Module 6

Spatial and Temporal Aspects of Signalling

Synopsis

The function and efficiency of cell signalling pathways are very dependent on their organization both in space and time. With regard to spatial organization, signalling components are highly organized with respect to their cellular location and how they transmit information from one region of the cell to another. This spatial organization of signalling pathways depends on the molecular interactions that occur between signalling components that use signal transduction domains to construct signalling pathways. Very often, the components responsible for information transfer mechanisms are held in place by being attached to scaffolding proteins to form macromolecular signalling complexes. Sometimes these macromolecular complexes can be organized further by being localized to specific regions of the cell, as found in lipid rafts and caveolae or in the T-tubule regions of skeletal and cardiac cells.

Another feature of the spatial aspects concerns the local and global aspects of signalling. The spatial organization of signalling molecules mentioned above can lead to highly localized signalling events, but when the signalling molecules are more evenly distributed, signals can spread more globally throughout the cell. In addition, signals can spread from one cell to the next, and such intercellular communication can co-ordinate the activity of cell communities This spatial organization of signalling is well illustrated by the elementary and global aspects of Ca^{2+} signalling.

The temporal aspects of signalling concern the way information is organized in the time domain. Many biological processes are rhythmical. Of particular importance are the cellular oscillators that set up oscillating intracellular signals that can operate over an enormous range of frequencies to drive a wide range of cellular processes. Membrane oscillators (millisecond to second range) set up rapid membrane potential oscillations that can drive neural processing of information and pacemaker activity in contractile systems such as the heart and smooth muscle. Cytosolic oscillators (second to minute range) set up oscillations in intracellular Ca^{2+} to control a large number of cellular processes, such as fertilization, contraction of smooth muscle cells, ciliary beat frequency and glycogen metabolism in liver cells. The circadian clock, which is responsible for driving the 24 h diurnal rhythm, is a transcriptional oscillator.

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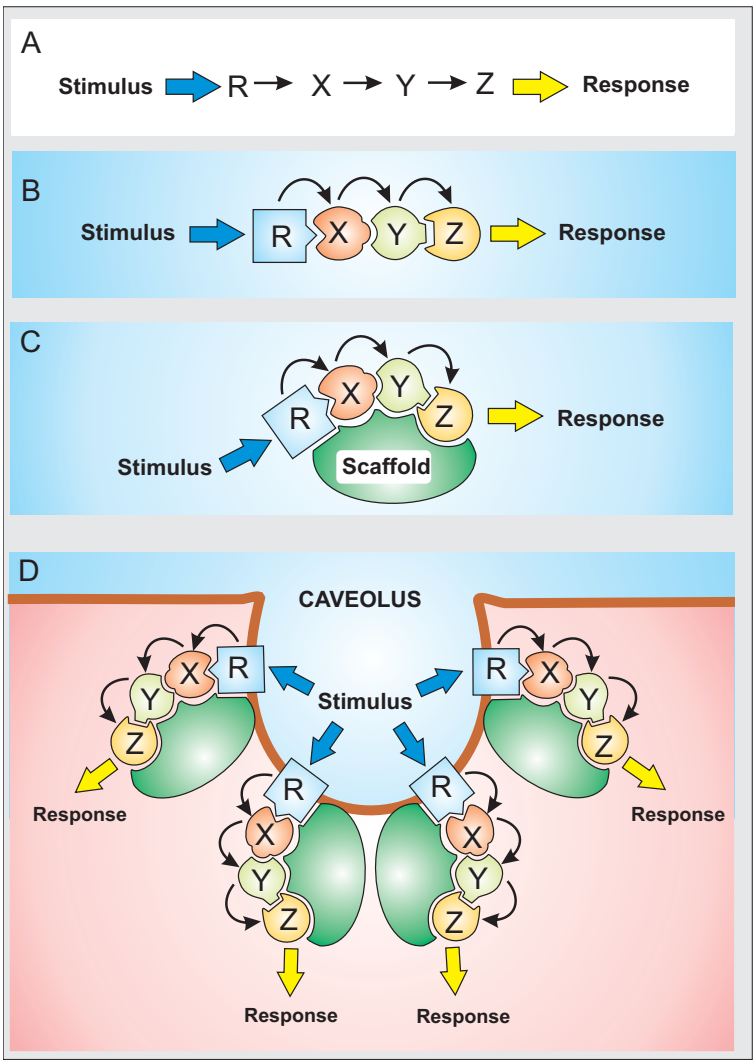
Another important temporal aspect is timing and signal integration, which relates to the way in which functional interactions between signalling pathways are determined by both the order and the timing of their presentations.

The organization of signalling systems in both time and space greatly enhances both their efficiency and versatility.

Spatial organization of signalling pathways

Most signalling pathways function by transmitting information from one component to the next (panel A in Module 6: Figure signalling hierarchies). The efficiency and speed of this vectorial flow of information is greatly facilitated by the spatial organization of the signalling components that are often linked together through signal transduction domains (panel B in Module 6: Figure signalling hierarchies). If all of the signalling components are in place and correctly aligned, information can flow quickly down the signalling cascade by avoiding the delays that would occur if the interacting partners had to find each other by diffusion during the course of each signal transmission sequence. Another important spatial feature is the location of signalling pathways within the cell. There are a variety of scaffolding/targeting proteins that function as anchors and adaptors to hold signalling components in place to form macromolecular signalling complexes (panel C in Module 6: Figure signalling hierarchies). These scaffolding systems can also function to direct macromolecular complexes to specific locations within the cell, such as the lipid rafts and caveolae (panel D in Module 6: Figure signalling hierarchies).

Module 6: | Figure signalling hierarchies



The spatial organization of signalling pathways.
The way in which information is transferred in cells is often highly organized, as illustrated in this highly schematic depiction of how components of signalling pathway are organized. A. The basic components of a typical signalling pathway, consisting of a receptor (R) and three signalling components (X, Y and Z). B. In those cases where the signalling components are proteins, information is transmitted through protein–protein interactions using signal transduction domains. For example, a motif on protein X recognizes a specific binding site on protein Y and so on. C. A variety of scaffolds function to hold together the individual components of signalling pathways to create macromolecular signalling complexes. D. These macromolecular signalling complexes can be aggregated in specific locations within the cell, as occurs in lipid rafts and caveolae.

In this hypothetical system, the signalling components have a fixed location both with regard to each other and to their location within the cell. However, there are numerous examples of signalling components being much more mobile and undergoing marked translocations during the operation of a signalling cascade. This mobility is particularly evident for proteins that have signal transduction domains that interact with various signalling lipids in cell membranes.

Signal transduction domains

A characteristic feature of many signalling proteins is that they contain signal transduction domains that enable them to interact with other signalling components to set up signalling pathways (Module 6: Figure signalling hierarchies). These domains participate either in protein–protein interactions or in protein–lipid interactions.

Protein–protein interactions

Protein–protein interactions depend upon the following modular protein domains:

- 14-3-3 domain
- CC domain
- CH domain
- EH domain
- FERM domain
- ITAM domain
- LIM domain
- PDZ domain
- PTB domain
- SAM domain

- SH2 domain
- SH3 domain
- WW domain

These protein domains bind to specific sequences on their target proteins, as summarized in Module 6: Figure modular protein domains.

Coiled-coil (CC) domain

The coiled-coil (CC) domain functions to interact with equivalent CC domains on other proteins to form either homo- or hetero-typic interactions.

Src homology 2 (SH2) domain

The Src homology 2 (SH2) domain binds to a phosphotyrosine group located within a specific sequence on the target protein (panel A in Module 6: Figure modular protein domains). The following are some examples of signalling molecules that use SH2 domains:

- Phospholipase Cγ (PLCγ) has two SH2 domains that are used during translocation of the enzyme from the cytoplasm to tyrosine kinase-linked receptors at the cell surface (Module 2: Figure PLC structure and function).
- The signal transducers and activators of transcription (STATs) transcription factors have an SH2 domain (Module 2: Figure JAK and STAT structure) that enables them to attach to the Janus kinases (JAKs) (Module 2: Figure JAK/STAT function).
- The regulatory subunits of the Class IA PtdIns 3-kinase have SH2 domains (Module 2: Figure PI 3-K family) that function to attach the catalytic subunits to various tyrosine kinase-linked receptors at the cell surface such as the platelet-derived growth factor receptor (PDGFR) (Module 1: Figure PDGFR activation) and the insulin receptor (Module 2: Figure insulin receptor).

Phosphotyrosine-binding (PTB) domain

A phosphotyrosine-binding (PTB) domain interacts with a unique sequence containing a phosphotyrosine group (panel B in Module 6: Figure modular protein domains). The PTB domain is an important feature of many signalling molecules, such as the insulin receptor substrate (IRS) (Module 6: Figure IRS domain structure).

14-3-3 domain

The 14-3-3 proteins belong to a family of adaptor proteins that recognize a phosphoserine residue embedded in a specific sequence within target proteins (panel C in Module 6: Figure modular protein domains). For example, the phosphorylated transcription factor TAZ is exported from the nucleus during activation of the hippo signaling pathway (Module 2: Figure hippo signalling pathway).

Src homology 3 (SH3) domain

The Src homology 3 (SH3) domain interacts with a polyproline motif on its target proteins (panel D in Module 6: Figure modular protein domains). The adaptor protein growth factor receptor bound protein 2 (Grb2) is a classical example of an SH3-containing protein that binds to the guanine nucleotide exchange factor (GEF) Son-of-sevenless (SoS) during the activation of Ras signalling (Module 1: Figure stimuli for enzyme-linked receptors).

PDZ domain

The PDZ (named after postsynaptic density 95, Discs large and zonula occludens 1) domain binds to its target via a short peptide sequence that has a C-terminal hydrophobic residue (panel E in Module 6: Figure modular protein domains). There are a large number of PDZ-containing proteins with a wide range of functions (Module 6: Figure PDZ-containing proteins). For example, many of the scaffolding proteins that contain PDZ domains function to assembly large macromolecular signalling complexes.

WW domain

The WW domain binds a sequence rich in proline residues (panel F in Module 6: Figure modular protein domains).

Sterile alpha motif (SAM) domain

The sterile alpha motif (SAM) domain is a protein–protein interaction region that has approximately 70 amino acids. It is 5 helices that are organized into a compact bundle with a conserved hydrophobic core. This SAM domain is found on many different proteins where it can function in both homo- and heterotypic interactions. The following are examples of proteins that interact through SAM domains:

- The C-terminal region of the Eph receptor has a SAM domain that participates in a homotypic interaction during receptor dimerization (Module 1: Figure Eph receptor signalling).
- The stromal interaction molecule (STIM), which is located in the endoplasmic reticulum (ER) where it functions to control Ca^{2+} entry, has a SAM domain in the N-terminal region (Module 3: Figure SOC signalling components).

Calponin homology (CH) domain

The calponin homology (CH) domain is particularly evident in proteins that function as part of the cytoskeleton. Tandem CH domains, such as those found in parvin, are particularly effective in binding actin as occurs in the focal adhesion complex (Module 6: Figure integrin signalling).

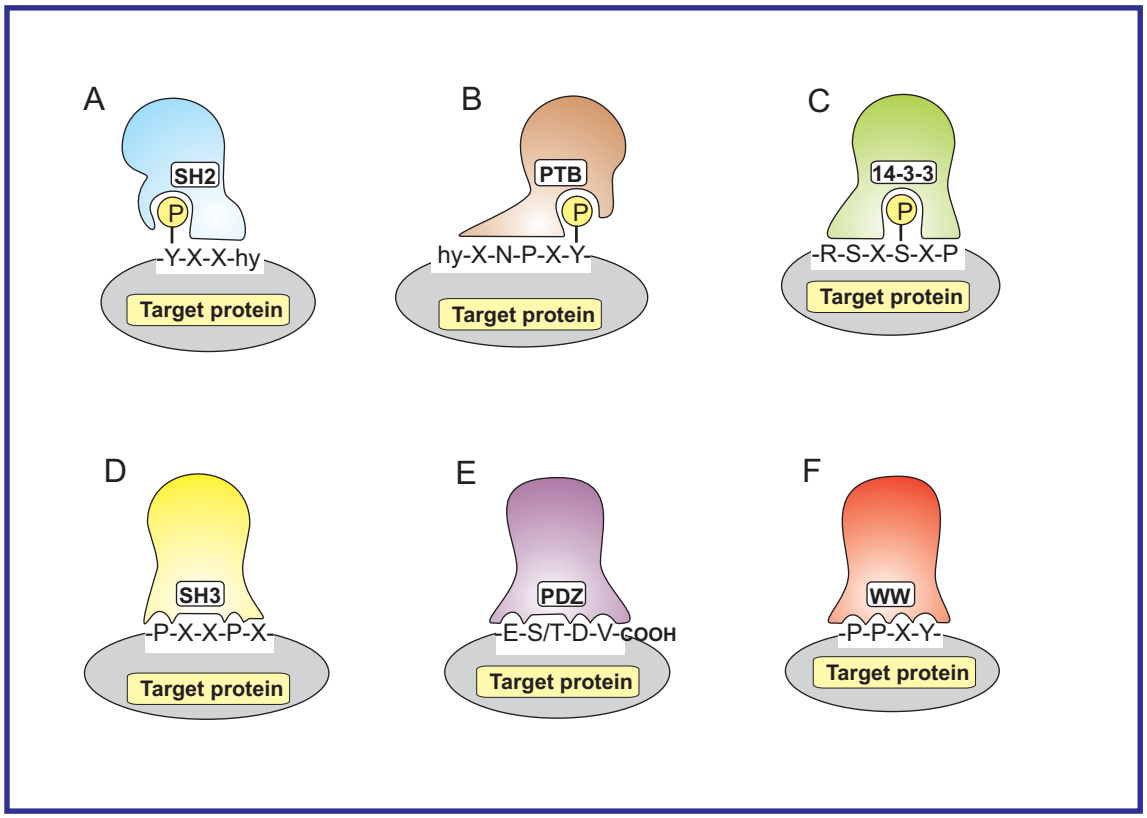
EH domain

The EH domain, which binds to the asparagine-proline-phenylalanine motif, contributes to a variety of protein–protein interactions. It has been identified on the scaffolding protein intersectin.

FERM domain

The FERM (named after four-point-one, ezrin, radixin and moesin) domain contains three compact modules (A–C), which has basic residues capable of binding PtdIns4,5P_2. FERM domains are particularly evident on some of the proteins located on adhesion complexes such as talin and focal adhesion kinase (FAK) (Module 6: Figure focal adhesion components).

Module 6: | Figure modular protein domains



Summary of some of the major protein modules used to assemble cell signalling pathways.
The fidelity of information transfer between signalling components depends upon highly precise interactions between a variety of signal transduction domains and corresponding specific signal sequences on the target protein (see the text for further details).

Immunoreceptor tyrosine-based activation motifs (ITAMs)

The immunoreceptor tyrosine-based activation motifs (ITAMs) are docking sites located on the cytoplasmic domains of various receptors. These ITAMs function to assemble the following signal transduction complexes:

- The Fc receptor γ (FcRγ) chains in blood platelets have ITAMs that are phosphorylated by Fyn to provide binding sites for phospholipase Cγ2 (PLCγ2) (see step 2 in Module 11: Figure platelet activation).
- The CD3 subunits (γ, δ and ε) and the ζ subunits of the T cell receptor (TCR) have long cytoplasmic chains that contain ITAMs (red bars in Module 9: Figure TCR signalling), which provide the docking sites to assemble the receptor scaffolds responsible for activating various signalling pathways.
- ITAMs on the FcεRI subunits of mast cells recruit various transducing elements, such as the non-receptor tyrosine kinases Fyn, Lyn and Syk (Module 11: Figure FcεRI mast cell signalling).
- Igα and Igβ signalling proteins recruit signalling components during the B-cell antigen receptor (BCR) activation process (Module 9: Figure B cell activation).

LIM domain

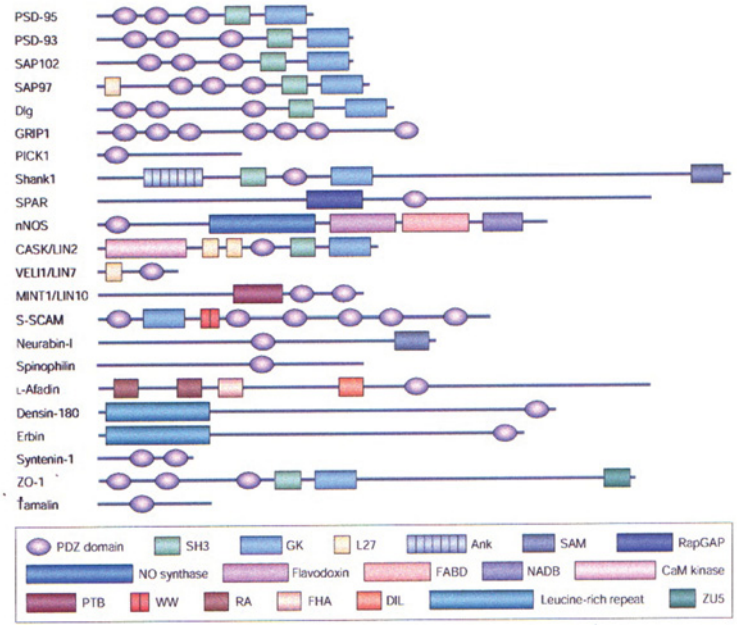
The LIM domain was first identified in the three transcription factors LIN 11, ISL 1 and MEC3 and the first letter of each one was used to produce the abbreviation LIM. This LIM domain consists of a tandem cysteine-rich Zn^{2+}-finger motif that is used for protein–protein interactions. Such LIM domains have been identified in various proteins that participate in junctional complexes such as the particularly interesting cysteine/histidine-rich protein (PINCH) and paxillin.

Lasp-1 is an example of an adaptor protein, which contains an N-terminal LIM domain, that binds to actin and may contribute to the reorganization of the cytoskeleton during the control of parietal cell secretion of acid (Module 7: Figure HCl secretion).

Protein–lipid interactions

A number of signalling molecules function by interacting with specific lipid messengers located in membranes. These protein–lipid interactions depend upon a number of modular protein domains (e.g. C2, FYVE, PH, PX and ENTH) that bind to specific membrane lipids such as diacylglycerol (DAG) and various phosphoinositides such as PtdIns4,5P_2, PtdIns3P, PtdIns3,4P_2 and PtdIns3,4,5P_3 (Module 6: Figure modular lipid-binding domains):

Module 6: | Figure PDZ-containing proteins



PDZ-containing proteins.
A large number of proteins contain either single or multiple PDZ domains. Reproduced by permission from Macmillan Publishers Ltd: *Nat. Rev. Neurosci.*, Kim, E. and Sheng, M. (2004) PDZ domain proteins of synapses. 5:771–781. Copyright (2004); http://www.nature.com/nrn; see Kim and Sheng (2004).

BAR domain

The C-terminal Bin, Amphiphysin, Rvs (BAR) domain is located on proteins that can bind to cell membranes. BAR domains dimerize with each other to form a concave structure that can bind to membranes with a positive curvature as found on vesicles and tubules. They are found on some members of the sorting nexin (SNX) family that function in the sorting of proteins such as the early endosome to plasma membrane trafficking of the transferrin receptor (TFR) (Module 4: Figure early endosome budding) or during the early endosome to *trans*-Golgi network (TGN) trafficking (Module 4: Figure endosome budding TGN).

C2 domain

C2 domains are found on many proteins, where they function to bind Ca^{2+} to induce a conformational change to form a lipid-binding domain that enables proteins to interact with membrane lipids. The C2 domains vary with regard to their lipid preference: some bind neutral lipids, whereas others prefer negatively charged phospholipids. Not all C2 domains bind Ca^{2+}. The tumour suppressor phosphatase and tensin homologue deleted on chromosome 10 (PTEN), which hydrolyses the lipid second messenger PtdIns3,4,5P_3, has such a Ca^{2+}-insensitive C2 domain, which still functions to attach the enzyme to the membrane so that it can reach its substrate. The more classical Ca^{2+}-sensitive C2 domains were originally described in protein kinase C (PKC), where they are found on both the conventional and novel PKCs (Module 2: Figure PKC structure and action). C2 domains are also found on the synaptotagmins that function in Ca^{2+}-dependent exocytosis (Module 4: Figure Ca^{2+}-induced membrane fusion) and on the otoferin that triggers hair cell transmitter release.

ENTH domain

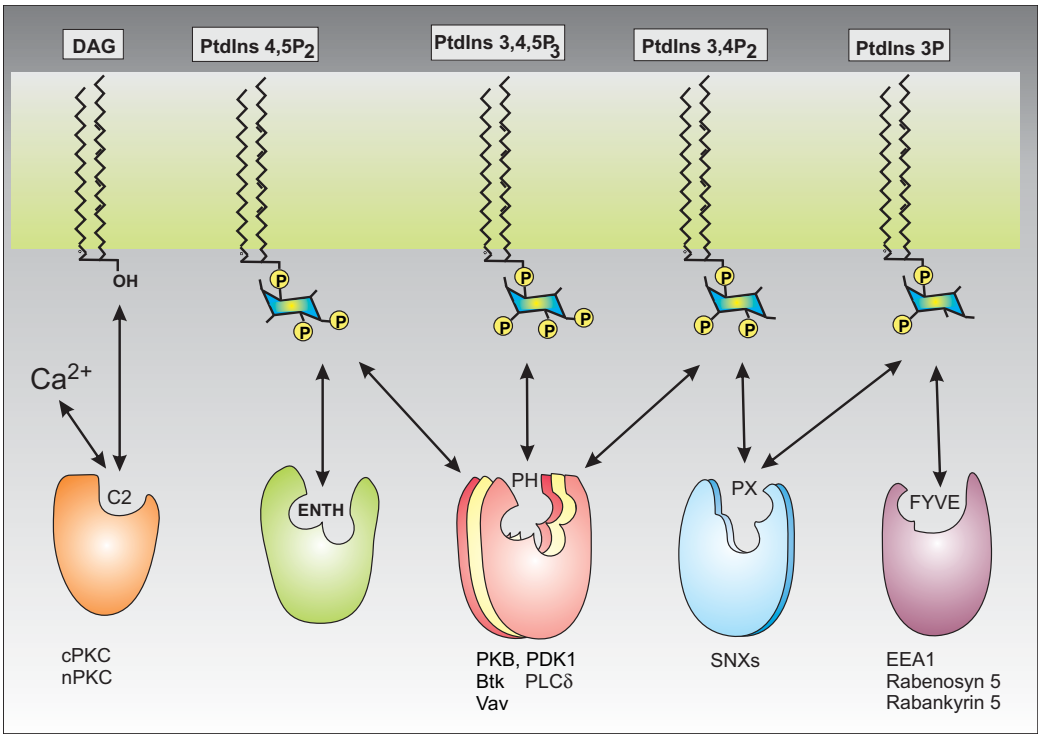
ENTH is a lipid-binding domain that recognizes PtdIns4,5P_2 (Module 6: Figure modular lipid-binding domains). This motif contains about 140 residues and is located on proteins that function in endocytosis and cytoskeletal organization. With regard to the latter, ENTH may play a role in mediating the PtdIns4,5P_2 regulation of actin remodelling.

Pleckstrin homology (PH) domain

The pleckstrin homology (PH) domain is capable of binding to a number of lipid messengers (Module 6: Figure modular lipid-binding domains). There are multiple PH domains that have 100–120 residues that have little sequence homology, but there is considerable similarity in their tertiary structure. These different PH domains are present on many signalling molecules:

- Many of the phospholipase Cs (PLCs) have PH domains, which help the enzyme to associate with the membrane (Module 2: Figure PLC structure and function).
- Protein kinase B (PKB).
- Phospholipase D (PLD) has a PH domain that binds to PtdIns4,5P_2 (Module 2: Figure PLD isoforms).
- Bruton's tyrosine kinase (Btk)
- The insulin receptor substrate (IRS) has a PH domain that is used to bind IRS to the insulin receptor (Module 2: Figure insulin receptor).

Module 6: | Figure modular lipid-binding domains



Summary of the lipid signal transduction domains found on proteins that interact with specific lipids in cell membranes.
Many proteins contain specific domains that enable them to interact with different signalling lipids located in cell membranes. See the text for details of the different proteins.

Phox homology (PX) domain

Phox homology (PX) domains are found in many different proteins, and have been divided into three classes:

- Class I contain small proteins where the PX domain represents most of the protein, and many of these belong to the sorting nexin (SNX) family (e.g. SNX3, SNX9, SNX10, SNX12, SNX22, SNX23, SNX24 and SNX26).
- Class II resemble the above, but have larger flanking regions. Many of these also are found within the SNX family (e.g. SNX1, SNX2, SNX4–SNX8, SNX11, SNX16, SNX20, SNX21 and SNX29).
- Class III represent proteins that contain PX domains, together with other protein domains such as the pleckstrin homology (PH) and HKD domains in phospholipase D 1 (PLD1) and 2 (PLD2) (Module 2: Figure PLD isoforms).

FYVE domain

FYVE is a membrane-targeting motif that recognizes PtdIns3P and is often found on proteins that function in membrane trafficking:

- The early endosome antigen 1 (EEA1) uses its FYVE domain to associate with the phagosome (Module 4: Figure phagosome maturation).
- The Class III PtdInsP kinase, which is also known as PIKfyve, functions in the PtdIns3,5P_2 signalling cassette (Module 2: Figure PIKfyve activation). The FYVE domain targets PIKfyve to endomembranes.

As a result of these protein–lipid interactions, proteins translocate from the cytoplasm on to the cell membrane, and this is a critical part of their signalling function. A critical event for many signalling molecules is their translocation to the plasma membrane, as found in the following examples:

- Translocation of conventional protein kinase C (cPKC) to the plasma membrane (Module 2: Figure PKC structure and activation).
- Translocation of phospholipase Cγ (PLCγ) to the plasma membrane during phosphoinositide signalling (Module 2: Figure PLC structure and function).
- Phosphoinositide-dependent kinase 1 (PDK1) and protein kinase B (PKB) translocate to the plasma membrane in response to the formation of the lipid messenger PtdIns3,4,5P_3.

There are other proteins that move from the cytoplasm to various intracellular membranes such as the endosomes. For example, translocation of the early endosome antigen (EEA1) on to the PtdIns3P on the phagosome membrane (Module 4: Figure phagosome maturation).

Scaffolding/targeting proteins

There are many examples of scaffolding/targeting proteins that function as adaptors to assemble macromolecular complexes and to target signalling complexes to specific locations in the cell:

- A-kinase-anchoring proteins (AKAPs)

- A subunit of protein phosphatase 2A
- Abelson-interactor (Abi)
- Arrestins
- Axin
- Caveolin is a scaffolding protein that organizes the signalling function of caveolae.
- Cbl
- Crk
- Dishevelled (Dsh)
- Fe65
- Glycogen scaffold
- Growth factor receptor-bound protein 2 (Grb2)
- Insulin receptor substrate (IRS)
- Intersectin
- Postsynaptic density (PSD) scaffolding and adaptor components
- Protein interacting with Cα-kinase 1 (PICK1)
- Membrane-associated guanylate kinase (MAGUK)
- Septins
- Shc
- Shank

Postsynaptic density (PSD) scaffolding and adaptor components

Many of the scaffolding proteins contain PDZ domains (Module 6: Figure PDZ-containing proteins). A particularly impressive example is provided by the postsynaptic density (PSD) scaffolding and adaptor components that have a number of PDZ-containing proteins co-operate to form the PSD (Module 10: Figure postsynaptic density).

Axin

Axin is a scaffolding protein that functions in the Wnt signalling pathway. It acts as a scaffold for a multiprotein complex that functions to phosphorylate β-catenin to target it for destruction by the proteasome (Module 2: Figure Wnt canonical pathway). The stability of axin is enhanced by sumolyation.

Cbl

The casitas B-lineage lymphoma (Cbl) family in mammals consists of three members: c-Cbl, Cbl-b and Cbl-3 (Module 6: Figure Cbl structure). Cbl has two very different functions. Firstly, it contains various protein–protein interaction domains that enable it to act as an adaptor protein that contributes to the assembly of signalling complexes. Secondly, it contains a ubiquitin ligase (E3) region responsible for terminating the activity of many signalling components by targeting them for degradation. Cbl structure and regulation reveals the presence of many domains that contribute to Cbl adaptor functions and Cbl down-regulation of signalling components. Some myeloid neoplasms are caused by mutations in Cbl.

