Sensors and Effectors

Synopsis

Signalling pathways regulate cellular processes by acting through sensors to stimulate the downstream effectors that are responsible for controlling different cellular processes. In some cases, these effectors might be relatively simple, consisting of a single downstream effector system, whereas there are more complicated effectors made up of multiple components such as those driving processes such as membrane and protein trafficking, endocytosis, exocytosis, phagocytosis, motor proteins, gene transcription, gene silencing and actin remodelling.

Sensors

Intracellular sensors detect the signals coming from the different signalling pathways (Module 2: Figure cell signalling pathways) and use the information to stimulate the effectors that bring about different cellular processes.

There are many different sensors:

- The Ca^{2+} signalling system employs a range of different Ca^{2+} sensors:

    Calmodulin (CaM)
    Troponin C (TnC)
    Neuronal Ca^{2+} sensor (NCS) proteins
    Ca^{2+}-binding proteins (CaBPs)
    Calcium and integrin binding protein 1 (CIB1)
    S100 proteins
    Annexins
    Synaptotagmins

- The regulatory subunit of protein kinase A (PKA) binds the second messenger cyclic AMP [Module 2: Figure protein kinase A (PKA)].
- In the case of protein kinase C (PKC), which responds to both diacylglycerol (DAG) and Ca^{2+}, the enzyme is both the sensor and the effector (Module 2: Figure PKC structure and activation).
- The sensors for cyclic GMP are located on its two main effectors: cyclic GMP-dependent protein kinase (cGK) and the cyclic nucleotide-gated channel (CNGC) (Module 3: Figure cyclic nucleotide-gated channels).
- The many sensors, which function in protein-lipid interactions, have lipid-binding domains capable of sensing different lipid messengers (Module 6: Figure modular lipid-binding domains).

Ca^{2+} sensors

For the Ca^{2+} signalling pathway, there are a number of Ca^{2+} sensors, such as troponin C (TnC), calmodulin (CaM), annexins, S100 proteins and the synaptotagmins.

Calmodulin (CaM)

Calmodulin (CaM) is one of the major Ca^{2+} sensors in cells. It contains four helix–loop–helix folding motifs containing EF-hand Ca^{2+}-binding sites (Module 4: Figure EF-hand motif). These sites are arranged into two lobes (the N- and C-terminal lobes) at either end of the molecule separated by a flexible linker (Module 4: Figure CaM structure). Each lobe has two EF-hands with sites I and II in the N-terminal domain and III and IV in the C-terminal domain. The latter two sites have a Ca^{2+} affinity that is 10-fold higher than for sites I and II. When Ca^{2+} binds to these lobes, the molecule undergoes a pronounced conformational change that exposes a hydrophobic pocket and increases its affinity for its various targets that have characteristic CaM-binding domains. In effect, the N- and C-terminal lobes wrap themselves around the CaM-binding domains of their effector proteins to induce the conformational changes responsible for altering the activity of many different signalling components:

- Opening of intermediate-conductance (IK) and small-conductance (SK) K^+ channels (Module 3: Figure IK/SK channel opening)
- $Ca_V1.2$ L-type channel (Module 3: Figure $Ca_V1.2$ L-type channel).
- Ca^{2+}/calmodulin-dependent protein kinases (CaMKs)
- Myosin light chain kinase (MLCK)
- Phosphorylase kinase
- Activation of NO synthase (Module 2: Figure NO synthase mechanism)
- Neuromodulin
- Neurogranin
- Ras guanine nucleotide release-inducing factors (RasGRFs)

Module 4: | Figure EF-hand motif
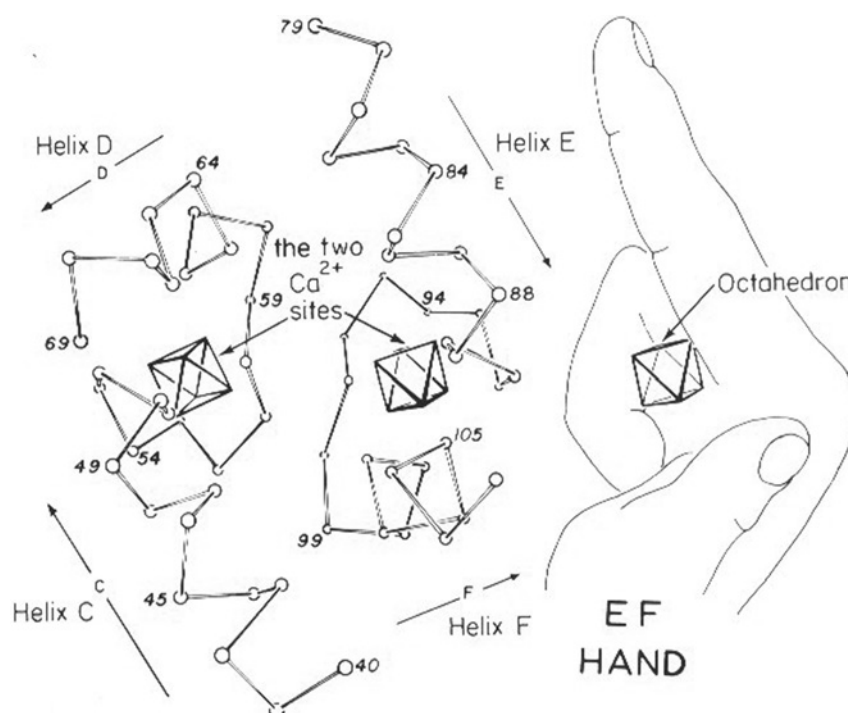
Structural organization of the EF-hand.
The orientations of the two helices that make up the EF-hand resemble a finger and a thumb as illustrated on the right. The small loop connecting these two helices contains aspartate and glutamate residues that provide the carbonyl-group oxygen atoms to co-ordinate the Ca^{2+} ion. Reproduced, with permission, from the Annual Review of Biochemistry, Volume 45 © 1976 by Annual Reviews; http://www.annualreviews.org; see Kretsinger 1976.

• Human vacuolar protein sorting 34 (hVps34)

EF-hand

The EF-hand is one of the major Ca^{2+}-binding regions found on many of the Ca^{2+} sensors. It takes its name from the fact that the binding site is located between an E and an F helix that are aligned like a thumb and a finger (Module 4: Figure EF-hand motif). A small loop of 12 amino acids, which connects the two helices, contains aspartate and glutamate side chains that have carbonyl groups that provide the oxygen atoms to co-ordinate the Ca^{2+}. The number of EF-hands on proteins can vary. In the case of the classical sensors such as calmodulin (CaM) and troponin C (TnC), there are four EF-hands.

The Stromal interaction molecule (STIM), which functions in the mechanism of store-operated channel (SOC) activation to sense the Ca^{2+} content of the endoplasmic reticulum (ER) has a modified EF-hand with an affinity matched to the higher concentrations of Ca^{2+} located within the lumen of the ER (Module 3: Figure SOC signalling components).

Neuromodulin

Neuromodulin, which is also known as GAP43, B-50 or P-57, is a neural-specific calmodulin (CaM)-binding protein that is located mainly in the presynaptic region. It is found at concentrations resembling that of CaM and may thus play a role in regulating the free level of CaM. Neuromodulin, which is tethered to the membrane by two palmitoylated cysteine residues, has the unusual property of being able to bind to CaM at resting concentrations of Ca^{2+}. In response to a local elevation in Ca^{2+}, the CaM is released from neuromodulin and diffuses away to carry out its signalling functions. The IQ domain on neuromodulin that binds CaM has a Ser-41 that is phosphorylated by protein kinase C (PKC) and this blocks CaM binding and thus represents another way in which the level of CaM might be regulated.

Neurogranin

Neurogranin is a neural-specific calmodulin (CaM)-binding protein that functions primarily in the postsynaptic region. It has some sequence homology to neuromodulin particularly within the IQ domain that binds CaM that also has a phosphorylation site for protein kinase C (PKC). Neurogranin may function to regulate CaM levels in postsynaptic regions just as neuromodulin does in presynaptic regions. In response to an increase in Ca^{2+}, the neurogranin/CaM complex will be disrupted and the released CaM will be free to carry out its many postsynaptic functions (Module 10: Figure neuronal gene transcription).

Troponin C (TnC)

Troponin C (TnC) resembles calmodulin in that it has two pairs of Ca^{2+}-binding EF-hands. It has a specific function during excitation-contraction (E-C) coupling in skeletal muscle and in cardiac muscle cells. Its function has been worked out in some detail in the case of skeletal

Module 4: | Figure CaM structure

The EF-hand Ca^{2+}-binding domain.
These ribbon diagrams illustrate the structure of calmodulin in the absence (left) and presence (red balls in the centre and right-hand panels) of Ca^{2+}. When Ca^{2+} binds, calmodulin (CaM) undergoes a remarkable change in conformation that enables it to wrap around parts of proteins (shown in green on the right) to alter their properties. Such an action is evident in the Ca^{2+}-dependent opening of K^+ channels (Module 3: Figure IK/SK channel opening). Reproduced, with permission, from the Annual Review of Pharmacology and Toxicology, Volume 41 ©2001 by Annual Reviews; http://www.annualreviews.org; see Hook and Means 2001.

muscle (Module 7: Figure skeletal muscle E-C coupling). The Ca^{2+} released from the sarcoplasmic reticulum (SR) acts through TnC to stimulate contraction. TnC is one of the components of a regulatory complex located on actin that determines its ability to interact with myosin. One of these proteins is tropomyosin, which has a long rod-like structure made up of two chains arranged in a coiled-coil that lies on the rim of the groove of the actin helix (Module 7: Figure skeletal muscle structure). These tropomyosin rods are separated at 40 nm intervals (corresponding to seven actin subunits) by a complex of regulatory troponin proteins that include troponin I (TnI), troponin T (TnT) and TnC. The TnI binds to both actin and TnC, whereas TnT interacts with tropomyosin and TnC. This troponin complex regulates the position of the tropomyosin rods relative to the groove of the actin helix in a Ca^{2+}-dependent manner. In the resting muscle, the tropomyosin is displaced out of the groove to provide a physical barrier (steric hindrance) preventing the myosin heads from interacting with the actin subunits. During E-C coupling, Ca^{2+} binds to the EF-hands on TnC to induce a conformational change in the troponin complex that is transmitted to tropomyosin, causing it to move a small distance (approximately 1.5 nm) towards the groove, and this then permits the myosin heads to interact with actin to begin the contractile process.

Neuronal Ca^{2+} sensor (NCS) proteins
The neuronal Ca^{2+} sensor (NCS) proteins are mainly found in neurons, where they were first discovered, but some are also found to function in other cell types. They have multiple functions in cells, including the regulation of ion channels, membrane trafficking, receptor modulation, gene transcription and cell survival. There are 14 members of the NCS family, which are typical EF-hand Ca^{2+}-binding proteins (Module 2: Table Ca^{2+} signalling toolkit). They possess four EF-hands (Module 4: Figure EF-hand motif), but only three of them bind Ca^{2+}. In the case of recoverin, only two of the EF-hands are functional. While most of the NCS proteins are fairly widely distributed throughout the neuronal population, some have a limited expression, such as hippocalcin, which is restricted mainly to the hippocampal pyramidal neurons. Hippocalcin may contribute to the Ca^{2+}-dependent activation of endocytosis during the process of long-term depression (LTD).

Many of the NCS proteins are multifunctional in that they can control different downstream effectors. For example, KChIP3/DREAM/calsenilin has three quite distinct functions. Likewise, NCS-1 also interacts with a number of downstream effectors. Most of the effectors controlled by the NCS proteins are located on membranes, and there is some variability concerning the way they locate these targets. A characteristic feature of many NCS proteins is that they have an N-terminal myristoyl group, which functions to attach them to membranes. In some cases, there is a Ca^{2+}-myristoyl switch that enables the protein to associate with membranes in a reversible manner, depending on the level of intracellular Ca^{2+}. For some of the NCS proteins, such as NCS-1 and K^+-channel-interacting protein 1 (KChIP1), the myristoyl group is exposed in the absence of a Ca^{2+} signal, which means that they are already associated with the membrane and can thus respond rapidly to brief Ca^{2+} transients. On

the other hand, the proteins that use the Ca^{2+}-myristoyl switch have slower response times and are thus tuned to respond to slower global changes in intracellular Ca^{2+}. The association of NCS proteins with membranes is fairly specific and is mainly confined to interactions with the plasma membrane or membranes of the *trans*-Golgi network, where they play a variety of roles as outlined in the following descriptions of individual NCS proteins:

Neuronal Ca^{2+} sensor-1 (NCS-1)

Neuronal Ca^{2+} sensor-1 (NCS-1) is widely expressed in neurons. It has an N-terminal myristoyl group, which attaches it to membranes even in the absence of Ca^{2+}, which means that NCS-1 can respond rapidly to Ca^{2+} transients. It has been implicated in the control of different cellular processes. One action is to modulate ion channels, and particularly members of the voltage-operated channels (VOCs) such as the P/Q-, N- and L-type channels. It can inhibit the internalization of dopamine D_2 receptors by interacting with both the receptor and the G protein-coupled receptor kinase 2 (GRK2). There is evidence that NCS-1 can have an effect on exocytosis, which may depend on its ability to activate the type III PtdIns 4-kinase (PtdIns 4-K) IIIβ. The formation of PtdIns4P is one of the steps in the PtdIns4,5P$_2$ regulation of exocytosis. NCS-1 also functions in Golgi to plasma membrane transfer at the *trans*-Golgi network (TGN) (See step 3 in Module 4: Figure membrane and protein trafficking).

NCS-1 has been implicated in inositol 1,4,5-trisphosphate receptor (InsP$_3$R) modulation where it acts to promote the release of Ca^{2+} from the endoplasmic reticulum (Module 3: Figure InsP$_3$R regulation).

NCS-1 has been found to associate with interleukin-1 receptor accessory protein-like (IL1RAPL) protein, which is mutated in X-linked mental retardation. There is an up-regulation of NCS-1 in the prefrontal cortex in patients with schizophrenia and bipolar disorders.

Guanylyl cyclase-activating proteins (GCAPs)

Expression of the guanylyl cyclase-activating proteins (GCAPs) is restricted to the retina, where they play an important role in light adaptation during phototransduction (Step 11 in Module 10: Figure phototransduction). In the light, the level of Ca^{2+} declines and this allows the GCAPs to stimulate guanylyl cyclase to restore the level of cyclic GMP.

K^+-channel-interacting proteins (KChIPs)

There are four members of the K^+-channel-interacting proteins (KChIPs), which were first identified as regulators of the K_v4 voltage-dependent K^+ (K_v) channels responsible for the A-type K^+ current in neurons. These K^+ channels have six membrane-spanning regions, with the N- and C-termini facing the cytoplasm (Module 3: Figure K^+ channel domains). The KChIPs bind to the N-terminal region, where they have two main effects. Firstly, they control the trafficking of K^+ channels to the plasma membrane. In the absence of KChIP, the channel remains in the Golgi. Secondly, binding of KChIP to channels in the plasma membrane can markedly influence channel prop-

erties by enhancing the current flow and by lowering the rate of recovery. The channel will then remain open for longer and will thus act to reduce excitability. In the case of KChIP2, which is expressed in heart cells, transgenic mice that lack this channel display ventricular tachycardia, thus emphasizing the importance of the KChIPs in regulating membrane excitability. This may have relevance for epilepsy, because patients suffering from this condition have reduced levels of KChIP/DREAM/calsenilin.

KChIP is an unusual protein in that it has multiple functions. Not only does it regulate K^+ channel activity but it also functions as a Ca^{2+}-sensitive transcription factor (DREAM) and as a regulator of the presenilins, and was thus called calsenilin. To avoid confusion, it has been referred to here as KChIP/DREAM/calsenilin.

KChIP/DREAM/calsenilin

KChIP/DREAM/calsenilin is a multifunctional neuronal Ca^{2+} sensor (NCS) protein that was discovered through three independent studies to regulate quite different processes, and for each one it was given a specific name:

- It was found to be one of the K^+ channel auxiliary subunits and was called $\underline{K}^+$-$\underline{ch}$annel-$\underline{i}$nteracting $\underline{p}$rotein (KChIP) (Module 3: Figure K^+ channel domains).
- It was also found to be a Ca^{2+}-sensitive transcription factor, and was called $\underline{d}$ownstream $\underline{r}$egulatory $\underline{e}$lement $\underline{a}$ntagonistic $\underline{m}$odulator (DREAM). DREAM binds constitutively to its promoter, and this transcriptional activity is inhibited by Ca^{2+}, which acts to remove DREAM (Mechanism 3 in Module 4: Figure transcription factor activation). In the absence of Ca^{2+}, DREAM binds to its promoter sites, where it functions as a transcriptional repressor. This gene repression is removed when Ca^{2+} binds to the EF-hands on DREAM to reduce its affinity for DNA.
- It was found to regulate the presenilins, and was thus called calsenilin. The KChIP3/DREAM/calsenilin binds to the γ-secretase complex responsible for the processing of the β-amyloid precursor protein (APP) resulting in the formation of the β-amyloids responsible for Alzheimer's disease (AD) (Module 12: Figure APP processing).

Having these three names has led to some confusion regarding its terminology and here I have referred to this protein as KChIP/DREAM/calsenilin to emphasize that it is the same protein with three very different functions.

Recoverin

Recoverin is located in the retina, where it functions to prevent inactivation of phototransduction by inhibiting the rhodopsin kinase that phosphorylates rhodopsin (Step 2 in Module 10: Figure phototransduction).

Ca^{2+}-myristoyl switch

For some of the neuronal Ca^{2+} sensor (NCS) proteins [hippocalcin, neurocalcin δ, visinin-like protein (VILIP)-1 and VILIP-3], the position of the myristoyl group is sensitive to Ca^{2+}, and this provides a mechanism for

the proteins to translocate to cell membranes in a Ca^{2+}-dependent manner. Under resting conditions, the myristoyl group is tucked away in a hydrophobic pocket. The binding of Ca^{2+} induces a conformational change that exposes the myristoyl group, which then inserts itself into membranes and pulls the protein on to the membrane surface.

Ca^{2+}-binding proteins (CaBPs)

The Ca^{2+}-binding proteins (CaBPs) are a small group of EF-hand proteins that are related to calmodulin (CaM) and the neuronal Ca^{2+} sensor (NCS) proteins. As is evident from Module 2: Table Ca^{2+} signalling toolkit, the CaBPs are found predominantly in the brain and retina. Caldendrin was the first member of this family to be characterized. There are two splice variants of caldendrin, known as long CaBP1 (L-CaBP1) and short CaBP1 (S-CaBP1). CaBP-1 has attracted interest because it seems to function as an ion channel modulator with effects on both P/Q voltage-operated channels and on the inositol 1,4,5-trisphosphate receptor ($InsP_3R$). Neurons also have two proteins called calneuron-1 and calneuron-2, which are closely related to caldendrin. The calneurons seem to act by controlling the PtdIns 4-KIIIα that functions in the PtdIns4P signalling cassette to regulate vesicle trafficking during the Golgi to plasma membrane transfer of proteins.

Calcium and integrin binding protein 1 (CIB1)

Calcium and integrin-binding protein 1 (CIB1), which is also known as calmyrin and kinase-interacting protein (KIP), is a 22 kDa EF-hand protein that resembles calmodulin and several other calcium-binding proteins. As its name implies, CIB1 was originally identified through its ability to bind to the cytoplasmic tail of platelet integrin IIb. It was found subsequently to be widely distributed and is able to bind to a number of other targets. CIB1 has four EF-hand motifs, but only two of these can bind Ca^{2+}. CIB1 can bind to membranes through an N-terminal myristoyl group.

The binding of Ca^{2+} to the EF hand motifs on CIB1 induces a conformational change that enables this effector to modulate the activity of its various target protein:

- CIB1 responds to an elevation of Ca^{2+} by activating p21-activated kinases (PAK1) to bring about the phosphorylation and inactivation of gelsolin that contributes to actin polymerization (Module 2: Figure Rac signalling).
- Apoptosis signal-regulating kinase 1 (ASK1), which is a component of the mitogen-activated protein kinase (MAPK) signalling toolkit (Module 2: Table MAPK signalling toolkit) where it acts to stimulate both the JNK signalling and p38 signalling pathways, is inhibited through its association with CIB1. In response to an elevation of Ca^{2+}, this inhibition is removed resulting in an activation of the ASK1-JNK and ASK1-p38 signalling pathways. CIB1 may thus act as a Ca^{2+}-sensitive antagonist of stress-induced signalling events.
- CIB1 has been implicated in the control of spermatogenesis.

- In cardiac cells, CIB1 functions to anchor calcineurin (CaN) to the sarcolemma where it responds to the Ca^{2+} signals that activate the cardiac NFAT shuttle responsible for the onset of cardiac hypertrophy.
- CIB1 has been shown to interact with presenilin 2 and could thus contribute to the onset of Alzheimer's disease (AD).
- Activity of the $\alpha IIb/\beta_3$, which functions in integrin signalling in blood platelets and megakaryocytes, is regulated by CIB1.
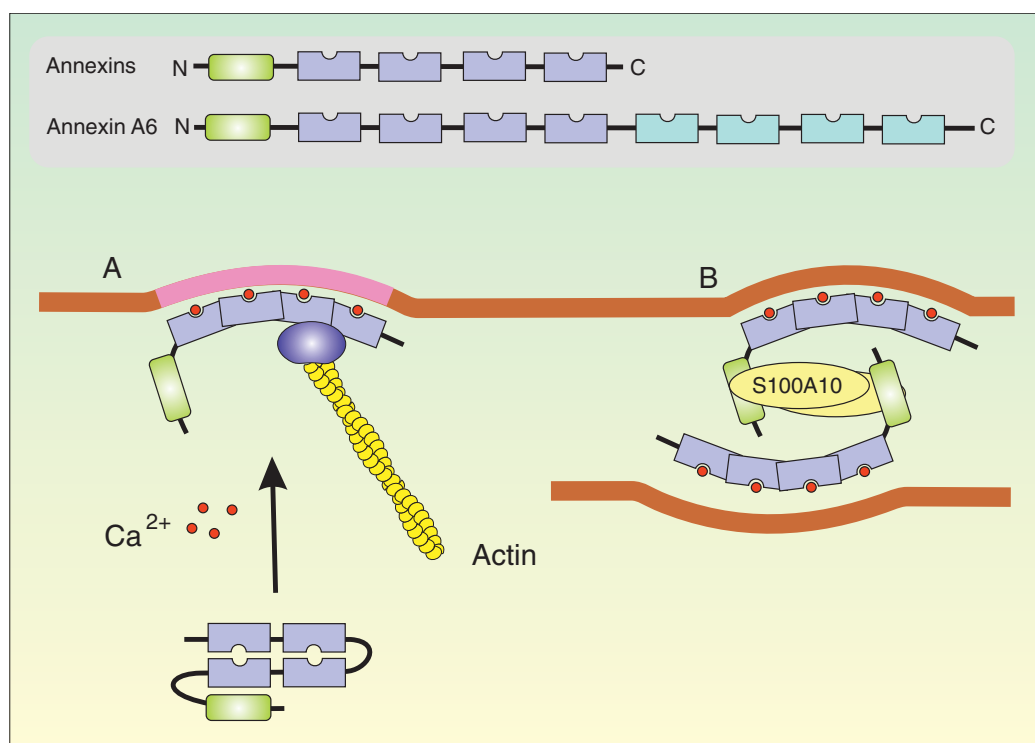
Annexins

The annexins are a large group of Ca^{2+} sensors that are also capable of binding to membrane phospholipids. There are 12 annexin subfamilies (Module 2: Table Ca^{2+} signalling toolkit). The annexins respond to an increase in Ca^{2+} by translocating to the membranes, both the plasma membrane and internal membranes. Annexin structure is dominated by the annexin core, which is the region responsible for the translocation to membrane surfaces.

When annexins bind to membranes, they can have a number of effects, such as the organization and attachment of the cytoskeleton, regulation of membrane trafficking including exocytosis, endocytosis and membrane transfer between intracellular compartments, linking membranes together and the regulation of ion fluxes. One of the problems has been to link these various effects, many of which have been uncovered in artificial membrane systems or in cultured cells, to specific cellular control mechanisms. However, various transgenic approaches have begun to reveal a number of cellular approaches that will be revealed in the description of individual annexins.
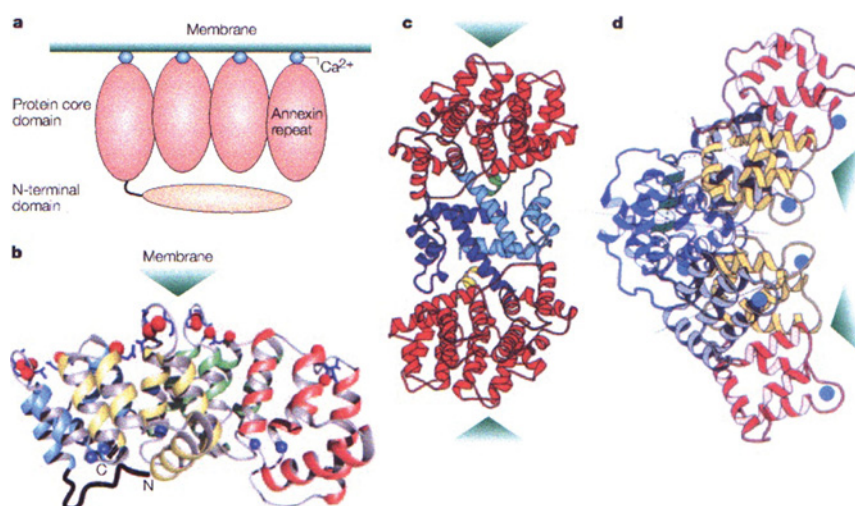
Annexin structure

The central feature of annexin structure is the annexin core, which is made up of four annexin regions, each of which has 70 amino acids with a large number of carbonyl and carboxy groups that make up the Ca^{2+}-binding regions (Module 4: Figure annexin structure). The affinity for Ca^{2+} is in the low-micromolar range, and this sensitivity can be altered following tyrosine phosphorylation of the N-terminal region. When Ca^{2+} binds to this site, it induces a conformational change that exposes a convex surface where the Ca^{2+} can form salt bridges with the membrane phospholipids. The molecular structure of this Ca^{2+}-bound form is shown in panel b in Module 4: Figure annexin molecular structures. It clearly illustrates the convex region that attaches to the membrane and the concave region where attachments to other proteins are made. Another consequence of Ca^{2+} binding is that the N-terminal region swings away and becomes accessible to phosphorylation by serine/threonine and tyrosine kinases, which not only alters Ca^{2+} sensitivity, but also can make these proteins susceptible to proteases.

The function of annexins is dependent on interactions with other proteins. For example, annexins 1 and 2 can interact with members of the S100A family to form symmetrical heteromeric complexes that have the potential to bind together different membrane surfaces (see panel B

Module 4: | Figure annexin structure

Annexin structure and membrane association.

The annexins have four annexin repeats (lilac), which makes up the annexin core domain. Each repeat has a Ca^{2+}-binding domain. Annexin A6 is unusual in that the core domain is duplicated, which has resulted from the fusion of annexins A5 and A10. When annexins bind Ca^{2+} (see A), a conformational change occurs to expose a hydrophobic concave surface that attaches to membranes through a salt bridge formed by Ca^{2+} interacting with the carbonyl and carboxy groups of the annexins and the negative charges on the phospholipid head groups in the membrane. The conformational change can also displace the N-terminal region (green) to open up the concave surface, which can then interact with other proteins such as actin. Annexin can also interact with S100A10 to form a symmetrical tetrameric structure capable of bringing together two membranes (see B).

Module 4: | Figure annexin molecular structures

Molecular structure of different annexins.

Panels a–d illustrate the molecular structure of annexins in their Ca^{2+}-bound forms, which associate with membranes depicted as the green triangles. a. This panel illustrates the Ca^{2+} ions (blue) attaching the convex surface of the core domain to the membrane. b. The protein core illustrating how the four annexin repeats (coloured differently) are composed of five α-helices. c. The symmetrical heteromeric complex composed of a central S100A10 dimer (blue) connected to two annexin A2 core domains attached to different membranes. A schematic diagram of this arrangement is shown in Module 4: Figure annexin structure. d. Structural organization of annexin A6, which has duplicate core domains connected by a flexible linker that enables each half to assume different orientations. The bound Ca^{2+} is shown in blue. Reproduced by permission from Macmillan Publishers Ltd: *Nat. Rev. Mol. Cell Biol.* Gerke, V., Creutz, C.E. and Moss, S.E. (2005) Annexins: linking Ca^{2+} signalling to membrane dynamics. 6:449–461. Copyright (2005); http://www.nature.com/nrm/; see Gerke et al. 2005.

in Module 4: Figure annexin structure and panel c in Module 4: Figure annexin molecular structures).

Annexin A1

Annexin A1 has all the hallmarks of a typical annexin. It is activated by Ca^{2+} (see panel A in Module 4: Figure annexin structure). The N-terminal tail can be phosphorylated by tyrosine kinases to alter its Ca^{2+}-sensitivity. Like annexin A2, it can segregate lipids within the plasma membrane, and there appears to be a particularly high affinity for the PtdIns4,5P$_2$ to form raft-like domains rich in this lipid. Since annexin A1 can also bind actin, it is thought to provide attachment points where the cytoskeleton links to the plasma membrane.

Annexin A1 can bind to S100A10 and S100A11 to form heteromeric complexes, as shown in panel B in Module 4: Figure annexin structure.

Annexin A2

Annexin A2 behaves much as annexin A1 with regard to its association with the plasma membrane and its ability to bind to PtdIns4,5P$_2$, and to provide membrane attachment points for the cytoskeleton. This annexin also associates with actin at the 'comet tails' that propel endocytic vesicles away from the plasma membrane.

The complex formed between annexin A2 and S100A10 has also been implicated in the control of plasma membrane Cl^- channels, the TRPV5 and TRPV6 channels and the TWIK-like, acid-sensitive K^+ channel-1 (TASK1).

Annexin A2 has a nuclear export signal (NES) and is normally excluded from the nucleus. Following phosphorylation of tyrosine residues on the N-terminal region, it can translocate into the nucleus. Since it has been shown to bind to RNA, it could play a role in RNA transport.

Annexin A4

Annexin A4 has been implicated in the control of Cl^- channels in the plasma membrane.

Annexin A5

An unusual feature of annexin A5 is that the binding of Ca^{2+} allows a tryptophan residue to insert itself into the membrane to interact with the hydrocarbon chains. This propensity to intercalate with the hydrophobic region of the membrane is enhanced further at low pH when the protein integrates into the membrane such that it can function as a Ca^{2+} channel in biophysical experiments. However, there is little evidence for such a channel function in cells under normal conditions.

Like annexin A2, annexin A5 can also enter the nucleus following its tyrosine phosphorylation.

Annexin A6

Annexin A6 is an unusual annexin in that it contains two core domains that arose from the fusion of annexins A5 and A10 (Module 4: Figure annexin structure). The two Ca^{2+}-binding core domains are connected by a flexible linker that enables it to bind different membrane regions (panel d in Module 4: Figure annexin molecular structures).

Annexin A7

Annexin A7 was originally identified in adrenal medulla cells, where it appeared to be associated with chromaffin granules. Subsequently, it was found to have an effect on release of Ca^{2+} from internal stores by both the inositol 1,4,5-trisphosphate receptor (InsP$_3$R) and the ryanodine receptor (RYR). In the case of the latter, it is of interest that annexin A7 associates with sorcin, which is known to act as a negative regulator of the coupling of L-type Ca^{2+} channels and RYR2s in heart cells. If annexin A7 contributes to such a negative regulation of Ca^{2+} release mechanisms, it might explain the phenotypes of some of the transgenic animal experiments. Astrocytic Ca^{2+} waves from annexin A7$^{-/-}$ mice were found to have higher velocities, consistent with an increased propensity to release Ca^{2+}. The increased sensitivity may also explain why the astrocytes displayed an increased rate of proliferation and were also more prone to cancer. Indeed, it is considered that annexin A7 could function as a tumour promoter gene.

Annexin A11

Annexin A11 may play an important role in the trafficking and insertion of vesicles. One role occurs during the process of cytokinesis, when the daughter cell separates into two at the time of cell division (Module 9: Figure cytokinesis). It appears to enter the nucleus at prophase and locates to the midbody, where it is in position to contribute to vesicle trafficking during cytokinesis. Annexin 11 may also play a role in the trafficking of vesicles during COPII-mediated transport from ER to Golgi (Module 4: Figure COPII-coated vesicles).

Annexin A11 binds to S100A6.

Annexin A13

Annexin A13 is unusual in that it can associate with membranes through an N-terminal myristoylation in a Ca^{2+}-independent manner.

S100 proteins

S100 proteins are the largest subgroup of the EF-hand Ca^{2+}-binding protein family. There are about 20 S100 proteins that are very divergent with regard to their distribution, function and Ca^{2+}-binding properties. An interesting aspect of their function is that they can operate both intra- and extra-cellularly. When functioning within the cell, they can have multiple effects on cytoskeletal dynamics, gene transcription, Ca^{2+} homoeostasis, cell proliferation and differentiation. With regard to their extracellular function, some of the S100 proteins (e.g. S100B and S100A12) are secreted and can act as extracellular ligands. One of their targets is the receptor for advanced glycation end-product (RAGE).

They have attracted considerable attention because there are a number of disorders, such as cancer, inflammation, cardiomyopathy and neurodegeneration, that have been linked to deregulation of these proteins.

One of the curious features of the S100 protein family is that many of the genes are clustered in a small region (1q21) on human chromosome 1 (Module 4: Figure S100 phylogenetic tree). This suggests that the S100 family expanded

Module 4: | Figure S100 phylogenetic tree
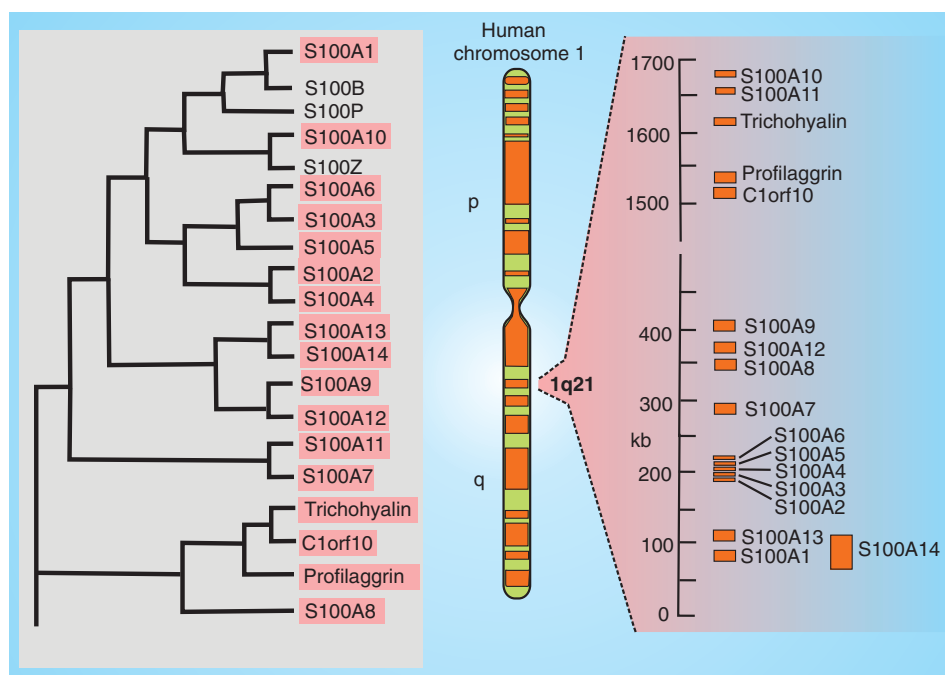
Phylogenetic tree and clustering of many S100 genes on human chromosome 1.

The phylogenetic tree shown on the left illustrates the relationships between the S100 protein family, which may have grown through gene duplication. The gene map on the right shows how many of the genes (i.e. those highlighted in orange on the phylogenetic tree) are clustered in the 1q21 region of human chromosome 1. The nomenclature of the S100 proteins, summarized by Heizmann et al. 2003, is based on this gene clustering (Module 2: Table Ca^{2+} signalling toolkit). Redrawn from *Handbook of Cell Signaling*, Volume 2 (R.A. Bradshaw and E.A. Dennis, eds), Heizmann, C.W., Schäfer, B.W. and Fritz, G., The family of S100 cell signalling proteins, pages 87–93. Copyright (2003), with permission from Elsevier; see Heizmann et al. 2003.

by gene duplication. The link between S100 proteins and cancer emerged from the observation that tumours arise from deletions or rearrangements within this chromosome region.

Members of the S100 proteins contain two EF-hand motifs (Module 4: Figure EF-hand motif). The C-terminal motif is similar to the canonical Ca^{2+}-binding site found on other EF-hand proteins, whereas the N-terminal motif has a slightly different structure that is characteristic of the S100 proteins and has been referred to as a 'pseudo-EF-hand'. The S100 proteins usually function as homo- or hetero-dimers. In response to an elevation of Ca^{2+}, the dimers usually bind four Ca^{2+} ions, which induce the conformational changes that expose an internal hydrophobic region responsible for activating its downstream effectors. Some of the S100 proteins can also bind Zn^{2+} and Cu^{2+}. For example, S100A3 has a much higher affinity for Zn^{2+} compared with that for Ca^{2+}.

The S100 proteins have been implicated in a very large number of cellular processes, and it has proved difficult to clearly identify their precise function in specific cell types. The following descriptions of some of the individual members illustrate how this large family has been implicated in many different cellular control mechanisms:

S100A1

S100A1 is preferentially expressed in cardiac cells, where it is located near the sarcoplasmic reticulum (SR) and the contractile filaments. There are indications that it might be a regulator of cardiac contractility. It is up-regulated during compensated hypertrophy, but reduced during cardiomyopathy. When overexpressed in cardiac cells, it markedly increases the amplitude of the Ca^{2+} transients, apparently by increasing the uptake of Ca^{2+} into the SR through some action on the sarco/endo-plasmic reticulum Ca^{2+}-ATPase (SERCA) pump.

S100A2

S100A2 is increased in various tumours, and the expression level has proved to have considerable diagnostic value in assessing the severity of laryngeal squamous carcinoma.

S100A4

The expression of S100A4 has been associated with cancer, where it seems to play a role in promoting metastasis. One proposal is that it may be released from cells to act as an extracellular ligand to activate tumour angiogenesis.

S100A6

This S100 protein is also known as calcyclin. Like many other members of the S100 family, S100A6 is often up-regulated in tumours, and especially in those showing high metastatic potential. Its expression is also increased during neurodegeneration in both Alzheimer's disease and in amyotrophic lateral sclerosis (ALS). In the case of the former, it is markedly increased in the astrocytes, especially

in the regions surrounding the amyloid plaques (Module 4: Figure S100A6 in AD neocortex). These astrocytes also express large amounts of S100B.

S100A8
S100A8 appears to have a role in inflammation. It is also thought to control wound healing by reorganizing the keratin cytoskeleton in the epidermis.

S100A9
S100A9 has a similar mode of action as S100A8 in inflammation and wound healing.

S100A10
S100A10 is somewhat unusual in that it is constitutively active even at low Ca^{2+} levels. It can thus bind to its targets independently of binding Ca^{2+}. Its action is intimately connected with that of annexin A1 and annexin A2. It forms a symmetrical heteromeric complex with these annexins (Module 4: Figure annexin structure).

S100B
S100B is located mainly in the brain astrocytes where it has both intra- and extracellular functions. It acts within the cell to modulate microtubule assembly and it also regulates the cell cycle by interacting with p53. S100B is also released to the extracellular space where it functions much like a cytokine to stimulate neurite outgrowth. However, at high concentrations it stops having this neurotrophic function and begins to activate apoptosis. Various neurodegenerative diseases, such as Alzheimer's disease, Down's syndrome and amyotrophic lateral sclerosis (ALS), are associated with an increased expression of S100B. The apoptosis may arise from an inflammatory response, because high levels of S100B are known to increase the production of nitric oxide (NO) and reactive oxygen species (ROS).

S100B is thought to act through the receptor for advanced glycation end-products (RAGE), which is a member of the immunoglobulin (Ig) superfamily of receptors. An S100B tetramer binds to the V domain of the RAGE receptor and this complex then interacts with another S100B/RAGE complex to form the functional dimer that then triggers cell signalling.

There also is strong evidence to suggest that S100B can inhibit the activity of the tumour suppressor p53. S100B interacts with both the p53 oligomerization domain and the C-terminal domain that is phosphorylated by protein kinase C (PKC). S100B can thus reduce p53 binding to DNA and its transcriptional activity. A Ca^{2+}-dependent activation of p53 may thus suppress the activity of p53, which will contribute to cancer progression. Such an action is consistent with the observation that S100B is overexpressed in many melanomas, astrocytomas and gliomas. Antibodies against S100B have been used for tumour typing and the diagnosis of melanoma.

Synaptotagmins
Synaptotagmins are a family of Ca^{2+}-binding proteins that function as Ca^{2+} sensors to control exocytosis (Module 4: Figure Ca^{2+}-induced membrane fusion). Some of the syn-

Module 4: | Figure S100A6 in AD neocortex
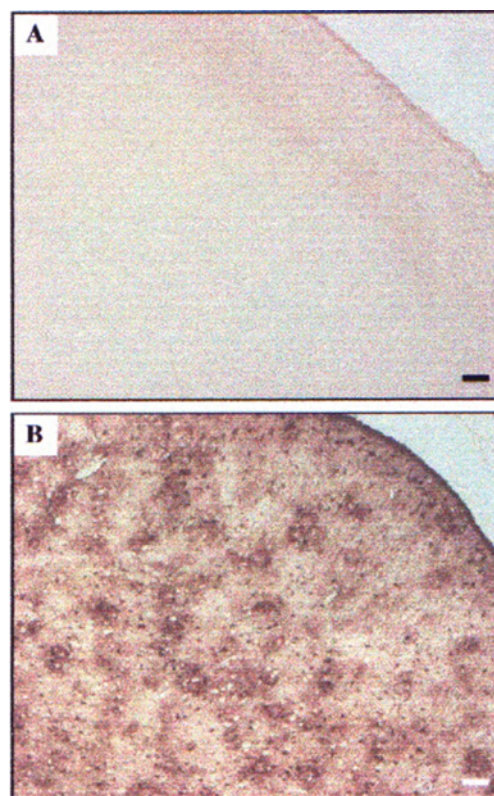
Comparison of S100A6 levels in the neocortex of normal and Alzheimer's disease-affected brain.
There is a large increase in the histochemical labelling of S100A6 in Alzheimer's disease (AD)-affected neocortex (B) compared with that of normal subjects (A). Reproduced from *Biochim. Biophys. Acta*, Vol. 1742, Broom, A., Pochet, R., Authelet, M., Pradier, L., Borghgraef, P., Van Leuven, F., Heizmann, C.W. and Brion, J.-P., Astrocytic calcium/zinc binding protein S100A6 over expression in Alzheimer's disease and in PS1/APP transgenic mice models, pages 161–168. Copyright (2004), with permission from Elsevier; see Broom et al. 2004.

aptotagmins are embedded in the vesicle, whereas others are found in the plasma membrane. For example, synaptotagmins I and II are embedded in the vesicle with their two C2 domains facing the cytosol, where they bind to Ca^{2+} and undergo a conformational change that helps to trigger Ca^{2+}-dependent exocytosis. On the other hand, synaptotagmin VII is embedded in the plasma membrane.

Effectors
'Effector' is a rather general term used to describe the cellular process responsible for carrying out the actions of signalling pathways. Information provided by the signalling systems instructs the effectors to control a variety of cellular processes. Some of these effectors are single entities, such as an ion channel or an enzyme regulating a metabolic process. However, there are more complex effector mechanisms that often are the targets of multiple signalling pathways. These effectors are then responsible for controlling a variety of cellular processes:

- Exocytosis
- Phagocytosis

- Gene transcription
- Ciliary beating
- Actin remodelling

Ca^{2+} effectors

Ca^{2+}-sensitive cellular processes in cells depend upon a wide range of Ca^{2+} effectors:

- Ca^{2+}-sensitive K$^+$ channels
- Ca^{2+}-sensitive Cl$^-$ channels (CLCAs)
- Ca^{2+}/calmodulin-dependent protein kinases (CaMKs)
- Calcineurin (CaN)
- Ca^{2+}-sensitive Ras-GAPs such as Ca^{2+}-promoted Ras inactivator (CAPRI) and RASAL (Ras GTPase-activating-like) (Module 2: Figure Ras signalling)
- Phosphorylase kinase
- Myosin light chain kinase (MLCK)

Ca^{2+}/calmodulin-dependent protein kinases (CaMKs)

The Ca^{2+} sensor calmodulin (CaM) activates a family of Ca^{2+}/CaM-dependent protein kinases (CaMKs) (Module 4: Figure structure of CaMKs). Some of these CaMKs are dedicated kinases in that they have a single substrate, such as phosphorylase kinase and myosin light chain kinase (MLCK). There are other multifunctional enzymes such as Ca^{2+}/calmodulin-dependent protein kinase I (CaMKI), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV) that phosphorylate a wide range of substrates. CaMKI and CAMKIV are monomeric and share a similar activation process. Ca^{2+} uses CaM as a sensor to activate these enzymes. Activation of CaMKI and CaMKIV depends upon a sequence of events beginning with the binding of Ca^{2+}/CaM, which is then followed by phosphorylation of their activation loop by a Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK). These enzymes are thus organized into cascades during which information is transferred to downstream targets. CAMKII, which is a multimer of eight to twelve subunits, has a different activation mechanism that depends solely on the binding of Ca^{2+}/CaM. However, this isoform has a complex autophosphorylation mechanism that gives it unique properties to function both as a frequency detector and as a long-term storage of information.

Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK)

This enzyme is located in both the cytoplasm and nucleus. It functions as part of a phosphorylation cascade to activate either Ca^{2+}/calmodulin-dependent protein kinase I (CaMKI) or IV (CaMKIV) (Module 4: Figure activation of CaMKs).

CaMKK may also function to phosphorylate other substrates such as AMP-activated protein kinase (AMPK) as part of the AMP signalling pathway (Module 2: Figure AMPK control of metabolism).

Ca^{2+}/calmodulin-dependent protein kinase I (CaMKI)

This ubiquitous enzyme is located in the cytosol. Its activation requires both Ca^{2+}/calmodulin (CaM) binding and phosphorylation of its activation loop on Thr-177 (Module 4: Figure activation of CaMKs).
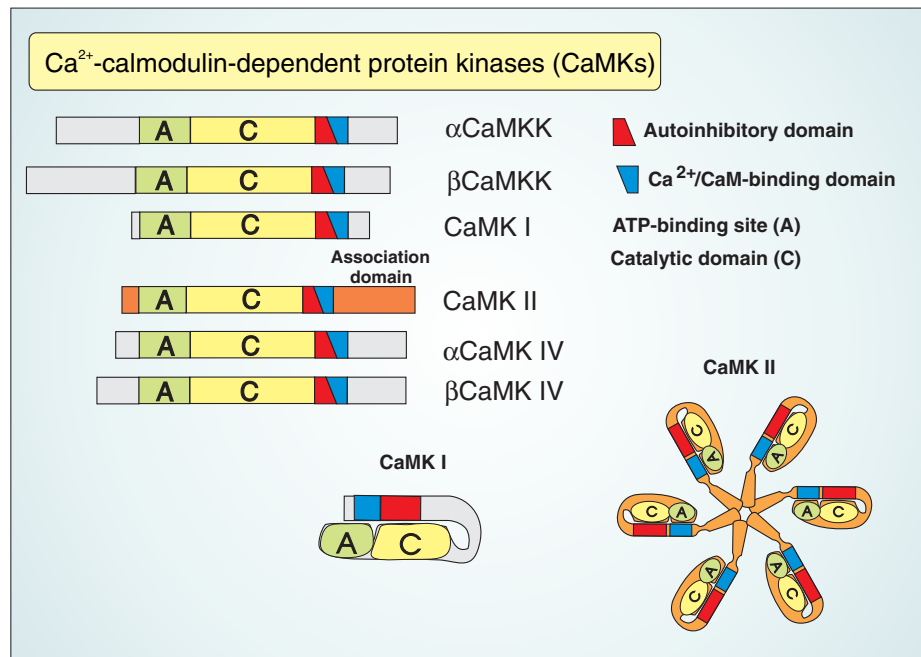
Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is a highly versatile enzyme that can phosphorylate a range of substrates. One of its proposed functions is to operate as a spike frequency detector to decode frequency-modulated (FM) Ca^{2+} signals (Module 6: Figure encoding oscillatory information). It also has a central role as a molecular switch in learning and memory.

The eight to twelve subunits that make up the holoenzyme are encoded by four separate genes: α, β, γ and δ). Structural analysis reveals that the subunits are organized in two-stacked hexameric rings (Module 4: Figure structure of CaMKII). The CaMKIIs expressed in different cells contain different proportions of these four isoforms. For example, the majority of brain CaMKII is present as the α/β isoforms in a ratio of 3:1, whereas the predominant isoform in the heart is CaMKIIδ, which exists as different spliced variants (e.g. δ$_B$ and δ$_C$). The CaMKIIδ$_B$ variant contains a nuclear localization signal and is found in both the cytoplasm and nucleus, whereas the CaMKIIδ$_C$ variant is confined to the cytoplasm. All of the isoforms are alternatively spliced to give up to 30 spliced versions. These subtle variations in the structure of the different isoforms are probably responsible for substrate targeting and subcellular localization. The catalytic headgroups that are arranged close to each other in the multimeric complex are activated by Ca^{2+} in a series of steps, as illustrated in Module 4: Figure CaMKII activation:

1. Ca^{2+} binds to calmodulin (CaM) to form the bilobed Ca^{2+}/CaM structure.
2. The bilobed Ca^{2+}/CaM wraps around the Ca^{2+}/CaM-binding domains (blue) so that the catalytic site (C) moves away from the autoinhibitory domain (red) and is now free to carry out two types of phosphorylation reactions (Steps 3 and 4).
3. In one type of phosphorylation reaction, the catalytic domain (C) phosphorylates substrates of the downstream effectors controlled by CaMKII. These substrates have a substrate domain (red) that resembles the autoinhibitory domain.
4. The other type of phosphorylation reaction is a kinase/kinase reaction in that the catalytic site (C) can also phosphorylate Thr-286 in the autoinhibitory domain (red) of a neighbouring subunit. In order for this intermolecular phosphorylation to occur, two neighbouring subunits have to be activated by each binding a Ca^{2+}/CaM, which then enables one to act as a kinase and the other as a substrate. In effect, there is an internal kinase cascade that operates between contiguous subunits. The phosphorylation of Thr-286 opens up the molecule to unveil new binding sites for CaM, and this can lead to the phenomenon of CaM trapping,
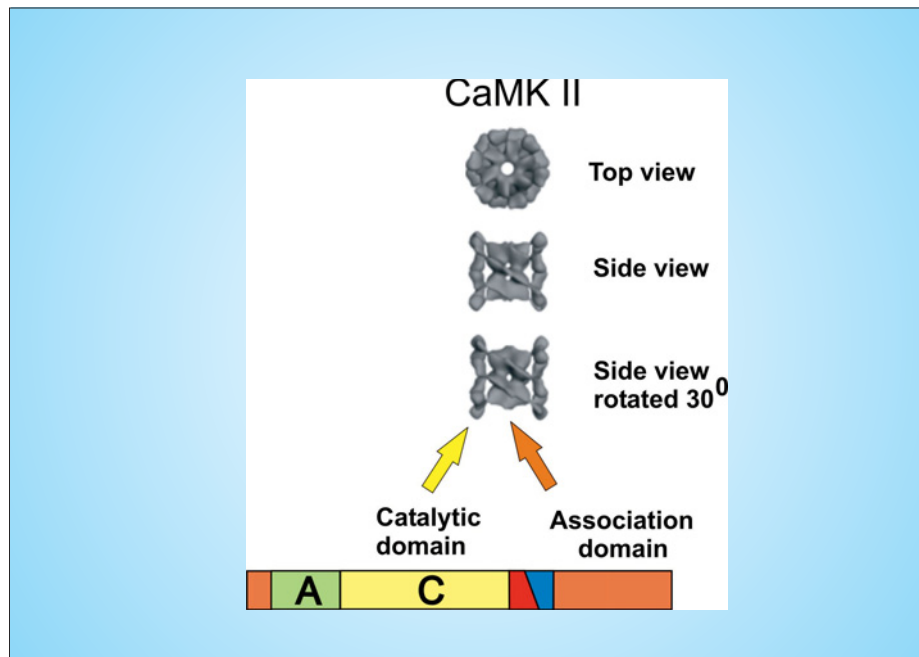
Module 4: | Figure structure of CaMKs

The domain structure and organization of Ca^{2+}/calmodulin-dependent protein kinases (CaMKs).

The Ca^{2+}/calmodulin-dependent protein kinases (CaMKs) share a number of similar domains. They all have an ATP-binding site (A), a catalytic domain (C) and an autoinhibitory domain (red) that overlaps the Ca^{2+}/CaM (calmodulin)-binding domain (blue). Most of the CaMKs function as monomers, as shown for CaMKI at the bottom of the figure. In the resting state, the catalytic site (C) bends around to interact with the autoinhibitory domain (red). CaMKII is a multimeric enzyme, and half of the complex is illustrated on the right, where six monomers connect together through their C-terminal association domains to create a wheel-like structure (Module 4: Figure structure of CaMKII).

Module 4: | Figure activation of CaMKs
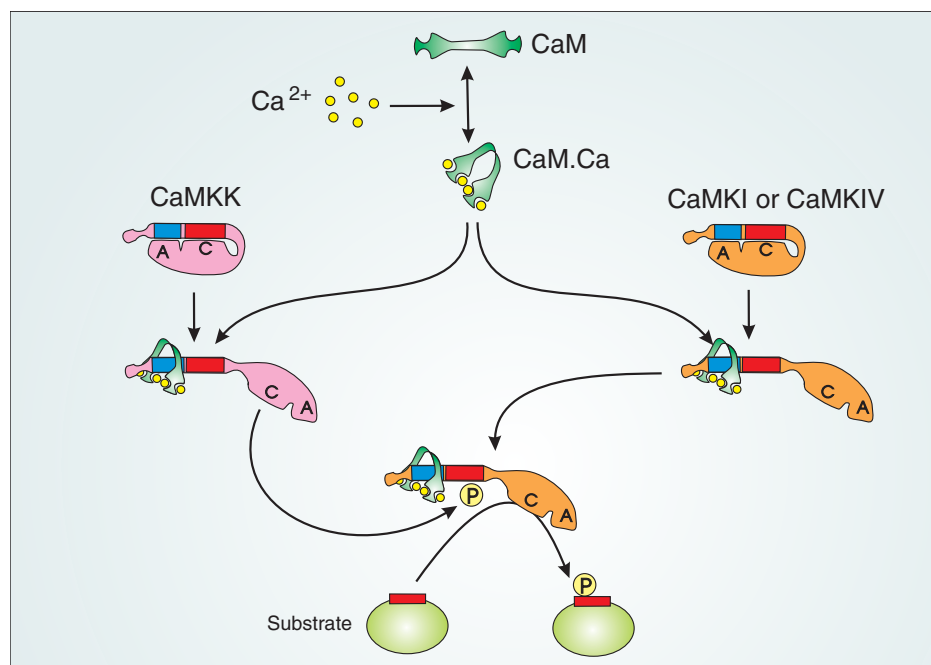
Ca^{2+} activation of Ca^{2+}/calmodulin-dependent protein kinases I and IV (CaMKI/CaMKIV).

Ca^{2+}/CaM (calmodulin)-dependent protein kinase I (CaMKI) and IV (CaMKIV) are described together because they share a similar activation mechanism. The action of Ca^{2+} begins with it binding to CaM to form the Ca^{2+}/CaM complex, which then has two separate actions. Firstly, it binds to the Ca^{2+}/CaM-binding site of CaMK kinase (CaMKK), resulting in a conformational change that leads to the activation of the catalytic domain. In its second action, Ca^{2+}/CaM binds to CaMKI or CaMKIV, causing a similar conformational change that then enables the activated CaMKK to phosphorylate a threonine residue on the activation loops of CaMKI (Thr-177) or CaMKIV (Thr-196), which are then able to phosphorylate their substrates.

Module 4: | Figure structure of CaMKII

Three-dimensional structure of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII).

The domain structure at the bottom illustrates the different structural regions of one of the Ca^{2+}/calmodulin-dependent protein kinase (CaMKII) monomers that make up the dodecameric structure. The structure of the multimeric holoenzyme was determined by reconstructing electron microscopic images. It has a barrel-shaped structure made up of the association domains forming the inner core, with the catalytic domains protruding as foot structures from either side. In effect, there will be a ring of six catalytic domains on either side of the molecule that are located close enough for the intermolecular interactions necessary for enzyme activation (Module 4: Figure CaMKII activation). The stain model-based reconstructions were reproduced from Kolodziej, S.J., Hudmon, A., Waxham, M.N. and Stoops, J.K. (2000) Three-dimensional reconstructions of calcium/calmodulin-dependent (CaM) kinase IIa and truncated CaM kinase IIa reveal a unique organisation for its structural core and functional domains. J. Biol. Chem. 275:14354–14359, with permission from the American Society for Biochemistry and Molecular Biology; see Kolodziej et al. 2000.

which becomes apparent during the recovery phase when Ca^{2+} is removed.

5. When Ca^{2+} returns to its resting level, CaM comes off those subunits that have not been phosphorylated, and these subunits return to their resting configurations. However, those that have been phosphorylated on Thr-286 remain in an active state. There are two types of autonomous activity: those molecules that have not trapped CaM, whereas others have a trapped CaM (Steps 6 and 7 respectively).

6 and 7. The phosphorylation of Thr-286 thus creates Ca^{2+}/CaM-independent states that remain competent to phosphorylate downstream substrates even though Ca^{2+} has returned to its resting level. It is this autonomous activity that gives the enzyme such interesting properties. This autonomous activity implies that CaMKII has a 'memory', and this long-lasting activity has been implicated as the molecular switch in learning and memory (Module 10: Figure Ca^{2+} control of LTD and LTP).

The fact that the holoenzyme has 12 subunits all capable of being switched into an autonomous state means that the enzyme is capable of 'counting'. Indeed, a role for CaMKII in frequency decoding may play a key role in the process of encoding and decoding of Ca^{2+} oscillations.

CamKII has multiple functions:

- It mediates the action of Ca^{2+} in triggering chromosome separation at anaphase (Module 9: Figure chromosome separation).
- It carries out the action of Ca^{2+} in stimulating PtdIns 3-kinase during phagosome maturation (Module 4: Figure phagosome maturation).
- It enhances inositol 1,4,5-trisphosphate (InsP3) formation by inhibiting the inositol polyphosphate 5-phosphatase.
- CaMKII functions as a molecular switch in learning and memory (Module 10: Figure Ca^{2+} control of LTD and LTP).
- Phosphorylation of phosphodiesterase 1B (PDE1B) by CaMKII results in a decrease in its sensitivity to Ca^{2+} activation.
- CaMKII is one of the enzymes that phosphorylates phospholamban (PLN) to control the pumping activity of the sarco/endo-plasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a) pump (Module 5: Figure phospholamban mode of action).
- CaMKII phosphorylates Syn-GAP, which is one of the GTPase-activating proteins (GAPs) responsible for switching off Ras signalling (Module 2: Figure Ras signalling).
- CaMKII stimulates cytosolic phospholipase A2 (PLA2), which is phosphorylated on Ser-515.

- CaMKII associates with the *N*-methyl-d-aspartate (NMDA) receptor and can alter its activity by phosphorylating sites on both NR2A and NR2B subunits (Module 3: Figure NMDA receptor).
- CaMKII activates the transcription factor cyclic AMP response element-binding protein (CREB) to modulate the circadian clock (Module 6: Figure circadian clock input-output signals).
- In parietal cells, CaMKII phosphorylates the Ca^{2+}-sensitive phosphoprotein of 28 kDa (CSPP-28) during the onset of acid secretion (Module 7: Figure HCl secretion).

Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV)

Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV) has a somewhat limited tissue distribution in that it is located mainly in the nucleus. CaMKIV is inactive until CaMK kinase (CaMKK) phosphorylates a single threonine residue (Thr-196) on its activation loop (Module 4: Figure activation of CaMKs). This activation process is reversed by protein phosphatase 2A (PP2A), which is constitutively active and is normally found closely associated with CaMKIV. There is some indication that Ca^{2+} can dissociate this complex, thus prolonging the active phosphorylated form of CaMKIV.

The CaMKIV located in the nucleus has a number of functions:

- CaMKIV phosphorylates the transcription factor cyclic AMP response element-binding protein (CREB) (Module 4: Figure CREB activation). Such a mechanism operates during the control of neuronal gene transcription (Module 10: Figure neuronal gene transcription).
- CaMKIV contributes to the differentiation of skeletal muscle. It phosphorylates histone deacetylase (HDAC), which is then exported from the nucleus in association with the 14-3-3 protein, thus terminating the deacetylation of chromatin as occurs during the activation of MyoD (Module 4: Figure MyoD and muscle differentiation).
- CaMKIV plays a prominent role in activating the transcription factor myocyte-enhancer factor 2 (MEF2) (Module 4: Figure MEF2 activation).
- The transcriptional repressor methyl-CpG-binding protein 2 (MeCP2) is phosphorylated by CaMKIV during the activation of neural genes such as that encoding brain-derived neurotrophic factor (BDNF) (Module 4: Figure MeCP2 activation).
- Cell depolarization produces Ca^{2+} signals that activate CAMKIV to regulate the alternative splicing of ion channels through a CAMKIV-responsive RNA element (CaRRE).

Phosphorylase kinase

Phosphorylase kinase is a Ca^{2+}-sensitive enzyme that is regulated by a resident calmodulin (CaM). The cyclic AMP signalling pathway can phosphorylate this enzyme and this can enhance the activity of the enzyme by increasing its sensitivity to Ca^{2+}. Phosphorylase kinase is particularly important in regulating the process of glycogenolysis both in skeletal muscle (Module 7: Figure skeletal muscle E-C coupling) and in liver cells (Module 7: Figure glycogenolysis and gluconeogenesis).

Myosin light chain kinase (MLCK)

Myosin light chain kinase (MLCK) is a Ca^{2+}-sensitive enzyme that functions to phosphorylate the myosin light chain that regulates the activity of myosin II (NMII) found in both smooth muscle and a variety of non-muscle cells:

- Control of smooth muscle contraction (Module 7: Figure smooth muscle cell E-C coupling).
- Control of cytokinesis during cell division (Module 9: Figure cytokinesis).
- Control of endothelial permeability (Module 7: Figure endothelial cell contraction).

Calcineurin (CaN)

Calcineurin (CaN), which is also known as protein phosphatase 2B (PP2B), is a member of the phosphoprotein phosphatase (PPP) family of serine/threonine protein phosphatases (Module 5: Table serine/threonine phosphatase classification). The function of calcineurin is inhibited by the immunosuppressant drugs cyclosporin A (CsA) and FK506, which act through the immunophilins. Calcineurin functions as a heterodimeric complex composed of a catalytic A subunit (CaNA), a regulatory B subunit (CaNB) and calmodulin (CaM). Both the B subunit and calmodulin confer the Ca^{2+}-sensitivity of the enzyme. There are three isomers of the A subunit (CaNAα, CaNAβ and CaNAγ). Whereas CaNAα and CaNAβ are expressed in many different cells, CaNAγ is restricted to the testis and certain regions of the brain. An alteration in the CaNAγ has been linked to schizophrenia.

CaN is activated by Ca^{2+} through a two-stage process (Module 4: Figure calcineurin):

1. As the cytosolic Ca^{2+} level rises, Ca^{2+} binds to the two low-affinity sites on CaNB to induce a conformational change in CaNA, resulting in the exposure of the CaM-binding domain (CaM-BD) site.
2. In the next step, Ca^{2+} activates CaM, which wraps around the CaM-BD site to induce a further conformational change that pulls the autoinhibitory domain (AID) away to free up the catalytic site so that it can begin to hydrolyse its phosphorylated substrates such as nuclear factor of activated T cells (NFAT) and protein phosphatase 1 (PP1).

In cardiac cells, CaN is anchored to the sarcolemma by binding to calcium and integrin-binding protein 1 (CIB1). During cardiac hypertrophy, there is an increased expression of CIB1 and this may contribute to the way CaN triggers the cardiac NFAT shuttle (Module 12: Figure hypertrophy signalling mechanisms).

The Ca^{2+}-dependent activation of CaN then acts by dephosphorylating a number of key signalling components:

Module 4: | Figure CaMKII activation
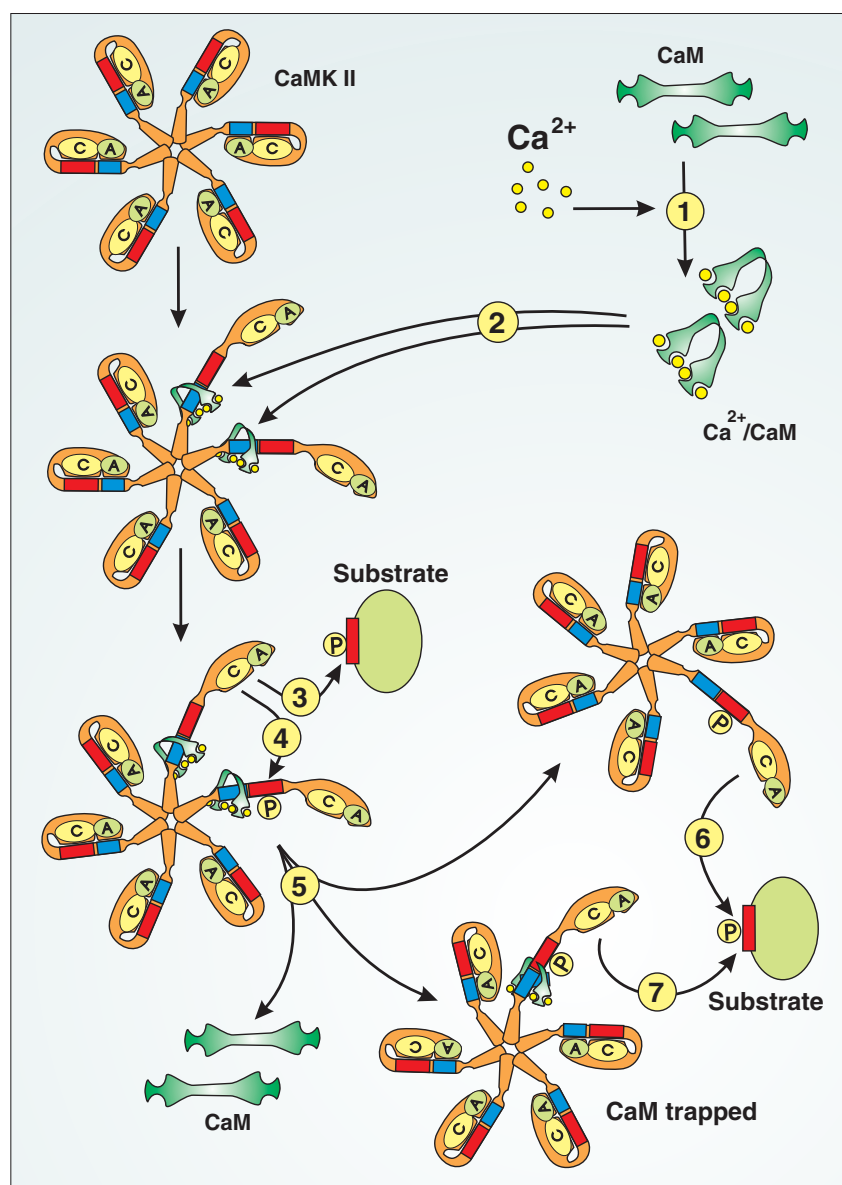
Ca^{2+} activation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII).

The activation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) by Ca^{2+} (half-maximal at a Ca^{2+} concentration of 0.5–1 μM) depends upon a sequential series of events that gives rise to a number of CaMKII activation states. See the text for details of Steps 1–7.

- One of the major targets is the transcription factor NFAT (Module 4: Figure NFAT activation).
- CaN acts together with PP1 during synaptic plasticity in neurons (Module 10: Figure Ca^{2+} control of LTD and LTP). It plays a central role in the mechanism of Ca^{2+}-induced synaptic plasticity where it acts to erase memories (Module 10: Figure Ca^{2+}-induced synaptic plasticity).
- CaN controls Ca^{2+}-dependent gene transcription in glucagon-secreting α-cells (Module 7: Figure α-cell signalling).
- CaN dephosphorylates the transducer of regulated CREB (TORC), thus enabling it to enter the nucleus to co-operate with CREB to switch on transcription (Module 4: Figure CREB activation).

The number of calcineurin molecules in cells can vary enormously: 5000 in lymphocytes, but 200 000 in hippocampal and cardiac cells.

Immunophilins

The immunophilins are a family of proteins that modulate a number of signalling components. They were first defined through their ability to bind to immunosuppressive drugs such as cyclosporin A (CsA) and FK506. The major immunophilins are cyclophilin A and the FK506-binding proteins (FKBPs).

Cyclophilin A

Cyclophilin A is the protein that binds cyclosporin A to regulate the activity of calcineurin (CaN). This inhibition

Module 4: | Figure calcineurin
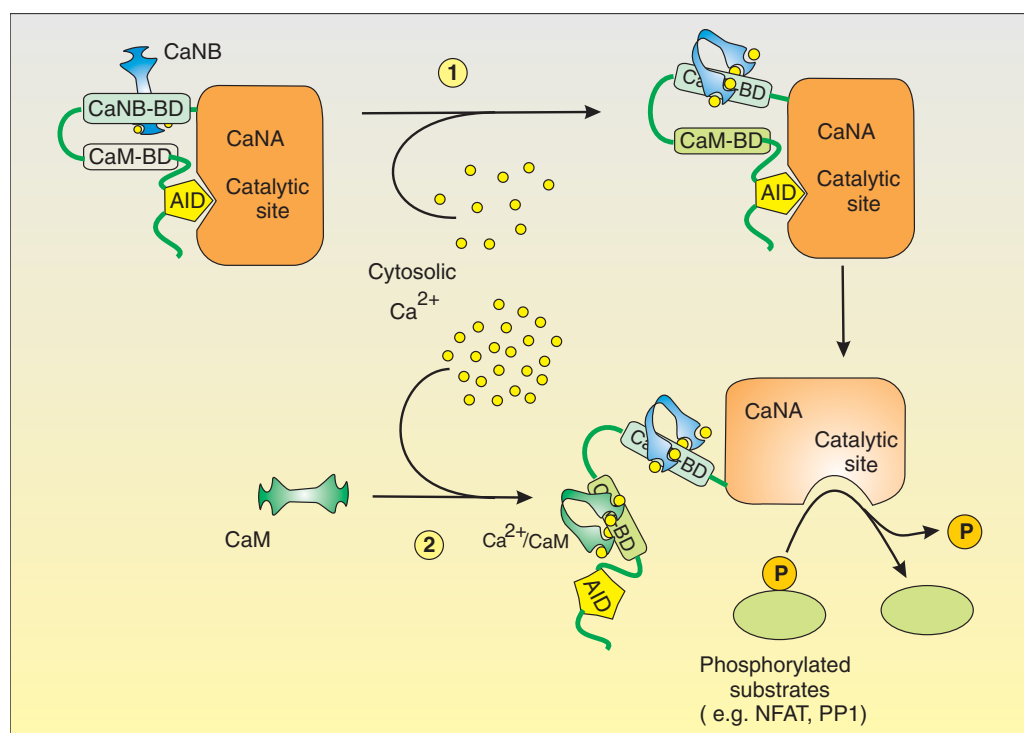
Calcineurin (CaN) is a Ca^{2+}/calmodulin-sensitive serine/threonine protein phosphatase.
Shown on the top is the catalytic calcineurin A (CaNA) subunit, which has the catalytic site, a calcineurin B-binding domain (CaNB-BD), a calmodulin (CaM)-binding domain (CaM-BD) and an autoinhibitory domain (AID) that inhibits the catalytic site in the inactive state. CaNB is an EF-hand Ca^{2+}-binding protein that has a similar structure to CaM and is bound to the enzyme under resting conditions. The stimulatory action of Ca^{2+} is carried out by two Ca^{2+} sensors, CaNB and CaM, which act by removing the inhibitory effect of AID that occurs in two steps, as described in the text.

of CaN is particularly evident in the inhibition of nuclear factor of activated T cells (NFAT) activation in T cells (Module 9: Figure T cell Ca^{2+} signalling), which is the basis of decreasing the rejection of transplanted organs by the immunosuppressant drug cyclosporin A (CsA). CsA is also able to inhibit the cardiac gene transcription responsible for hypertrophy.

FK506-binding proteins (FKBPs)
The FK506-binding proteins (FKBPs) were first recognized through their ability to bind the immunosuppressant drugs FK506 and rapamycin. There are approximately eight family members in mammals, with most attention being focused on FKBP12 and FKBP12.6. These members of the immunophilin family have peptidylpropyl *cis–trans* isomerase (PPIase) activity.

One of the functions of FKBP12 is to regulate the activity of the target of rapamycin (TOR) (Module 9: Figure target of rapamycin signalling). Another important function of the FKBPs is to regulate the Ca^{2+} release channels. In the case of muscle cells, FKBP12 (calstabin1) regulates the ryanodine receptor 1 (RYR1), whereas FKBP12.6 (calstabin2) modulates the activity of ryanodine receptor 2 (RYR2) in cardiac cells.

Calcineurin inhibitors
The activity of calcineurin (CaN) is sensitive to a number of inhibitors, both endogenous and exogenous:

- Cyclosporin A (CsA)
- FK506
- Down's syndrome critical region 1 (DSCR1)
- Calcineurin homologous protein (CHP)
- Cain/Cabin
- Carabin

Cyclosporin A (CsA)
Cyclosporin A (CsA) is a potent immunosuppressant drug that acts to inhibit a family of cyclophilins. CsA binds to one of the cyclophilins, and this CsA/cyclophilin complex then binds to the active site of calcineurin (CaN) to block enzymatic activity. By inhibiting the activation of nuclear factor of activated T cells (NFAT) in T cells (Module 9: Figure T cell Ca^{2+} signalling), CsA is capable of reducing the rejection of transplanted organs. CsA is also able to inhibit the cardiac gene transcription responsible for hypertrophy.

CsA is also a potent inhibitor of apoptosis. It binds to the cyclophilin-D (CyP-D), which is a component of the mitochondrial permeability transition pore (mPTP) (Module 5: Figure ER/mitochondrial shuttle).

FK506
Like cyclosporin A, FK506 is a potent immunosuppressant drug capable of reducing the rejection of transplanted organs. It acts by binding to the FK506-binding protein (FKBP), and this FK506/FKBP complex then binds to the

active site of calcineurin (CaN) to block enzymatic activity. FK506 has been shown to reverse Ca^{2+}-dependent neuro-degeneration by preventing the memory loss in a mouse model of Alzheimer's disease (AD) (Module 12: Figure amyloids and Ca^{2+} signalling).

Down's syndrome critical region 1 (DSCR1)

The Down's syndrome critical region 1 (DSCR1) gene is found in the critical region of chromosome 21 that is amplified by trisomy. There are approximately 230 supernumary genes on this extra region of DNA. DSCR1, which is also known as the regulator of calcineurin 1 (RCAN1) or the modulator calcineurin interacting protein 1 (MCIP1), is thus one of the candidate genes that may be responsible for Down's syndrome. DSCR1 is part of a family that includes ZAKI-4 and DSCR1L2 (DSCR1-like 2), which also act to inhibit calcineurin (CaN). These inhibitors are strongly expressed in the brain, and DSCR1 and ZAKI-4 are also found in the heart and skeletal muscle.

One of the interesting features of DSCR1 is that its expression is induced by Ca^{2+} acting through a calcineurin (CaN)/nuclear factor of activated T cells (NFAT)-dependent mechanism, thus representing a negative-feedback loop to limit the activity of CaN (Module 4: Figure NFAT control of Ca^{2+} signalling toolkit).

Calcineurin homologous protein (CHP)

Calcineurin (CaN) homologous protein (CHP) shares some homology with CaN regulatory B subunit (CaNB) and competes with the latter to inhibit the activity of CaN.

Cain/Cabin

Cain/Cabin are non-competitive inhibitors of calcineurin. Cain/Cabin can reduce the cardiac gene transcription responsible for cardiac hypertrophy. It can act as a repressor to inhibit the activity of the transcription factor MEF2 (Module 4: Figure MEF2 activation)

Carabin

Carabin has 446 amino acid residues and has a putative Ras/Rab GAP domain at the N-terminus and a calcineurin-binding domain at the C-terminus. It is strongly expressed in spleen and peripheral blood lymphocytes. During **T cell receptor (TCR) signalling**, there is a marked up-regulation of carabin that may thus exert a negative-feedback loop to inhibit T cell signalling (**Module 9: Figure T cell Ca^{2+} signalling**). Carabin also inhibits Ras signalling through its Ras GTPase activity and may thus provide a cross-talk mechanism between the Ras and Ca^{2+} signalling pathways.

Membrane and protein trafficking

Membranes and their protein components are constantly being turned over through a mechanism that has multiple components and pathways. Most emphasis will be focused on the proteins that are synthesized on the endoplasmic reticulum and then begin their journey through the cell through a number of pathways some of which are illustrated in Module 4: Figure membrane and protein trafficking:

1. Endoplasmic reticulum/Golgi transport mechanisms describe the two-way protein transport pathways that operate between the ER and the Golgi. The coat protein complex II (COPII) vesicles carry cargo from the ER to the Golgi, whereas the COPI vesicles return certain ER proteins from the Golgi back to the ER.
2. Golgi protein sorting and packaging.
3. Golgi-to-plasma membrane transfer
   The *trans*-Golgi network (TGN) is a major protein sorting organelle functioning to direct newly synthesized proteins either to the plasma membrane or to various endosomal compartments. Control of vesicle formation at the TGN depends on Ca^{2+} regulation of the PtdIns 4-KIIIβ that forms the PtdIns4P necessary for this trafficking process. In addition, it can also receive cargo back from the endosomes. This bidirectional transfer between the TGN and endosomes is particularly important for sorting and directing lysosomal hydrolases towards the lysosome. These hydrolases are carried on the cation-independent mannose 6-phosphate receptor (CI-MPR), which is then returned to the TGN by the early endosome to *trans*-Golgi network (TGN) trafficking pathway (Module 4: Figure endosome budding TGN).
4. Exocytosis
5. Endocytosis and the transport of vesicles to the early endosome by myosin motors such as myosin VI.
6. Endosome vesicle fusion to early endosomes
7. Early endosome protein sorting and intraluminal vesicle formation.
   The endocytic vesicles that bud off from the plasma membrane fuse with the early endosome to deliver a number of different proteins. These proteins have to be sorted and then packaged for transport to a number of other cellular locations.
8. Early endosome to plasma membrane trafficking
9. Early endosome to *trans*-Golgi network (TGN) trafficking
10. Early endosome maturation to lysosomes
    As the early endosome begins to accumulate intralumenal vesicles it matures into a multivesicular endosome (MVE). At this stage, there is a large accumulation of PtdIns3,4,5P$_2$, which is a phosphoinositide lipid signalling molecule (Module 2: Figure localized inositol lipid signalling) that activates the TRPML1 channels to create the local domains of Ca^{2+} that triggers the fusion events to form the lysosomes.
11. There is an alkaline phosphatase (ALP) pathway responsible for transporting ALP from the Golgi to the endosomal–lysosomal complex. The ALP and another cargo protein Vam3 have di-leucine sorting signals that direct them into adaptor protein-3 (AP-3)-coated vesicles. The AP-3 is associated with hVps41 that helps to transport the vesicles to the endosomal–lysosomal complex. The SORT1 trafficking protein may use a similar pathway to transport proteins from the Golgi to the lysosomes.

Module 4: │ Figure membrane and protein trafficking
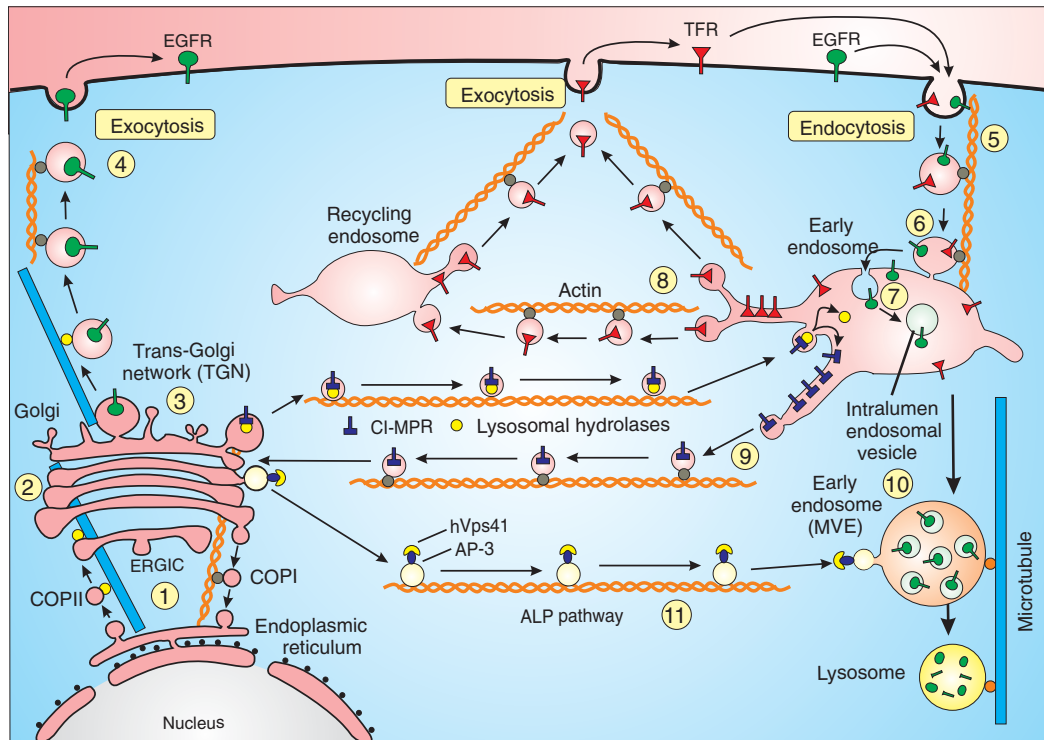
Membrane and protein trafficking pathways.
The turnover of membrane components and proteins depends on a constant flux between the plasma membrane and an array of intracellular organelles such as the endoplasmic reticulum, Golgi, various endosomal compartments and the lysosome. The lipid signalling pathways responsible for controlling these trafficking processes is described in Module 2: Figure localized inositol lipid signalling. The major pathways that connect these different organelles are described in the text. CI-MPR, cation-independent mannose 6-phosphate receptor.

There are two hVps41 SNPs that may increase the susceptibility of developing Parkinson's disease.

Endoplasmic reticulum/Golgi transport mechanisms

The first step in membrane and protein trafficking is the transfer of proteins from the ER to the Golgi (see step 1 in Module 4: Figure membrane and protein trafficking). The Golgi is a highly dynamic organelle that processes large amounts of protein that not only is being exported to the plasma membrane, but is also constantly being exchanged with the ER and the endosomal system. To carry out these dynamic Golgi functions, it is essential that this organelle maintains its characteristic morphology. The PtdIns4 signalling cassette (Module 2: Figure localized inositol lipid signalling) seems to play an important role in orchestrating both the morphology and function of the Golgi. The PtdIns4P binds to proteins such as oxysterol-binding protein (OSBP), phosphatidylinositol-Four-P AdaPtor Protein (FAPP) and the ceramide transfer protein (CERT). CERT functions in the generation and function of ceramide and sphingosine 1-phosphate (S1P) (see Step 2 in Module 2: Figure sphingomyelin signalling). In addition, GOLPH3 binds to PtdIns4P to provide an anchor, which is linked to myosin 18A and then to actin to provide a tensile force that stretches out the membrane stacks to maintain the characteristic shape of the Golgi.

In this section, we will consider the two-way transport between the ER and the Golgi that is orchestrated by coat protein complex I and II (COPI and COPII). COPII-mediated transport from ER to Golgi is responsible for the anterograde transport system (Module 4: Figure COPII–coated vesicles), whereas COPI-mediated transport from Golgi to ER takes care of the retrograde transport of certain proteins that are returned to the ER (Module 4: Figure COPI-coated vesicle).

COPII-mediated transport from ER to Golgi
The anterograde transport of newly synthesized proteins from the ER to the ER–Golgi intermediate compartment (ERGIC) is carried out by coat protein complex II (COPII) through the following sequence of events (Module 4: Figure COPII-coated vesicles):

1. The ER exit sites (ERES), which are specialized to communicate with the Golgi, have the secretory 12 (Sec12) type II transmembrane protein that functions as a guanine nucleotide-exchange factor (GEF) for the small GTPase Sar-1. When cytoplasmic Sar-1 interacts with Sec12, it exchanges its GDP for GTP, which then induces a conformational change causing the protrusion of an N-terminal amphipathic α-helix that attaches Sar-1 to the membrane.
2. The membrane-bound Sar-1.GTP complex then recruits components of the COPII complex beginning

with the Sec23/Sec24 dimer. The Sec23 has two functions. First, it is a GTPase-activating protein (GAP) that will come into play later when the COPII coat is removed (see step 6 below). Secondly, it functions to attach the vesicle to the Golgi surface by binding to the tethering complex trafficking protein particle 1 (TRAPP1). The Sec24 is responsible for capturing the cargo that is to be transported to the Golgi.

3. The Sec13/Sec31 heteromeric complex then attaches to the Sec23/Sec24 dimer to complete the formation of the COPII complex. As these COPII complexes accumulate, they induce a localized curvature of the membrane that then matures into a bud. SNARE proteins, which will be used for vesicle fusion to the Golgi, are also incorporated into the maturing vesicle.

4. The mechanism of bud scission is still not fully understood. There are indications that the hydrolysis of phosphatidylcholine (PC) by phospholipase D (PLD) to form phosphatidic acid (PA) may play a role in deforming the membrane during bud scission. PA may also be formed from diacylglycerol (DAG) through the activity of DAG kinase (DAGK). Both DAG and PA are cone-shaped lipids that can bring about negative curvature of the membrane that can facilitate scission of the vesicle. The BFA-induced ADP-ribosylation substrate (BARS), whose activity seems to depend on PA, has also been implicated in the process of cutting off the vesicle.

5. Once the vesicle has been released from the ER, it is carried along microtubules towards the Golgi by the dynein motor. It is the p50 component of dynactin, which is a dynein adaptor (Module 4: Figure dynein), that uses the golgin bicaudal D and Rab6 to attach the motor to the COPII vesicle.

6. As the COPII-coated vesicle approaches the ER–Golgi intermediate compartment (ERGIC) membrane system, it begins to shed its COPII coat. Part of this shedding process seems to be driven by the inactivation of the Sar-1.GTP complex when the GTP is hydrolysed to GDP through a process facilitated by the GAP activity of Sec23 (see step 2 above) (Module 4: Figure COPII–coated vesicles).

7. The Sec23 has an additional function of attaching the vesicle to the TRAPP1 tethering complex, which contains six main subunits. It is the Bet3 subunit that is responsible for binding to the Sec23 subunit on the vesicle. The TRAPP1 is also a guanine nucleotide-exchange factor (GEF) for Rab1 and thus converts inactive Rab1.GDP into active Rab1.GTP that functions in vesicle tethering. Sec31 can bind the penta-EF-hand protein apoptosis-linked gene 2 (ALG-2), which also interacts with Annexin-11 (ANX11). Alg-2 and ANX11 are Ca^{2+}-binding proteins that may carry out a Ca^{2+}-dependent regulatory step to modify the membrane events related to either vesicle formation or the later function of COPII vesicles to the Golgi. To what extent Ca^{2+} plays a role in the regulation of this ER-to-Golgi transport system remains to be determined.

8. The next step is for the SNARE complexes on the two membranes to begin to interact with each other. This final approach may be facilitated by the golgin protein p115, which attaches to Rab1 on the Golgi membrane.

9. Once the SNAREs begin to interact with each other, they drive vesicle fusion thus enabling the ER cargo proteins to reach the Golgi

COPI-mediated transport from Golgi to ER

The COPI retrieval pathway functions to return those ER-resident proteins that escaped to the Golgi through the COPII-mediated transport from ER to Golgi (Module 4: Figure COPII-coated vesicles). This retrieval pathway (see step 2 in Module 4: Figure membrane and protein trafficking) is somewhat more complex than the anterograde pathway because it can originate from multiple locations within the Golgi. ER-resident proteins that move along the Golgi as it matures can be removed at all levels and moved backwards to be returned to the Golgi. This retrograde transport to the ER, which depends on the coat protein complex I (COPI), occurs through the following sequence of events (Module 4: Figure COPI-coated vesicles):

1. The initial step in this retrograde pathway depends on the activation of the small GTPase Rab1b, which then recruits the golgin protein p115.

2. The Rab1b/p115 complex helps to recruit the Golgi-specific brefeldin A-resistant factor 1 (GBF1), which is a guanine nucleotide-exchange factor (GEF) for the small monomeric G protein ADP-ribosylation factor 1 (Arf1) (Module 2: Table monomeric G protein toolkit). The Arf signalling pathway has an important role in controlling key events that occur during coat formation, actin polymerization and Golgi vesicle budding (Module 2: Figure Arf signalling).

3. When cytoplasmic Arf1.GDP interacts with GBF1, it exchanges its GDP for GTP, which then induces a conformational change causing the protrusion of an N-terminal amphipathic α-helix capped by a myristoyl group that attaches Arf1.GTP to the membrane that sets the stage for assembling the COPI coat.

4. The membrane-bound Arf1.GTP complex then begins to assemble components of the COPI complex beginning with the p23/p24 heterodimer and this is then followed by the large COPI complex that consists of multiple subunits organized into two groups: F-COP (β, γ, δ, and ζ) and B-COP (α, β'and ε). It is the β-COP and the γ-COP that bind to the Arf1.GTP. At this stage, the cargo marked out for transport also associates with this large COPI coat.

5. As these COPI complexes begin to accumulate, they induce a localized curvature of the membrane that then matures into a bud. SNARE proteins, which will be used for vesicle fusion to the Golgi, are also incorporated into the maturing vesicle. It is during this budding stage that the ArfGAP1 and the two closely related ArfGAPs 2 and 3 (ArfGAP2/3) associate with the developing bud through separate mechanisms. ArfGAP1 recognizes the curvature of the bud using an ArfGAP1 lipid sensory (ALPS) motif, which is normally unstructured, but when it detects a curved

Module 4: | Figure COPII-coated vesicles
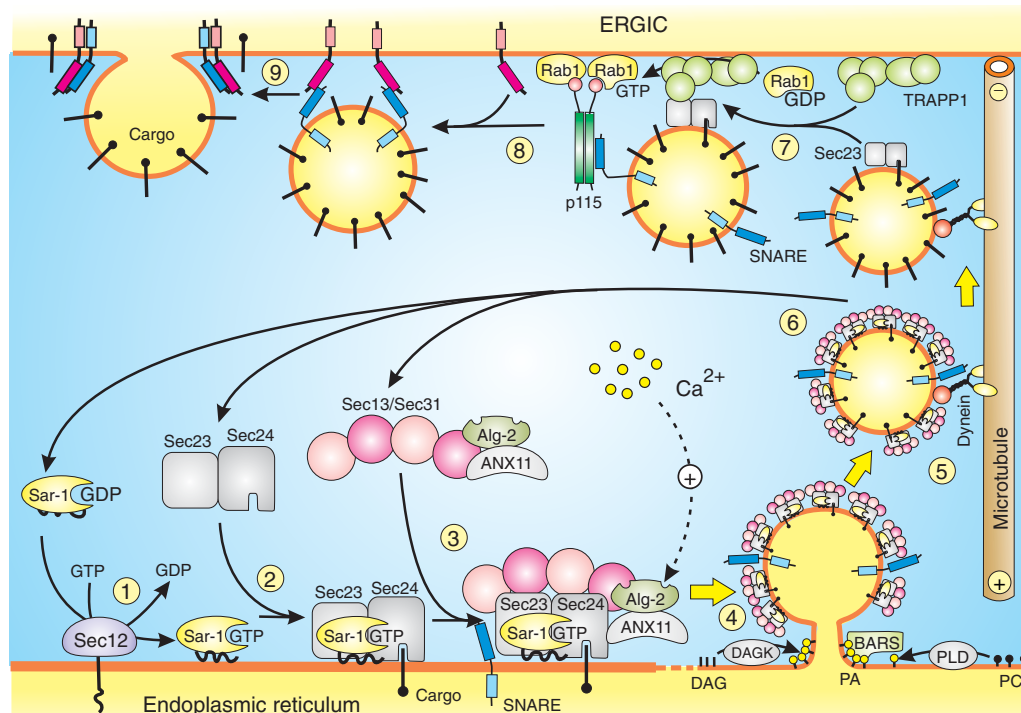
Cargo transport from the ER to the Golgi using COPII-coated vesicles.

The small GTPase Sar-1 initiates the transport process by bringing together the COPII coat made up of the Sec23/Sec24 and Sec13/Sec31 complexes that bind to the cargo. The COPII, perhaps helped by the Ca^{2+}-binding proteins apoptosis-linked gene 2 (ALG-2) and annexin-11 (ANX11), then deforms the membrane to form a bud that is cut off and is transported along microtubules towards the ER–Golgi intermediate compartment (ERGIC) using the dynein motor. The COPII coat is removed allowing the vesicle to be captured by tethers such as trafficking protein particle 1 (TRAPP1), which enables the SNARE proteins to engage with each other and to fuse with the early Golgi compartment. Note that there is a change in scale between steps 1 and 4 and the subsequent steps in the sequence. See the text for further details.

membrane it forms an amphipathic α-helix enabling the molecule to bind to the developing vesicle.

The mechanism of bud scission is still not fully understood. There are indications that the hydro-lysis of phosphatidylcholine (PC) by phospholipase D (PLD) to form phosphatidic acid (PA) may play a role. PA may also be formed from diacylglycerol (DAG) through the activity of DAG kinase (DAGK). Both DAG and PA are cone-shaped lipids that can bring about negative curvature of the membrane that can facilitate scission of the vesicle. The BFA-induced ADP-ribosylation substrate (BARS), whose activity seems to depend on PA, has also been implicated in the process of cutting off the vesicle.

6. Once the vesicle has been released from the Golgi, it is carried along actin filaments towards the ER by a kinesin-2 motor.

7. As the COPI-coated vesicle approaches the ER, it begins to shed it's COPI coat. Part of this shedding process seems to be driven by the inactivation of the Arf1.GTP complex when the GTP is hydrolysed to GDP through a process facilitated by the GAP activities of both ArfGAP1 and ArfGAP2/3.

8. The initial contact between the vesicle and the ER depends on a tethering complex called syntaxin 18, which may also act to draw in the SNARE proteins necessary for subsequent membrane fusion.

9. The next step is for the SNARE complexes on the two membranes to begin to interact with each other.

10. This SNARE interaction finally drives vesicle fusion thus enabling the Golgi proteins to reach the ER.

Golgi sorting and protein packaging

The Golgi consists of a series of flattened cisternal membranes, which are stacked on top of each other (Module 4: Figure membrane and protein trafficking). The Golgi is polarized with the *cis*-face exchanging proteins and lipids with the endoplasmic reticulum while the *trans*-face sends secretory proteins to the plasma membrane and also communicates with the endosomal system.

This stack like organization appears to be held together by interactions with the cytoskeleton and also through the action of the coiled-coil proteins of the golgin family.

Golgins

The Golgins are a family of coiled-coil proteins that operate within the Golgi during the process of Golgi sorting and protein packaging. The golgins help to maintain the structural organization of the Golgi and may also function as membrane tethers during vesicle transfer between different vesicle compartments. The golgins function as dimers held together by their coiled-coil regions, and some of the family members have interaction domains that enable them to interact with various small GTPases such as

Module 4: | Figure COPI-coated vesicle
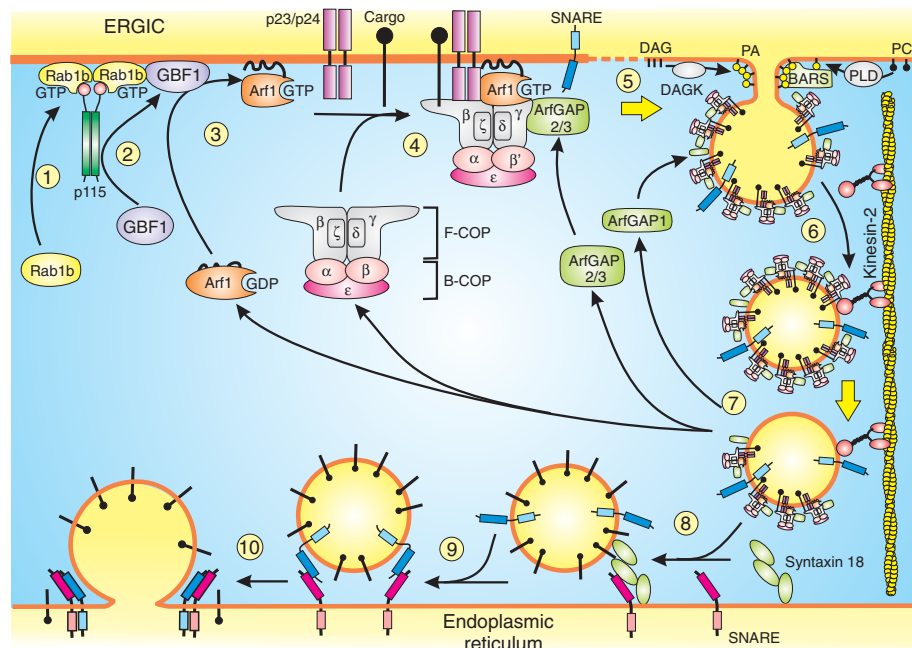
Retrograde cargo transport from the Golgi to the ER using COPI-coated vesicles.
The small GTPase Rab1b initiates the transport process by activating Arf1 that then acts to bring together the p23/p24 dimer, the COPI coat made up of multiple subunits and the cargo protein that is to be returned to the ER. The COPI then helps to deform the membrane to form a bud that is then cut off and transported along actin filaments towards the ER using kinesin-2 motors. The COPI coat components are removed allowing the vesicle to be captured by tethers such as syntaxin 18, which then enables the SNARE proteins to engage with each other and to fuse with the ER. Note that there is a change in scale between steps 1 and 4 and the subsequent steps in the sequence. See the text for further details.

Rab1, ADP-ribosylation factor (Arf) and Arf-like (ARL) (Module 2: Table monomeric G protein toolkit) that appear to control their interaction with the Golgi membranes. The following are some of the main golgin family members:

1. Transmembrane golgins such as Giantin, Golgin-84 and CCAAT-displacement protein (CDP) alternatively spliced product (CASP) are attached to membranes through a C-terminal transmembrane domain.
2. Adaptor-associated golgins are attached to membranes through the Golgi reassembly stacking protein (GRASP) adaptors, which associate with membranes through an N-terminal myristoyl group. For example, GM130 is attached to GRASP65, whereas Golgin-45 is attached to GRASP55.
3. Rab-associated golgins are recruited to membranes through the small Rab GTPases. Rab1 recruits p115 during COPI-mediated transport from Golgi to ER (Module 4: Figure COPI-coated vesicle). The Rab1/p115 complex also functions as a tether on the Golgi surface during COPII-mediated transport from ER to Golgi (Module 4: Figure COPII-coated vesicles). Rab6 binds to the golgin Bicaudal D, which functions in vesicle transport between the Golgi and the ER, to attach the dynein motor complex to the vesicle (Module 4: Figure dynein) for its transfer from the ER to the Golgi membranes.
4. ARL-associated golgins are recruited to membranes through the Arf-like (ARL) small GTPase. The golgin-

97 and golgin-245 attach to ARL1 through their GRIP domains.
5. Arf-associated golgins, such as GMAP-210, are recruited to membranes through the small GTPase ADP-ribosylation factor (Arf).

Golgi to plasma membrane transfer

The *trans*-Golgi network (TGN) is a major protein-sorting organelle functioning to direct newly synthesized proteins either to the plasma membrane or to various endosomal compartments (see step 3 in Module 4: Figure membrane and protein trafficking). The formation of vesicles at the TGN appears to be regulated by a local Ca^{2+} signal that stimulates the PtdIns4 signalling cassette (Module 2: Figure localized inositol lipid signalling). A key component of this Golgi lipid signalling system is PtdIns 4-KIIIα. At resting levels of Ca^{2+}, this lipid kinase is kept inactive when bound to the Ca^{2+}-sensing proteins calneuron-1 or calneuron-2. In response to a local pulse of Ca^{2+}, the inhibitory calneurons are replaced by neuronal Ca^{2+} sensor 1 (NCS-1) that stimulates PtdIns 4-KIIIα to begin to produce the PtdIns4P necessary for vesicle formation.

Exocytosis

Many aspects of cell communication depend upon the process of exocytosis to release signalling molecules such as hormones and neurotransmitters. These signalling molecules are stored in membrane vesicles that are released during cell–cell communication. This regulated release

of stored vesicles occurs through a process of Ca^{2+}-dependent exocytosis. There are two exocytotic mechanisms: the classical exocytotic/endocytotic cycle and a briefer kiss-and-run vesicle fusion mechanism. In both cases, the problem is to understand how an elevation in Ca^{2+} can trigger the initial event of membrane fusion. Since there is a natural reluctance for membranes to fuse with each other, special exocytotic machinery is used to force the two membranes together so that fusion occurs.

There are two types of Ca^{2+}-dependent exocytosis (Module 4: Figure Ca^{2+}-dependent exocytosis):

- Exocytosis triggered by Ca^{2+} entry through voltage-operated channels (VOCs)
- Exocytosis triggered by Ca^{2+} release from internal stores

Delivery of AMPARs to the postsynaptic membrane, which plays an important role in Ca^{2+}-dependent synaptic plasticity (Module 10: Figure Ca^{2+}-induced synaptic plasticity), is also carried out by exocytosis, but how this is controlled remains to be determined.

Exocytotic mechanisms

Membrane vesicles lying close to the plasma membrane are primed to fuse with the plasma membrane in response to a pulse of Ca^{2+}. The neuronal Ca^{2+} sensor-1 (NCS-1) can enhance exocytosis and this may depend upon its ability to facilitate the priming step. NCS-1 activates the PtdIns 4-kinase (PtdIns 4-K), which contributes to the PtdIns4,5P$_2$ regulation of exocytosis. Once the vesicles are primed, membrane fusion is triggered by a brief pulse of Ca^{2+}. When fusion occurs, the contents of the vesicles are free to diffuse out through the fusion pore. Just how much of the content is released depends upon the subsequent events. In the case of the classical exocytotic/endocytotic cycle, all of the contents are released. On the other hand, the kiss-and-run vesicle fusion mechanism is much briefer and can be repeated a number of times, thus enabling the same vesicle to function repeatedly.

Exocytotic/endocytotic cycle

The classical exocytotic/endocytotic cycle (Module 4: Figure vesicle cycle) depends upon sequential processes of docking, priming, exocytosis and endocytosis. During this process, the vesicle fuses with the plasma membrane to release all of its contents, and this is then followed by the membrane of the empty vesicle being taken up again through the process of endocytosis. The scaffolding protein intersectin may play an important role in co-ordinating the processes of exocytosis and endocytosis,

Kiss-and-run vesicle fusion

As its name implies, the kiss-and-run vesicle fusion process depends upon individual vesicles fusing repeatedly with the membrane, during which process they release a small proportion of their contents. This mechanism is particularly evident in the small synaptic endings found in the brain, which have 20–30 synaptic vesicles that can be re-used repeatedly to give transient pulses of neurotransmitter during synaptic transmission. Another example of

kiss-and-run fusion has been described in chromaffin cells (Module 7: Figure chromaffin cell exocytosis).

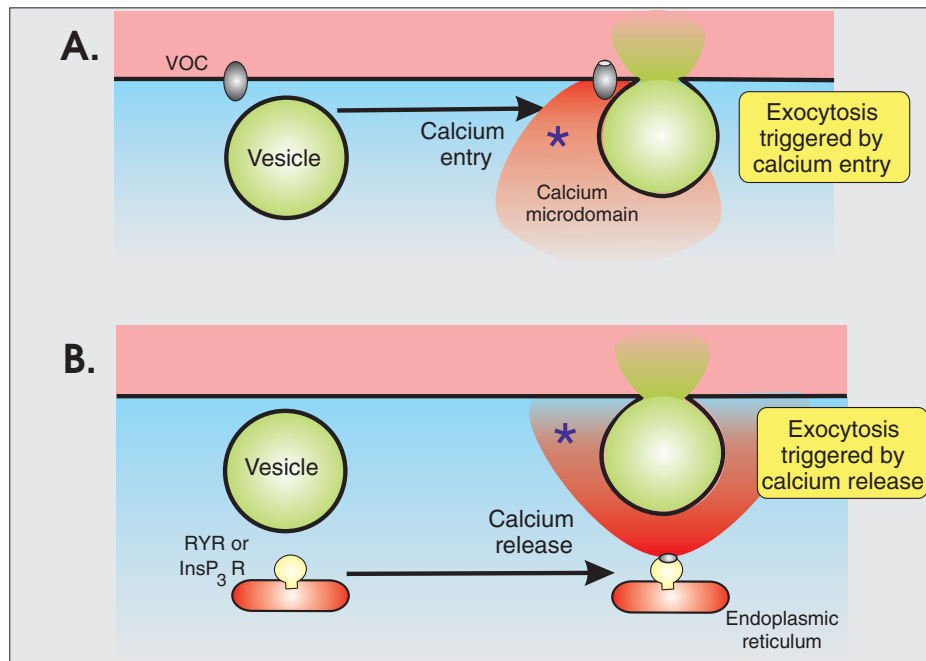### Exocytosis triggered by Ca^{2+} entry through voltage-operated channels (VOCs)

The most extensively studied form of exocytosis is the release of synaptic vesicles at neuronal presynaptic endings, which depends upon Ca^{2+}-dependent exocytosis triggered by the entry of Ca^{2+} through the Ca_V2 family of N-type, P/Q-type and R-type channels. A remarkable aspect of this process is its rapid kinetics. The phenomenal computational ability of the brain depends upon neurons being able to communicate with each other in less than 2 ms (Module 10: Figure kinetics of neurotransmission). During this process of synaptic transmission, the arrival of an action potential at the synaptic ending can trigger the release of neurotransmitter in less than 200 μs. The organization of the exocytotic machinery appears to be specially designed to achieve these high reaction rates.

Exocytosis is part of an orderly vesicle cycle (Module 4: Figure vesicle cycle). Vesicles move from a reserve pool to dock with the membrane, during which the exocytotic machinery is assembled and primed to respond to the final event of Ca^{2+}-induced exocytosis. The key to achieving such rapid responses is therefore to have the exocytotic machinery assembled and primed prior to the arrival of the Ca^{2+} signal, which then functions just to trigger the final step of membrane fusion.

### Exocytosis triggered by Ca^{2+} release from internal stores

Some cells seem to be capable of stimulating exocytosis by releasing Ca^{2+} from internal stores (Module 4: Figure Ca^{2+}-dependent exocytosis). The concentration of Ca^{2+} within the microdomains that form around the opening of internal release channels, such as the inositol 1,4,5-trisphosphate receptors (InsP$_3$Rs) and ryanodine receptors (RYRs), is very high and thus will be capable of triggering the exocytotic process. There are a number of examples of vesicle release being triggered by release of Ca^{2+} from internal stores:

- Astrocytes use InsP$_3$-induced release of Ca^{2+} to trigger the exocytosis of vesicles containing glutamate as part of the astrocyte-neuronal communication system (Module 7: Figure astrocyte tripartite synapse).
- Mossy fibre presynaptic Ca^{2+} release.
- Cerebellar basket cell presynaptic Ca^{2+} release.
- Hypothalamic neuronal presynaptic Ca^{2+} release.
- Neocortical glutamatergic presynaptic Ca^{2+} release.
- Release of luteinising hormone (LH) and follicle-stimulating hormone (FSH) from gonadotrophs (see step 5 in Module 10: Figure gonadotroph regulation).
- Release of thyroid-stimulating hormone (TSH) from the thyrotrophs is triggered by Ca^{2+} released from the internal store (Module 10: Figure thyrotroph regulation).
- Release of 5-HT from type III presynaptic cells during taste reception (Module 10: Figure taste receptors).

Module 4: | Figure Ca^{2+}-dependent exocytosis


Two types of Ca^{2+}-dependent exocytosis.

A. Exocytosis triggered by Ca^{2+} entry through voltage-operated channels (VOCs). B. Exocytosis triggered by Ca^{2+} released through ryanodine receptors (RYRs or inositol 1,4,5-trisphosphate receptors (InsP3Rs from the endoplasmic reticulum. The asterisks indicate the local microdomains of Ca^{2+} responsible for triggering membrane fusion.

Module 4: | Figure vesicle cycle
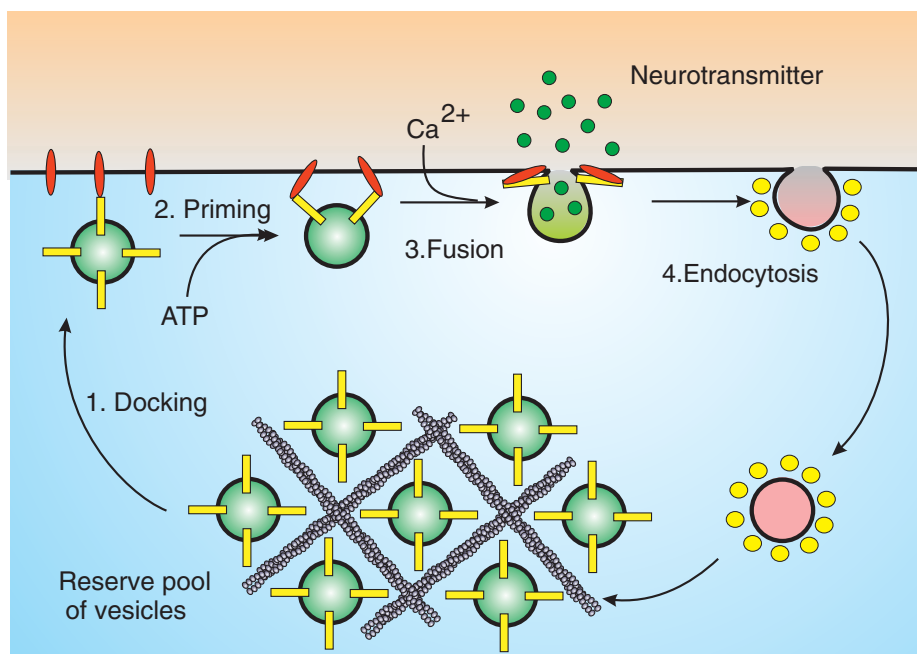
The exocytotic vesicle cycle.

1. Docking. A vesicle held within the reserve pool of vesicles embedded in a cytoskeletal matrix moves towards the membrane and uses its vesicle soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptors (v-SNAREs) to dock with the target SNAREs (t-SNAREs) in the plasma membrane. 2. Priming. The vesicle is primed through an ATP-dependent process that prepares the exocytotic machinery for fusion. 3. Fusion. Upon membrane depolarization, Ca^{2+} entering through a voltage-operated channel (VOC) triggers membrane fusion: the process of exocytosis (Module 4: Figure Ca^{2+}-induced membrane fusion). 4. Endocytosis. The membrane of the empty vesicle is retrieved through a process of endocytosis.

Module 4: | Figure Ca^{2+}-induced membrane fusion
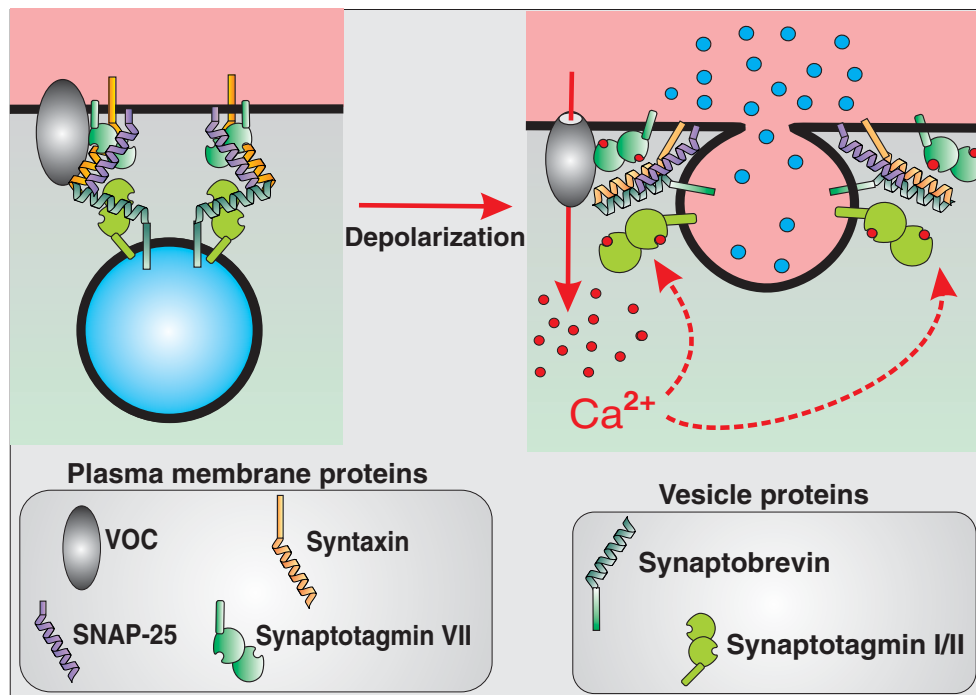
Exocytotic machinery: a hypothetical mechanism for Ca^{2+}-induced membrane fusion.

The major proteins that function in exocytosis are the vesicle soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptor (v-SNARE) synaptobrevin and the target SNAREs (t-SNAREs) syntaxin and 25 kDa synaptosome-associated protein (SNAP-25). These three SNAREs interact with each other in the docked state (left) to form a ternary complex. This complex may be prevented from interacting further by regulatory proteins such as the Ca^{2+}-sensitive proteins synaptotagmin I/II. A single voltage-operated channel (VOC), a member of the CaV2 family of N-type, P/Q-type and R-type channels, is anchored to syntaxin or SNAP-25 and opens in response to depolarization to introduce a pulse of trigger Ca^{2+} that acts through the synaptotagmins to enable the SNAREs to drive the two membranes to fuse with each other. These synaptotagmins have two adjacent C2 domains (C2A and C2B), which have Ca^{2+}-binding pockets with the low affinities (40 μM to 1 mM) necessary to respond to the high levels of Ca^{2+} found in the microdomains surrounding the vesicles. The site where the SNAREs interact with the CaV2 family of VOCs is shown in Module 3: Figure Ca$_V$2 channel family.

Exocytotic machinery

The exocytotic machinery is made up of many different components that are distributed between the plasma membrane and the synaptic vesicle. The latter are encrusted by a large number of proteins that include those that function in exocytosis such as synaptobrevin, and synaptotagmin (Module 10: Figure synaptic vesicle). Membrane fusion is driven by an interaction between these vesicle proteins and the plasma membrane proteins (Module 4: Figure Ca^{2+}-induced membrane fusion). Some of the key players in this membrane fusion mechanism are the soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptors (SNAREs), composed of the vesicle SNAREs (v-SNAREs) and the target SNAREs (t-SNAREs) located on the plasma membrane (Module 4: Figure Ca^{2+}-induced membrane fusion):

- Synaptobrevin [also known as vesicle-associated membrane protein 2 (VAMP2)] is a v-SNARE.
- Syntaxin 1a and 25 kDa synaptosome-associated protein (SNAP-25) are examples of t-SNAREs.

These three SNAREs are weakly homologous proteins, especially with regard to a SNARE motif consisting of a coiled-coil domain, that bind to each other with high affinity through hydrophobic interactions to form parallel arrays (Module 4: Figure Ca^{2+}-induced membrane fusion). The current models of exocytosis consider that the SNARE proteins zipper up with each other, thereby inducing fusion by driving the two membranes together.

Mutation in the gene encoding PICALM, which functions in the trafficking of synaptobrevin, has been linked to familial Alzheimer's disease (FAD).

The rapidity of the fusion process described in the section on exocytosis triggered by Ca^{2+} entry through voltage-operated channels (VOCs) can be accounted for by the fact that the fusion proteins may begin the zippering processes during the docking/priming events, and are thus poised for completion when given the appropriate signal through the process of Ca^{2+}-dependent exocytosis.

Ca^{2+}-dependent exocytosis

The process of membrane fusion during exocytosis in neurons is driven by an influx of Ca^{2+} through the Ca$_V$2 family of N-type, P/Q-type and R-type channels. A characteristic feature of these voltage-operated channels (VOCs) is that they possess a binding site that tightly anchors them to the exocytotic machinery (Module 3: Figure Ca$_V$2 channel family), and they are thus positioned to provide the rapid, high-intensity pulse of Ca^{2+}

necessary to trigger membrane fusion (Module 4: Figure Ca^{2+}-induced membrane fusion). In many endocrine cells, activation of L-type Ca^{2+} channels produces the global elevation of Ca^{2+} necessary to trigger exocytosis. Just how this Ca^{2+} triggers fusion is still somewhat of a mystery. The synapse has a number of Ca^{2+} sensors that may play different functions, as they seem to be sensitive to different Ca^{2+} concentrations. The zipper model implies that, once the exocytotic machinery has been primed, it is prevented from going to completion by inhibitory mechanisms that are removed by the pulse of Ca^{2+}. It therefore seems reasonable to imagine that the Ca^{2+}-sensitive synaptotagmin family of proteins might mediate this inhibition. Different synaptotagmins seem to play a role in exocytosis. Synaptotagmins I and II are integral membrane proteins anchored in the vesicle through membrane-spanning regions. On the other hand, synaptotagmin VII is located in the plasma membrane. These synaptotagmins are ideally suited to regulate fusion in that they can bind to syntaxin and their C2 domains can bind to phospholipids in a Ca^{2+}-dependent manner. These Ca^{2+}-sensitive C2 domains, which are separated from each other by a flexible linker, contain a β-barrel and bind multiple Ca^{2+} ions during which there is a conformational switch that might activate the exocytotic machinery. One possibility is that the conformational change in synaptotagmin relieves the inhibition on the exocytotic machinery, thus enabling fusion to occur (Module 4: Figure Ca^{2+}-induced membrane fusion). The plasma membrane and vesicular synaptotagmins appear to have different affinities for Ca^{2+}: those on the plasma membrane are high-affinity sensors involved in slow exocytosis, whereas those on the vesicle have low-affinity sensors capable of fast Ca^{2+}-dependent exocytosis.

Another protein called piccolo/aczonin, which has C-terminal C2A and C2B domains, may be a low-affinity Ca^{2+} sensor that functions as a regulator when Ca^{2+} accumulates during repetitive activity.

Endocytosis

Cells take up a wide range of molecules through a number of mechanisms such as clathrin-mediated endocytosis (CME), caveolin-mediated endocytosis, clathrin/caveolin-independent endocytosis and macropinocytosis (Module 4: Figure membrane and protein trafficking). The endocytic vesicles, which carry molecules away from the plasma membrane, are directed towards the early endosome and the subsequent process of endosome vesicle fusion to early endosomes, enabling the molecules taken up from the plasma membrane to enter the intracellular protein trafficking system (Module 4: Figure membrane and protein trafficking). A number of signalling mechanisms function in the control of endocytosis.

