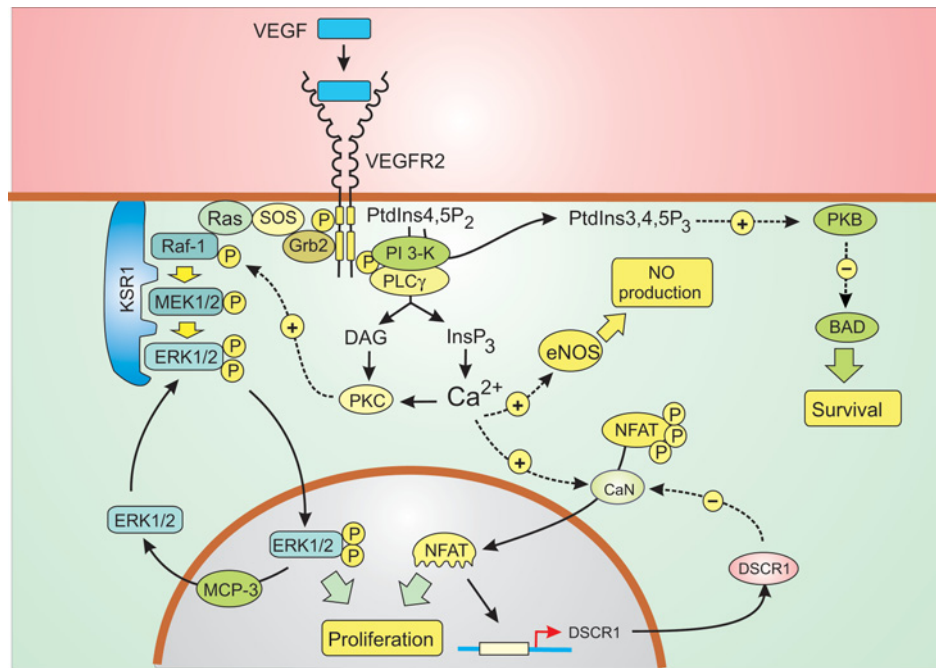
cells (coloured red) to begin to differentiate into a tip cell). VEGF is also released during wounding and inflammation (Module 11: Figure inflammation). Not only does VEGF activate angiogenesis, but it also contributes to the endothelial regulation of paracellular permeability.

- B. Despite the fact that a number of endothelial cells begin to sense the VEGF-A, only one turns into a tip cell and this rather precise specification is enabled through a process of lateral inhibition whereby the tip cell switches off the ability of neighbouring cells to become tip cells. This inhibition depends on the tip cell activating the Notch signalling pathway to inhibit the conversion into tip cells. The extracellular matrix (ECM) is degraded to provide an opening for the outward growth of the sprout.
- C. The tip cell releases platelet-derived growth factor (PDGF) that then acts locally to induce endothelial cell proliferation to initiate sprouting through the growth of stalk cells.
- D. As the endothelial cells proliferate to form stalk cells, the sprout grows out towards the tumour led by the tip cells that have filopodia that appear to provide the guidance system that senses the source of the VEGF-A gradient. The PDGF also stimulates the proliferation of the pericytes. As the sprout lengthens, branching can occur through a process that recapitulates that of sprouting. One cell (shown in red) becomes a tip cell and this then leads the way for the developing branch. As the new vessel develops, deposition of a new ECM begins to restore the external covering of the new blood vessel.

**Module 9:** | Figure angiogenesis signalling**Angiogenesis signalling mechanisms.**

Angiogenesis, begins when one of the endothelial cells responds to vascular endothelial growth factor (VEGF) by differentiating into a tip cell. The latter then releases platelet-derived growth factor B (PDGF-B) to stimulate the proliferation of neighbouring endothelial cells and pericytes and it recruits the Notch signalling pathway to inhibit these other cells from becoming tip cells. This signalling is restricted to a local region around the tip cell because the PDGF is prevented from diffusing away by being bound to heparan sulphate proteoglycans (HSPGs). The activated endothelial cells release sphingosine 1-phosphate (S1P) and angiopoietin 2 (Ang-2). See text for further details.