Cbl structure and regulation

Two of the Cbls are highly homologous (c-Cbl and Cbl-b), whereas Cbl-3 is much smaller with a large part of the C-terminal region missing. Cbl contains numerous structural domains related to its adaptor and protein degradation functions (Module 6: Figure Cbl structure). It has a highly conserved N-terminal tyrosine kinase-binding (TKB) domain, which is made up of three elements: a four-helix bundle (4H), a Ca^{2+}-binding EF-hand and a modified Src homology (SH2) domain. In addition to binding protein tyrosine kinase-linked receptors (PTKRs) [e.g. epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), colony-stimulating factor-1 receptor (CSF-1R)] and non-receptor protein tyrosine kinases (e.g. Src and Syk), TKB can also interact with other proteins such as adaptor protein-containing pleckstrin homology (PH) and Src homology 3 (SH3) domains (APS), Src-like adaptor protein (SLAP), Sprouty 2 (Spry2) and tubulin. The TKB is attached through a short linker (L) to the RING finger domain, which has the E3 ubiquitin ligase activity. An important aspect of the ubiquitination activity of Cbl is the ability of the RING domain to associate with an E2 ubiquitin-conjugating enzyme that functions in the Cbl down-regulation of signalling components (Module 1: Figure receptor down-regulation).

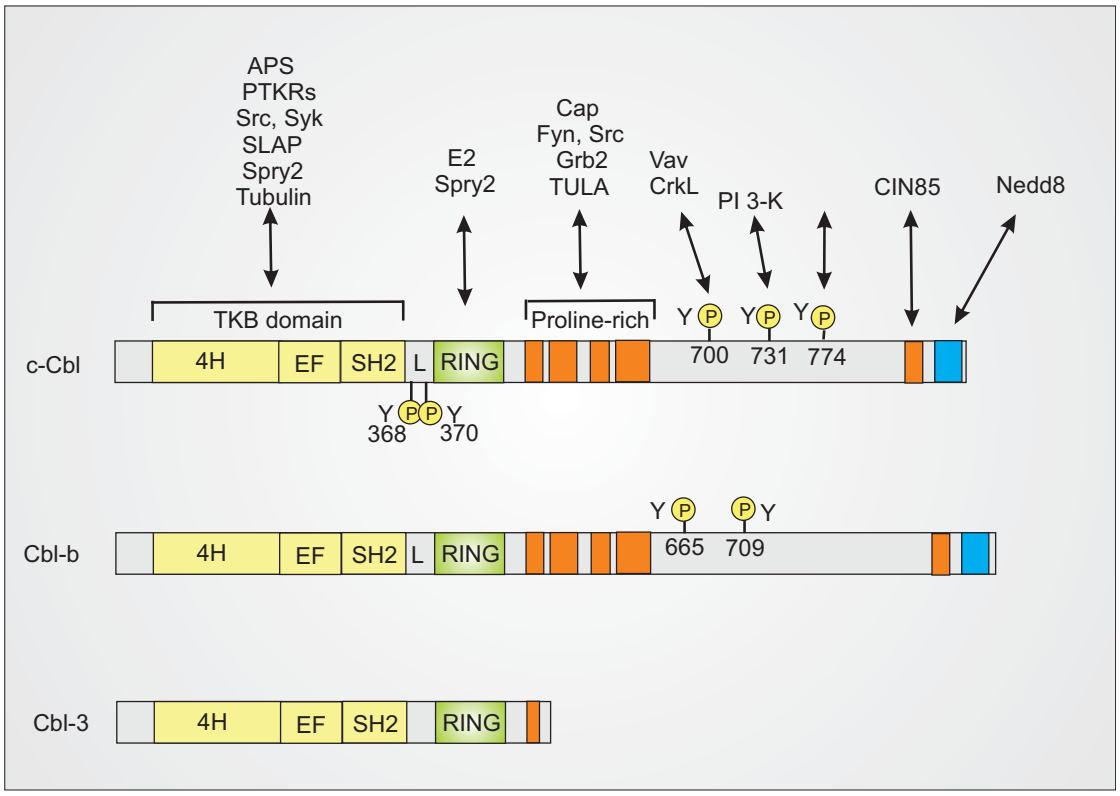
Both c-Cbl and Cbl-b have an extensive region of proline-rich motifs that can bind to proteins that have Src homology 3 (SH3) domains such as non-receptor protein tyrosine kinases (e.g. Src and Fyn), Cbl-associated protein (Cap), growth factor receptor-bound protein 2 (Grb2) and T cell ubiquitin ligand (TULA). A proline-rich sequence located close to the C-terminus binds to Cbl-interacting protein of 85 kDa (CIN85), and functions to target receptor complexes to the clathrin-coated vesicles by binding to the endophilins (Module 1: Figure receptor down-regulation).

Following the proline-rich region, there are a number of tyrosine residues that are phosphorylated and contribute to the regulation of Cbl activity (see below). The C-terminal region has a ubiquitin-associated (UBA) domain, which can link to either ubiquitin (primarily for Cbl-b) or to the ubiquitin-like domains found on other E3 ligases such as neuronal-expressed developmentally down-regulated gene 8 (Nedd8).

The activity of Cbl is regulated in a number of ways. Phosphorylation of Cbl by other signalling elements, such as the non-receptor protein tyrosine kinases (e.g. Src), plays a critical regulatory role. For example, phosphorylation of tyrosine residues in the N-terminal region provide binding sites for various proteins that have Src homology 2 (SH2) domains such as the Crk-like (CrkL), PtdIns 3-kinase (PtdIns 3-K) and Vav (Module 6: Figure Cbl structure). In addition, phosphorylation of two tyrosine residues (Tyr-368 and Tyr-371) in the L region plays a critical role in switching on the ubiquitination activity of Cbl.

Cbl is also regulated by interacting with other proteins. TULA, which is also known as suppressor of T cell signalling-2 (Sts-2), inhibits Cbl by binding constitutively to the proline-rich region. One action of TULA is to induce the ubiquitination and degradation of c-Cbl. Another regulator is Spry2, an inducible inhibitor of Cbl which binds to the RING finger domain. By binding to RING, it prevents the binding of the E2 enzyme. Upon activation of tyrosine kinase-linked receptors, Spry2 is phosphorylated, and this causes its displacement from the RING domain

Module 6: | Figure Cbl structure



Structure of the Cbl family of adaptor proteins.
The c-Cbl and Cbl-b isoform are closely homologous. The Cbl-3 isoform resembles the other two in its N-terminal region, but is missing the C-terminal regions. The double-headed arrows illustrate the ability of c-Cbl to interact with a large number of signalling components. Many of these interactions are also evident for Cbl-b. See the text for a description of the abbreviations.

to the TKB domain. The RING domain is now free to bind E2 enzymes, which are then able to begin the ubiquitination of the receptor and Spry2. The subsequent proteasomal degradation of Spry2 is compensated for by an EGFR-dependent up-regulation of Spry2 expression. These ubiquitination reactions that occur at receptors contribute to the Cbl down-regulation of signalling components (Module 1: Figure receptor down-regulation).

Cbl adaptor functions

Cbl functions as an adaptor in a number of processes including cell adhesion, spreading and motility. The multidomain Cbl structure enables Cbl to interact with a large number of signalling and structural proteins. Cbl proteins are primarily cytosolic, but they are able to translocate to different cellular sites, such as the plasma membrane and cytoskeleton, following activation of various signalling pathways. One of the important adaptor functions of Cbl is to contribute to the skeletal and signalling events that occur during cell motility, as illustrated by events that occur during focal adhesion integrin signalling (Module 6: Figure integrin signalling) and formation of osteoclast podosomes (Module 7: Figure osteoclast podosome).

Another adaptor role for Cbl occurs at protein-tyrosine kinase linked receptors (PTKRs). For example, during osteoclastogenesis, the phosphorylation of the colony-stimulating factor-1 receptor (CSF-1R) provides a binding site for c-Cbl, which then functions as an adaptor to bind the p85-subunit of PtdIns 3-kinase (PtdIns 3-K) (Module 8: Figure osteoclast differentiation). A similar sequence occurs at Tyr-1003 on the Met receptor. This association between Cbl and protein tyrosine-linked receptors is also relevant to the Cbl down-regulation of signalling components (Module 1: Figure receptor down-regulation).

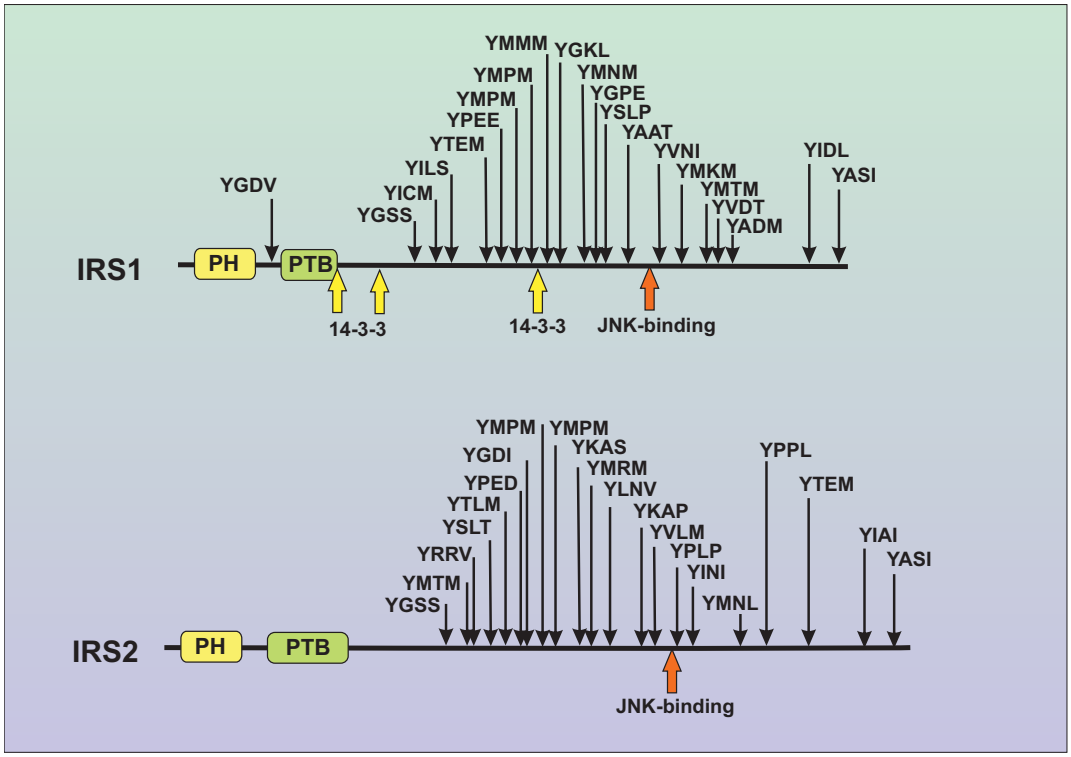
A subunit of protein phosphatase 2A

The A subunit of protein phosphatase 2A (PP2A) assembles the functional holoenzyme by binding both the catalytic subunit as well as the regulatory B subunit that targets the enzyme to specific cellular locations (Module 5: Figure PP2A holoenzyme).

Fe65

The Fe65 family has three members: Fe65, Fe65L1 and Fe65L2. Fe65 is an adaptor protein that seems to have a primary function in neurons where it regulates the trafficking of integral membrane proteins such as the β-amyloid precursor protein (APP) (Module 12: Figure APP processing). Fe65 has three protein–protein interaction domains: an N-terminal WW domain and two PTB domains. It is the C-terminal PTB2 domain that binds to APP, whereas PTB1 interacts with the CP2/LSF/LBP1 transcription factor.

Module 6: | Figure IRS domain structure



Domain structure of the insulin receptor substrate (IRS).

There are three insulin receptor substrate (IRS) isoforms. The domain structures of the main IRS1 and IRS2 isoforms illustrate the position of the phosphotyrosine-binding (PTB) domain and the pleckstrin homology (PH) domain. The latter is unusual in that it has a low affinity for lipids, but resembles the structure of PTB. The sequence motifs are potential tyrosine phosphorylation sites that enable IRS to recruit various signalling molecules. A typical example is PtdIns 3-kinase, which associates with IRS through its Src homology 2 (SH2) domains (Module 2: Figure insulin receptor).

Insulin receptor substrate (IRS)

The insulin receptor substrate (IRS) was one of the first scaffolding proteins to be identified. There are three IRS proteins in humans (IRS1, IRS2 and IRS4). The first two are expressed widely, whereas IRS4 is restricted to the brain, kidney, thymus and β-cells. Like other scaffolding proteins, IRS contains a number of interaction domains that enable it to interact with various signalling components (Module 6: Figure IRS domain structure). One of the most important is the phosphotyrosine-binding (PTB) domain that interacts with a unique sequence containing a phosphotyrosine group (Module 6: Figure modular protein domains). Such an interaction occurs at the juxtamembrane phosphotyrosine residue of the insulin receptor, which is responsible for recruiting IRS into the receptor complex (Module 2: Figure insulin receptor).

Glycogen scaffold

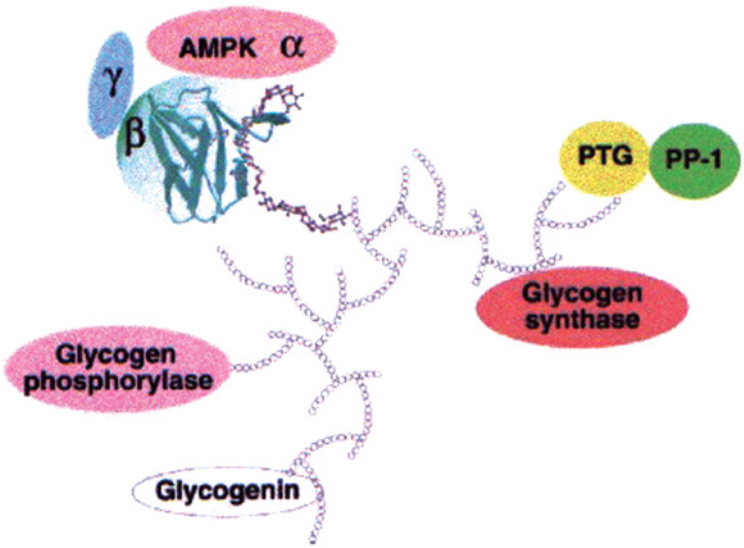
Glycogen functions as a scaffold to bring together many of the proteins that function in glycogen metabolism, such as AMP-activated protein kinase (AMPK), glycogen phosphorylase, glycogen synthase and protein phosphatase 1 (PP1) (Module 6: Figure glycogen scaffold). For some of these proteins, their attachment to glycogen is facilitated by various adaptors, such as the protein targeting to glycogen (PTG), G_M and G_L in the case of PP1 (Module 5: Figure PP1 targeting to glycogen).

Growth factor receptor-bound protein 2 (Grb2)

Growth factor receptor-bound protein 2 (Grb2) is an adaptor protein that functions to link tyrosine kinase-linked receptors to the mitogen-activated protein kinase (MAPK) signalling system (Module 1: Figure stimuli for enzyme-linked receptors). It is particularly important for the extracellular-signal-regulated kinase (ERK) pathway (Module 2: Figure ERK signalling). The central Src homology 2 (SH2) domain binds to the pTyr-Xaa-Asn motifs found on activated receptors or on cytoplasmic scaffolding proteins. The N-terminal Src homology 3 (SH3) domain binds the Pro-Xaa-Xaa-Pro motif on the Ras guanine nucleotide exchange factor Son-of-sevenless (SoS). Typical examples of this adaptor function occur on the platelet-derived growth factor (PDGF) receptor (Module 1: Figure PDGFR activation) and on the vascular endothelial growth factor (VEGF) receptor (Module 9: Figure VEGF-induced proliferation).

Grb2 contributes to the Cbl down-regulation of signalling components by helping to attach Cbl to activated receptors (Steps 2 and 3 in Module 1: Figure receptor down-regulation).

Module 6: | Figure glycogen scaffold



The scaffolding function of glycogen.
Glycogen acts to bring together a number of signalling components, such as AMP-activated protein kinase (AMPK), glycogen phosphorylase, glycogen synthase, glycogenin, protein targeting to glycogen (PTG) and protein phosphatase 1 (PP1). Reproduced from *Curr. Biol.*, volume 13, Polekhina, G., Gupta, A., Michell, B.J., van Denderen, B., Murthy, S., Feil, S.S.C., Jennings, I.G., Campbell, D.J., Witters, L.A., Parker, M.W., Kemp, B.E. and Stapleton, D., AMPKβ subunit targets metabolic stress sensing to glycogen, pp. 867–871. Copyright (2003), with permission from Elsevier; see Polekhina et al. (2003).

Interesectin

There are two intersectins (ITSN1 and ITSN2), which are multidomain scaffolding proteins that function in both cell signalling and in endocytosis. Beginning at the N-terminus, there are two EH domains, a CC domain and five SH3 domains. The presence of multiple protein interaction domains means that intersectin can interact with different proteins and this is particularly evident with regard to its role in exocytosis and endocytosis. Since it can interact with SNAP25, Eps15, synaptojanin 1 and dynamin, it may play a role in integrating the exocytotic/endocytotic cycle process during synaptic vesicle recycling in neurons (Module 4: Figure vesicle cycle).

There also is a neuron-specific long ITSN isoform (ITSN-L), which has an additional DH domain, a PH domain and a C2 domain. This long isoform can function as a GEF for Cdc42 as occurs in the ephrin (Eph) receptor signalling pathway (Module 1: Figure Eph receptor signalling).

The *ITSN1* gene is located on the Down's syndrome (DS) critical region on chromosome 21. The resulting increase in the levels of ITSN1 may thus contribute to Down's syndrome.

Septins

The septins are a family of GTP-binding proteins that have diverse cytoskeletal, scaffolding and diffusion barrier functions. The human genome contains 13 septins (*SEPT1–SEPT12* and *SEPT14*; *SEPT13* is a pseudogene). All the septins have a characteristic structure consisting of an N-terminal proline-rich region, a polybasic region that can bind membrane phosphoinositides, a central GTP-binding region and a C-terminal coiled-coil domain. The septins can interact with each other to form hetero-oligomeric complexes to form bundles, filaments or rings. Such structures have been implicated in many cellular processes such as ciliogenesis, cytokinesis and neurogenesis.

There is increasing evidence that they may act as diffusion barriers to restrict proteins to specific regions of the cell. For example, they play a major role in the mechanism of store-operated channel (SOC) activation by facilitating the formation and stabilization of the STIM1/Orai 1 interaction (Module 3: Figure SOC signalling components).

Shc

The Src homology 2 (SH2)-domain-containing protein (Shc) is a highly versatile adaptor protein that can associate with a number of signalling components. There are three Shc genes (*ShcA*, *ShcB* and *ShcC*). ShcB and ShcC are mainly confined to the nervous system. There is an N-terminal phosphotyrosine-binding (PTB) domain and the C-terminal region has an adaptin-binding domain (ABD) followed by an Src homology (SH2) domain. Located between the PTB and ABD domains there are tyrosine residues that are phosphorylated to provide additional binding sites for adaptors such as growth factor receptor-bound protein 2 (Grb2) (Module 2: Figure ERK signalling).

Shank

The Shank family (Shank1–3) are scaffolding proteins that are major binding partners for Homer in the postsynaptic density (Module 10: Figure postsynaptic density). The three members of the Shank family are located in different regions of the brain: Shank1 is found in most regions of the brain except in the striatum, Shank2 is located in the hippocampus, cortex and Purkinje cells in the cerebellum but not in the thalamus and striatum, and Shank3 is found in the hippocampus, cortex, thalamus, striatum and in the granule cells in the cerebellum. The N-terminus has a domain of ankyrin repeats, an SH3 domain, a PDZ domain, a long proline-rich sequence that has binding sites for Homer and cortactin. These multiple domains enable Shank to interact with other scaffolding molecules such as adaptor protein Homer and with many signalling components such as inositol 1,4,5-trisphosphate receptor ($InsP_3R$), the metabotropic glutamate receptor (mGluR) and the canonical transient receptor potential 1 (TRPC1). Through these multiple protein–protein interactions, the Shank proteins are master organizers of the postsynaptic density (PSD) in that they bring together many of the major components that function in the dendritic spines (Module 10: Figure postsynaptic density).

Disruption of *Shank3* has been identified as a candidate gene for Phelan–McDermid syndrome and autisim spectrum disorders (ASD).

A-kinase-anchoring proteins (AKAPs)

The A-kinase-anchoring proteins (AKAPs) are a diverse family of scaffolding proteins that function to locate **protein kinase A (PKA)** (primarily PKA II) and other signalling components to specific cellular targets. PKA is attached to a specific binding region on the AKAP, which also has targeting sequences that enable it to associate with different cellular structures [**Module 2: Figure protein kinase A (PKA)**]. In addition to binding PKA, many of the AKAPs [e.g. **AKAP350**, **AKAP220** and **Wiskott–Aldrich syndrome protein (WASP) verprolin homologous 1 (WAVE1)**] also bind to a range of other components related to both cyclic AMP and other signalling systems. By establishing large macromolecular signalling complexes, the AKAPs provide a platform where the parallel processing of information and the cross-talk between different signalling pathways can occur.

There are a number of different AKAPs that function in specific locations within the cell:

- Plasma membrane (AKAP79/150, AKAP18, Yotiao)
- Mitochondria [Wiskott–Aldrich syndrome protein (WASP) verprolin homologous 1 (WAVE1), D-AKAP1, Rab32)
- Centrosome (AKAP350, pericentrin)
- Microtubules [microtubule-associated protein-2 (MAP2)]
- Cytoskeleton [Wiskott–Aldrich syndrome protein (WASP) verprolin homologous 1 (WAVE1), AKAP-Lbc (AKAP13), gravin (AKAP12)]

Some of the AKAPs function in a context-dependent manner in that they assemble a different set of signalling components depending on where they are expressed. For example,Wiskott–Aldrich syndrome protein (WASP) verprolin homologous 1 (WAVE1) can act in neurons to regulate actin remodelling during the outgrowth of neurons, whereas in the liver, it associates with the mitochondria, where PKA acts to phosphorylate Bad to inhibit apoptosis.

In the following list of AKAPs, their common names have been used and many of these refer to their molecular masses. The name in parentheses is that given by the HUGO Gene Nomenclature Committee:

D-AKAP1 (AKAP1)

D-AKAP1 is a scaffolding protein for **protein kinase A (PKA)** and **protein phosphatase 1 (PP1)**. It is targeted to the mitochondria by a conventional mitochondrial targeting sequence.

AKAP150 (AKAP5)

The human isoform is AKAP79, so the protein is often referred to as AKAP79/150. It is attached to the plasma membrane by binding to phospholipids. AKAP79, which binds protein kinase A (PKA) and protein phosphatase 2B (PP2B), which is also known as calcineurin (CaN), is linked to α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors through synapse-associated protein 97 (SAP97) (Module 10: Figure postsynaptic density).

mAKAP (AKAP6)

Muscle AKAP (mAKAP) associates with both **protein kinase A (PKA)** and the phosphodiesterase **PDE4D3**. One of the functions of mAKAP is to link PKA to the type 2 **ryanodine receptor (RYR2)** (**Module 3: Figure ryanodine receptor structure**).

AKAP18 (AKAP7)

Dual palmitoyl groups target AKAP18 to the plasma membrane, where it brings protein kinase A (PKA) into close association with various voltage-operated channels (VOCs) such as the L-type Ca^{2+} channels in skeletal muscle (**Module 3: Figure $Ca_V1.1$ L-type channel**) and in cardiac muscle (**Module 3: Figure $Ca_V1.2$ L-type channel**). AKAP 18 has an important role in the **modulation of the $Ca_V1.1$ L-type channel**.

AKAP350 (AKAP9)

AKAP350 exists in different splice variants with different names and different functions. For example, AKAP350 is also known as centrosome- and Golgi-localized protein kinase N (PKN)-associated protein (CG-NAP), which is targeted to the centrosome (microtubule-organizing centres), where it functions as a scaffold to assemble a complex containing **protein kinase A (PKA)**, **protein phosphatase 2A (PP2A)**, **protein kinase Cε (PKCε)**, **protein phosphatase 1 (PP1)**, **phosphodiesterase (PDE)** and **casein kinase I (CKI)**. On the other hand, one of the splice variants, called Yotiao, is found in synaptic regions, where it binds to the cytoplasmic region of the NR1 subunit of ***N*-methyl-d-aspartate (NMDA) receptors** (**Module 10: Figure postsynaptic density**).

D-AKAP2 (AKAP10)
Associates with protein kinase A (PKA).

AKAP220 (AKAP11)
This scaffolding protein brings together three of the enzymes [protein kinase A (PKA), protein phosphatase 1 (PP1) and glycogen synthase kinase-3β (GSK-3β)] that function in glycogen metabolism (Module 7: Figure skeletal muscle E-C coupling).

Gravin (AKAP12)
Gravin is thought to associate with the plasma membrane through both phospholipid binding and an N-terminal myristoyl group. It functions to target protein kinases A (PKA) and C (PKC) to the neuromuscular junction, and can also associate with the β-adrenergic receptor.

AKAP-Lbc (AKAP13)
This AKAP functions in the assembly of stress fibres, where it acts to bring together protein kinases A (PKA), C (PKC) and D (PKD) and Rho.

Membrane-associated guanylate kinases (MAGUKs)
In humans there are 22 membrane-associated guanylate kinases (MAGUKs), which are a heterogeneous group of modular scaffolding proteins with multiple cellular functions. They often participate in the assembly of multiprotein complexes on the inner surface of the plasma membrane where they contribute to junctional complexes and the regulation of receptors and ion channels. There are a number of MAGUK family groups:

- Membrane-associated guanylate cyclase kinase, WW and PDZ domain-containing (MAGI)
- Calcium/calmodulin-dependent serine protein kinase (CASK)
- Membrane protein, palmitoylated (MPP)
- Zona occludens (ZO)
- Disc, large homology (DLG)
- CARMA1
- Cytoplasmic Ca^{2+} channel β subunits (CACNBs)

Membrane-associated guanylate cyclase kinase, WW and PDZ domain containing (MAGI)
There are three human membrane-associated guanylate cyclase kinase, WW and PDZ domain containing (MAGI) proteins (MAGI1–3) (Module 6: Figure MAGUKs).

Protein interacting with Cα-kinase 1 (PICK1)
The protein interacting with Cα-kinase 1 (PICK1) is a scaffolding protein that contains a single PDZ domain, a coiled-coil (CC) domain and an acidic C-terminal domain. It can interact with a number of cell signalling molecules such as protein kinase Cα (PKCα), AMPAR subunits GluA2/3, Arp2/3, dopamine transporters and the prolactin-releasing peptide receptor. In neurons, PICK1 functions in synaptic plasticity by facilitating the disassembly of the actin cytoskeleton during the process of long-term depression (LTD) (Step 7 in Module 10: Figure Ca^{2+}-induced synaptic plasticity).

Calcium/calmodulin-dependent serine protein kinase (CASK)
Calcium/calmodulin-dependent serine protein kinase (CASK), which is the paralogue of the LIN-2 protein in *Caenorhabditis elegans,* belongs to the membrane-associated guanylate kinase (MAGUK) family of scaffolding molecules (Module 6: Figure MAGUKs). CASK seems to have two functions: it is a membrane-associated scaffold protein associated with intercellular junctions and it can also function as a transcriptional co-regulator. CASK consists of an N-terminal PDZ domain, a central SH3 domain and a C-terminal guanylate-kinase homology domain. It is unusual in that it has an N-terminal Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) domain. The C-terminal guanylate-kinase domain of CASK is a pseudokinase that is involved in targeting the protein to the nucleus in neuronal cells where it interacts with the T-brain (TBR1) transcription factor and the CASK-interacting nucleosome-assembly protein (CINAP), which regulates the expression of neuronal genes.