Clathrin-mediated endocytosis (CME)

Most attention has focused on clathrin-mediated endocytosis (CME), which has multiple functions, such as down-regulation of surface receptors, nutrient uptake and synaptic vesicle recycling. The uptake of integral membrane proteins, such as the transferrin receptor (TFR), occurs through various stages (Module 4: Figure endocytosis):

- Cargo selection by sorting proteins
- Membrane invagination and scission
- Vesicle transport
- Coat removal

Cargo selection by sorting protein

The initial step of cargo sorting depends on the assembly of various sorting proteins that recognize the cargo. The sorting proteins function as adaptors to connect cargo proteins to the clathrin coat during the process of endocytosis (Module 4: Figure endocytosis). In order to carry out this adaptor function, the sorting proteins such as the adaptor proteins (APs) and the clathrin-associated sorting proteins (CLASPs) have to bind multiple partners. For example adaptor protein 2 (AP2) associates with the membrane by binding to PtdIns4,5P$_2$, formed by the PtsIns4P 5-kinase Iγ (PIPKIγ), and to the sorting signals located on the cytoplasmic domain of the cargo proteins (Module 4: Figure cargo sorting signals). These sorting proteins then form a molecular platform that binds clathrin, which is an essential feature of the coated vesicles. The cytoplasmic domain of the cargo proteins has the sorting signals that enable them to be recognized by the sorting proteins. Many of these sorting signals are short amino acid sequences that are found on cargo proteins that are taken up constitutively. However, the endocytosis of some proteins is regulated through a post-translational modification such as the ubiquitination that occurs during the Cbl down-regulation of cell signalling components (Module 1: Figure receptor down-regulation).

The adaptor protein (AP) family are particularly important sorting proteins, with AP2 playing a major role in endocytosis. In addition to AP2, there are a number of clathrin-associated sorting proteins (CLASPs) that function in cargo recognition during endocytosis.

Adaptor protein (AP)

The adaptor protein family has three members: AP1, AP2 and AP3. Most attention has focussed on adaptor protein 2 (AP2), which has a primary role to play in clathrin-mediated endocytosis (CME) (Module 4: Figure endocytosis). AP2 consists of four subunits (α, β2, μ2 and σ2) that form a heterotetrameric complex that has a large trunk and two appendage domains located on flexible linkers that come from the α- and β2-subunits (Module 4: Figure cargo sorting signals). The trunk region attaches AP2 to the cargo and the membrane, whereas the appendages bind to various accessory proteins that contribute to forming the molecular layer that coats the vesicle.

The YXXØ sorting signal, which is located on proteins such as the transferrin receptor (TFR), CD-M6PR, LAMP1, LRP1, PAR1, P2X$_4$ receptor and the γ2-subunit of the GABA$_A$ receptor, is recognized by a region on the μ2-subunit of AP2. The latter also binds to phosphatidyl 4,5-bisphosphate (PtdIns4,5P$_2$), which also functions as an

Module 4: | Figure endocytosis
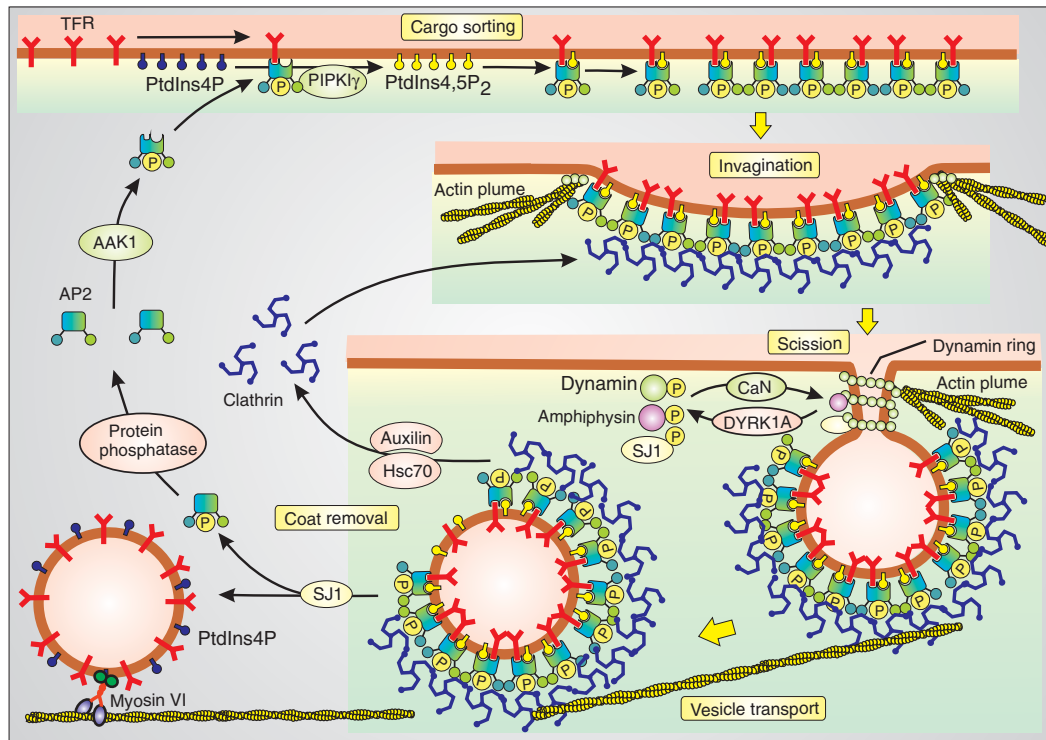
Control of clathrin-mediated endocytosis.
The uptake of plasma membrane proteins by the process of endocytosis is regulated by two main mechanisms. First, the adaptor protein 2 (AP2) is phosphorylated by adaptor-associated kinase 1 (AAK1), which helps it to bind to cargo proteins such as the transferrin receptor (TFR). Secondly, the PtdIns4P 5-kinase γ (PIPKIγ) phosphorylates PtdIns4P to PtdIns4,5P$_2$, which contributes to the binding of cargo. As the AP2–TFR complexes aggregate, they begin to bind clathrin and the membrane starts to invaginate. Various proteins, which bind to the neck region, are responsible for the process of scission during which the vesicle is budded off into the cytoplasm. Dephosphorylation of AP2 and PtdIns4,5P$_2$ by protein phosphatases and synaptojanin-1 (SJ1) respectively result in disassembly of the coat components that are re-used for further rounds of endocytosis.

adaptor to 'glue' AP2 to the membrane. A separate group of cargo proteins such as CD4, CD3γ, LIMP2 and Nef have the sorting signal [DE]XXXL[LI], which recognizes the σ2-subunit.

**Clathrin-associated sorting proteins (CLASPs)**
The clathrin-associated sorting proteins (CLASPs) function as adaptors to select cargo during endocytosis (Module 4: Figure cargo sorting signals). Some of these CLASPs, such as disabled 2 (DAB2), autosomal recessive hypercholesterolemia (ARH) and Numb, have a PTB domain, which recognizes the [FY]XNPX[YF] sorting signal found on cargo proteins such as the LDL receptor, LRP1, LRP2 (megalin), P-selectin and β1A integrin1 and 2. These CLASPs contribute to the coat by binding to clathrin and AP2.

The epsins and the epidermal growth factor receptor substrate 15 (EPS15) contribute to endocytosis by functioning as sorting proteins to detect ubiquitinated cargo. They have ubiquitin-interacting motifs (UIMs) that bind to the ubiquitinated cargo such as the epidermal growth factor receptor (EGFR) as occurs during the Cbl down-regulation of cell signalling components (Module 1: Figure receptor down-regulation). Epsin 1 and EPS15 can recognize the polyubiquitination of Lys-63 on the EGFR.

The receptor down-regulation of G protein-coupled receptors (GPCRs) is carried out by β-arrestins that behave like CLASPs (Module 1: Figure homologous desensitization). When the arrestins bind to the hyperphosphorylated GPCRs, they reveal calthrin- and AP2-binding motifs that guide the complex into the coated pits ready for internalization (Module 4: Figure cargo sorting signals).

Membrane invagination and scission
One sorting protein, such as the adaptor protein 2 (AP2), have trapped and concentrated cargo proteins, clathrin begins to coat the macromolecular complexes and the membrane invaginates to form a concentric coated bud (Module 4: Figure scission of endocytic vesicles). The clathrin, which consists of two subunits, an elongated heavy chain and a light chain, polymerize to form triskelia. These clathrin triskelia, which have three legs radiating out from a hub, interact with each other to form a web that coats the vesicular bulb. This bulb is then cut off through a scission process that is not fully understood, but some of the main players have been identified. A key event appears to be the formation of a macromolecular spiral that wraps around the neck of the vesicular bud. The main components of this spiral are SNX9 and the large GTPase dynamin. The PX domain on SNX9 binds to PtdIns4,5P$_2$, which is present at high levels, to induce a conformation change resulting

Module 4: | Figure cargo sorting signals
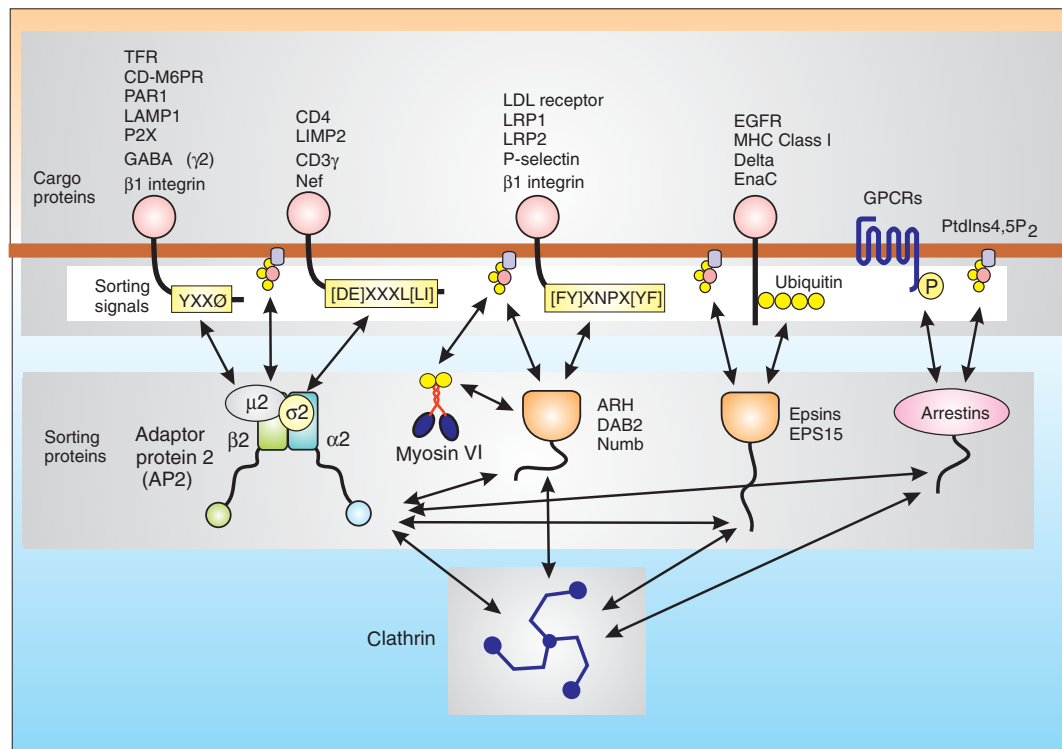
Cargo recognition by sorting proteins.

A wide range of cargo proteins located in the plasma membrane have specific sorting signals that are recognized by various sorting proteins. These sorting proteins have a variety of motifs that recognize both the sorting signals on the cargo proteins as well as clathrin. These sorting proteins also have lipid-binding motifs enabling them to bind to phosphatidyl 4,5-bisphosphate (PtdIns4,5P$_2$). The ability of the sorting proteins to connect cargo proteins to the clathrin coat is an essential part of the process of endocytosis (Module 4: Figure endocytosis).

in its oligomerization and this then provides a platform to draw in other proteins. For example, dynamin binds to both SNX9 and to PtdIns4,5P$_2$ to form part of the spiral.

The SNX9/dynamin spiral provides a platform to assemble actin remodelling proteins such as cortactin, N-WASP and Arp2/3 responsible for the nucleation of actin filaments (Module 4: Figure scission of endocytic vesicles). In addition, the SNX9 also provides another connection to actin by binding to myosin 1E. Another molecular motor (myosin VI) is connected to the vesicular region of the bulb by binding to both the sorting protein DAB2 and to PtdIns4,5P$_2$. As the clathrin-coated pit matures, the phospholipid composition of the membrane begins to change to prepare the vesicle for its transfer to the endocytic system (Module 2: Figure localized inositol lipid signalling). Firstly, the PtdIns4,5P$_2$ is dephosphorylated to PtdIns4P, which is then phosphorylated to PtdIns3,4P$_2$ by PI3KC2α. This PtdIns3,4P$_2$ is then dephosphorylated by inositol polyphosphate 4-phosphatase type II (INPP4B) to form PtdIns3P, which is the characteristic inositol lipid found in endosomes.

As the various scission molecules bind, the neck begins to thin and is then severed (scission) through a process that seems to depend on the GTP-dependent action of dynamin. In addition, the two motor proteins might help to pull the vesicle into the cytoplasm through their interaction with actin (Module 4: Figure scission of endocytic

vesicles). Myosin 1E is a plus-end motor that will pull the dynamin ring towards the plasma membrane, whereas the minus-end motor myosin VI will pull the vesicle in the opposite direction towards the cytoplasm.

Dynamin

Dynamin is a large GTPase that functions in clathrin-mediated endocytosis (CME) (Module 4: Figure scission of endocytic vesicles). At the time of scission, dynamin is part of a macromolecular complex containing amphiphysin, endophilin, epsin, Eps15, synaptojanin, syndapin, N-WASP, cortactin, mammalian actin-binding 1 (mAbp1), intersectin and profilin. One of its functions is to provide a protein scaffold that assembles many of the proteins required for scission. One of its scaffolding functions is to link the neck of the pit to the actin cytoskeleton. It can regulate F-actin dynamics by binding to accessory proteins such as cortactin, mammalian actin-binding 1 (mAbp1), intersectin and profilin. At the time of scission, dynamin hydrolyses GTP and this induces a conformational change of the spiral that stretches out the neck resulting in release of the coated vesicle.

Dynamin is part of a dynamin superfamily of large GTPases, such as the dynamin-like proteins, Mx proteins, OPA1, Mitofusins and GBP/atlastin-related proteins, which all seem to function in either membrane tubulation or scission.

Amphiphysins

There are two genes encoding the amphiphysins: amphiphysin 1 expressed in the brain and amphiphysin 2, which has a wider distribution and has numerous spliced isoforms. They have an N-terminal Bar domain that enables two molecules to dimerize to form a positively-charged concave surface that enables the dimers to interact with membranes. The C-terminal region has a SH3 domain that enables it to interact with its binding partners such as dynamin and synaptojanin 1 (SJ1) during the late stages of endocytosis. Amphiphysin is found in a 1:1 stoichiometry with dynamin and may function to facilitate the recruitment of dynamin to form the spiral responsible for scission (Module 4: Figure scission of endocytic vesicles). The ability of amphiphysins to associate with dynamin and the cell membrane is controlled by a phosphorylation/dephosphorylation system (Module 4: Figure endocytosis). Phosphorylation by the dual-specificity tyrosine–phosphorylation regulated kinase 1A (DYRK1A) inhibits binding, whereas dephosphorylation by calcineurin (CaN) removes this inhibition to allow amphiphysins to participate in endocytosis.

Amphiphysins have also been implicated in the formation of the T-tubules found in muscle cells.

Endophilin

The endophilin family has three members: endophilin 1 (SH3p4), endophilin 2 (SH3p8) and endophilin 3 (SH3p13). Endophilin 1 is particularly abundant in nerve terminals in the brain, whereas endophylin 2 is the main isoform in non-neuronal cells. Endophilin has a structure resembling that of the amphiphysins in that it has an N-terminal BAR domain and a C-terminal SH3 domain. The latter binds to the proline-rich regions of dynamin and synaptojanin. One of the primary functions of endophilin is to recruit synaptojanin, which plays a major role in coat removal following scission (Module 4: Figure scission of endocytic vesicles).

In non-neuronal cells, endophilin 2 plays a major role in the Cbl down-regulation of cell signalling components during the endocytosis of various tyrosine kinase-coupled receptors such the Trk receptors and EGF receptors. In these examples, Cbl functions to recruit both endophilin and Cbl-interacting protein of 85 kDa (CIN85) to control receptor internalization (Module 1: Figure receptor down-regulation). Phosphorylation of endophilin by Rho kinase inhibits endocytosis.

Vesicle transport

Following scission of the bud, the coated vesicles enters the cytoplasm where the various coat proteins are removed and the endosomal vesicles move towards the early endosome where it fuses to deliver its membrane cargo (see step 5 in Module 4: Figure membrane and protein trafficking).

Coat removal

When the coated vesicles enter the cytoplasm and begin to move towards the early endosomes, the various coat proteins are removed (Module 4: Figure endocytosis). This uncoating process depends on proteins such as Hsc70 and

its cofactor auxilin. The auxilin is recruited by a lipid signal, which appears to be the PtdIns4P that is formed when PtdIns4,5P$_2$ is hydrolysed by synaptojanin 1. Cells express two auxilins, brain-specific auxilin 1 and the more ubiquitous auxilin 2, which is also known as cyclin G-associated kinase (GAK). Auxilin is rapidly recruited just before scission occurs and enables the 70 kDa heat shock protein (Hsc70) to uncoat the membrane through an ATP-dependent process. The energy derived from ATP hydrolysis enables Hsc70 to drive disassembly of the clathrin coat to liberate the individual triskelions (Module 4: Figure scission of endocytic vesicles).

Control of endocytosis

The process of endocytosis is regulated at a number of different stages (Module 4: Figure endocytosis). During the initial step of cargo selection by sorting proteins, the sorting protein adaptor protein 2 (AP2) is phosphorylated by adaptor-associated kinase 1 (AAK1), which helps it to bind to cargo proteins such as the transferrin receptor (TFR). Association with the membrane also depends on the enzyme PtdIns4P 5-kinaseγ (PIPKIγ) that phosphorylates PtdIns4P to PtdIns4,5P$_2$, which contributes to the binding of cargo. As the AP2/TFR complexes aggregate, they begin to bind clathrin and this coincides with membrane invagination.

These processes are then reversed during the later stages of scission and coat removal. The large GTPase protein dynamin helps to assemble the actin fibres and plays a critical role in the scission process. The action of dynamin and its various endocytic accessory proteins such as amphiphysin and synaptojanin 1 (SJ1) is regulated by a phosphorylation/dephosphorylation cycle (Module 4: Figure scission of endocytic vesicles). Phosphorylation of these proteins by the dual-specificity tyrosine-phosphorylation regulated kinase 1A (DYRK1A) and by the neuronal cyclin-dependent kinase 5 (CDK5) prevents them from being recruited into the macromolecular complexes that drive scission and subsequent coat removal (Module 4: Figure scission of endocytic vesicles). These inhibitory phosphate groups are removed by the Ca^{2+}-sensitive phosphatase calcineurin (CaN) and this can account for the Ca^{2+}-sensitivity of endocytosis, particularly in the case of synaptic vesicle retrieval at synaptic endings during the events related to Ca^{2+} and synaptic plasticity (Module 10: Figure Ca^{2+}-induced synaptic plasticity).

After the coated vesicle has been formed, the coat is removed using various mechanisms. First, the auxilin/Hsc70 system removes clathrin (Module 4: Figure scission of endocytic vesicles). Secondly, the removal of sorting proteins such as AP2 follows the hydrolysis of PtdIns4,5P$_2$ to PtdIns4P by synaptojanin 1 (SJ1) (Module 4: Figure endocytosis). The coat components are then re-used for further rounds of endocytosis.

Dual specificity tyrosine-phosphorylation regulated kinase 1A (DYRK1A).

The gene *DYRK1A* encodes the dual-specificity tyrosine-phosphorylation regulated kinase 1A (DYRK1A), which

Module 4: | Figure scission of endocytic vesicles
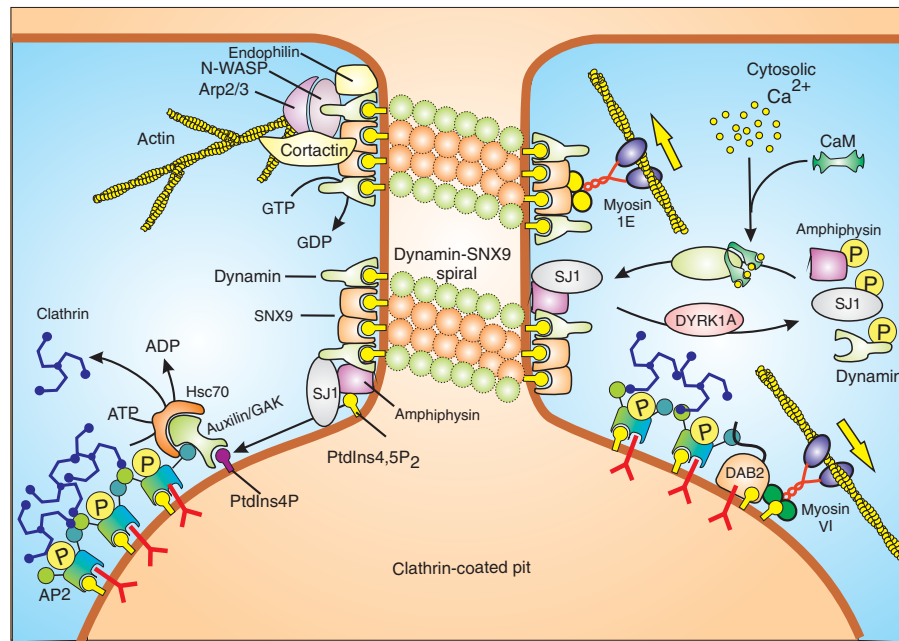
Endocytic vesicle scission and removal of the clathrin coat.

Once the clathrin-coated pit has formed, the neck is cut off to release the vesicle into the cytoplasm. The sequence of events responsible for scission remains to be established. This Figure attempts to illustrate the structural organization of some of the major components responsible for scission. Once the vesicle is formed, the clathrin coat is removed by an ATP-dependent process activated by Hsc70. See the text for further details.

is an orthologue of *Drosophila* minibrain kinase (MNB). This kinase not only functions in early brain development, but it continues to operate in the adult brain. DYRK1A is a serine/threonine protein kinase that has multiple functions both in the nucleus and in the cytoplasm. The bipartite nuclear targeting sequence enables it to operate in both locations. The DYRK1A located in the nucleus is responsible for phosphorylating the transcription factor NFAT to promote its export from the nucleus (Module 4: Figure NFAT control of Ca^{2+} signalling toolkit). DYRK1A also phosphorylates various endocytic accessory proteins that function during membrane invagination and scission during the process of endocytosis (Module 4: Figure scission of endocytic vesicles). For example, it phosphorylates the proline-rich domains (PRDs) of dynamin, amphiphysin and synaptojanin 1 (SJ1). The phosphorylation of these proteins seems to inhibit their recruitment at endocytic sites. Dephosphorylation of these proteins by calcineurin (CaN) activates the recruitment and assembly of these endocytic accessory proteins.

DYRK1A is also located on the Down syndrome critical region 1 (DSCR1) on human chromosome 21, where trisomy occurs resulting in an elevation of this protein that could contribute to the Down's syndrome phenotype.

Phagocytosis

The process of phagocytosis, which is particularly evident in haematopoietic cells such as macrophages, is a specialized form of endocytosis. During phagocytosis, the cell engulfs large particles such as bacteria through two main mechanisms determined by the nature of the receptors that

are activated by proteins on the particle cell surface. Engagement of the Fcγ receptors results in the formation of pseudopodia that rise up to engulf the particle. On the other hand, particles coated with complement C3 receptors simply sink into the cell.

The process of Fcγ-mediated phagocytosis depends upon the formation of pseudopodia that engulf the particle. The PtdIns4,5P$_2$ regulation of phagocytosis brings about the actin remodelling necessary to form the pseudopodia. In addition, there is a process of 'focal exocytosis', during which membrane vesicles are added to the growing tips of the pseudopodia. The large GTPase protein dynamin-2 appears to play a role in regulating this process of exocytosis. This exocytosis is revealed by an increase in capacitance that precedes the rapid decrease when the particle is finally internalized.

Phagosome maturation

When a pathogenic organism has been engulfed within the phagosome, a complex cascade of events drives a maturation process whereby the phagosome is converted into a phagolysosome (Module 4: Figure phagosome maturation). During phagosome maturation, there is a dramatic change both in the composition of the surrounding membrane and in the contents of the phagosome brought about primarily by an orderly fusion of vesicles from the endocytic pathway. A large number of signalling molecules and accessory proteins collaborate in this maturation process, and the precise sequence of events remains to be worked out. The process begins when Fcγ receptors (FcγRs) on the cell surface recognize an opsonized

micro-organism to initiate phagocytosis to form a phagosome. After the phagosome has formed, one of the earliest events to occur is the activation of the Class III PtdIns 3-kinase (PtdIns 3-K), which converts PtdIns into PtdIns3P that builds up rapidly on the surface of the phagosome (Module 4: Figure PtdIns3P formation in phagosomes). The PtdIns3P appears within minutes and persists for at least 30 min. This PtdIns3P then has a crucial role in recruiting endocytic vesicles (EVs) by binding to proteins such as Rab5 and the early endosome antigen 1 (EEA1). EEA1 contains a FYVE motif that specifically recognizes PtdIns3P (Module 6: Figure modular lipid-binding domains). Once the endosome is docked near the phagosome, a family of membrane-tethered coiled-coil proteins, resembling those of the exocytotic machinery [e.g. soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptor (SNARE) and synaptosome-associated protein (SNAP)], are used to drive fusion of the endocytic vesicle with the phagosome (Module 4: Figure phagosome maturation). A phagolysosome forms when the late endosome/lysosome vesicles fuse with the phagosome.

Just how the PtdIns 3-K on the phagosome is activated remains to be established, but there appears to be an important role for Ca^{2+} that is generated through an interaction between the phospholipase D (PLD) signalling pathway (Module 2: Figure PLD signalling) and the sphingomyelin signalling pathway (Module 2: Figure sphingomyelin signalling). Just how the FcγR activates PLD1 is unknown, but it is likely to depend upon some of the known activators such as RhoA, ADP-ribosylation factor (Arf) or protein kinase Cα (PKCα). Activation of PLD1 located on either the sorting endosome (SE) or the endoplasmic reticulum (ER) produces phosphatidic acid (PA), which then activates sphingosine kinase (SPHK) to convert sphingosine into sphingosine 1-phosphate (S1P). The latter then releases Ca^{2+} from internal stores using channels that remain to be identified. The pulses of Ca^{2+} appear to have two roles: either they act through CaMKII to stimulate the PtdIns 3-K, or they may also trigger the endosome/phagosome fusion events (Module 4: Figure phagosome maturation).

One of the interesting aspects of phagosome maturation is the way that it is blocked by certain pathogens:

- *Mycobacterium tuberculosis*, which causes tuberculosis, is able to survive within the phagosomes of the host by switching off the signalling processes responsible for converting it into a phagolysosome. *M. tuberculosis* produces lipoarabinomannan (LAM), which then acts to inhibit both the PLD1 and the SPHK that generate the Ca^{2+} signals responsible for driving various aspects of the maturation process (Module 4: Figure phagosome maturation).
- The bacterium *Chlamydia trachomatis*, which causes chlamydial diseases, is also able to inhibit the process of phagosome maturation.
- The bacterium *Listeria monocytogenes*, which causes listeriosis, uses the bacterial virulence factor listeriolysin O (LLO) to escape from the phagosome.

Endosome vesicle fusion to early endosomes

Endocytic vesicles coming from the plasma membrane travel inwards to fuse with the early endosome through an intracellular exocytotic mechanism (Step 6 in Module 4: Figure membrane and protein trafficking). The fusion machinery is controlled by the Rab signalling mechanism (Module 2: Figure Rab signalling). The GTP-binding protein Rab5, which is a member of the Rab family of monomeric GTP-binding proteins (G proteins) (Module 2: Table monomeric G protein toolkit). Like other Rabs, Rab5 exists in two forms. It is either soluble in the cytoplasm where it is associated with GDP-dissociation inhibitor (GDI) or it is associated with the membranes of vesicles or intracellular organelles. This association with the membrane is assisted by the prenylated Rab acceptor 1 (PRA1), which acts as a GDI displacement factor. Rab5 recruits various tethering components and effectors that orchestrate the close apposition of the membranes necessary for SNARE proteins to induce membrane fusion (Module 4: Figure endosome vesicle fusion). This assembly of a fusion complex depends on the Rab5-dependent recruitment and activation of the Class III PtdIns 3-kinase called hVps34 to produce a local accumulation of PtdIns3P. Three of the Rab5 effectors [early endosome antigen 1 (EEA1), rabenosyn 5 and rabankyrin 5] not only bind Rab5, but they also have FYVE domains that enable them to bind PtdIns3P. The EEA1 has N- and C-terminal Rab5-binding sites that may help to tether the incoming vesicles to the early endosome.

The Rab5 and its associated effector proteins interact with the SNAREs to position them such that they can interact with each other to bring about membrane fusion. The EEA1 and rabenosyn associate with the target-SNAREs syntaxin-6 and syntaxin-13 on the early endosome whereas the v-SNARE VAMP4 associates with Rabex-5 and rabankyrin-5. The N-ethylmaleimide sensitive factor (NSF), the soluble attachment protein α (α-SNAP) and hVPS45 are accessory factors that contribute to the priming of the SNARE complexes for fusion to occur.

Early endosome protein sorting and intraluminal vesicle formation

The early endosome is the initial clearing house for proteins that it receives following the fusion of endocytic vesicles and the first task is to sort them out so that they can be sent to different locations (Step 7 in Module 4: Figure membrane and protein trafficking). One sorting mechanism deals with proteins such as the EGFR that are destined to be degraded by the lysosome (Module 1: Figure receptor down-regulation). The first step in the degradation pathway is for the EGFRs to be corralled within an intraluminal endosomal vesicle (Module 4: Figure intraluminal endosomal vesicle formation). An endosomal sorting complex required for transport (ESCRT) complex functions to sort proteins and to form the intraluminal vesicles. There are four ESCRT complexes (ESCRT0–ESCRTIII) that cooperate with each other to sort cargo into a specific

Module 4: | Figure PtdIns3P formation in phagosomes
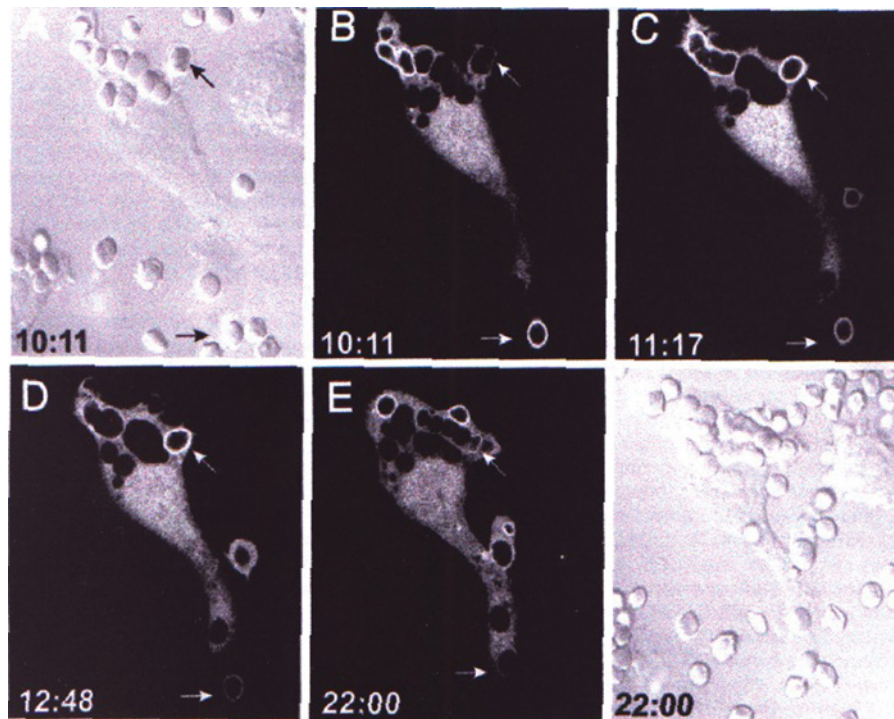
Rapid accumulation of PtdIns3P in the early phagosome following ingestion of IgG-opsonized red blood cells.
The appearance of PtdIns3P was detected using green fluorescent protein (GFP) fused to two FYVE domains that binds to this lipid. The progress of two engulfed particles (black arrows in the interference contrast image A) illustrates the time course of the PtdIns3P response. At the beginning of the fluorescence recording, the bottom particle is already brightly labelled (B), but this begins to wane 6 min later (C). The particle at the top had just been engulfed in B and shows no evidence of PtdIns3P, but this is clearly evident 6 min later, and is still present 37 min later (D). Reproduced from Vieira et al. 2002, with permission from the Biochemical Society.

Module 4: | Figure phagosome maturation
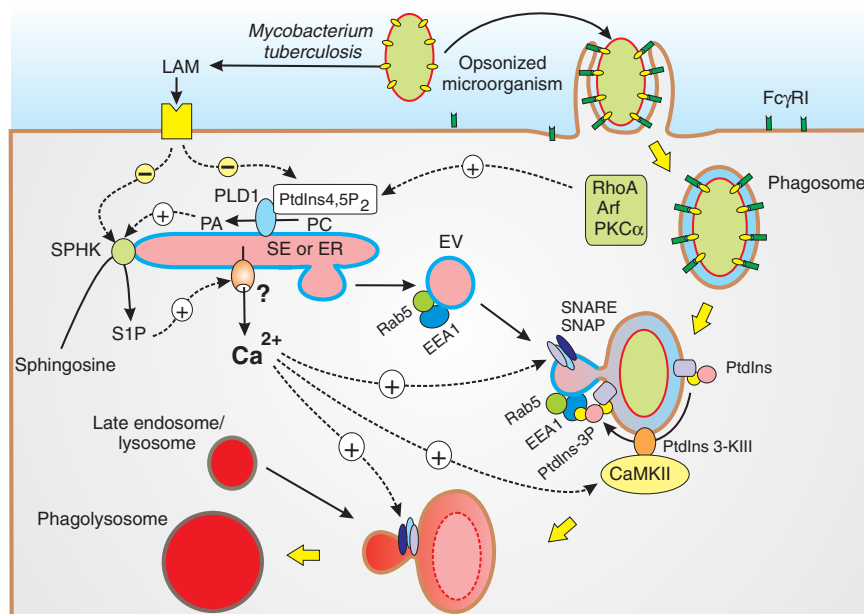
Phagosome maturation.
This working hypothesis summarizes some of the major signalling events that are thought to regulate the orderly conversion of a phagosome into a phagolysosome. See the text for further details.

Module 4: | Figure endosome vesicle fusion
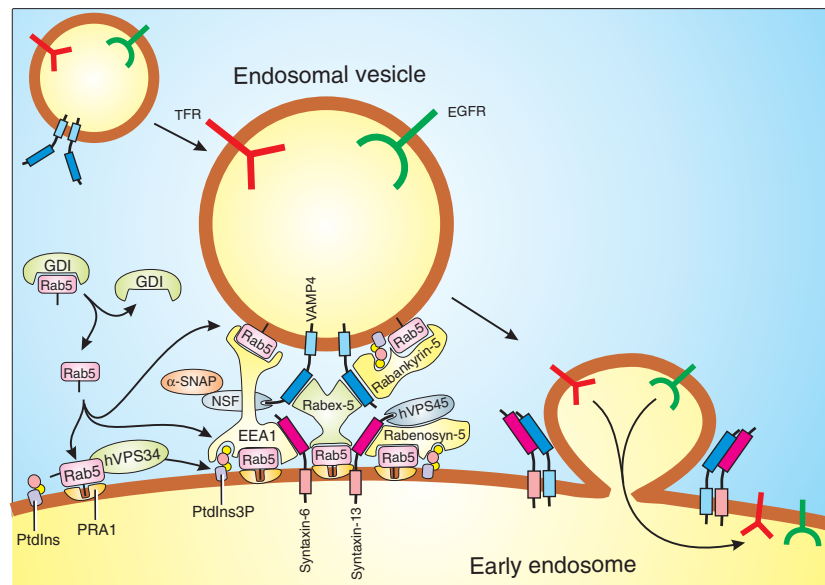
Endocytic vesicle fusion to early endosomes.
Endocytic vesicles that have budded off the plasma membrane travel to the early endosome where they fuse through a mechanism resembling the exocytosis seen at the plasma membrane. This 'exocytotic mechanism' depends on a number of components whose activity is orchestrated by Rab5 and the local formation of phosphatidyl 3-phosphate (PtdIns3P), which functions to recruit proteins that regulate the fusion process.

membrane region where an inward deformation of the membrane buds off to form the intraluminal vesicle.

Just how these complexes interact with each other remains to be worked out, and one hypothesis is that they operate as a conveyer belt to both sort the cargo and to form the bud. As for many other endosomal functions, the local formation of PtdIns3P by the Class III PtdIns 3-kinase called hVps34 plays a role in initiating the sorting process by the ESCRT-0 complex. The hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) has a FYVE domain that targets it to the PtsIns3P. The HRS then binds to the signal transducing adaptor molecule 1 (STAM1) that recognizes the ubiquitin (UB) moiety on the cytoplamic tail of EGFR that marks it out for this degradative pathway. ESCRTI and ESCRTII have proteins with ubiquitin-binding domains, such as tumour susceptibility gene 101 (TSG101) and VPS36p that feed cargo to the final step that depends on components of ESCRTIII that form the bud. The initial deformation of the membrane seems to depend on lysobisphosphatidic acid (LBPA) and the LBPA-binding protein ALIX [apoptosis-linked gene 2 (ALG-2)-interacting protein X]. Before the vesicle is formed, ubiquitin hydrolases remove the ubiquitin group that is re-used for further cycles of receptor internalization and degradation. In mammals, this deubiquitinization is carried out by deubiquitinating enzymes (DUBs) such as associated molecule with the Src homology 3 (SH3) domain of STAM (AMSH) and ubiquitin-specific protease Y (UBPY).

Once the vesicle and its cargo have been internalized, the function of the ESCRT complexes is complete and they are then dissociated through an ATP-dependent process that is driven by the ATPase Vps4p.

Early endosome to plasma membrane trafficking

The early endosome can rapidly recycle certain membrane proteins, such as the transferrin receptor (TFR), back to the plasma membrane through two pathways (Step 8 in Module 4: Figure membrane and protein trafficking). A rapid recycling pathway transports TFR back to the membrane directly, whereas in the slower pathway the TFR passes initially to the recycling endosome before being transferred to the plasma membrane. In both cases, the initial sorting of the cargo is carried out by various members of the large family of sorting nexins (SNXs). Assembly of the SNX4 complexes depend on activation of the Class III PtdIns 3-kinase called hVps34 to produce a local accumulation of PtdIns3P (Module 4: Figure early endosome budding). Once the TFR has been sorted, the cytoskeletal-associated recycling or transport (CART) complex, which consists of actinin-4, brain-expressed RING finger protein (BERP) and myosin V, directs the vesicle to the plasma membrane. The molecular motor myosin V and Rab4 are responsible for moving the vesicle along the actin filaments.

Similar processes are responsible for sorting cargo such as TFR for its transfer to the recycling endosome. As for the fast recycling mechanism described above, sorting depends on SNX4. The Lemur tyrosine kinase 2 (LMTK2) appears to have a role in controlling this sorting process.

Module 4: | Figure intraluminal vesicle formation
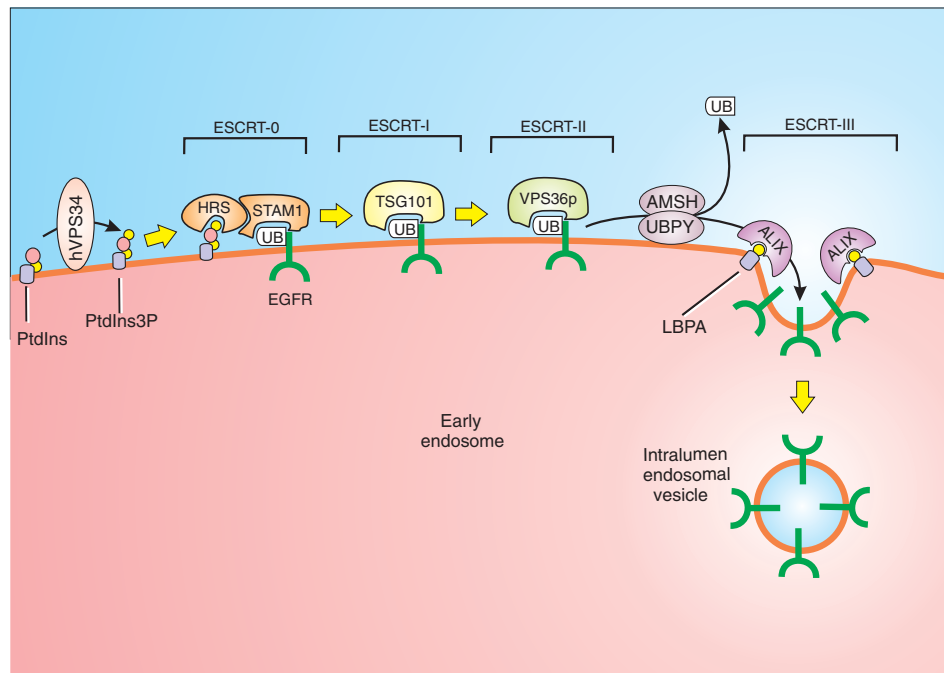
Protein sorting and formation of intraluminal vesicles in early endosomes.
Proteins destined for degradation are sorted into intraluminal endosomal vesicles. The sequence of events is orchestrated by an endosomal sorting complex required for transport (ESCRT) complexes (0–III). The main ESCRT components are illustrated on the basis of the 'conveyer belt' hypothesis that proposes that the ESCRT complexes act in sequence to sort and transport the proteins into the internal vesicle.

The vesicles that bud off from the end of the tubules are transported along actin using myosin VI and Rab4.

Early endosome to *trans*-Golgi network (TGN) trafficking

Certain proteins, such as the cation-independent mannose 6-phosphate receptor (CI-MPR) move between the *trans*-Golgi network (TGN) and the early endosome (see step 3 in Module 4: Figure membrane and protein trafficking). Once CI-MPR has released its cargo of lysosomal hydrolases to the early endosome, it is returned to the LGN through a specific trafficking pathway (Module 4: Figure endosome budding to TGN). The term retromer has been used to describe the retrieval complex that sorts cargo and orchestrates the tubulation and subsequent budding to form the vesicles that returns cargo to the TGN. Activation of the Class III PtdIns 3-kinase called hVps34 produces a local accumulation of PtdIns3P that contributes to the assembly of various members of the family of sorting nexins (SNXs). The BAR domains of SNX1 and SNX4 dimerize to form the concave structure that facilitate formation of the tubules where sorting occurs through a cargo selection complex consisting of VPS26, VPS29 and VPS35. The VPS35 binds to the C-terminal tail of CI-MPR, which has a phosphoserine motif that was placed there by the sorting retromer at the TGN in order to direct the CI-MPR towards the early endosome. The removal of this phosphate by VPS29 enables the CI-MPR to cycle back to the TGN.

The last sequence of this retrieval process is vesicle excision when the tips of the tubules bud off vesicles. The scaffolding protein Eps15p homology (EH) domain-containing protein 1 (EDH1), which has an ATPase domain, may function to pinch off the end of the tubule. The phosphofurin acid cluster sorting protein 1 (PACS1), which binds acidic clusters on cargo proteins such as CI-MRP and furin, has also been implicated in the endosome to TGN trafficking process.

Phosphofurin acid cluster sorting protein 1 (PACS1)

The phosphofurin acid cluster sorting protein 1 (PACS1) has been implicated in early endosome to *trans*-Golgi network (TGN) trafficking. It plays a role in the trafficking of both furin and mannose-6-phosphate receptor by connecting their acidic-cluster-containing cytoplasmic domains to the adaptor-protein complex-1 (AP-1).

A related phosphofurin acid cluster sorting protein 2 (PACS2) may contribute to the stability of mitochondria-associated ER membranes (MAMs).

Sorting nexins (SNXs)

The family of sorting nexins (SNXs) are characterized by having a PX domain, which enables them to bind to lipid messengers such as phosphatidylinositol 3-phosphate (PtdIns3P) during certain events that occur during membrane and protein trafficking. Some members of the family also possess BAR domains that dimerize with adjacent domains to form a concave structure that binds to the

Module 4: | Figure early endosome budding
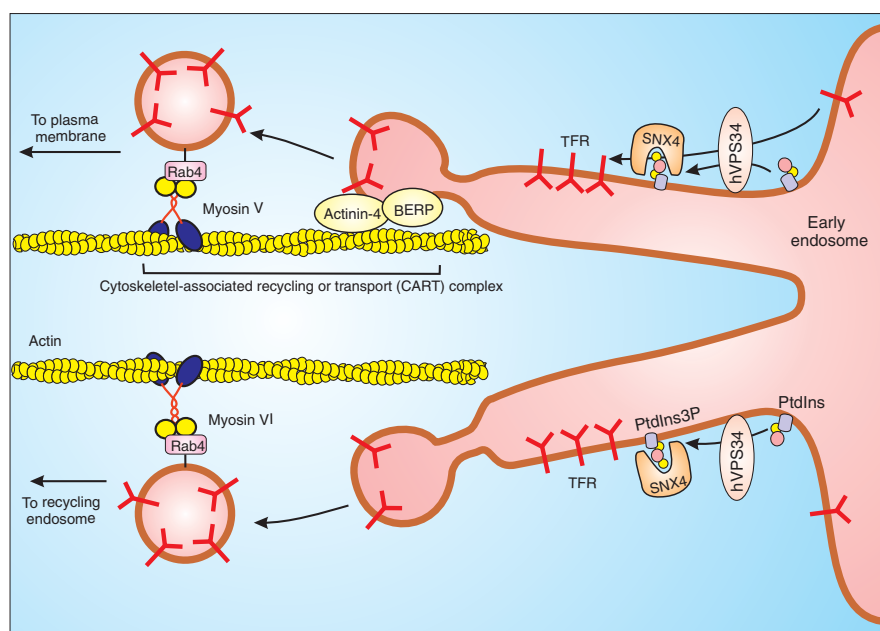
Early endosome budding.

One of the functions of the early endosome is to recycle various membrane proteins such as the transferrin receptor (TFR) back to the plasma membrane. These proteins are either returned directly to the plasma membrane or they are recycled via the recycling endosome (See Module 4: Figure membrane and protein trafficking). In both cases, the proteins are sorted at tubular extension before being budded off into vesicles that are carried away by various motor proteins.

Module 4: | Figure endosome budding to TGN
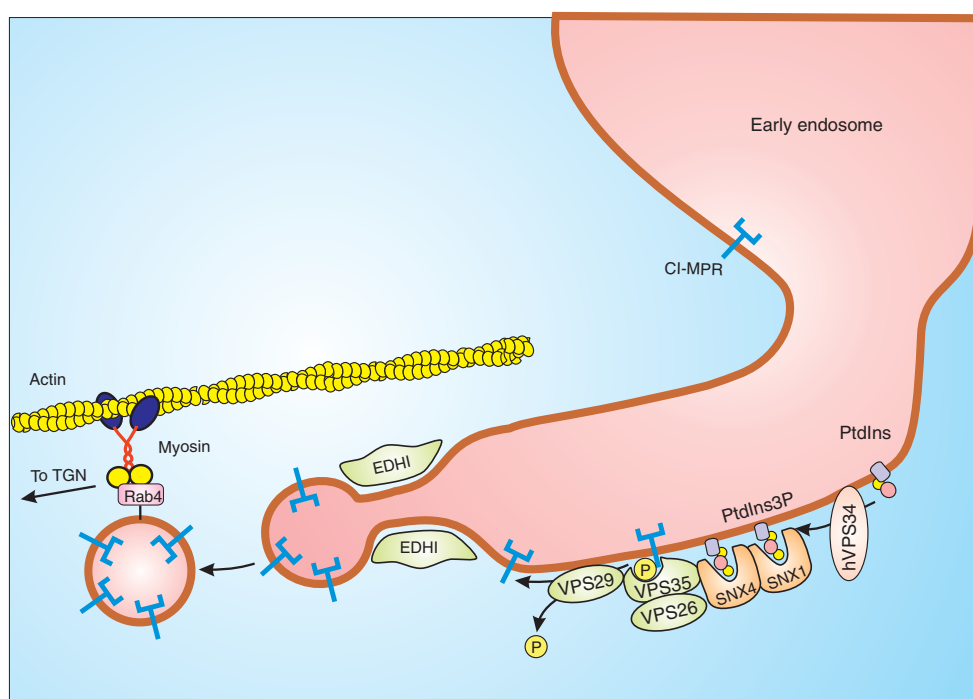
Early endosome budding and transport to TGN.

The early endosome receives vesicles from the Golgi carrying proteins such as the cation-independent mannose 6-phosphate receptor (CI-MPR), which is a carrier of various hydrolases. This CI-MPR carrier is then returned to the *trans*-Golgi network (TGN) once it has been sorted and transferred into vesicles by the mechanism illustrated in this figure.

surface of membrane tubules as occurs during early endosome to plasma membrane trafficking (Module 4: Figure early endosome budding) or during the early endosome to *trans*-Golgi network (TGN) trafficking (Module 4: Figure endosome budding to TGN).

Early endosome maturation to lysosomes

During the process of early endosome protein sorting and intraluminal vesicle formation proteins are sorted into different groups that are then sent off to different locations. Some proteins are recycled by being sent back to either the plasma membrane or to the recycling endosome (see Step 8 in Module 4: Figure membrane and protein trafficking). Others, such as the cation-independent mannose 6-phosphate receptor (CI-MPR) are sent back to the Golgi (see Step 9 in Module 4: Figure membrane and protein trafficking). Finally, proteins such as the EGFR that are destined to be degraded by lysosomes (Module 1: Figure receptor down-regulation) are isolated into intraluminal endosomal vesicle (Module 4: Figure intraluminal endosomal vesicle formation). As the internal vesicles accumulate, the early endosome gradually matures into a multivesicular endosome (MVE) and eventually end up as lysosomes (see Step 10 in Module 4: Figure membrane and protein trafficking). The PtdIns3,5P$_2$ signalling cassette (Module 2: Figure PIKfyve activation) plays an import role in this late endosome-lysosome transformation.

Sortilin 1 (SORT1)

Sortilin 1 (SORT1) is a transmembrane protein that binds a number of molecules to direct their transport within the *trans*-Golgi network (TGN). It is a type-1 receptor protein that consists of a large luminal domain, a single transmembrane domain and a short C-terminal cytoplasmic domain that resembles that on the mannose 6-phosphate receptor (CI-MPR). SORT1 can traffick proteins from the Golgi apparatus either to the plasma membrane or in the opposite direction towards the lysosomes.

It is a multifunctional receptor capable of transporting many different proteins such as low-density lipoprotein (LDL), neurotensin, progranulin (PGRN), proBDNF and proNGF. Its role in transporting LDL has implications for hypercholesterolaemia and atherosclerotic lesion formation.

SORT1 can operate independently of CI-MPR by functioning as a clearance receptor that directs proteins from the cell surface through the Golgi apparatus to the lysosomes (step 11 in Module 4: Figure membrane and protein trafficking). For example, progranulin (PGRN) may be metabolized by this SORT1-clearance pathway.

Motor proteins

The kinesin or dynein motors, which travel along microtubules, are responsible for long-range transport from the nucleus to the cell periphery and back, such as the ante- and retro-grade process of axonal transport in neurons.

Once cargo reaches the vicinity of its final destination, it is often transferred to the actin-dependent myosin motors for the final transfer to the plasma membrane.

Myosin

A large myosin superfamily is responsible for different forms of cell motility. These myosins emerged early in evolution and are widely distributed throughout the eukaryotes. Some myosins, such as myosin VIII and myosin IX are found only in plants. Most of the myosins move towards the plus-end of actin with the exception of myosin VI, which is a minus-end directed motor. The conventional myosin II family operates in muscle cells to bring about large scale cellular contractions, whereas the unconventional myosin motor proteins, which are exemplified by myosin Va, transport a great variety of cargoes (synaptic vesicles, secretory granules, melanosomes, InsP$_3$-sensitive Ca^{2+} stores and mRNA–protein complexes) along actin tracks to different intracellular destinations.

Despite their multiple functions, all of the myosins have a highly conserved N-terminal motor domain, whereas the C-terminal region is highly divergent and enables the myosin motors to interact with many different cargo proteins.

Myosin I

Myosin IE plays a role in the process of membrane invagination and scission during the endocytosis of clathrin-coated vesicles (Module 4: Figure scission of endocytic vesicles).

Myosin II

The myosin II subfamily consists of two main types: the conventional type II myosins and the non-muscle myosin II (NMII). All of the type II myosins have the same basic structure. Two myosin heavy chains form dimers that are held together by their α-helical coiled-coil N-terminal globular heads that have the ATPase catalytic region that converts the energy from ATP hydrolysis into mechanical force. There are also two myosin light chains (MLCs) located between this head domain and the long coiled-coil tail. Each myosin II dimer aggregates to form myosin filaments, with the head regions lined up precisely opposite the actin filaments. In skeletal muscle, each filament contains approximately 200 myosin molecules that lie in the middle of the sarcomere between the actin fibres (Module 7: Figure skeletal muscle structure). By contrast, the non-muscle filaments are smaller comprising approximately 12–20 myosin molecules.

The main difference between the type II myosins lies in their mode of activation. The conventional type II myosins, which function in skeletal and cardiac muscle, are controlled by Ca^{2+} acting on troponin C (TnC) as described in the process of excitation-contraction (E-C) coupling in skeletal muscle cells (Module 7: Figure skeletal muscle structure). In the case of smooth muscle type II myosins and the non-muscle type II myosins, the primary regulation is exerted by phosphorylation of the 20 kDa MLC. This phosphorylation can occur either through activation of myosin light chain kinase (MLCK) or through a smooth muscle Rho/Rho kinase signalling pathway that

inhibits a myosin phosphatase (MYPT) as illustrated by smooth muscle cell excitation-contraction coupling (Module 7: Figure smooth muscle cell E-C coupling). In the case of the non-muscle myosin II motors, phosphorylation of the MLCKs can also be carried out by a number of other kinases including citron kinase, leucine zipper interacting kinase (ZIPK) and myotonic dystrophy kinase-related CDC42-binding kinase (MRCK).

There are three non-muscle type II myosin heavy chains: NMHCIIA, NMHCIIB and NMHCIIC coded for by the *Myh9*, *Myh10* and *Myh14* genes respectively. Like the conventional myosin II, these non-muscle myosins also have two MLCs that regulate their function. These non-muscle myosins (NMIIA, NMIIB and NMIIC) differ in their kinetic properties and particularly with regard to their 'duty ratio', which is defined by the time that the myosin head remains attached to actin during the course of a typical contraction cycle. NMIIA has the shortest duty ratio in that it has the highest rate of ATP hydrolysis and thus moves over actin at the highest rate. By contrast, NMIIB has the longest duty ratio and maintains tension on the actin filaments for longer periods enabling it to contribute to the tonic contraction of smooth muscle cells. These non-muscle myosins have multiple functions in cells such as cell migration, adhesion and mitosis:

- In endothelial cells, the endothelial regulation of paracellular permeability depends on non-muscle myosin II providing the contractile force to opens up the paracellular pathway (Module 7: Figure regulation of paracellular permeability).
- Non-muscle myosin II functions in neutrophil chemotaxis during actin assembly, pseudopod formation and uropod contraction. At the front of the cell, inactive non-muscle myosin IIA (NMIIA) helps to form the cables that attach to the integrin receptors. At the back of the cell, Rho stimulates the NMIIA to contract the uropod actin network to propel the cell forward (Module 11: Figure neutrophil chemotactic signalling).
- The actin fibres attached to integrin receptors are stabilized by binding to non-muscle myosin II filaments (Module 6: Figure integrin signalling).
- The activation of contraction during the process of cytokinesis depends on phosphorylation of the myosin light chains (MLCs) of myosin II filaments (Module 9: Figure cytokinesis).
- Clot retraction, which is the final stage in blood platelet activation (step f in Module 11: Figure thrombus formation), may be driven by contraction of non-muscle myosin II filaments (Module 11: Figure platelet activation).

Myosin V
There are three myosin V genes (*MYO5A*, *MYO5B* and *MYO5C*) that are widely expressed, particularly in the nervous system. These three motor proteins have similar motor domains, but variable C-terminal domains that recognize different cargo proteins. The N-terminal region of Myosin Va (MyoVa) has the motor domain that binds to actin and is responsible for motility (see panel A in Module 4: Figure myosin motors). The motor domains

are connected to the coiled-coil regions via an α-helical 24 nm lever arm that has six IQ motifs that bind to calmodulin (CaM). These long lever arms enable MyoV to take 36 nm steps, which is approximately equal to the pseudo-repeat distance of the actin helix. This arrangement enables the motor to move straight along the actin without having to spiral around the helical actin filament. The long coiled-coil forms the region where the two molecules of MyoV are tied together to form the functional motile unit. The C-terminal region contains the globular domains that bind to different cargo proteins and it is this region that mainly defines the three Myo5 motors.

MyoV exists in two states. A compact inactive state where the two cargo-binding globular tail domains fold over to interact with the motor head domains (see panel B in Module 4: Figure myosin motors). In this inactive state, one of the motor heads can bind to actin so that the motor is positioned on the actin track waiting for the activation signal, which converts the folded molecule into its extended open state capable of transporting cargo. Just how this activation is triggered is somewhat of a mystery and there appear to be two mechanisms. First, there are indications that activation is triggered by Ca^{2+} acting on the resident calmodulin (CaM) molecules. The concentration of Ca^{2+} appears to be critical because very high levels will displace some of the CaMs resulting in the lever arms becoming too inflexible to participate in the motile cycle. Secondly, the presence of cargo binding to the C-terminal globular domains may pull the latter away from the motor domains allowing the molecule to extend out into its active state.

Myosin Va
Myosin Va (MyoVa) is a multifunctional motor protein capable of transporting a number of different cargoes. One of the functions of MyoVa is to transport melanosomes into the dendrites of melanocytes (Module 7: Figure melanogenesis). The C-terminal globular domains bind to the adaptor protein melanophilin, which is also known as synaptotagmin-like protein homologue lacking C2 domain a (Slac2-a). The melanophilin is attached through the GTPase Rab27a to the melanosome (see panel C in Module 4: Figure myosin motors). The MyoVa then transports the melanosomes down the dendrites towards the periphery where they are transferred across to the keratinocytes (see steps 8 and 9 in Module 7: Figure melanogenesis). Myo5a also functions to transport secretory granules in both chromaffin cells and insulin-secreting β cells. The interaction between this motor and the granules is carried out by Rab27a and the myosin- and Rab-interacting protein (Myrip), which is also known as synaptotagmin-like protein homologue lacking C2 domain c (Slac2-c) (see panel C in Module 4: Figure myosin motors). Both Slac2-c/MyRIP and synaptotagmin-like protein 4-a play a role together with Rab27 in the control of amylase release from rat parotid acinar cells.

During the process of early endosome to plasma membrane trafficking, myosin Va transports recycling vesicles from the early endosome to the plasma membrane (Module 4: Figure early endosome budding).