- E. As the sprout and branch develops, the pericytes begin to migrate up the new vessel.
  - F. As the new vessel reaches its destination and invades the tumour, the pericytes assist in the maturation process by stabilizing the endothelial cells by flattening out over their surface and by developing various cell surface junctions some of which are concentrated in the peg–socket junctional complexes where the N-cadherin complexes are located (Module 9: Figure angiogenesis signalling).
- The sequence of events that occur during angiogenesis are orchestrated by a number of cell–cell signalling mechanisms (Module 9: Figure angiogenesis signalling):
1. A gradient of vascular endothelial growth factor A (VEGF-A), which is produced either at a site of inflammation, damage or by tumour cells, initiates angiogenesis by acting on the VEGFR2 on an endothelial cell to induce it to transform in to a tip cell ( see stages A and B in Module 9: Figure angiogenesis).
  2. As part of this differentiation, the tip cell increases the expression of the Delta-like-4 (DLL4) ligand that activates Notch receptors on the neighbouring endothelial cells to prevent them from becoming tip cells- a process known as lateral inhibition (Module 9: Figure angiogenesis signalling). Activation of this Notch signalling pathway in the endothelial cells appears to act by reducing the expression of VEGFR2 thus preventing the induction of tip cells but the endothelial cells that retain the ability to respond to VEGF-A by proliferating.
  3. The endothelial cells located close to the tip cells are induced to proliferate by VEGF-A to produce the stalk cells of the sprout. Activation of the VEGFR2 on endothelial cells recruits a number of signalling pathways associated with the onset of proliferation (Module 9: Figure VEGF-induced proliferation).
  4. The tip cells release platelet-derived growth factor B (PDGF-B) that acts on the PDGF receptor  $\beta$  (PDGFR- $\beta$ ) to stimulate proliferation of the pericytes using the signalling pathways activated by this growth factor (Module 1: Figure PDGFR activation). The PDGF-B binds to heparan sulphate proteoglycans (HSPGs) and this prevents it from diffusing away and thus restricts its action to a local region around the tip cell. Indeed, the HSPGs not only restrict PDGF-B diffusion but it facilitates its binding to the PDGFR- $\beta$  (Module 9: Figure angiogenesis signalling).
  5. The endothelial cells that have been induced to proliferate by VEGF-B, release sphingosine 1-phosphate (S1P), which is a component of the sphingomyelin signalling pathway (Module 2: Figure sphingomyelin signalling).
  6. The pericytes respond to the S1P using EDG receptors that can activate a number of signalling pathways that appear to target the N-cadherin complex that functions in the peg–socket junctional complex (Module 9: Figure angiogenesis signalling). The S1P signalling pathways may interact with the signalling pathways that are associated with such N-cadherin complexes (Module 6: Figure classical cadherin signalling). Since S1P is known to activate cell proliferation in other cell types, it is

**Module 9:** | Figure VEGF-induced proliferation**Control of endothelial cell proliferation, NO production and survival by the vascular endothelial growth factor (VEGF).**

Endothelial cell activation by vascular endothelial growth factor (VEGF) depends upon its binding and dimerization of two VEGF receptor 2 (VEGFR2) monomers. The latter are tyrosine kinase-linked receptors that have an extracellular domain composed of seven immunoglobulin homology domains and an intracellular tyrosine kinase domain split by a kinase insert sequence. Following receptor dimerization, these kinase regions phosphorylate each other to provide the phosphotyrosine docking sites responsible for initiating the activation of a number of signalling pathways.

possible that it may act together with PDGF- $\beta$  to drive the proliferation of the pericytes.

- In addition to the N-cadherin complex, the pericytes communicate with the endothelial cells through an angiopoietin-TIE2 signalling pathway (Module 9: Figure angiogenesis signalling). The pericytes release angiopoietin 1 (Ang1) that normally acts through TIE2 receptors to control the maturation and stability of the endothelial cells.
- At the time of angiogenesis, such local cell–cell contacts are reduced to enable cells to proliferate and migrate to form a sprout. The proliferating endothelial cells facilitate this loosening of cell contacts by releasing angiopoietin 2 (Ang2), which acts by inhibiting the activation of the TIE2 receptors by Ang1.

Alterations in angiogenesis have a number of severe pathological consequences, such as diabetic retinopathy and age-related macular degeneration. Tumour angiogenesis plays a vital role in tumour growth and metastasis.

**Vascular endothelial growth factor (VEGF)**

Vascular endothelial growth factor (VEGF) is one of the main growth factors responsible for regulating both angiogenesis (Module 9: Figure angiogenesis) and the endothelial regulation of paracellular permeability. VEGF is also released during wounding and inflammation (Module 11: Figure inflammation).

The VEGF family, which are glycoproteins secreted in the vicinity of blood vessels to induce angiogenesis, has

five members: VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PLGF).

Abnormal activation of choroidal neovascularization by VEGF may be responsible for age-related macular degeneration (AMD).

**Vascular endothelial growth factor receptors (VEGFR)**

There are three vascular endothelial growth factor receptor isoforms [VEGFR-1 (Flt-1), VEGFR-2 (Flk-1) and VEGFR-3 (Flt-4)] that play a role in regulating angiogenesis and vasculogenesis.