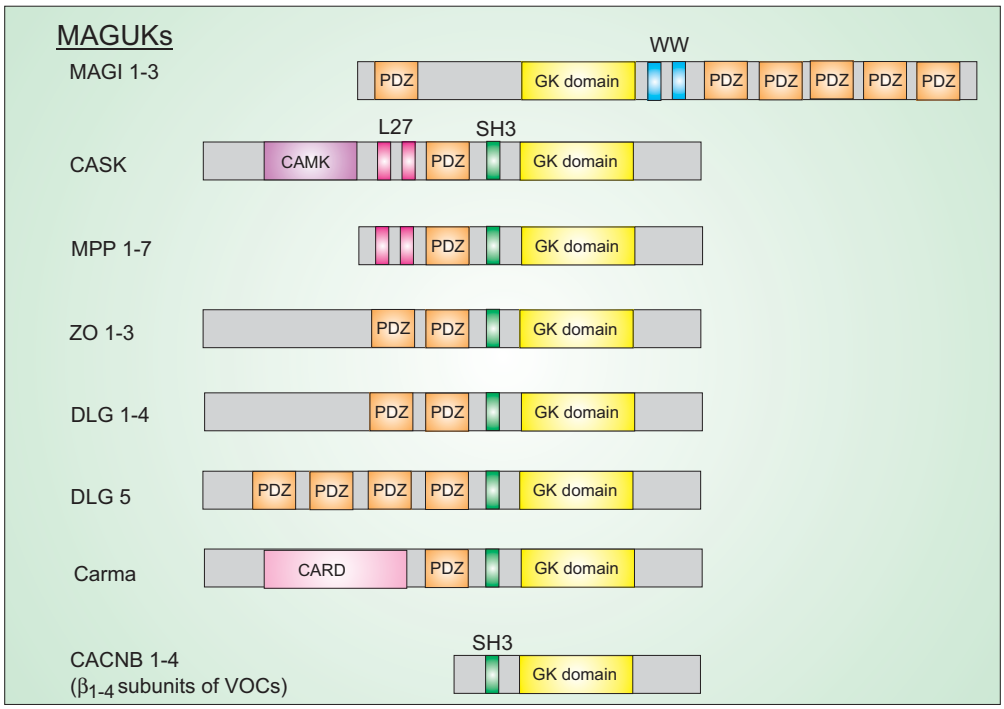
TBR1, which has been linked to autism spectrum disorders (ASDs), also controls some other candidate ASD genes such as *RELN* and the autism susceptibility candidate 2 (*AUTS2*). The *RELN* gene encodes the extracellular matrix glycoprotein reelin that plays a role in neuronal migration and also contributes to dendritic and synapse formation.

Membrane protein, palmitoylated (MPP)
There are seven human membrane protein, palmitoylated (MPP) proteins (MPP1–7), which belong to the membrane-associated guanylate kinase (MAGUK) family (Module 6: Figure MAGUKs). MPP1 may function in neutrophil chemotaxis by regulating the phosphorylation of PKB.

Disc, large homology (DLG)
There are five human Disc, large homology (DLG) proteins (DLG1–5), which belong to the membrane-associated guanylate kinase (MAGUK) family (Module 6: Figure MAGUKs). The DLGs have multiple scaffolding functions especially for orchestrating the positioning of signalling components such as receptors and ion channels in discrete cellular domains. For example, DLG1, which is also known as synapse-associated protein 97 (SAP97), is one of the postsynaptic density (PSD) signalling elements (see 1 in Module 10: Figure postsynaptic density). DLG1/SAP97 plays a role in AMPA receptor trafficking during synaptic plasticity. The expression of Kv4 and Kv1.5 channels can also be regulated by DLG1.

Zona occludens (ZO)
The three zona occludens (ZO1–3) proteins, which belong to the membrane-associated guanylate kinase (MAGUK) family (Module 6: Figure MAGUKs), are scaffolding proteins located within the tight junctions (TJs) that form between epithelial cell layers, such as endothelial cells (Module 7: Figure endothelial cell), and in the myelin sheaths formed by oligodendrocytes and Schwann cells. Tight junctions consist of multiple proteins that

Module 6: | Figure MAGUKs



Domain organization of the MAGUK family.
Members of the membrane-associated guanylate kinases (MAGUKs) family of scaffolding proteins have a number of protein–protein interaction domains.

fall into three main groups: integral membrane proteins, cytoplasmic plaque proteins and the cytoskeletal elements. Claudin, occludin, tricellulin, junctional adhesion molecule-A (JAM-A), JAM4, coxsackie adenovirus receptor (CAR) and endothelial cell-selective adhesion molecule (ESAM) are some of the key integral proteins. They function to hold together the two membranes to form a tight seal. The ZO1–3 proteins provide a link between the integral membrane proteins and the cytoskeleton. For example, they can bind both to claudin and to F-actin.

CARMA1

The full name for CARMA1 is caspase-recruitment domain (CARD) membrane-associated guanylate cyclase (MAGUK) protein 1. As its name implies, CARMA1 belongs to the membrane-associated guanylate kinase (MAGUK) family of scaffolding proteins. It contains a caspase-recruitment domain (CARD), a coiled-coil domain, a PDZ domain, an SH3 domain and a C-terminal guanylate kinase (GK) domain (Module 6: Figure MAGUKs). CARMA1 plays a major role in the activation of the NF-κB signalling pathway in both T cells and B cells. In T cells, for example, one of the signalling components activated by the T-cell receptor (TCR) signalling system is protein kinase Cθ (PKCθ), which is responsible for phosphorylating CARMA1, which is then recruited into the immunological synapse (Module 9: Figure TCR signalling). CARMA1 then associates with a pre-existing complex that consists of the CARD protein Bcl10 and the mucosa-associated lymphoid tissue protein-1 (MALT1, which is also known as paracaspase) to form the CARMA–Bcl10–Malt1scaffolding complex. CARMA1 and Bcl10 interact through their CARD domains. MALT1 has an N-terminal death domain followed by two Ig-like domains and a C-terminal caspase-like domain. This complex then activates the IκB kinase (IKK) resulting in activation of the NF-κB signalling pathway.

Alterations in the MALT and Bcl10 genes have been linked with MALT lymphomas.

Cytoplasmic Ca^{2+} channel β subunits (CACNBs)

The cytoplasmic Ca^{2+} channel β subunits (CACNBs) are a sub-family of the membrane-associated guanylate kinase (MAGUK) proteins (Module 6: Figure MAGUKs). There are four CACNB genes (*CACNB1–4*) with additional splice variants that code for the β_{1-4} subunits that control voltage-operated Ca^{2+} channels.

The CACNBs are made up of a core Src homology domain 3 (SH3) and a guanylate kinase (GK) domain joined together by a variable linker. The GK domain interacts with high affinity to the Ca^{2+} channel α-subunits to regulate channel opening and closing (Module 3: Figure $Ca_V1.1$ L-type channel).

A mutation of the *CACNB2* gene that codes for the β2 subunit of the $Ca_V1.2$ channel is a schizophrenia-associated gene and has been linked to bipolar disorder and other psychiatric disorders.

Microtubule-associated protein-2 (MAP2)

The microtubule-associated protein-2 (MAP2) protein functions to links together protein kinase A (PKA) and tubulin.

Pericentrin

Pericentrin is a large coiled-coil scaffolding protein that is a major component of the centrosome, also known as the **microtubule organizing centre (MTOC)**.

Pericentrin attaches to the centrosome through a pericentrin–AKAP350 centrosomal targeting (PACT) domain. It is included in this list of AKAPs because it is a scaffold for protein kinase A (PKA) and also binds protein kinase C (PKC).

Wiskott–Aldrich syndrome protein (WASP) verprolin homologous 1 (WAVE1)

Wiskott-Aldrich syndrome protein (WASP) verprolin homologous 1 (WAVE1) is a multifunctional scaffolding protein that brings together different sets of signalling molecules depending on where it is located in the cell. When it is associated with the mitochondria, it brings together protein kinase A (PKA), protein phosphatase 1 (PP1), Bad and glucokinase. However, when it is operating to control actin polymerization, it recruits a different set of proteins, such PKA, Abl, Rac, WAVE-associated Rac Gap protein (WRP) and the actin-related protein 2/3 complex (Arp2/3 complex) (Module 4: Figure actin remodelling).

Rab32

Rab32 is a scaffolding protein that binds protein kinase A (PKA) and is associated with the mitochondria-associated ER membranes (MAMs).

Macromolecular signalling complexes

Components of many signalling pathways are often collected together to form large molecular complexes (panel C in Module 6: Figure signalling hierarchies). The close apposition of signalling components that are often arranged on molecular scaffolds greatly enhances the efficiency of information transfer. There are numerous examples of such macromolecular signalling complexes:

- The T cell receptor (TCR) uses its receptor subunits and scaffolding elements such as the proteins LAT (linker for activation of T cells) and Src homology 2 (SH2)-domain containing leukocyte protein of 76 kDa (SLP-76) to assemble a large group of signalling molecules (Module 9: Figure TCR signalling).
- The platelet-derived growth factor receptor (PDGFR) provides phosphorylated residues to assemble the components of a number of signalling pathways (Module 1: Figure PDGFR activation).
- In the Wnt signalling pathway there is a large β-catenin degradation complex that functions to regulate the phosphorylation and degradation of β-catenin (Module 2: Figure Wnt canonical pathway).
- The scaffolding protein KSR1 holds together components of the extracellular-signal-regulated kinase (ERK) signalling pathway (Module 2: Figure ERK signalling).
- The ryanodine receptors (RYRs) not only function as Ca^{2+} channels, but also assemble many of the signalling components responsible for modulating their activity (Module 3: Figure ryanodine receptor structure).
- The postsynaptic density (PSD) in neurons is a large collection of different, but interacting macromolecular signalling complexes (Module 10: Figure postsynaptic density).
- Integrin receptors located in the focal adhesion complex assemble a large number of signalling components many of which function to assemble the actin cytoskeleton (Module 6: Figure integrin signalling).
- Gene transcription is regulated by transcriptosomes that consist of transcription factors, co-regulators such as the co-activators and co-repressors that recruit chromatin remodelling enzymes such as histone acetyltransferases (HATs), histone deacetylases (HDACs) and protein methylases.

Lipid rafts and caveolae

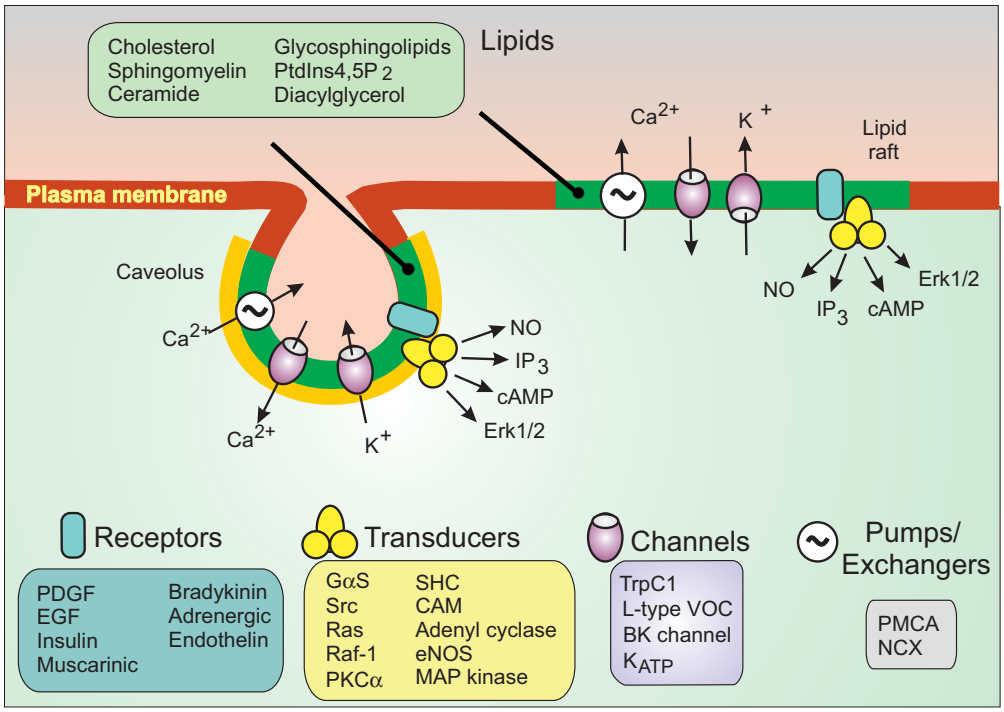
Lipid rafts and caveolae are specialized regions of the membrane that have a number of signalling functions. They provide a plasma membrane-associated scaffolding system for organizing signalling components. The lipid composition of rafts and caveolae provides a unique membrane microenvironment rich in lipids, such as cholesterol and sphingomyelin, which create a liquid-ordered phase domain where a variety of signalling components aggregate. Caveolae structure depends upon the coat protein caveolin, which has a caveolin-scaffolding domain capable of binding to the many components responsible for the signalling function of caveolae. Lipid rafts closely resemble that of the caveolae and may perform a similar function of organizing signalling components, and this may be an important feature of the immunological synapse.

Lipid composition of rafts and caveolae

Lipid rafts and caveolae are characterized by having a lipid composition quite different from that of the surrounding plasma membrane (Module 6: Figure caveolae organization). These special domains are particularly rich in cholesterol and sphingomyelin, but they also contain high levels of glycosphingolipids, ceramide, $PtdIns4,5P_2$ and diacylglycerol (DAG). Many of these lipids are closely associated with various signalling pathways and highlight the importance of these zones as sites of information transfer across the plasma membrane. The surrounding plasma membrane, which is rich in phospholipids with kinked unsaturated fatty acid tails, forms a highly fluid 'liquid-disordered phase', within which there is considerable lateral movement of membrane proteins. In contrast, the high concentration of saturated hydrocarbons within the lipid rafts and caveolae form a 'liquid-ordered phase' because the straight fatty acid chains and the cholesterol pack tightly together to give a highly ordered structure (Module 6: Figure caveolae molecular organization). In effect, the plasma membrane is separated into spatial domains.

There is a phase separation in the membrane, with the bulk of the membrane being in a fluid state, while the lipids are much more ordered in the rafts and caveolae. The semi-crystalline state of the latter makes them resistant to detergents, which can dissolve away the

Module 6: | Figure caveolae organization



The organization and signalling function of lipid rafts and caveolae.
The rafts and caveolae are specialized regions of the plasma membrane (green zones), which are characterized by having a lipid composition (particularly rich in cholesterol and sphingomyelin) distinct from that of the remaining plasma membrane. Apart from its distinct shape, the caveolus differs from the raft by having a cytoplasmic coat of caveolin molecules (yellow). These specialized regions of the plasma membrane contain a number of signalling components (receptors, transducers, channels, pumps and exchangers) responsible for initiating many of the major cell signalling pathways.

liquid-disordered regions of the bulk of the membrane to leave behind the rafts and caveolae. Many of the earlier names for these membrane domains reflected this low solubility in detergents, i.e. detergent-resistant membranes (DRMs), detergent-insoluble, glycolipid-enriched membranes (DIGs), glycolipid-enriched membranes (GEMs) or the Triton X-100-insoluble floating fraction (TIFF). These structures are now usually referred to as lipid rafts and caveolae. However, there remains some debate as to the functional equivalence of these two structures. While caveolae structure is clearly different to that of the rafts (Module 6: Figure caveolae organization), these two domains do have many similarities, especially with regard to their role in signalling. It is important to appreciate, however, that the rafts and caveolae may carry out some different functions. Here most attention will be focused on the caveolae.

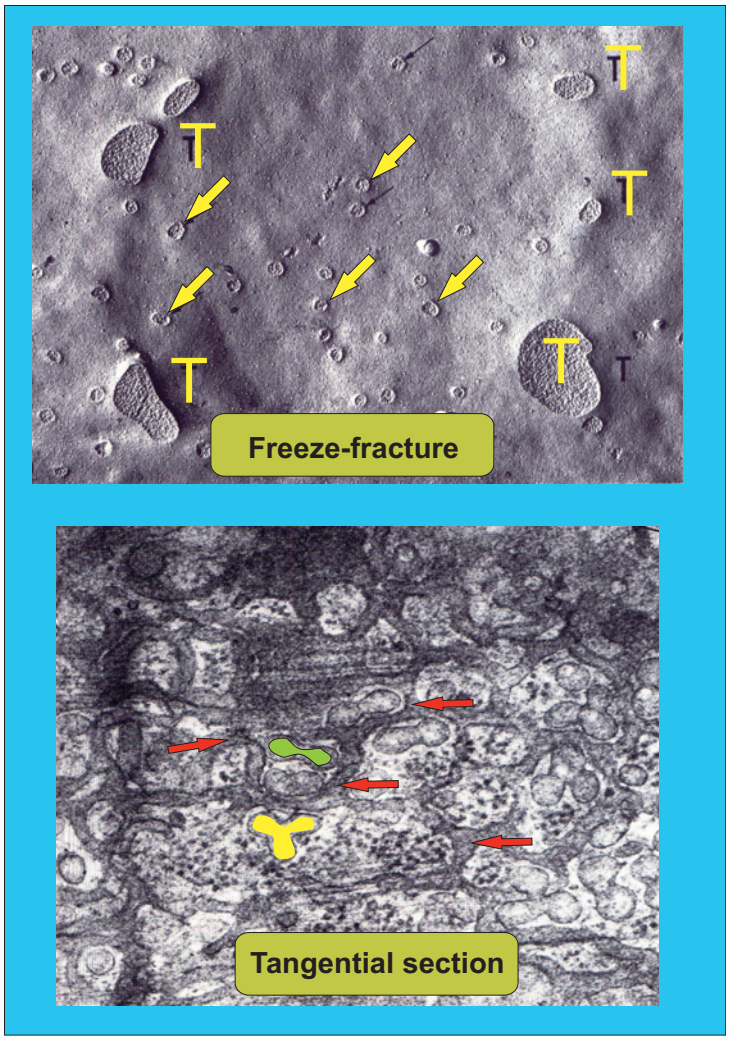
Caveolae structure

Caveolae are flask-shaped invaginations of the plasma membrane. The organization of the caveolae is maintained by a cytoplasmic coat of integral membrane proteins (the yellow layer in Module 6: Figure caveolae organization), of which caveolin is the major component. Caveolae have been observed in many cell types and are particularly evident in endothelial cells and various muscle cells. In cardiac muscle, the numerous openings of the caveolae are clearly evident in freeze–fracture images (Module 6: Figure cardiac caveolae). An important feature of caveolae, which is particularly evident in cardiac and smooth muscle cells, is their close association with the sarcoplasmic reticulum (SR). The full extent of these close associations is very evident in tangential sections, where the caveolae lie in the interstices between the highly reticulated SR. What is not clear from these early electron microscopic studies is whether this peripheral SR near the plasma membrane is connected to the SR that lies deeper within the cell that is responsible for excitation–contraction coupling in muscle cells. An interesting possibility is that this peripheral SR located close to the caveolae might have a separate function, such as the control of store-operated Ca^{2+} entry (Module 3: Figure capacitative Ca^{2+} entry).

In the case of endothelial cells, caveolae function in a transcellular pathway to transport large molecules such as albumin from the plasma to the interstitial space (Module 7: Figure endothelial cell).

A similar association between the caveolae and the SR has been described in smooth muscle cells (Module 6: Figure smooth muscle caveolae). As for the cardiac cell, the caveolae can be seen lying in holes in the flat SR sheet. In addition to associating with the caveolae, portions of the SR also come into close contact with the plasma membrane to form junctional zones that could have various functions. They might function like the junctional zones in cardiac muscle to trigger the release of internal Ca^{2+}. Alternatively, they might represent regions where the SR

Module 6: | Figure cardiac caveolae



Caveolae in rat cardiac ventricular cells.
In the freeze–fracture image shown at the top, there are numerous openings of the caveolae (yellow arrows) in the membrane between the T-tubule invaginations. In the tangential section shown at the bottom, the caveolae have different shapes – rounded, dumb-bells (green) or trilobed (yellow) – and are surrounded by a network of the sarcoplasmic reticulum (red arrows). Reproduced from *J. Ultrastruct. Res.*, Vol. 65, Gabella, G., Inpocketings of the cell membrane (caveolae) in the rat myocardium, pp. 135–147. Copyright (1978), with permission from Elsevier; see Gabella (1978).

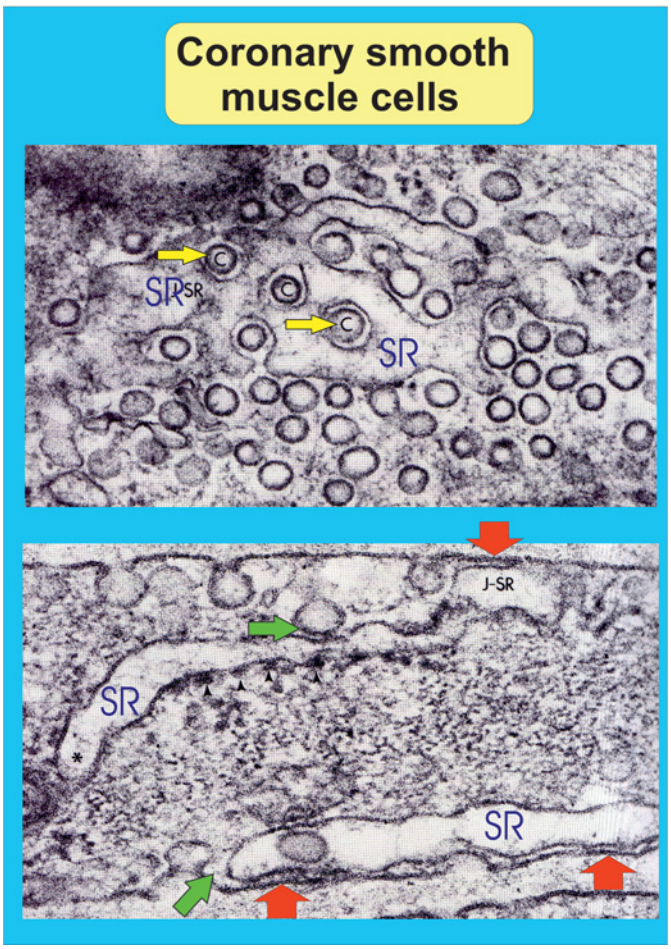
functions to control the opening of plasma membrane ion channels such as the store-operated channels (SOCs), or they might be regions where Ca^{2+} sparks activate Ca^{2+}-sensitive K^+ channels such as the large-conductance (BK) channels (Module 7: Figure smooth muscle cell spark) and the ATP-sensitive K^+ (K_{ATP}) channels (Module 6: Figure caveolae organization). In the case of arterial smooth muscle cells, the $K_{ir}6.1$ subunit of the K_{ATP} channel is located in the caveolae where it appears to be associated with caveolin-1. There are indications that the BK channels found in the caveolae of uterine smooth muscle cells appear to be regulated by cholesterol within the caveolar membrane. An excessive build-up of cholesterol that occurs during obesity may increase the risk of complications in pregnancy. By enhancing BK channel activity, uterine contractility will be reduced during labour and this may account for the increased incidence of Caesarean sections in obese women.

Signalling function of caveolae

The caveolae have been implicated in a number of signalling processes.

Caveolae contain a high concentration of the lipid precursors that are used for signalling. For example, the sphingomyelin signalling pathway is thought to be localized to the caveolae by virtue of the fact that most of sphingomyelin in the plasma membrane is concentrated at these sites. Components of a number of signalling pathways are located at caveolae (Module 6: Figure caveolae molecular organization). Many of these pathways are associated with the caveolins, which are integral membrane proteins that have two important functions. Firstly, they bind to the 'liquid-ordered phase', forming a dense mat beneath the membrane that is responsible for maintaining the flask-like shape of the caveolae. Secondly, they have a scaffolding function, which depends on a caveolin scaffolding domain in the N-terminal region that binds a

Module 6: | Figure smooth muscle caveolae



Caveolae in coronary smooth muscle cells of the mouse.
The tangential section at the top reveals the large number of concentric caveolae (yellow arrows), many of which are surrounded by an extensive interconnected network of the sarcoplasmic reticulum (SR). In the longitudinal section at the bottom, the caveolae are shown opening to the surface. The SR makes close contact with both the caveolae (green arrows) and the plasma membrane, where it forms a typical junctional zone (red arrows). Such junctions may play a role in store-operated Ca^{2+} entry (Module 3: Figure capacitative Ca^{2+} entry). Reproduced from *J. Ultrastruct. Res.*, Vol. 67, Forbes, M.S., Rennels, M.L. and Nelson, E., Caveolar systems and sarcoplasmic reticulum in coronary smooth muscle cells of the mouse, pp. 325–339. Copyright (1979), with permission from Elsevier; see Forbes et al. 1979.

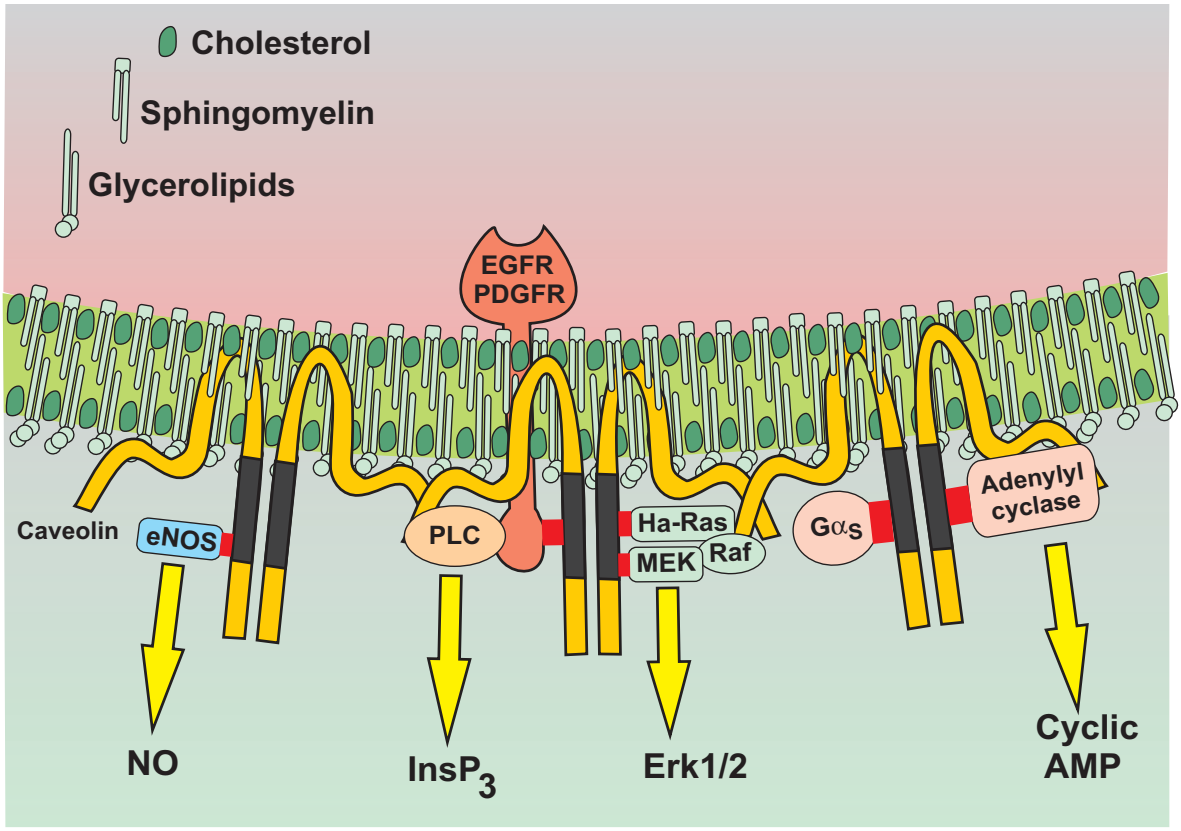
number of signalling components [e.g. epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), $G\alpha_S$, $G\alpha_{i1}$, $G\alpha_{i2}$, adenylyl cyclase (AC), Ha-Ras, Src, Fyn, endothelial nitric oxide synthase (eNOS), protein kinase Cα (PKCα) and mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated protein kinase (ERK) kinase (MEK)], which have distinct caveolin-binding motifs. Some protein tyrosine phosphatases (PTPs), i.e. PTP1B, PTP1C, SHP-2 [Src homology 2 (SH2) domain-containing PTP-2], phosphatase and tensin homologue deleted on chromosome 10 (PTEN) and leucocyte common antigen-related (LAR), have caveolin-1-binding motifs and may function by being recruited into lipid rafts or caveolae.

The attachment of signalling components to caveolin brings these elements together, thus increasing the efficiency of information transfer. In some cases, the close association can lead to inactivation of certain signalling pathways.