Module 4: | Figure myosin motors
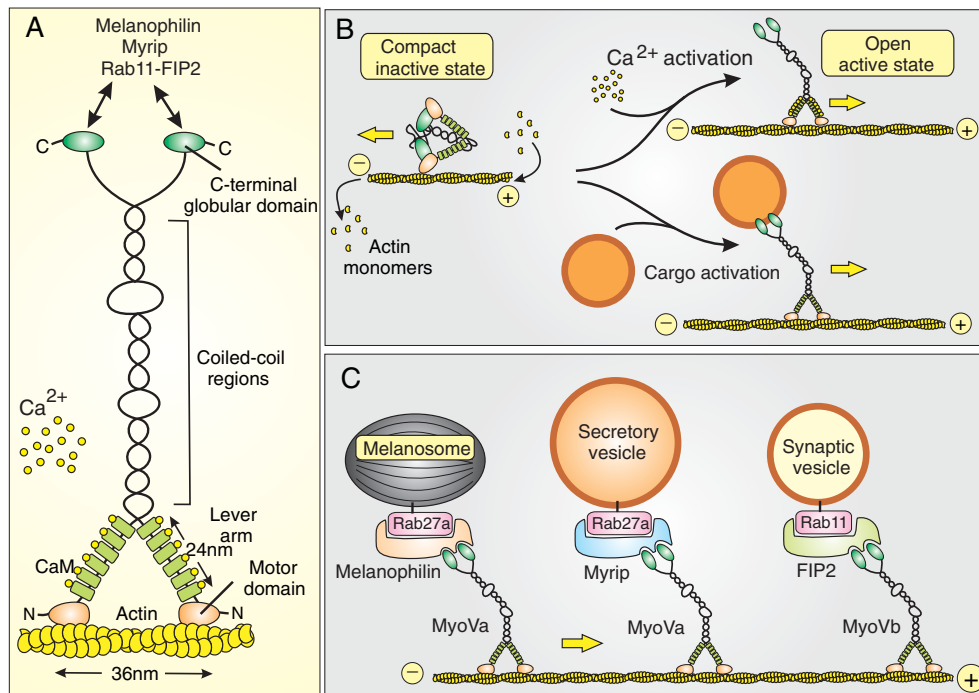
Myosin V motor structure and function.
The myosin V family exemplifies the activity of many of the unconventional myosin motors. A: myosin V functions as a dimer with the two heavy chains joined together through a coiled coil region. The N-terminal motor domains are attached to actin and the C-terminal globular domains are linked to various cargoes by different adaptors. Six calmodulins (CaMs) are attached to each of the 24 nm lever arms. B: activation of myosin V is driven either by Ca^{2+} or the binding of cargo. C. The Myosin V family members bind to different cargoes using a variety of adaptors (melanophilin, myrip and FIP$_2$) and Rab GTPases (Rab11 and Rab27a).

When the secretory and synaptic vesicles leave the actin filaments and begin to approach the plasma membrane in preparation for exocytosis, the myosin Va may contribute to the docking process by the molecular motor interacting with syntaxin-1 on the plasma membrane.

Myosin Va plays an important role in transporting endoplasmic reticulum vesicles containing InsP$_3$ receptors along the dendrites to their location in the dendritic spines. Another function of myosin Va is to transport messenger RNA ribonucleoprotein that have mRNA-binding proteins.

Mutation of the *MYO5a* has been linked to Griscelli syndrome (GS).

Myosin Vb

Myosin Vb (MyoVb) is highly enriched in neuronal synaptic spines where it functions to transport endosomes carrying AMPARs to the exocytotic sites (Module 10: Figure Ca^{2+}-induced synaptic plasticity).

The myosin Vb also plays a role in non-clathrin-dependent endocytosis that is driven by Rab8A.

Mutations in myosin Vb has been linked to microvillus inclusion disease.

Myosin VI

Myosin VI is unusual in that it moves backwards towards the minus-end of actin filaments. Just how the myosin IV moves along the actin is still debated.

Myosin VI has been implicated in a number of vesicular transport mechanisms during protein trafficking where it functions in both exocytosis and endocytosis. The C-terminal tail has binding domains enabling it to associate with various vesicle components such as the phospholipid PtdIns4,5P$_2$ and Disabled-2 (Dab2) that enables it to bind clathrin-coated vesicles. With regard to the latter, it functions in the membrane invagination and scission of clathrin-coated vesicles and then helps to transport these vesicles from the cell surface towards the early endosome (Module 4: Figure scission of endocytic vesicles).

Various other vesicle proteins can bind to myosin VI, such as glucose-transporter binding protein (GIPC) and FIP2 (also known as optineurin).

Myosin VI has a role in hair cells where it is located at the base of the stereocilia and transports components that are essential for the structural integrity of the mechano-sensitive mechanisms. In stereocilia, the myosin VI moves toward the base and this helps to maintain stability by increasing the internal tension.

Mutations in FIP2/optineurin have been linked to some forms of glaucoma and amyotrophic lateral sclerosis (ALS).

Kinesin

The superfamily of kinesin motors transports a great variety of intracellular targets along microtubules. The kinesin motor domain uses the energy of ATP to provide the force

to drag cargos through the cytoplasm. The large kinesin superfamily, which contains 45 *KIF* genes, has been divided into three separate families that are defined on the basis of the location of kinesin motor domain (Module 4: Table kinesin superfamily). The motor domain is located at the N-terminus of the 12 N-kinesins (kinesin 1–12), in the middle of the molecule for the M-kinesins (kinesin-13) and at the C-terminus in the C-kinesins (kinesin-14). Each of the 14 kinesin families has variable numbers of motors, which are referred to as KIFs.

The structure of the KIFs has three main regions: the globular motor and cargo-binding domains are connected through variable stalk regions. These linker regions often form coiled-coil segments when subunits dimerize to form either homo- or hetero-dimers (Module 4: Figure kinesin motor structure). The globular motor domains of all of the KIFs display a high degree of homology. It is this region that has the specific microtubule-binding domain and the ATP-binding domain. Most variability is found in the cargo-binding domain responsible for interacting with the different cargos that are transported through the cell. Considering this variability, it is difficult to generalize and each kinesin has to be considered separately.

Kinesin uses its two heads to progress along the surface of the microtubule with the two heads alternating with each other as they bind and then detach from the tubulin dimers (Module 4: Figure kinesin and dynein motor mechanisms). The motor moves in steps of approximately 8 nm as the heads take turns to move over the tubulin surface. ATP is used to drive each attachment/detachment cycle.

The ability of these motors to transport cargo around the cell is controlled by a number of kinesin motor regulatory mechanisms.

Kinesin-1

Kinesin-1 is a typical N-kinesin and has three family members (KIF5A, KIF5B and KIF5C) (Module 4: Table kinesin superfamily). The KIF5 motors, which function as dimers, have the typical N-terminal globular motor domain that is connected via a stalk to the C-terminal cargo-binding domain (Module 4: Figure kinesin motor structure). The cargo-binding domain associates with two kinesin light chains (KLCs) to form a fan-like structure responsible for binding a number of different cargos that attach to either the KLCs or the cargo-binding domains, often using specific scaffolding proteins. The processivity of kinesin-1 is enhanced by the acetylation of the α-tubulin subunits.

The KIF5 motors are particularly active in carrying different cargos down neuronal axons and dendrites (Module 4: Figure kinesin cargo transport in neurons). For example, the cargo-binding domain uses glutamate receptor-interacting protein 1 (GRIP1) to bind to AMPARs that are transported down the dendrites. The cargo-binding domain of KIF5 also functions to transport a large oligomeric complex of proteins and mRNAs into the dendrites. This ribonucleoprotein (RNP) particle has approximately 40 proteins, such as fragile X syndrome protein 1 (FXRP1), mStaufen and cofactors for mRNP localization in dendrites such as PURα and PURβ. The FMRP1 links the particle to KIF5. The mRNAs that are attached to

Module 4: | Table kinesin superfamily
The superfamily of kinesin motors.

Kinesin superfamily	
N-terminal kinesins	The N-kinesins superfamily (39
(N-kinesins)	*KIF* genes) have the globular
	motor domain located at the
	N-terminus and usually
	transport cargo towards the
	plus end of microtubules
	(Module 4: Figure kinesin
	cargo transport in neurons)
Kinesin-1	KIF5 plays a major role in
	transporting cargo in neurons
	(Module 4: Figure kinesin
	cargo transport in neurons)
KIF5A	
KIF5B	
KIF5C	
Kinesin-2	
KIF3A	
KIF3B	
KIF3C	
KIF17	
Kinesin-3	
KIF1A	
KIF1Bα, KIF1Bβ	
KIF1C	
KIF13A	
KIF13B	
Kinesin-4	
KIF4A	
KIF4B	
KIF21A	
KIF21B	
Kinesin-5	
KIF11	
Kinesin-6	
KIF20	
KIF23	
Kinesin-7	
KIF10	
Kinesin-8	
KIF18	
KIF19	
Kinesin-9	
Kinesin-10	
KIF22	
Kinesin-11	
Kinesin-12	
KIF12	
KIF15	
Middle kinesins (M-kinesins)	The M-kinesins superfamily (3
	KIF genes) usually transport
	cargo towards the plus end
	of microtubules
Kinesin-13	
KIF2A	
KIF2B	
KIF2C	
C-terminal kinesins	
(C-kinesins)	
Kinesin-14	
KIFC1	
KIFC2	
KIFC3	

this complex code for proteins, such as activity-regulated cytoskeletal-associated protein (ARC) and the α-subunit of CAMKII that function within the postsynaptic spines (Module 10: Figure Ca^{2+}-dependent synaptic plasticity). On the other hand, the KLCs use the JIPs to interact with the β-amyloid precursor protein (APP) and apolipoprotein E receptor 2 (APOER2) that is moved down the axons.

Module 4: | Figure kinesin motor structure
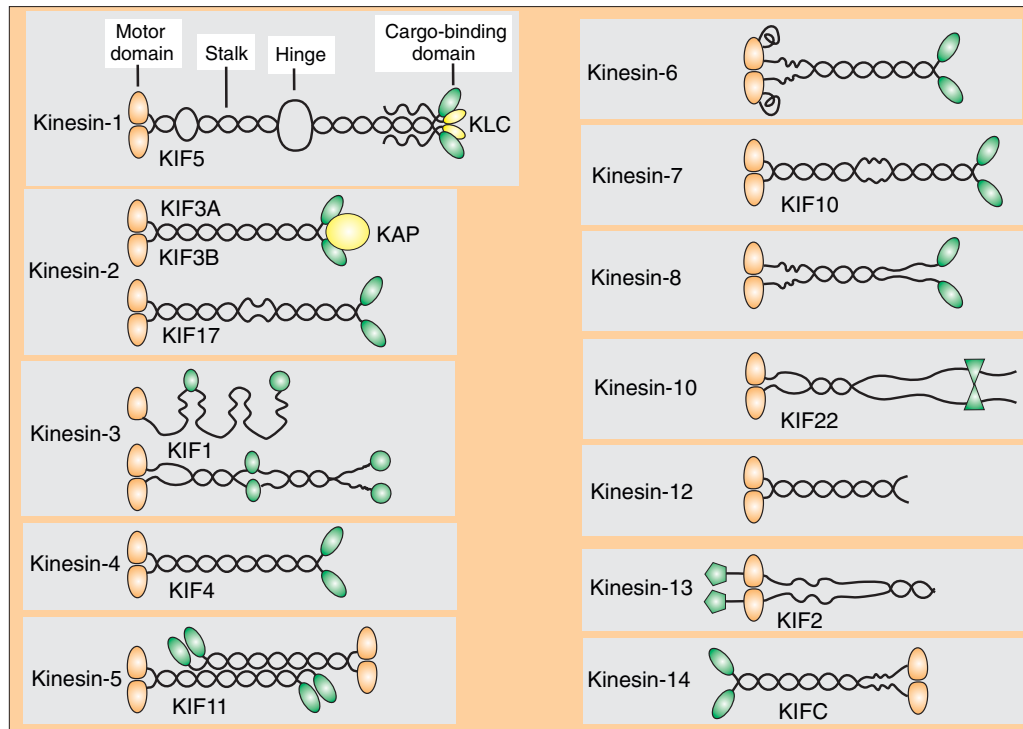
Structure of kinesin motors.

The kinesin superfamily of motor proteins, which has 45 *KIF* genes, has been divided into 14 families. The structure of some of the KIFs illustrates their three main regions: globular motor domain (orange), cargo-binding domain (green) and a stalk region (black). Many of the motors form dimers through coiled-coil segments of these stalk regions. Some of these stalks are interrupted by hinge regions that enables the cargo-binding domain to bend around to interact with the motor domain to set up an autoinhibitory interaction in the absence of any cargo. See the text for further details. Information for this Figure was taken from Box 1 in Verhey and Hammond (2009).

Kinesin-2

Kinesin-2 is an N-kinesin, which has four family members (KIF3A–C and KIF17) (Module 4: Table kinesin superfamily). KIF3A can form heterodimers with KIF3B and KIF3C, which then form heterotrimeric complexes when their N-terminal region interacts with kinesin superfamily-associated protein 3 (KAP3) (Module 4: Figure kinesin motor structure). One of the functions of this KIF3A/KIF3B/KAP3 complex is to transport large vesicles (90–150 nM) that are associated with fodrin (Module 4: Figure kinesin cargo transport in neurons). KIF3A also transports the partitioning protein 3 (PAR3), which is part of the polarity complex (PAR3/PAR6/atypical PKC) that helps to establish which neurites will grow into axons.

The KIF17 motor transports vesicles containing NM-DARs down dendritic microtubules at a rate of approximately 0.75 µm/s towards the postsynaptic spines (Module 4: Figure kinesin cargo transport in neurons). The attachment between the KIF17 motor domain and the NR2B subunit of the NMDAR is carried out by a trimeric scaffolding complex containing the Munc18-interacting protein (MINT1), calcium/calmodulin-dependent serine protein kinase (CASK) (LIN-2) and the vertebrate LIN-7 homologue (VELIS), which is also known as mammalian LIN-7 protein (MALS). These proteins have PDZ domains that enable Velis/MALS to bind to the NR2B subunit and MINT1 to attach the whole complex to KIF17.

Kinesin-2 transports vesicles during COPI-mediated transport from Golgi to ER (Module 4: Figure COPI–coated vesicle).

Fodrin

Fodrin, which is also known as non-erythroid spectrin, consists of α- and β-subunits that bind together to form filaments that bind to actin at both ends to create a network just beneath the plasma membrane. The α-fodrin is cleaved by caspases during apoptosis.

In adipocytes, fodrin might play a role in the translocation and fusion of the GLUT4 storage vesicles (GSVs). The GSVs have VAMP2 that interacts with syntaxin 4 to trigger membrane fusion. The cortical fodrin–actin network may play a role in moving GSVs to the plasma membrane.

Kinesin-3

Kinesin-3 is a N-kinesin that has a number of family members (KIF1A, KIF1Bα and KIF1Bβ, KIF1C, KIF13A and KIF13B) (Module 4: Table kinesin superfamily). These kinesin-3 motors can function either as monomers, as for KIF1A and the two alternatively spliced KIF1Bα and KIF1Bβ, or as homodimers (Module 4: Figure kinesin motor structure).

In neurons, KIF1A and KIF1Bβ transport organelles containing synaptic vesicle precursors (Module 4: Figure kinesin cargo transport in neurons), such as

synaptotagmin, synaptophysin and Rab3A. On the other hand, the KIF1Bα isoform can transport mitochondria down axons.

A point mutation in the ATP-binding site of the motor domain of KIF1Bβ has been linked to a hereditary peripheral neuropathy called Charcot-Marie-Tooth disease (CMT) type 2A.

KIF13A is used to transport vesicles containing the mannose 6-phosphate receptor (M6PR), which recognizes the sorting signal located on adaptor protein 1 (AP-1). AP-1 functions as an adaptor/scaffold that binds to both clathrin and to the motor protein KIF13A to transport coated vesicles from the *trans*-Golgi network to the early endosome (see step 3 in Module 4: Figure membrane and protein trafficking). AP-1 consists of four adaptin subunits (β1, γ, μ1 and δ1) and it is the β1-adaptin subunit that binds to the KIF13A subunit.

Kinesin-4
Kinesin-4 is an N-kinesin that has a number of family members (KIF4A, KIF4B, KIF21A and KIF21B). The two KIF4 members carry the cargo poly(ADP-ribose) polymerase 1 (PARP1). CaMKII acts to release PARP1 from KIF4 and the PARP1 then enters the nucleus to control transcription and DNA repair.

Kinesin-5
Kinesin-5 is a typical N-kinesin and has a single member KIF11 (Module 4: Table kinesin superfamily). KIF11 forms homodimers that then interact with each other to form homotetramers that line up with their globular domains facing in opposite directions (Module 4: Figure kinesin motor structure). KIF11 is activated by phosphorylation of its C-terminal cargo-binding domain by cyclin-dependent kinase 1 (CDK1).

Kinesin-6
The kinesin-6 family of motor proteins has two members: KIF20A [also known as Rab6 kinesin or mitotic kinesin-like protein 2 (MKLP2)] and KIF23 [also known as mitotic kinesin-like protein 1 (MKLP1)].

Kinesin-7
Kinesin-7 is a typical N-kinesin and has a single member KIF10 (Module 4: Table kinesin superfamily), which is also known as centromere-associated protein E (CENPE). Like some other kinesins, KIF10 is autoinhibited when the C-terminal cargo-binding domain bends over to interact with the motor domain. This inactivation is reversed following phosphorylation of the cargo-binding domain by various kinases such as CDK1/cyclin B or by monopolar spindle protein 1 (MPS1). The latter is located on the kinetochore where KIF10 functions during mitosis.

Kinesin-8
Kinesin-8, which is a typical N-kinesin that has two members KIF18 and KIF19 (Module 4: Table kinesin superfamily), has a unique ability to both walk along and depolymerize microtubules.

Kinesin-13
Kinesin-13 is an M-kinesin, which has three members KIF2A, KIF2B and KIF2C (Module 4: Table kinesin superfamily), and is unusual because the motor domain is located in the middle of the molecule (Module 4: Figure kinesin motor structure). While it is not capable of directed movement, it is capable of destabilizing microtubules. Kinesin-13 is strongly expressed in the developing brain where it has an important role in controlling microtubule dynamics within the growth cones.
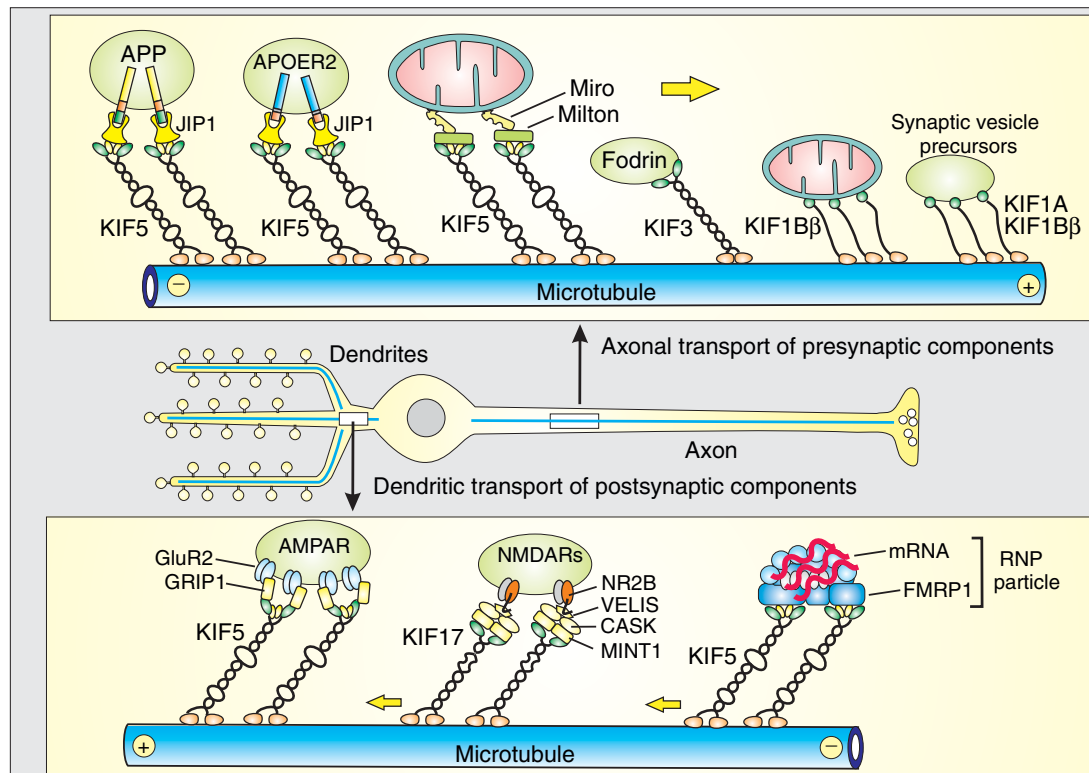
Kinesin-14
Kinesin-14 is a C-kinesin, which has three members KIFC1, KIFC2 and KIFC3 (Module 4: Table kinesin superfamily), and is unusual because the motor domain is located at the C-terminus (Module 4: Figure kinesin motor structure). It has the ability of moving along and destabilizing microtubules.

Kinesin motor regulation
The regulation of kinesin motor activity depends on a number of mechanisms that can operate during the course of a typical transport cycle. This cycle has three main processes: it begins with the selection of cargo and attachment to the microtubule, processivity (co-ordinated movement along the microtubule) and ends with motor detachment.

The selection of cargo and attachment to the microtubule is an important site of control and varies somewhat between the different motors. Many of the motors, such as kinesin-1, kinesin-2 (KIF17) and kinesin-7 (KIF10), exist in an inactive folded state where the C-terminal cargo-binding domain bends over to interact with the motor domain thereby blocking the nucleotide pocket and thus inhibits the binding and hydrolysis of the ATP required for movement. Various mechanisms are used to relieve this autoinhibitory state. In the case of kinesin-1, autoinhibition is relieved by binding two proteins: fasciculation and elongation protein-ζ1 (FEZ1, also known as zygin1), which binds to the C-terminal cargo-binding domain and Jun N-terminal kinase (JNK)-interacting protein 1 (JIP1), which attaches to the kinesin light chains (KLCs) (Module 4: Figure kinesin cargo transport in neurons). The disrupted in schizophrenia 1 (DISC1) protein is also known to interact with FEZ1. Another way of activating the motors is through phosphorylation as occurs for kinesin-5 and kinesin-7. Following phosphorylation, these motor proteins unfold to a more extended state thus enabling them to interact and move along the microtubules.

Once motors attach themselves to the microtubule their rate of movement tends to depend on the state of the microtubules that can be modified in different ways usually in the form of a post-translational modification that not only provide a mechanism for regulating the rate of movement, but may also mark out microtubules to direct kinesins to carry cargo to specific cellular destinations. For example, acetylation of the α-tubulin subunits can enhance the processivity of kinesin-1, whereas detyrosination of the same subunits has the effect of directing kinesin-1 to transport cargo to specific destinations. Polyglutamylation of tubulin subunits seems to enhance the ability of KIF1 to

Module 4: | Figure kinesin cargo transport in neurons

Transport of cargo by kinesin motors in neurons.
In the case of the KIF5 motors, the C-terminal cargo-binding domain (green) carries cargo (AMPARs) down the dendrites whereas cargo that is attached to the KLCs (yellow), such as the amyloid precursor protein (APP) and apolipoprotein E receptor 2 (APOER2) moves down the axons. This Figure is based on the information shown in Figure 3 in Hirokawa and Takemura (2005).

transport synaptic vesicle precursors (Module 4: Figure kinesin cargo transport in neurons).

Finally, there are a number of mechanisms that terminate processivity by enhancing the detachment of the kinesin motors. One is a physical mechanism whereby the various microtubule-associated proteins (MAPs), such as tau, may act to block motors such as kinesin-1. Displacement of the motors may also be controlled by phosphorylation. The JIP1 scaffolding protein, which attaches cargo to KIF5 (Module 4: Figure kinesin cargo transport in neurons), is also an activator of the MAP kinase signalling pathway that is known to dissociate JIP1 from its motor thus terminating transport. A similar action of the MAP kinase signalling may disrupt cargo transport by the kinesin-2 motor proteins that operate in cilia and flagella.

The Ca^{2+} signalling system may also play a role in regulating the interaction between cargo and kinesin motors. For example, phosphorylation of the C-terminal cargo-binding domain region of the Kinesin-2 family member KIF17 releases the NMDAR cargo vesicles that are carried down the microtubules to the dendrites. In this way, local elevations of Ca^{2+} in the presynaptic region will serve to recruit NMDARs as part of the process of synaptic remodelling responsible for learning and memory. The Ca^{2+}-sensitive mitochondrial Rho-GTPase (MIRO) responds to local elevations of Ca^{2+} by releasing mitochondria (Module 5: Figure mitochondrial motility).
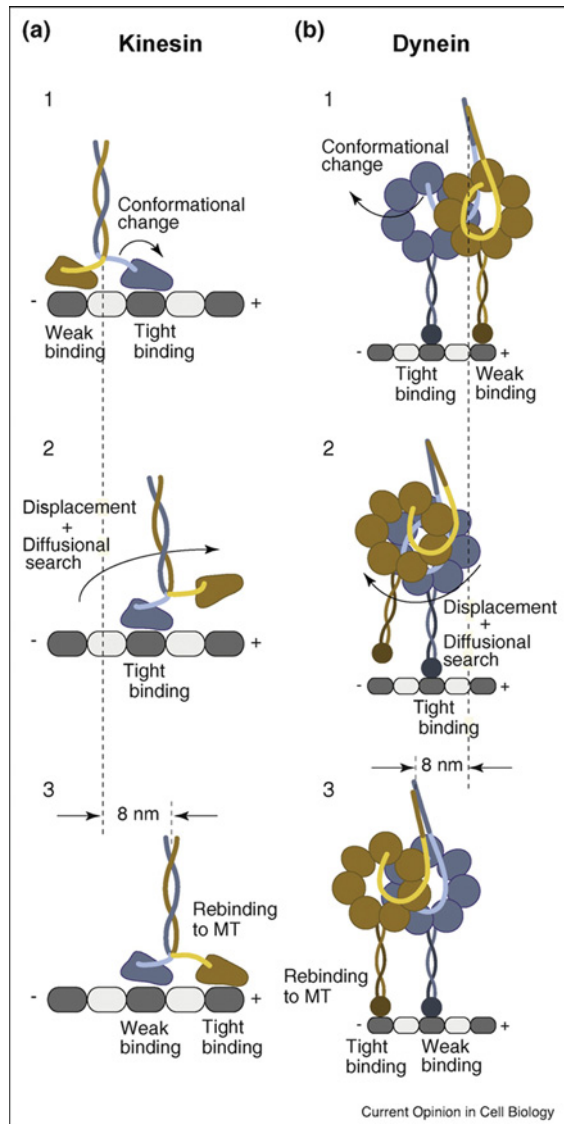
Dynein

Dynein is a motor protein that carries cargo along microtubules in the minus-end direction. It has important functions during membrane and protein trafficking as it moves cargo from the cell periphery towards the cell centre. This is particularly evident in neurons where dynein transports various components from the synapses back to the cell body. Dynein also moves proteins down the axoneme of cilia. There are a large number of dynein heavy chains, but only two of these function as motors to transport materials around the cell. Intraflagellar transport (IFT) dynein transports cargo down the axoneme whereas cytoplasmic dynein transports cargo such as vesicles, proteins, mRNA and also functions during mitosis. The large dynein complex can be divided into separate functional components (Module 4: Figure dynein):

- Dynein catalytic motor protein has the catalytic component that converts the energy of ATP to move dynein
- Dynein non-catalytic subunits link the dynein motor to various adaptors
- Dynein adaptors link dynein to various cargos

Dynein catalytic motor protein
The dynein catalytic motor protein consists of a single protein, a large catalytic heavy chain that has three main regions: a motor domain, linker domain and an N-terminal

Module 4: | Figure kinesin and dynein motor mechanisms

Motile mechanisms of kinesin and dynein motors
The proposed motile mechanism for kinesin and dynein motors. A. For kinesin, one head is always firmly attached whereas the othe head detaches and then moves forward to attach to tubulin 8 nM away. Once it is bound the trailing head can detach to repeat the sequence. Reprinted from Curr. Opin. Cell Biol., 21, Gennerich, A. and Vale, R.D., Walking the walk: how kinesin and dynein coordinate their steps, 59–67, © 2009, with permission from Elsevier.

tail where the non-catalytic subunits are bound (Module 4: Figure dynein). The C-terminal motor domain has two main regions: the ATP-binding modules (1–6) and the microtubule-binding domain located at the end of a short stalk. These heavy chains belong to the superfamily of ATPase associated with various cellular activities (AAA + ATPase), but is somewhat unusual in that its six AAA domains are all part of the same polypeptide chain. These six AAA domains form a catalytic ring responsible for binding and hydrolysing ATP. Hydrolysis of ATP by AAA1 and AAA3 are mainly responsible for providing the energy to drive the motor along the tubulin subunits of the microtubule. A long loop, which connects AAA4 and AAA5, forms a stalk that has the microtubule-binding domain that attaches the heavy chain to the tubulin subunits. The chain that emerges from AAA1 forms the linker domain that connects to the coiled-coil region where the N-terminal tails of the two heavy chains dimerize with each other. The dynein non-catalytic subunits are bound to this N-terminal tail.

### Dynein non-catalytic motor subunits

A number of subunits are associated with the coiled-coil region of the N-terminal tail (see the green box in Module 4: Figure dynein). The light intermediate chain (LIC) and the dynein intermediate chain (IC) bind directly to the heavy chain, whereas the smaller dynein light chain 7 (LC7), dynein light chain 8 (LC8) and T-complex testis-specific protein 1 (TCTEX1) are all attached to IC. These subunits function by interacting with various dynein adaptor proteins, as described below.

Dynein adaptors

There are a number of adaptors (see pink boxes in Module 4: Figure dynein). The most important adaptor is dynein activator (dynactin), which is made up of 11 subunits. The interaction between dynein intermediate chain (IC) and dynactin is particularly important in controlling the motor function of dynein. A major component of dynactin is the p150 subunit that links to the dynein catalytic motor protein complex through the IC. At its other end, p150 has a cytoskeleton-associated protein glycine-rich (CAP-Gly) domain that can bind to tubulin. The interaction between tubulin and p150 is facilitated by the latter interacting with two plus-end-binding proteins called end-binding 1 (EB1) and CAP-Gly domain-containing linker protein 170 (CLIP170). A short filament made up from actin-related protein 1 (ARP1) plays an important role in binding to some cargos especially those that are membrane-bound. This ARP1 filament of dynactin binds to the filamentous protein βIII spectrin that is often found on the surface of many membranes including those of the Golgi. At the pointed end of the ARP1 filament, there is a complex of proteins consisting of actin-related protein 11 (ARP11), p62, p25 and p27. The barbed end of the ARP1 filament has a number of proteins. There is an actin-capping protein, a p24 subunit and a p50 tetramer, which is also known as dynamitin. The p50 interacts with other dynein adaptors, such as Bicaudal D1 and Rod-ZW10-Zwilch (RZZ). Bicaudal D was originally discovered in *Drosophila* where it functions to transport mRNA during pattern formation in early development. The two mammalian homologues (Bicaudal D1 and Bicaudal D2) belong to the golgin family and appear to function in vesicle transport between the Golgi and the ER. The GTPase Rab6 binds to Bicaudal D and thus provides a mechanism to attach the dynein motor complex to the vesicle for the COPII-mediated transport from ER to Golgi (Module 4: Figure COPII-coated vesicles). Bicaudal D1 may also interact with LC8.

In addition to the large dynactin complex, there are some other adaptors that can attach to the dynein complex. Lissencephaly (LIS1), which interacts with either nuclear distribution protein (NUDE) or the closely related

Module 4: | Figure dynein
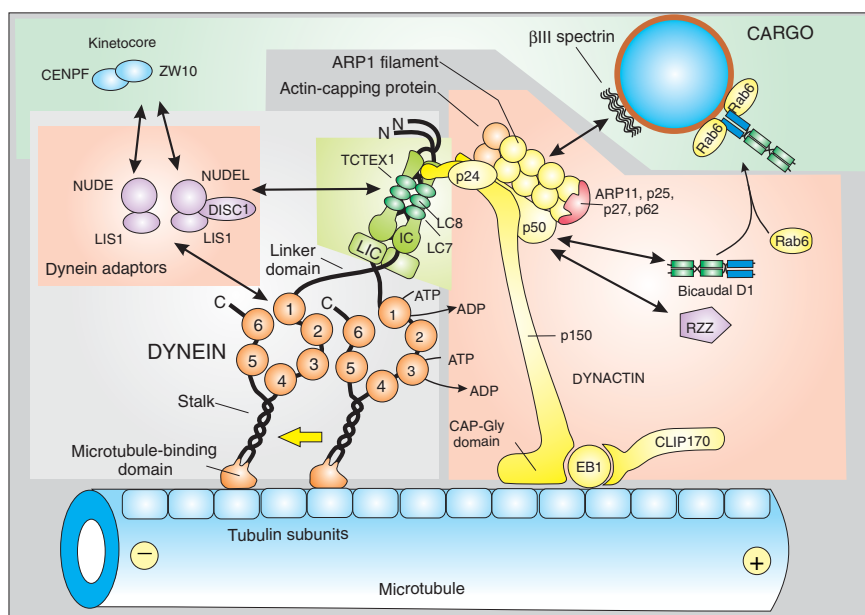
Structure and function of dynein.
The dynein motor complex consists of the large catalytic dynein heavy chain (thick black line) that has six ATP-binding modules (1–6), a microtubule-binding domain located at the end of a short stalk and an N-terminal region that forms a coiled-coil with a neighbouring chain. Attached to this N-terminal region of the heavy chain, there are a number of dynein non-catalytic subunits (shown in green). A number of dynein adaptors (shown in the pink boxes) provide links to various cargoes. The main adaptor is the dynactin complex (shown in yellow). See text for further details.

NUDE-like (NUDEL), is attached to the kinetochore by interacting with centromere protein F (CENPF) and ZW10 (Module 4: Figure dynein). LIS1 has the unique ability to bind to the AAA1 domain and this may function to regulate ATPase activity and hence motor activity. The centrosomal proteins NUDE and NUDEL also interact with disrupted in schizophrenia 1 (DISC1) as part of a complex that functions in mitosis, neuronal migration and microtubule organization during brain development.

The human disease lissencephaly may be caused by mutations in the gene that encodes lissencephaly 1 (LIS1).

Disrupted in schizophrenia 1 (DISC1)
The gene for disrupted in schizophrenia 1 (DISC1) confers an increased risk for various mental illnesses, including bipolar disorder and schizophrenia. DISC1 is one of the most highly associated susceptibility genes for schizophrenia. A chromosomal translocation between chromosome 11 and chromosome 1 (where DISC1 is located) results in the truncation of DISC1 and the subsequent loss of function seems to be responsible for disrupting both brain structure and function. These widespread changes can be explained by the fact that DISC1 has many binding partners responsible for carrying out multiple physiological processes. Some of these processes are shown in Module 12: Figure schizophrenia:

- DISC1 is part of the NUDEL/LIS1 complex that functions as one of the dynein adaptors for the dynein motor protein (Module 4: Figure dynein) to carry out processes such as mitosis, neuronal migration, neurite formation and axon elongation. With regard to the

last process, DISC1 may function by binding to the actin-binding protein fasciculation and elongation protein zeta-1 (FEZ-1), which seems to function in kinesin motor regulation.

- The hydrolysis of cyclic AMP by PDE4B is inhibited by DISC1. The antidepressant Rolipram inhibits PDE4B.
- DISC1 may also function to regulate gene transcription through different mechanisms. It can interact with transcription factors such as ATF4 and ATF5. DISC1 can also interact with glycogen synthase kinase 3β (GSK3β), which is part of the PtdIns 3-kinase signalling pathway (Module 2: Figure PtdIns 3-kinase signalling). Modification of GSK3β activity will then influence the operation of the canonical Wnt/β-catenin pathway that controls gene transcription through activation of β-Catenin (Module 2: Figure Wnt canonical pathway).

Gene transcription
A major function of many signalling pathways is to activate gene transcription. This regulation of gene expression operates throughout the life history of a typical cell. Developmentally regulated transcription factors begin to function early in development to define developmental axes, and they contribute to cellular differentiation to form specialized cells. An important component of differentiation is signalsome expression, during which each cell type expresses the specific signalling components that are necessary to meet its functional requirements (Module 8: Figure signalsome expression). Once cells have differentiated, transcription plays an important role in maintaining both

the stability of the differentiated state and its signalling pathways. An important aspect of this phenotypic stability is the way in which the signalling and transcriptional systems co-operate to create a quality assessment system that ensures signalsome stability.

All of these processes require making transcripts of individual genes, which is carried out by RNA polymerase II (pol II). Gene regulation depends upon careful control of polymerase II by a host of transcription factors and transcriptional co-regulators. The way in which transcription factors communicate with pol II is carried out by a mediator complex, which can exert a significant role in controlling whether or not specific genes are transcribed. While most attention will focus on the transcription factors, it is clear that co-regulators such as the co-activators and co-repressors also play a significant role by recruiting chromatin remodelling enzymes such as histone acetyltransferases (HATs), histone deacetylases (HDACs) and protein methylases to form the large macromolecular signalling complexes called transcriptosomes that regulate transcription. There also is an important relationship between ubiquitin signalling and gene transcription that operates at many different levels.

Most attention will be focused on the transcription factor activation mechanisms used by signalling pathways to control gene expression.

Transcription factors

There is a bewildering variety of transcription factors that can either function as activators or repressors of gene transcription. These activators and repressors do not function in isolation, but are usually part of a multi-protein transcriptosome made up of transcriptional co-regulators and associated factors.

A transcription factor classification has been introduced to provide a framework for understanding the diverse functions of the following transcription factors:

- Activating protein 1 (AP-1) (Fos/Jun)
- Activating transcription factor 6 (ATF6)
- APP intracellular domain (AICD)
- β-Catenin
- BCL6
- CCAAT/enhancer-binding protein (C/EBP)
- CSL (CBF-1, Suppressor of Hairless, Lag-1)
- Cyclic AMP response element-binding protein (CREB)
- Downstream regulatory element antagonistic modulator (DREAM)
- E2F family of transcription factors
- E twenty-six (ETS)
- Forkhead box O (FOXO)
- Glucocosteroid receptor (GR)
- Hepatocyte nuclear factor 4α (HNF4α)
- Hypoxia-inducible factor (HIF)
- Interferon-regulatory factors (IFNs)
- Krüppel-like factors (KLFs)
- LBP1 family of transcription factors
- c-Maf
- Methyl-CpG-binding protein 2 (MeCP2)
- Microphthalmia transcription factor (MITF)

- Mitochondrial transcription factor (Tfam)
- Myocyte enhancer factor-2 (MEF2)
- Myc
- MyoD
- Nuclear factor of activated T cells (NFAT)
- Nuclear factor κB (NF-κB)
- Nuclear factor erythroid 2 related factor 2 (NRF-2)
- Nuclear transcription factor Y (NF-Y)
- Nuclear response factor-1 (NRF-1)
- p53
- Parkin-interacting substrate (PARIS)
- Peroxisome-proliferator-activated receptors (PPARs)
- Peroxisome-proliferator-activated receptor γ (PPARγ) coactivator-1α (PGC-1α)
- Pituitary-specific transcription factor (Pit-1)
- Single-minded 1(Sim1)
- Smads
- Specificity protein 1 (Sp1)
- Sterol regulatory element-binding proteins (SREBPs)
- Signal transducers and activators of transcription (STATs)
- Serum response factor (SRF)
- Transcription initiation factor IB (TIF-IB)
- Vitamin D receptor (VDR)
- X-Box binding protein 1 (XBP-1)

Transcriptional co-regulators

The action of transcription factors often depends on co-regulators such as the transcriptional co-activators and transcriptional co-repressors. These additional components facilitate the activity of the transcriptional activators and repressors in different ways. In many cases, they function by recruiting chromatin remodelling proteins that alter the way transcription factors access gene promoter sites.

Transcriptional co-activators

Co-activators, which function to enhance gene expression, usually act by binding to transcriptional activators. Many of the coactivators function by regulating the transcription of genes that control cellular differentiation, migration, and proliferation. Some of these coactivators are histone acetyltransferases (HATs) that add acyl groups to the lysine groups on histones resulting in an increase in the accessibility of DNA to transcription factors. The following are examples of such co-activators:

- CREB binding protein (CBP)
- Myocardin family
- p300
- Peroxisome-proliferator-activated receptor γ (PPARγ) coactivator-1 (PGC-1)
- p300/CBP association factor (PCAF)
- p53-binding protein 1 (53BP1) is a p53 co-activator whose binding is facilitated by p53 methylation on K370me2.
- Tat interactive protein 60 (TIP60)

CREB binding protein (CBP)
CREB binding protein (CBP) and the closely related protein p300 are histone acetyltransferase (HAT) paralogues that arose through gene duplication. Since they share a similar structure and function, they are often referred to as p300/CBP. Details of how these two proteins function is described in the section on p300.

Mutations in CBP are responsible for Rubinstein-Taybi syndrome (RTS).

Myocardin family
The myocardin family consists of closely related transcriptional coactivators such as myocardin itself, which is located mainly in the nucleus, myocardin-related transcription factor-A (MRTF-A) and myocardin-related transcription factor-B (MRTF-B). These three coactivators have a similar structure consisting of an N-terminal RPEL domain, which enables MRTF-A and MRTF-B to bind to actin. The RPEL domain of myocardin cannot bind to actin. In the middle of these myocardin family molecules there is a basic (+), a glutamine-rich (Q), a SAP (SAF-A/B, Acinus, and PIAS) and an LZ domain with a transcriptional activation domain (TAD) domain at the C-terminal region. The association between SRF and these myocardin family members is mediated by the basic (+) and glutamine-rich (Q) regions.

Myocardin
Myocardin is a potent coactivator that binds to serum response factor (SRF) that controls the expression of cytoskeletal and contractile proteins. It is located mainly in the nucleus and acts to control cardiac development (Module 8: Figure cardiac development) and smooth muscle cells (Module 8: Figure smooth muscle cell differentiation).

Myocardin-related transcription factor-A (MRTF-A)
Myocardin-related transcription factor-A (MRTF-A), which is also known as MAL, MKL-1 and BSAC, is strongly expressed in mesenchymal, epithelial and muscle cells during embryogenesis. The MRTF-A plays an important role in mediating the link between actin dynamics and gene transcription where it acts as a transcriptional coactivator of the serum response factor (SRF) (Module 4: Figure actin dynamics and gene transcription).

Myocardin-related transcription factor-B (MRTF-B)
Myocardin-related transcription factor-B (MRTF-B) is also known as MKL-2. During embryogenesis, MRTF-B functions in the branchial arch arteries and in the developing nervous system.

p300
The transcriptional co-activator p300 and the closely related CREB binding protein (CBP) are histone acetyltransferase (HAT) paralogues that arose through gene duplication. Since they share a similar structure and function, they are often referred to as p300/CBP. The structure of p300/CBP is dominated by a central HAT domain that is flanked by other protein interaction domains. There is an N-terminal KIX domain that promotes interactions with CREB and MYB. There are three cysteine/histidine re-

gions (CH1-3); the CH3 domain enables p300/CBP to bind to PCAF, p53, MyoD, E2F and c-fos.

Once p300/CBP is recruited to the transcriptional activator it can acetylate various proteins responsible for driving transcription. In most cases, p300/CBP acetylates histones to relax chromatin structure to facilitate gene transcription. In addition, it can also acetylate transcription factors such as p53. The following examples illustrate the action of p300/CBP in controlling gene transcription:

- Phosphorylation of Ser-133 on cyclic AMP response element-binding protein (CREB) results in the recruitment of p300/CBP (Module 4: Figure CREB activation).
- Activation of CREB has a central role in controlling neuronal gene transcription (Module 10: Figure neuronal gene transcription).
- During muscle differentiation, activated MyoD recruits p300 to induce histone acetylation (Module 4: Figure MyoD and muscle differentiation).
- A p53 acetylation reaction activates the transcriptional activity of p53 (Module 4: Figure p53 function).
- Activation of transcription by FOXO is facilitated by p300 (Module 4: Figure FOXO control mechanisms).
- Activation of the transcription factor MEF2 depends on the recruitment of p300 (Module 4: Figure MEF2 activation).
- Acetylation of peroxisome-proliferator-activated receptor γ (PPARγ) coactivator-1 (PGC-1), which is one of the main transcriptional regulators of uncoupling protein 1 (UCP-1) expression during differentiation of brown fat cells, depends on p300 (Module 8: brown fat cell differentiation).

Mutations in CBP are responsible for Rubinstein-Taybi syndrome (RTS)

p300/CBP association factor (PCAF)
p300/CBP association factor (PCAF) is a transcriptional coactivator that associates with p300 and CBP. It is a histone acetyltransferase (HAT) that acetylates proteins (Module 1: Figure protein acetylation). PCAF activity is regulated by acetylation either through autoacetylation or by p300. One of the important functions of PCAF is to acetylate and activate transcription initiation factor IB (TIF-IB) that regulates the activity of RNA polymerase I (Pol I).

General control of amino-acid synthesis (GCN5)
General control of amino-acid synthesis (GCN5), which is also known as lysine acetyltransferase 2A (KAT2A), is a typical histone acetylase (HAT). One of its activities is to acetylate and inhbit peroxisome-proliferator-activated receptor γ (PPARγ) coactivator-1α (PGC-1α) that regulates genes that contribute to ATP generation, such as those that function in fatty acid oxidation, glycolysis and mitochondrial biogenesis (see Step 4 in Module 2: Figure AMPK control of metabolism).

Tat interactive protein 60 (TIP60)
Tat interactive protein 60 (TIP60), which is also known as lysine acetyltransferase 5 (KAT5), is a typical histone acetyltransferase (HAT) that acetylates proteins (Module 1: Figure protein acetylation). TIP60 is inactivated by proteosomal degradation following its ubiquitinylation by the ubiquitin ligase Mdm2.

One of the functions of TIP60 is to acetylate and activate various components of the DNA damage repair mechanism (Module 9: Figure G1 checkpoint signalling). It contributes to the arrest of growth by acetylating p53 on lysine-120 (Module 4: Figure p53 domains). By acetylating the histones H2A and H4, it opens up the chromatin for the various repair processes. For example, it acetylates ataxia telangiectasia mutated (ATM), which is a key component of the repair mechanism (Module 9: Figure G1 checkpoint signalling).

TIP60 can also function to control autophagy by activating ULK1 (Module 11: Figure autophagy).

Transcriptional co-repressors
Transcriptional co-repressors, which function to decrease gene expression, usually act by binding to transcriptional repressors such as Mad, some members of the E2F family of transcription factors (E2F4–E2F7), methyl-CpG-binding protein 2 (MeCP2) and CSL (CBF-1, Suppressor of Hairless, Lag-1). One of their actions is to recruit histone deacetylases (HDACs) that remove acyl groups from the lysine groups on histones resulting in the DNA being less accessible to transcriptional activators. There are a number of such co-repressors:

- C-terminal binding protein (CtBP)
- Nuclear receptor co-repressor (NCoR)
- Ligand-dependent co-repressor (LCOR)
- Silencing mediator of retinoic and thyroid hormone receptor (SMRT)
- Switch independent 3 (SIN3)
- Transcriptional intermediary factor 1 (TIF1)

C-terminal binding protein (CtBP)
C-terminal binding protein (CtBP) is a transcriptional co-repressor that is recruited to target promoter sites by binding to PxDLS motifs on various repressors. NADH, which is part of the NAD signalling pathway, plays a role in dimerization of CtBP monomers. It was originally identified through its interaction with the adenovirus A1A oncoprotein. CtBP functions as a bridging molecule by recruiting various histone deacetylases (HDACs) and histone methyltransferases. The two CtBP members (CtBP1 and CtBP2) are closely homologous. One difference is that CtBP2 lacks the Lys-428 sumolyation residue found on CtBP1. Translocation of CtBP from the cytoplasm in to the nucleus is controlled by sumoylation.

One of the functions of CtBP is to contribute to the transcriptional repression of the E-cadherin gene, which is one of the classical cadherins.

Switch independent 3 (SIN3)
The switch independent (SIN3) co-repressor was first identified in yeast where it functioned in mating-type switching and hence its name. SIN3 lacks DNA binding activity and is drawn into transcription complexes by interacting with various repressors such as Mad that silences genes normally controlled by the proto-oncogene Myc (Module 4: Figure Myc as a gene activator). The transcription factor myocyte enhancer factor-2 (MEF2) is also regulated by SIN3 (Module 4: Figure MEF2 activation). SIN3 carries out its co-repressor activities through its targeting and scaffolding functions. For example, it has a PAH domain that binds to the SIN3 interaction domain (SID) located on repressors such as Mad. With regard to its scaffolding function, SIN3 assembles a large number of proteins. The core SIN3/HDAC complex contains histone deacetylases (HDAC1 and 2), retinoblastoma-associated proteins 46 and 48 (RbAp46/48), SIN-associated protein 18 and 30 (SAP18 and SAP30) and SDS3. The HDACs function to deacetylate histones that tightens the histone–DNA interaction to prevent transcription. The RbAp46/48 proteins provide a bridge to the nucleosomes by binding to histones H4 and H2A. The SAP proteins have a stabilizing role particularly with regard to the interaction between SNI3 and HDAC1. The SDS3 protein stabilizes the complex by binding both SIN3 and the HDACs.

Other enzymes such as the DNA and histone methylases can be added to this SIN3/HDAC core complex to expand its chromatin remodelling function. One such protein methylation enzyme is ERG-associated protein with SET domain (ESET) is a histone H3-specific methyltransferase. Another example is ALL-1, which is a histone 3 lysine 4 (H3K4) methyl transferase.

Mediator complex
The mediator complex consist of a collection of proteins that play a role in initiating gene transcription, which consists of a group of initiation factors (e.g. TFIIB etc.) that are associated with RNA polymerase II (pol II) at the core promoter. The gap between the transcription factors located on their specific promoters and this initiation complex is bridged by this mediator complex as illustrated for the regulation of peroxisome-proliferator-activated receptor γ (PPARγ) action (Module 4: Figure PPARγ activation). The terminology of the large number of proteins making up this complex is confusing, because some of the names refer to the different complexes whereas others refer to specific proteins. Examples of the former are the MED, thyroid hormone receptor-associated complex (TRAP), vitamin D receptor interacting protein (DRIP) complex, ARC, CRSP and PC2. As far as the individual proteins within these complexes are concerned, there are Mediator (MED1-31) proteins and the mediator-associated kinases such as CDK8 and CDK11. Cyclin 7 usually associates with CDK8 when it binds to the mediator complex to phosphorylate various components of the initiator complex.

Transcription factor classification
Transcription factors (TFs) can be classified on the basis of their functional characteristics and their mode of activation (Module 4: Figure transcription factor classification). Most

Module 4: | Figure transcription factor classification
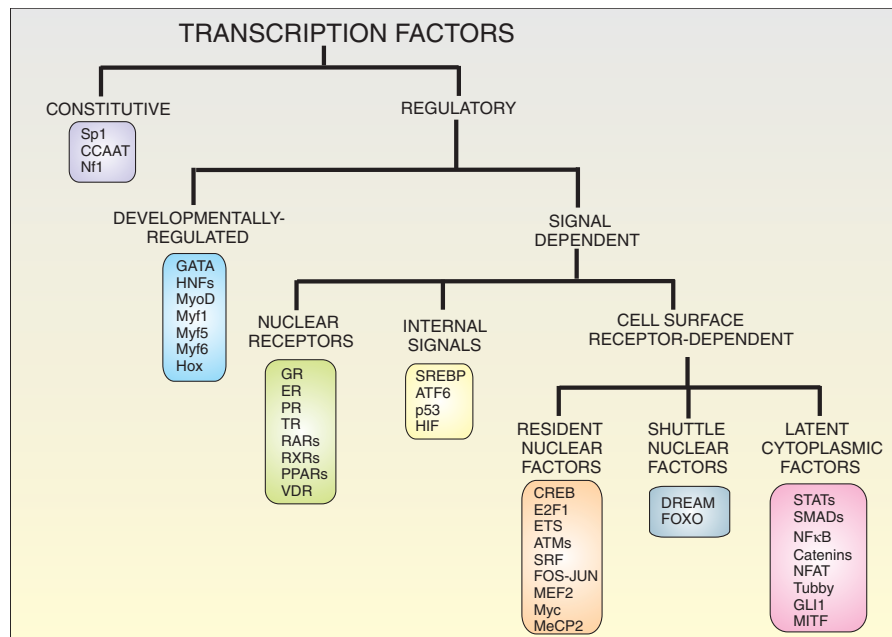
Classification of transcription factors based on their function and mode of activation.
Some transcription factors (TFs) are always present in the nucleus and are constitutively active. The regulatory transcription factors fall into two groups. The developmentally regulated TFs are often cell-type-specific, such as those that regulate muscle differentiation (MyoD and Myf5) and those that are signal-dependent. The latter can be subdivided further on the basis of whether they are regulated by steroid hormones, internal signals or by cell-surface receptors. The cell-surface receptor-dependent TFs can be divided further into those that are resident within the nucleus, those that can shuttle between the nucleus and the cytoplasm, and the latent cytoplasmic TFs that migrate into the nucleus upon receptor activation. Modified with permission from Brivanlou, A.H. and Darnell, Jr, J.E. (2002) Signal transduction and the control of gene expression. Science 295:813–818. Copyright (2002) American Association for the Advancement of Science; see Brivanlou and Darnell 2002.

attention will be focused here on the signal-dependent transcription factors, with special attention on how their transcriptional activity is regulated. The nuclear receptors belong to a superfamily containing approximately 48 human transcription factors, which are located mainly in the nucleus, where they are activated by steroids or by other lipids such as fatty acids. Internal signal-dependent transcription factors are activated by signals generated within the cell. Many of these are activated by the endoplasmic reticulum (ER) stress signalling pathway (Module 2: Figure ER stress signalling). Most of the transcription factors described so far are the cell-surface receptor-dependent transcription factors that are activated following the stimulation of cell-surface receptors. These receptors activate cell signalling pathways that then stimulate either resident nuclear factors or latent cytoplasmic factors that are then induced to enter the nucleus to initiate transcription.

Transcription factor activation mechanisms

Cells employ a great variety of activation mechanisms to control the transcription factors that regulate gene transcription. As illustrated in Module 4: Figure transcription factor activation, the signalling mechanisms that control transcription can act both in the cytoplasm and within the nucleus:

1. One of the functions of the endoplasmic reticulum stress signalling mechanisms is to activate latent transcription factors such as activating transcription factor 6 (ATF6) and sterol-regulatory-element-binding proteins (SREBPs), which are then released and imported into the nucleus (Module 2: Figure ER stress signalling).

2. Receptors on the cell surface generate cytosolic signals that activate latent transcription factors in the cytoplasm, which are then imported into the nucleus. Some receptor-dependent cytosolic signals enter the nucleus to activate different types of transcription factors.

3. Nuclear signals can inactivate repressors such as downstream regulatory element antagonistic modulator (DREAM), Forkhead box O (FOXO) and methyl-CpG-binding protein 2 (MeCP2). Once removed from the DNA, some of these such as DREAM and FOXO are then exported from the nucleus.

4. Nuclear signals activate or inhibit latent transcription factors that are already attached to DNA.

5. Nuclear signals activate latent transcription factors in the nucleoplasm that then bind to DNA.

Gene silencing

When considering transcription factor activation mechanisms, it is appropriate to include the process of gene silencing that can occur through different mechanisms. First, the expression of gene mRNA transcripts can be regulated by microRNAs. Secondly, the genes themselves can be switched off for long periods. Such gene silencing is responsible for the process of imprinting. One of the major

Module 4: | Figure transcription factor activation
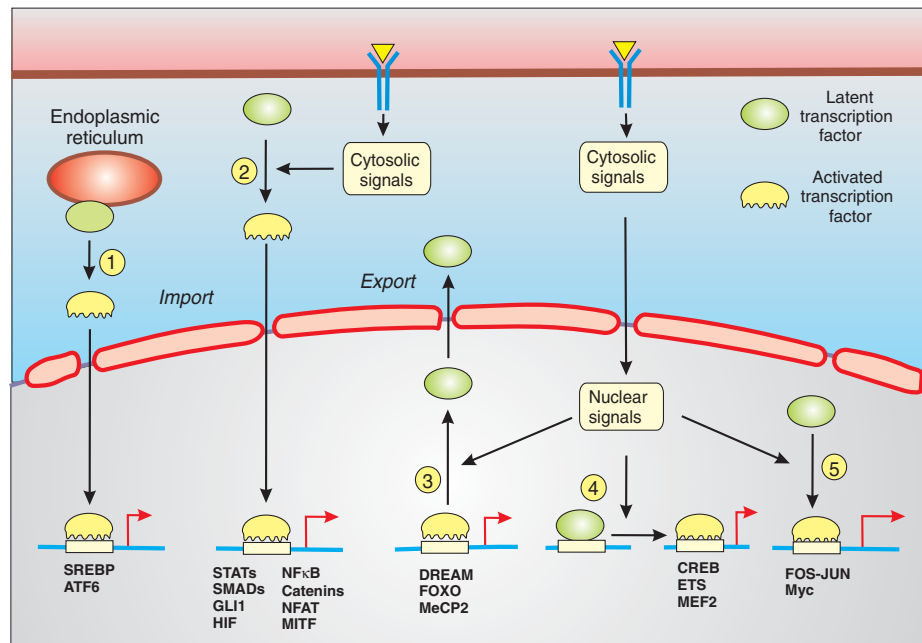
Location and mechanism of transcription factor activation.
Transcription factors are activated or inhibited by a variety of cell signalling pathways that act either within the cytosol or within the nucleus (see the text for details of the different mechanisms).

mechanisms of gene silencing depends upon the epigenetic mechanism of DNA methylation, which is a global phenomenon in that the methylation occurs throughout the genome. DNA methyltransferases, such as DNA methyltransferase 1 (DNMT1), carry out this methylation, which occurs on cytosine (C) residues that are followed by guanine residues (CpGs). There are a large number of such CpG dinucleotides distributed throughout the DNA sequence, which is thus 'marked' when the cytosine residues are methylated. Of particular interest are those methyl-CpGs that occur within 5' regulatory regions where they are responsible for gene silencing as they bring about a localized alteration in chromatin structure that shuts genes down for prolonged periods.

The alteration in chromatin structure depends upon a number of proteins that have methyl-CpG-binding domains that attach to the CpG islands and recruit various transcriptional repressors, such as methyl-CpG-binding protein 2 (MeCP2) and MBD1–4 to silence transcriptional activity. Much attention has focused on MeCP2 because mutations in the *MECP2* gene are responsible for Rett syndrome. MeCP2 may also play an important role in repressing the expression of neuroligin 1 (NLGN 1) and this may play a significant role in mediating the effect of inflammation in Alzheimer's disease (AD) (see Step 6 in Module 12: Figure Inflammation and Alzheimer's disease).

Imprinting
Gene silencing is responsible for the process of imprinting whereby only the paternal or maternal copy of certain genes are expressed. Such imprinting has interesting consequences with regard to genetic diseases. For a gene that is

normally imprinted with paternal silencing, any mutation in the functional maternal copy of the gene will result in disease. In contrast, there is no effect from mutations in the silenced paternal copy. Relatively few of the approximately 85 human imprinted genes have been associated with a human disease:

- Prader-Willi syndrome (PWS),
- Angelman syndrome (AS)
- Mental retardation
- Beckwith–Wiedemann syndrome (BWS)
- Russell–Silver (SRS)

Ubiquitin signalling and gene transcription
The ubiquitin signalling system, which is characterized by the reversible ubiquitination of cell signalling components (see top panel in Module 1: Figure protein ubiquitination), is particularly important in regulating gene transcription at a number of levels as illustrated by the following examples:

- The activity of the transcription factor p53 is regulated by its p53 ubiquitination and degradation (Module 4: Figure p53 function).
- The ubiquitin system responsible for Myc degradation is carefully controlled to regulate the turnover of this transcription factor (Module 4: Figure Myc as a gene activator).
- The FOXO4 transcription factor is activated by monoubiquitination (Module 4: Figure FOXO control mechanisms)

Developmentally regulated transcription factors

The developmentally regulated transcription factors (TFs) are often cell-type-specific and function to control the differentiation of the many different cell types found in the body (Module 7: Table cell inventory). The myogenic regulatory factors (MRFs) that function in the development of skeletal muscle are examples of such developmentally regulated TFs. One of these factors is MyoD, which is responsible for activating a large number of muscle-specific genes during the differentiation of skeletal muscle.