These VEGFRs are typical protein tyrosine kinase-linked receptors (Module 1: Figure stimuli for enzyme-linked receptors) that have slightly different functions. VEGFR-1, which responds to VEGF-A, VEGF-B and placental growth factor (PLGF), functions in monocyte migration and the organization of proper vessels. VEGFR-2, which is activated mainly by VEGF-A, plays a major role in stimulating the endothelial cells to proliferate, to migrate and to differentiate into new vessels during angiogenesis (Module 9: Figure angiogenesis). It is also responsible for stimulating the formation of nitric oxide (NO). VEGFR-3, which is activated by VEGF-C and VEGF-D, is mainly located on lymphatic vessels, where it functions to stimulate lymphangiogenesis. Physical interactions between endothelial cells and the extracellular matrix (ECM) are responsible for the mechanosensitive control of VEGF receptor expression.

These VEGFRs are capable of activating a number of signalling pathways (Module 9: Figure VEGF-induced proliferation):

- The extracellular-signal-regulated kinase (ERK) pathway (Module 2: Figure ERK signalling) that controls proliferation.
- The PtdIns 3-kinase signalling cassette (Module 2: Figure PtdIns 3-kinase signalling) that promotes survival by inhibiting the pro-apoptotic factor Bad.
- The inositol 1,4,5-trisphosphate (InsP<sub>3</sub>)/Ca<sup>2+</sup> signalling cassette that acts on endothelial nitric oxide synthase (eNOS) and co-operates with the diacylglycerol (DAG)/protein kinase C (PKC) signalling cassette to help activate the ERK pathway. The elevation of Ca<sup>2+</sup> also acts on calcineurin (CaN) to dephosphorylate the transcription factor NFAT thus enabling it to enter the nucleus to activate proliferation. NFAT also induces the transcription of the Down's syndrome critical region 1 (DSCR1) gene, which sets up a negative-feedback loop because DSCR1 is a potent inhibitor of CaN. The increased expression of DSCR1 in a Down's syndrome individual can protect against cancer by suppressing the activity of VEGF and thus reducing the flow of blood to tumours.

The miR-126 plays a role in maintaining angiogenesis by reducing the expression of SPRED1, which inhibits the MAP kinase signalling pathway and the PtdIns 3-kinase regulatory p85 $\beta$  subunit that inhibits the PtdIns 3-kinase signalling pathway.

#### *Mechanosensitive control of VEGF receptor expression*

The physical interaction between endothelial cells and the underlying extracellular matrix (ECM) may be responsible for regulating transcription of the vascular endothelial growth factor receptor 2 (VEGFR2). This mechanical signal appears to be detected by p190RhoGAP, which normally acts to inhibit the Rho signalling mechanism by enhancing the hydrolysis of GTP (Module 2: Figure Rho signalling). Rho appears to influence the transcription of VEGFR2 by altering the balance between two competing transcription factors: TFII-I and GATA2

#### **Astrocyte proliferation**

Astrocytes are induced to grow at the sites of brain injury.

#### **Insulin-secreting $\beta$ -cell proliferation**

Fully differentiated insulin-secreting  $\beta$ -cells have been shown to proliferate in response to pancreatectomy in mice, which raises the possibility of devising strategies to replace damaged cells in diabetics. The survival and proliferation of  $\beta$ -cells is regulated by glucose and by various hormones such as glucagon-like peptide-1 (GLP-1), which appear to act synergistically to activate gene transcription by stimulating the transducers of regulated cyclic AMP response element-binding protein (CREB) (TORC) (Module 7: Figure  $\beta$ -cell signalling).  $\beta$ -Cell proliferation is also controlled by Ca<sup>2+</sup> acting through the nuclear factor of activated T cells (NFAT) transcriptional cascade.

#### **Parathyroid gland hyperplasia**

Hyperplasia of the parathyroid gland occurs when the gland is being stimulated excessively due either to hypocalcaemia or to a decrease in the level of vitamin D. Under normal conditions, there is little proliferation of the chief cells, but this is rapidly reversed during intense stimulation as occurs during the fall in plasma Ca<sup>2+</sup> that occurs during the secondary hyperparathyroidism brought on by kidney disease.

The hyperplasia induced by a fall in level of vitamin D provides some clues as to the mechanisms responsible for controlling proliferation of the chief cells. One of the actions of the hormone 1,25-dihydroxyvitamin D<sub>3</sub> [1,25(OH)<sub>2</sub>D<sub>3</sub>] is to promote the expression of cyclin-dependent kinase (CDK) inhibitors such as p21, which inhibit cell proliferation (Module 7: Figure PTH secretion). The onset of hyperplasia can be rapid and may be accelerated by expression of both the epidermal growth factor (EGF) receptor and its normal ligand TGF $\alpha$ .

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