Caveolae and Ca^{2+} entry

A number of Ca^{2+} channels are found either in or attached to the caveolae (Module 6: Figure caveolae molecular organization). Examples of the former are the Ca_V1 family of L-type channels and the canonical transient receptor potential TRPC1 channels. The excitability and contractility of vascular smooth muscle cells is controlled by a complex consisting of caveolin-1, the Cav1.2 Ca^{2+} channels and the BK channels ($K_{Ca}1.1$). There is an interaction between caveolin-1 and the TRPC1 entry channels. Somewhat more problematical are the inositol 1,4,5-trisphosphate receptors ($InsP_3Rs$), which have been localized to the caveolae region, but their exact topology is uncertain. There are suggestions that they might be embedded in the plasma membrane and can thus function in Ca^{2+} entry. Alternatively, they might be embedded in endoplasmic reticulum (ER) regions that are tightly associated with the caveolae (Module 6: Figure smooth muscle caveolae). The $InsP_3R$ can be immunoprecipitated by either anti-caveolin-1 or

Module 6: | Figure caveolae molecular organization



A schematic diagram to illustrate how caveolin is thought to organize various signalling components.
The caveolin molecules that oligomerize with each other are embedded in the membrane through a hydrophobic region. Another region that is close to this hydrophobic domain has the caveolin scaffolding domain (shown in black). A number of signalling components have caveolin-binding motifs (shown in red), which enable them to associate with this scaffolding region.

anti-TRPC1 antibodies, suggesting that all these components may interact with each other at the caveolae to form a store-operated Ca^{2+} entry complex, consistent with the conformational coupling hypothesis (Module 3: Figure conformational coupling hypothesis).

Caveolin inactivation of signalling pathways

Many signalling components are inactivated when they are bound to caveolin. This inactivation phenomenon may be particularly important during the onset of cell transformation and cancer, during which there is a down-regulation of caveolin. As the expression of caveolin declines, the caveolae disappear and the associated signalling elements are no longer inactivated and begin to signal constitutively; a characteristic of many cancer cells. Examples of the signalling systems that are inactivated by caveolin include endothelial nitric oxide (NO) synthase (eNOS) (Module 2: Figure eNOS activation), the mitogen-activated protein kinase (MAPK) signalling pathway, Src family kinases and PKCα. In the case of the $Cav1^{-/-}$ mouse, the inhibition of eNOS was removed, resulting in a dramatic increase in systemic NO levels.

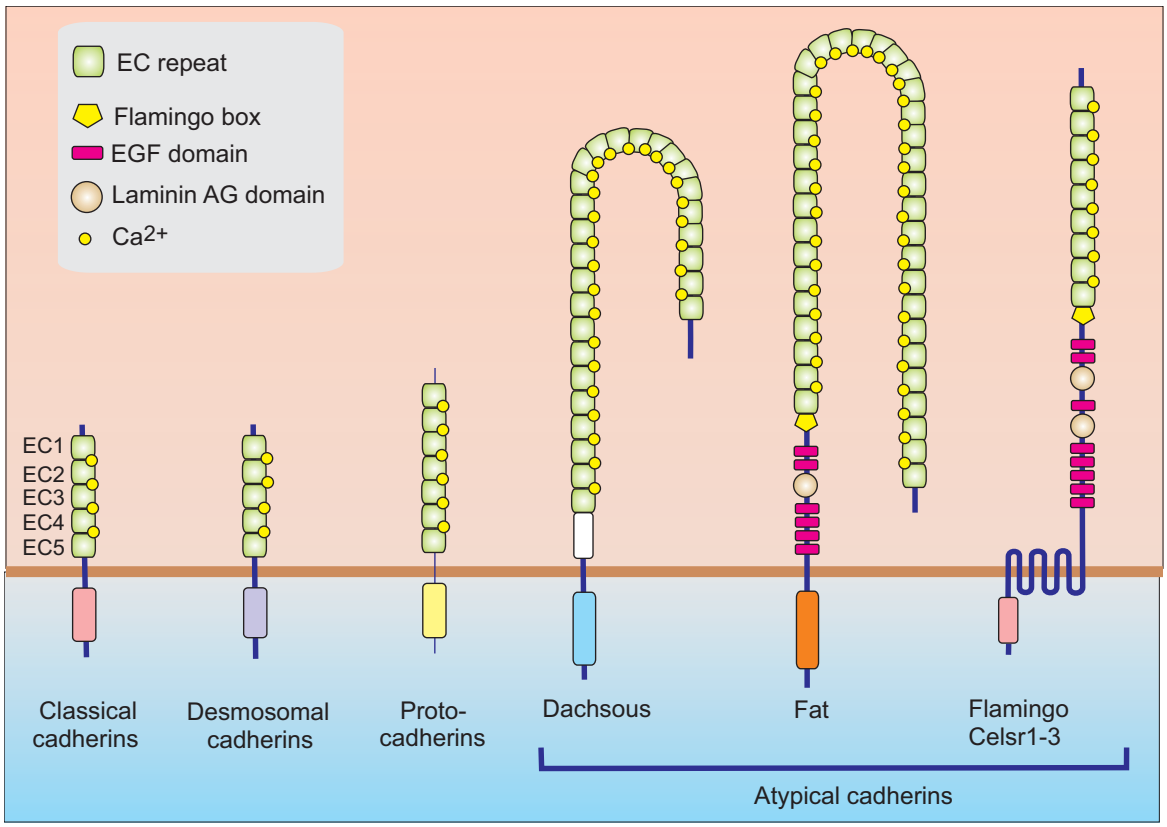
The fact that caveolin can inhibit the MAPK cascade may be particularly important with regard to the role of mitogen-activated protein kinase (MAPK) signalling in cardiac hypertrophy. Removal of the caveolin 3, which is specifically expressed in cardiac cells, resulted in an increase in the MAPK signalling pathway and an increase in cardiac hypertrophy in mice.

Cadherins

There is a large superfamily of cadherins, which are highly dynamic cell–cell adhesion molecules. From a signalling point of view, the cadherins are of interest in that they are the target of various signalling pathways that can adjust cell–cell interactions by altering cadherin adhesiveness. Conversely, there is increasing evidence that some of the cadherins may initiate cell signalling pathways to control various cellular pathways such as the establishment of planar cell polarity (PCP) during development.

Cadherins have a wide range of functions both during development and in the turnover of adult tissues. With regard to the former, they are responsible for many of the morphogenic processes that occur during development. They play a role in separating cells into distinct layers by forming tissue boundaries and they contribute to cell migration and the formation of synapses during brain development. After tissues have formed, they continue to function in adult life. In the brain, they contribute

Module 6: | Figure cadherin superfamily



Cadherin superfamily of cell–cell adhesion molecules.

Most of the cadherins are single-membrane-spanning proteins characterized by having large extracellular domains containing multiple Ca^{2+}-binding extracellular cadherin (EC) domains (shown in green). The exception is the *Drosophila* cadherin called Flamingo and its mammalian homologues Celsr1–Celsr3, which have seven membrane-spanning regions. The way in which some of these cadherin interact with each other during cell signalling is shown in Module 6: Figure classical cadherin signalling and in Module 8: Figure planar cell polarity signalling.

to learning and memory by strengthening new synapses. Cadherins orchestrate the replacement of cells in tissues that turnover rapidly, such as the gut and epidermis. In order for new cells to move into the regenerating tissues, the cadherins must strike a fine balance by allowing cells to move while maintaining tissue integrity. When such tissue integrity breaks down, cells dissociate and changes in cadherin profiles are a feature of metastatic cells. In the endothelium, they regulate junctional permeability where there is a controlled cell–cell separation to allow the passage of neutrophils.

These multiple and diverse functions are carried out by the cadherin superfamily that has been divided into different groups:

- Classical cadherins
- Desmosomal cadherins
- Protocadherins
- Atypical cadherins

Classical cadherins

The classical cadherins are a large family of multifunctional proteins. They tend to be named after the tissue where they were first discovered as illustrated below:

- E-cadherin (epithelial cadherin) is expressed in epithelial cells where it is mainly associated with the zonula adherens that functions to hold cells together. The expression of E-cadherin is regulated by 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), which is also known as Smad-interacting protein 1 (SIP1). The expression of ZEB1 and ZEB2 is regulated by miR-200.

Mutations in the ZEB1 gene are associated with posterior polymorphous corneal dystrophy-3 (PPC3) and late-onset Fichs endothelial corneal dystrophy.

- N-cadherin (neural cadherin) is expressed mainly in the nervous system where it contributes to synapse formation (see step 7 in Module 10: Figure postsynaptic density). The fourth extracellular cadherin domain (EC4) of N-cadherin interacts with thefibroblast growth factor receptor (FGFR) thus enabling this cadherin to contribute to mitogenic signalling. The N-cadherin complex also functions in the peg-socket junctional complex that forms between pericytes and endothelial cells (Module 9: Figure angiogenesis signalling).

- M-cadherin functions in the interaction between muscle cells and satellite cells (Module 8: Figure satellite cell activation).
- R-cadherin (retinal) was original identified in the retina, but also functions in other regions of the brain. It interacts with cadherin-6 during brain development.
- VE-cadherin (vascular endothelial cadherin).

This nomenclature can be confusing because it soon became clear that many of cadherins shown above were not tissue-specific. This lettering system was switched to a numbering system for many of the remaining cadherins, of which some are shown below:

- Cadherin-6 is mainly expressed in the kidney and brain. During brain development, differential expression of cadherin-6 and R-cadherin may set up the compartment boundary between the cerebral cortex and the striatum.
- Cadherin-23 appears to form the helical tip link filament that connects the ends of stereocilia on hair cells (Module 10: Figure tip link). Mutation of cadherin-23 is one of the causes of the deaf/blindness Usher syndrome.

Most attention has focused on E-cadherin, which is used as the basis for the following general description of cadherin function. As part of their role in cell adhesion, cadherins provide a membrane anchor for actin and they can also assemble a complex of signalling components that can relay information into the cell. Like many other cadherins, the classical cadherins are single membrane-spanning proteins (Module 6: Figure cadherin superfamily). The extracellular part of the molecule has Ca^{2+}-binding extracellular cadherin (EC) domains. There are five EC domains for E-cadherin, but this number varies for some of the others. The binding of Ca^{2+} serves to link the EC subunits together to form a rod-like structure. The cytoplasmic region contains binding sites for a variety of components that function in both actin attachment and cell signalling (Module 6: Figure classical cadherin signalling).

Various catenins play a key role in these dual functions of adhesion and signalling. β-Catenin has a core of 12 Armadillo repeats that bind to a region of the cytoplasmic tail of cadherin between residues 625 and 723. The interaction between β-catenin and cadherin is stabilized by phosphorylation of three serine residues (684, 686 and 692) on this cadherin tail. Since phosphorylation of these sites provides a signal for degradation, cadherin is stabilized by having these sites covered up by β-catenin.

The cadherin/β-catenin/actin complex is highly dynamic in that it can be assembled and disassembled in a controlled manner as occurs during cell migration. The stability of the complex depends upon the balance between tyrosine phosphorylation and dephosphorylation. An increase in tyrosine phosphorylation destabilizes the complex and this is counteracted by a battery of tyrosine phosphatases. Tyrosine phosphorylation of residues 142 and 654 on β-catenin are critical for this dynamic behaviour of the complex. Phosphorylation of the Tyr-142 residue on β-catenin by Fyn, Src or c-Met regulate its interaction with α-catenin. Similarly, phosphorylation of Tyr-654 by the epidermal growth factor receptor (EGFR) or Src reduces the interaction between β-catenin and cadherin. Constitutive activation of these tyrosine kinases during cancer will help to dismantle the cell–cell adhesion role of the cadherins and contributes to the onset of metastasis.

The β-catenin tyrosine phosphorylations are counteracted by tyrosine phosphatases (Module 6: Figure classical cadherin signalling). One of the major phosphatases is protein tyrosine phosphatase 1B (PTPB1), which is one of the non-transmembrane protein tyrosine phosphatases (Module 5: Figure tyrosine phosphatase superfamily). In order to dephosphorylate the phosphotyrosine residues on β-catenin, PTP1B must first bind to cadherin where β-catenin is located. This binding is dependent on Tyr-152 on PTP1B being phosphorylated by Fer, which is one of the non-receptor protein tyrosine kinases (Module 1: Figure non-receptor tyrosine kinases). Fer thus occupies a pivotal role in regulating the stability of the cadherin/β-catenin/actin complex.

The link between the cadherin/β-catenin complex and the cytoskeleton is provided by α-catenin, which is another member of the catenin family (Module 6: Figure classical cadherin signalling). There is a dynamic interaction between α- and β-catenin as described in the section on α-catenin. The latter also interacts with many of the components known to play a role in actin remodelling at the focal adhesion complex, such as vinculin and α-actinin (Module 6: Figure integrin signalling). In addition, α-catenin binds to formin-1 (FMN1), which belongs to the formin family and functions in actin assembly in response to the monomeric G proteins such as Rac and Cdc42. Indeed, the p120 catenin seems to play a role in activating these two G proteins through a mechanism that may depend upon the inhibition of Rho. p120 catenin can bind and activate the p190RhoGAP which functions to inactivate Rho (Module 2: Figure Rho signalling). This inhibition of Rho will prevent its inhibition of Rac and Cdc42 that can then contribute to the assembly of actin.

Catenins

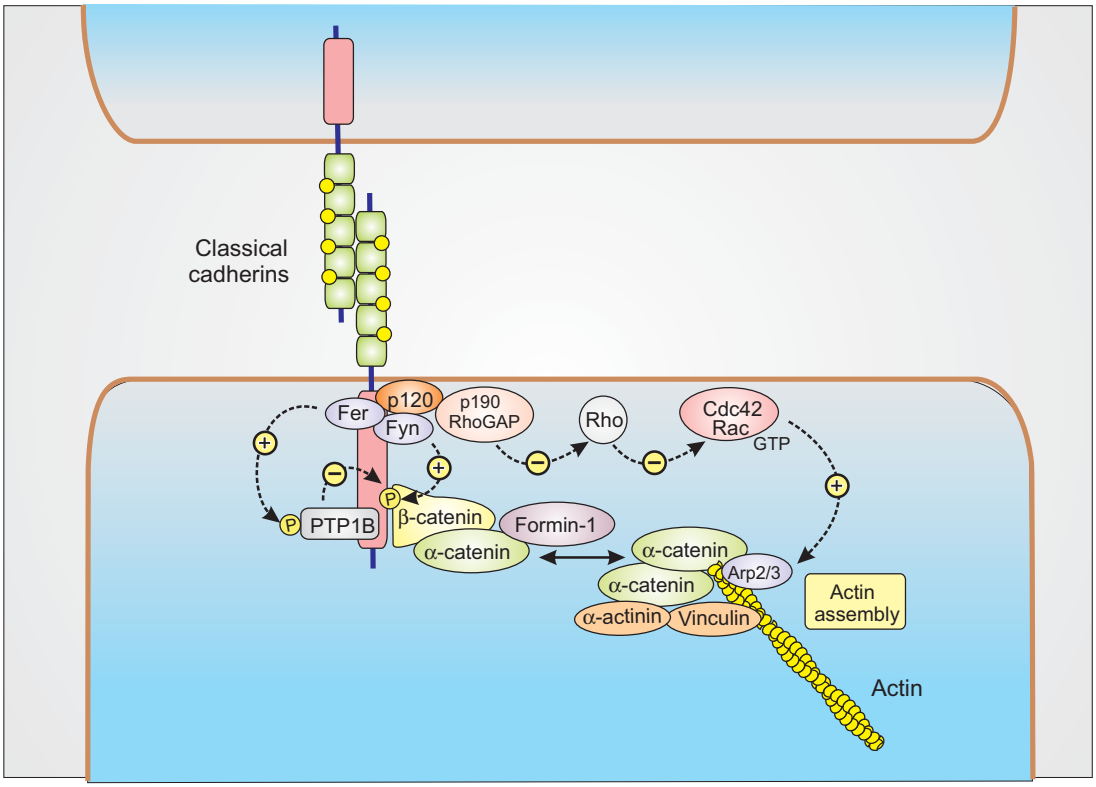
The catenins are a family of molecules with diverse functions ranging from cell–cell adhesion to the regulation of gene transcription. Catenin is the Greek word for link because these molecules were first discovered in cell junctions where they linked the cadherins embedded in the plasma membrane to the actin cytoskeleton. Subsequently, it was found that their cytoskeletal and signalling functions were carried out by the following family of catenins:

- α-Catenin
- β-Catenin
- γ-Catenin (plakoglobin)
- p120 catenin

α-Catenin

α-Catenin functions as a cytoskeletal protein that plays a central role in linking the classical cadherins/β-catenin

Module 6: | Figure classical cadherin signalling



Classical cadherin cytoskeletal and signalling functions.
Classical cadherins on neighbouring cells interact with each through their extracellular cadherin (EC) domains. The cytoplasmic domain interacts with both p120 catenin, which has a regulatory role by interacting with signalling components such as Fer and the p190RhoGAP that controls G protein signalling, and β-catenin. The latter provides a link to the cytoskeleton through its interaction with α-catenin.

complex to the actin cytoskeleton (Module 6: Figure classical cadherin signalling). This appears to be a dynamic interaction because when α-catenin is bound to β-catenin it cannot bind actin. The latter binds to the α-catenin homodimer. The role of α-catenin in actin attachment is also evident from the fact that it can associate with other actin-binding proteins such as vinculin and α-actinin.

β-Catenin

β-Catenin is unusual because it has two distinct functions: it has a structural role as part of the cadherin complex (Module 6: Figure classical cadherin signalling) and there also is an action of β-catenin as a transcription factor where it regulates gene transcription as part of the canonical Wnt/β-catenin pathway (Module 2: Figure Wnt canonical pathway). The core of the β-catenin protein is made up of 12 Armadillo repeats, each of which consists of three tightly packed α-helices. This core region binds to a specific region of the cadherin cytoplasmic domain located between residues 625 and 723 (Module 6: Figure classical cadherin signalling). Tyrosine phosphorylation of β-catenin regulates the stability and operation of the skeletal function of the classical cadherins.

In addition to this structural role, β-catenin can also function as a transcription factor for the canonical Wnt/β-catenin pathway. 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 that regulate development and cell proliferation (Module 2: Figure Wnt canonical pathway).

There also is a role for β-catenin as an oncogene.

γ-Catenin (plakoglobin)

γ-Catenin has a similar cytoskeletal role to β-catenin in that it associates with the same cytosolic region on cadherin. It is particularly important in forming the desmosomes, where it provides the link to the intermediate filaments. Unlike β-catenin, however, it does not appear to double up as a transcription factor.

p120 catenin

p120 catenin binds to the distal region of the cadherin cytoplasmic domain (Module 6: Figure classical cadherin signalling). It appears to have a regulatory role by coordinating some of the signalling events associated with the cadherin complex. For example, p120 can bind and activate the p190RhoGAP that inhibits Rho, which will indirectly enhance signalling through Rac and Cdc42. In addition, p120 catenin associates with Fer, which controls the protein tyrosine phosphatase 1B (PTP1B), which dephosphorylates β-catenin

Desmosomal cadherins

The desmosomal cadherins are somewhat specialized in that they are restricted to the desmosomes, which are adhesive junctions that are linked to the cytoskeleton through intermediate filaments. The main desmosomal cadherins are the desmocollins and the desmosgleins, which are linked to intermediate filaments by γ-catenin (plakoglobin).

Protocadherins

The protocadherins (Pcdhs) are the largest group of the cadherin superfamily containing approximately 60 members. Most of these have been classified into three clusters (Pcdh-α, Pcdh-β and Pcdh-γ) and the remaining form a smaller Pcdh-δ cluster. The gene organization is complex with multiple variable exons and a set of constant exons that are mixed and matched to create a large number of isoforms, which is very reminiscent of immunoglobulin genes. This enormous isoform diversity has created much interest in the possibility that protocadherins might provide a mechanism for creating the specific synaptic connections necessary for wiring up the nervous system during development. This is of special interest because these protocadherins are mainly located in the nervous system.

Protocadherin structure resembles that of the classical cadherins, but there are a number of differences (Module 6: Figure cadherin superfamily). They have six or seven EC domains, and the cytoplasmic region is not linked to the catenins, but appears to interact with other signalling molecules. They have rather weak adhesive properties and appear not to form the homophilic and heterophilic adhesions characteristic of the classical cadherins. It has been suggested that they may function to relay signals to cells in response to cell recognition, but the signalling mechanisms remain to be worked out.

Isoforms of protocadherin-15 contribute to the organization of the stereocilia found on the tips of the hair cells responsible for hearing (Module 10: Figure tip link). Mutation of protocadherin-15 is one of the causes of the deaf/blindness Usher syndrome.

Atypical cadherins

There are a number of atypical cadherins that have also been described as cadherin-like signalling proteins. The extracellular domains, which can be very large, have cadherin-type extracellular cadherin (EC) domains (Module 6: Figure cadherin superfamily). Members of this group such as Dachsous (Ds), Fat (Ft) and Flamingo (Fmi) were first described in *Drosophila*. They appear to play an important role during early development and particularly during planar cell polarity (PCP).

Dachsous (Ds)

Dachsous (Ds), which is one of the atypical cadherins, has been implicated in both cell proliferation as well as planar cell polarity (PCP). The structure of Ds is dominated by the 27 extracellular cadherin (EC) domains that make up most of the large extracellular part of the molecule (Module 6: Figure cadherin superfamily). During the cell–cell interactions that occur when PCP is established in *Drosophila*, Ds appear to interact with and activate Fat (Ft) (Module 8: Figure planar cell polarity signalling). Ds may be considered to be a tethered agonist for Ft because the extracellular domain of Ds is sufficient to transfer information to Ft during PCP.

Fat (Ft)

Fat (Ft), which was first described in *Drosophila*, is one of the atypical cadherins that is characterized by its very large extracellular domain (Module 6: Figure cadherin superfamily). The structure of Fat is dominated by the 34 extracellular cadherin (EC) domains that make up most of the large extracellular part of the molecule. Located in the region between the EC repeats and the transmembrane region, there are epidermal growth factor (EGF)-like repeats, laminin AG domains and sometimes a flamingo box. The cytoplasmic domain has some sequence homology with classical cadherins, but, unlike the latter, they do not bind β-catenin.

In mammals, there are four Ft homologues, Fat1, Fat2, Fat3 and Fat-j. The cytoplasmic regions of the insect and mammalian homologues show little sequence conservation, suggesting that they may function through different mechanisms. The precise function of Ft remains to be determined. In the case of *Drosophila*, Ft has a role in planar cell polarity (PCP), but exactly how it functions remains to be determined. Genetic analysis of the effects of having an island of cloned cells in a background of wild-type cells (Module 8: *Drosophila* planar cell polarity) suggests that the Ds/Ft pathway operates separately, but in parallel with the PCP signalling pathway initiated by the Frizzled (Fz) receptor (Module 8: Figure planar cell polarity signalling). There are indications that Dachsous (Ds) on one cell functions as a ligand to activate Ft on the neighbouring cell. Just how this heterophilic interaction between Ds and Ft is transduced into a change in polarity is still a mystery.

A challenge for the future is to uncover the nature of the signalling components associated with these two signalling pathways and how they act on the downstream effector systems responsible for the morphological manifestations of PCP. There already are indications that both pathways are linked to the cytoskeleton (Module 8: Figure planar cell polarity signalling). The cytoplasmic domain of mammalian Fat1 appears to bind to the Ena/vasodilator-stimulated phosphoprotein (VASP) complex, and this could explain how it functions to regulate the dynamics of actin assembly.

In addition to its role in PCP, Ft is also considered to be a tumour suppressor because it can inhibit cell proliferation.

Flamingo (Fmi)

Flamingo (Fmi), which is also known as starry night (Stan), was first described in *Drosophila* where it functions in planar cell polarity (PCP). It is unusual in that it has seven membrane-spanning regions compared with the single one found in most of the other members of the cadherin superfamily (Module 6: Figure cadherin superfamily). It is included in this superfamily because it has nine typical extracellular cadherin (EC) domains. The extracellular do-

main also has epidermal growth factor (EGF)-like repeats, laminin AG domains and a Flamingo box. Fmi has a central role to play in one of the pathways that regulates insect bristle polarity (Module 8: Figure planar cell polarity signalling).

Three mammalian homologues of Fmi have been described: Celsr1, Celsr2 and Celsr3. Celsr1 contributes to the PCP process that result in the orientation of hair cell stereociliary bundles during development of the cochlea.

Cell adhesion complexes

There are a number of cell adhesion complexes that enable individual cells to interact either with the extracellular matrix (ECM) or with each other. These adhesion complexes have different structures and carry out very different functions:

- Gap junctions enable cells to communicate directly with each other (Module 1: Figure cell communication).
- Adherens junctions, which contain the classical cadherins, function to hold cells together
- Tight junctions occur in epithelia to restrict the passive transfer of ions and molecules between cells.
- The focal adhesion complex is a specialized localized region that functions to attach the cell to the underlying ECM or to cell-surface molecules on neighbouring cells.
- The podosome is a specialized cell-matrix adhesion complex that functions in cell spreading and motility.

Focal adhesion complex

The focal adhesion complex is a specialized attachment site where the cell makes close contact with either the extracellular matrix (ECM) or to cell-surface molecules expressed on neighbouring cells. This adhesion process depends on the integrin receptors embedded in the plasma membrane. These integrin receptors have two main functions. Firstly, they provide the molecular link between the actin cytoskeleton and the adhesion molecules located in the ECM or the cell surface. Secondly, they participate in a process of integrin signalling (Module 1: Figure integrin receptor), which is intimately linked to their skeletal/adhesive function. As described in the section on integrin signalling, the integrin receptors provide a mechanism for the two-way transmission of information. The *outside-in* and the *inside-out* signalling processes carry out different signalling functions and greatly enhance the versatility of the integrin signalling mechanism.

One of the problems of trying to understand the operation of the focal adhesion complex is its enormous complexity (Module 6: Figure integrin signalling). The following list summarizes some of the major enzymes and adaptor molecules that come together to form the functional complex:

- Abelson-interactor (Abi)
- α-Actinin
- Crk
- Crk-associated substrate (Cas)
- Filamin
- Focal adhesion kinase (FAK)
- Integrin-linked kinase (ILK)
- Kindlin
- Migfilin
- Particularly interesting cysteine/histidine-rich protein (PINCH)
- Parvin
- Paxillin
- Talin
- Tensin
- Vinculin

These focal adhesion molecules fall into three main groups: adaptors, kinases, and GTPase-modulating proteins (Module 6: Figure focal adhesion components). All of these different components come together to form the large complex that provides the platform for the focal adhesion actin attachment and the focal adhesion integrin signalling systems.

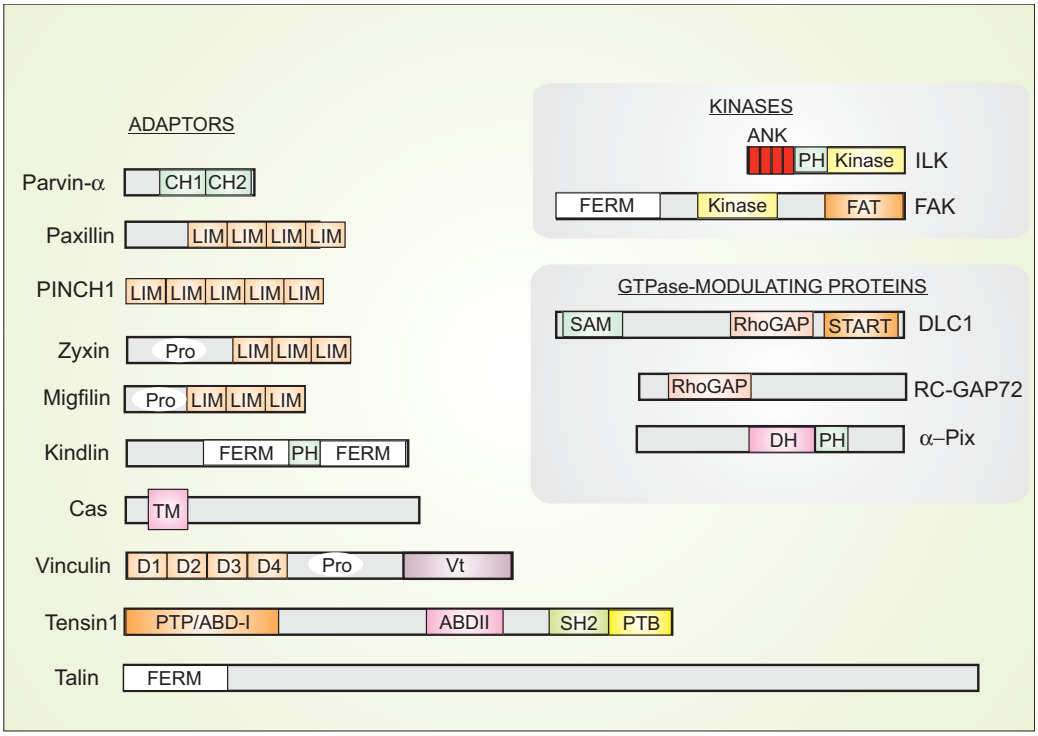
Focal adhesion actin attachment

There are at least three ways of attaching actin to the cytoplasmic tail of the integrin β-subunit (Module 6: Figure integrin signalling). The precise organization of the focal adhesion complex is unknown, and this figure was constructed based on the binding properties of the major components (Module 6: Figure focal adhesion components). Many of these are adaptors that contain the protein–protein interaction domains [e.g. phosphotyrosine-binding (PTB), FERM, LIM and calponin homology (CH)] that enable them to link together into a functional complex. With regard to actin attachment, there is no direct link between actin and the integrin β-subunit. The interaction is provided by various bridging proteins that provide the link either to the β-subunit directly or through other intermediaries (Module 6: Figure integrin signalling). Construction of this adhesion complex depends upon a few proteins that provide the basic scaffold.