Myogenic regulatory factors (MRFs)

The myogenic regulatory factors (MRFs) play a key role early in skeletal muscle myogenesis (Module 8: Figure skeletal muscle myogenesis). They belong to a family of basic helix–loop–helix (bHLH) transcription factors:

- MyoD (also known as Myf3)
- Myf5
- Myogenin (also known as Myf1)
- MRF4 also known as Myf6/herculin

The way in which such myogenic factors function is typified by MyoD.

MyoD

Like the other myogenic regulatory factors, MyoD is expressed exclusively in skeletal muscle, where it functions to activate a large number of muscle-specific genes (Module 4: Figure MyoD and muscle differentiation). MyoD has two important domains, a DNA-binding region and the basic helix–loop–helix (bHLH). The latter enables it to form heterodimers with regulators such as E2A. One of the important functions of MyoD is its ability to remodel chromatin by forming complexes with acetylating enzymes such as p300 and the p300/cyclic AMP response element-binding protein (CREB)-binding protein (CBP)-associated protein (PCAF).

MyoD acts at a critical phase during the proliferation-differentiation switch. It is therefore not surprising to find that there are interactions between MyoD and proteins of the cell cycle machinery.

The way in which the myoblasts are maintained in a proliferative state and then switched into the process of differentiation are summarized in Module 4: Figure MyoD and muscle differentiation. The inhibitory mechanisms that operate on MyoD during proliferation are shown in the upper panel:

1. The cyclin-dependent kinase 4 (CDK4), which is activated early during cell proliferation (Module 9: Figure cell cycle signalling mechanisms), inhibits the activity of MyoD.
2. The cyclin D/CDK4 complex hyperphosphorylates, and thus inactivates, the Rb protein, which is prevented from stimulating MyoD (see lower panel) (Module 4: Figure MyoD and muscle differentiation).
3. MyoD is inhibited by activating protein 1 (AP-1), the Jun/Fos heterodimer.
4. The coactivator myocyte enhancer factor-2 (MEF2) binds to MyoD and draws in histone deacetylase (HDAC), which deacetylates chromatin to inhibit transcription.
5. The inhibitor of DNA binding (Id) protein , which is a dominant-negative inhibitory protein, binds to E2A that promotes differentiation by binding to MyoD as a heterodimer.

All of these mechanisms ensure that MyoD is prevented from switching on the differentiation programme while the myoblasts continue to proliferate. These inhibitory processes are reversed when the myoblasts stop growing and MyoD begins to activate the differentiation programme by stimulating the expression of muscle-specific genes (lower box in Module 4: Figure MyoD and muscle differentiation):

6. During differentiation, there is an induction of cell cycle inhibitors such as p21, which inactivate CDK4, thus removing its inhibitory action on MyoD.
7. The Rb protein, which is dephosphorylated when cells stop proliferating, is able to bind to MyoD to facilitate its activation.
8. Activation of Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV) phosphorylates HDAC, which is then exported from the nucleus in association with 14-3-3 protein, thus terminating the deacetylation of chromatin.
9. The activated MyoD associated with MEF2 now binds the histone acetyltransferases (HATs) p300/CBP and PCAF, which then facilitates transcription by remodelling chromatin by histone acetylation.
10. The inhibitory Id proteins leave E2A, which is then able to associate with MyoD to form an active heterodimer capable of stimulating the transcription of muscle-specific genes.

Paired box (Pax)

A family of paired box (Pax) transcription factors function during development to orchestrate the development of specific tissues and organs. For example, Pax3 and Pax7 are activated early during skeletal muscle myogenesis where they control the expression of myogenic regulatory factors (MRFs) such as MyoD (Module 8: Figure skeletal muscle myogenesis).

Pax expression also plays an important role in maintaining stem cell progenitor cell populations:

- Pax3 functions in the self-maintenance of melanocyte stem cells. It functions again during the process of melanogensis (see step 3 in Module 7: Figure melanogenesis). Mutations in Pax3 have been linked to Waardenburg syndrome 3 (WS3).
- Pax7 functions in the self-maintenance of satellite cells, which are skeletal muscle stem cells (Module 8: Figure Satellite cell function).

The Pax family has been divided into four subfamilies (Module 4: Table Pax transcription factors).

Sex-determining region Y (SRY)-box (SOX) transcription factors

The sex-determining region Y (SRY)-box (SOX) functions to control the differentiation of different cell types:

Module 4: | Figure MyoD and muscle differentiation
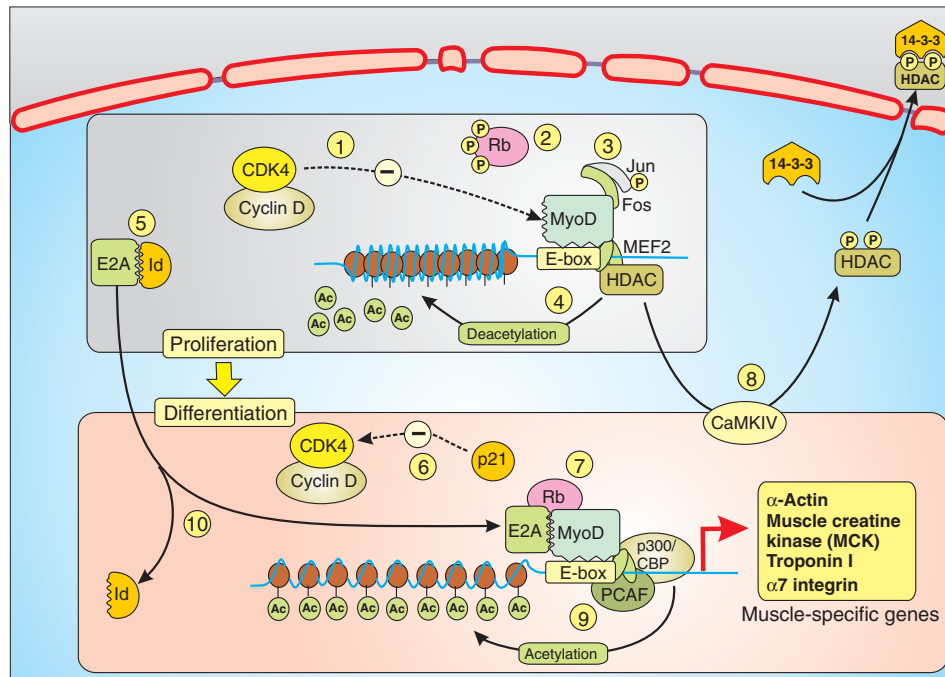
The role of MyoD in skeletal muscle differentiation.
MyoD is one of the myogenic regulatory factors (MRFs) that control muscle differentiation. MyoD binds to the E-box, which is a DNA motif (CANNTG) found on the promoters of many muscle-specific genes. MyoD is expressed in myoblasts early in muscle development when mesodermal cells acquire a myogenic lineage. Since these myocytes continue to proliferate early in development, differentiation is kept in abeyance by various mechanisms that inhibit the activity of this potent myogenic factor. (See the text for details of the mechanisms in the two panels.)

Module 4: | Table Pax transcription factors
Paired box (Pax) transcription factors.

Paired box (Pax) transcription factors	Developmental expression in tissues/organs
Subfamily 1	
Pax3	CNS, craniofacial tissue, trunk neural crest (peripheral nervous system, melanocytes, endocrine glands, connective tissue), skeletal muscle somites
Pax7	CNS, craniofacial tissue, skeletal muscle somites
Subfamily 2	
Pax4	Pancreas, gut
Pax6	CNS, pancreas, gut, nose eye
Subfamily 3	
Pax 2	CNS, kidney, ear
Pax5	CNS, kidney, thyroid
Pax8	CNS, B-lymphocytes
Subfamily 4	
Pax1	Skeleton, thymus, parathyroid
Pax9	Skeleton, thymus, craniofacial tissue, teeth

The paired box (Pax), which are divided into four subfamilies, control the development of many tissues and organs. Information contained in this table was taken from Table 1 in Buckinham and Relaix (2007).

- In embryonic stem cells (ES), Sox2 functions together with Oct4 and Nanog to maintain pluripotency.
- In melanocytes Sox10 contributes to the expression of the transcription of the microphthalamia-associated transcription factor (MITF) to control melanogenesis (Module 7: Figure melanogenesis).

- The specific transcription factor Sox9 and its two targets Sox5 and Sox6 controls the conversion of mesenchymal stem cells (MSCs) to chondrocytes.

Nuclear receptors

The nuclear receptors belong to a lipid-activatable super-family that contains approximately 48 human transcription factors. The nuclear receptor toolkit reveals the presence of a variety of transcription factors, coactivators and repressors (Module 4: Table nuclear receptor toolkit). In many cases, the nuclear receptors act to inhibit gene transcription and this effect depends on their association with various co-repressors such as silencing mediator for retinoid and thyroid hormone receptor (SMRT) and nuclear receptor co-repressor (N-CoR). These co-repressors act by recruiting chromatin remodelling complexes containing histone deacetylases (HDACs). These nuclear receptors function to control many different cellular processes:

- Peroxisome-proliferator-activated receptors (PPARs) are particularly important in regulating energy metabolism by controlling the expression of many of the metabolic components that regulate lipid and carbohydrate metabolism.
- Liver X receptors (LXRs) function in the control of lipid metabolism.
- Thyroid hormone receptor (TR) mediates the action of thyroid hormone in many different cell types.

Module 4: | **Table nuclear receptor toolkit**
The toolkit for the nuclear factor superfamily of transcription factors.

Nuclear receptor superfamily	Comments
Transcription factors	
Oestrogen receptor-α (ERα)	ERα controls expression of enzymes that function in adipogenesis and mitochondrial biogenesis (Module 5: Figure mitochondrial biogenesis)
Oestrogen receptor-β (ERβ)	
Hepatocyte nuclear factor 4α (HNF4α)	This signalling pathway operates to regulate insulin biosynthesis (Module 7: Figure β-cell signalling)
Glucocorticoid receptor (GR)	See Module 10: Figure corticotroph regulation
Liver X factor (LXR)	
Thyroid hormone receptors (TRs)	See Module 8: Figure brown fat cell differentiation and Module 10: Figure thyrotroph regulation
TRα	Also known as NR1A1
TRβ	Also known as NR1A2
Retinoid X receptors (RARs)	
RARα	Also known as NR1B1
RARβ	Also known as NR1B2
RARγ	Also known as NR1B3
Vitamin D receptor (VDR)	Functions in PTH secretion (Module 7: Figure PTH secretion)
Peroxisome-proliferator-activated receptors (PPARs)	
PPARα	Also known as NR1C1
PPARγ	Also known as NR1C2; contains two isoforms created by gene splicing
PPARγ1	Expressed in liver, muscle, macrophages, colon
PPARγ2	Expressed mainly in fat cells
PPARδ	Also known as NR1C3
Nuclear receptor cofactors	
Androgen-associated protein (ARA70)	
Glucocorticoid receptor interacting protein 1 (GRIP1)	
Cyclic AMP response element-binding protein (CREB)-binding protein (CBP)/p300	
PPAR-binding protein (PBP)	
PPARγ coactivator-1 (PGC-1)	
PGC-1α	Contributes to action of PPARα in liver
PGC-1β	
PPARγ coactivator-2 (PGC-2)	
Steroid receptor coactivator-1 (STC-1)	Contributes to the activity of the VDR (Module 7: Figure vitamin D receptor activation)
Steroid receptor coactivator-2 (STC-2)	
Receptor-interacting protein 140 (RIP140)	Acts with PGC-1α to induce uncoupling protein 1 (UCP-1) in brown fat cells
Nuclear receptor co-repressors	
Silencing mediator for retinoid and thyroid hormone receptor (SMRT)	Binds to PPARγ
Nuclear receptor co-repressor (N-CoR)	Binds to PPARα and PPARγ

- The glucocorticoid receptor (GR) exerts negative feedback control in corticotrophs (see step in 7 in Module 10: Figure corticotroph regulation)

CCAAT/enhancer-binding protein (C/EBP)

A family of transcription factors (C/EBPα, C/EBPβ, C/EBPγ and C/EBPδ) are expressed in preadipocytes where they function in the differentiation of white fat cells (Module 8: Figure white fat cell differentiation). They appear very early during differentiation and function to switch on the peroxisome-proliferator-activated receptors (PPARs) responsible for the expression of adipose genes.

Glucocorticoid receptor (GR)

The glucocorticoid receptor (GR) belongs to the nuclear receptor family (Module 4: Table nuclear receptor toolkit). The inactive receptor is present in the cytoplasm in an oligomeric complex together with Hsp90, FKBP52 and p23. Upon binding its ligands cortisol or corticosterone it dissociates from this complex, forms a dimer and translocates into the nucleus where it binds to the glucocorticoid response element (GRE) on the promoter of glucocorticoid genes. The transcriptional activity of the GR is often facilitated by transcriptional coactivator complexes such as CREB binding protein (CBP), p300, PCAF, and SRC1 (steroid receptor coactivator-1).

Once GR has bound to GRE, it can either activate or repress genes and this can depend on the phosphorylation of GR on specific sites. For example, phosphorylation of GR on serine-211 by cyclin-dependent kinase 5 (CDK5) results in activation of the *Hdac2* gene and the resulting increase in HDAC2 may contribute to the neurodegeneration seen in Alzheimer's disease.

The glucocorticoid receptor (GR) exerts negative-feedback control in corticotrophs (see step in 7 in Module 10: Figure corticotroph regulation).

Activation of the GR with glucocorticoids such as prednisone, dexamethasone and hydrocortisone has been used to treat asthma.

Liver X receptor (LXR)

One of the functions of the liver X receptors (LXRs), which come in two isoforms LXRα and LXRβ, is to control the expression of various components that function in lipid metabolism such as apolipoprotein E (ApoE) and ABCA1.

Thyroid hormone receptor (TR)

The thyroid hormone receptor TR is a typical example of a nuclear receptor (Module 4: Figure transcription factor classification). There are various isoforms: TR-α1, TR-β1 and TR-β2. The TR mediates the action of thyroid hormone in a number of cells:

- The TR-β2 is restricted to the nervous system and anterior pituitary where it functions as part of a negative-feedback loop enabling T3 to inhibit both the expression of thyrotropin-releasing hormone (TRH) by the hypothalamic neurons and the expression of thyroid-stimulating hormone (TSH) by the thyrotrophs (Module 10: Figure thyrotroph regulation).
- The TR controls the expression of uncoupling protein-1 (UCP-1), which contributes to the differentiation of brown fat cells (Module 8: brown fat cell differentiation).

Retinoid X receptor (RXR)
Retinoid X receptors (RXRs), also known as retinoic acid receptors are members of the nuclear receptor family (Module 4: Table nuclear receptor toolkit) and are widely distributed in many different cell types. There are three RXRs, RXRα, RXRβ and RXRγ, that normally function by forming heterodimers together with other nuclear receptors such as liver X receptor (LXR), peroxisome-proliferator-activated receptors (PPAR), thyroid hormone receptor (TR) and the vitamin D receptor (VDR) (Module 7: Figure vitamin D receptor activation).

The cancer drug bexarotene acts by stimulating RXR and may thus increase the activity of the vitamin D receptor (VDR) (Module 7: Figure vitamin D receptor activation). Such an action might explain the reported beneficial effects on both Parkinson's disease and Alzheimer's disease, but these findings are somewhat controversial.

Nuclear receptor co-repressor (N-CoR)
The nuclear receptor co-repressor 1 (N-CoR1) and the closely related silencing mediator for retinoid and thyroid hormone receptor (SMRT), which is also known as N-CoR2, are co-repressors of the nuclear receptors. They are large multidomain proteins that associate with the nuclear receptors to repress gene transcription. N-CoR1 and SMRT have deacetylase activation domains (DADs) that enable them to associate with histone deacetylase 3 (HDAC3) to enhance gene transcription. One of the functions of the N-CoR1–HDAC3 complex is to help control the circadian clock molecular mechanisms.

Chromatin remodelling
In its condensed form, DNA is wrapped around histones to form nucleosomes and other higher-order compact chromatin structures. Remodelling of chromatin opens up this compact structure to enable gene transcription, DNA replication and DNA repair to occur. This chromatin remodelling is carried out by a number of different types of chromatin modifying mechanisms. There are enzyme-based complexes that modify histones through protein acetylation, methylation, phosphorylation or ubiquitinylation. There also are a number of ATP-dependent chromatin-remodelling complexes that use the energy of ATP to restructure the nucleosome to open up the DNA and make it accessible for the transcriptional machinery. Examples of these remodelling complexes are SWI/SNF, ISWI, NuRD/Mi-2/CHD, INO80 and SWR1.

There also is a chromodomain helicase DNA-binding (CHD) family that plays an important role in regulating gene transcription by acting as a repressor. One of these is CHD8 that binds directly to β-catenin and suppresses signalling through the canonical Wnt/β-catenin pathway (Module 2: Figure Wnt canonical pathway). The CHD8, which appears to act by binding to histone H1, is expressed mainly during embryonic development. Such an action may explain why mutations in the CHD9 gene have been linked to autism spectrum disorders (ASDs).

Apolipoprotein E (ApoE)
Apolipoprotein E (ApoE) is the major apolipoprotein in the brain where it functions to distribute lipids between the glial cells and neurons. ApoE is synthesized by the astrocytes and microglia and once it is released, the ABCA1 transporter functions in its lipidation. The *APOE* gene encodes three isoforms ApoE2, ApoE3 and ApoE4. One of the functions of ApoE is to prevent the build-up of the β-amyloids by enhancing their hydrolysis and by regulating the processing of the β amyloid precursor protein (APP) (see step 7 in Module 12: Figure amyloid cascade hypothesis).

A polymorphism in the ApoE4 isoform increases the risk of developing Alzheimer's disease (AD).

Estrogen-related receptor α (ERRα)
Estrogen-related receptor α (ERRα) belongs to the nuclear receptor superfamily but is an orphan receptor in that there is no known agonist. One of the functions of EERα is act together with PGC-1α to regulate some of the components required for fatty acid oxidation during mitochondrial biogenesis and maintenance (Module 5: Figure mitochondrial biogenesis).

Hepatocyte nuclear factor 4α (HNF4α)
Hepatocyte nuclear factor 4α (HNF4α) belongs to the nuclear receptor superfamily (Module 4: Table nuclear receptor toolkit). It usually functions as a homodimer and can bind a number of coactivators such as GRIP1, p300/CBP, DRIP205 and PGC-1α. Like many other nuclear receptors, HNF4α can bind a lipid and, in this case, it is linoleic acid. It is not clear whether such ligand binding influences the activity of this transcription factor.

HNF4α is strongly expressed in the liver where it functions to control expression of the glycolytic genes. The activity of HNF4α is regulated by the AMP signalling pathway (Module 2: Figure AMPK control of metabolism). When the level of the metabolic messenger AMP is high the AMP-activated protein kinase (AMPK) represses gene transcription by phosphorylating and inactivating the hepatocyte nuclear factor 4α (HNF4α). This signalling pathway operates to regulate insulin biosynthesis (Module 7: Figure β-cell signalling).

Mutations in HNF4α have been linked to diabetes.

Peroxisome-proliferator-activated receptors (PPARs)
The peroxisome-proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily (Module 4: Table nuclear receptor toolkit). They play a particularly important role in controlling both lipid and glucose homoeostasis. The PPARs always function as heterodimers bound to the retinoid X receptor (RXR). This heterodimer then binds to a peroxisome-proliferator-response element (PPRE), which has a six-nucleotide motif (AGATCA). There usually are two PPRE elements located close together to provide binding sites for the two components of the PPAR/RXR dimer. The PPAR is activated by lipid moieties such as free fatty acids or by eicosanoids, whereas the RXR element can be activated by 9-*cis*-retinoic acid. It

is not necessary for both components to bind their ligands for the dimer to be active.

The PPAR group of transcription factors has three members with different cellular distributions and functions:

- PPARα
- PPARγ
- PPARδ

PPARα

Peroxisome-proliferator-activated receptor α (PPARα) is mainly expressed in liver, heart, kidney proximal tubule cells and enterocytes at the tips of the villi. All of these cells are characterized by having high levels of mitochondrial and peroxisome β-oxidation. Like other members of the family, PPARα is stimulated by free fatty acids, and its activity can also be modulated by insulin released from the insulin-secreting β-cell and glucocorticoids released from the adrenal cortex.

The activity of PPARα is markedly enhanced by the peroxisome-proliferator-activated receptor γ (PPARγ) co-activator-1 (PGC-1), which is particularly important during the transcriptional cascade that up-regulates gluconeogenesis in liver cells (Module 7: Figure liver cell signalling).

PPARγ

Peroxisome-proliferator-activated receptor γ (PPARγ) is mainly expressed in white and brown fat cells, but is also found in other tissues such as the brain, intestinal mucosa, liver and heart. While its primary role is as a physiological lipid sensor in white fat cells, it also has been implicated in a number of pathophysiological processes, such as atherosclerosis, kidney oedema and tumours.

Like other nuclear receptors, PPARγ is inhibited by the co-repressors nuclear receptor co-repressor 1 (N-CoR1) and the closely related silencing mediator for retinoid and thyroid hormone receptor (SMRT) (Module 4: Figure PPARγ activation). PPARγ is also inhibited by FOXO1. The sirtuins also play a key role in inactivation by de-acylating both PPARγ and FOXO1.

The activation of PPARγ is driven by a number of factors that are all related to a build-up of excessive nutrients. Of particular importance is the positive-feedback loop whereby an increase in free fatty acids (FFAs) stimulates the white fat cells to increase their propensity to store fat (Module 7: Figure metabolic energy network). The increased exposure to FFAs activates PPARγ directly. Insulin also plays a role in that it phosphorylates FOXO1 to reduce its inhibitory effect and it strongly promotes the expression of the co-activator PGC-1α (Module 4: Figure *PGC-1α* gene activation). PPARγ is activated further following its acetylation by p300/CBP, which is drawn into the complex by binding to the N-terminal transcriptional activation domain of PGC-1α (Module 4: Figure PPARγ activation). This activation of PPARγ sets up a feed-forward mechanism by increasing the expression of a number of genes that promote lipogenesis. Some of the genes that are activated include adipocyte lipid-binding protein (aP2), fatty acid transport protein 1 (FATP1), a fatty acid translocase (FAT) also known as FAT/CD36, ste-

aroyl CoA desaturase 1 (SCD-1) and oxidized low-density lipoprotein (oxLDL) receptor 1, all of which contribute to an increase in the fat content of white fat cells (Module 7: Figure white fat cell metabolism). In addition, there also is an increase in phosphoenolpyruvate carboxykinase (PEPCK), glycerol kinase and aquaporin 7 (a glycerol transporter), all of which enhance the recycling of FFAs within the fat cells.

The activation of PPARγ is also responsible for driving differentiation of white fat cells (Module 8: Figure white fat cell differentiation). While this process is normally confined to the period of development, strong activation of fibroblast-like preadipocytes in adults can result in the differentiation of new white fat cells and this may be particularly important during the onset of obesity.

PPARδ

Peroxisome-proliferator-activated receptor δ (PPARδ) is expressed ubiquitously and is particularly abundant in the nervous system, skeletal muscle, cardiac cells, placenta and large intestine. Like the other PPAR isoforms, it plays an important role in controlling energy metabolism, but its action is subtly different. It seems to play a particular role in the metabolism of fatty acids and in the enzymes that function in adaptive thermogenesis. With regard to the latter, it appears to act antagonistically to PPARγ by promoting the burning of fat rather than its storage, a property that is attracting considerable attention as a possible therapeutic target for controlling obesity and diabetes.

Peroxisome-proliferator-activated receptor γ (PPARγ) coactivator-1α (PGC-1α)

The peroxisome-proliferator-activated receptor γ (PPARγ) coactivator-1α (PGC-1α) is the founder member of a family that also contains PGC-1β and PGC-1 related coactivator (PRC). They all functions to control major metabolic functions in cells by acting as transcriptional co-activators to strongly potentiate the activity of many of the nuclear receptors (NRs) (Module 4: Table nuclear receptor toolkit) such as the PPAR transcription factors (PPARα and PPARγ), thyroid hormone receptor, retinoid receptors, hepatic nuclear factor-4 (HNF-4), liver X receptor (LXR), and the estrogen-related receptor α (ERRα). In addition, PGC-1α can bind to other types of transcription factors such as FOXO1, SREBP1 and myocyte enhancer factor-2 (MEF-2). The PGC-1α performs its co-activation function by binding to these multiple transcriptional partners to provide a platform to bring in other regulatory factors such as p300/CBP, nuclear respiratory factor1 (NRF-1) and -2 (NRF-2) and the Mediator complex. In addition to functioning as a transcriptional coactivator, it can also act to increase the expression of transcription factors such as ERRα.

The PGC-l family members are strongly expressed in cells that have a high oxidative capacity and are rich in mitochondria such as muscle (heart and skeletal muscle), brown fat cells, brain and kidney. Expression PGC-1α seems to be driven by stimuli such as cold temperatures, exercise and nutritional status that will require cells

Module 4: | Figure PPARγ activation
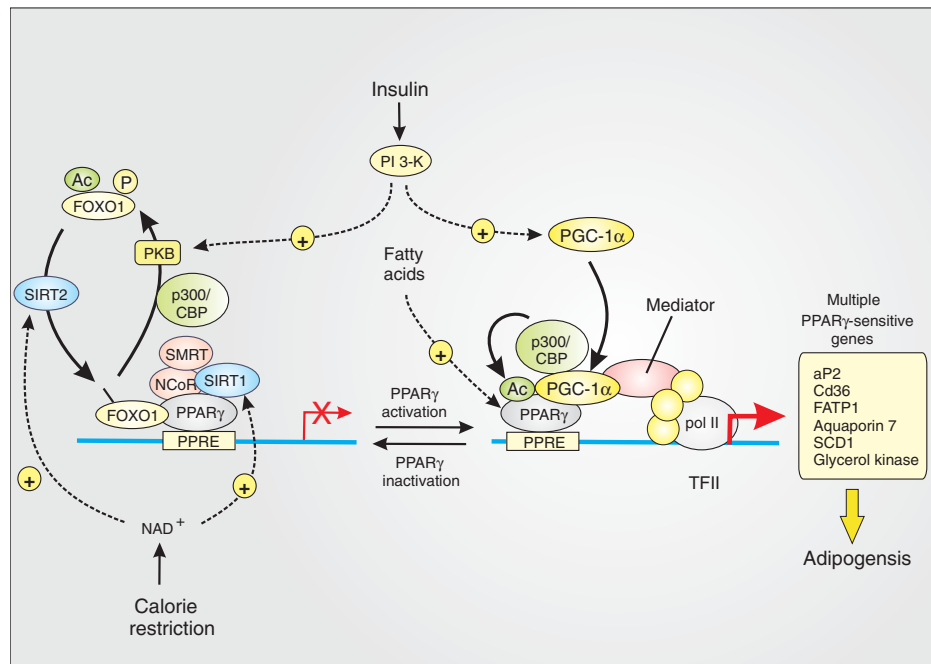
PPARγ activation.
The peroxisome-proliferator-activated receptor γ (PPARγ) is a regulator of lipid metabolism in cells such as the white and brown fat cells. It binds to the PPRE promotor site and is inactivated by corepressors such as NcoR and SMRT and is also inhibited by FOXO1. In response to insulin and an elevation of fatty acids, PPARγ is activated and begins to stimulate transcription of multiple genes associated with the activation of adipogenesis.

to enhance mitochondrial energy production (Module 4: Figure *PGC-1α* gene activation). A number of signalling pathways are used to translate these external stimuli into the activation of different transcription factors that control *PGC-1α* gene expression. In muscle, the elevation in Ca^{2+} associated with exercise stimulates calcineurin (CaN) that dephosphorylates MEF2. In addition, Ca^{2+} stimulates Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV) that acts to phosphorylate HDAC and CREB.

The activity of PGC-1α is inhibited following its acetylation by the general control of amino-acid synthesis (GCN5). This inactivation is reversed by the sirtuin SIRT1 that deacetylates PGC-1α. This activation by SIRT1 plays an important role in the maintenance of energy metabolism and antioxidant defences (Module 12: Figure ageing mechanisms) and has been implicated in the process of ageing.

The multiple actions of PGC-1α contribute to the operation of the metabolic energy network by coactivation of genes that function in both lipid and glucose metabolism, as indicated by the following examples:

- PGC-1α is rapidly induced in liver cells during fasting or following stimulation with glucagon, and then acts as a cofactor with PPARα to increase the expression of a number of the components that function in gluconeogenesis (Module 7: Figure liver cell signalling).
- In brown fat cells, PGC-1α plays a central role in the differentiation of brown fat cells by inducing the expression of uncoupling protein-1 (UCP-1) (Module 8: Figure brown fat cell differentiation). It also plays a role

in enhancing thermogenesis by switching on genes responsible for increasing fuel uptake, mitochondrial oxidation and the expression of the uncoupling protein-1 (UCP-1) to ensure that this oxidation is converted into heat.
- In dopaminergic neurons, PGC-1α stimulates the expression of the transcription factor nuclear response factor-1 (NRF-1), which is responsible for maintaining the levels of both antioxidants and detoxifying enzymes that protect against the deleterious effects of ROS (Module 12: Figure signalling pathways in Parkinson's disease).

Parkin interacting substrate (PARIS)
Parkin-interacting substrate (PARIS) is a transcriptional repressor that controls the peroxisome-proliferator-activated receptor γ (PPARγ) coactivator-1α (PGC-1α) that has a number of important metabolic functions. The level of PARIS is regulated by the ubiquitin E3 ligase Parkin, which is one of the genes known to be mutated in Parkinson's disease (Module 12: Figure signalling pathways in Parkinson's disease).

Nuclear transcription factor Y (NF-Y)
Nuclear transcription factor Y (NF-Y), which is composed of three subunits (NF-YA, NF-YB and NF-YC) to form a heterotrimeric transcription factor that binds to the CCAAT sequence in the regulatory regions of many different genes. The NF-YA binds to DNA, whereas the B and C subunits have histone-fold motifs.

Module 4: | Figure *PGC-1α* gene activation
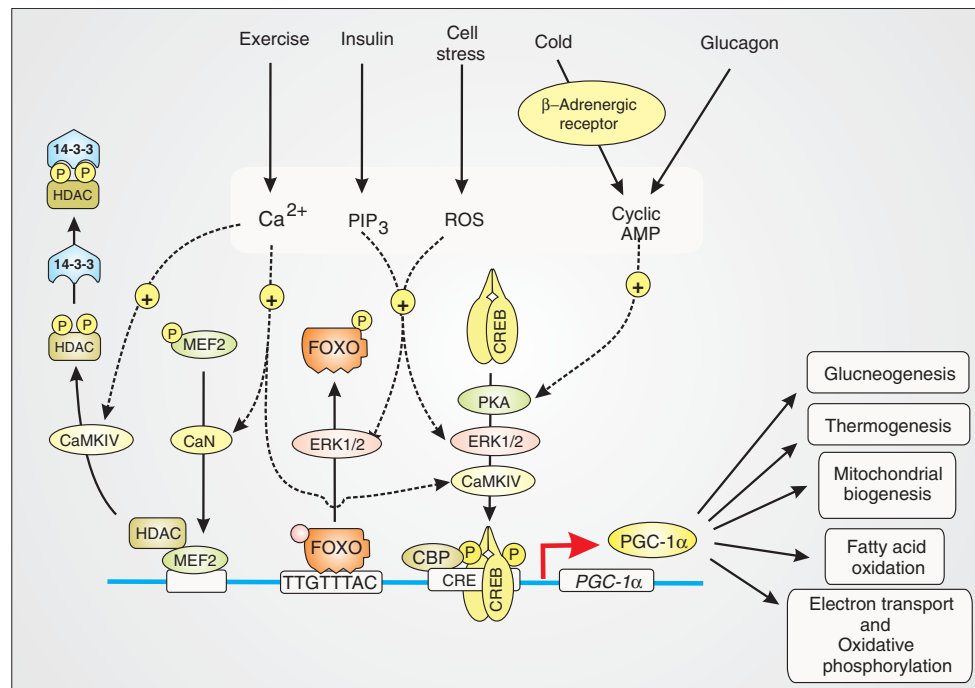
Activation of the *PGC-1α* gene.
The *PGC-1α* gene is activated by a number of external stimuli that act through various signalling pathways to regulate the activity of three main transcription factors: MEF2, FOXO and CREB. The expressed PGC-1α functions by binding to and coactivating many other genes that regulate cellular energy metabolism.

Many of these NF-Y target genes express proteins that regulate a number of different cellular processes:

- The cell cycle [e.g. topoisomerase II alpha (topo IIα), cyclins (cyclin A and cyclin B1) and cdc25B and cdc25C, p21]
- Protein degradation (proteasome proteins)
- The biological clock through control of the BMAL1 regulatory loop.
- The checkpoint function of p53 that prevents mitotic cell death may depend on an interaction between p21, the transcription factor NF-Y and the Polo-like kinases 1 (Plk1).
- NP-Y controls the T-cell leukaemia homeobox 3 (TLX3 also known as Hox11L2) transcription factor that regulates the development of the visceral nervous system and the noradrenergic neurons found in the brainstem that control the respiratory and cardiovascular systems.

NP-Y may also regulate human diseases by regulating expression of proteins such as laminin-1 (muscular dystrophy) and transforming growth factor β type II receptor (TβRII) (cancer).

Nuclear respiratory factor-1 (NRF-1)
Nuclear response factor-1 (NRF-1) is a transcription factor that functions to co-ordinate the induction of genes that encode a number of detoxifying enzymes that protect against oxidative stress. The expression of NRF-1 is controlled by peroxisome-proliferator-activated receptor γ (PPARγ) coactivator-1α (PGC-1α) (Module 12: Figure

signalling pathways in Parkinson's disease). The NRF-1 binds to an antioxidant response element (ARE) located in the promoter region of these antioxidant and detoxifying genes. Mutation of the Parkin gene, which controls the expression of PGC-1α, regulates this signalling pathway, resulting in a decrease in the formation of antioxidants and is thus consistent with the calcium and ROS hypothesis of Parkinson's disease.

The NRF-1 also acts to increase the expression of the mitochondrial transcription factor (Tfam) that has a vital role in regulating the replication and transcription of mitochondrial DNA during mitochondrial biogenesis (Module 5: Figure mitochondrial biogenesis).

Nuclear factor erythroid 2 related factor 2 (NRF-2)
The nuclear factor erythroid 2 related factor 2 (NRF-2) is a stress-sensing transcription factor that responds to reactive oxygen species (ROS) by enhancing the cells antioxidant defences. The transcriptional activity of NRF-2 is regulated by a number of mechanisms that determines its nuclear import/export balance and its degradation (Module 4: Figure NRF-2 antioxidant function).

NRF-2 is continuously formed and enters the nucleus to maintain the expression of the antioxidants. In the absence of cell stress and ROS, some of the NRF-2 binds to Kelch-like ECH-associated protein 1 (Keap1), which represses NRF2 activity and is associated with the ubiquitin ligase Cullin 3 that ubiquitinates NRF-2 resulting in its degradation by the proteasome (Module 4: Figure NRF-2 antioxidant function). When ROS levels rise in

response to cell stress, the Keap1 is oxidized and is no longer capable of binding NRF-2, which means that more of the NRF-2 can enter the nucleus to boost antioxidant formation. This nuclear entry of NRF-2 can also be enhanced following its phosphorylation by a number of protein kinases such as the MAP kinases (ERK1/2, JNK and p38) and protein kinase C (PKC). NRF-2 can also be phosphorylated by PERK following endoplasmic reticulum (ER) stress signalling (Module 2: Figure ER stress signalling). In addition, there are two important regulators of NRF-2: p62 and DJ-1, which seem to act by inhibiting the interaction between NRF-2 and Keap1 and thus reducing the degradation of NRF-2, which then enters the nucleus (Module 4: Figure p62 function).

NRF-2 binds to the antioxidant response element (ARE) to enhance the expression of a large number of antioxidant and detoxifying enzymes. This transcriptional activity of NRF-2 depends on its binding to MafG, which is one of the small musculo-aponeurotic fibrosarcoma Mafs. Transcription through the ARE site can be enhanced by valproate, which inhibits histone deacetylase (HDAC) indicating that histone acetylation has an important role in maintaining cellular antioxidant levels. In addition, the transcriptional activity of NRF-2 is also facilitated by interacting with a number of other factors such as AP-1, ATF-1, PPARγ and RARα. This NRF-2 redox regulator controls the expression of a large number of proteins such as the enzyme glutamate cysteine ligase (GCL) that synthesizes the redox buffer glutathione (GSH), glutathione S-transferase, haemoxygenase 1 (HO1), NAD(P)H quinone oxidase 1 (NQO1), peroxyredoxins and thioredoxin (TRX).

The export of NRF-2 from the nucleus is regulated by glycogen synthase kinase-3β (GSK-3β) (Module 4: Figure NRF-2 antioxidant function). The GSK-3β activates this export either by phosphorylating NRF-2 directly or by acting indirectly by stimulating the tyrosine kinase Fyn. Inhibition of GSK-3β by lithium can markedly enhance the expression of NRF-2.

There is considerable evidence to suggest that a decline in NRF-2 activity may contribute to numerous diseases such as cancer, Alzheimer's disease (AD), Parkinson's disease (PD) (Module 12: Figure signalling pathways in Parkinson's disease), amyotrophic lateral sclerosis, chronic pulmonary obstructive disease, and various inflammatory disorders.

A decrease in the activity of NRF-2 is also an important component of the ROS hypothesis of ageing.

p62

The p62 protein, which is also known as sequestosome-1, has a number of different functions. Its domain structure has all the hallmarks of a scaffolding protein capable of interacting with different cellular functions and signalling systems (Module 4: Figure p62 function). One of its important functions is to regulate the stability of the nuclear factor erythroid 2 related factor 2 (NRF-2) by inhibiting its interaction with Keap1, which directs NRF-2 to the protein degradation pathway (Module 4: Figure NRF-2 antioxidant function). This interaction sets up a feedback loop, because the NRF-2 functions to activate the expression of p62.

The C-terminal region has an ubiquitin-association domain (UBA) that enables p62 to form aggregates of ubiquitinylated proteins that are then removed from the cytoplasm by autophagy (Module 11: Figure autophagy) and proteasomal degradation.

There are indications that a decrease in the expression and cytosolic levels of p62 may contribute to various neurodegenerative diseases and particularly those associated with the accumulation of misfolded protein aggregates as seen in Alzheimer's disease (AD) and Parkinson's disease (PD). In addition, a decline in p62 activity will also result in a decrease in NRF-2 activity and in the NF-κB signalling pathway that could contribute to oxidative stress and inflammation respectively.

Internal signal-dependent transcription factors

There are a number of transcription factors that are stimulated by signals generated from within the cell. Classical examples are activating transcription factor 6 (ATF6) and p53, which are generated by various forms of cell stress. Other examples include sterol regulatory element-binding proteins (SREBPs), which function in sterol sensing and cholesterol biosynthesis, and hypoxia-inducible factor (HIF), which is an oxygen-sensitive transcription factor.

Activating transcription factor/cyclic AMP response element binding protein (ATF/CREB) family
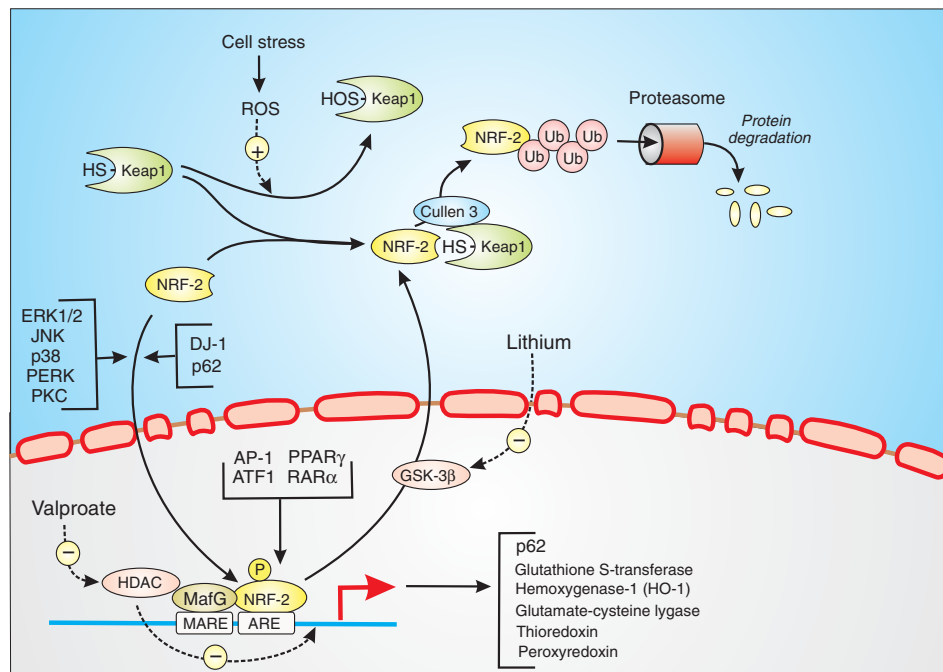
The activating transcription factor/cyclic AMP response element binding protein (ATF/CREB) family are members of a leucine zipper family of transcription factors (ATF1-7), which have the consensus binding site cAMP responsive element (CRE). Both ATF4 and ATF6 play prominent roles in the endoplasmic reticulum (ER) stress signalling (Module 2: Figure ER stress signalling).

### Activating transcription factor 4 (ATF4)

Activating transcription factor 4 (ATF4) is a member of the activating transcription factor/cyclic AMP response element binding protein (ATF/CREB) family of leucine zipper transcription factors. ATF4 is induced by endoplasmic reticulum (ER) stress signalling (Module 2: Figure ER stress signalling). ATF4 is a universal stress-responsive gene that seems to have a protective role by regulating cellular adaptation to adverse conditions. However, it is also capable of inducing the transcription of factors such as CHOP, PUMA and Noxa that promote cell death.

Activating transcription factor 6 (ATF6)

Activating transcription factor 6 (ATF6) is part of a transcriptional pathway that is activated by the endoplasmic reticulum (ER) stress signalling pathway. ATF6 normally resides in the ER/sarcoplasmic reticulum (SR) membrane through a single transmembrane domain located in the centre of the molecule. Upon Ca^{2+} depletion, the cytosolic N-terminal domain is released by proteolysis to enter the nucleus, where it interacts with the ER stress response element of the C/EBP (CCAAT/enhancer-binding

Module 4: | Figure NRF-2 antioxidant function

NRF-2 antioxidant function.
The nuclear factor erythroid 2 related factor 2 (NRF-2) is a stress-sensing transcription factor that responds to reactive oxygen species (ROS). In the absence of cell stress and ROS, NRF-2 binds to Kelch-like ECH-associated protein 1 (Keap1), which is associated with the ubiquitin ligase Cullin 3 that ubiquitinylates NRF-2 resulting in its degradation by the proteasome. When ROS levels rise, the Keap1 is oxidized and no longer binds to NRF-2, which then enters the nucleus where it binds to the antioxidant response element (ARE) to enhance the expression of a large number of antioxidant and detoxifying enzymes.

Module 4: | Figure p62 function
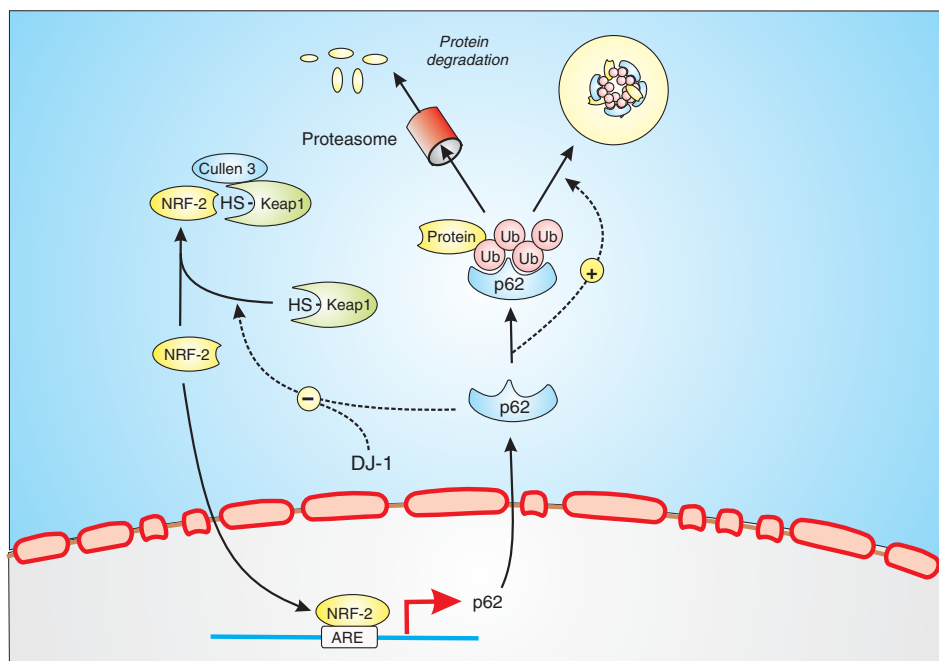
p62 function.
p62 is a multifunctional protein. Its expression is controlled by nuclear factor erythroid 2 related factor 2 (NRF-2) and it then feeds back to enhance the stability of NRF-2 by inhibiting Keap1, which directs it towards proteasomal degradation (Module 4: Figure NRF-2 antioxidant function).

protein)-homologous protein 10 (CHOP) gene (Module 2: Figure ER stress signalling). This complex activates various stress-response proteins, such as 78 kDa glucose-regulated protein (GRP78)/immunoglobulin heavy-chain-binding protein (BiP). As part of this stress response, there is a compensatory increase in the expression of sarco/endo-plasmic reticulum Ca^{2+}-ATPase 2 (SERCA2), which plays an important role in signalsome stability of the Ca^{2+} signalling system.

Specificity protein 1 (Sp1)

The specificity protein 1 (Sp1) is a ubiquitous zinc finger transcription factor that regulates the expression of a large number of proteins that function in cell growth, differentiation, immune responses, regulation and antioxidant defences. With regard to the latter, Sp1 appears to control the expression of the antioxidant protein DJ-1, which is often mutated in Parkinson's disease (PD) (Module 12: Figure signalling pathways in Parkinson's disease).

One of its actions is to remodel chromatin by binding p300 and histone deacetylases (HDACs).

Sp1 plays a role in regulating the transcription of the type 1 inositol 1,4,5-trisphosphate (InsP$_3$) receptor in response to the action of tumour necrosis factor α (TNFα) during inflammation in Alzheimer's disease (AD) (Module 12: Figure inflammation in Alzheimer's disease).

Sterol regulatory element-binding proteins (SREBPs)

The sterol regulatory element-binding proteins (SREBPs) function in sterol sensing and cholesterol biosynthesis. SREBPs are integral membrane proteins located in the endoplasmic reticulum (ER). The N-terminal region is a latent transcriptional regulator, which is cleaved by a sterol-regulated system of proteases (Module 2: Figure sterol sensing). Once released into the cytosol, these transcription factors dimerize through a basic loop and are imported into the nucleus.

AMP-activated protein kinase (AMPK) can inhibit the activity of SREBP (Module 2: Figure AMPK control of metabolism).

p53

The transcription factor p53 is part of a family of similar proteins consisting of p53 itself, p63 and p73. Most attention has been focused on p53, which is often referred to as the 'guardian of the genome' as it occupies a pivotal position right at the centre of the processes that regulate the cell cycle (Module 9: Figure cell cycle network). Its particular function is to maintain DNA integrity, especially in the face of stress stimuli such as oncogene activation, UV light and ionizing radiation. In healthy individuals, p53 is continually being produced, but its level is kept low as it is rapidly degraded. In response to various forms of DNA damage, p53 is activated to begin its protective role by contributing to the checkpoint signalling system. One of the components of this checkpoint signalling is the acetylation of p53 by TIP60 (Module 9: Figure G$_1$ checkpoint signalling). The p53 domain structure and function is characteristic of other transcription factors, with regions specialized to bind DNA and other regions that respond

to the signalling pathways that determine its activation. Under normal conditions, its level is kept low through p53 ubiquitination and degradation. This rapid turnover is prevented by genotoxic stimuli that act through a variety of post-translational modifications, such as p53 phosphorylation, p53 acetylation and p53 SUMOylation, to stabilize its level and to promote its accumulation in the nucleus resulting in p53-induced cell cycle arrest and p53-induced apoptosis. Since the major functions of p53 are to regulate the cell cycle and to promote apoptosis, there is a major role for p53 in tumour suppression. The importance of its role in tumour suppression is evident by the fact that there is a strong relationship between alterations in p53 and cancer.

p53 domain structure and function

The p53 protein contains a number of domains that determine its function as a regulator of gene transcription (Module 4: Figure p53 domains). Beginning at the N-terminal region, there is a transactivation domain (TAD) that interacts with a number of cofactors including other transcription factors, mouse double minute-2 (MDM2) ubiquitin ligase responsible for p53 ubiquitination and degradation, and acetyltransferases such as cyclic AMP response element-binding protein (CREB)-binding protein (CBP)/p300. Next, there is a proline-rich Src homology 3 (SH3)-like domain, which enables p53 to bind Sin3 to protect it from degradation. The middle of the molecule has a DNA-binding domain (DBD). Many of the mutations in p53 that result in the onset of cancer are located in the DBD region that interacts with DNA (Module 4: Figure p53/DNA complex). The C-terminal end contains nuclear localization signals (NLSs) and nuclear export signals (NESs) responsible for the translocation of p53 into and out of the nucleus. There is a tetramerization (TET) domain that enables the p53 monomers to oligomerize into the tetramers that function in gene transcription. Finally, there is a C-terminal regulatory domain (REG), which contains a large number of basic amino acids.

Under normal conditions, the activity of p53 is somewhat benign; it is kept in a quiescent state by virtue of the persistent p53 ubiquitination and degradation processes, which is controlled by its negative regulator mouse double minute-2 (MDM2) (Module 4: Figure p53 function). However, in response to a variety of stress stimuli, it is rapidly activated to begin the processes of p53-induced cell cycle arrest and p53-induced apoptosis. There is a close relationship between these p53 functions and microRNAs in this regulation of cell cycle arrest and apoptosis. The former seems to take precedence over the latter, such that the cell stops proliferating to allow the repair mechanism to restore normal function. If this repair process fails, the cell then induces apoptosis. The activation of p53 to begin the processes of cell cycle arrest and apoptosis is driven by multisite post-translational modifications carried out by a number of processes, including p53 phosphorylation, p53 acetylation, p53 methylation and p53 sumoylation (Module 4: Figure p53 domains). The modifications are located primarily at the two ends of the molecule. One of the functions of these modifications is to pull p53 away from

the MDM2 that is normally responsible for degrading it through p53 ubiquitination and degradation.

The list below provides details of the enzymes shown in Module 4: Figure p53 domains:

- Ataxia telangiectasia mutated (ATM)
- Ataxia telangiectasia mutated (ATM) and Rad3-related (ATR)
- Cyclin-dependent kinase (CDK)
- Casein kinase I (CKI)
- COP9 signalsome-associated kinase complex (CSN-K)
- DNA-dependent protein kinase (DNAPK)
- Extracellular-signal-regulated kinase (ERK); see Module 2: Figure ERK signalling
- Glycogen synthase kinase-3 (GSK-3)
- c-Jun N-terminal kinase (JNK); see Module 2: Figure JNK signalling
- p38: part of a mitogen-activated protein kinase (MAPK) signalling pathway; see Module 2: Figure MAPK signalling
- Protein kinase C (PKC)
- Double-stranded RNA-activated protein kinase (PKR)
- TATA-box-binding protein-associated factor 1 (TAF1)

Ataxia telangiectasia mutated (ATM)

This is an example of a sensor kinase that functions in the checkpoint signalling response of cells to DNA damage induced by either ionizing radiation or UV. In the case of the latter, ATM is sensitive to a specific wavelength of UV light, which has been separated into UVA (UVAI, 340–400 nm, and UVAII, 320–340 nm), UVB (280–320 nm) and UVC (180–280 nm). ATM responds to UVA light. In addition to phosphorylating Ser-15 of p53 directly, ATM also acts on other components. It can activate other kinases, such as checkpoint kinase 2 (CHK2) (Module 9: Figure G_1 checkpoint signalling). It can also phosphorylate mouse double minute-2 (MDM2), which results in inhibition of MDM2 and thus a corresponding increase in the level of p53.

This mutation of ATM is responsible for the human genetic disorder ataxia-telangiectasia (AT) syndrome.

Ataxia telangiectasia mutated (ATM) and Rad3-related (ATR)

The Ataxia telangiectasia mutated and Rad3-related (ATR) is a sensor kinase that functions in S and G_2/M checkpoint signalling to single-stranded DNA (Module 9: Figure S/G_2 phase checkpoint signalling).

DNA-dependent protein kinase (DNA-PK)

DNA-dependent protein kinase (DNA-PK) is a sensor kinase that plays an important role in the G_1 checkpoint signalling to DNA double-strand breaks (DSBs), which is an important response of cells to DNA damage induced by either ionizing radiation or UV (Module 9: Figure G_1 checkpoint signalling). It is one of the kinases that is activated at the site DNA strand breaks that occur following DNA damage. One of its actions is to phosphorylate p53 on Ser-15 and Ser-37 (Module 4: Figure p53 domains). In response to DNA damage, DNA-PK also activates the non-receptor protein tyrosine kinase Abl, which contributes to the process of DNA repair (Module 1: Figure Abl signalling).

p53 ubiquitination and degradation

Degradation of p53 is carried out by various ubiquitin ligases such as mouse double minute-2 (MDM2), Pirh2 and COP1. MDM2 is classified as an oncogene because it causes tumours when overexpressed in cells. The action of MDM2 is facilitated by a related protein called, Mdmx, which binds to MDM2 to form heterodimers. MDM2 has dual functions: it is responsible for both the nuclear export of p53, and its polyubiquitination and degradation by the 26S proteasome (Module 4: Figure p53 function). MDM2 also facilitates the nuclear export of p53 by entering the nucleus, where it carries out the mono-ubiquitination of p53 to expose the nuclear export signal (NES) resulting in its translocation into the cytoplasm, where it is polyubiquitinated prior to its degradation by the 26S proteasome.

The ubiquitination of p53, which is an example of the relationship between ubiquitin signalling and gene transcription, is a dynamic process because a ubiquitin-specific protease Usp7, which is also known as herpes virus-associated ubiquitin-specific protease (HAUSP), is a p53-binding protein that functions to de-ubiquitinate p53. The activity of Usp7/HAUSP is regulated by the actin-modulating protein supervillin. The Usp7/HAUSP also plays an important role in stabilizing MDM2, which can be autoubiquitinated leading to its degradation. The Usp7/HAUSP stabilizes the level of MDM2 thus helping it to prevent the increases in p53 that would lead to cell cycle arrest.

The negative regulator MDM2 and p53 are connected together by a negative-feedback loop whereby p53 induces the transcription of MDM2, whereas the latter acts to inhibit p53 action by inducing its degradation. This feedback mechanism has to be modified in order for stressful stimuli to increase the activity of p53. This feedback loop operating between p53 and MDM2 can be adjusted by a number of mechanisms:

- One of the main mechanisms depends upon p53 phosphorylation.
- The tumour suppressor alternative reading frame (ARF) stabilizes p53 by binding to MDM2 in the nucleus, and thus prevents p53 from leaving the nucleus to be degraded in the cytoplasm.
- p53 activity is prolonged through Abl inhibition of mouse double minute-2 (MDM2) (Module 1: Figure Abl signalling).

Mdmx

Mdmx functions together with mouse double minute-2 (MDM2) to regulate p53 ubiquitination and degradation. Mdmx has some sequence homology with MDM2, but unlike the latter, it lacks E3 ligase activity. Mdmx forms heterodimers with MDM2 and may contribute to the action of MDM2 in inhibiting p53. There is some indication that Mdmx binds to the transactivation domain of p53.

Module 4: | Figure p53 domains
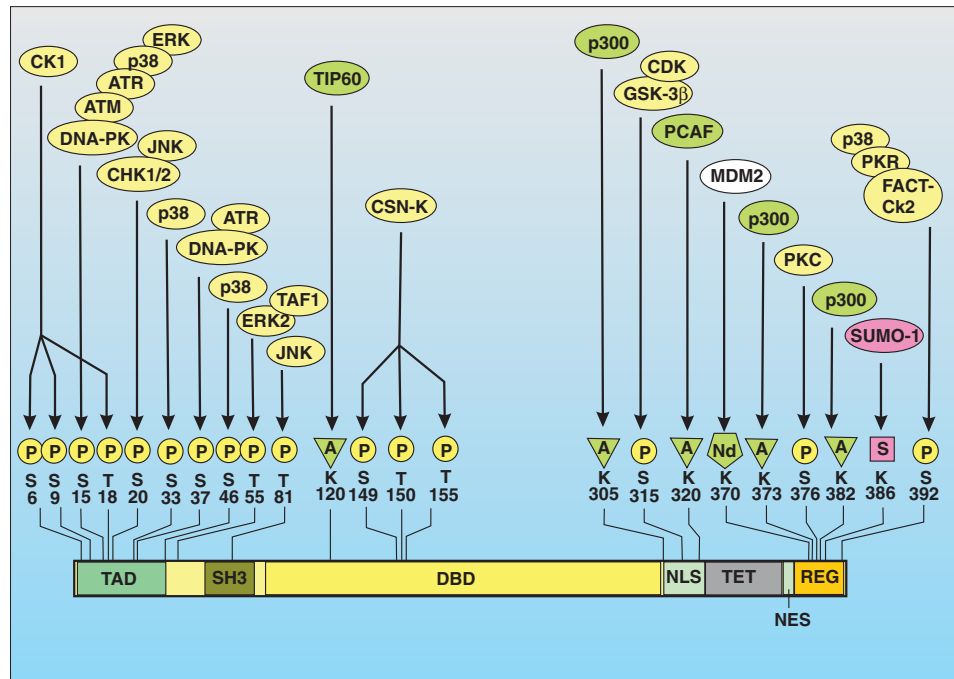
The domain structure and post-translational modification sites of the transcription factor p53.

The major domains of p53 consist of an N-terminal transactivation domain (TAD), followed by a Src homology 3 (SH3)-like domain. In the middle of the molecule there is a large DNA-binding domain (DBD). The C-terminal region has a nuclear localization signal (NLS), a tetramerization domain (TET) and a regulatory (REG) domain. There are a large number of post-translational sites that are modified by phosphorylation (P), acetylation (A), sumoylation (S) and neddylation (Nd) by a large number of signalling pathways. (See the text for a description of the different enzymes.)

Module 4: | Figure p53/DNA complex
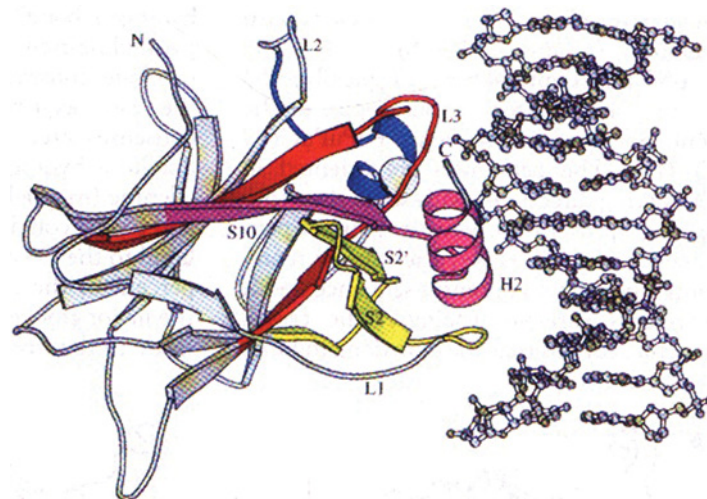
A ribbon drawing of p53 complexed to DNA.