Two of the key proteins are the focal adhesion kinase (FAK) and the integrin-linked kinase (ILK), which not only contribute to focal adhesion integrin signalling, but also have a scaffolding role by assembling complexes that contain the main actin-binding proteins such as α-actinin, parvin, talin and vinculin. Because of the large number of interactions, the FAK and ILK scaffolds are drawn separately in Module 6: Figure integrin signalling, but in reality, they might all be connected together in a cluster of integrin receptors. As shown on the right of the figure, actin is connected to talin, α-actinin and vinculin.

Vinculin has a particularly important role in stabilizing the actin attachment complex (Module 6: Figure vinculin function). There appears to be a dynamic equilibrium between the auto-inhibitory and active states of vinculin. In the former state, it is free in the cytoplasm, but as the focal adhesion complex forms, it begins to open up by forming bridges between actin, talin and the phosphoinositide PtdIns4,5P_2, and between actin, talin and the actin-related protein 2/3 complex (Arp2/3 complex), which plays a role in actin remodelling (Module 4: Figure actin remodelling).

Module 6: | Figure focal adhesion components



Some of the major components of focal adhesions.
The focal adhesion complex contains a large number of components that belong to three main groups: adaptors, kinases and GTPase-modulating proteins. Module 6: Figure integrin signalling illustrates the location and function of many of these proteins within the focal adhesion complexes. CH, calponin homology domain; Pro, proline-rich domain; TM, transmembrane domain.

Another actin attachment site is based on the integrin-linked kinase (ILK) (shown on the left of Module 6: Figure integrin signalling). The ILK attaches to both the integrin β-subunit and to the actin-binding protein parvin. Paxillin may contribute to this complex since it can bind to both ILK and parvin.

The final actin attachment mechanism depends on the protein filamin, which can bind to both the integrin β-subunit and actin. Filamin also associates with the protein migfilin, which is also attached to kindlin-2.

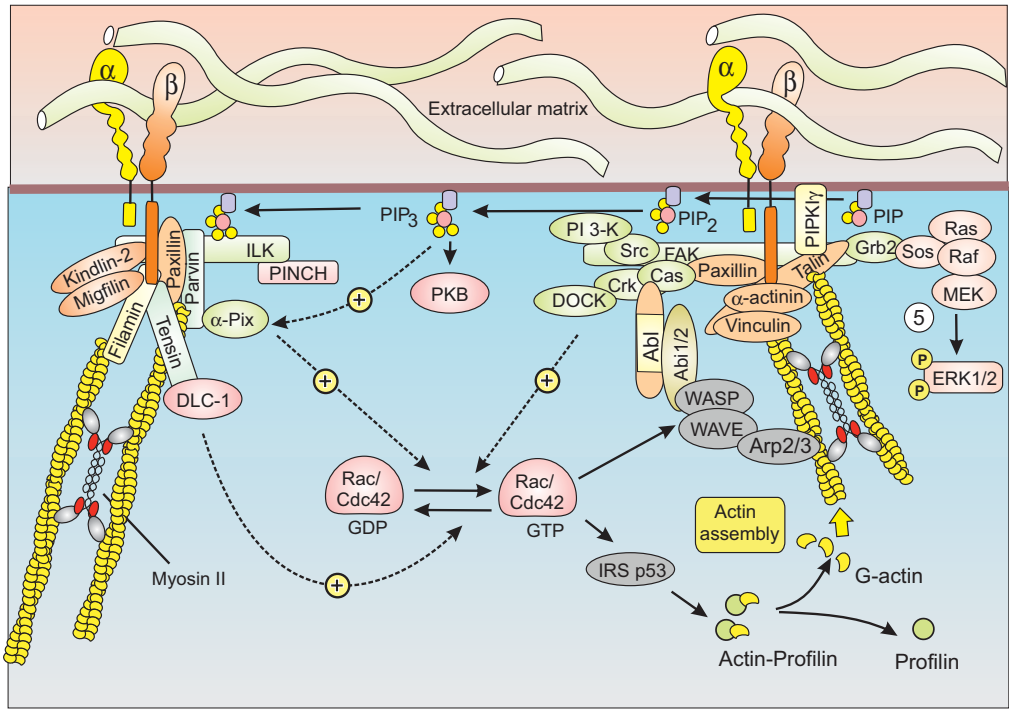
Focal adhesion integrin signalling

In addition to providing a scaffolding complex to link into actin, the integrins also have a signalling role. This integrin signalling complex has a number of different signalling pathways (Module 1: Figure integrin receptor). The integrin receptor also has an unusual ability to signal in both directions. In addition to a more conventional *outside-in* mode, integrins also have an *inside-out* mode, which depends on the ability of other receptors (such as growth factor receptors) being able to switch the receptor from a low- to a high-affinity state. This mode may be particularly important in controlling the way in which cells interact with the extracellular matrix (ECM) or with other cells, as occurs in blood platelets (Module 11: Figure platelet activation). In this section, attention will focus on *outside-in* signalling that is activated when the integrins engage components of the ECM (Module 6: Figure integrin signalling).

Key elements in integrin signalling are the focal adhesion kinase (FAK) and the integrin-linked kinase (ILK), which provides the structural platforms to assemble a variety of transducing elements. When the integrins engage an external signal, there is a conformation change that adjusts the position of the short cytoplasmic tails enabling the latter to interact with and activate FAK and ILK. As a part of the activation, FAK undergoes autophosphorylation at several sites, which provide binding sites for signal transducers. For example, phosphorylation of Tyr-925 provides a binding site for the adaptor growth factor receptor-bound protein 2 (Grb2), which enables FAK to relay information out to the mitogen-activated protein kinase (MAPK) signalling pathway. Phosphorylation of Tyr-397 in the N-terminal region provides a binding site for both Src and the p85 subunit of PtdIns 3-kinase (PtdIns 3-K).

Phosphoinositides play a number structural and signalling roles in the adhesion complex. Activation of the p85 subunit of PtdIns 3-kinase increases the level of the lipid messenger PtdIns3,4,5P_3 that contributes to the activation of ILK. The central pleckstrin homology (PH) domain of ILK binds to PtdIns3,4,5P_3. Formation of PtdIns4,5P_2 also plays an important role. The Iγ isoform of PtdIns4P 5-kinase (PtdIns4P 5-K), which binds to the FERM domain of talin, is thus drawn into the complex, where it functions to create a local increase in the PtdIns4,5P_2, that contributes to the process of PtdIns4,5P_2 regulation of actin remodelling. Actin assembly within the adhesion complex

Module 6: | Figure integrin signalling



Structural and signalling functions of integrins within the focal adhesion complex.
Integrins provide an anchor for attaching actin to the plasma membrane. Attachment of actin to the cytoplasmic domain of the integrin β-subunit depends upon a number of proteins such as α-actinin, filamin, paxillin, talin and vinculin. The integrin receptor also assembles a number of signalling components for the mitogen-activated protein kinase (MAPK) pathway, the PtdIns 3-kinase signalling pathway and the guanine nucleotide exchange factors (GEFs) such as α-Pix and downstream of Crk-180 homologue (DOCK) that activate G proteins such as Rac and Cdc42 to initiate actin assembly. This signalling aspect depends on two main kinases: focal adhesion kinase (FAK) and integrin-linked kinase (ILK), which are shown separately to simplify the diagram.

is regulated by a number of signalling pathways many of which are orchestrated by the monomeric G proteins. Both Rac signalling mechanisms (Module 2: Figure Rac signalling) and Cdc42 signalling mechanisms (Module 2: Figure Cdc42 signalling) appear to play important roles. In the case of the focal adhesion complex, these G proteins are activated by guanine nucleotide exchange factors (GEFs) such as α-Pix and downstream of Crk-180 homologue (DOCK), which are drawn into the complex. The formation of PtdIns3,4,5P_3 described above is also able to activate α-Pix.

The activated Rac and Cdc42 then stimulate Wiskott-Aldrich syndrome protein (WASP) and Wiskott-Aldrich syndrome protein (WASP) verprolin homologous (WAVE), which are critical for stimulating the actin-related protein 2/3 complex (Arp2/3 complex) responsible for actin assembly (Module 4: Figure actin remodelling). WASP can also be activated by a signalling pathway controlled by Abl (Module 1: Figure Abl signalling). As the actin fibres begin to form, they are stabilized by binding to non-muscle myosin II filaments (Module 6: Figure integrin signalling).

Abelson-interactor (Abi)

The Abelson-interactor (Abi) was first identified as an Abl-binding protein. Abi was subsequently found to be an important adaptor protein with a particularly important function in assembling the Wiskott-Aldrich syndrome protein (WASP) verprolin homologous (WAVE) complex that controls actin remodelling (Module 4: Figure actin remodelling). There are two isoforms, Abi1 and Abi2, which have multiple domains enabling it to interact with Abl, WAVE and the actin-related protein 2/3 complex (Arp2/3 complex). It contains a WAVE-binding domain (WAB), a Src homology 3 (SH3) domain and proline rich sequences. Abi contributes to Abl signalling (Module 1: Figure Abl signalling) as is illustrated in the focal adhesion complex (Module 6: Figure integrin signalling).

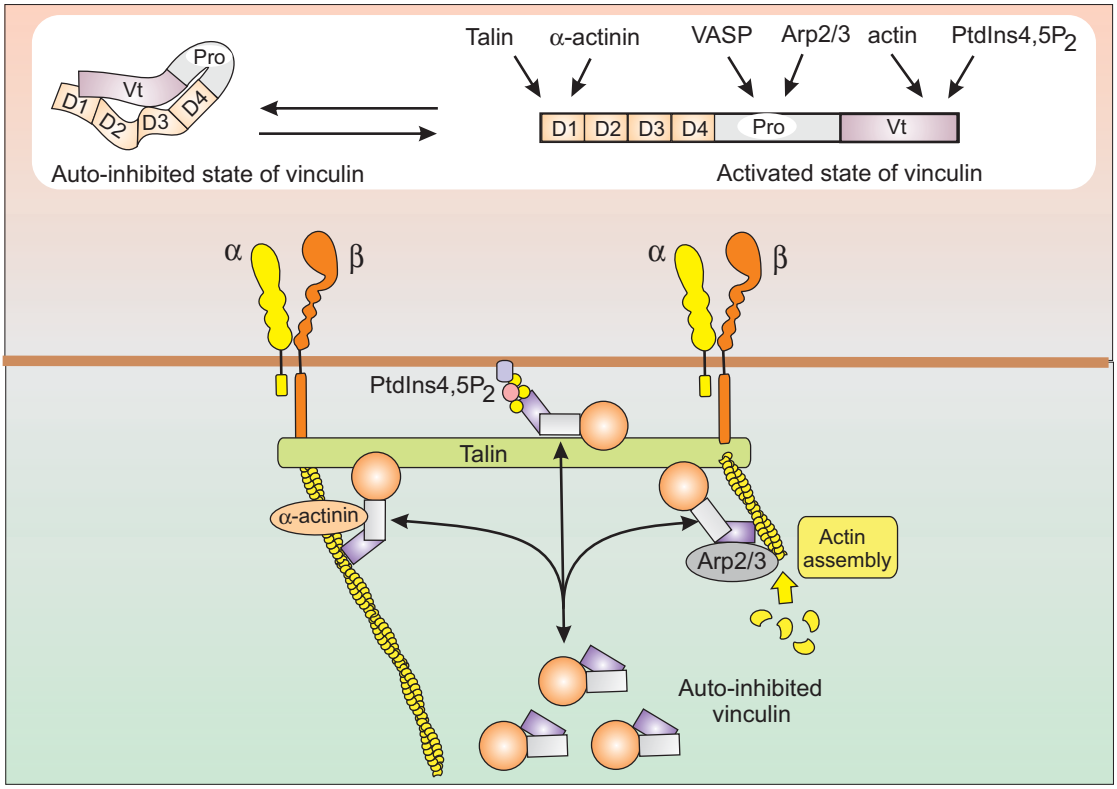
α-Actinin

α-Actinin is one of the major proteins found in focal adhesions where it provides the link between the β-integrin subunit and the actin filament (Module 6: Figure integrin signalling). It can also bind to vinculin, which helps to stabilize the interaction between α-actinin and actin (Module 6: Figure vinculin function).

Crk

The C10 regulator of kinase (Crk) family has two members: Crk, which occurs in two spliced forms CrkI and CrkII, and a second gene encoding Crk-like (CrkL). These are adaptor proteins that have both Src homology 2 (SH2) and 3 (SH3) domains. The SH2 domain binds to the tyrosine-phosphorylated form of Cas and paxillin.

Module 6: | Figure vinculin function



Proposed role of vinculin in stabilizing focal adhesion complexes.
Vinculin exists in two main states: an auto-inhibited state, where the Vt domain bends round to interact with the N-terminal D1 and D3 regions, and an activated state, where the molecule opens out to unveil binding sites for many of the components of the adhesion complex. By interacting with different components, vinculin functions to stabilize the complex. For simplicity, most of the other adhesion molecules have been left off. A more detailed picture is shown in Module 6: Figure integrin signalling.

They function in the transduction of signals by receptors [epidermal growth factor receptor (EGFR), neurotrophic growth factor and FGFR (fibroblast growth factor receptor)] and they also play a role in remodelling the cytoskeleton at focal adhesions (Module 6: Figure integrin signalling).

Crk-associated substrate (Cas)

Crk-associated substrate (Cas) is an adaptor protein that binds to both Src and focal adhesion kinase (FAK). In the case of the osteoclast podosome, Cas binds to the proline-rich tyrosine kinase 2 (Pyk2) (Module 7: Figure osteoclast podosome). Cas contributes to the recruitment of other signalling molecules such as Abl, Crk and Nck. The interaction between FAK/Pyk2, Src, Cas and Crk seems to be critical for cell migration.

Filamin

Filamin is an adaptor protein that can connect actin to the cytoplasmic domain of the β integrin (Module 6: Figure integrin signalling). Filamin is also known to associate with the Ca^{2+}-sensing receptor (CaR).

Focal adhesion kinase (FAK)

Focal adhesion kinase (FAK) is a typical non-receptor protein tyrosine kinase. As its name implies, its primary function is to control the function of junctional complexes. Its domain structure emphasizes its role in interacting with many of the other components found at cell junctions (Module 6: Figure focal adhesion components). There is an N-terminal FERM domain, a kinase domain is located in the middle and a focal adhesion targeting (FAT) domain located in the C-terminal region that can bind to talin and paxillin. Phosphorylation of Tyr-397 in the N-terminal region provides a binding site for both Src and the p85 subunit of PtdIns 3-kinase (PtdIns 3-K) (Module 6: Figure integrin signalling). At the other end of the molecule, phosphorylation of Tyr-925 provides a binding site for growth factor receptor-bound protein 2 (Grb2), which enables FAK to relay information out to the mitogen-activated protein kinase (MAPK) signalling pathway. There are two proline-rich regions located between the kinase and FAT domains that are able to bind to Crk-associated substrate (Cas) and to a GTPase-activating protein for Rho (GRAF).

The primary role of FAK is to link integrin receptors to a number of downstream signalling pathways.

Integrin-linked kinase (ILK)

The integrin-linked kinase (ILK) has an N-terminal region containing three ankyrin repeats, a central pleckstrin homology (PH) domain that binds to PtdIns3,4,5P_3 and

a C-terminal kinase domain (Module 6: Figure focal adhesion components). ILK plays both a structural role by linking to other components, but it also uses its kinase domain to phosphorylate key signalling components to relay information into the cell. A central feature of this structural and signalling role is the complex that ILK forms with the particularly interesting cysteine/histidine-rich protein (PINCH) and parvin (Module 6: Figure integrin signalling). ILK is responsible for attaching this ILK/Pinch/parvin (IPP) complex to the cytoplasmic domain of the β_1 and β_3 integrins.

Kindlin

Kindlins function in protein–protein interactions with other components of focal adhesion complexes such as integrin-linked kinase (ILK), migfilin and integrin (Module 6: Figure integrin signalling). The kindlin family contains three members:

- Kindlin-1, which is also known as kindlerin, FERMT1 and UNC-112-related protein 1 (URP1). Transforming growth factor β1 (TGFβ1) increases the expression of kindlin-1, and is up-regulated in various cancers (lung and colon).
- Kindlin-2, which is also known as the mitogen-inducible gene-2 (Mig-2), contributes to a complex with migfilin and filamin and thus contributes to the attachment of integrin-linked kinase (ILK) to actin (Module 6: Figure integrin signalling). In addition to this skeletal function, kindlin-2 also plays a role in gene transcription. It binds to the cardiac homeobox transcription factor CSX/Nkx2-5 through its LIM domains, and the complex enters the nucleus through a process that appears to be driven by an increase in intracellular Ca^{2+}.
- Kindlin-3, which is also known as UNC-112-related protein 2 (URP2). Not much is known about this isoform, except that its expression seems to be restricted to cells of the immune system.

All three of the kindlins have a similar structure that has a split FERM domain separated by a pleckstrin homology (PH) domain (Module 6: Figure focal adhesion components).

Kindler syndrome is linked to mutations in kindlin-1.

Migfilin

Migfilin, which is also known as filamin-binding LIM protein-1 (FBLP-1) or CSX-associated LIM protein (Cal), is an adaptor protein that was first identified in focal adhesions where it co-localizes with kindlin-2. The structure of migfilin reveals two major protein–protein interaction domains: a proline-rich domain in the middle that binds vasodilator-stimulated phosphoprotein (VASP) and three LIM domains at the C-terminal end that bind to kindlin-2 (Module 6: Figure focal adhesion components).

Particularly interesting cysteine/histidine-rich protein (PINCH)

There are two particularly interesting cysteine/histidine-rich protein (PINCH) family members, PINCH1 and PINCH2, which function as adaptors. They have five LIM domains (Module 6: Figure focal adhesion components). The N-terminal LIM domain is used to attach PINCH to integrin-linked kinase (ILK) (Module 6: Figure integrin signalling).

Parvin

There is a family of parvin proteins (α-, β- and γ-parvin). The α- and β-isoforms have overlapping expression patterns, whereas the γ-parvin seems to be restricted to haematopoietic cells. The parvins have two calponin homology (CH) domains, which function to bind to actin and also serve to link parvin to the integrin-linked kinase (ILK) (Module 6: Figure integrin signalling).

Paxillin

Paxillin is located at sites of cell adhesion where integrins make contact with components of the extracellular matrix, as occurs at focal adhesions (Module 6: Figure integrin signalling). It functions as a multidomain adaptor protein that is phosphorylated by focal adhesion kinase (FAK). It forms part of a scaffold that organizes a number of signalling molecules such as Src, Crk, vinculin and integrin-linked kinase (ILK). Paxillin can also associate with protein phosphatase 2A (PP2A).

The domain structure reveals the presence of four tandem LIM domains (Module 6: Figure focal adhesion components).

Proline-rich tyrosine kinase 2 (Pyk2)

The proline-rich tyrosine kinase 2 (Pyk2) is related to the focal adhesion kinase (FAK). It has a similar domain structure to FAK (Module 1: Figure non-receptor tyrosine kinases). It is strongly expressed in the CNS and in the haematopoietic lineage (Module 8: Figure haematopoiesis). It plays an important role in osteoclasts, where it functions in the formation of the osteoclast podosome (Module 7: Figure osteoclast podosome). Pyk2 is a Ca^{2+}-sensitive enzyme. Elevations in Ca^{2+} result in autophosphorylation of Tyr-402, which then provides a binding site for other signalling components such as Src. The latter then recruits Cbl and PtdIns 3-kinase to stimulate the formation of the lipid second messenger PtdIns3,4,5P_3, which contributes to the processes that control actin assembly.

During the assembly of the podosome, Pyk2 also phosphorylates Crk-associated substrate (Cas), which is constitutively bound to Pyk2.

Talin

The talin family (talin1 and talin2) are adaptors that contribute to the formation of adhesion complexes. Most attention has focused on talin1. Talin2 is closely homologous with talin1, but appears to have a more restricted expression.

One of their functions is to link the integrins to actin (Module 6: Figure integrin signalling). Their adaptor function depends on their ability to bind to the cytoplasmic tails of the β_1, β_2 and β_3 integrins, actin, vinculin and focal adhesion kinase (FAK). The FERM domain of talin can also bind to the Iγ isoform of PtdIns4P 5-kinase (PtdIns4P 5-K), where it functions to create a local increase in the

PtdIns4,5P_2, which controls the formation of adhesion complexes.

Tensin

There is a family of four tensin molecules (tensin1–tensin3 and Cten), which were originally identified as components of focal adhesion complexes. Tensin1–tensin3 have very similar domain structures (Module 6: Figure focal adhesion components). The N-terminal region contains a phosphatase and tensin homologue deleted on chromosome 10 (PTEN)-related protein tyrosine phosphatase (PTP) domain located next to an actin-binding 1 (ABD-1) region. The C-terminal region has an Src homology 2 (SH2) domain and a phosphotyrosine-binding (PTB) domain. Although the PTB domain normally interacts with a phosphotyrosine residue, it has been shown to bind to the cytoplasmic domain of the β integrin independently of a prior phosphorylation.

The C-terminal tensin-like (Cten) protein differs from the other tensins in that it lacks the PTP/ABD-1 region. It has a somewhat restricted expression (prostate and placenta).

Vinculin

Vinculin plays a critical role in the formation of cell–cell or cell–matrix attachment structures, such as the focal adhesion complex, where it is one of the most abundant proteins. It is an actin-binding protein that can also bind many of the other components of the adhesion complex (Module 6: Figure integrin signalling). Vinculin has a globular head region made up of four C-terminal D domains, which is linked via a proline-rich region to an N-terminal tail domain (Vt). The molecule can undergo considerable conformational changes linked to its role in various cell attachment complexes (Module 6: Figure vinculin function). It may be recruited into such complexes by binding to talin, actin and phosphoinositides such as PtdIns4,5P_2 and PtdIns3,4,5P_3. Its primary function appears to be stabilization of the other molecular interactions that bind actin to the cytoplasmic tail of the β-integrin subunit.

Podosome

The podosome is a specialized region of the cell that functions in the cell adhesion events associated with cell motility and cell spreading (Module 7: Figure osteoclast podosome). It contains many of the same elements found in focal adhesion complexes, but unlike the latter, podosomes are much more labile. They are characterized by having an aggregate of integrin receptors that can rapidly assemble and disassemble an actin core that is used for cell movement. The structure and function of this dynamic motility assembly is well-illustrated by the osteoclast podosome. Neutrophils may use podosomes to force a passage across endothelial cells as they migrate from the blood to sites of inflammation (see Step 8 in Module 11: Figure inflammation).

Local and global aspects of signalling

The spatial organization of signalling pathways reveals that the components of cell signalling pathways are often highly organized and often localized to discrete cellular areas such as the caveolae (Module 6: Figure signalling hierarchies). This is particularly the case for the plasma membrane, since it has the receptors responsible for initiating most cell signalling pathways. In those cases where the signalling pathway begins with the generation of a second messenger, the immediate vicinity of the receptor will be a focal point, where the concentration will be at a maximum and this will then decline away exponentially as the messenger diffuses into the cytosol. This will create a second messenger microdomain, which is beginning to attract increasing attention as a key component of cell signalling mechanisms. Most attention has focused on the elementary and global aspects of Ca^{2+} signalling, where it is now evident that signalling can occur within highly localized regions or it can be spread more globally by the formation if both intra- and inter-cellular Ca^{2+} waves. However, there is increasing evidence that they may be a feature of other signalling systems such as the cyclic AMP microdomains and reactive oxygen species (ROS) microdomains.

One of the exciting aspects of signalling microdomains is the way they are used by neurons to increase their capacity to process information. The fact that information processing can be confined to very small volumes within the spines means that each neuron is capable of simultaneously processing large amounts of information. Such input-specific signalling is particularly relevant to the process of synaptic modifications during learning and memory (Module 10: Figure input-specific signalling). The high concentrations of neuronal Ca^{2+} buffers, such as calbindin D-28k (CB), play a major role in restricting Ca^{2+} signals to individual spines, which are the smallest units of neuronal integration.

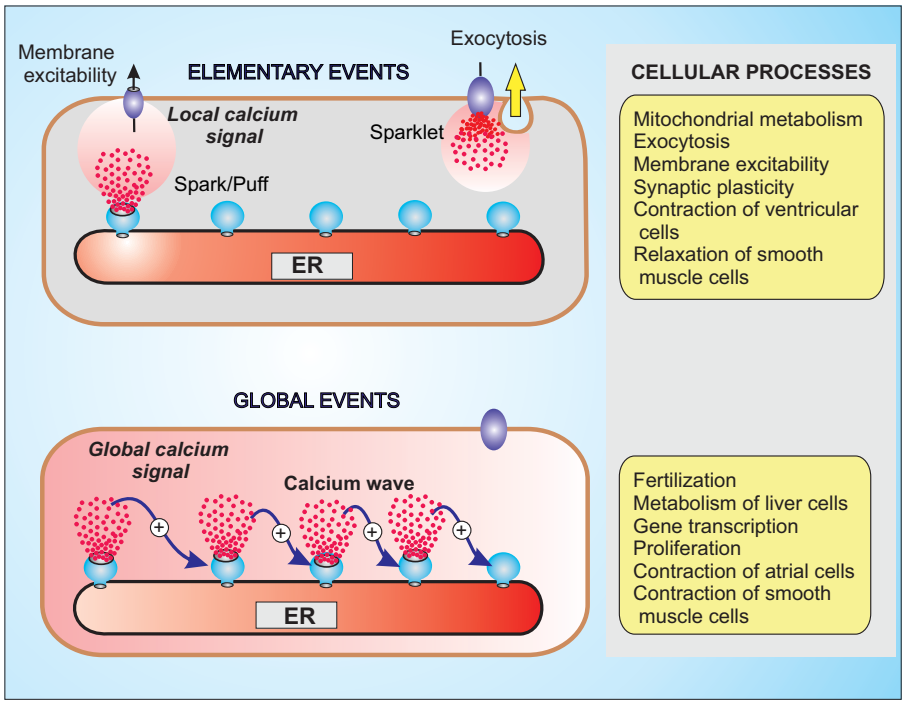
The size of these signalling microdomains will depend upon a number of aspects, such as the rate of signal generation, the rate of signal diffusion, the rate of signal removal by the OFF mechanisms and the degree of buffering. Second messenger buffers play an important role in determining the volume of these signalling microdomains, and this may have been a particularly important feature for the miniaturization of Ca^{2+} signalling within the brain.

Atrial muscle cells manipulate Ca^{2+} microdomains as part of the mechanism for the modulation of atrial Ca^{2+} signals (Module 7: Figure atrial Ca^{2+} domains).

Elementary and global aspects of Ca^{2+} signalling

The development of fluorescent indicators to visualize Ca^{2+} in real time in living cells has revealed a spatial dimension to its action that can account for both the universality and the versatility of Ca^{2+} signalling. The two main ways that Ca^{2+} acts is through elementary and global events (Module 6: Figure elementary and global Ca^{2+} events). The elementary events, which are produced by the brief opening of either entry channels in the plasma membrane or release channels in the endoplasmic

Module 6: | Figure elementary and global Ca^{2+} events



Elementary and global aspects of Ca^{2+} signalling.

Opening of entry channels in the plasma membrane or release channels in the endoplasmic reticulum (ER) give rise to elementary events. Sparklets at the cell surface trigger exocytosis, whereas sparks and puffs can activate a number of cellular processes. These elementary events can also be recruited to create regenerative Ca^{2+} waves that spread through the cell to produce a global Ca^{2+} signal. These elementary events are described in more detail in Module 6: Figure elementary events.

reticulum (ER), have two functions. Firstly, elementary Ca^{2+} events can control highly localized cellular processes, such as exocytosis or membrane excitability. Secondly, these elementary events are the building blocks for the formation of global Ca^{2+} signals. If the release channels are sufficiently sensitive, they can respond to an elementary event in one part of the cell to set up Ca^{2+} waves that lead to a global Ca^{2+} signal responsible for activating a separate set of cellular processes.

Elementary Ca^{2+} events

Elementary events are the basic building blocks of Ca^{2+} signalling. They can either perform highly localized signalling functions, or they can be recruited to generate global Ca^{2+} signals (Module 6: Figure elementary and global Ca^{2+} events). Most of these elementary events are due to the brief opening of Ca^{2+} channels located either in the plasma membrane or in the endoplasmic reticulum (ER) and thus result in localized pulse of Ca^{2+}. The presence of Ca^{2+} buffers helps to restrict these brief pulses to small microdomains within the cytoplasm. In the case of the ER, release of Ca^{2+} causes a corresponding fall in the level of Ca^{2+} within the lumen, and this has been called a blink. It is included in the following list of elementary Ca^{2+} events because it may have an important signalling function, as described below:

- Blink
- Puff
- Flicker
- Sparklet
- Spark
- Syntilla

Sparklet

A sparklet is formed as a result of the brief opening of a voltage-operated channel (VOC) (Module 6: Figure elementary events). Details of the opening mechanism that results in a sparklet are described in the section on voltage-operated channel (VOC) properties (Module 3: Figure VOC properties). Two important signalling functions have been identified for these sparklets:

- Sparklets have been visualized in ventricular heart cells (Module 3: Figure Ca^{2+} sparklet). These sparklets play a critical role in ventricular cell E-C coupling because they provide the trigger Ca^{2+} that activates the type 2 ryanodine receptors (RYR2s) in the junctional zone (Module 7: Figure ventricular cell E-C coupling).
- Detrusor smooth muscle cell activation is triggered by ATP to control bladder emptying. During the process of excitation–contraction coupling, sparklets activate ryanodine receptors to produce the wave of Ca^{2+} that triggers contraction (Module 7: Figure bladder SMC activation).
- Another function for sparklets is to control exocytosis, particularly at synaptic endings, where a localized pulse of Ca^{2+} is responsible for transmitter release (Module 4: Figure Ca^{2+}-induced membrane fusion).

- An elementary event, which is equivalent to a sparklet, is formed in the stereocilia of hair cells upon opening of the transient receptor potential TRPA1 channel (Module 10: Figure stereocilia Ca^{2+} signals).

Spark

A Ca^{2+} spark is formed by the opening of a group of ryanodine receptors (RYRs) (Module 6: Figure elementary events). They were first described in cardiac cells, where they are responsible for excitation–contraction (E-C) coupling. However, they have now been described in many other cell types, where have a variety of different functions:

- In ventricular cardiac cells, the spark is the unitary Ca^{2+} signal that is produced at each junctional zone (Module 7: Figure ventricular cell E-C coupling). There are approximately 10000 junctional zones in each cell, and to get a rapid contraction, the individual sparks must all be fired simultaneously. An electrical recruitment process is used for this synchronization (Module 7: Figure ventricular and atrial cell kinetics). The neat rows of Ca^{2+} sparks ignited along the T-tubules can be seen in panel C in Module 12: Figure CSQ-induced hypertrophy.
- In atrial cells, the sparks have a different function from those in the ventricular cells (Module 7: Figure ventricular and atrial cell kinetics). The initial sparks activated by membrane depolarization are restricted to the junctional zones at the cell surface, where they provide a signal to ignite a Ca^{2+} wave that spreads into the cell by triggering a progressive series of sparks through a process of Ca^{2+}-induced Ca^{2+} release (CICR).
- In mossy fibre presynaptic endings, spontaneous Ca^{2+} sparks can trigger transmitter release (Module 10: Figure mossy fibre presynaptic Ca^{2+} release).
- Spontaneous Ca^{2+} transients (SCaTs), which resemble sparks, have been recorded in cerebellar basket cell presynaptic endings (Module 10: Figure basket cell Ca^{2+} transients).
- Smooth muscle cell Ca^{2+} sparks function to control both contraction and relaxation (Module 7: Figure smooth muscle cell spark). In the case of relaxation, the spark activates the large-conductance (BK) K^+ channel to produce an outward current that hyperpolarizes the membrane (Module 3: Figure smooth muscle cell Ca^{2+} sparks).

Syntilla

A syntilla is an elementary event produced by ryanodine receptors (thus equivalent to a spark) (Module 10: Figure hypothalamic Ca^{2+} syntilla). These syntillas function in hypothalamic neuronal presynaptic Ca^{2+} release.

Blink

Blinks have been visualized in ventricular muscle cells (Module 7: Figure sparks and blinks). Since release channels such as the ryanodine receptors (RYRs) have a very high conductance, they can gate sufficient Ca^{2+} to cause a temporary depletion of Ca^{2+} within the lumen of the junctional SR (Module 7: Figure ventricular cell Ca^{2+} blink). These blinks are attracting considerable interest, because they may play a role in the inactivation of ventricular type 2 ryanodine receptors (RYR2s).

Mitochondrial blinks may also occur during mPTP and mitochondrial Ca^{2+} homoeostasis (Module 5: Figure mitochondrial flickers).

Puff

A puff is a unitary event that results from the release of Ca^{2+} from a small group of inositol 1,4,5-trisphosphate receptors ($InsP_3Rs$) (Module 6: Figure elementary events). Puffs are very similar to the Ca^{2+} spark. Puffs are the building blocks of many of the intracellular Ca^{2+} waves in cells that result in global Ca^{2+} signals. There are examples of localized Ca^{2+} signals that may well be puffs or collections of puffs forming microdomains of Ca^{2+} to regulate localized cellular processes:

- Microdomains of Ca^{2+} occur in the astrocyte endings that form part of the tripartite synapse (Module 7: Figure astrocyte Ca^{2+} signalling).
- $InsP_3Rs$ contribute to a microdomain of Ca^{2+} responsible for neocortical glutamatergic presynaptic Ca^{2+} release (Module 10: Figure neocortical Ca^{2+} release).
- Release of Ca^{2+} by $InsP_3Rs$ produce the microdomains of Ca^{2+} that are confined to individual spines in Purkinje neurons (Module 10: Figure Purkinje cell input-specific Ca^{2+} signals).
- Puffs are an essential feature of the ICC cytosolic Ca^{2+} oscillator (Module 7: Figure ICC pacemaker) responsible for the pacemaker activity that drives a number of smooth muscle cells.

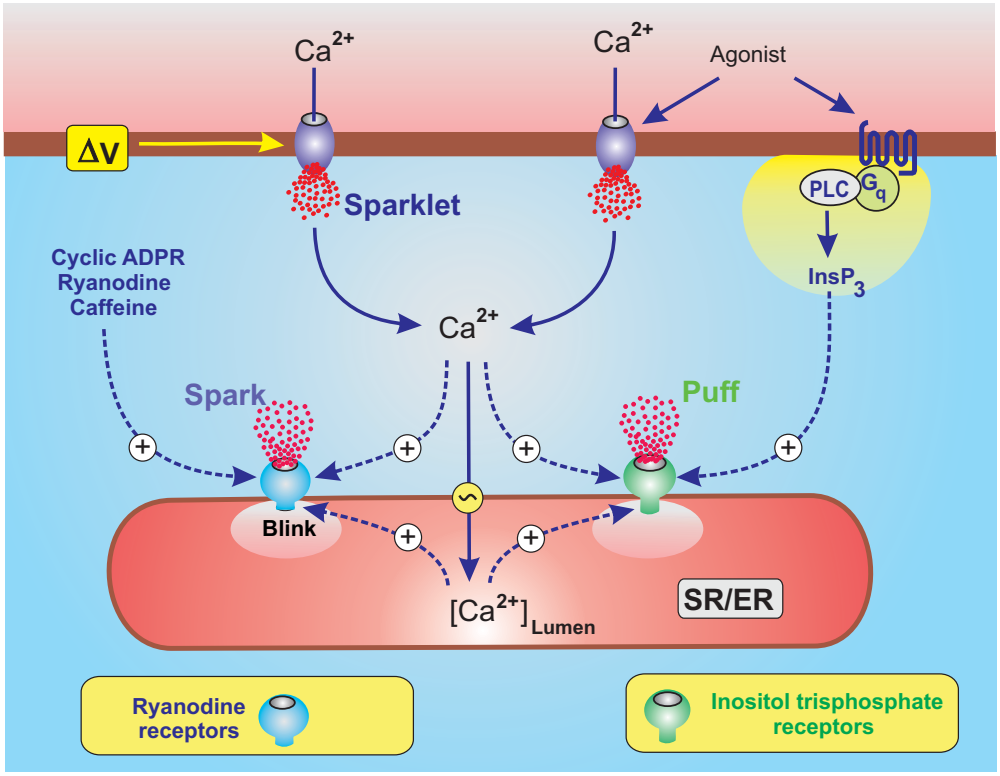
Flicker

Flickers are elementary Ca^{2+} events that have been recorded in neutrophils during chemotaxis (Module 11: Figure neutrophil chemotaxis). They may contribute to the Ca^{2+} signalling microdomains and chemotactic orientation mechanism that enables neutrophils to migrate along a chemotactic gradient. Since these flickers seem to be generated by $InsP_3$ receptors ($InsP_3Rs$), they are probably equivalent to Ca^{2+} puffs.

Global Ca^{2+} signals

Most of the global Ca^{2+} signals in cells are produced by the release of Ca^{2+} from internal stores (Module 6: Figure elementary and global Ca^{2+} events). The intracellular release channels, such as the inositol 1,4,5-trisphosphate ($InsP_3Rs$) and the ryanodine receptors (RYRs), can create such global signals if their release activity can be synchronized. There are two mechanisms of synchronization, which is illustrated nicely by the way ventricular and atrial cardiac cells are controlled (Module 7: Figure ventricular and atrial cell kinetics). In ventricular cells, electrical recruitment by the action potential is used to activate all the sparks in the junctional zones simultaneously. By contrast, atrial cells use a process of diffusional recruitment, whereby Ca^{2+} sparks at the cell surface trigger Ca^{2+} waves that spread inwards through a process of Ca^{2+}-induced Ca^{2+} release (CICR) by recruiting RYRs located deeper within the cell. There are many examples of such intracellular Ca^{2+} waves.

Module 6: | Figure elementary events



The elementary events of Ca^{2+} signalling.

Elementary events are the localized Ca^{2+} signals that arise from either individual or small groups of ion channels. The localized plumes of Ca^{2+} have been given different names, depending on the channels that produce them. Voltage-operated channels (VOCs) in the plasma membrane produce sparklets; ryanodine receptors (RYRs) on the sarcoplasmic reticulum (SR) create sparks (and syntillas), whereas the inositol 1,4,5-trisphosphate receptors produce puffs. These intracellular channels have a large conductance and gate so much Ca^{2+} that results in a local depletion of Ca^{2+} within the lumen immediately below the channel. This local emptying of the endoplasmic reticulum (ER) store has been visualized and has been called a blink.

There also are examples where intracellular waves can spill across into neighbouring cells to set up intercellular Ca^{2+} waves, which thus provide a mechanism for co-ordinating the activity of a local population of cells.

Intracellular Ca^{2+} waves

Intracellular Ca^{2+} waves can be generated by both inositol 1,4,5-trisphosphate ($InsP_3Rs$) and ryanodine receptors (RYRs), which are Ca^{2+}-sensitive channels and can thus contribute to the positive-feedback process of Ca^{2+}-induced Ca^{2+} release (CICR) responsible for forming Ca^{2+} waves (Module 2: Figure Ca^{2+}-induced Ca^{2+} release). The main condition that has to be met for Ca^{2+} waves to form is that the Ca^{2+}-sensitivity of these release channels must be increased so that they can respond to the local Ca^{2+} spark or puff produced by their neighbours. In both cases, the level of Ca^{2+} within the lumen of the endoplasmic reticulum (ER) is critical. This ER loading is achieved by entry of external Ca^{2+} by various cell-surface channels. As the lumen loads up with Ca^{2+}, the $InsP_3Rs$ and RYRs gradually increase their sensitivity so that they can participate in the regenerative processes that result in a Ca^{2+} wave. In effect, this increase in Ca^{2+}-sensitivity of the release channels converts the cytoplasm into an "excitable medium" capable of spawning these regenerative waves. In the case of the $InsP_3Rs$, this loading process and wave generation are explored more fully in the section on the mechanism of Ca^{2+} oscillations (Module 6: Figure Ca^{2+} oscillation model).

These intracellular waves are an integral part of many cellular control processes:

- Intracellular Ca^{2+} waves provide the global Ca^{2+} signal that activates mammalian oocytes at fertilization (Module 8: Figure fertilization-induced Ca^{2+} oscillations).
- Excitation–contraction (E-C) coupling in atrial cardiac cells depends on Ca^{2+} waves that spread into the cell from the periphery (Module 7: Figure atrial cell Ca^{2+} signalling).
- Intracellular Ca^{2+} waves are responsible for excitation–contraction coupling in a number of smooth muscle cells such as vas deferens (Module 7: Figure vas deferens), detrusor smooth muscle (Module 7: Figure bladder SMC activation), vascular and airway smooth muscle cells (Module 7: Figure SMC cytosolic oscillator) and interstitial cells of Cajal (Module 7: Figure ICC pacemaker).
- Astrocyte excitability depends on an intracellular wave that spreads from the tripartite synapses down to the

endfoot processes (Module 7: Figure astrocyte Ca^{2+} signalling).
- The respiratory pacemaker mechanism located in neurons of the pre-Bötzinger complex depends on a positive-feedback mechanism based on a dendritic Ca^{2+} wave (Module 10: Figure respiratory pacemaker mechanism).
- Macrophages generate waves in response to both ADP and membrane depolarization. The wave spreads rapidly around the periphery, where there is a tight packing of the $InsP_3R3$s.
- During acetylcholine-induced pancreatic secretion, $InsP_3Rs$ in the apical region initiate the wave that then spreads through to the basal region via RYRs.

Intercellular Ca^{2+} waves

There are a number of instances of Ca^{2+} waves travelling from one cell to the next. Such intercellular waves may act to co-ordinate the activity of a local population of cells. There is still some uncertainty concerning the way in which the wave is transmitted from one cell to the next (Module 6: Figure intercellular Ca^{2+} waves). One mechanism proposes that low-molecular-mass components such as inositol 1,4,5-trisphosphate ($InsP_3$) or Ca^{2+} spill through the gap junctions to ignite waves in neighbouring cells. In order for a cell to set up an intracellular wave, the internal release channels have to be sensitized, so it seems likely that all the cells in the population have to be in a similar state in order for an intercellular wave to pass from one cell to the next. In such a scenario, Ca^{2+} is the most likely candidate to be the stimulus that passes from one cell to the next. An alternative model proposes that the intracellular wave in one cell stimulates the release of ATP by hemichannels (See Module 3: Figure hemichannels and gap junctions) that then diffuses across to neighbouring cells, where it acts on P2Y receptors to increase $InsP_3$, which then acts to trigger a new wave (Mechanism B in Module 6: Figure intercellular Ca^{2+} waves).

The function for such intercellular waves has not been clearly established. Much of the work on these waves has been done on cultured cells, but there are a number of reports showing that such intercellular waves do occur between cells *in situ*:

- Intercellular waves have been described in astrocytes, where they are a consequence of astrocyte excitability (Module 7: Figure astrocyte Ca^{2+} signalling). The physiological function of these waves is unclear. Since they only seem to appear following intense stimulation, they may be a manifestation of some pathological change. In this respect, the astrocyte wave moves at approximately the same rate as spreading depression that appears to be linked to the onset of migraines.
- Intercellular waves have been recorded in the intact perfused liver that appears to travel in a periportal to pericentral direction (Module 6: Figure liver intercellular Ca^{2+} wave). This directionality has led to the suggestion that the wave might function to regulate a peristaltic contraction wave to control the flow of bile.
- During development, there are pan-embryonic intercellular waves that sweep around the blastoderm margin in the late gastrula of zebrafish (see Stage d in Module 8: Figure developmental Ca^{2+} signalling).
- Endothelial cells and smooth muscle cells (SMCs) appear to communicate through an intercellular wave.
- An intercellular Ca^{2+} wave passing through the cell community of the juxtaglomerular apparatus (JGA) (cells connected by double-headed red arrows in Module 7: Figure juxtaglomerular apparatus) may function to transfer information during the operation of the tubuloglomerular feedback (TGF) mechanism.
- The activity of endocrine cells in the anterior pituitary may be co-ordinated by an intercellular Ca^{2+} wave that spreads through the folliculostellate (FS) cells (Module 10: Figure FS Ca^{2+} wave).

Cyclic AMP microdomains

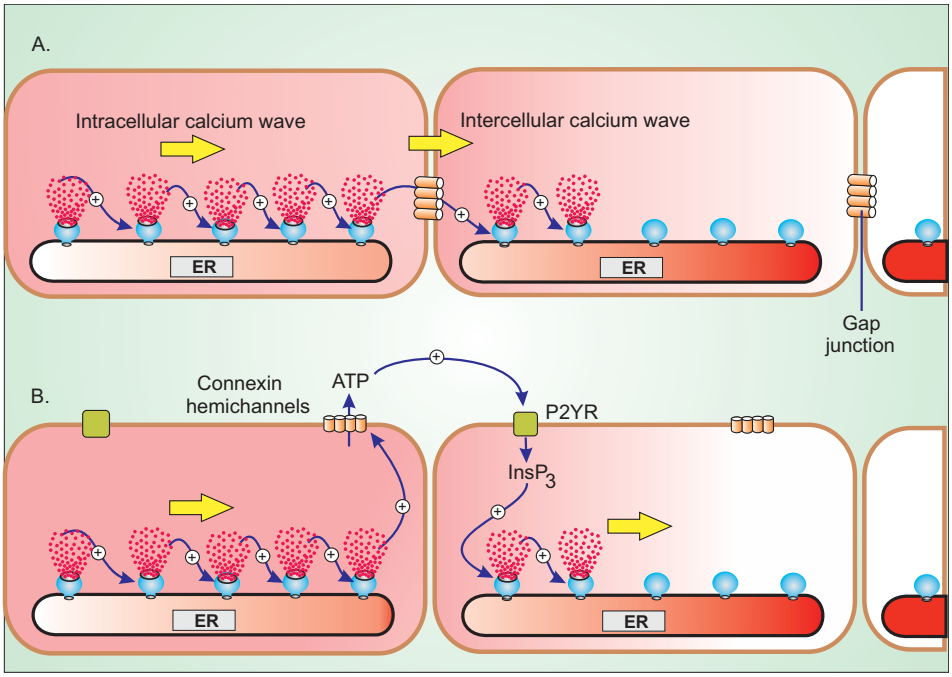
The cyclic AMP signalling pathway uses the second messenger cyclic AMP to carry information from cell-surface receptors to internal effector systems (Module 2: Figure cyclic AMP signalling). Since cyclic AMP has a relatively high diffusion rate of approximately 5000 $\mu m^2/s$, it was thought that this messenger would rapidly equilibrate throughout the cytosol as a global signal. However, there is now evidence that some of the actions of cyclic AMP might be restricted to microdomains (Module 6: Figure cyclic AMP microdomains). In the cardiac cell, these cyclic AMP microdomains were clearly lined up in striations along the Z lines. This localization indicates that the generation, metabolism and action of cyclic AMP are highly localized to the T-tubule invaginations. Within this T-tubule region, there are indications that the action of cyclic AMP may be divided further into additional microdomains (Module 6: Figure ventricular cyclic AMP microdomains).

The microdomains located around the T-tubule of the ventricular cells function in the modulation of ventricular Ca^{2+} signals. Pharmacological studies have shown that stimulation of the β-receptors located on the T-tubule facing the junctional sarcoplasmic reticulum (SR) can enhance the activity of the L-type Ca^{2+} channels without having any effect on the activity of the sarco/endo-plasmic Ca^{2+}-ATPase (SERCA) pumps lined up on the free SR. This local domain of cyclic AMP also phosphorylates the ryanodine receptors (RYRs). A separate microdomain set up by the T-tubule β-receptors spreads out over the free SR and the myofibrils to control two important functions. First, the phosphorylation of phospholamban removes its inhibition of the SERCA pumps enabling them to increase their pump rates. Secondly, the sensitivity of the contractile system is enhanced by the phosphorylation of troponin I. For details of these different actions of cyclic AMP, see Module 7: Figure ventricular cell E-C coupling.

Reactive oxygen species (ROS) microdomains

Reactive oxygen species (ROS) such as superoxide ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2) are the second messengers

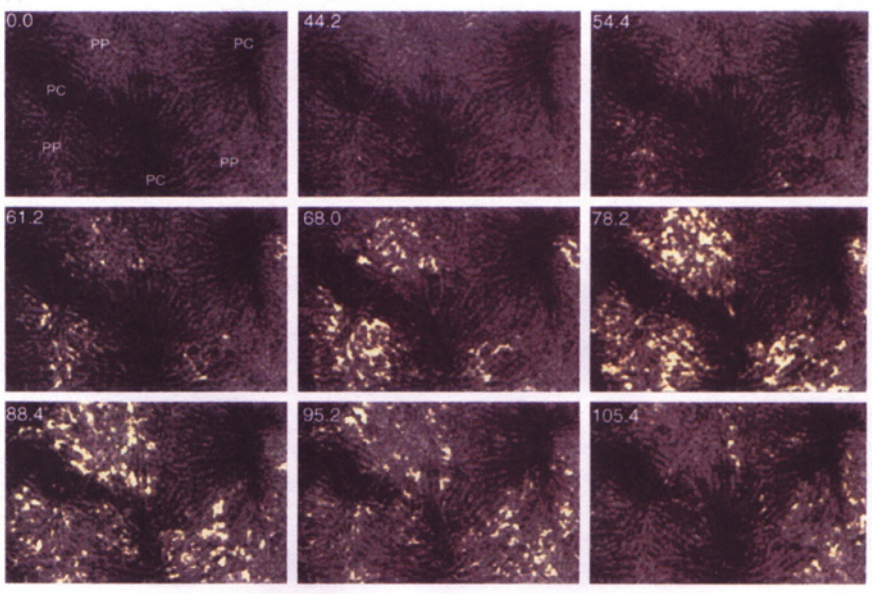
Module 6: | Figure intercellular Ca^{2+} wave



Proposed mechanisms of intercellular wave propagation.

There are numerous examples of Ca^{2+} waves that travel from cell to cell. There is still some debate about the way the wave travels between cells. A. One mechanism proposes that, when the intracellular wave reaches the cell boundary, some low-molecular-mass component, most likely to be Ca^{2+}, diffuses across the gap junction to ignite another intracellular wave in the neighbouring cell. B. An alternative mechanism suggests that the intracellular wave in one cell stimulates the release of ATP through hemichannels, which then diffuses across to ignite a wave in neighbouring cells by acting on P2Y receptors to produce inositol 1,4,5-trisphosphate ($InsP_3$).

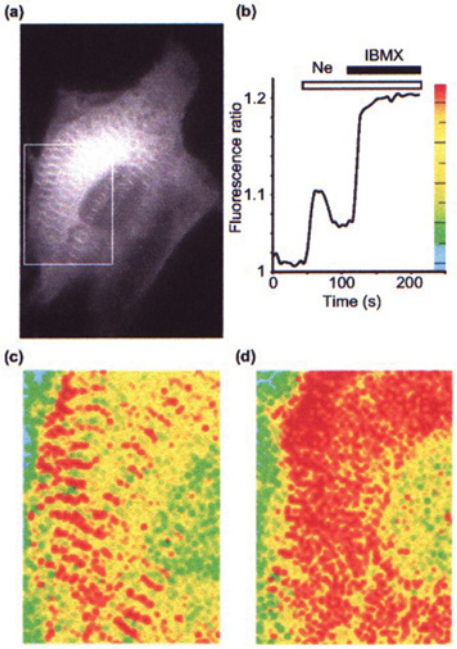
Module 6: | Figure liver intercellular Ca^{2+} wave



An intercellular Ca^{2+} wave recorded in the intact liver.

The intact liver was loaded with the Ca^{2+} indicator Fluo3, which showed up the sheets of liver cells in a number of lobules. When the liver was perfused with vasopressin, Ca^{2+} signals originated in the periportal (PP) regions and then moved out as an intercellular wave towards the pericentral (PC) regions. Reproduced from Robb-Gaspers, L.D. and Thomas, A.P. (1995) Coordination of Ca^{2+} signalling by intercellular propagation of Ca^{2+} waves in the intact liver. J. Biol. Chem. 270:8102–8107, with permission from the American Society for Biochemistry and Molecular Biology; see Robb-Gaspers and Thomas 1995.

Module 6: | Figure cyclic AMP microdomains



Visualization of cyclic AMP microdomains in cardiac myocytes.
A fluorescence resonance energy transfer (FRET)-based protein kinase A (PKA) sensor was used to detect cyclic AMP microdomains in a cardiac myocyte. (a) The fluorescence produced by the FRET partners, which where the catalytic and regulatory subunits of PKA, formed striations along the Z line. (b) Addition of noradrenaline (Ne) caused an increase in fluorescence, indicating the formation of cyclic AMP, which was increased further upon addition of the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX). (c) The boxed region in panel a was imaged at 60 s (i.e. at the peak of the Ne response), revealing that the increase in cyclic AMP was localized to the Z lines. (d) When imaged at 200 s (i.e. after the addition of IBMX), there was a large increase in fluorescence that was now more spread out. Reproduced from *Curr. Opin. Cell Biol.*, Vol. 14, Zaccolo, M., Magalhães, P. and Pozzan, T., Compartmentalisation of cAMP and Ca^{2+} signals, pp. 160–166. Copyright (2002), with permission from Elsevier; see Zaccolo et al. 2002.

used by the redox signalling pathway (Module 2: Figure summary of redox signalling). The ROS generated at both the plasma membrane and at the mitochondria may occur in microdomains to provide a highly localized signalling system. At the plasma membrane, the ROS produced by NADPH oxidase functions within a limited microdomain (Module 2: Figure ROS microdomains). Similarly, brief superoxide flashes have been recorded in individual mitochondria. Such flashes are particularly evident during the course of mitochondrial flickers (Module 5: Figure mitochondrial flickers). Such superoxide flashes can spread throughout the cell through a process of ROS-induced ROS release (RIRR).

The size of these microdomains is regulated by a high concentration of redox buffers, which act to maintain the redox balance within the cell. The high concentration of glutathione (GSH), which is the major redox buffer, functions to restrict the size of ROS microdomains. Similarly, the peroxiredoxins can limit the size of H_2O_2 microdomains (see step 8 in Module 2: Figure peroxiredoxin catalytic cycles).

Temporal aspects of signalling

Cellular oscillators

Oscillations are a characteristic feature of many cellular control systems. Such oscillatory activity can have a wide range of frequencies (**Module 6: Figure cellular oscillators**). There are three main types of oscillatory activity: **membrane oscillators, cytosolic oscillators** and the **circadian clock**. These oscillators span very different time domains. **Brain rhythms** (0.2–200 Hz) contain the highest frequency oscillations, and many of these emerge as a property of the neural network and have been referred to as network oscillators. However, it is evident that neurons and many other cells can also generate endogenous oscillations that can be divided into membrane oscillators or cytosolic oscillators (**Module 6: Figure membrane and cytosolic oscillators**). The output from membrane oscillators usually sets up a regular train of action potentials that provide the rhythmical pacemaker activity responsible for driving many cellular processes, such as contraction, neuronal activity and secretion. These membrane potential oscillations can open voltage-operated channels (VOCs) that gate Ca^{2+} and can thus result in intracellular Ca^{2+} oscillations. Such Ca^{2+} oscillations can also be generated in many cell types through the operation of cytosolic oscillators. Most attention has concentrated on such **Ca^{2+} oscillations** because this messenger can be monitored in real time using a range of indicators. Information transfer within the Ca^{2+} signalling systems is critically dependent on understanding the **encoding and decoding of Ca^{2+} oscillations**. The **circadian clock** has a very different oscillatory mechanism that is based on a transcription cascade with multiple feedback loops.

Brain rhythms

The electroencephalogram (EEG) is characterized by a complex series of oscillations that reflect the on-going electrical activity occurring in different circuits within the brain during sleep and consciousness. These sleep/wake states are characterized by different types of brain rhythms (Module 10: Figure sleep phases). Sleep is divided into two main phases: non-rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep phases that cycle with an ultraradian frequency of approximately 90 min. During NREM sleep, there are slow oscillations (<1 Hz) and delta oscillations (1–4 Hz), whereas there are faster rhythms such as the theta oscillations (4–10 Hz) and gamma oscillations (20–80 Hz).