This figure illustrates the proposed interaction between p53 (shown on the left) and DNA in its vertical orientation on the right. Many of frequently mutated residues of p53 are located on the surface that interacts with DNA. Reproduced with permission from Cho, Y., Gorina, S., Jeffrey, P.D. and Pavletich, N.P. (1994) Crystal structure of a p53 tumor suppressor–DNA complex: understanding tumorigenic mutations. Science 265:346–355. Copyright (1994) American Association for the Advancement of Science; http://www.sciencemag.org/; see Cho et al. 1994.

p53 phosphorylation

Phosphorylation of p53 plays a key role in its transcriptional activation. Many of the phosphorylation sites are located in the same N-terminal region where ubiquitin ligase MDM2 is bound. Phosphorylation of these sites blocks this binding of MDM2 and thus prevents p53 degradation and allows its level to rise such that it can begin to induce gene transcription (Module 4: Figure p53 function). There are at least 16 sites on p53 that are phosphorylated by a large variety of serine/threonine protein kinases (Module 4: Figure p53 domains). Certain sites are phosphorylated by a single kinase; for example, Ser-6, Ser-9 and Thr-18

Module 4: | Figure p53 function
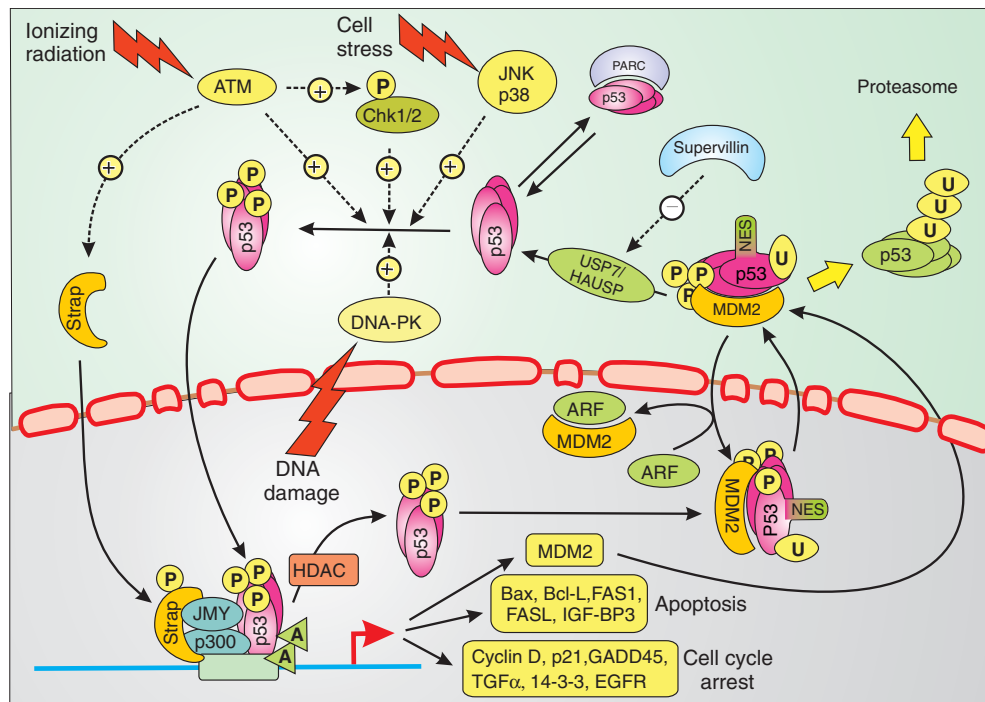
The activation and function of p53.

The transcriptional activity of p53 is regulated by a complex set of processes that control its stability, activation and translocation. The transcriptional activity of p53 is normally kept at a low level either by its binding to various proteins such as p53-associated parkin-like cytoplasmic protein (PARC), which effectively acts as a buffer, or by its rapid turnover. The ubiquitin ligase mouse double minute-2 (MDM2) is responsible for the degradation and for the nuclear export of p53. The activity of MDM2 is inhibited by alternative reading frame (ARF), which acts to stabilize p53. A variety of stressful stimuli such as DNA damage and ionizing radiation stimulate the protein kinases and acetylases responsible for the post-translational modifications (Module 4: Figure p53 domains) that activate p53. Once activated, it accumulates in the nucleus where it binds to the p53-responsive element found on a large number of genes, many of which function to control cell cycle arrest or apoptosis. One of the genes switched on by p53 codes for MDM2, thus setting up a negative-feedback loop to curb the activity of p53.

are specific for casein kinase I (CKI). However, for some of the other sites, there is considerable redundancy in that they can be phosphorylated by a number of kinases, as is the case for Ser-15. It seems that p53 integrates a large number of input signals, and the sum of the modifications then determines the specificity and the magnitude of its transcriptional activity. There also are indications that the degree of p53 phosphorylation fluctuates during the course of the cell cycle, which is in keeping with the process of p53-induced cell cycle arrest.

p53 acetylation

Acetylation is an important post-translational modification that regulates the transcriptional activation of p53. The histone acetyltransferases (HATs) p300 and CREB binding protein CBP (p300/CBP), p300/CBP-associated factor (PCAF) and Tat-interactive protein 60 (TIP60) function to acetylate specific lysine residues located in the C-terminal region of p53 (Module 4: Figure p53 domains). This acetylation enhances the stability of p53, thus contributing to its function in gene transcription. Conversely, the deacylation of p53 by histone deacetylases (HDACs), such as the sirtuin Sirt1, may initiate the process of degradation, because some of the deacylated residues appear to be those used for the mono-ubiquitination by mouse double minute-2 (MDM2) that results in the export of p53

from the nucleus and its degradation (Module 4: Figure p53 function).

The cofactor function of p300 is augmented by junction-mediating and regulatory protein (JMY). The interaction of p300 and JMY is facilitated by a scaffolding protein called serine/threonine kinase receptor associated protein (STRAP), which has a tandem series of tetratricopeptide repeats that function in protein–protein interactions. The scaffolding function is regulated by its phosphorylation by ataxia telangiectasia mutated (ATM), which enables STRAP to enter the nucleus, where it binds to the p300/JMY complex to acetylate p53. In addition to acetylating p53, p300 will also acetylate histones to open up the chromatin for transcription to occur.

p53 methylation

The transcriptional activity of p53 is regulated by protein methylation. The Lys-370 site undergoes both mono-methylation (K370me1) carried out by Smyd-2 and di-methylation (K370me2) through an unknown methyltransferase. The K370me1 represses transcriptional activity whereas the K370me2 enhances transcription by promoting association of p53 with the transcriptional co-activator p53-binding protein 1 (53BP1). This activation step is reversed by histone lysine-specific demethylase (LSD1).

p53 sumoylation

The transcriptional activity of p53 can be modulated by sumoylation. This is a post-translational modification that depends on the formation of an isopeptide bond between the ubiquitin-like protein SUMO1 and the ε-amino group of Lys-386 on p53. The effect of this sumoylation is still not clear, but recent evidence seems to indicate that it may repress the transcriptional activity of p53.

p53-induced cell cycle arrest

One of the functions of p53 is to arrest the cell cycle to enable damaged DNA to be repaired. This arrest is achieved by stimulating the transcription of many of the cell cycle signalling components, such as p21, growth-arrest and DNA-damage-inducible protein 45 (GADD45), Wip1, proliferating-cell nuclear antigen (PCNA), cyclin D1, cyclin G, transforming growth factor α (TGFα) and 14-3-3σ (Module 4: Figure p53 function). One of the main mechanisms used by p53 is to activate the G_1 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). Another important checkpoint control mechanism is regulated by a protein called GADD45a, which is another of the proteins up-regulated by p53 acting together with another tumour suppressor Wilms' tumour suppressor (WT1). GADD45a acts at the G_2/M checkpoint by dissociating the cyclin B/CDK1 complex by binding to CDK1 (Module 9: Figure proliferation signalling network).

p53-induced apoptosis

One of the main tumour suppressor functions of p53 is to induce the apoptosis of damaged cells (Module 9: Figure proliferation signalling network). An increase in tumorigenesis occurs when this pro-apoptotic function is inactivated. p53 promotes apoptosis through transcription-dependent and transcription-independent mechanisms.

p53 function and microRNAs

There is a close relationship between the functions of p53 and microRNAs (Module 4: Figure microRNAs and p53 function). Translation of the *TP53* gene transcript to form p53 is regulated by miR-125b and by miR-380-5p. Once formed, p53 phosphorylation by various stimuli, such as ionizing radiation, cell stress and DNA damage, results in the activation of p53 to bring about p53-induced cell cycle arrest and p53-induced apoptosis. Regulation of these processes by p53 is carried out by two main transcriptional mechanisms. First, p53 increases the transcription of components that control the cell cycle (e.g. p21 and GADD45) and apoptosis (e.g. Bax, FAS1 and FASL) (Module 9: Figure proliferation signalling network). Secondly, p53 regulates the transcription of a number of microRNAs (miRs), such as the miR-34 family, that can contribute to the regulation of both cell cycle arrest and apoptosis. In some cases (e.g. miR-16-1, miR-143 and miR-145), p53 not only activates the initial transcription but it also enhances Drosha

that controls a key step in microRNA biogenesis (Module 4: Figure microRNA biogenesis). These three miRs then help to switch off the cell cycle.

Transcription-dependent mechanism.

The primary action of p53 is to activate the transcription of many of the key components of apoptosis (Module 4: Figure p53 function). It acts by up-regulating components of both the extrinsic pathway (e.g. Fas) and the intrinsic pathway (e.g. Bax, Bid, Apaf-1, Puma and Noxa). Alternatively, p53 suppresses some of the anti-apoptotic genes such as Bcl-2, and Bcl-X_L. In addition, it can also promote the expression of phosphatase and tensin homologue deleted on chromosome 10 (PTEN), which thus will reduce the cell survival signalling mechanisms controlled by the PtdIns 3-kinase signalling pathway (Module 2: Figure PtdIns 3-kinase signalling).

Transcription-independent mechanism.

The transcription-independent mechanism depends upon an effect of p53 at the mitochondrion, where it binds to the anti-apoptotic proteins Bcl-2, Bcl-X_L and Mcl-1, thereby contributing to the activation of the pro-apoptotic proteins Bax and Bak. The manganese superoxide dismutase (MnSOD) might be another target for p53 in the mitochondria.

p53 in tumour suppression

p53 is one of the main tumour suppressors that functions by preventing the emergence of cancer cells. Under normal circumstances, this function of p53 is held in abeyance and is only called into action by various sensor kinases, such as ataxia telangiectasia mutated (ATM) and DNA-dependent protein kinase (DNAPK), which detect DNA damage and begin to phosphorylate p53 (Module 4: Figure p53 function). The activated p53 then begins its suppression of tumour formation through the twin-track approach of p53-induced cell cycle arrest and p53-induced apoptosis. If this function of p53 as a negative regulator of cell growth is reduced either by mutation or by its binding to various oncogenic viruses, such as simian virus 40 (SV40) large T antigen, adenovirus E1B 55 kDa protein and human papillomavirus E6 (HPV E6), the causative link between p53 and cancer emerges.

A role for p53 in cancer may depend in part on its interaction with the Ca^{2+}-binding protein S100B. Elevated levels of Ca^{2+} will increase the S100B-dependent inhibition of p53, and this could contribute to the progression of tumours. Such an interaction is another example of the relationship between Ca^{2+} signalling and cancer.

A germline mutation that results in a single copy of the *TP53* gene that codes for p53 is the cause of Li-Fraumeni syndrome.

p63

The p53 tumour suppressor family consists of three members: p53, p63 and p73. The primary function of p63 seems to be related to maintenance and development of stem cells.

Module 4: | Figure microRNAs and p53 function
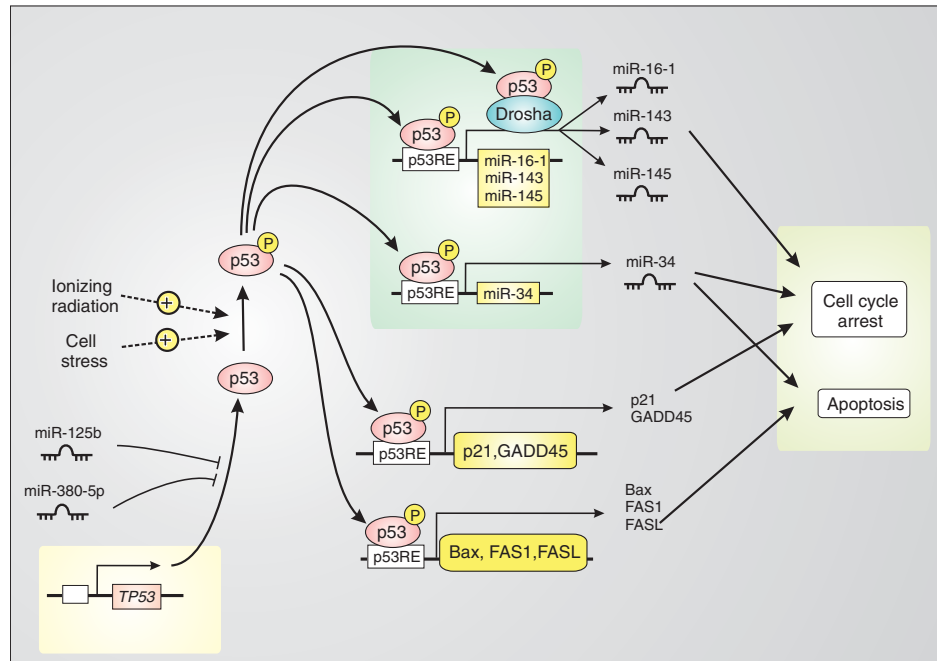
MicroRNAs and p53 function.
There are intimate relationships between the functions of microRNAs (miRs) and p53. The expression of p53 is regulated by miR-125b and miR-380-5p, whereas activated p53 can control the transcription of a number of miRs that contribute to regulation of cell cycle arrest and apoptosis.

p73

The p53 tumour suppressor family consists of three members: p53, p63 and p73. The function of p73, which resembles that of p53, is complicated in that it exists as multiple isoforms. The TAp73 isoform promotes apoptosis and this might be related to its ability to inhibit tumorigenesis. TAp73 can also promote the expression of TWIK-1, which is also known to inhibit tumour growth.
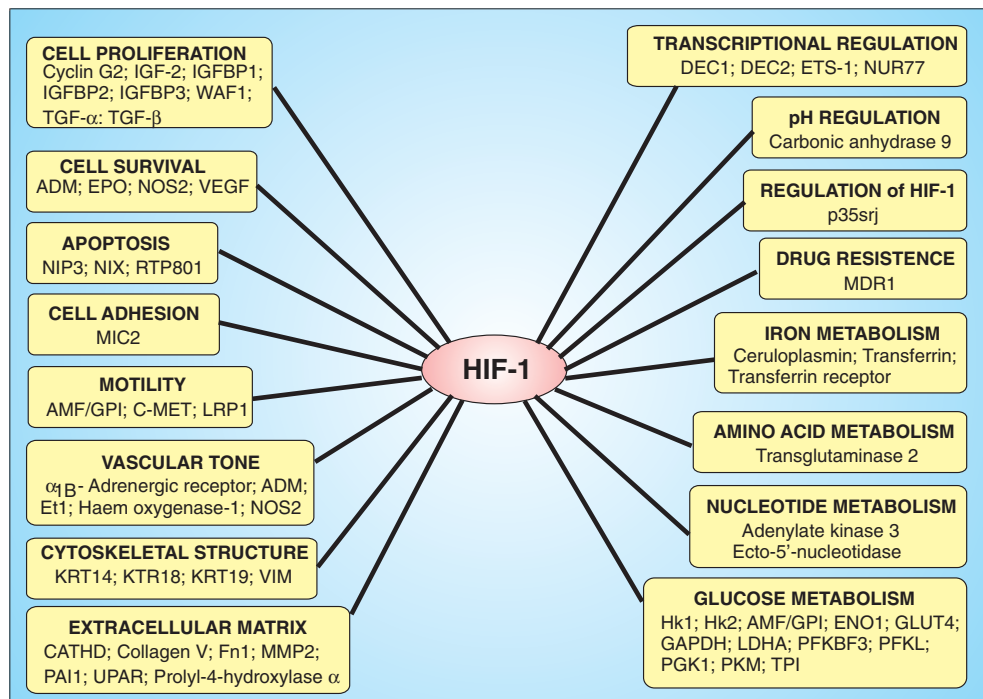
Hypoxia-inducible factor (HIF)

Hypoxia-inducible factor (HIF) functions in one of the O_2-sensing responses that cells employ when facing prolonged hypoxia. This is particularly important in the growth of tumours where new cells become starved of O_2 as they divide and move away from the existing vasculature. These new cells respond to the lack of O_2 by sending out signals to activate angiogenesis, which creates the new blood vessels required to oxygenate the growing tumour. An important component of this angiogenic response is the activation of HIF that then increases the expression of a large number of components that function in one way or another in the regulation of cell proliferation (Module 4: Figure HIF functions). For example, one of these components is vascular endothelial growth factor A (VEGF-A), which promotes the angiogenic response. HIF also regulates the expression of chemokines, such as CXCL12 and its receptor CXCR4 that contributes to a relationship between chemokines and cancer. Hypoxia-inducible factor (HIF) structure reveals that this transcription factor is hydroxylated by O_2-sensitive enzymes that play a key

role in hypoxia-inducible factor (HIF) activation when cells are subjected to low O_2 tensions.

The transcription factor NF-κB plays a role in linking innate immunity and inflammation to the hypoxic response by increasing expression of HIF-1α (Module 4: Figure NF-κB activation and function).
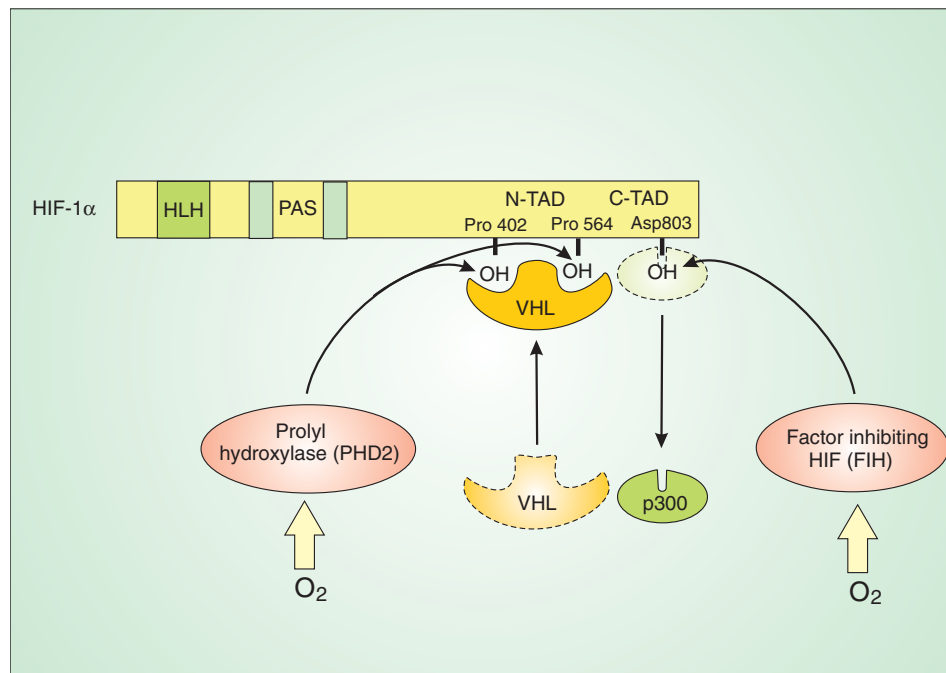
Hypoxia-inducible factor (HIF) structure

There are a number of hypoxia-inducible factors (HIFs), which belong to the basic helix–loop–helix (bHLH) group of proteins. Most attention has focused on HIF-1α and HIF-1β, which interact with each other to form the heterodimer, which is the active form of this transcription factor. In addition, there is HIF-2α, which seems to act like HIF-1α, but acts on different target genes. Finally, there is HIF-3α, which seems to function as an inhibitor of HIF-1α. The N-terminal region of HIF-1α has the bHLH motif responsible for binding to HIF-1β to form the functional heterodimer (Module 4: Figure HIF structure). The N-terminal transactivation domain (N-TAD) contains the two proline residues that are hydroxylated by the prolyl hydroxylase domain (PHD2) protein to produce the binding sites for the von Hippel–Lindau (VHL) protein. The C-terminal transactivation domain (C-TAD) has the Asp-803 residue that is hydroxylated by the factor inhibiting HIF (FIH) to displace the p300 that facilitates the transcription of HIF target genes. These hydroxylation events play a critical role in hypoxia-inducible factor (HIF) activation under hypoxic conditions.

Module 4: | Figure HIF functions

Hypoxia-inducible factor 1 (HIF-1) activates the transcription of multiple genes.
There are a large number of genes that are regulated by hypoxia-inducible factor 1 (HIF-1). Some of these target genes function in the regulatory networks that control cell proliferation, survival and apoptosis. (Redrawn from Figure 3 from Semenza 2003.)

Module 4: | Figure HIF structure

The structure and activation of hypoxia-inducible factor 1α (HIF-1α).
The O2-sensitive hypoxia-inducible factor 1α (HIF-1α) has a basic helix–loop–helix (HLH) domain that enables it to form a heterodimer with the O2-insensitive HIF-1β subunit to form the functional transcription factor. The activity of HIF-1α depends on the hydroxylation of proline residues at 402 and 564 and an asparagine residue at 803. The proline residues are hydroxylated by a prolyl hydroxylase (PHD2), whereas a factor inhibiting HIF (FIH) hydroxylates the asparagine residue. These hydroxy groups provide binding sites for the von Hippel–Lindau (VHL) protein that directs HIF-1α to the proteasomal degradation pathway and for p300 that regulates its transcriptional activity. See Module 4: Figure HIF activation for details of how O2 regulates the activity of HIF-1α.

Module 4: | Figure HIF activation
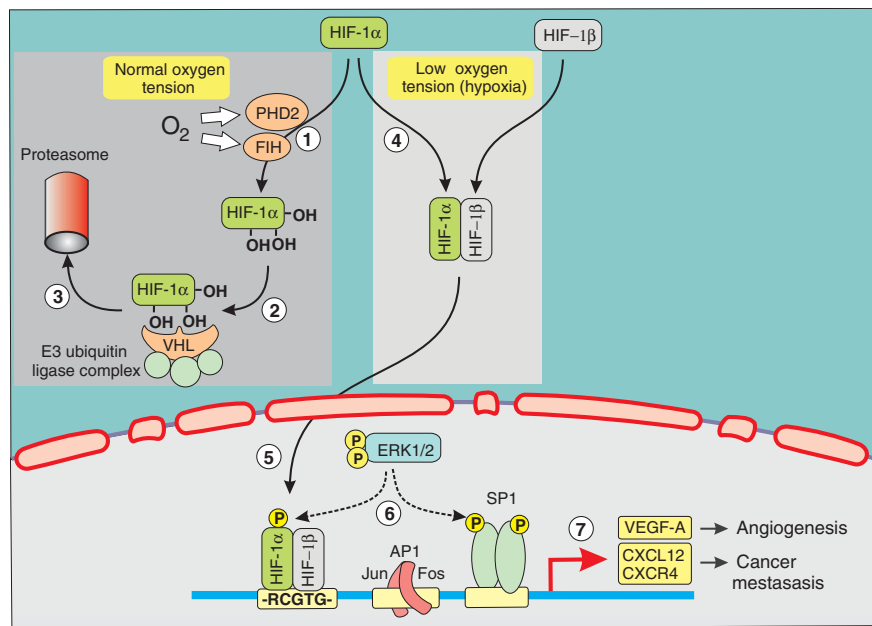
Activation of the hypoxia-inducible factor (HIF).

1. At the normal oxygen tension of blood, the hypoxia-inducible factor 1α (HIF-1α) isoform is constantly being degraded by a process that is driven by O2, which activates the O2-sensitive prolyl hydroxylase domain (PHD2) protein and factor inhibiting HIF-1 (FIH). These two proteins hydroxylate HIF-1α. 2. Two of these hydroxy groups provide binding sites for the von Hippel–Lindau (VHL) protein, which is a component of the E3 ubiquitin ligase complex. 3. Ubiquitinated HIF-1α is degraded by the proteasome. 4. At low O2 tensions, PHD2 and FIH are inactive. 5. HIF-1α is free to associated with HIF-1β to form a heterodimer. 6. The heterodimer translocates into the nucleus to initiate the transcription of genes such as vascular endothelial growth factor A (VEGF-A) that function in angiogenesis.

Hypoxia-inducible factor (HIF) activation

The activation of hypoxia-inducible factor (HIF) during hypoxia depends upon two O_2-sensitive hydroxylases that control both the stability and transcriptional activity of the HIF-1α subunit. At normal oxygen tensions, the HIF-1α subunit is unstable and is constantly being degraded through the following sequence of events (Module 4: Figure HIF activation):

1. The O_2 activates two hydroxylases. A prolyl hydroxylase domain (PHD2) protein hydroxylates Pro-402 and Pro-564 in the N-terminal transactivation domain (N-TAD) (Module 4: Figure HIF structure).
2. These two hydroxylations provide a binding site for the von Hippel–Lindau (VHL) protein, which is a component of the E3 ubiquitin ligase complex (Module 1: Figure ubiquitin-proteasome system), which ubiquitinates HIF-1α and thus marks it out for destruction.
3. The ubiquitinated HIF-1α is then degraded by the proteasome (Module 4: Figure HIF activation). Under normal oxygen tensions, therefore, the HIF-1α is unstable and thus unable to activate transcription.
4. At low oxygen tensions, the hydroxylation enzymes are inactive and the HIF-1α is stabilized and now can bind to HIF-1β to form the active heterodimer.
5. The activated HIF-1α translocates into the nucleus where it binds to the hypoxia response element (HRE), which contains the core sequence -RCGTG-, on the promoter region of the large number of HIF target genes.

6. For many of these target genes, such as the vascular endothelial growth factor A (VEGF-A) gene, there are additional binding sites for transcription factors such as activating protein (AP-1) and specificity protein 1 (SP1).
7. The HIF target genes include VEGF-A that controls angiogenesis and the chemokine CXCL12 and its receptor CXCR4 that contribute to the relationship between chemokines and cancer metastasis.

Cell-surface receptor-dependent transcription factors

A variety of mechanisms are used to activate receptor-dependent transcription factors (Module 4: Figure transcription factor activation). Receptors on the cell surface employ various signalling pathways to stimulate transcription factors located either in the cytosol or in the nucleus. In the case of some transcription factors, activation depends upon their expression, as is the case for the Myc family.

Activation of transcription by cytosolic signals

There are a large number of transcription factors that lie dormant within the cytoplasm until they are activated by a variety of signalling pathways (Mechanism 2 in Module 4: Figure transcription factor activation):

- APP intracellular domain (AICD)
- β-Catenin

- Interferon-regulatory factors (IFNs)
- LBP1 family of transcription factors
- Notch intracellular domain (NICD)
- Signal transducers and activators of transcription (STATs)
- Nuclear factor of activated T cells (NFAT)
- Nuclear factor κB (NF-κB)
- Smads

APP intracellular domain (AICD)

The APP intracellular domain (AICD) is formed when mutant APP is hydrolysed to form amyloid β42 (Aβ$_{42}$) (see steps 4 and 5 in Module 12: Figure amyloid cascade hypothesis). The AICD then functions as a transcription factor that may play a role in controlling the expression of some of the Ca^{2+} signalling components such as the SERCA pump and the ryanodine receptor (RYR). This link between amyloid processing and the remodelling of the Ca^{2+} signalling system may be a significant step in the progression of Alzheimer's disease (AD).

β-Catenin as a transcription factor

β-Catenin is the transcription factor that is activated by the Wnt signalling pathways. Under resting conditions, the cytosolic level of β-catenin is kept low by proteasomal degradation. Following Wnt activation, the β-catenin degradation complex is inhibited allowing β-catenin to accumulate and to enter the nucleus, where it induces the transcription of the Wnt genes responsible for regulating development and cell proliferation (Module 2: Figure Wnt canonical pathway).

Interferon-regulatory factors (IFRs)

The interferon-regulatory factors (IFRs) are a family of transcription factors that contribute to the function of the type I interferons (IFNs) [(interferon-α (IFN-α) and interferon-β (IFN-β)]. There are at least nine members of the family. These IRFs are activated when cells respond to IFNs and also during the induction of the type I IFNs. The latter is illustrated by the role of IRF3 and IRF7 during the response of cells to viral infections (Module 2: Figure viral recognition).

LBP1 family of transcription factors

The leader-binding protein-1 (LBP1) family has two genes that give rise to different splice variants. One gene gives rise to LBP1a and LBP1b, whereas the other gene produces LBP1c and LBP1d. These different isoforms can form both homo- and heterodimers. The LBC1c variant, which is also known as CP2 and LSF, has been implicated in the onset of Alzheimer's disease because it may interact with the APP intracellular domain (AICD) to regulate gene transcription (Module 12: APP processing).

c-Maf

c-Maf is considered to be a proto-oncogene. It belongs to the family of small musculoaponeurotic fibrosarcoma Mafs, which are basic region leucine zipper domain transcription factors consisting of MafF, MafG and MafK. These Mafs bind to Maf-recognition elements (MAREs) of target genes that have many functions. They have been implicated in the control of interleukin-4 (IL-4) and also interleukin-21 (IL-21). The latter is important in driving the final stages of B-cell differentiation in the lymph node (Module 8: Figure B cell maturation signalling). MafA activates the insulin gene promoter in β-cells. MafG combines with the nuclear factor erythroid 2 related factor 2 (NRF-2) to control the expression of antioxidant enzymes (Module 4: Figure NRF-2 antioxidant function).

The oncogenic v-Maf, which was identified in the genome of the acute transforming avian retrovirus AS42, induces musculoaponeurotic fibrosarcoma (Maf).

Signal transducers and activators of transcription (STATs)

The Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signalling pathway is another example of transcriptional activation by cytosolic signals (Module 2: Figure JAK/STAT function). Following receptor activation, the JAKs are activated to phosphorylate tyrosine residues on the STATs, which then dimerize before migrating into the nucleus to activate transcription.

Mutations in STAT3 have been linked to hyper-IgE syndrome (HIES).

Notch intracellular domain (NICD)

The Notch intracellular domain (NICD) is the cytoplasmic domain of the Notch protein that functions in the Notch signalling pathway. When Notch is hydrolysed by the γ-secretase complex, NICD is released into the cytoplasm and then diffuses into the nucleus where it induces the transcription of multiple Notch target genes (steps 4 and 5 in Module 2: Figure Notch signalling).
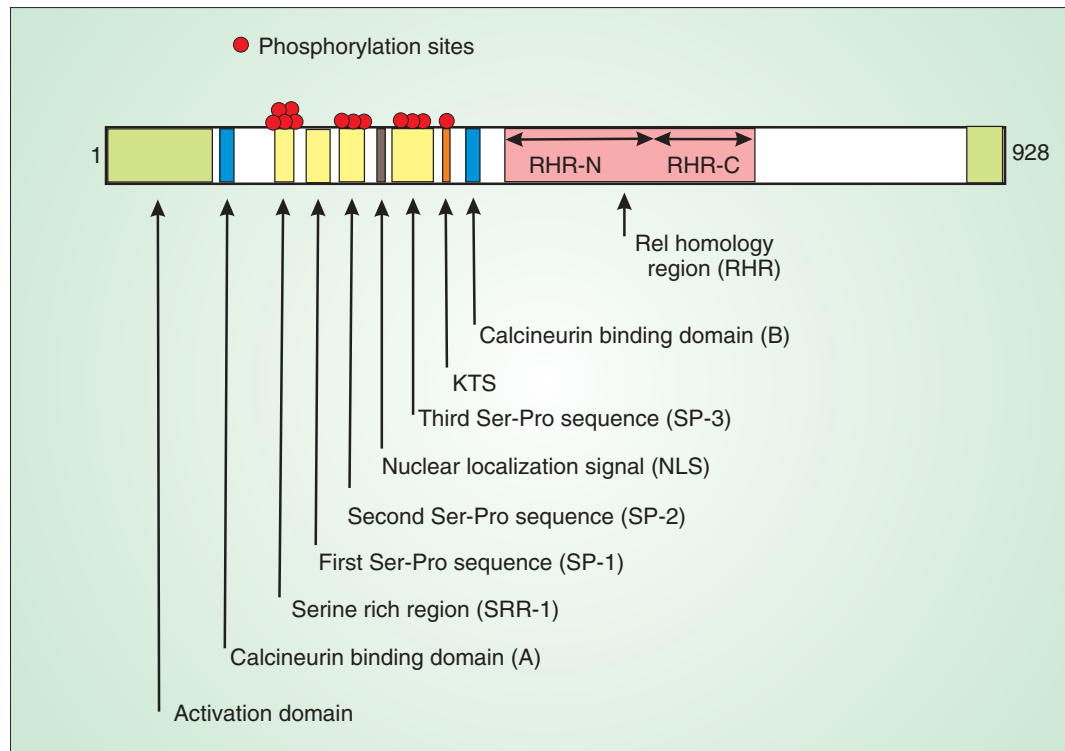
Nuclear factor of activated T cells (NFAT)

The nuclear factors of activated T cells (NFATs) are a family of cytosolic transcription factors (NFAT1–NFAT4) that function not only during early development, but also in the subsequent response of cells to external signals that activate processes such as cell proliferation, neural control of differentiation and cardiac hypertrophy. **Nuclear factor of activated T cells (NFAT) structure** reveals the many features that are required for NFATs to respond to cytosolic signals and to bind to DNA. **Nuclear factor of activated T cells (NFAT) activation** depends upon cytosolic Ca^{2+} signals that activate **calcineurin (CaN)** to dephosphorylate NFAT, thereby enabling it to enter the nucleus. **Nuclear factor of activated T cells (NFAT) function** is very diverse. It operates during early development and during the subsequent process of differentiation into specialized cells. NFAT also continues to function in fully differentiated cells, where it controls adaptation and maintenance of the differentiated state.

Nuclear factor of activated T cells (NFAT) structure

The four nuclear factor of activated T cells (NFAT) family members share similar structural domains (**Module 4: Figure NFAT structure**). The Rel-homology region (RHR) is very similar to that found in the nuclear factor κB

Module 4: | Figure NFAT structure

The structural domains of nuclear factor of activated T cells (NFAT).
The various domains located along the length of the nuclear factor of activated T cells (NFAT) molecule perform different functions during its activation of transcription. Starting with the N-terminal region, there is an activation domain. The interaction between NFAT and calcineurin depends on two calcineurin-binding domains (A and B, shown in blue). Calcineurin then removes the 13 phosphate groups (red dots) that are clustered in four regions: a serine-rich region (SRR-1), two Ser-Pro sequences (SP-2 and SP-3) and one at a KTS site. Removal of these phosphates unveils a nuclear localization signal (NLS) that enables NFAT to enter the nucleus. Once in the nucleus, NFAT binds to DNA through the Rel-homology region (RHR), which has two parts: an N-terminal specificity domain (RYR-N), which binds to DNA, and a C-terminal dimerization domain (RHR-C), which binds to other transcription factors such as activating protein 1 (AP-1) (Module 4: Figure NFAT/AP-1/DNA complex). Modified, with permission, from Hogan, P.G., Chen, L., Nardone, J. and Rao, A. (2003) Transcriptional regulation by calcium, calcineurin, and NFAT. Genes Dev. 17:2205–2232. Copyright (2003) Cold Spring Harbor Laboratory Press; see Hogan et al. 2003.

(NF-κB)/Rel family. It is responsible for DNA binding. While NFAT can bind to DNA as a homodimer, the interaction with DNA is weak. Under normal circumstances, its DNA-binding is facilitated by binding to other transcription factors, such as **activating protein 1 (AP-1)** (Fos/Jun), c-Maf or GATA4, to form a high-affinity DNA-binding complex (**Module 4: Figure NFAT/AP-1/DNA complex**). The formation of such complexes greatly expands the versatility of the NFAT transcriptional system and also serves to enhance its integrative capacity by providing additional inputs from other signalling pathways (**Module 4: Figure NFAT activation**).

Nuclear factor of activated T cells (NFAT) activation
Nuclear factor of activated T cells (NFAT) is an example of a transcription factor that is activated in the cytoplasm before being imported into the nucleus (Mechanism 2 in Module 4: Figure transcription factor activation). Activation of NFAT is controlled by an increase in the concentration of cytosolic Ca^{2+}, which then stimulates the enzyme calcineurin to dephosphorylate NFAT (Module 4: Figure NFAT activation). It is this dephosphorylated NFAT that is imported into the nucleus as part of an NFAT nuc-

lear/cytoplasmic shuttle. The control of this shuttle depends upon the balance between Ca^{2+} stimulating the import process and the action of various kinases such as glycogen synthase kinase-3β (GSK-3β) and mitogen-activated protein kinase (MAPK) p38 (Module 2: Figure MAPK signalling) that stimulate export back into the cytosol. The activation sequence that begins with an elevation of cytosolic Ca^{2+} is illustrated in Module 4: Figure NFAT activation.

1. Ca^{2+} binds to calmodulin (CaM), which then interacts with calcineurin (CaN).
2. The activated CaN then removes the multiple phosphates located on NFAT to unveil a nuclear localization signal (NLS) that promotes import into the nucleus, where it can have two actions.
3. The dephosphorylated NFAT can interact with other transcription factors such as activating protein 1 (AP-1) (Fos/Jun), and the ternary Fos/Jun/NFAT complex then binds to DNA sites on the promoters of NFAT target genes.
4. The other action of NFAT, which is confined to NFAT2, is to bind to the myocyte enhancer factor-2 (MEF2), where it helps to recruit the p300 coactivator.

Module 4: | Figure NFAT/AP-1/DNA complex
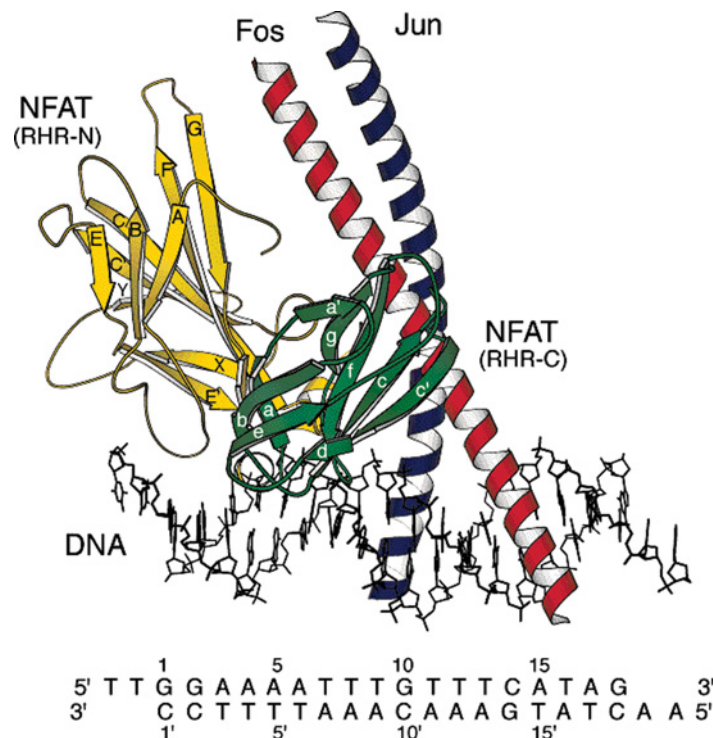
Structure of the nuclear factor of activated T cells (NFAT)/activating protein 1 (AP-1)/DNA complex.
The N-terminal region of nuclear factor of activated T cells (NFAT) is shown in yellow, whereas the C-terminal region that interacts with Fos (red) and Jun (blue) is shown in green. Reproduced by permission from Macmillan Publishers Ltd: *Nature*, Chen, L., Glover, N.M., Hogan, P.G., Rao, A. and Harrison, S.A. (1998) Structure of the DNA-binding domains from NFAT, Fos and Jun bound specifically to DNA. 392:42–48. Copyright (1998); http://www.nature.com; see Chen et al. 1998.

5. The recruitment of p300 contributes to chromatin remodelling and gene transcription by histone acetylation.
6. The inactivation of NFAT depends upon its export from the nucleus following its rephosphorylation by various 'NFAT kinases'.
7. One of these kinases is glycogen synthase kinase-3β (GSK-3β), which appears to initiate the rephosphorylation cascade by phosphorylating sites at the N-terminal serine-rich region (SRR-1) (Module 4: Figure NFAT structure). The nuclear activity of NFAT is prolonged by the PtdIns 3-kinase signalling cassette, which uses protein kinase B (PKB) to phosphorylate GSK-3β.
8. NFAT is also phosphorylated by p38, one of the MAPK signalling pathways.

The Ca^{2+}-dependent translocation of NFAT from the cytoplasm into the nucleus can be visualized by expressing a green fluorescent protein (GFP)/NFAT construct in baby hamster kidney (BHK) cells (Module 4: Figure NFAT translocation).

The time that NFAT resides within the nucleus is critically dependent on the balance between the rates of import and export, which can vary considerably between cell types. The other characteristic feature of this NFAT shuttle is that it is a dynamic process whose equilibrium is very dependent on the temporal properties of the Ca^{2+} signal. This becomes particularly evident when the trans-location process is examined at different frequencies of Ca^{2+} signalling (Module 6: Figure NFAT nuclear translocation). This NFAT shuttle may thus represent a mechanism whereby cells can decode oscillatory information through integrative tracking (Module 6: Figure decoding oscillatory information). In general, low-frequency high-amplitude transients are ineffective, whereas more frequent transients are very effective. This is evident in skeletal muscle, where stimulation at 1 Hz had little effect, whereas stimulation at 10 Hz resulted in a gradual transfer of NFAT from the cytosol to the nucleus (Module 8: Figure nuclear import of NFAT).

Nuclear factor of activated T cells (NFAT) function
Nuclear factor of activated T cells (NFAT) has a multitude of functions, since it is a transcription factor that operates throughout the life of an animal. One of its earliest developmental functions is in axis formation, as has been shown for dorsoventral specification. It is employed again during differentiation, where it is particularly significant in controlling the expression of tissue-specific genes (Module 4: Figure NFAT activation). As part of this differentiation process, it has a special role in signalsome expression when the signalling pathways characteristic of each cell type is being established. Finally, NFAT continues to function in the maintenance of fully differentiated cells and in their adaptation to external stimuli. An example of the latter is signalsome stability, where the transcription of genes such

Module 4: | Figure NFAT translocation
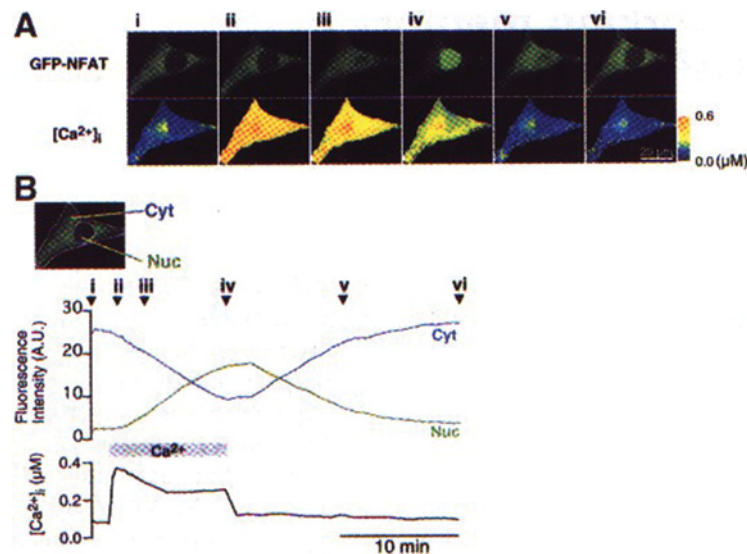
The Ca^{2+}-dependent translocation of nuclear factor of activated T cells (NFAT) from the cytoplasm into the nucleus.
When a green fluorescent protein (GFP)/nuclear factor of activated T cells (NFAT) construct is expressed in baby hamster kidney (BHK) cells, it is located in the cytoplasm at rest (see panel A,i). Upon an increase in Ca^{2+} as shown in the lower panels of A, the GFP/NFAT moves from the cytoplasm into the nucleus as shown in panel A,iv. The time course of these changes is shown in B. Note how the increase in Ca^{2+} induces a fall in the cytosolic level of GFP/NFAT, with a corresponding increase in the nucleus. Reproduced by permission from Macmillan Publishers Ltd: *EMBO J.*, Tomida, T., Hirose, K., Takizawa, A., Shibasaki, F. and Iino, M. (2003) NFAT functions as a working memory of Ca^{2+} signals in decoding Ca^{2+} oscillation. EMBO J., 22:3825–3832. Copyright (2003); http://www.nature.com/emboj; see Tomida et al. (2003).

as NFAT may contribute to a feedback mechanism that ensures stability of the differentiation state of cells, including their signalling pathways (Module 4: Figure NFAT control of Ca^{2+} signalling toolkit).

NFAT thus plays a role in the regulation development, proliferation and cellular adaptation to changing circumstances:

- Remodelling the cardiac signalsome during compensatory hypertrophy (Module 12: Figure hypertrophy signalling mechanisms).
- Regulating the biosynthesis of glucagon (Module 7: Figure α-cell signalling).
- T cell activation is critically dependent on the activation of NFAT (Module 9: Figure T cell Ca^{2+} signalling).
- The formation of slow-twitch skeletal muscle during the neural control of differentiation, where it contributes to gene activation by the transcription factor myocyte enhancer factor-2 (MEF2).
- Development of the vasculature, which depends upon cross-talk between the developing vessels and the supporting tissues, requires the activation of NFAT.
- β-Cell secretagogues that elevate Ca^{2+} maintain the supply of insulin by stimulating the proliferation of β-cells through the NFAT pathway (Module 7: β-cell signalling).
- NFAT regulates the expression of many components of the Ca^{2+} signalling system, such as endothelin, transient-receptor potential-like channel 3 (TRPC3), inositol 1,4,5-trisphosphate receptor 1 ($InsP_3R1$), NFAT2 and Down's syndrome critical region 1 (DSCR1) (Module 4: Figure NFAT control of Ca^{2+} signalling toolkit).

There appears to be a link between Down's syndrome and NFAT function. Down's syndrome may result from a dysregulation of NFAT resulting from an increased expression of two of the proteins that function to regulate the NFAT shuttle (Module 4: Figure NFAT control of Ca^{2+} signalling toolkit).

Nuclear factor κB (NF-κB)
The nuclear factor κB (NF-κB) is a multifunctional transcription factor that is used to regulate a large number of processes, such as inflammation, cell proliferation and apoptosis (Module 4: Figure NF-κB activation and function). NF-κB belongs to the group of transcription factors that lie latent in the cytoplasm and then translocate into the nucleus upon activation (mechanism 2 in Module 4: Figure transcription factor activation). The way in which NF-κB is activated is described in the section on the nuclear factor κB (NF-κB) signalling pathway (Module 2: Figure NF-κB activation).

Single-minded 1 (Sim1)
A hypothalamic transcription factor located in neurons that function in the neural network for the control of food intake and body weight (Module 7: Figure control of food intake). Sim1 is strongly expressed in the paraventricular nuclei (PVN) which are innervated by the POMC/CART neurons located in the arcuate nucleus (ARC). These POMC/CART neurons release α-MSH, which acts on the melatonin receptor MC4R responsible for stimulating Sim1. Haploinsufficiency of the *Sim1* gene causes hyperphagic obesity indicating that the activation of this transcription factor functions in a signalling pathway

Module 4: | Figure NFAT activation
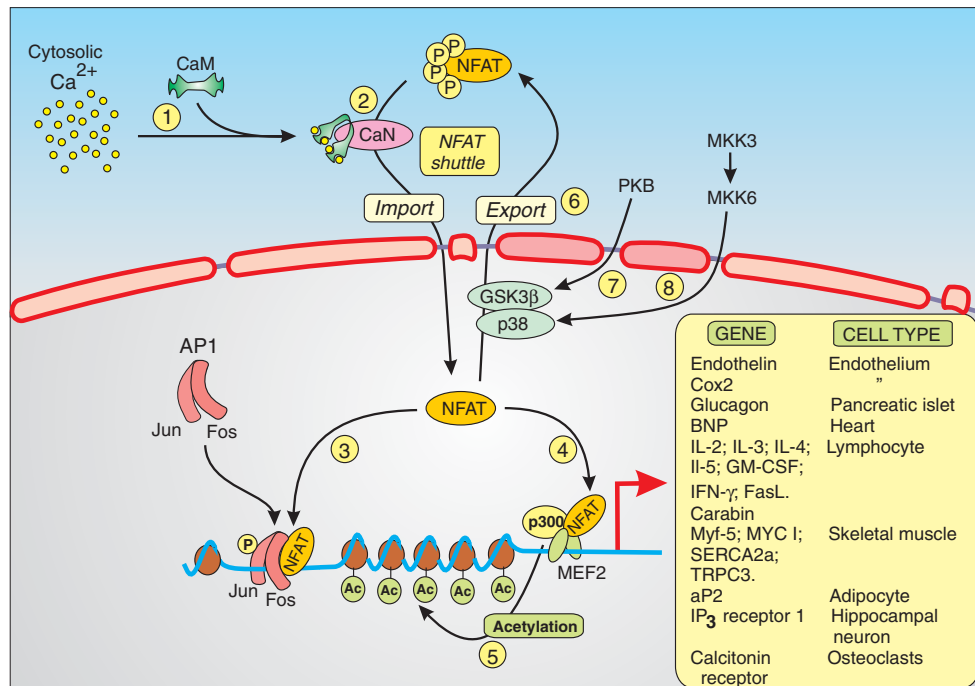
The Ca^{2+}-dependent activation of nuclear factor of activated T cells (NFAT).

An increase in the concentration of cytosolic Ca^{2+} initiates a series of events that culminate in the transcriptional activation of a large number of genes. [See the text for details of the Ca^{2+}-dependent activation of nuclear factor of activated T cells (NFAT).]

Module 4: | Figure NF-κB activation and function
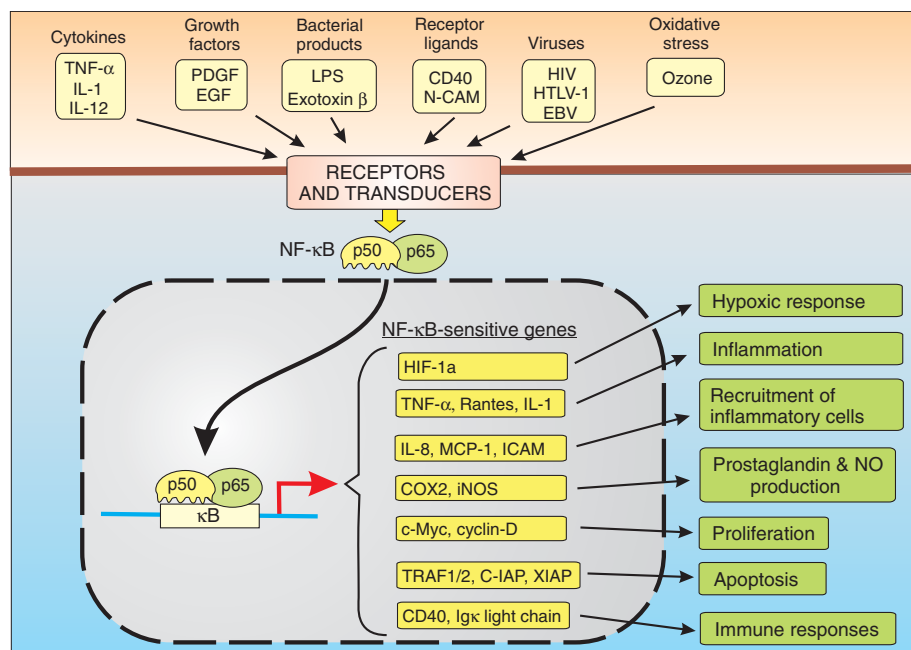
Activation and function of the transcription factor nuclear factor κB (NF-κB).

A number of different stimuli acting through various receptors and transducing mechanisms activate nuclear factor κB (NF-κB), which then enters the nucleus, where it binds to κB promoter elements to induce the expression of many genes that function in many different cellular processes. Adapted from *Handbook of Cell Signaling*, Vol. 3, (Bradshaw, R.A. and Dennis, E.A., eds), Westwick, J.K., Schwamborn, K. and Mercurio, F., NFκB: a key integrator of cell signalling, pp. 107–114. Copyright (2003), with permission from Elsevier; see Westwick et al. 2003.

that carries information to the satiety centres to terminate feeding.

Smads

In the case of the Smad signalling pathway, the activation process is relatively simple. Smads are activated directly through serine/threonine phosphorylation by the receptor-associated kinases (Module 2: Figure TGF-βR activation). The activated Smads then diffuse into the nucleus to activate transcription (Module 2: Figure Smad signalling).

Activation of transcription by nuclear signals

Many of the signalling pathways in the cytosol are capable of entering the nucleus, where they operate to activate different transcription factors (Mechanisms 3–5 in Module 4: Figure transcription factor activation). There are transcriptional activators and repressors that can shuttle between the nucleus and cytoplasm, and whose activity is altered by these nuclear signals:

- Activating protein 1 (AP-1) (Fos/Jun)
- Cyclic AMP response element-binding protein (CREB)
- Downstream regulatory element antagonistic modulator (DREAM)
- E twenty-six (ETS)
- E2F family of transcription factors
- Forkhead box O (FOXO)
- Methyl-CpG-binding protein 2 (MECP2)
- Myocyte enhancer factor-2 (MEF2)
- Serum response factor (SRF)

Cyclic AMP response element-binding protein (CREB)

The cyclic AMP response element-binding protein (CREB) is a ubiquitous multifunction transcription factor. CREB belongs to a family of transcription factors that have a series of leucine residues that function as a leucine zipper to form both homo- and hetero-dimers. CREB is activated by a number of signalling pathways (Module 4: Figure CREB activation). CREB activation is also dependent on Ca^{2+} signalling, which has two main actions. Firstly, it activates the nuclear Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV), which is one of the kinases that phosphorylate CREB. Secondly, it activates calcineurin (CaN), which dephosphorylates the transducer of regulated CREB (TORC) thus enabling it to enter the nucleus to co-operate with CREB to switch on transcription.

The phosphorylation of CREB enables it to bind the transcriptional co-activator proteins p300 and CBP, which are histone acetyltransferases (HATs) that acetylates histones thereby remodelling the chromatin so that transcription can proceed.

CREB functions in the control of many different cellular processes:

- The survival and proliferation of β-cells is regulated by glucose and by various hormones such as glucagon-like peptide-1 (GLP-1), which appear to act synergistically to activate the CREB co-activators called TORCs (Module 7: Figure β-cell signalling).

- Regulating the biosynthesis of glucagon (Module 7: Figure α-cell signalling).
- CREB is particularly important in activation of neuronal gene transcription responsible for learning and memory (Module 10: Figure neuronal gene transcription), the expression of clock genes (Module 6: Figure circadian clock input-output signals), the expression of brain-derived neutrophic factor (BDNF) (Module 4: Figure MeCP2 activation) and to activate the genes that defines the phenotype of fast-spiking interneurons (Module 12: Figure schizophrenia).
- The CREB/TORC transcriptional duo play a critical role in energy metabolism by controlling the expression of the peroxisome-proliferator-activated receptor γ (PPARγ) coactivator-1 (PGC-1), which acts together with the PPAR transcription factors to control the expression of components that function in liver cell gluconeogenesis (Module 7: Figure liver cell signalling).
- CREB controls the transcription of the aquaporin (AQP) channels AQP2 and AQP3 during vasopressin-induced antidiuresis (Module 7: Figure collecting duct function).
- CREB is one of the major transcription factors that is activated during the process of melanogenesis (step 3 in Module 7: Figure melanogenesis).
- NMDA receptor hypofunction in schizophrenia is caused by a decrease in the Ca^{2+}-dependent phosphorylation and activation of CREB that is responsible for maintaining the phenotypic stability of inhibitory interneurons in the brain (Module 12: Figure schizophrenia).

B cell lymphoma 6 (BCL-6)

B cell lymphoma 6 (BCL-6) is a member of the BTB/POZ zinc-finger family of transcription factors. It functions as a repressor to control the very rapid proliferation of centroblasts during B-cell differentiation in the lymph node (Module 8: Figure germinal centre). This repressor role can have different outcomes depending on the ability of BCL-6 to recruit different co-repressor complexes. For example, BCL-6–co-repressor (BCoR)/nuclear-receptor co-repressor (NCoR)/silencing mediator for retinoid and thyroid receptor (SMRT) complex seem to play a role in suppressing the genes that control growth and apoptosis. On the other hand, recruitment of the nucleosome remodelling and deacetylase (Mi-2-NuRD) complex switches off the genes that control the differentiation of the germinal centre B-cells.