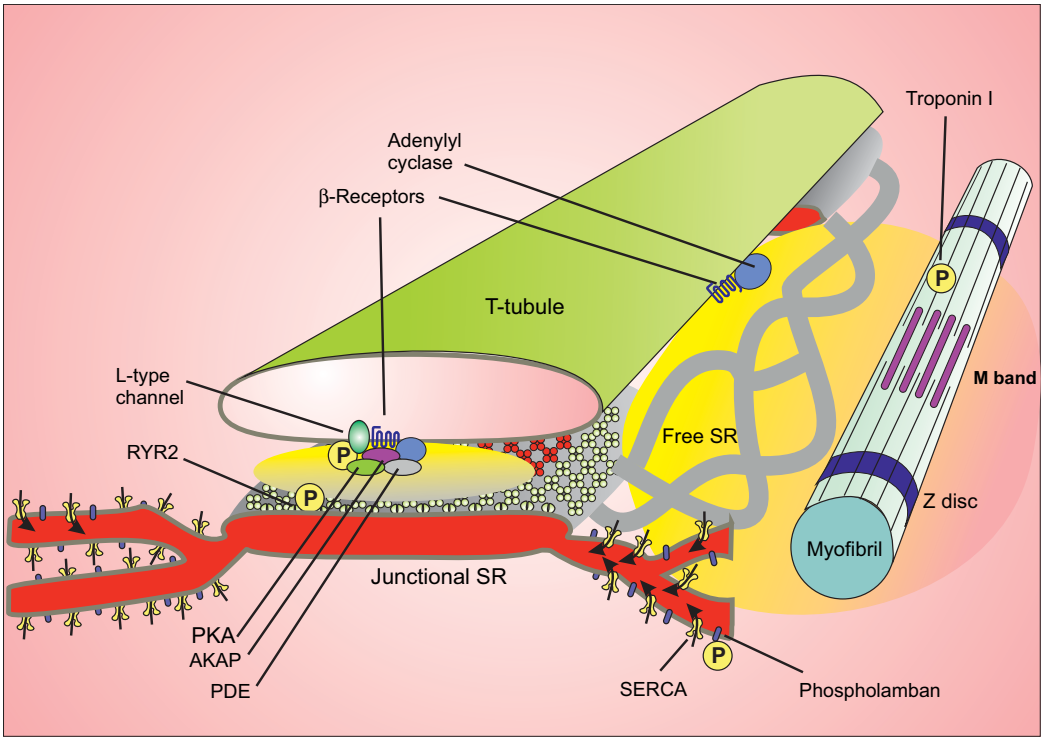
Brain rhythm synchronization is critical for the role of these brain oscillations in driving a large variety of brain functions.

Alterations in these brain rhythms, particularly the gamma oscillations, have been linked to schizophrenia.

Slow oscillations (<1 Hz)

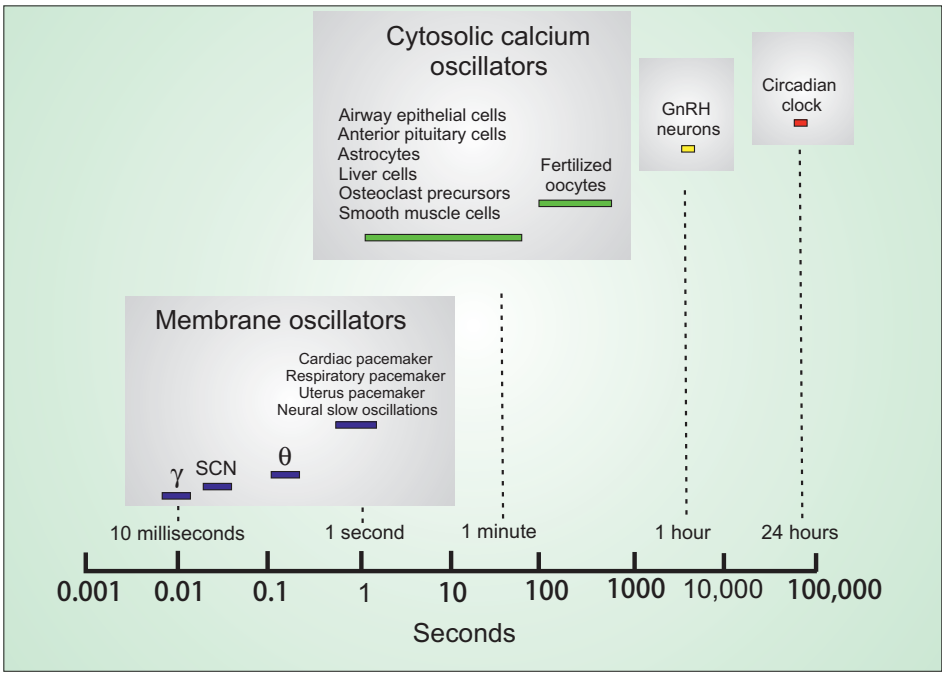
Slow oscillations that occur during non-rapid eye movement (NREM) sleep are characterized by two membrane states (Module 10: Figure sleep phases). An Up state where the membrane is depolarized to about 65 mV alternates with a Down state where the membrane in hyperpolarized

Module 6: | Figure ventricular cyclic AMP microdomains



Microdomains of cyclic AMP in ventricular cardiac cells.
This three-dimensional drawing illustrates the relationship of the T-tubule, the junctional sarcoplasmic reticulum (SR), the free SR and the contractile myofibril. The T-tubule has β-adrenergic receptors on the surface facing the junctional SR and on the surface facing the free SR. Both groups of receptors appear to be capable of setting up separate microdomains of cyclic AMP (shown in yellow).

Module 6: | Figure cellular oscillators



Temporal distribution of cellular oscillators.
Cellular oscillators span a wide temporal range. Membrane oscillators operate in the sub-second range. Cytosolic Ca^{2+} oscillators have periodicities in the second to minute range. Gonadotropin-releasing hormone (GnRH) neurons oscillate in the 1–2 h range, whereas the circadian clock has a periodicity of approximately 24 h.

by 10–15 mV (Module 10: Figure slow oscillation mechanism). During the Up state, there often are bursts of action potentials that fire at rates comparable with those seen in the wake state and have been implicated in the process of memory consolidation that occurs during NREM sleep. A slow oscillation mechanism is responsible for generating these oscillations.

These slow oscillations and the delta oscillations (described below) display a remarkable degree of brain rhythm synchronization in that most of the neurons in the brain are oscillating in synchrony with each other during the NREM sleep period. A slow oscillation synchronization and wave propagation mechanism is responsible for this large scale synchronization of the slow oscillations.

Delta (0.5–4 Hz) oscillations (δ)

The very regular patterns that appear in the electroencephalogram (EEG) records during sleep result from the fact that vast arrays of neurons are firing rhythmically and in synchrony. There are two main patterns: the slow waves that predominate in the early phase of sleep, which are interrupted by rapid eye movement (REM) sleep that becomes more frequent towards the end of sleep. Such REM sleep has been considered as the gateway to wakefulness. Of these two activities, it is slow-wave sleep that appears to be most important with regard to the beneficial aspects of sleep.

The slow-wave patterns of the EEG recordings have two main components that originate from different regions of the brain: the delta (0.5–4 Hz) waves originate in the cortex, and the spindle oscillations (7–14 Hz) originate in the thalamus. However, these two brain regions do not operate in isolation, because there is a continuous dialogue between them, which serves to create the synchrony that is such a feature of these large neuronal networks. The dialogue is carried out through reciprocal connections between the cortical and thalamic neurons. Additional players are the reticular cells, which receive inputs from these two neurons and thus 'eavesdrop' on the corticothalamic dialogue. However, they are not innocent bystanders in that they can influence this dialogue through an inhibitory input on to the cortical cells. The highly synchronized behaviour of slow-wave sleep emerges from this relatively simple neuronal circuit containing the usual excitatory and inhibitory connections. All the cortical neurons fire together at intervals of about 1 s.

Theta (4–10 Hz) oscillations (θ)

Rhythmic theta oscillations (4–10 Hz) are particularly prevalent in the hippocampus and cortex (Module 10: Figure brain circuitry and rhythms). These relatively slow oscillations seem to reflect the activation state of the hippocampus where they function as a temporal organizer for a number of processes such as the encoding and retrieval of spatial and episodic memories. Each circuit has characteristic patterns of activity. For example, the pyramidal neurons within the hippocampus are quiescent for much of the time, but when animals explore new environments, there are brief bursts of spikes (4–10 Hz), called the theta (θ) rhythm, separated by long quiescent periods of approximately 60 s.

A critical feature of these theta oscillations is their synchronization between the hippocampus and the cortex. The theta oscillation generated within the hippocampus is 'exported' to the other cortical regions to provide a mechanism to co-ordinate the theta oscillations in these disperse brain regions.

Hippocampal theta oscillations are generated by a typical network oscillator (Module 10: Figure theta oscillatory mechanisms).

Gamma (20–80 Hz) oscillations (γ)

Gamma oscillations represent the fast (20–80 Hz) neuronal oscillations that have been recorded in various brain regions (Module 10: Figure brain circuitry and rhythms). The hippocampal gamma oscillations are produced by a typical network oscillator (Module 10: Figure gamma oscillatory mechanisms). Since these gamma oscillations sweep through large assemblies of neurons, they are thought to function as a synchronization signal that enables neurons to fire together during processes such as working memory and visual attention. Since the neurons responsible for a particular memory, such as a persons face, are distributed throughout the brain, they all have to be activated synchronously to recall that particular memory. When an image is stored in a computer, the individual pixels are all stored in the same file. In the brain, however, the information is stored by many different neurons (roughly equivalent to pixels in a computer) that are widely distributed throughout the brain and have to be brought together to recall a memory. This has been referred to as the 'binding problem' and it is thought that gamma oscillations provide a mechanism to synchronize the firing of widely dispersed neurons.

There is still much uncertainty as to how these gamma oscillations are controlled. Cholinergic activation induces persistent gamma oscillations in the hippocampus and in the somatosensory cortex. Cortical neurons in the visual system respond to coherent visual stimuli by discharging synchronously at frequencies of around 40 Hz. The synchronization may reflect a transient binding together of reverberating groups of neurons, each of which responds to a different feature of the same perceptual object. These 40 Hz oscillations can be induced in slices by a combination of a γ-aminobutyric acid type A ($GABA_A$) inhibitory and a glutamate [(1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylate (ACPD)] metabotropic excitatory transmitters that are known to be important components of the network oscillator in the hippocampus (Module 10: Figure gamma oscillatory mechanisms).

High-frequency (200 Hz) rhythm

These high-frequency transmissions are mediated through direct electronic coupling of neurons. The hippocampus has high-frequency network oscillations (similar to 200 Hz 'ripples'), whereas neocortex has low-frequency (1–4 Hz) and spindle (7–14 Hz) oscillations. There are temporal correlations between hippocampal ripples and cortical spindles, and this may play a role in cortico-

hippocampal communication during sleep. The coactivation of these two pathways may be important for memory consolidation, during which information is gradually translated from short-term hippocampal to longer-term neocortical stores.

Network oscillators

Network oscillators are found within the nervous system. The **network gamma oscillation mechanism** is an example of how excitatory and inhibitory neurons can interact with each other to generate theta and gamma oscillations. For example, such network oscillators are responsible for generating **hippocampal oscillations**. In this case, **hippocampal local circuits**, consisting of inhibitory interneurons interacting with the excitatory pyramidal neurons, set up both **hippocampal gamma oscillations** (**Module 10: Figure gamma oscillatory mechanisms**) and **hippocampal theta oscillations** (**Module 10: Figure theta oscillatory mechanisms**).

In the case of the **respiratory centre**, the oscillator that regulates breathing appears to combine elements of both a cytosolic oscillator and a network oscillator (**Module 10: Figure respiratory pacemaker mechanism**). The latter provides a mechanism to synchronize the individual cytosolic oscillators to provide a discrete output signal to set up the regular pattern of breathing.

Membrane oscillators

Membrane oscillators are usually generated through an interplay between outward currents, usually carried by K^+, that induce membrane hyperpolarization that inhibits the inward currents (e.g. Na^+ and Ca^{2+} currents) that cause depolarization (Module 6: Figure membrane and cytosolic oscillators). The entry of Ca^{2+} during the depolarizing phase is responsible for the phasic elevation of Ca^{2+} that is returned to the cytoplasm by the plasma membrane Ca^{2+}-ATPase (PMCA) during the OFF reaction. In some cases, the Ca^{2+} that enters the cell during the depolarizing phase can contribute to the membrane oscillator by activating Ca^{2+}-sensitive K^+ channels to switch on the hyperpolarizing phase. The Ca^{2+} signal generated by the membrane oscillator is often amplified by releasing Ca^{2+} from the internal store.

There are a number of cell types that set up pacemaking activity using such membrane oscillators:

- Sinoatrial node pacemaker cells establish the regular trains of action potential that drive cardiac contraction (Module 7: Figure cardiac pacemaker).
- Thalamocortical neurons have a neuronal rhythmicity that displays oscillations at a frequency of 0.5–4 Hz using a combination of different inward and outward currents.
- Neurons that reside within the suprachiasmatic nucleus (SCN) have the biological clock. In addition there is an SCN membrane oscillator that is responsible for generating the output signals from the biological clock (Module 6: Figure circadian clock input–output signals).
- There is evidence for individual neurons within the respiratory centre having a membrane oscillator as part of the network oscillator that controls breathing.
- Contractions of the uterus during labour are driven by a uterus smooth muscle cell membrane oscillator (Module 7: Figure uterus activation).
- Corticotrophs in the anterior pituitary have a membrane oscillator that generates the periodic action potentials responsible for the release of adrenocorticotropic hormone (ACTH) (Module 10: Figure corticotroph regulation).
- A membrane oscillator drives the spontaneous activity of lactotrophs that release the hormone prolactin (PRL) (Module 10: Figure lactotroph regulation).
- Somatotrophs have a membrane oscillator that controls the release of growth hormone (GH) (Module 10: Figure somatotroph regulation).

Cytosolic oscillators

Many cell signalling mechanisms have complex feedback control mechanisms, so it is likely that their output systems will oscillate. So far, most attention has been focused on Ca^{2+} oscillations because this internal messenger can be monitored in single cells in real time. Cells can also generate cyclic AMP oscillations that have been implicated in the control of insulin-secreting β-cells and neural gene transcription.

Ca^{2+} oscillations

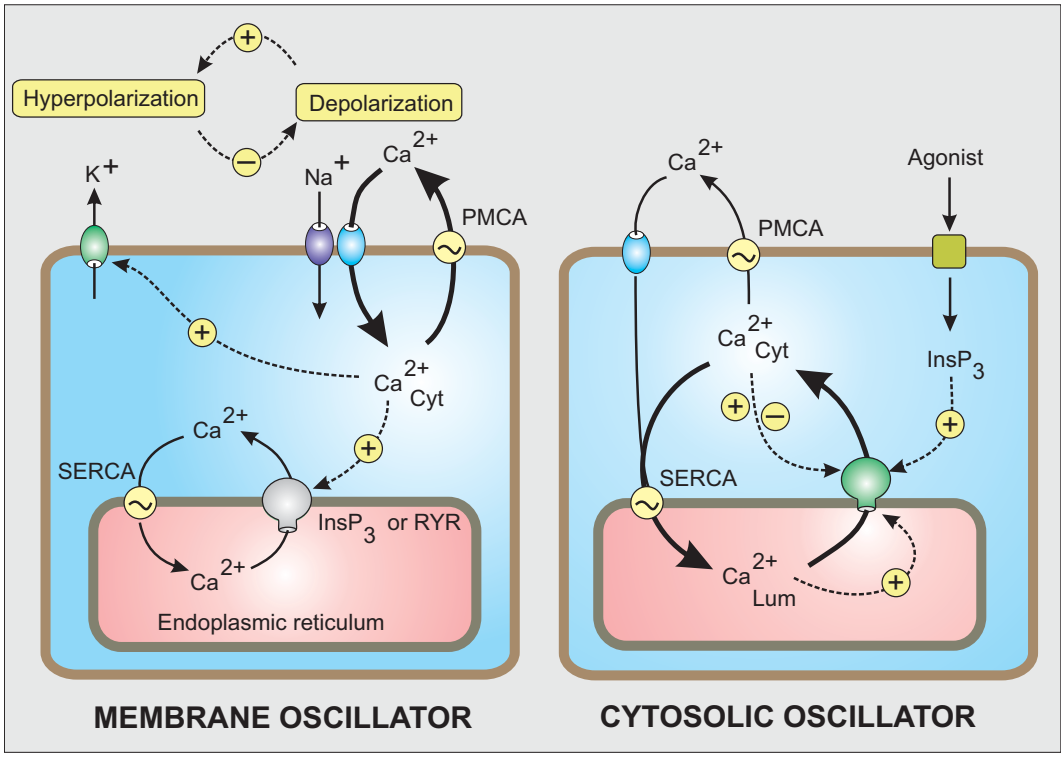
A characteristic feature of most Ca^{2+} signals is that they are presented as a brief Ca^{2+} transient (Module 2: Figure Ca^{2+} transient mechanisms). These transients can either be produced on demand by periodic stimulation, as occurs in muscle or neurons, or they can appear as part of an oscillation (Module 2: Figure temporal aspects). There are two main mechanisms for generating such Ca^{2+} oscillations. For those cells that have membrane oscillators, or are driven by membrane oscillators as occurs in the heart, regular Ca^{2+} transients are created by the periodic entry of Ca^{2+} across the plasma membrane (Module 6: Figure membrane and cytosolic oscillators).

Alternatively, oscillations can occur through the periodic release of internal Ca^{2+} through the operation of a cytosolic oscillator. This mechanism of Ca^{2+} oscillations depends upon the release of Ca^{2+} from intracellular stores. In the case of agonist-induced oscillations, the inositol 1,4,5-trisphosphate ($InsP_3$)/Ca^{2+} signalling cassette is primarily responsible for initiating oscillatory activity. The information concerning encoding and decoding of Ca^{2+} oscillations depends upon the ability to modulate the different parameters of Ca^{2+} oscillations.

Such agonist-dependent cytosolic Ca^{2+} oscillations are a major feature of Ca^{2+} signalling in many cell types:

- Liver cells display Ca^{2+} oscillations with agonist concentration-dependent changes in frequency

Module 6: | Figure membrane and cytosolic oscillators



The main features of membrane and cytosolic oscillators.

The fluctuations in membrane potential that characterize membrane oscillators are generated by the periodic opening of K^+ channels that cause hyperpolarization and the opening of Na^+ or Ca^{2+} channels responsible for depolarization. By contrast, cytosolic oscillators depend upon the periodic release of Ca^{2+} from the endoplasmic reticulum by inositol 1,4,5-trisphosphate ($InsP_3$) receptors. The continuation of this oscillator is critically dependent upon a constant input of Ca^{2+} entering from the outside (further details of the mechanism are given in Module 6: Figure Ca^{2+} oscillation model).

(Module 5: Figure cytosolic and mitochondrial Ca^{2+} transients).

- Ca^{2+} oscillations are evident during the acquisition of meiotic competence during development (Module 8: Figure meiotic Ca^{2+} signalling).
- Ca^{2+} oscillations are responsible for oocyte activation during mammalian fertilization (Module 8: Figure fertilization-induced Ca^{2+} oscillations). Such Ca^{2+} oscillations are also observed following artificial insemination by intracytoplasmic spermatozoa injection (ICSI) (Module 8: Figure ICSI-induced Ca^{2+} oscillations).
- Smooth muscle cells surrounding cortical arterioles display spontaneous Ca^{2+} oscillations (Module 7: Figure smooth muscle cell Ca^{2+} oscillations).
- Astrocytes display spontaneous Ca^{2+} oscillations (Module 7: Figure astrocyte Ca^{2+} oscillations).
- Vascular and airway smooth muscle cells are activated by a smooth muscle cytosolic oscillator (Module 7: Figure SMC cytosolic oscillator).
- Airway epithelial cells respond to ATP to generate the Ca^{2+} oscillations that control ciliary beat frequency (CBF) (Module 7: Figure airway cell oscillations).
- The osteoclast-associated receptor (OSCAR) induces Ca^{2+} oscillations in osteocyte precursor cells that activate the nuclear factor of activated T cells (NFAT) necessary for the differentiation of osteoclasts (Module 8: Figure osteoclast Ca^{2+} oscillations).
- Ca^{2+} oscillations drive the amino acid-dependent activation of cell growth control (Module 9: Figure target of rapamycin signalling).

Mechanism of Ca^{2+} oscillations

Most cytosolic Ca^{2+} oscillations are driven by agonists that are coupled to the **inositol 1,4,5-trisphosphate ($InsP_3$)/ Ca^{2+} signalling cassette**. The increase in $InsP_3$ is then responsible for setting up repetitive cycles of Ca^{2+} release from the endoplasmic reticulum (ER) This regenerative phase of Ca^{2+} release depends on the release channels ($InsP_3$ and ryanodine receptors) acting separately or in combination with each other. Such agonist-dependent Ca^{2+} oscillations have a number of important properties that have to be considered when attempting to design a model to explain such oscillatory activity:

1. Ca^{2+} oscillations occur at very low agonist concentrations that usually correspond to the range of concentrations that are normally responsible for activating physiological responses.
2. Oscillation frequency increases with agonist concentration, often without causing any change in the amplitude of the individual spikes.
3. Oscillation frequency is often, but not always, sensitive to changes in the external concentration of Ca^{2+}.

4. Ca^{2+} itself seems to play a critical role in setting up the oscillatory release of Ca^{2+} by the $InsP_3$ receptor.

The exact mechanism responsible for these $InsP_3$-induced oscillations is still a matter of some debate. There are two main models. One model proposes that the oscillator depends upon periodic fluctuations in the level of $InsP_3$. These $InsP_3$ fluctuations are thought to be driven by a feedback effect of Ca^{2+} on phospholipase C (PLC).

The other model proposes that oscillations occur at constant levels of $InsP_3$ that act by sensitizing either the $InsP_3$ receptor or the ryanodine receptor (RYR), enabling them to set up regular cycles of Ca^{2+} release that are regulated by the positive- and negative-feedback effects of Ca^{2+} acting from both the cytoplasm and the lumen of the endoplasmic reticulum (Module 6: Figure membrane and cytosolic oscillators). The major cycling of Ca^{2+} occurs across the ER, whereas a minor cycle, which operates at a constant rate across the plasma membrane, is responsible for ensuring that the lumen of the ER remains topped up with Ca^{2+}.

The different processes that occur during an oscillatory cycle are described below (as illustrated in Module 6: Figure Ca^{2+} oscillation model). This model applies to $InsP_3$-dependent oscillations, but a similar mechanism could operate using ryanodine receptors or a combination of the two release channels:

- Store loading. A critical feature of this model is the luminal regulation of Ca^{2+} release channels. This regulation depends upon an effect of luminal loading on the sensitivity of the $InsP_3$ receptors to the stimulatory effect of Ca^{2+}. It is argued that at the low agonist concentrations that give oscillations, the amount of $InsP_3$ being produced is very small and its site of action may be limited to small domains (yellow shell) near the plasma membrane (Module 3: Figure capacitative Ca^{2+} entry). This localized domain may cause a small release of Ca^{2+} from the ER in the immediate vicinity of the membrane, whereas the bulk of the ER store is unaffected. This local depletion will promote Ca^{2+} entry either through store-operated channels (SOCs) or some other entry channel, and the Ca^{2+} flowing into the cell will begin to load up the store leading to $InsP_3$ receptor sensitization and spike initiation.
- Spike initiation. An important and, as yet, ill-understood process is responsible for initiating the spike. It is proposed that the build-up of Ca^{2+} within the lumen of the ER sensitizes the $InsP_3$ receptors or ryanodine receptors (RYRs) such that they begin to release Ca^{2+} in the form of puffs or sparks respectively, which often begin at a discrete initiation site and result in spike development (Module 6: Figure Ca^{2+} oscillation model). It is the concentration of Ca^{2+} within the lumen of the ER that sets the stage for the periodic release of Ca^{2+}. This model predicts that the concentration of Ca^{2+} must decline rapidly during the course of the spike and then build up gradually towards the onset of the next spike, which is exactly what is found experimentally when the level of luminal Ca^{2+} is measured in pancreatic cells (Module 6: Figure pancreatic Ca^{2+} oscillations). Such store loading may also explain the sperm-induced Ca^{2+} oscillations that occur during fertilization (Module 8: Figure mammalian fertilization).
- Spike development. The spike develops through a process of Ca^{2+}-induced Ca^{2+} release (CICR), during which Ca^{2+} acts together with $InsP_3$ to set up a regenerative wave that spreads Ca^{2+} throughout the cell to give a global signal.
- Spike recovery. During the recovery process, a proportion of the Ca^{2+} is pumped back into the ER, and some is pumped out of the cell. The latter has to be replaced by Ca^{2+} entry before another spike can be triggered. One of the difficulties with trying to understand the mechanism of Ca^{2+} oscillations is to explain how changes in agonist concentration can alter oscillation frequency. The model outlined in Module 6: Figure Ca^{2+} oscillation model suggests that it is the relatively slow process of store loading that determines the period between spikes. Since the rate of loading depends upon the rate of Ca^{2+} entry, which in turn depends upon agonist concentration, it is possible to see how changes in agonist concentration are translated into changes in spike frequency.

Encoding and decoding of Ca^{2+} oscillations

There are various ways in which information is encoded through Ca^{2+} oscillations. Since Ca^{2+} signals usually appear as brief transients that are the digital signals used to transmit information, different bits of information can be encoded within Ca^{2+} oscillations by modulating the frequency, amplitude and width of these digital signals (**Module 6: Figure encoding oscillatory information**). There are a number of examples of cellular processes that are regulated by frequency-modulated (FM) Ca^{2+} oscillations:

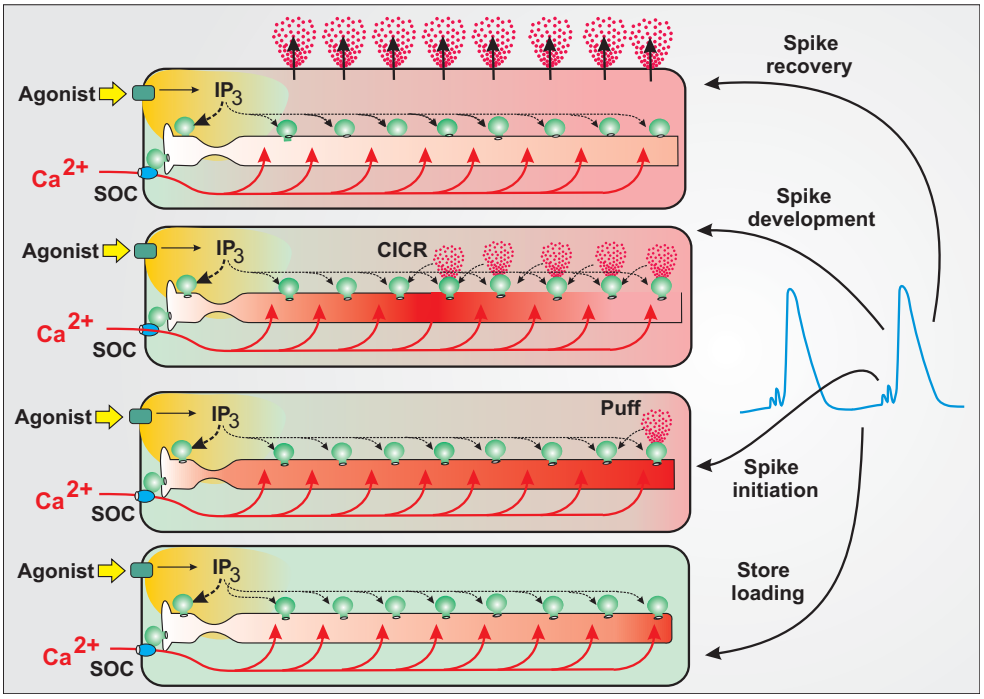
- The frequency of Ca^{2+} oscillations determines the type of transmitter that is expressed during the differentiation of neurons.
- Ca^{2+} control of ciliary beat frequency (CBF) in airway epithelial cells (Module 7: Figure airway cell oscillations).
- The frequency of Ca^{2+} pulses determines the muscle phenotype during the neural control of differentiation.

An important question therefore arises as to how the information encoded in the digital Ca^{2+} signals alters the activity of the effectors that bring about the changes in cellular activity. There appear to be two main mechanisms for information decoding (Module 6: Figure decoding oscillatory information). One is through a mechanism of digital tracking whereby the nature of the downstream response closely tracks each Ca^{2+} transient. Examples of such digital tracking include the following:

- Ventricular cell Ca^{2+} release (Module 12: Figure decoding cardiac Ca^{2+} spikes).
- Ca^{2+} control of ciliary beat frequency (CBF) in airway epithelial cells (Module 7: Figure airway cell oscillations).