Pituitary-specific transcription factor (Pit-1)

Pituitary-specific transcription factor (Pit-1) controls the development of some of the anterior pituitary endocrine cells such as the somatotrophs, lactotrophs and thyrotrophs. In the case of the somatotrophs, Pit-1 functions to control the expression of growth hormone (GH) and perhaps also the growth hormone-releasing hormone receptor (GHRH-R) (Module 10: Figure somatotroph regulation).
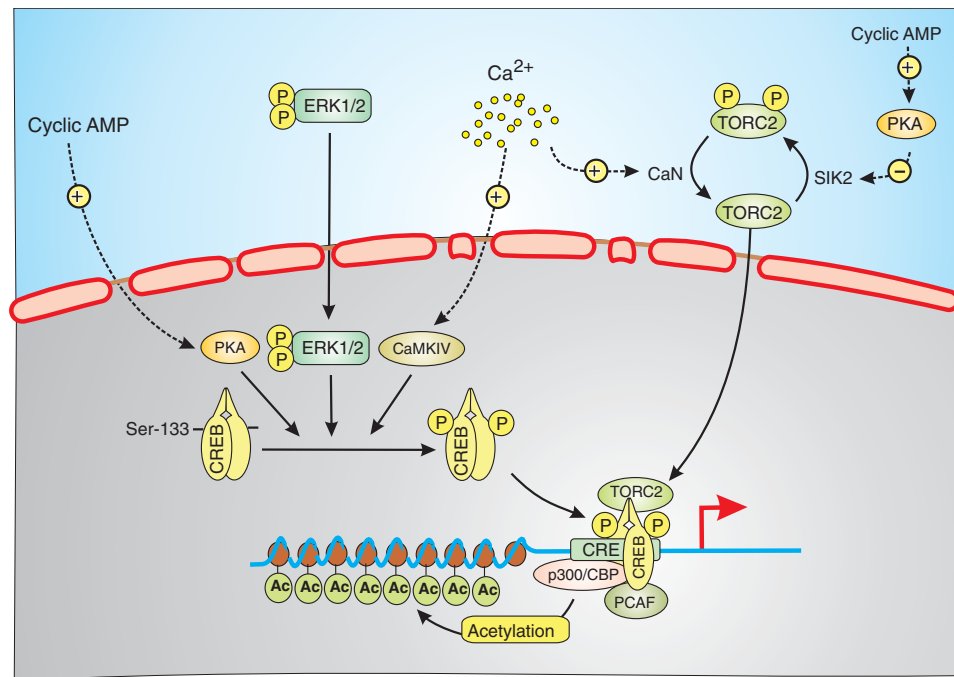
Activation of the cyclic AMP response element-binding protein (CREB).
Cyclic AMP response element-binding protein (CREB) is phosphorylated by a number of signalling pathways that employ different kinases to phosphorylate the critical residue Ser-133, which is essential for its transcriptional activity. In addition to activating CREB within the nucleus, some of these signalling pathways also regulate the activity of cofactors called transducers of regulated CREB (TORCs). Under resting conditions, TORC is phosphorylated and resides in the cytoplasm, but upon dephosphorylation, it enters the nucleus, where it acts together with CREB to promote transcription. TORC phosphorylation depends upon a Ca^{2+}-dependent dephosphorylation mediated by calcineurin (CaN) and the cyclic AMP/protein kinase A (PKA)-dependent inhibition of the salt-inducible kinase 2 (SIK2) that phosphorylates TORC.
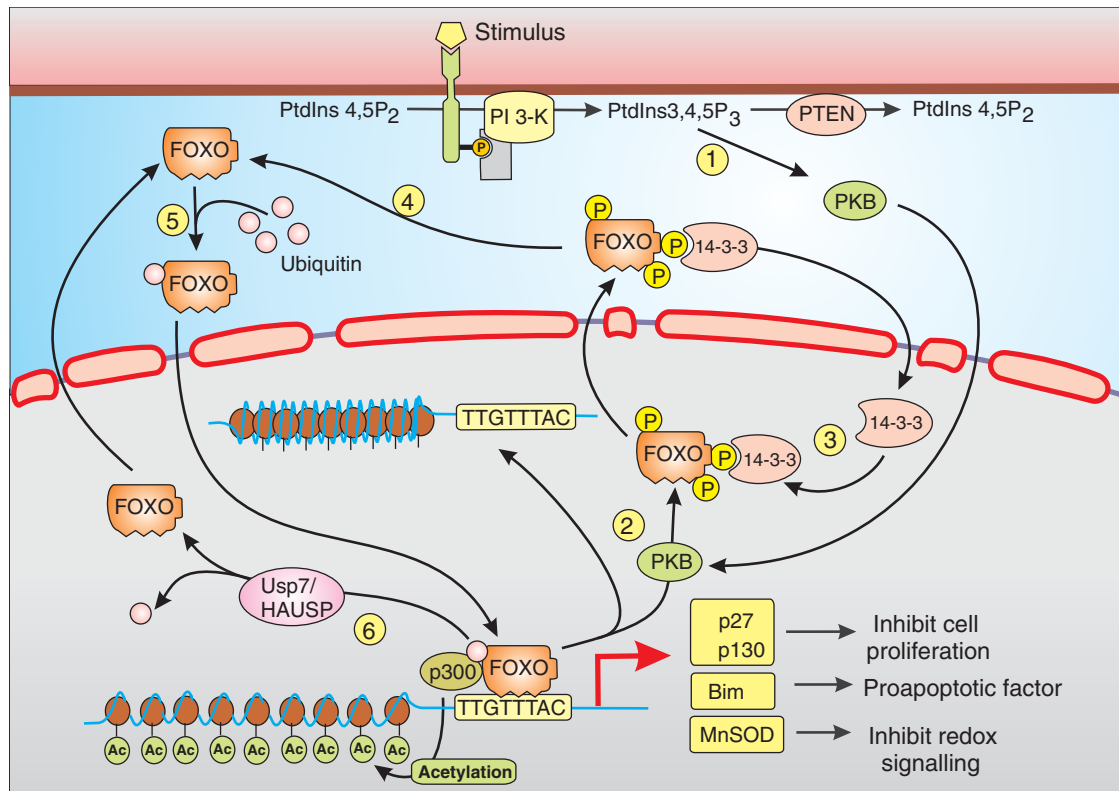
Forkhead box O (FOXO)

This family of forkhead transcription factors has been re-named as the forkhead box O (FOXO1, FOXO3a and FOXO4) factors. These resident nuclear factors, which are constitutively active, are examples of shuttle nuclear factors (Module 4: Figure transcription factor classification) that are regulated within the nucleus. The FOXO factors have a DNA-binding domain and their movement across the nuclear pores is mediated by a nuclear localization signal (NLS) and a nuclear export signal (NES). The PtdIns 3-kinase signalling cassette (Module 2: Figure PtdIns 3-kinase signalling) is responsible for inhibiting the transcriptional activity of FOXO, as illustrated in the sequence of events shown in Module 4: Figure FOXO control mechanisms:

1. External signals operating through the PtdIns 3-kinase signalling cassette generate the lipid second messenger PtdIns3,4,5P3, which then activates protein kinase B (PKB) (Module 2: Figure PtdIns 3-kinase signalling).
2. Activated PKB enters the nucleus and phosphorylates FOXO on threonine and serine residues, some of which lie within the NLS.
3. The phosphorylated FOXO is recognized by 14-3-3 protein, or by the exportin Crm1, which facilitate its export into the cytoplasm.
4. When the PtdIns 3-kinase signalling system is switched off, the phosphorylated FOXO in the cytoplasm can be dephosphorylated, enabling it to return to the nucleus.
5. Once it has been dephosphorylated, the FOXO is free to re-enter the nucleus to begin gene transcription. During stress, the FOXO4 isoform is monoubiquitinated and this facilitates its entry into the nucleus. The ubiquitin group may also facilitate its interaction with various transcriptional co-activators such as p300 (Module 4: Figure FOXO control mechanisms).
6. Such stress-induced activation is reversed by the ubiquitin-specific protease Usp7/HAUSP. Removal of ubiquitin enables FOXO4 to return to the cytoplasm.

The function of FOXO is linked to decisions related to cell proliferation/quiescence. Cells that are quiescent, i.e. at G_0, have various mechanisms for suppressing the cell cycle, and FOXO may play a role in this because it is known to code for components such as the cyclin-dependent kinase (CDK) inhibitor p27 and the retinoblastoma (Rb) family protein p130, which play a role inhibiting the cell cycle (Module 9: Figure cell cycle signalling mechanisms). FOXO3a regulates the transcription of manganese superoxide dismutase (MnSOD), which contributes to redox signalling in apoptosis by providing greater protection against reactive oxygen species (ROS)-induced apoptosis. FOXO can regulate the expression of Bim, which is a pro-apoptotic factor that contributes to the intrinsic

Module 4: | Figure FOXO control mechanisms

Control of forkhead box O (FOXO) proteins by the PtdIns 3-kinase signalling cassette.

The forkhead box O (FOXO) family are typical shuttle nuclear transcription factors that bind to a specific promoter sequence to activate the transcription of genes such as p27, p130, Bim and manganese superoxide dismutase (MnSOD). Like many other transcription factors, FOXO can bind p300, thereby acetylating histone to make DNA more accessible to transcription. The transcriptional activity of FOXO is inhibited by phosphorylation through the PtdIns 3-kinase signalling cassette, resulting in export from the nucleus into the cytoplasm. (See the text for details of the signalling pathway.)

pathway of apoptosis (Module 11: Figure Bcl-2 family functions).

The inactivation of FOXO by the PtdIns 3-kinase signalling cassette thus contributes to the sequence of molecular events that control the cell cycle and apoptosis when cells are stimulated to proliferate (Module 4: Figure FOXO control mechanisms). The ability of phosphatase and tensin homologue deleted on chromosome 10 (PTEN) to function as a tumour suppressor may well depend upon its ability to reduce the level of PtdIns3,4,5P$_3$, thus removing the inhibitory effect on PKB activation to allow FOXO to express the cell cycle regulators that prevent proliferation. The onset of tumorigenesis in renal and prostate carcinoma cells that have a defect in PTEN may thus result from an uncontrolled elevation of PtdIns3,4,5P$_3$ and inhibition of FOXO activity.

E twenty-six (ETS)

The E twenty-six (ETS) domain transcription factors are typical resident nuclear factors (Module 4: Figure transcription factor classification) that are activated primarily through the mitogen-activated protein kinase (MAPK) signalling pathway. They act together with the serum response factor (SRF) to activate many of the early response genes, such as Fos (Module 4: Figure ETS activation).

These ETS transcription factors are made up of a family of so-called ternary transcription factors that form a complex with DNA and with SRF. Typical members are ETS-1, ETS-2, the ETS-like transcription factor-1 (Elk-1), the SRF accessory protein-1 (Sap-1) and Net. These ETS transcription factors have four main domains They all have a winged helix–turn–helix DNA binding domain (ETS domain), which recognizes ETS-binding sites (EBS) that contain a conserved CGAA/T sequence. Some of the ETS family also has a Pointed (PNT) domain responsible for interactions with other proteins such as SRF. There is also an activation domain (AD) which is phosphorylated by the MAPK signalling pathway. The Elk-1 transcription factor regulates a variety of cellular processes:

- Phosphorylation of Elk-1 plays an important role in reversing the **differentiation of smooth muscle** when cells are switched back into a proliferative state.
- Elk-1 plays a role in the transcriptional events that occur in medium spiny neurons during drug addiction (**Module 10: Figure medium spiny neuron signalling**).
- KLF4 plays a role in regulating the differentiation of smooth muscle cells (Module 8: Figure smooth muscle cell differentiation).

Module 4: | Figure ETS activation
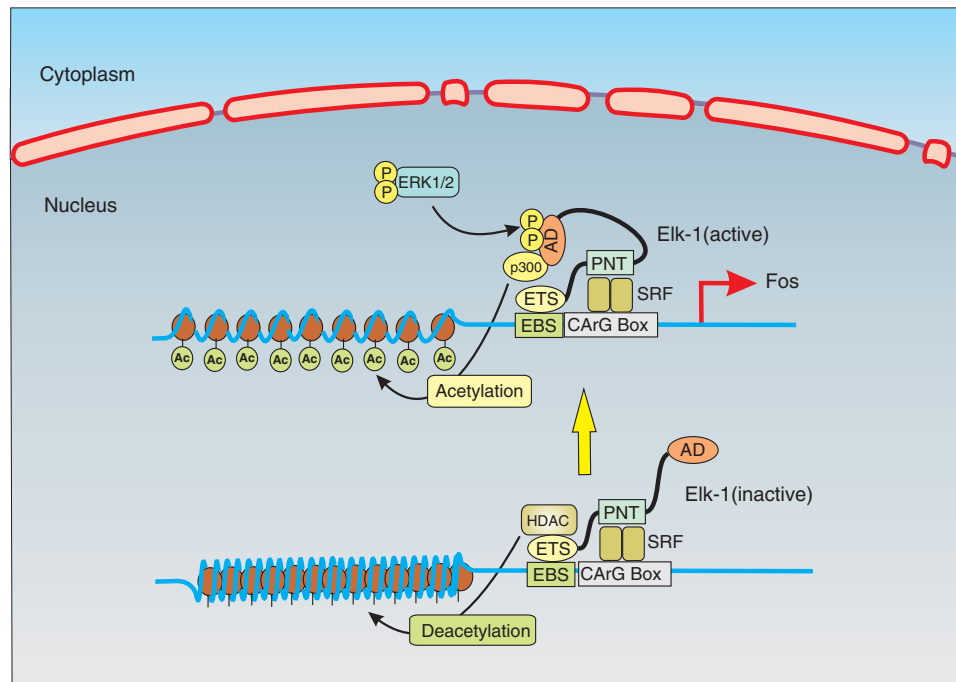
Gene activation through co-operation of the E twenty-six (ETS) and serum response factor (SRF) transcription factors.
A ternary complex is formed through DNA, the serum response factor (SRF) bound to the CArG box and the E twenty-six (ETS) transcription factors such as Elk-1, which bind to the ETS-binding site (EBS) on DNA and to SRF through their Pointed (PNT) domains. In the inactive state, as shown at the bottom, ETS functions as a repressor by recruiting inhibitors such as the histone deacetylases (HDACs), which deacylate histone to increase chromatin condensation. A major activator of transcription is the extracellular-signal-regulated kinase (ERK) pathway of the mitogen-activated protein kinase (MAPK) signalling pathway, with the effector ERK1/2 phosphorylating sites on the activation domain (AD). Just how this phosphorylation induces transcription is unclear, but there does appear to be an activation of histone acetyltransferases (HATs), such as p300, to increase histone acetylation to decondense the chromatin.

CSL (CBF-1, Suppressor of Hairless, Lag-1)

CSL (CBF-1, Suppressor of Hairless, Lag-1) is a transcriptional repressor that functions in the Notch signalling pathway (Module 2: Figure Notch signalling). It functions to repress Notch target genes by providing a framework to recruit co-repressors such as SMRT, SHARP, SKIP and CIR.

Methyl-CpG-binding protein 2 (MeCP2)

Methyl-CpG-binding protein 2 (MeCP2) is a transcriptional repressor that functions in gene silencing (Module 4: Figure MeCP2 activation). It has the methyl-CpG-binding domain that enables it to associate with those methyl-CpG islands that are found in the promoter regions of many genes.

MeCP2 is found in a number of cell types, but is mainly expressed in brain where it appears in postmitotic neurons where it functions as a regulator of neuronal gene expression. Levels of MeCP2 are low during early development but then increase progressively as neurons mature preceding the onset of synapse formation. Microarray analysis has begun to identify some of the MeCP2-regulated genes, which include brain-derived neurotrophic factor (BDNF), insulin-like growth factor binding protein 3 (IGFBP3), the ubiquitin ligase UBE3A, γ-aminobutyric acid (GABA) receptor and the inhibitor of DNA binding (Id) proteins. The latter function in the proliferation-dif-

ferentiation switch (Module 8: Figure proliferation-differentiation switch) and may thus be important in neuronal differentiation. In immature neurons, expression of the Ids suppresses a number of the differentiation genes. During neuronal maturation, MeCP2 represses the expression of the Ids and this unmasks the neuronal differentiation genes such as Neurod1.

MeCP2 is normally located in the nucleus associated with the methyl-CpG islands through its methyl-CpG-binding domain. It also has a transcriptional repression domain (TRD) and two nuclear localization signals (NLSs). The activity of MeCP2 is sensitive to various signalling pathways as illustrated in the following sequence of events (Module 4: Figure MeCP2 activation):

1. During neural activity, membrane depolarization (ΔV) activates voltage-operated channels (VOCs) that introduce Ca^{2+} into the cell.
2. Ca^{2+} acts through Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV) to phosphorylate the MeCP2 that is bound to mCpG islands causing it to leave the DNA. In addition, CaMKIV also phosphorylates the transcription factor CREB as part of the gene activation mechanism.
3. MeCP2 is a multifunctional protein that interacts with a number of other proteins such as DNA methyl transferases 1 (DNMT1) and the transcriptional

co-repressors switch independent (SIN3) that recruits histone deacetylase (HDAC) to function in chromatin remodelling. Histone deacetylation by HDAC inhibits gene transcription. Once MeCP2 is removed, these remodelling complexes also leave the DNA, preparing the way for the onset of gene transcription.

4. An important aspect of this onset of gene transcription is the activation of CREB by phosphorylation of Ser-133 by CaMKIV or by the cyclic AMP signalling pathway.

5. CREB binds to the CRE element and helps to build up a transcriptional complex that includes chromatin remodelling components such as p300 that acetylate the histones to open up the chromatin so that transcription can proceed.

6. One of the functions of MeCP2 is to regulate the gene coding for brain-derived neurotrophic factor (BDNF), which is essential for neural development especially the changes in synaptic plasticity associated with learning and memory such as long-term potentiation (LTP). MeCP2 also regulates the expression of neuroligin 1, which plays an important role in synaptic plasticity by forming and stabilizing the contact between pre- and post-synaptic endings (Module 10: Figure postsynaptic density).

Mutations in the *MECP2* gene that codes for MeCP2, which is the primary cause of Rett syndrome, have also been found in a number of other neurological disorders such as autism, Angelman syndrome (AS), neonatal encephalopathy and X-linked mental retardation. Microglial inflammation in Alzheimer's disease results in MeCP2 activation and a decrease in the expression of neuroligin 1, which may play a role in the loss of memory associated with this neurodegenerative disorder (see step 6 in Module 12: Figure Inflammation and Alzheimer's disease).

Myocyte enhancer factor-2 (MEF2)
The myocyte enhancer factor-2 (MEF2) plays a role in cell proliferation and differentiation. As its name implies, the MEF2 family, which belongs to the MADS family of transcription factors that includes serum response factor (SRF), was originally discovered in muscle cells, but is now known to regulate important decisions in cell proliferation and differentiation in many other cell types. The family consists of four members (MEF2A, MEF2B, MEF2C and MEF2D), all encoded by separate genes. The N-terminal region has a MADS domain responsible for dimerization and for DNA binding. Next to the MADS domain there is a MEF2-specific domain that enables it to bind to a number of other transcription factors and cofactors, such as MEF2 itself, to form a homodimer. The function of MEF2 is very much defined by this ability to associate with other factors, some of which are activators, such as MyoD (Module 4: Figure MyoD and muscle differentiation) and the nuclear factor of activated T cells (NFAT) (Module 4: Figure NFAT activation), whereas others are inhibitory [e.g. the histone deacetylases (HDACs) and Cabin1].

The function of MEF2 is particularly sensitive to Ca^{2+} signalling. Since MEF2 is a resident nuclear factor (Module 4: Figure transcription factor classification), it is activated by a sequence of Ca^{2+}-sensitive steps within the nucleus (Module 4: Figure MEF2 activation):

1. In the absence of Ca^{2+} signals, MEF2 acts as a repressor to inhibit transcription by associating with various negative regulators. It binds the class II histone deacetylases (HDAC4, HDAC5, HDAC7 and HDAC9), which induce chromatin condensation and inhibit transcription by deacetylating the N-terminal tails of histones.

2. An increase in nuclear Ca^{2+} binds to calmodulin (CaM) within the nucleus.

3. The Ca^{2+}/CaM activates the resident nuclear Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV).

4. The Ca^{2+}/CaM/CaMK IV complex phosphorylates the class II HDACs on two conserved serine residues.

5. The phosphorylated HDAC is recognized by 14-3-3 protein, which removes it from MEF2 and assists its export from the nucleus into the cytosol.

6. In some cells, such as T cells, CaM can have a more direct role in activating MEF2 when it is bound through Cabin1 to the class I HDACs (HDAC1 and HDAC2) through the co-repressor switch independent (SIN3). The Ca^{2+}/CaM binds to Cabin1 to release both the Cabin1/SIN3 complex and the class I HDACs.

7. Once the HDACs have been removed (Steps 5 and 6), the MEF2 is able to bind activators such as NFAT, CREB and p300. The CaMK IV facilitates this process by phosphorylating CREB.

8. The calcineurin (CaN) that is associated with NFAT helps to activate MEF2 by removing an inhibitory phosphate.

9. The recruitment of p300 acetylates the histones to facilitate transcription by opening up the chromatin.

10. Activation of the MEF2 promotor results in an increase in the transcription of PGC-1α and many other genes responsible for multiple processes such as mitochondrial biogenesis and fatty acid oxidation.

MEF2 functions in the regulation of a number of processes, many of which are related to morphogenesis:

- The formation of slow-twitch skeletal muscle during the neural control of differentiation.
- Remodelling the cardiac signalsome during the onset of hypertrophy (Module 12: Figure hypertrophy signalling mechanisms).
- Smooth muscle cell proliferation.

Activating protein 1 (AP-1) (Fos/Jun)
The activating protein 1 (AP-1) family has an important function in regulating cell proliferation. It is a heterodimer formed by the association between members of the Jun family (c-Jun, Jun B and Jun D) and the Fos family (c-Fos, Fos B, Fra-1 and Fra-2). These two genes are classical examples of 'immediate early genes' that are activated rapidly following growth factor stimulation. This Fos/Jun dimer is held together by a leucine zipper (Module 4: Figure SRF and AP-1).

Module 4: | Figure MeCP2 activation
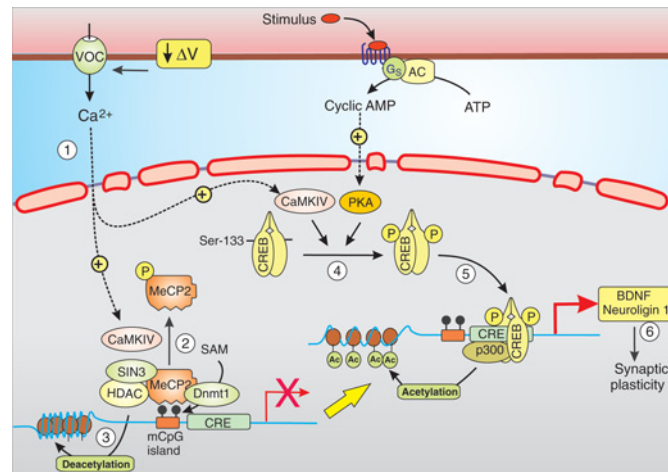
Activation of methyl-CpG-binding protein 2 (MeCP2).

Many genes that contain methyl CpG (mCpG) islands are silenced by MeCP2 that recruits histone deacetylases (HDACs) and DNA methyl transferases 1 (DNMT1) to alter chromatin structures through histone deacylation and DNA methylation respectively. An increase in Ca^{2+} leads to phosphorylation of MeCP2 that leaves the DNA enabling gene transcription to proceed through transcription factors such as CREB. One of the genes controlled by this mechanism is brain-derived neurotrophic factor (BDNF) that functions in synaptic plasticity.

Module 4: | Figure MEF2 activation
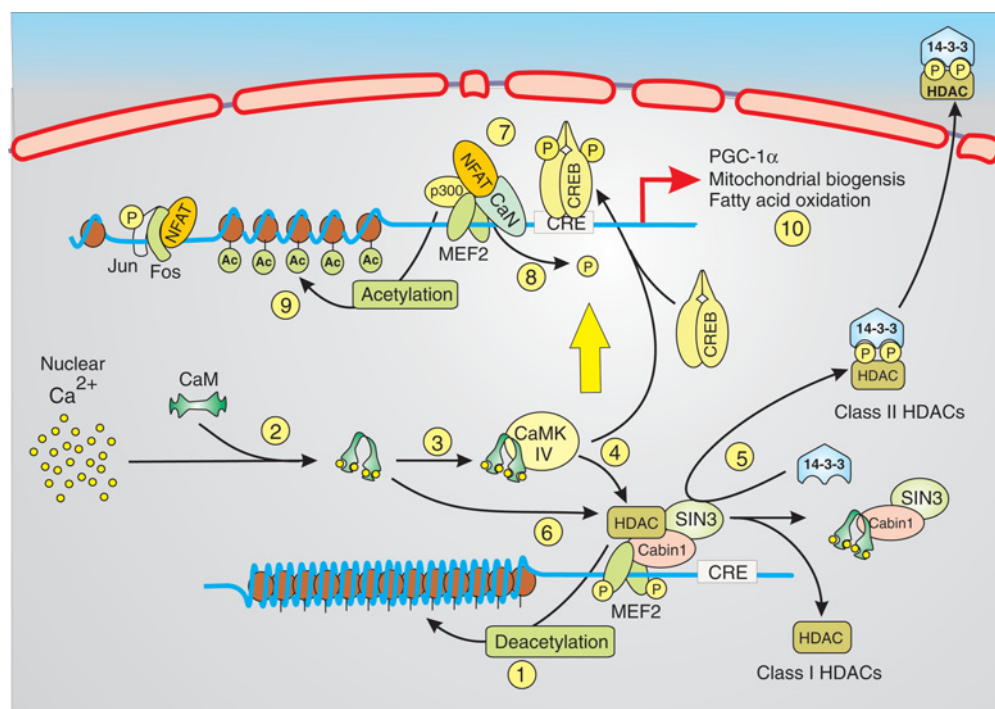
Function of nuclear Ca^{2+} in myocyte enhancer factor-2 (MEF2) activation.

There are a number of ways in which an increase in the level of Ca^{2+} within the nucleus can influence the conversion of MEF2 from a repressor into an activator of transcription. (See the text for the sequence of Ca^{2+}-activated events.)

The activation of AP-1 (Fos/Jun) has two components. Firstly, growth factors and other stimuli can induce a rapid transcription of both Fos and Jun family members. Fos transcription is activated by the serum response factor (SRF), whereas Jun is controlled by a combination of Jun and activating transcription factor (ATF). Once these two transcription factors are formed, they zipper up to form the dimer that then goes on to activate transcription of a set of genes that have a 12-*O*-tetradecanoylphorbol 13-acetate (also called phorbol 12-myristate 13-acetate) (TPA)-responsive element (TRE). Secondly, the transcriptional activity of AP-1 (Fos/Jun) is regulated by both phosphorylation and by redox signalling. The c-Jun N-terminal kinase (JNK) pathway plays a major role by

Module 4: | Figure SRF and AP-1
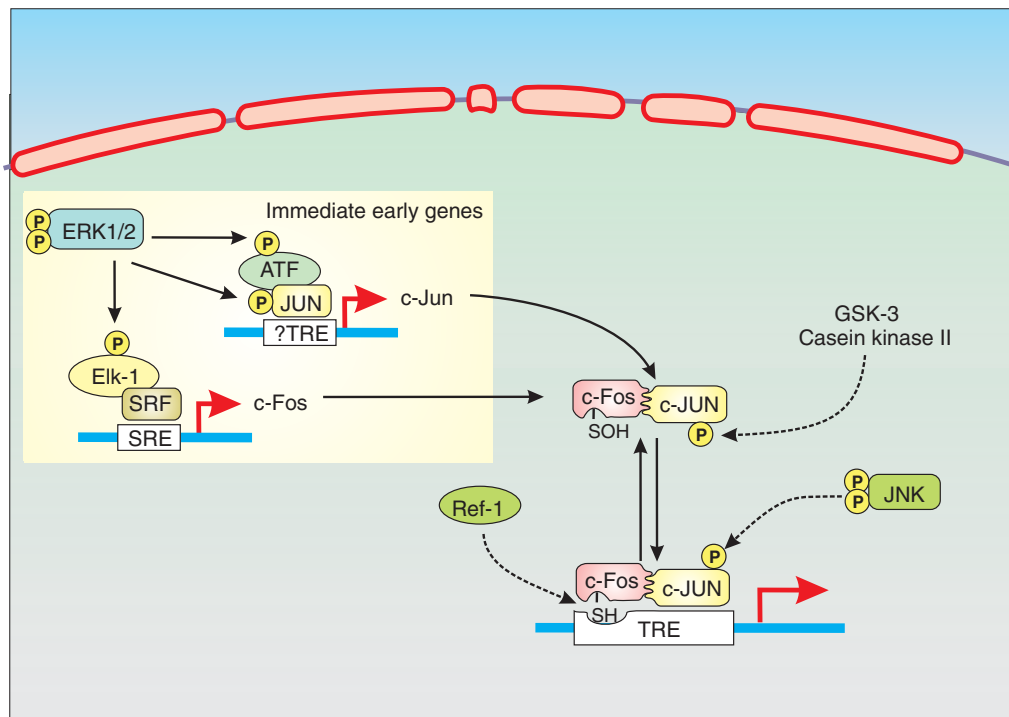
Activation of serum response factor (SRF) and activating protein-1 (AP-1).
One of the earliest consequences of growth factor activity is to stimulate the transcription of the transcription factors c-Fos and c-Jun. The latter then forms a dimer through a leucine zipper and binds to the 12-O-tetradecanoylphorbol 13-acetate (also called phorbol 12-myristate 13-acetate) (TPA)-responsive element (TRE). The interaction with TRE is regulated both by phosphorylation and by the oxidation state of a highly conserved cysteine residue located in the DNA-binding domain. A redox factor 1 (Ref-1) controls the redox state of a cysteine residue in the DNA-binding domain.

phosphorylating Jun (Module 2: Figure JNK signalling). Transcription can also be inhibited by glycogen synthase kinase-3 (GSK-3) and casein kinase II (CK2), which phosphorylate sites on the DNA-binding domain. A link between redox signalling and gene transcription has also been identified for AP-1 (Fos/Jun) (Module 4: Figure SRF and AP-1). Binding to DNA is very dependent on the oxidation state of a critical cysteine residue located in the DNA-binding domain. The nucleus contains a redox factor 1 (Ref-1), which functions to control transcription by reducing this cysteine residue.

The binding of AP-1 (Fos/Jun) can also support the binding of other transcription factors to form larger transcriptional complexes. An example is the interaction of AP-1 with nuclear factor erythroid 2 related factor 2 (NRF-2) (Module 4: Figure NRF-2 antioxidant function) and nuclear factor of activated T cells (NFAT) (Module 4: Figure NFAT activation). The organization of the NFAT/AP-1 complex is shown in Module 4: Figure NFAT/AP-1/DNA complex. AP-1 also contributes to the transcriptional activity of hypoxia-inducible factor (HIF) (Module 4: Figure HIF activation).

Serum response factor (SRF)
Serum response factor (SRF) belongs to a small family of MADS (MCM1, Agamous, Deficiens, SRF) transcription factors that include the myocyte enhancer factor-2

(MEF2) family. The 57-amino-acid MADS-box enables these transcription factors to dimerize and bind to DNA to regulate approximately 160 genes that control processes such as cell growth, migration and muscle cell differentiation. SRF may also regulate survival through its control of the anti-apoptotic Bcl-2 gene. The activity of SRF is very dependent on coactivators, particularly members of the myocardin family.

SRF is stimulated by growth factors and plays an important role by activating the transcription of c-Fos, which is a component of activating protein 1 (AP-1) (Fos/Jun). The SRF, which is associated with p62TCF, binds to the serum response element (SRE) of the c-Jun promoter. The extracellular-signal-regulated kinase (ERK) pathway plays an important role in stimulating transcription by phosphorylating the ETS-like transcription factor-1 (Elk-1) (p62TCF), which is a cofactor of SRF (Module 4: Figure SRF and AP-1).

One of the functions of SRF is to participate in the action of either myocardin or the E twenty-six (ETS) transcription factors (Module 4: Figure ETS activation), as occurs during the proliferation/differentiation switch during the differentiation of smooth muscle (Module 8: Figure smooth muscle cell differentiation). In the presence of the transcriptional coactivator myocardin, the SRF stimulates the genes responsible for smooth muscle cell differentiation. SRF is also activated by the myocardin-related

transcription factors (MRTFs), which provide a critical link between actin dynamics and gene transcription (Module 4: Figure actin dynamics and gene transcription). SRF regulates the expression of a large number of genes and many of these encode proteins that function in actin formation and in the coupling of actin to the integrins.

SRF also plays a role in the differentiation of cardiac cells (Module 8: Figure cardiac development) and in the regulation of miR-133a to control Ca^{2+} signalling and cardiac hypertrophy (Module 12: Figure miRNA and cardiac hypertrophy).

Transcription initiation factor IB (TIF-IB)

Cell growth requires protein synthesis, which in turn depends on ribosomal RNA (rRNA) transcription and ribosome formation. Transcription of rRNA is carried out by RNA polymerase I (Pol I), which is controlled by various transcription factors such as transcription initiation factor IB (TIF-IB), which is a multimeric protein complex containing TATA-binding protein (TBP) and four TBP-associated factors (TAFs) that are specific for polymerase I transcription. One of these TAFs is TAF$_1$68, which is regulated by reversible acetylation. When it is acetylated by PCAF, there is an increase in its DNA-binding activity and this increases Pol I transcription. This activation is reversed following deacylation of TAF$_1$68.

X-Box binding protein 1 (XBP-1)

The X-Box binding protein 1 (XBP-1) acts to regulate the transcription of genes that function in both immune responses and in the endoplasmic reticulum (ER) stress signalling pathway (for details see step 3 in Module 2: Figure ER stress signalling).

Krüppel-like factors (KLFs)

The Krüppel-like factors (KLFs), which take their name from the *Drosophila* Krüppel protein, function in a number of cellular processes such as proliferation, differentiation and survival. The C-terminus has three zinc fingers (Cys2 His2) that are separated from each other by a highly conserved H/C link.

In the case of the differentiation of white fat cells, KLF2 inhibits transcription of the *Pparg* gene in the preadipocytes but is replaced by stimulatory KFL5 and KFL15 isoforms during the process of differentiation (Module 8: Figure white fat cell differentiation). KLF4 plays a role in regulating the differentiation of smooth muscle cells (Module 8: Figure smooth muscle cell differentiation).

Activation of transcription factors by regulating their expression

The activity of some transcription factors is determined by adjusting their intracellular concentration. In the case of Myc, this is achieved through a growth factor-dependent increase in expression.

Myc

Myc was identified as a component of the myelocytomatosis transforming virus (v-Myc) and was subsequently found to be a normal human gene, which plays a central role in the control of cell proliferation (Module 9: Figure G$_1$ proliferative signalling). Myc structure reveals that it is a member of the basic helix–loop–helix leucine zipper (bHLH-Zip) group of transcription factors. Since Myc has a short half-life (approximately 20 min), its level in quiescent cells is low. When cells are stimulated to grow, Myc formation is markedly increased by growth factor signalling pathways that activate Myc transcription. Myc action depends upon it binding to another bHLH-Zip protein called Max to form a Myc/Max heterodimer, which then activates a large number of Myc targets. Most of these targets function in the control of cell proliferation. Given this central role of Myc in the regulation of cell proliferation, it is not surprising to find a strong link between Myc dysregulation and cancer development.

Myc structure

The Myc family of transcription factors is composed of four members (c-Myc, N-Myc, L-Myc and S-Myc). Most attention has focused on the first three members, which have been strongly implicated in the genesis of human cancers. Myc belongs to the basic helix–loop–helix leucine zipper (bHLH-Zip) group of transcription factors (Module 4: Figure Myc structure).

Myc formation

The formation of Myc is dependent on the action of growth factors that act to rapidly stimulate its transcription. Myc is thus a typical immediate early gene in that it is rapidly switched on when cells are stimulated to grow and proliferate. Myc has a short half-life (20 min), and its function in the cell is determined by its rate of formation. It is the increase in Myc concentration that enables it to bind to its partner Max to form the active dimers that are responsible for Myc action to stimulate transcription. The level of Myc is determined by a balance between Myc formation and Myc degradation.

The Myc promoter contains binding sites for the nuclear factor of activated T cells c1 (NFATc1) isoform of the Ca^{2+}-sensitive transcription factor NFAT, suggesting that the Ca^{2+} signalling pathway may play a role in stimulating the expression of Myc. This activation pathway may be relevant to Myc dysregulation and cancer development, because large amounts of NFATc1 are expressed in pancreatic cancer cells.

Myc degradation

The stability of Myc is regulated by various cell signalling pathways. For example, the mitogen-activated protein kinase (MAPK) pathway acts to stabilize Myc by phosphorylating Ser-62 (Module 4: Figure Myc as a gene activator). On the other hand, glycogen synthase kinase-3 (GSK-3) phosphorylates Thr-58, which destabilizes Myc by initiating its degradation through the ubiquitin-proteasome system. The PtdIns 3-kinase signalling pathway can prevent this degradation by inhibiting the phosphorylation of Ser-58 by GSK-3.

A prolyl isomerase (PIN1) recognizes the phosphorylated Thr-58 and this then leads to isomerization of Pro-59, which in turn allows the protein phosphatase 2A (PP2A) to remove the stabilizing phosphate on Ser-58.

Module 4: | Figure Myc structure
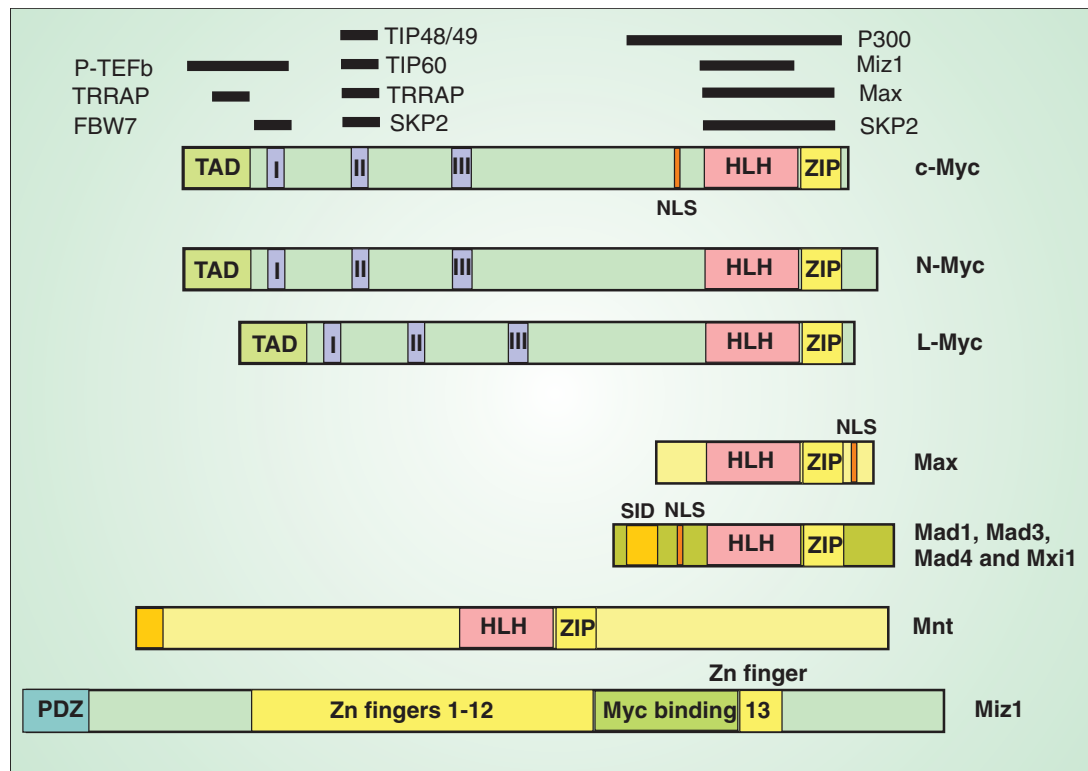
Structure of the Myc family and their associated proteins Max, Mad, Mnt and Miz.
The three main Myc proteins are shown at the top. The C-terminal region contains the basic helix–loop–helix (HLH) and the associated leucine zipper (ZIP) domains. The N-terminal region contains a transactivation domain (TAD) and the conserved Myc-boxes (I–III), which play an important role in binding to various transcriptional coactivators (denoted by the black bars shown above the c-Myc structure). The HLH-ZIP domains of Myc bind to similar domains on its partner Max. Max also binds to the Mads, Mxi1 and Mnt, which also have HLH-ZIP domains. Miz1 is a zinc-finger protein that also binds to Myc through a Myc-binding domain that does not depend on the HLH-ZIP domain. A nuclear localization signal (NLS) is located on c-Myc, Max and Mad1.

The phosphate that remains at Thr-58 is then recognized by the SCF ubiquitin ligases Fbw7 and Skp2 (SCF^{Fbw7} and SCF^{Skp2}), which label it for degradation by the proteasome. The ubiquitin-specific protease Usp28 can stabilize Myc by removing the ubiquitin groups that target it for degradation by the proteasome.

Myc action
Myc appears to be a master gene in that it can regulate up to 15% of all genes. The action of Myc is complex in that it can function by either activating or repressing genes, depending on its various binding partners. In general, it activates those genes that promote the cell cycle, such as the cyclins D_1 and D_2, and cyclin dependent kinase 4 (CDK4) (Module 4: Figure Myc as a gene activator), whereas it represses those genes that inhibit the cell cycle, such as the various CDK inhibitors p27, p21 and p15 (Module 4: Figure Myc as a gene repressor).

The transcriptional activity of Myc depends upon it forming a dimer by combining with another basic helix–loop–helix zipper (bHLH-Zip) protein, Max (Module 4: Figure Myc structure), to form a Myc/Max heterodimer that functions to regulate a large number of gene targets that control both cell growth and the cell cycle. When Myc functions in gene activation, the Myc/Max dimer binds to

the E-box of those genes that function in cell growth or the cell cycle (Module 4: Figure Myc as a gene activator). When cells are either quiescent (G_0) or differentiated, these genes are silenced by the binding of dimers formed between Max and its other partners, the transcriptional repressors such as Mad or Mnt. These repressors act by recruiting the transcriptional co-repressor switch independent (SIN3), which assembles a chromatin remodelling complex that contains histone deacetylases that remove acetyl groups from the histones. When the level of Myc rises, it displaces the repressors (Mads and Mnt) by binding to Max to form the Myc/Max activation complex.

The repressor action of the Myc/Max complex is achieved by interfering with the activation of genes that are normally induced by the transcription factor Miz1 (Module 4: Figure Myc as a gene repressor). Some of these genes code for the CDK inhibitors such as p15 and p21, which function to inhibit events during the G_1 phase of the cell cycle (Module 9: Figure cell cycle signalling mechanisms). In the case of the p21 gene, which is activated by p53 (Module 4: Figure p53 function), the Myc/Max dimer inactivates the Miz1 bound to the initiator (INR) site (Module 4: Figure Myc as a gene repressor). The transforming growth factor β (TGF-β) inhibition of cell proliferation depends upon the Smad signalling pathway that uses the activated

Smad1/4 complex to bind to the serum response factor (SRF) site on the promoter of the p15 gene (Module 4: Figure Myc as a gene repressor).

Many of the Myc targets code for components that act to regulate the cell cycle.

Myc targets

Many of the targets activated or repressed by Myc play a critical role in promoting cell cycle progression and cell growth during the early G_1 phase (Module 9: Figure proliferation signalling network). Myc functions in a transcriptional pathway that results in the expression of various components of the proliferative signalling pathways, such as activators of the cell cycle [cyclin D_1, cyclin D_2 and cyclin-dependent kinase 4 (CDK4)] that operate during G_1 to drive the cell into S phase (Module 4: Figure Myc as a gene activator). Myc also increases the expression of the inhibitor of DNA binding (Id) proteins (Ids) that help promote proliferation by switching off the cyclin-dependent kinase (CDK) inhibitor p16^{INK4a} (Module 8: Figure proliferation-differentiation switch). In addition, it can repress genes that normally inhibit cell cycle progression, such as the CDK inhibitors p15 and p21 (Module 4: Figure Myc as a gene repressor).

Another important target for Myc is to activate the expression of the alternative reading frame (ARF) tumour repressor, which inhibits the mouse double minute-2 (MDM2) E3 ligase that degrades p53 (Module 9: Figure proliferation signalling network). In this way, Myc activates the p53 surveillance mechanism, which can thus result in apoptosis. This indicates that Myc can activate both proliferation and apoptosis. This apparent paradox can probably be explained by a concentration effect of Myc. Under normal growth stimulation, the level of Myc may not increase enough to activate ARF to trigger apoptosis, but this pathway may come into play when Myc levels are abnormally increased as might occur during Myc dysregulation and cancer development.

With regard to cell growth, Myc plays a central role by controlling the biogenesis of ribosomes. Myc has been shown to increase the transcription of all three of the RNA polymerases (RNA Pol I, RNA Pol II and RNA Pol III).

Associated with this activation of cell growth, Myc can also stimulate cell metabolism by increasing the expression of lactate dehydrogenase (LDH), which may be particularly important during cancer development. Myc can also increase the expression of serine hydroxymethyl transferase (SHMT), which results in an increase in the metabolic pathway that generates one-carbon units.

MicroRNA (miRNA)

The microRNAs (miRs) are a large class of highly conserved non-coding small RNAs (approximately 20–26 nucleotides) that function as post-transcriptional regulators. They are key regulators of gene silencing by binding to the 3' untranslated region (UTR) of specific target mRNAs to influence both their stability and translation. Uncovering the processes responsible for microRNA biogenesis have revealed a highly regulated sequence of events with numerous positive- and negative-feedback loops that enhances the robustness of this regulatory mechanism. So far, approximately 650 human miRNAs have been identified. The task of establishing microRNA properties and function of individual miRs is ongoing and already there are indications that each miR can modulate the activity of up to 100 mRNAs to influence a large number of key biological processes:

- Maintenance of embryonic stem (ES) cell pluripotency.
- MicroRNA modulation of cell-cycle regulatory mechanisms
- p53 functions and microRNAs
- MicroRNA regulation of differentiation
    Differentiation of cardiac cells
    Differentiation of smooth muscle cells
- Cell proliferation
- Apoptosis
- Stress responses.
- MicroRNA dysregulation and cancer

MicroRNA biogenesis

The first step in microRNA biogenesis is the transcription of the miR gene by RNA polymerase II (Pol II) to form the primary miRNA (pri-miRNA) transcripts, which has a characteristic stem-loop structure and a 5' capped polyadenylated (poly A) tail (Module 4: Figure microRNA biogenesis). This pri-miRNA is recognized by Drosha, which is a double-stranded RNA-specific nuclease that acts together with DiGeorge syndrome critical region 8 (DGCR8, also known as Pasha) to cleave pri-miRNA to form pre-miRNA. This pre-miRNA is then exported from the nucleus by Exportin 5, which is a RAN-GTP-dependent nuclear transport receptor. Once it enters the cytoplasm, the pre-miRNA is recognized by the trans-activator RNA-binding protein (TRBP) and the ribonuclease Dicer, which cleaves the precursor to form the mature microRNA (miR).

The miR acts by binding to Argonaute (Ago1–4) proteins to form a RNA-silencing complex (RISC) that recognizes and inhibits target messenger RNAs (mRNAs). A short 'seed region' between bases 2 and 7 at the 5' end of the miRNA is responsible for recognizing and binding to the 3' untranslated region (UTR) of their target mRNAs to inhibit protein synthesis through a number of mechanisms such as translational repression, mRNA deadenylation and mRNA degradation. The translational repression of protein synthesis can occur through inhibition of either EIF4E to prevent initiation or the ribosomes to block elongation. Expression can also be inhibited by deadenylation of the poly-A tail by activation of the CCR4-NOT. The RISC complex can also bring about direct degradation of mRNA.

MicroRNA properties and function

The standard process of microRNA biogenesis generates approximately 650 microRNAs (miRs) with widely different properties and functions. The properties and functions of some of the well established miRs are described below:

Module 4: | Figure Myc as a gene activator
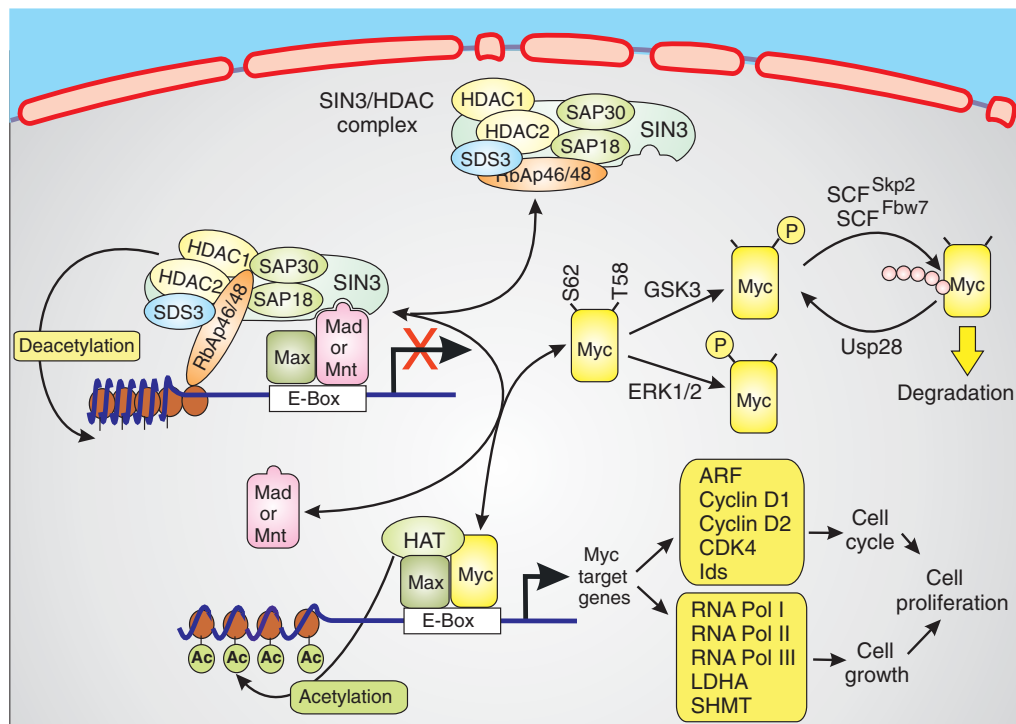
Myc activation of target genes that function in cell proliferation.
There are a large number of Myc target genes that are activated by an increase in the level of Myc. Many of these Myc targets have an E-box element containing the 5'-CACGTG-3' sequence. In quiescent G0 or differentiated cells, the many Myc target genes are repressed by Max/Mad or Max/Mnt dimers, together with the SIN3/HDAC transcriptional co-repressor complex. The HDACs deacetylate chromatin, making it inaccessible to transcription. Myc activates transcription by replacing the Mad/Mnt, which removes the repressor complex. The Myc/Max dimer bind to the E-box and provides a complex to bring in various coactivators. The histone acetyltransferases (HATs), such as CBP/p300, CGN5 and TIP60, acetylate histones to open up the chromatin to make it accessible so that the Myc target genes can be transcribed. These target genes contribute to cell growth and activation of the cell cycle.

Let-7

The family of let-7 miRNAs has twelve members (let-7-a1, let-7-a2, let-7-a3, let-7-b, let-7-c, let-7-d, let-7-f1, let-7-f2, let-7-g, let-7-I and miR-98). They are located on eight different chromosomal locations and are related to each other by having identical seed sequences and may thus act on similar targets. The level of let-7 is very low in embryonic stem (ES) cells and progenitor cells, but is an important contributor to the microRNA regulation of differentiation (Module 8: Figure ES cell miRNAs). The level of let-7 in ES cells is kept low through the RNA-binding proteins LIN28 and LIN28B through a double-negative-feedback loop. Down-regulation of let-7 by LIN28 and LIN28B has an important role maintaining the pluripotency of embryonic stem cells (ES).

The increase in let-7 that occurs during differentiation is reversed during the development of cancer, where a progressive decline in the level of let-7 coincides with an increase in the expression of the genes that control proliferation. There appears to be a direct correlation between these events because many of the let-7 target proteins are proliferative signals such as Ras and cMyc and cell-cycle components such as CDC25A and CDK6. They also regulate various embryonic genes such as high mobility group box A2 (HMGA2), insulin-like growth factor II mRNA binding protein 1 (IMP-1) and Mlin-41.

### Insulin-like growth factor II mRNA binding protein 1 (IMP1)

The insulin-like growth factor II mRNA-binding protein 1 (IMP) family has three members, IMP1–3, that function in post-transcriptional regulation including processes such as RNA trafficking, stabilization and translation. They have been identified as a target of the miRNA let-7 that helps to orchestrate developmental processes by controlling the expression of various embryonic genes. IMP1, which is also known as the coding region determinant-binding protein (CRD-BP), plays a role in cell proliferation and survival by stabilizing various target mRNAs (e.g. IGF-II, c-myc, tau, FMR1, semaphorin and βTrCP1) by shielding them against degradation.

IMP1 is overexpressed in various human neoplasias

Mlin41

Like *C. elegans* lin-41, mouse lin41 (Mlin41) is regulated by let-7 and miR-125 miRNAs. In a reciprocal manner, Mlin41 co-operates with the pluripotency factor Lin-28 in suppressing let-7 activity, revealing a dual control mechanism regulating let-7 in stem cells. The ability on Mlin41 to silence let-7 may depend on its interaction with Dicer and the Argonaute proteins.

The human homologue of lin41 (Hlin41), which is also known as tripartite motif-containing 71 (TRIM71), is a

Module 4: | Figure Myc as a gene repressor
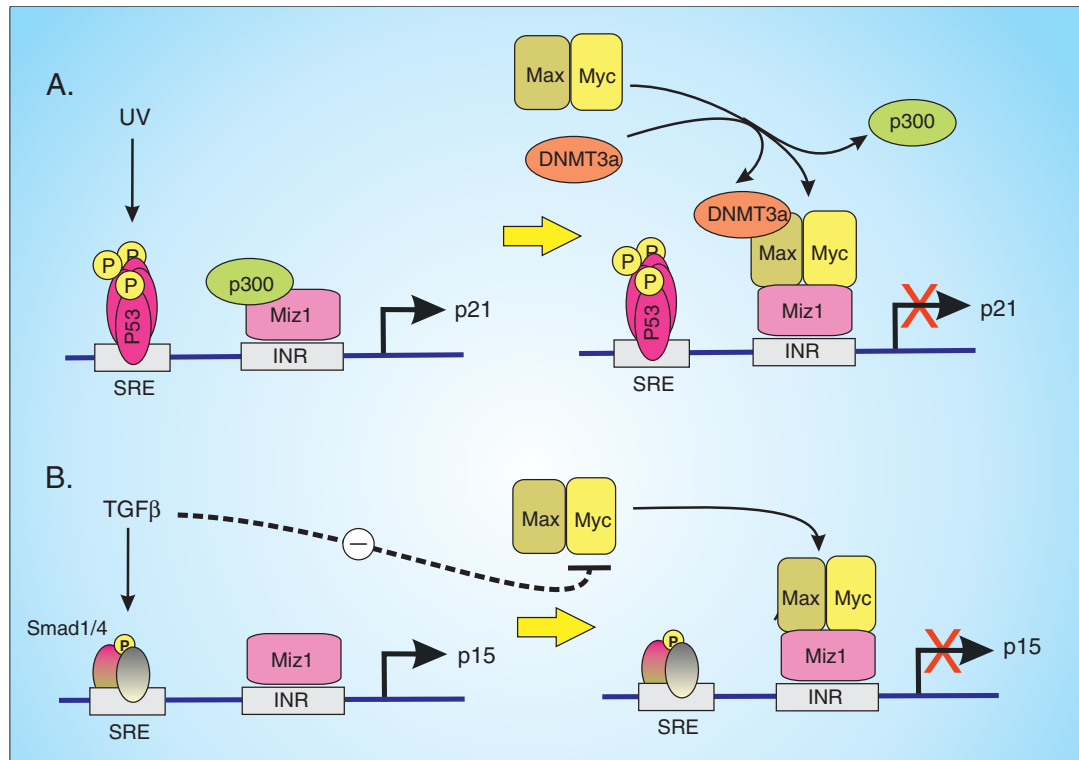
Myc represses genes activated by Miz1.
Activation of some of the cyclin-dependent kinase (CDK) inhibitors, such as p15 and p21, are repressed by Myc. A. The p21 gene, which is activated through UV irradiation and p53, is also activated at the initiator (INR) site by Miz1, which binds p300 that acts to acetylate chromatin to open up the locus for transcription. Myc/Max dimers inhibit this transcription by binding to Miz1 to displace the p300, and it also brings in the DNA methyltransferase DNMT3a. B. The p15 gene is also activated by Miz1. This gene is also controlled by transforming growth factor β (TGF-β), which has two actions. It uses activated Smad1/4 to bind to the serum response element (SRE) site, and it also inhibits Myc, which is a potent repressor of Miz1. An increase in Myc levels can silence this gene.

potential human developmental and disease gene because Mlin41 is required for neural tube closure and survival.

miR-1 and miR-133
Closely related forms of miR-1 and miR-133 occur as clusters on the same chromosomal locus and are transcribed together to control muscle differentiation. For example, the *miR-1-1/miR-133a-2* gene cluster is transcribed together during the differentiation of cardiac cells (Module 8: Figure cardiac development). The miR-133a continues to function in differentiated cardiac muscle cells where it restrains the expression of Ca^{2+} signalling components such as the type 2 inositol 1,4,5-trisphosphate receptor ($InsP_3R2$), calcineurin (CaN) and the nuclear factor of activated T cells 3 (NFAT3) (Module 12: Figure miRNA and cardiac hypertrophy). However, in response to signals that trigger hypertrophy, the Ca^{2+} released by the $InsP_3R2$ reduces the expression of miR-133a and results in a co-ordinated increase in the expression of the $InsP_3R2s$, CaN and NFAT3 to set up a positive-feedback loop that strongly promotes the onset of hypertrophy.

During the differentiation of skeletal muscle, MyoD enhances the expression of these miRNAs that then have different roles in promoting the proliferation-differentiation switch in that miR-133 inhibits proliferation whereas miR-

1 helps to promote differentiation (see step **2** in Module 8: Figure skeletal muscle myogenesis).

miR-15 and miR-16
One of the functions of these two miRNAs is to inhibit the activin receptor type IIA (ACVRIIA) (Module 2: Table Smad signalling toolkit), which is stimulated during early development by Nodal that acts through the Smad signalling pathway (Module 2: Figure Smad signalling). Expression of miR-15 and miR-16 is inhibited by the canonical Wnt/β-catenin signalling pathway, which thus increases the Nodal-ACVRIIA activation gradient that contributes to dorsoventral specification.

Another role for miR-15 and miR-16 is to regulate apoptosis by repressing the expression of Bcl-2. These two miRs are often deleted or down-regulated in many cases of chronic lymphocytic leukaemia (CLL). The level of miR-16-1 is enhanced by p53 (Module 4: Figure microRNAs and p53 function).

miR-21
miR-21 has a general role to enhance signalling through protein tyrosine kinase-linked receptors (PTKRs) by inhibiting both PTEN and sprouty (SPRY). Up-regulation of miR-21, which will enhance signalling through these

Module 4: | Figure microRNA biogenesis
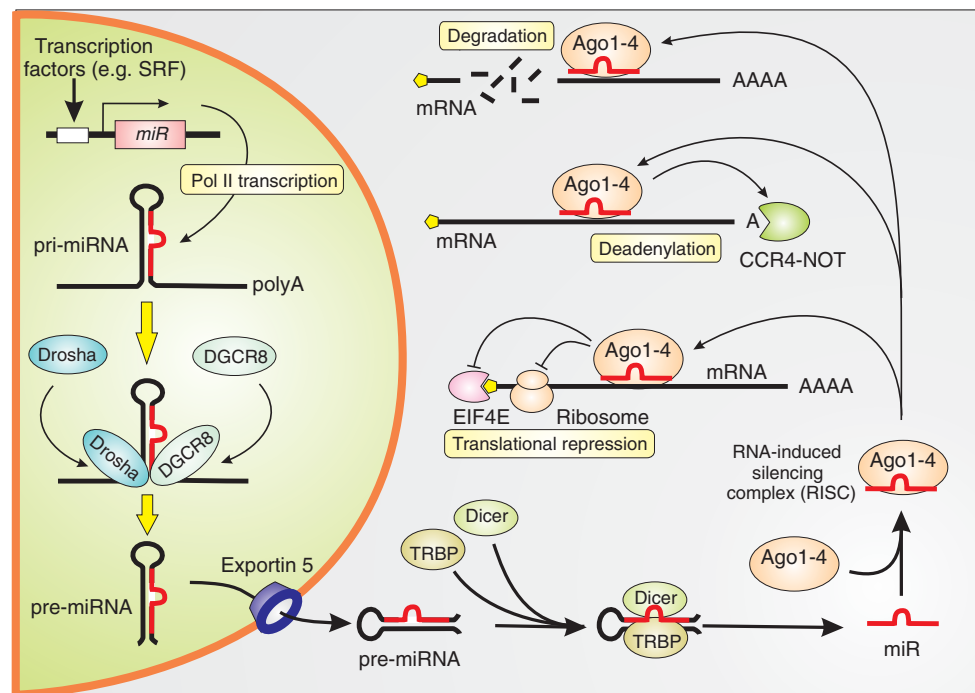
Biogenesis and function of microRNA.
RNA polymerase II (Pol II) transcribes miRNA genes to form primary miRNA (pri-miRNA) transcripts, which has a characteristic stem-cell structure. The latter is recognized by the ribonuclease Drosha that acts together with DiGeorge syndrome critical region 8 (DGCR8, also known as Pasha) to cleave pri-miRNA to form pre-miRNA, which is then exported from the nucleus by Exportin 5. Once it enters the cytoplasm, the pre-miRNA is recognized by TAR RNA-binding protein (TRBP) and the ribonuclease called Dicer, which cleaves the precursor to form the mature microRNA (miR). The miR acts by binding to Argonaute (Ago1–4) proteins to form a RNA-silencing complex (RISC) that recognizes and inhibits target messenger RNAs (mRNAs) through a number of mechanisms, such as translational repression, mRNA deadenylation and mRNA degradation.

receptors, has been found in various tumours and may also contribute to heart disease by increasing the proliferation of cardiac fibroblasts leading to fibrosis.

miR-23b
The miR-23b cluster acts to enhance formation of Smad3, Smad4 and Smad5, which operate in the TGF-β-sensitive Smad signalling pathway (Module 2: Figure Smad signalling). This control mechanism may operate during liver stem cell differentiation.

miR-26a
This miRNA acts specifically to inhibit PTEN to enhance signalling through the PtdIns 3-kinase signalling pathway and this was shown to have pathological consequences by enhancing the emergence of tumours that are derived from glial cells in brain.

miR-29b
The anti-apoptotic protein Mcl-1 is regulated by miR-29b. A decrease in the expression of mir-29b would result in an up-regulation of Mcl-1 which could have implications for cancer in much the same way as miR-15 and miR16 regulate apoptosis in various cancers.

miR-34
The expression of Sirtuin 1 (SIRT1) is regulated by miR-34.

miR-125b
A component of the relationship between p53 function and microRNA is the role of miR-125b to suppress the activity of p53 (Module 4: Figure microRNAs and p53 function). During genotoxic stress associated with DNA damage, miR-125b is repressed and this then allows p53 to arrest the cell cycle by inhibiting G1 (Module 9: Figure G_1 checkpoint signalling).

miR-126
There is a major role for miR-126 in controlling vascular development by modulating VEGF-dependent angiogenesis. Endothelial cells have large amounts of miR-126, which acts to maintain VEGF signalling by inhibiting the expression of the Class I PtdIns 3-kinase regulatory p85β subunit (also known as PIK3R2) that inhibits PtdIns 3-kinase signalling and sprouty-related, EVH1 domain-containing protein 1 (SPRED1) that inhibits the MAP kinase signalling pathway. In this way, miR-126 will inhibit two of the main signalling mechanisms that regulate angiogensis (Module 9: Figure VEGF-induced proliferation).

miR-137
miR-137 functions in neurogenesis and neuronal maturation. *MIR137* is a schizophrenia-associated gene.

miR-143 and miR-145

The *miR-143* and *miR-145* genes, which are arranged as a cluster, are transcribed together as occurs during the differentiation of smooth muscle (Module 8: Figure smooth muscle cell differentiation). In the case of smooth muscle cells, miR-145 and miR-143 regulate the genes responsible for initiating and maintaining the differentiated state. What is remarkable about these two microRNAs is that they can single-handedly direct the proliferation–differentiation switch, which is such a feature of the smooth muscle cell phenotype. When arteries are injured, there is a decline in the levels of miR-145 and miR-143 and this may result in the differentiated contractile cells switching back into a proliferative state and could account for the development of atherosclerotic blood vessels with thickened walls.

These two microRNAs also have a role in embryonic stem (ES) cells where they are of critical importance in regulating the microRNA regulation of differentiation (Module 8: Figure ES cell miRNAs). One primary target of miR-145 is the proto-oncogene c-Myc, whose enhanced expression is associated with aggressive tumors.

The fact that miR-145 mRNA is markedly reduced in many cancer cells could explain Myc dysregulation and cancer development.

miR-152

One of the functions of miR-152 is to repress the activity of the type 2 sarco/endo-plasmic reticulum Ca^{2+}-ATPase 2 (SERCA2), which functions in Ca^{2+} homoeostasis as part of the Ca^{2+} OFF reactions (Module 2: Figure Ca^{2+} signalling dynamics).

miR-192

The transcription of miR-192, which acts to inhibit expression of the zinc-finger E-box binding homeobox 2 (ZEB2), is enhanced by transforming growth factor β (TGF-β) that acts through the Smad signalling pathway. One of the actions of ZEB2 is to repress transcription of miR-216a and miR-217 that inhibit the activity of PTEN resulting in an increase in the activity of the PtdIns 3-kinase signalling pathway. This action of miR-192 illustrates how the miRNAs can function in the cross-talk that occurs between signalling pathways. Such a mechanism might account for the hypertrophy and survival of mesangial cells during diabetic nephrology.

miR-199

The expression of Sirtuin 1 (SIRT1) is regulated by miR-199.

miR-200

There is a miR-200 family that has five members (miR-200a, miR-200b, miR-200c, miR-141 and miR-429). Some of the main targets of the miR-200 family are zinc finger E-box binding homeobox 1 (ZEB1), which is also known as transcription factor 8 (TCF8), and zinc finger E-box binding homeobox 2 (ZEB2), also known as Smad-interacting protein 1 (SIP1). These two transcription factors regulate the expression of E-cadherin and are of central importance for the epithelial to mesenchymal transition (EMT).

One of the functions of ZEB1 is to regulate the expression of IL-2

In the mouse, miR-200c represses gene activity by acting on TCF and on evi, which is required for the secretion of Wnt.

Mutations in the ZEB1 gene have been associated with posterior polymorphous corneal dystrophy-3 (PPCD) and late-onset Fuchs endothelial corneal dystrophy.

miR-302

The miR-302 cluster has eight related miRNAs that are regulated by the stem cell transcription factors Oct4 and Sox2 as part of the ES cell cycle miRNA regulatory mechanisms (Module 8: Figure ES cell miRNAs). Expression of miR-302 can convert human skin cancer cells back into pluripotent ES cells.

miR-324-5p

The miR-324-5p suppresses the GLI1 transcription factor that operates in the Hedgehog signalling pathway (Module 2: Figure Hedgehog signalling pathway).One of the functions of GLI1 is to control the proliferation of cerebellar granule progenitor cells.

Mutations in miR-324-5p result in the development of medulloblastomas.

miR-372 and miR-373

These two microRNAs act to reduce the inhibitory effect of the large tumour suppressor (Lats), which is a serine/threonine protein kinase that phosphorylates the Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ), also known as Tafazzin, that are transcription factors which operate in the Hippo signalling pathway (Module 2: Figure hippo signalling pathway).

Signalsome stability

During the final phase of development, each cell type begins a process of differentiation during which a specific set of genes are expressed that define the phenotype of the different cells found in the body. A key component of this process of differential gene transcription is signalsome expression (Module 8: Figure signalsome expression), during which the cell puts in place a cell-type-specific signalling system. One of the features of the differentiated state is its relative stability, which includes stability of the cell-type-specific signalsomes. This stability depends on the turnover of signalsome components being tightly regulated, which depends on a balance between the degradation of signalsome components and their replacement. In the light of continuous signalsome degradation, stability is maintained by ongoing transcription processes. However, signalsomes are not fixed in stone, but can be remodelled during both normal and pathological conditions. In the latter case, phenotypic remodelling of the signalsome is a major cause of disease and may contribute to the process of ageing.

How do cells regulate the transcription of signalsome components in order to maintain the stability of their signalling systems?It seems that they may operate a quality assessment system whereby the properties of the output signals are constantly monitored and any deviations are fed back to the transcriptional system so that adjustments can be made to various signalling components. Such autoregulatory mechanisms are beginning to emerge for the Ca^{2+} signalling system.

What is remarkable about the stability of the Ca^{2+} signalsome is that Ca^{2+} itself plays a key role in regulating the phenotypic expression of its signalling pathway (Module 4: Figure signalsome transcription). The Ca^{2+} signalsome is a self-regulatory system with an inherent compensatory mechanism that enables the signalsome to adapt to imposed changes. Each cell-specific signalsome is set up to deliver a characteristic Ca^{2+} transient, and any alteration in this output signal tends to induce subtle alterations in the signalsome so that normal delivery is restored. In general, a decline in the level of Ca^{2+} signalling results in an up-regulation of the signalsome, and vice versa.

Similar regulatory mechanisms may function to control other signalling systems. For example, the section on mitogen-activated protein kinase (MAPK) signalling properties reveals that the extracellular-signal-regulated kinase (ERK) pathway (Module 2: Figure ERK signalling) and the c-Jun N-terminal kinase (JNK) pathway (Module 2: Figure JNK signalling) can induce the transcription of their own signalling components. In the case of the Ca^{2+} signalling system, there are numerous examples of Ca^{2+}-induced transcription of Ca^{2+} signalling components operating to compensate for alterations in the signalling pathway (Module 4: Figure signalsome transcription). This Ca^{2+}-dependent regulation of Ca^{2+} signalling pathways is particularly evident in the relationship between Ca^{2+} signalling and cardiac hypertrophy.

Support for such a mechanism is evident from the fact that the genes that encode components of Ca^{2+} signalling pathways are themselves regulated by Ca^{2+}-dependent transcription factors such as the calcineurin/nuclear factor of activated T cells (NFAT) system (Module 4: Figure NFAT control of Ca^{2+} signalling toolkit):

1. Activated NFAT binds to a site on the promoter of endothelin, which is a potent activator of receptors that generate Ca^{2+} signals.
2. The transient receptor potential (TRP) family member TRPC3 is up-regulated by NFAT.
3. Activated NFAT induces the transcription of the inositol 1,4,5-trisphosphate receptor type 1 ($InsP_3R1$) responsible for releasing internal Ca^{2+}.
4. NFAT is capable of inducing its own transcription in that it can increase the expression of NFAT2.
5. Activated NFAT increases the expression of Down's syndrome critical region 1 (DSCR1) gene, which encodes a protein that inhibits the activity of calcineurin (CaN), thereby setting up a negative-feedback loop. This gene is located within the critical region of human chromosome 21 where trisomy occurs in patients with Down's syndrome.

6. The gene *DYRK1A* encodes the dual-specificity tyrosine-phosphorylation regulated kinase 1A (DYRK1A), which is a serine/threonine protein kinase located in the nucleus and is responsible for phosphorylating NFAT to promote its export from the nucleus. Like DSCR1 described above, DYRK1A is also located on human chromosome 21, where trisomy occurs in patients with Down's syndrome.
7. NFAT induces the transcription of the sarco/endo-plasmic reticulum Ca^{2+}-ATPase (SERCA) pump that transports cytosolic Ca^{2+} back into the endoplasmic reticulum (ER)/sarcoplasmic reticulum (SR) store (Module 2: Figure Ca^{2+} signalling toolkit).
8. Expression of the large-conductance (BK) channels, which are activated by Ca^{2+} (Module 3: Figure K^+ channel domains), are controlled by NFAT3.
9. NFAT reduces the expression of the $K_{Ca}\beta1$ subunit that functions to control the Ca^{2+} sensitivity of the large-conductance (BK) channels (Module 3: Figure K^+ channel domains).

There are a number of examples of such a homoeostatic mechanism based on Ca^{2+}-induced transcription of Ca^{2+} signalling components:

- Overexpressing calsequestrin (CSQ) in mouse cardiac myocytes strongly reduces the amplitude of Ca^{2+} spikes that activate contraction and results in congestive heart failure (CHF). This increase in CSQ greatly enhances the amount of stored Ca^{2+}, and this would result in an increase in Ca^{2+} signal amplitude if it were not for a marked down-regulation of components of the release mechanism [ryanodine receptor type 2 (RYR2), triadin and junctin].
- A reduction in the Ca^{2+} content of the ER/SR results in the activation of the activating transcription factor 6 (ATF6), which then acts to increase the expression of the SERCA2 pump as a compensatory mechanism to restore the level of luminal Ca^{2+}. Disruption of one copy of the SERCA2 gene results in altered Ca^{2+} homoeostasis, as shown by a decrease in the amplitude of the Ca^{2+} transient in isolated cardiomyocytes (Module 4: Figure Ca^{2+} signals in SERCA$^{+/-}$ cardiac cells). This decline in spike amplitude was due to a 50% decline in the Ca^{2+} content of the SR. However, the rate of recovery was normal, and this seems to result from compensatory alterations in both phospholamban (PLN) and the Na^+/Ca^{2+} exchanger (NCX). The amount of PLN was reduced and there was an increase in its phosphorylated state; both changes would reduce its inhibitory activity on SERCA2. In addition, the Na^+/Ca^{2+} exchanger was up-regulated.
- When triadin 1 is overexpressed in mice, there is a compensatory decline in the expression of both RYR2 and junctin (Module 12: Figure Ca^{2+} in triadin-1-overexpressing mice).
- Overexpression of the L-type channel in cardiac cells results in an up-regulation of the NCX accompanied by a modest hypertrophy.

Module 4: | Figure signalsome transcription
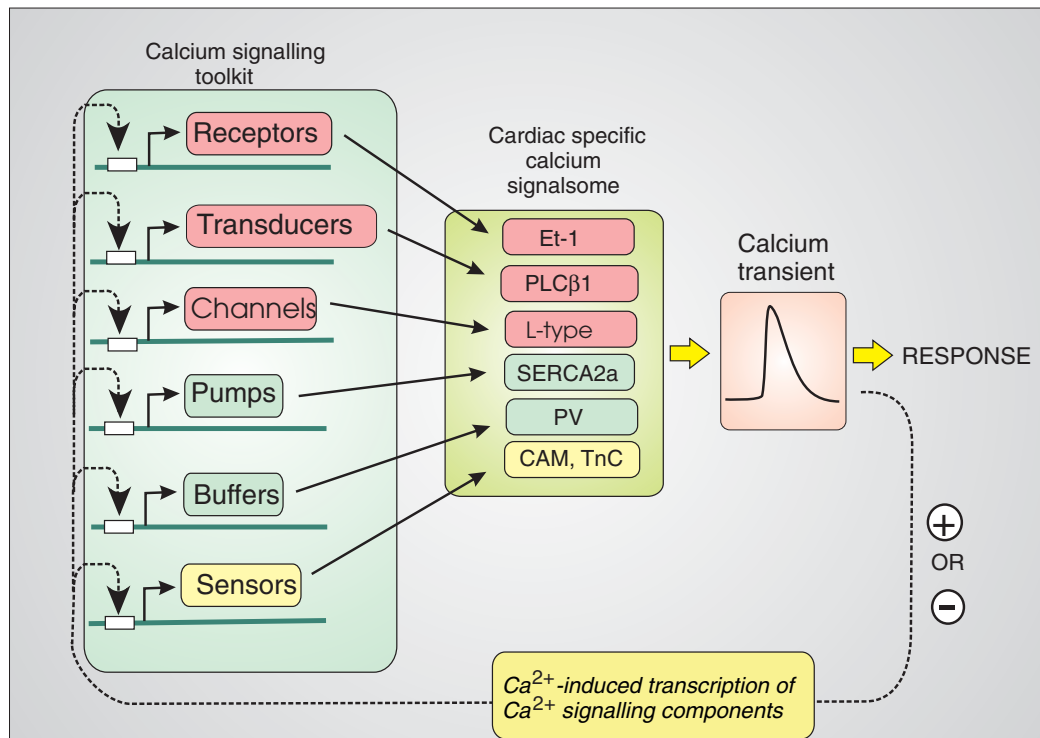
Ca^{2+} regulates the phenotypic expression of the Ca^{2+} signalsome.

During differentiation, each cell type assembles a specific Ca^{2+} signalsome, which delivers a Ca^{2+} transient exactly suited to control its particular cellular responses. In addition, this Ca^{2+} transient may also contribute to a feedback loop whereby a process of Ca^{2+}-induced transcription of Ca^{2+} signalling components functions to stabilize the signalsome.

Module 4: | Figure NFAT control of Ca^{2+} signalling toolkit
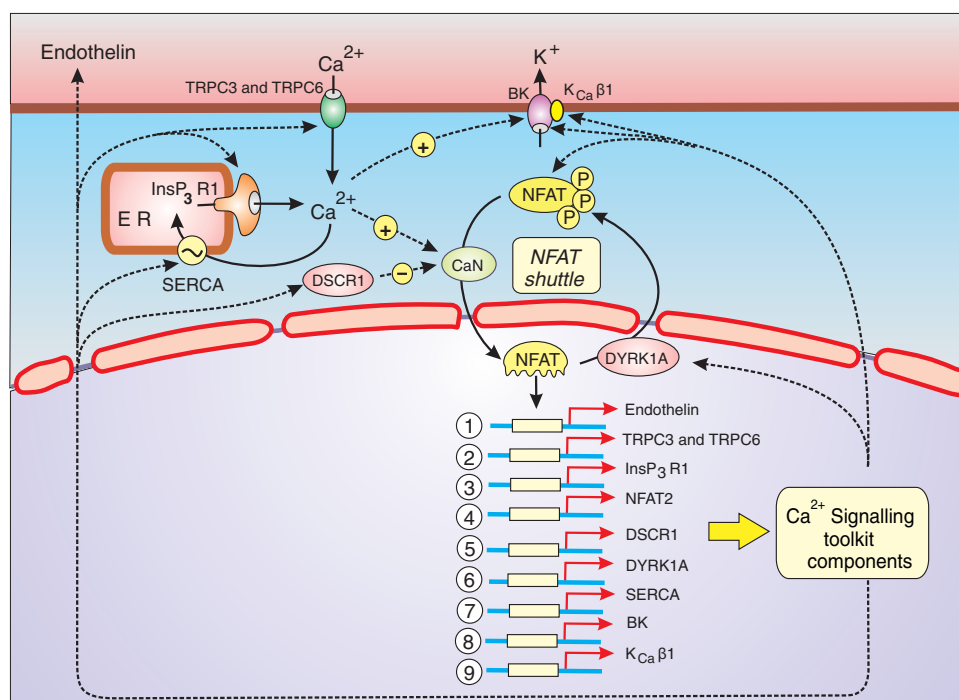
Regulated expression of the Ca^{2+} signalling toolkit by nuclear factor of activated T cells (NFAT).

The calcineurin (CaN)/nuclear factor of activated T cells (NFAT) transcriptional cascade plays a direct role in a process of Ca^{2+}-induced transcription of components of the Ca^{2+} signalling toolkit. (See the text for details of the different components.)

Module 4: | Figure Ca^{2+} signals in SERCA$^{+/-}$ cardiac cells
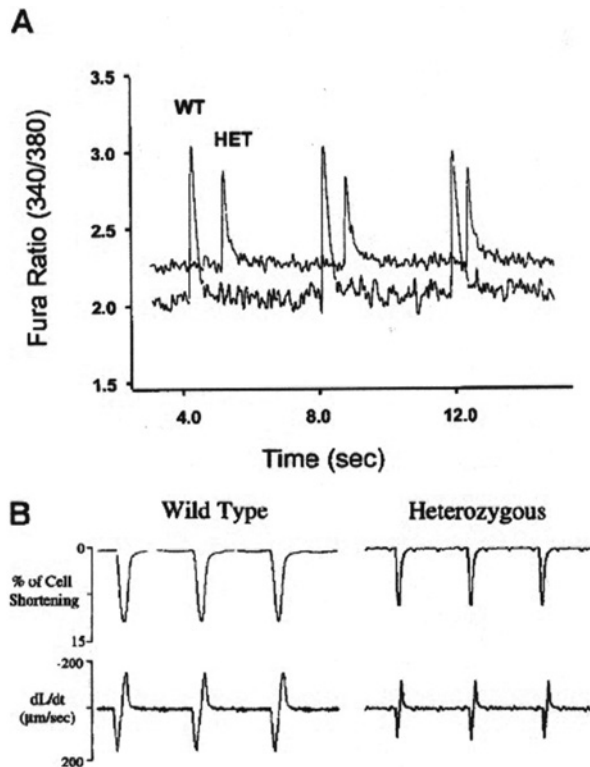
Contractions and Ca^{2+} transients in wild-type (WT) and sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA)$^{+/-}$ heterozygous (HET) ventricular myocytes.

The myocytes from the heterozygous mice had smaller Ca^{2+} transients (A) and contractions as measured by the percentage of cell shortening (B). Reproduced from Ji, Y., Lalli, M.J., Babu, G.J., Xu, Y., Kirckpatrick, D.L., Liu, L.H., Chiamvimonvat, N., Walsh, R.A., Shull, G.E. and Periasamy, M. (2000) Disruption of a single copy of the *SERCA2* gene results in altered Ca^{2+} homeostasis and cardiomyocyte function. J. Biol. Chem. 275:38073–38080, with permission from the American Society for Biochemistry and Molecular Biology; see Ji et al. 2000.

Ciliary beating

There are two types of cilia, as determined by the organization of the axoneme, which can be arranged into either a '9 + 2' pattern or a '9 + 0' pattern, as found in the primary cilium. The former are found on ciliated epithelial cells, where they beat rhythmically. This form of ciliary beating can be regulated, and this has been studied in some detail in airway epithelial cells.

Primary cilium

The '9 + 0' cilia, also known as primary cilia, are usually immotile except when located within the nodal region of the developing embryo, where they have a twirling motion that sets up the fluid flow responsible for determining left-right asymmetry (Module 8: Figure nodal flow hypothesis).

The immotile primary cilia ('9 + 0'), at least one of which is present in most cells (Module 4: Figure primary cilium), have important sensory functions involved in development, liquid flow in the kidney, mechanosensation, sight, and smell. In the case of the mechanotransduc-

tion signalling pathway in kidney cells, the primary cilia respond to fluid flow by a large increase in intracellular Ca^{2+}. This process of mechanotransduction in kidney cells depends upon the activation of the polycystin-2 channels, which are highly concentrated on these primary cilia where they function as mechanosensors.

The formation and function of primary cilia requires a tight integration of the microtubule cytoskeleton with the processes of membrane and protein trafficking. The Rab signalling mechanism (Module 2: Figure Rab signalling) controls the transport of key ciliary components that are carried into the cilium by various molecular motors. Rab8a, Rab17, and Rab23 appear to have a role in many of these trafficking events. One of the functions of Rab8a is to interact with cenexin/ODF2, which is a microtubule-binding protein that is essential for cilium biogenesis.

The developmental and multiple cellular function of the primary cilium is very dependent on inositol polyphosphate 5E-phosphatase (INPP5E), which removes the 5-phosphate from PtdIns4,5P$_2$ and PtdIns3,4,5P$_3$ (Step 12 in Module 2: Figure phosphoinositide metabolism). Mutations in the *INNP5E* gene have been linked to MORM syndrome. Other mutations that affect primary cilia have been linked to a number of diseases, such as Bardet-Biedl syndrome, neural tube defects, polycystic kidney disease and retinal degeneration.
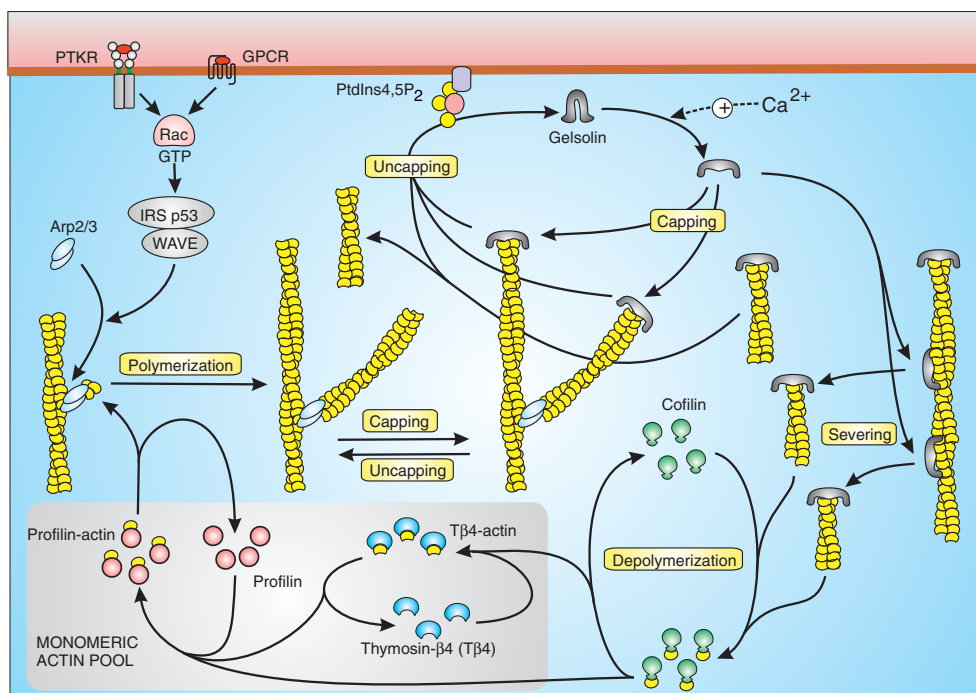
Actin remodelling

Reorganization of the cytoskeleton, which depends in part on a process of actin remodelling, is controlled by a number of signalling systems. An important aspect of actin remodelling is the process of actin tread milling, which depends on the polymerization of ATP-G-actin into the end of existing actin filaments at the barbed end (plus end) and the removal of ADP-actin at the pointed-end (minus-end). Such remodelling of the actin cytoskeleton has multiple functions such as the regulation of cell shape, adhesion, cytokinesis, gastrulation, cell migration and morphogenesis. The ERM protein family have a special role in attaching actin filaments to the plasma membrane. There also is an interesting relationship operating between actin dynamics and gene transcription. A number of signalling mechanisms contribute to regulation of the many processes that control this polymerization and depolymerization of actin (Module 4: Figure actin remodelling).

The monomeric G proteins, such as the Rho family members Rho, Rac and Cdc42, are of particular importance for such actin remodelling. For example, the Rac signalling mechanism is activated by a number of stimuli that act through guanine nucleotide exchange factors (GEFs) to convert inactive Rac-GDP into active Rac-GTP, which then has a number of functions (Module 2: Figure Rac signalling). One of these functions is to activate the Wiskott–Aldrich syndrome protein (WASP) verprolin homologous (WAVE), which orchestrates the actin-related protein 2/3 complex (Arp2/3 complex) (Module 4: Figure actin remodelling proteins). Cdc42 is another monomeric G protein that functions in actin remodelling

Module 4: | Figure primary cilium
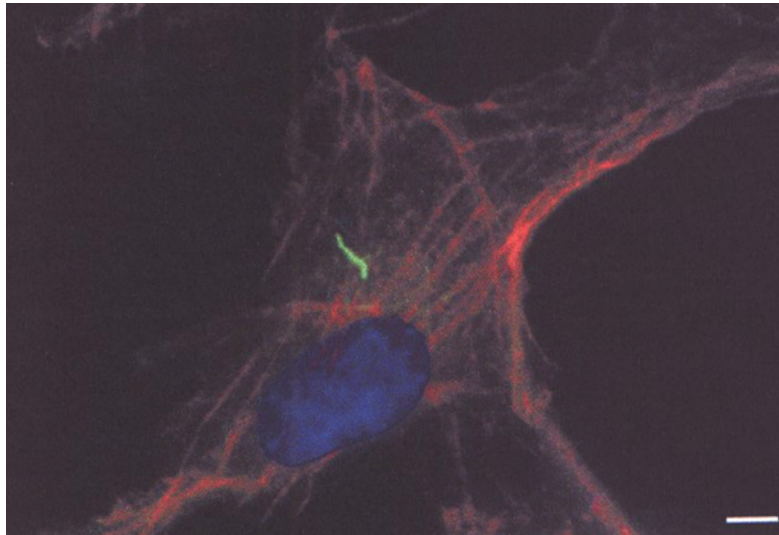
Example of a single primary cilium on a cultured cell.

This human retinal pigmented epithelial cell was labelled with an antibody against acetylated tubulin that has picked out the single primary cilium shown in green. Reproduced from *Curr. Opin. Cell Biol.*, Vol. 15, Pazour, G.J. and Witman, B.G., The vertebrate primary cilium is a sensory organelle, pp. 105–110. Copyright (2003), with permission from Elsevier; see Pazour and Witman 2003.

Module 4: | Figure actin remodelling

Actin remodelling mechanisms.

Actin remodelling is regulated by different signalling pathways. Protein tyrosine kinase-linked receptors (PTKRs) and G protein-coupled receptors (GPCRs) act through the Rac signalling pathway (Module 2: Figure Rac signalling) and initiate polymerization by recruiting the Arp2/3 complex. An increase in Ca^{2+} activates Gelsolin that inhibits polymerization by capping the growing end and also functions to sever existing filaments into short capped fragments that have two fates. The pointed-end can be depolymerized with the help of cofilin that then donates actin monomers to the pool of actin bound to profilin and thymosin-β4 (Tβ4). Some of these short fragments can also be uncapped through a mechanism that depends on the formation of PtdIns4,5P$_2$ that inactivates gelsolin resulting in uncapping such that polymerization can continue. This Figure is based on the information shown in Figure 10 in Shalini et al. (2013).

(Module 2: Figure Cdc42 signalling). In this case, a key component of the action of the active Cdc42/GTP complex is Wiskott–Aldrich syndrome protein (WASP) that controls the actin assembly (Module 4: Figure actin remodelling proteins).

The cytoplasm contains a monomeric actin pool in which individual actin molecules are bound to various actin-binding proteins. The profilin–actin pool is used for the polymerization reaction. The actin monomers bound to thymosin-β4 (Tβ4) act as a reservoir that can transfer actin into the profilin–actin pool as required (Module 4: Figure actin remodelling). These profilin–actin complexes then feed actin monomers into the growing barbed end during the processive actin polymerization reaction.

This polymerization process is modulated by gelsolin through two important reactions. In response to an elevation in Ca^{2+}, gelsolin undergoes a conformation change that exposes its actin-binding region that can then either cap or sever actin. By capping the growing barber ends it prevents further polymerization. Gelsolin can also bind to regions down the length of the filaments to produce a pool of shorter capped segments that can influence subsequent actin formation through two pathways. Firstly, these shorter segments can be uncapped through the $PtdIns4,5P_2$ signalling cassette. The $PtdIns4,5P_2$ disrupts the gelsolin–actin interaction to provide uncapped barbed ends that can nucleate the formation of new filaments. Since this uncapping occurs near the membrane, it will enable actin filaments to be formed rapidly near the surface to change the shape of the membrane and to promote cell movement. Alternatively, the pointed end of these short segments can be depolymerized through a mechanism facilitated by cofilin to replenish the pool of actin monomers.

Remodelling of the cortical actin cytoskeleton plays an important role in a number of cellular responses:

- Control of cytokinesis at the time of cell division (Module 9: Figure cytokinesis).
- Formation of pseudopodia during phagocytosis.
- Construction of the focal adhesion complex (Module 6: Figure integrin signalling).
- Formation of the osteoclast podosomes (Module 7: Figure osteoclast podosome).
- Actin assembly, pseudopod formation and uropod contraction during neutrophil chemotaxis, which is a classic example of cell migration (Module 11: Figure neutrophil chemotactic signalling).
- Growth of the spine during the action of Ca^{2+} in synaptic plasticity (Module 10: Figure Ca^{2+}-induced synaptic plasticity).
- Regulation of hormone release by pituicytes in the posterior pituitary (Module 10: Figure magnocellular neurons).

Gelsolin/villin superfamily
The gelsolin/villin superfamily of actin-modulating proteins contains eight members: adseverin, CapG, gelsolin, flightless I, advillin, villin, villin-like protein, and supervillin. Much of our current information on how these pro-

teins function has come from the study of gelsolin that has a characteristic structure consisting of six (G1–G6) closely similar gelsolin domains (97–118 amino acids) that are fairly equally spaced along the length of the molecule. Many of the other members of the gelsolin superfamily have a similar distribution of domains whereas others have either fewer domains or domains that are fused to other protein domains. These molecular variations result in the family members having slightly different properties to control a variety of process in different cell types.

Adseverin
Adseverin, which is also known as scinderin (SCIN), is a member of the gelsolin/villin superfamily of actin-modulating proteins. It is closely related to gelsolin and is expressed in various endocrine cells and in the skin. In the case of chromaffin cells located within the adrenal medulla, it functions to disintegrate the layer of cortical actin filaments to enable the secretory vesicles to approach the membrane where they fuse to release adrenalin (Module 7: Figure chromaffin cell secretion). Adseverin has also been implicated in the differentiation of chondrocytes.

Gelsolin
Gelsolin belongs to the gelsolin/villin superfamily of actin-modulating proteins. It is a Ca^{2+}-sensitive protein that modulates actin by promoting the nucleation of new actin filaments, capping of the barbed ends of growing filaments and the severing of existing filaments (Module 4: Figure actin remodelling).

The gelsolin protein contains six (G1–G6) closely similar gelsolin domains (97–118 amino acids) that are fairly equally spaced along the length of the molecule. The Ca^{2+}-binding sites of gelsolin are distributed down the length of the molecule: the G1 and G4 domains contain the Type-1 Ca^{2+}-binding site whereas a Type 2 site is located on each of the six gelsolin domains. The calcium-binding affinities (K_d) for these different sites vary between 0.2 and 600 μM, which indicates that gelsolin may be able to respond to a wide range of Ca^{2+} concentrations.

The activity of gelsolin is regulated by either the Ca^{2+} signalling pathway or the $PtdIns4,5P_2$ signalling cassette. In its inactive state, gelsolin is folded up in such a way as to shield the major actin-binding regions located on domains G1, G2 and G4. Elevation in Ca^{2+} induces a major conformational change in gelsolin that exposes these actin-binding regions thus enabling it to carry out its actin remodelling functions of either capping or severing actin filaments (Module 4: Figure actin remodelling). Capping of the growing filaments terminates polymerization. The severing activity of gelsolin results in the formation of large numbers of shorts capped actin filaments that have two fates. They can either be depolymerized through the activity of cofilin that then feeds actin monomers into the monomeric actin store where they are bound to either profilin or thymosin-β4 (Tβ4) or they can be uncapped to provide short segments that can be polymerized to form new filaments.

The uncapping of actin filaments is driven by formation of $PtdIns4,5P_2$, which binds to three binding sites

located in the linker region between G1 and G2, a region on G2 that overlaps the actin-binding site and the third is found in the linker region between G5 and G6. When PtdIns4,5P$_2$ binds to these sites it disrupts the interaction beween gelsolin and actin.

Familial amyloidosis is caused by a mutation in the type-2 calcium-binding site of domain G2.

Flightless I

Flightless I is a member of the gelsolin/villin superfamily of actin-modulating proteins. Its name reflects the fact that flightless I was first identified in *Drosophila* linked to a mutation causing defects in flight. Typical of many members of the gelsolin/villin superfamily, the mammalian flightless I protein has six gelsolin domains (G1–G6), but is unusual in that it has an extensive N-terminal leucine-rich repeat (LRR) domain, which normally functions in protein–protein interactions. This LRR domain enables flightless I to bind to other proteins such as LRR Flightless I-interacting proteins 1 and 2 (LLRFIP1 and LRRFIP2) and the TAR RNA-interacting protein (TRIP), which is a double-stranded RNA-binding protein. Flightless I and LLRFIP1 seem to be a component of the blood platelet cytoskeleton.

Flightless I is expressed in a large number of cell types and has been implicated in many different functions such as development, control of gene transcription (coactivation of nuclear hormone receptors and regulation of β-catenin-dependent transcription), inflammation, cell migration and wound healing. In the case of wound healing, flightless I seems to impair cellular proliferation and the re-epithelialization necessary to repair large wounds. Some of these effects may be caused by an increase in inflammation and this may be a significant factor in the reduced healing of foot ulcers in diabetic patients. Flightless I may contribute to this enhanced inflammation through its ability to sequester the adaptor protein MyD88 that is a component of the Toll receptor signalling pathway (Module 2: Figure Toll receptor signalling).

Supervillin

Supervillin is a member of the gelsolin/villin superfamily of actin-modulating proteins. Supervillin has five of the gelsolin domains (G2–G6) and also contains the types 1 and 2 Ca^{2+}-binding sites. There is a C-terminal headpiece resembling that of villin, but it may not bind actin. There are a number of isoforms: Isoform 1 (a canonical non-muscle 200 kDa isoform), isoform 2 also known as archvillin (striated muscle), isoform 3 (smooth muscle archvillin) and isoform 4 (non-muscle isoform). Supervillin has an important property of being able to link actin filaments to membranes. It can also increase myosin II contractility and can induce rapid integrin recycling by reducing integrin-mediated cell adhesion. It is widely distributed and has been implicated in multiple processes such as cytokinesis, myogenesis, cell-substrate adhesion and spreading, regulation of the tumour suppressor protein p53 levels to control cell survival and formation of podosomes and invadosomes.

These multiple functions of supervillin depend on its ability to bind to many different proteins:

- The ability of supervillin isoforms 1 and 4 to promote cell survival depends on its ability to decrease levels of the tumour suppressor protein p53. Supervillin acts on the ubiquitin ligase that participates in p53 ubiquitination and degradation (Module 4: Figure p53 function). In particular, it reduces the activity of the ubiquitin-specific protease Usp7, which is also known as herpes virus-associated ubiquitin-specific protease (HAUSP), which is a p53-binding protein that functions to deubiquitinate p53. The ability of supervillin to interact with USP7/HAUSP results in its own deubiquitination and stability.

- Supervillin can regulate the reactivity of blood platelets. For example, it inhibits the rapid activation and spreading of platelets that occurs during thrombus formation (Module 11: Figure thrombus formation). Supervillin may act to link together the GP1b–GPIX–GPV complex on the platelet surface to the actin cytoskeleton (See step 1 in Module 11: Figure platelet activation).

- Supervillin has a myosin II regulatory N-terminus that seems to have a role in regulating the turnover of podosomes, which are specialized cell adhesion complexes used by cells to form attachments with the extracellular matrix (ECM) or to interact with other cells. Osteoclast podosomes are an example of such a podosome (Module 7: Figure osteoclast podosome) that contain many of the same components that are found in the focal adhesion complex (Module 6: Figure integrin signalling). However, podosomes differ from the latter in that they are much more labile in that they have a lifetime of 2–12 min. This rapid turnover may depend, in part, on the ability of supervillin to modulate the dynamics of actomyosin-dependent contractility. Supervillin can interact with cytoskeletal proteins such as actin, non-muscle myosin II (NMII) and myosin light chain kinase (MLCK) (Module 7: Figure osteoclast podosome). Dissolution of the podosomes may be triggered when supervillin activates myosin II.

Villin

Villin is a member of the gelsolin/villin superfamily of actin-modulating proteins. It is closely related to gelsolin. It is expressed in various epithelial tissues such as gastrointestinal tract, gall bladder and kidney. It seems to be located primarily within the microvilli where it contributes to the reorganization of the actin bundles. Like some of the other members of the superfamily it contains the six (G1–G6) closely similar gelsolin domains, which contain the Ca^{2+}-binding domains.

A characteristic feature of villin is a C-terminal headpiece that has an F-actin-binding domain that is responsible for the cross-linking and bundling of actin especially at low concentrations of Ca^{2+}. At higher concentrations of Ca^{2+}, villin both caps and severs actin much as gelsolin does during actin remodelling (Module 4: Figure actin remodelling). Villin also has three phosphatidylinositol 4,5-bisphosphate (PtdIns4,5P$_2$) binding sites: one is located

at the head piece and the other two are in the core. The binding of PtdIns4,5P$_2$ inhibits actin capping and severing to enhance actin bundling cross-linking. Tyrosine phosphorylation of villin can enhance the Ca^{2+} sensitivity of villin and this is likely to play a significant role in regulating its ability to modulate actin dynamics.

Villin-like protein

Villin-like protein is a member of the gelsolin/villin superfamily of actin-modulating proteins. It has a domain structure that resembles that of advillin and villin. It is strongly expressed in epithelia (gall-bladder and intestine). There are indications that it may also play a role in spermatogenesis.

Advillin

Advillin is a member of the gelsolin/villin superfamily of actin-modulating proteins. It is closely related to adseverin and villin and is expressed in various tissues such as uterus, testis, taste buds, brain, gastrointestinal tract and skeletal muscle. Like villin, advillin has a C-terminal headpiece that is responsible for actin binding.

It seems to play an important role in the outgrowth of neurons from the dorsal root ganglia and trigeminal ganglia and may also function in neuronal regeneration.

Wiskott–Aldrich syndrome protein (WASP)

The Wiskott–Aldrich syndrome protein (WASP) family of proteins plays a critical role in cytoskeletal remodelling by functioning as intermediaries between upstream signalling events and the downstream regulators of actin. One of the first components to be identified was the protein WASP. A closely related neural WASP (N-WASP) is now known to be ubiquitous. In addition, there are three Wiskott–Aldrich syndrome protein (WASP) verprolin homologous (WAVE) isoforms that have similar functions. IRSp53, an insulin receptor substrate (IRS), functions as an intermediary between Rac and WAVE during the process of membrane ruffling (Module 2: Figure Rac signalling). The adaptor protein Abelson-interactor (Abi), which functions in the Abl signalling pathway (Module 1: Figure Abl signalling), plays an important role in linking Rac to WAVE.

The WASP family plays a critical role in orchestrating the processes of actin remodelling. The N-termini of both WAVE and WASP have a verprolin (V) homology region, a cofilin-like (C) and an acidic (A) region, which play key roles in promoting actin polymerization. The V region binds the profilin–actin complex to release free actin monomers that are then used by the Arp2/3 complex attached to the C/A region to polymerize actin. The WAVE complex favours actin branching, as occurs during membrane ruffling, whereas the WASP complex forms long actin filaments that produce filopodia.

WASP and N-WASP are fairly similar proteins with respect to their domain structure (Module 4: Figure actin remodelling protein). In the case of N-WASP, the N-terminal region begins with a WASP homology 1 (WH1) domain, followed by a basic (B) region and a GTPase-binding domain (GBD). The latter is also called the Cdc42- and Rac-

interactive binding (CRIB) domain, reflecting the fact that it is this site that binds to Cdc42 or Rac. The GBD domain is followed by a proline-rich region (Pro), two verprolin (V) homology regions, a cofilin-like (C) and an acidic (A) region.

WASP seems to be particularly important for carrying out the actin remodelling function of Cdc42 (Module 2: Figure Cdc42 signalling). Following cell stimulation, the GTP-bound form of Cdc42 binds to WASP and appears to open up the molecule such that the C-terminal region becomes free to associate with the actin-related protein 2/3 complex (Arp2/3 complex), which binds to the C/A region and begins to polymerize actin (Module 4: Figure actin remodelling protein). The actin monomers are brought in as a complex with profilin, which binds to the verprolin homology region. The profilin/actin complex dissociates, and the actin monomer is added to the growing tail, whereas the profilin is released to the cytoplasm.

The PtdIns4,5P$_2$ regulation of actin remodelling can be accommodated in this mechanism because this lipid binds to the basic (B) region and may act together with Cdc42 to activate WASP.

WASP plays an important role in T cell cytoskeletal reorganization during formation of the immunological synapse (Module 9: Figure immunological synapse structure). Mutations in WASP cause Wiskott-Aldrich syndrome.

Wiskott–Aldrich syndrome protein (WASP) verprolin homologous (WAVE)

There are three Wiskott–Aldrich syndrome protein (WASP) verprolin homologous (WAVE) isoforms that closely resemble each other with regard to their domain structure. The N-terminal region begins with a WAVE homology domain (WHD), followed by a basic (B) region, a proline-rich region (Pro), a verprolin (V) homology region, a cofilin-like (C) and an acidic (A) region (Module 4: Figure actin remodelling protein). The main difference from Wiskott–Aldrich syndrome protein (WASP) is that WAVE lacks the GTPase-binding domain (GBD). The activator Rac is connected to WAVE through a number of adaptors. The insulin receptor substrate (IRS) protein has a Src homology 3 (SH3) domain that binds to the proline-rich (Pro) region and a Rac-binding domain (RCB) that links it to Rac. In addition, Abelson-interactor (Abi) is an adaptor that links WAVE to the actin-related protein 2/3 complex (Arp2/3 complex) (Module 1: Figure Abl signalling).

WAVE seems to be particularly important for carrying out the actin remodelling function of Rac (Module 2: Figure Rac signalling). Following cell stimulation, the GTP-bound form of Rac binds to IRSp53, which functions as an adaptor protein, to link Rac to WAVE (Module 4: Figure actin remodelling). This binding of the Rac/IRSp53 complex appears to open up the molecule such that the C-terminal region becomes free to associate with the Arp2/3 complex, which binds to the C/A region to begin actin polymerization. The actin monomers are brought in as a complex with profilin, which binds to the verprolin (V) homology region. The profilin/actin complex dissociates, and

Module 4: | Figure actin remodelling proteins
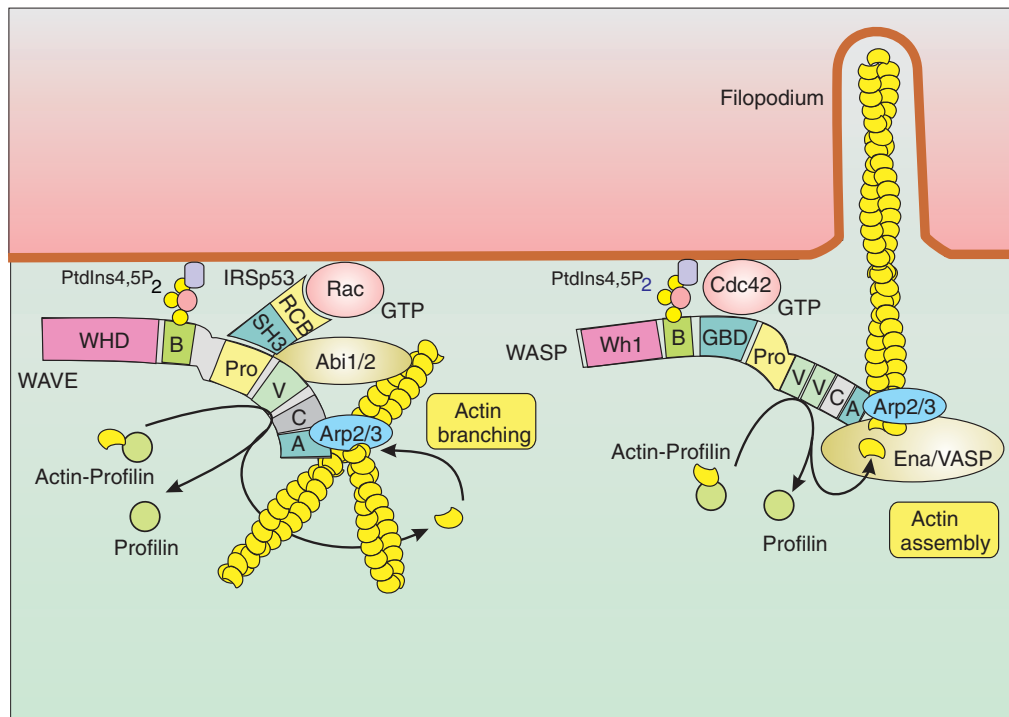
Wiskott–Aldrich syndrome protein (WASP) and WASP verprolin homologous (WAVE) proteins orchestrate remodelling of the actin cytoskeleton.
The Wiskott–Aldrich syndrome protein (WASP) verprolin homologous (WAVE) protein is activated by both the phospholipid PtdIns4,5P$_2$ and by the G protein Rac, which is linked to WAVE through the insulin receptor substrate IRSp53. The cofilin (C) homology and the acidic (A) regions bind actin-related protein 2/3 complex (Arp2/3 complex), which is responsible for actin polymerization that favours a branching pattern as is seen during membrane ruffling. The related WASP protein is regulated by PtdIns4,5P$_2$ and the G protein Cdc42, which binds to the GTPase-binding domain (GBD). The N-terminal C/A domain binds Arp2/3 to initiate actin polymerization, as does the WAVE protein. However, the WASP configuration seems to favour the formation of long filaments to form filopodia.

the actin monomer is added to the growing tail, whereas the profilin is released to the cytoplasm.

The PtdIns4,5P$_2$ regulation of actin remodelling can be accommodated in this mechanism because this lipid binds to the basic (B) region and may act together with Rac to activate WAVE. This WAVE-dependent remodelling of the actin cytoskeleton plays a critical role in regulating the store-operated entry of Ca^{2+} into T cells (Module 3: Figure STIM-induced Ca^{2+} entry). If the level of WAVE2 is reduced in Jurkat T cells, there is a marked reduction in the amplitude of Ca^{2+} (Module 3: Figure WAVE2 effects on Ca^{2+} entry).

### Actin-related protein 2/3 complex (Arp2/3 complex)

The actin-related protein 2/3 complex (Arp2/3 complex) is made up of a collection of seven proteins that initiate actin polymerization to form Y-branched actin filaments. In effect, it attaches to a pre-existing filament to form a nucleation site from which a new actin filament begins to polymerize (Module 4: Figure actin remodelling). Two of the proteins are actin-related proteins (Arp2 and Arp3), whereas the others are called the actin-related protein complex (Arpc-1–Arpc-5).

The Arp2/3 complex plays a central role in the regulation of a number of cellular processes that depend upon actin remodelling:

- Formation of the actin cytoskeleton during integrin signalling (Module 6: Figure integrin signalling).
- Assembly of actin filaments in the postsynaptic density (PSD) of neurons (Module 10: Figure postsynaptic density). Remodelling of this complex plays a role in synaptic plasticity (See steps 6 and 7 in Module 10: Figure Ca^{2+}-induced synaptic plasticity).
- Regulation of the cytoskeleton during ephrin (Eph) receptor signalling (Module 1: Figure Eph receptor signalling).
- Formation of actin during endothelial cell contraction (Module 7: Figure endothelial cell contraction).
- Assembly of actin in the osteoclast podosome (Module 7: Figure osteoclast podosome).
- Assembly of actin in the pseudopod during neutrophil chemotaxis (Module 11: Figure neutrophil chemotactic signalling).
- Assembly of actin during membrane invagination and scission during the process of endocytosis (Module 4: Figure scission of endocytic vesicles).

Cortactin

Cortactin functions as an activator of the actin-related protein 2/3 complex (Arp2/3 complex). It plays an important role in assembling the plume of actin that is attached to

Module 4: | Figure Ena/VASP family
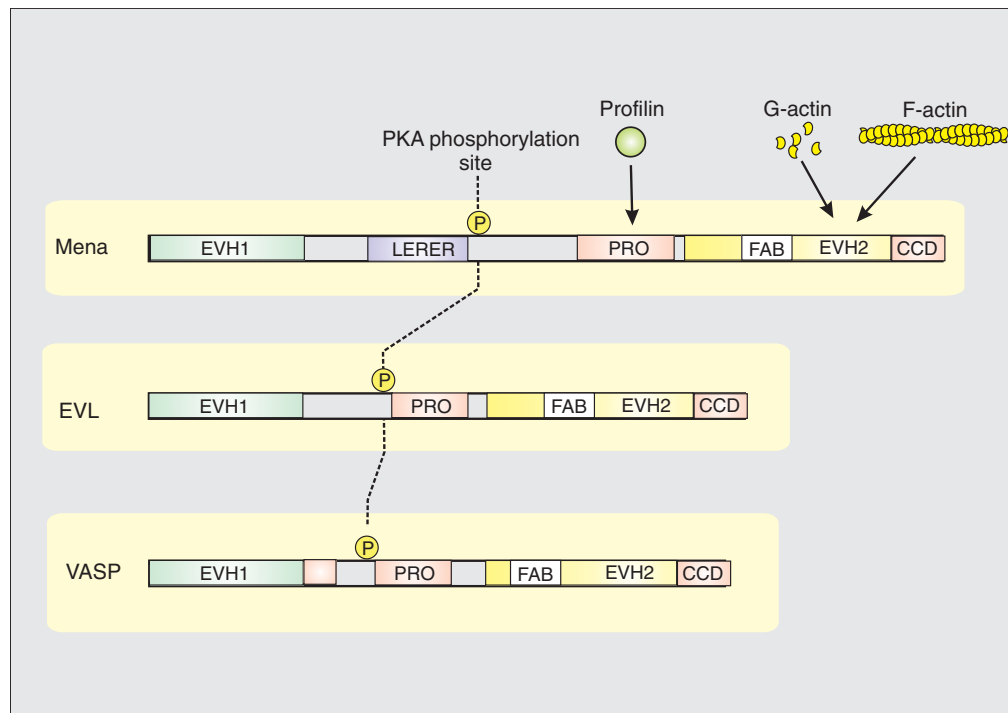
Structural organization of the Ena/vasodilator-stimulated phosphoprotein (VASP) family.
The three members of the Ena/vasodilator-stimulated phosphoprotein (VASP) family function in remodelling the actin cytoskeleton. They are particularly important in linking various cell signalling pathways to the processes responsible for actin assembly. EVH, Ena/VASP homology; PRO, proline-rich domain; CCD, coiled-coil region.

the neck of the vesicular bulb during membrane invagination and scission (Module 4: Figure scission of endocytic vesicles). It also is one of the postsynaptic density (PSD) signalling elements that play an important role in neuronal actin remodelling (Module 10: Figure postsynaptic density).

Ena/vasodilator-stimulated phosphoprotein (VASP) family

The Ena/VASP family contains three closely related family members: Mena (mammalian Enabled), EVL (Ena-VASP-like) and VASP (vasodilator-stimulated phosphoprotein) (Module 4: Figure Ena/VASP family). Ena/VASP functions in the dynamics of actin assembly during both cell–cell interactions and during the protrusion of lamellipodia and filopodia during cell migration. There is an N-terminal Ena/VASP homology 1 (EVH1) domain, a central proline-rich (PRO) domain and a C-terminal EVH2 domain, which binds to both G- (globular) and F- (filamentous) actin. The central PRO domain binds to profilin. All members of the family have a conserved protein kinase A (PKA) site.

Ena/VASP proteins have been shown to interact with many of the proteins associated with actin assembly such as Wiskott–Aldrich syndrome protein (WASP) and profilin. One way in which it might alter actin dynamics is to bind to the barbed ends of actin, where it antagonizes the activity of capping proteins while promoting addition of actin monomers (Module 4: Figure actin remodelling).

One of the functions of VASP is to promote actin remodelling during clot formation in blood platelets. Phosphorylation of VASP by protein kinase A (PKA) is one of the cyclic AMP-dependent inhibitory mechanisms for blocking clot formation (Step 12 in Module 11: Figure platelet activation). Ena/VASP binds to FAT1, which is the mammalian orthologue of the *Drosophila* atypical cadherin Fat (Ft) that functions in planar cell polarity (PCP) (Module 8: Figure planar cell polarity signalling).

ERM protein family

The ERM protein family consists of ezrin, radixin and moesin that act as molecular cross-linkers between actin filaments and proteins in the cell membrane. They are characterized by their N-terminal FERM domain, which enables these ERM proteins to interact with the proteins in the membrane. The C-terminal region, which is highly charged, binds to actin.

Actin dynamics and gene transcription

There is a close relationship between actin dynamics, which occurs during actin remodelling, and gene transcription. It is the balance between actin assembly and disassembly that acts to regulate gene transcription that depends on the transcriptional coactivator myocardin-related transcription factor (MRTF), which is an actin-binding protein (Module 4: Figure actin dynamics and gene transcription). When bound to actin, MRTF is located in the cytoplasm, but when actin is removed by its assembly into actin
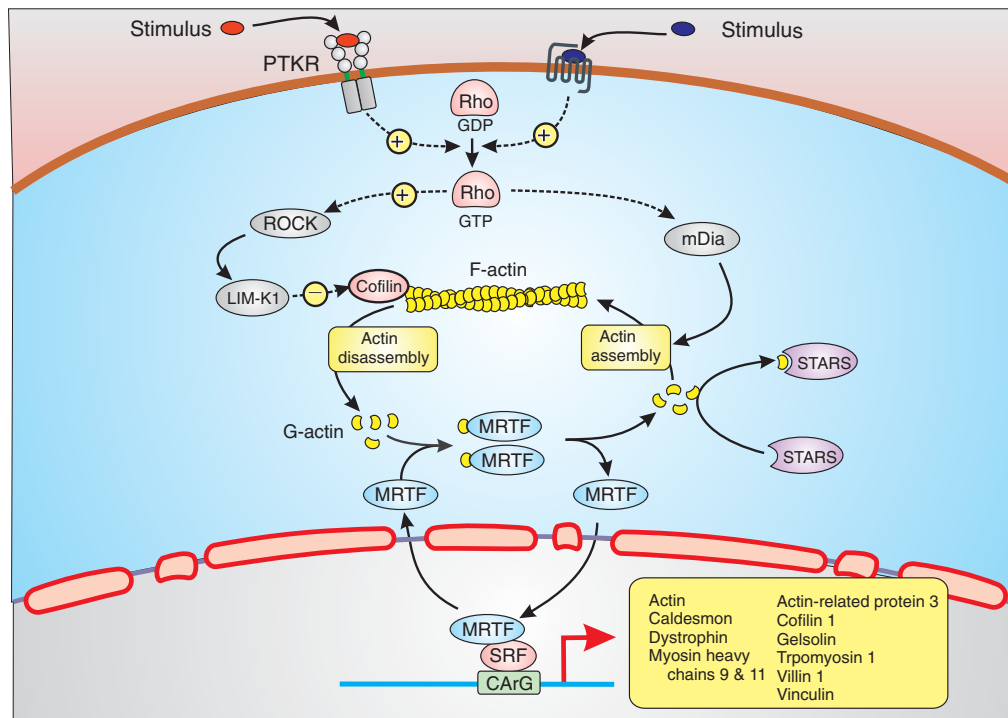
Actin dynamics and gene transcription.
The relationship between actin dynamics and gene transcription depends on the transcriptional coactivator myocardin-related transcription factor (MRTF), which is an actin-binding protein. When bound to actin, MRTF is located in the cytoplasm, but when actin is removed by assembly into actin filaments, the free MRTF is released and enters the nucleus where it acts as a coactivator of the serum response factor (SRF) that regulates expression of a large number of proteins that regulate the function of actin. The striated muscle activator of Rho signalling (STARS) contributes to this gene activation by binding to free actin thus contributing to the liberation of MRTF.

filaments, the free MRTF is released and enters the nucleus where it acts as a coactivator of the serum response factor (SRF) which regulates expression of a large number of proteins that regulate the function of actin. The striated muscle activator of Rho signalling (STARS) contributes to this gene activation by binding to free actin thus contributing to the liberation of MRTF.

Striated muscle activator of Rho signalling (STARS)
The striated muscle activator of Rho signalling (STARS), which is also known as Myocyte Stress 1 (MS1) or actin-binding Rho-activating protein (ABRA), is expressed mainly in cardiac and skeletal muscle. One of its functions is to bind to actin, which facilitates the release of myocardin-related transcription factors (MRTFs) that contribute to the activation of serum response factor (SRF) (Module 4: Figure actin dynamics and gene transcription).

Formins
The formins, which consist of 15 members, are a widely expressed family of proteins that contribute to actin remodelling. This large formin family consists of large multidomain proteins that associate with a variety of other cellular

factors to nucleate actin polymerization. Most of them have an N-terminal GTPase-binding domain (GBD) followed by a diaphanous inhibitory domain (DID), a coiled-coil domain (CC), a variable number of three formin-homology domains (FH1, FH2 and FH3) and finally a diaphanous autoregulatory domain (DAD). Under resting conditions, the C-terminal DAD interacts with the DID to induce a conformational change that effectively blocks the FH domains. Activation by the Ras family of small G proteins (e.g. Rho, Rac and Cdc42), which bind to the GBD region, disrupts the DID–DAD interaction thereby opening up the molecule such that the FH2 domain can initiate filament assembly. The formins remain associated with the fast-growing barbed end, enabling rapid insertion of actin subunits while protecting the end from capping proteins. Elongation proceeds as profilin–actin complexes are recruited by the adjacent FH1 domain.

The formin family is divided into the formins and Diaphanous-related formins (DRFs).

The formins consist of Formin-1 (FMN1), Formin-2 (FMN2), delphin, FHDC1 (INF1) and INF2.

The Diaphanous-related formins (DRFs) consist of dishevelled-associated activator of morphogenesis 1 (DAAM1), Formin-related gene in leukocytes 1 (FRL1)

and mammalian diaphanous-like formin proteins (mDia1, mDia2 and mDia3).

Formin-1 (FMN1)

Formin-1 (FMN1) belongs to the formin family of actin remodelling proteins. FMN1 plays a role in the formation of both dendrites and synapses. Another important role for FMN1 is in cell adhesion where it regulates the actin filaments that bind to members of the classical cadherins (Module 6: Figure classical cadherin signalling).

Dishevelled-associated activator of morphogenesis 1 (DAAM1)

Dishevelled-associated activator of morphogenesis 1 (DAAM1) belongs to the formin family of actin remodelling proteins. DAAM1 has an unusual activation mechanism in that it is linked to some of the Wnt signalling pathways (Module 2: Figure Wnt signalling pathways). In particular, it seems to play a role in the Wnt/planar cell polarity (PCP) pathway (Module 2: Figure Wnt signalling pathways) as illustrated in the Frizzled (Fz)/Flamingo (Fmi) polarity signalling pathway (Module 8: Figure planar cell polarity signalling). The ability of DAAM1 to promote actin formation seems to play a central role in tissue morphogenesis. For example, it controls cardiac morphogenesis by assembling the actin filaments of the contractile machinery.

Mammalian diaphanous-like formin 1 (mDia1)

There are three mammalian diaphanous-like formin proteins (mDia1, mDia2 and mDia3) that belong to the Diaphanous-related formin (DRFs) family. They contain three formin homology domains that are used to bind to various effectors. mDia1 is activated by theRho signalling mechanism and functions to control actin polymerization by binding to profilin (Module 2: Figure Rho signalling). mDia1 has also been implicated in the control of polycystin 2.

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