The other major decoding mechanism is integrative tracking whereby each transient has a small effect on

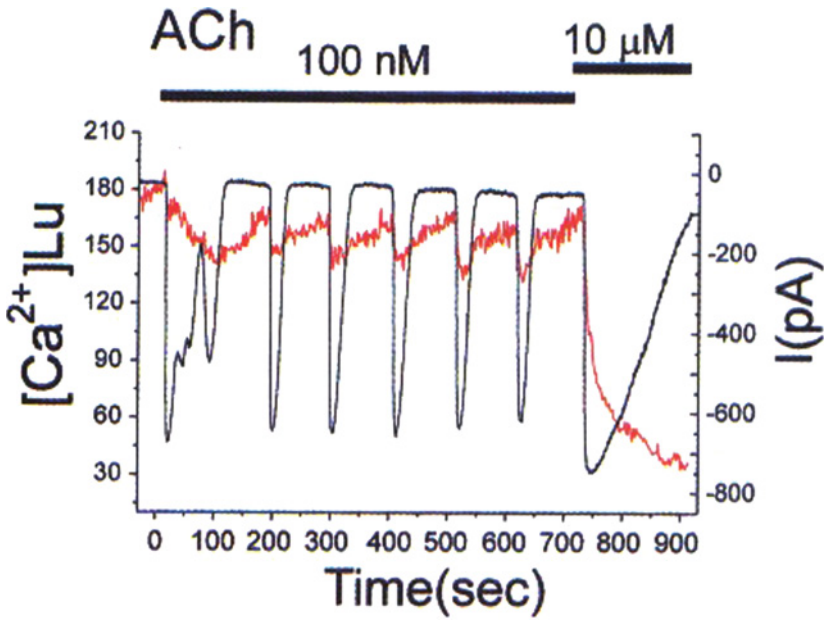
Module 6: | Figure Ca^{2+} oscillation model



A model to explain agonist-dependent Ca^{2+} oscillations.

The emphasis of this model is that oscillations depend upon the accumulation of Ca^{2+} within the lumen of the endoplasmic reticulum (ER), which then sensitizes the inositol 1,4,5-trisphosphate ($InsP_3$) receptors to initiate each spike. The site sensitive to luminal Ca^{2+} is located close to the Ca^{2+} gate (Module 3: Figure $InsP_3R$ activation). Oscillation frequency is thus determined by the rate at which the lumen is loaded with Ca^{2+}, which in turn is determined by the agonist-dependent rate of Ca^{2+} entry across the plasma membrane. The sequences of events that occur during a typical oscillatory cycle are described in more detail in the text. An animated version of this figure is available.

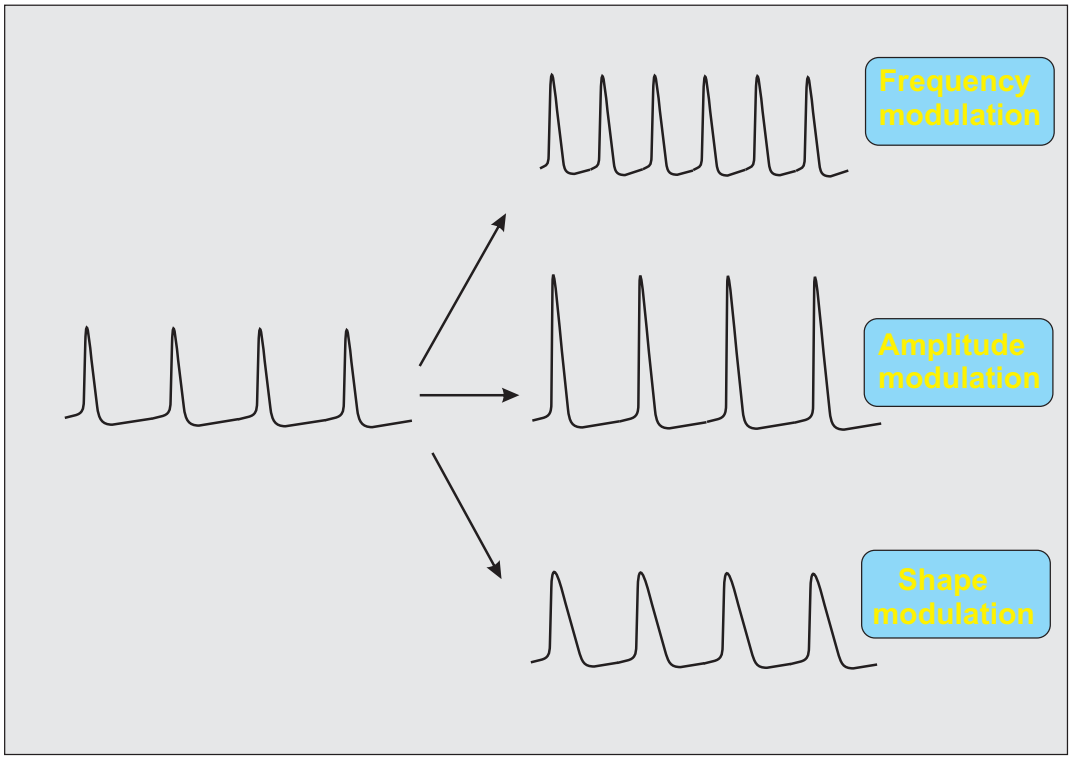
Module 6: | Figure pancreatic Ca^{2+} oscillations



Oscillations of Ca^{2+} in the endoplasmic reticulum (ER) lumen of pancreatic acinar cells.

The Ca^{2+} oscillations were recorded indirectly by monitoring the Cl^- current (I), which is the black trace and is inverted. Each downward deflection reflects an increase in the intracellular level of Ca^{2+}. Upon addition of 100 nM acetylcholine (ACh), the level of Ca^{2+} began to oscillate. Note how each Ca^{2+} transient resulted in a small decrease in the level of Ca^{2+} within the lumen of the endoplasmic reticulum (ER) (red trace). It is clear that each transient caused a small depletion, because upon addition of a larger concentration of Ach, there was a much larger release of Ca^{2+} and a corresponding large fall in the level of luminal Ca^{2+} ($[Ca^{2+}]Lu$). Reproduced by permission from Macmillan Publishers Ltd: *EMBO J.* Park, M.K., Petersen, O.H. and Tepikin, A.V. (2000) The endoplasmic reticulum as one continuous Ca^{2+} pool: visualization of rapid Ca^{2+} movements and equilibration. 19:5729–5739. Copyright (2000); http://www.embojournal.org; see Park et al. 2000).

Module 6: | Figure encoding oscillatory information



Different modes of information encoding Ca^{2+} oscillations.
There appear to be three main mechanisms for encoding information:

- Frequency modulation (FM). One of the important features of many Ca^{2+} oscillations is that frequency varies with agonist concentration, indicating that cells may employ FM as a mechanism for encoding information.
- Amplitude modulation (AM). Another way of encoding information is to vary the amplitude of the Ca^{2+} transients.
- Shape modulation. Information may also be included in the width of the individual transients.

some equilibrium processes, and the small changes are then integrated over time to provide a significant change in some cellular process (Module 6: Figure decoding oscillatory information). There are a number of processes that might operate to decode oscillatory information through integrative tracking. The one that has attracted most attention is Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), which has biochemical properties that are well suited to function in frequency decoding (Module 4: Figure CaMKII activation). The holoenzyme contains 12 identical subunits all capable of being activated by Ca^{2+}, which means that the enzyme can function as a Ca^{2+} transient counter. This function is made more sophisticated by the fact that these subunits can become autonomously active. There already is evidence that the degree of autonomous activity is related to the frequency of Ca^{2+} spiking. In dorsal root ganglion (DRG) neurons, CaMKII was able to decode frequencies between 0.1 and 1 Hz, but other mechanisms seem to take over at higher frequencies.

CaMKII thus seems to be well designed to decode information contained in Ca^{2+} oscillations.

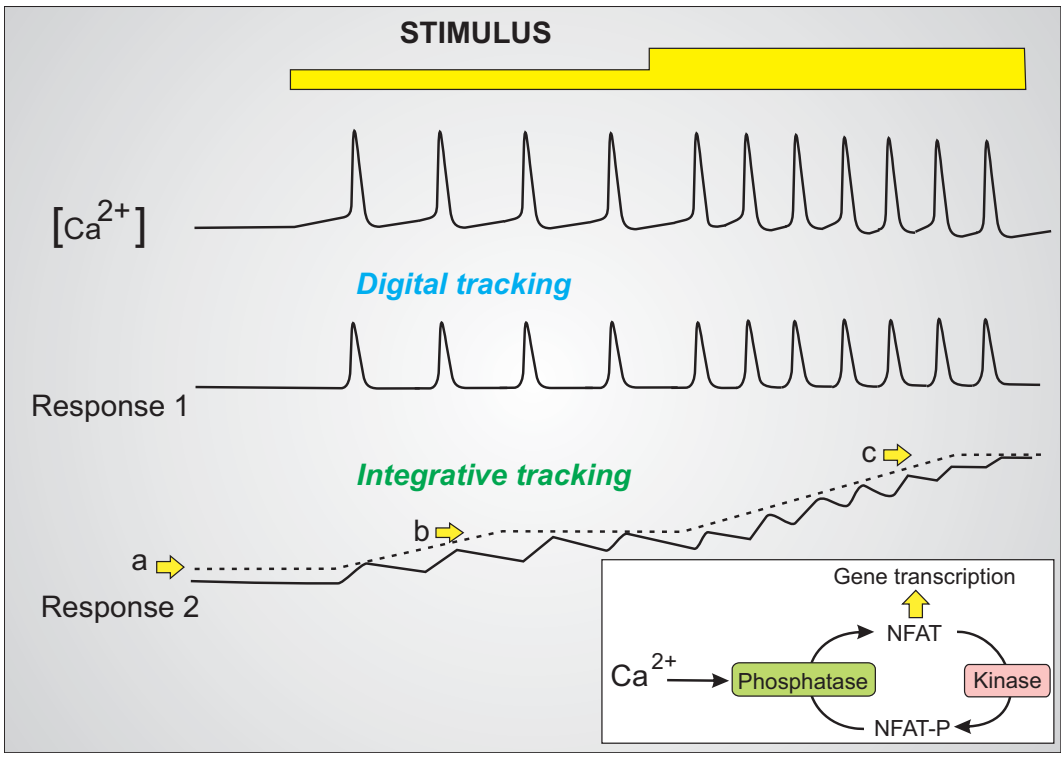
Another example of integrative tracking is the nuclear factor of activated T cells (NFAT) shuttle (Module 4: Figure NFAT activation). The basis of this shuttle is that NFAT is imported into the nucleus in response to an increase in Ca^{2+} and is exported back into the cytoplasm when Ca^{2+} returns to its resting level (Module 4: Figure NFAT translocation). In this experiment, the cell was subjected to a prolonged elevation to Ca^{2+}. However, it is more interesting to see what happens when the cells are subjected to the more typical oscillatory Ca^{2+} transients delivered at different frequencies (Module 6: Figure NFAT nuclear translocation). Notice how the translocation response to each frequency takes time to reach a new equilibrium, exactly in line with a mechanism of integrative tracking. Another example where NFAT translocation is sensitive to Ca^{2+} oscillation frequency has been described in skeletal muscle fibres (Module 8: Figure nuclear import of NFAT).

The cardiac nuclear factor of activated T cells (NFAT) shuttle is particularly important during Ca^{2+} signalling in cardiac hypertrophy (Module 12: Figure hypertrophy signalling mechanisms).

Cyclic AMP oscillations

It has been known for some time that chemotaxis in the slime mould *Dictyostelium* is driven by oscillations in the level of cyclic AMP. Recently, such cyclic AMP oscillations have been recorded in the embryonic neurons of *Xenopus* and in mammalian insulin-secreting β-cells. In the latter case, the onset of these oscillations was induced by either glucagon or glucagon-like peptide-1 (GLP-1). When Ca^{2+}

Module 6: | Figure decoding oscillatory information



Different modes of information decoding of Ca^{2+} oscillations.

There are two main ways of decoding information contained in Ca^{2+} oscillations. 1. Digital tracking. In the case of Response 1, there is a close correspondence between the Ca^{2+} transient and the downstream effector system. Examples of digital tracking occur in contractile cells and nerve terminals, where each Ca^{2+} transient triggers an all-or-none response. 2. Integrative tracking. In Response 2, each transient has a small effect on some dynamic processes that can adopt different equilibrium positions, as indicated by the broken line and yellow arrows. a. The resting position. b. At low frequencies, the process moves through a series of steps to a new equilibrium. c. The equilibrium moves to a higher level as the frequency is increased still further. The nuclear factor of activated T cells (NFAT) shuttle (see inset at the bottom) is an example of a process that might function in such integrative tracking (see Module 6: Figure NFAT nuclear translocation).

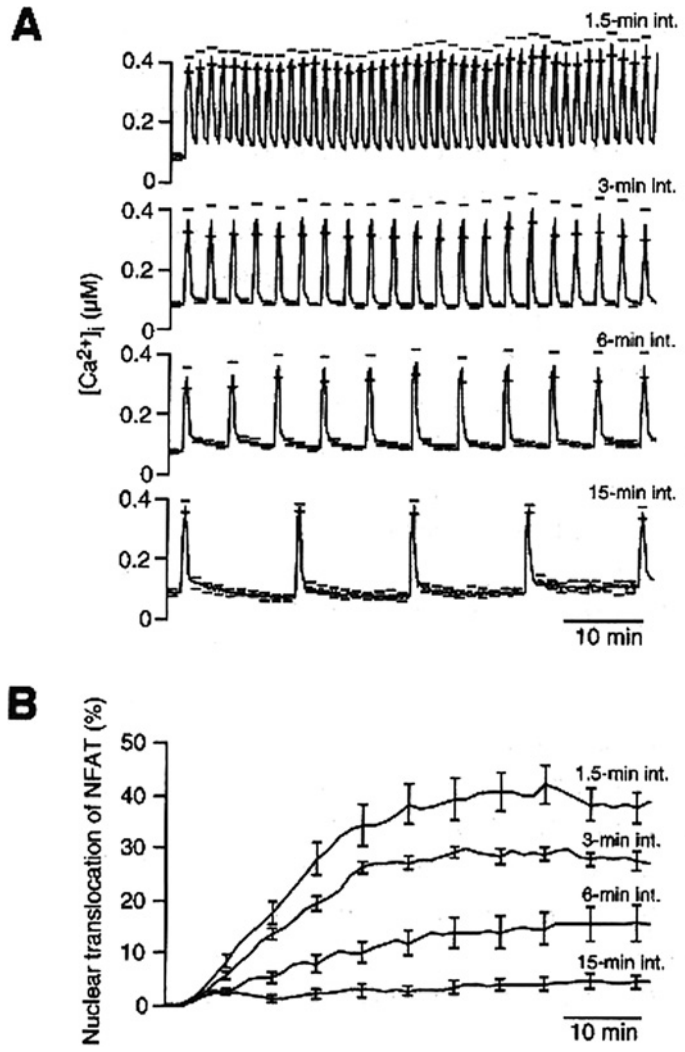
was monitored together with cyclic AMP, these two messengers were found to oscillate in synchrony with each other. An interesting feature of the cyclic AMP oscillations is that they were totally dependent on the presence of external Ca^{2+}. The oscillations induced by GLP-1 ceased when Ca^{2+} was removed from the bathing medium and promptly returned when Ca^{2+} was restored. It therefore seems that the Ca^{2+} and cyclic AMP signalling systems interact with each other to produce these co-ordinated oscillations.

It has been proposed that cyclic AMP oscillations could play a role in encoding information to control different cellular processes. Some evidence for this has come from the translocation of protein kinase A (PKA) into the nucleus when cyclic AMP was presented either as an oscillation or as a prolonged plateau. Only the latter was able to induce translocation. Oscillations in cyclic AMP may act to control processes within the cytoplasm, whereas prolonged elevation is required to control nuclear processes such as gene transcription.

Circadian clock

The name circadian is derived from two Latin words *circa* (about) and *dies* (day), which refers to a cycle that occurs once every day. We are probably most aware of this daily rhythm through our sleep/wake cycle, but there are many other aspects of our physiology that are controlled by our circadian clock. It controls the timing of the cell cycle and it can regulate the release of haematopoietic stem cells (HSCs) from the bone marrow (see Step 1 in Module 8: Figure bone marrow). It regulates many other physiological functions such as the cardiovascular system, body temperature, renal plasma flow, liver metabolism and detoxification. It also tracks the annual light cycle to provide information to control cycles of reproduction and hibernation. Ideas about the location of the circadian clock have undergone a major revision in the last few years. Originally it was thought that the clock was located in the suprachiasmatic nucleus (SCN), but recently it was discovered that almost all cells in the body have circadian clocks, and these peripheral clocks are then synchronized by the master clock in the SCN. The circadian clock molecular mechanism appears to be the same for both the central SCN and peripheral clocks; it is based on complex feedback interactions operating between gene transcription and protein expression that take approximately 24 h to complete each cycle. Processes of circadian clock synchronization and entrainment play an important role in ensuring that the autonomous clock mechanism in each cell is synchronized to ensure that there is a uniform sharp

Module 6: | Figure NFAT nuclear translocation



The effect of Ca^{2+} oscillation frequency on the nuclear translocation of nuclear factor of activated T cells (NFAT).

(A) Recordings of the Ca^{2+} oscillations that were used to study the nuclear translocation of nuclear factor of activated T cells (NFAT). (B) When these oscillations were applied to cells, the low-frequency oscillations had little effect, but translocation increased progressively as oscillation frequency was increased. The way in which NFAT translocation was measured is described in Module 4: Figure NFAT translocation. 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. 22:3825–3832. Copyright (2003); http://www.embojournal.org; see Tomida et al. 2003.

circadian clock output signal. There are various circadian clock entrainment mechanisms that are responsible for carrying out the processes of synchronization that occurs at two levels. Firstly, the central SCN clock must entrain to the light/dark cycle. Secondly, the individual cells must synchronize themselves with each other.

Circadian clock location

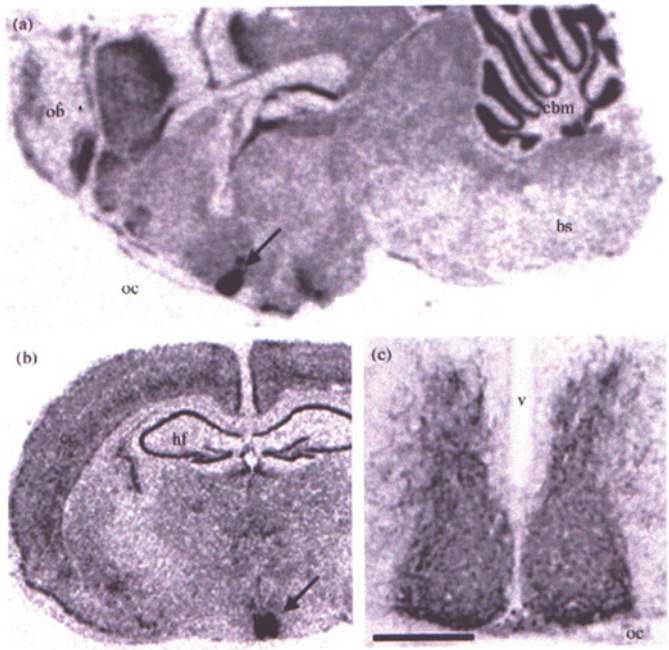
Most cells in the body contain a circadian clock. Most attention has focused on the suprachiasmatic nucleus (SCN) circadian clock, but much interest is beginning to focus on the peripheral circadian clocks.

Suprachiasmatic nucleus (SCN) circadian clock

The primary circadian clock is located in the anterior hypothalamus in the suprachiasmatic nuclei (SCN), which are paired structures each containing about 10000 neurons (Module 6: Figure suprachiasmatic nucleus). The SCN lies in the ventral region of the hypothalamus at the bottom of the brain, just above the optic chiasm, where they are ideally situated to receive information about the day/night light cycle. This information comes in from the retina passing down the retinohypothalamic tract (RHT) that terminates on the ventral core SCN neurons (Module 6: Figure circadian clock location).

The SCN contains the clock neurons responsible for setting up the diurnal rhythm. However, these SCN neurons are not homogeneous, but are divided into two main groups of neurons, the ventral core SCN neurons and the dorsal shell SCN neurons, which carry out different functions that are co-ordinated to produce a stable circadian oscillation. The ventral core neurons receive most of the photic stimuli coming in from the eyes. The intrinsically photoreceptive ganglion cells in the retina send out axons along the RHT to innervate the ventral core neurons. They release the neurotransmitter glutamate and the

Module 6: | Figure suprachiasmatic nucleus



Location of the suprachiasmatic nucleus (SCN) in the hypothalamic region of the brain.
The location of the suprachiasmatic nucleus (SCN) in mouse (arrows in a and b) was identified using autoradiography to detect radioactive labelled mRNA transcribed from the clock gene *Per*. (a) In the parasagittal section, the arrow points to the SCN located above the optic chiasm (oc). (b) In this coronal section, the paired SCN are clearly located at the bottom of the brain. (c) In this higher magnification, the SCN was stained with an antibody directed against vasoactive intestinal peptide (VIP), which is produced by the ventral core neurons. The latter are mainly confined to the ventral region close to the optic chiasm (oc), but they send out axons to innervate the dorsal shell neurons, as illustrated diagrammatically in Module 6: Figure circadian clock location. Reproduced from *Mutat. Res.*, Vol. 574, Reddy, A.B., Wong, G.K.Y., O'Neill, J., Maywood, E.S. and Hastings, M.H., Circadian clocks: neural and peripheral pacemakers that impact upon the cell division cycle, pp. 76–91. Copyright (2005), with permission from Elsevier; see Reddy et al. 2005.

neuromodulator pituitary adenylyl cyclase-activating peptide (PCAP). The glutamate and PCAP are mainly responsible for the circadian clock synchronization and entrainment mechanisms that adjust the circadian clock of the core neurons to the light/dark cycle.

The dorsal shell SCN neurons receive less innervation from the RHT, but it does receive input from the core neurons. The axons of these core neurons release γ-aminobutyric acid (GABA), vasoactive intestinal peptide (VIP) and substance P (SP), and it is these peptides that are responsible for synchronizing the circadian clocks of the dorsal shell neurons. The dorsal shell neurons have the main circadian clock responsible for the output signals that leave the SCN. They express various transmitters, such as vasopressin, which is one of the main output signals from the SCN (red arrow in Module 6: Figure circadian clock location).

In summary, both regions of the SCN contain clock neurons, but with subtly different properties. The core neurons have an oscillator that is uniquely responsive to photic stimuli and is responsible for entraining the oscillator in the shell neurons that relay information out to the rest of the body using both endocrine and neural signals. Despite these different entrainment mechanisms, both sets of neurons have the same circadian clock molecular mechanism.

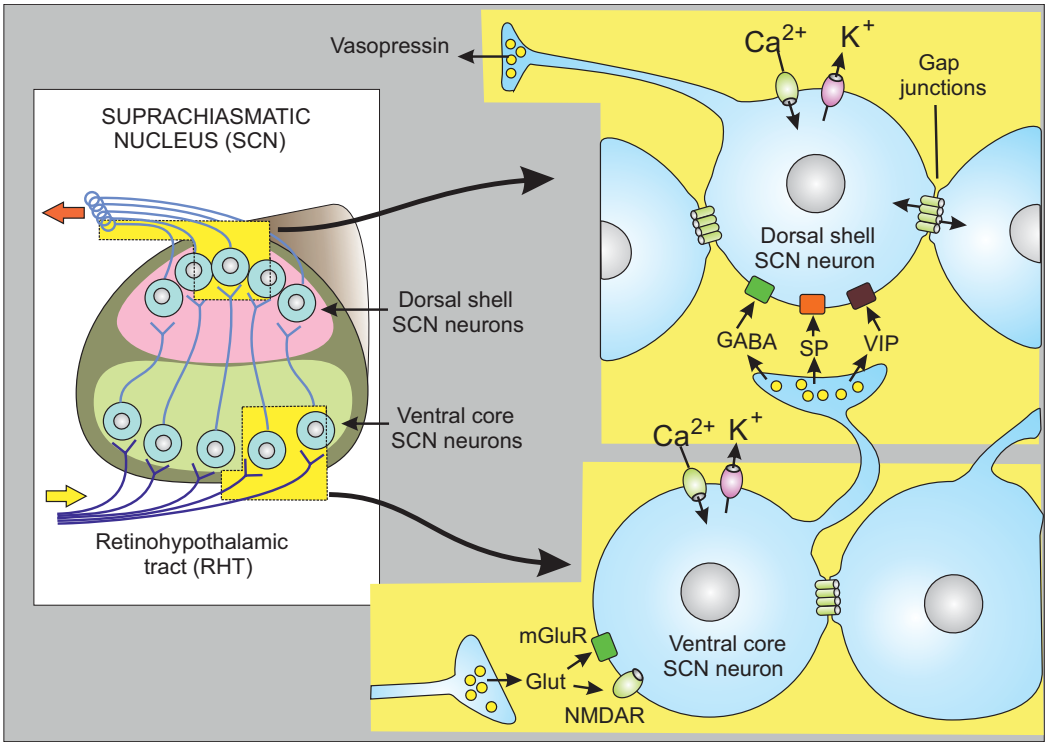
Peripheral circadian clocks

Most cells in the body contains a robust circadian clock that is dedicated to controlling the activity of specific cellular processes so that they remain in synchrony with those being carried out elsewhere in the body. It was originally thought that these diurnal rhythms in peripheral cells were controlled by information coming from the suprachiasmatic nucleus (SCN) circadian clock. Now it seems that the function of the central clock in the brain is to synchronize the activity of the peripheral clocks. The circadian clock molecular mechanism for these peripheral clocks is the same as that found in the SCN.

The most interesting aspect of these peripheral clocks is how they act to control different cellular functions:

- In the case of osteoblasts, the stimulation of proliferation by activating β_2-adrenergic receptors depends on the activation of transcription factors such as activating protein 1 (AP-1), cyclic AMP response element-binding protein (CREB) and Myc. However, this activation is severely reduced by the simultaneous activation of the *Per* gene by CREB and the expression of PER then feeds back to reduce the level of proliferation. This is an example of how the circadian clock can act to entrain the cell cycle.
- Another example has been described during liver regeneration where the proliferating cells enter mitosis

Module 6: | Figure circadian clock location



Location of the circadian clocks in the suprachiasmatic nucleus (SCN).
The clock neurons within the suprachiasmatic nucleus (SCN) are not homogenous, but fall into two main groups. There are ventral core neurons that receive most of the input (yellow arrow) from the retina that travels along the retinohypothalamic tract (RHT). These neurons send out projections to the dorsal shell SCN neurons, which are mainly responsible for the output signals (red arrow) that leave the SCN. The insets on the right illustrate a simplified version of the neural circuit within the SCN. The RHT neurons release glutamate that acts through the *N*-methyl-D-aspartate receptor (NMDAR) and metabotropic glutamate receptor 1 (mGluR1) on the core neurons. The latter express a number of transmitters such as γ-aminobutyric acid (GABA), vasoactive intestinal peptide (VIP) and substance P (SP), which act on the dorsal shell neurons. These neurons express vasopressin, which is one of the output signals they release from the neurons that leave the SCN. Both the core and shell neurons have Ca^{2+} and K^+ channels that generate the electrical activity responsible for releasing these neurotransmitters. The ventral neurons stained with an antibody against VIP are shown in panel c in Module 6: Figure suprachiasmatic nucleus.

at the same time during the day. The ability of the circadian clock to regulate the cell cycle seems to depend on the ability of the BMAL1/CLOCK heterodimer to stimulate the expression of Wee1. When the level of Wee1 is high, progress through the G_2/M boundary is inhibited (Module 9: Figure mitotic entry). When the level of BMAL1 declines late in the day, the Wee1 level will also fall and cells will be able to enter mitosis.

Circadian clock molecular mechanism

The circadian clock mechanism is responsible for setting up the diurnal oscillation with a periodicity of approximately 24 h. The remarkable feature of this autonomous oscillator is its ability to keep time even when cells are isolated from any obvious external input. For example, if neurons are dispersed into a culture dish, they will continue to produce bursts of action potentials that appear with a 24 h frequency. While such isolated cells start off roughly in synchrony, with time they lose the precise 24 h frequency and drift apart to become highly asynchronous. In considering this circadian oscillator, it is necessary to understand not only the nature of the endogenous circadian clock mechanism, but also how this mechanism is synchronized in the cell population.

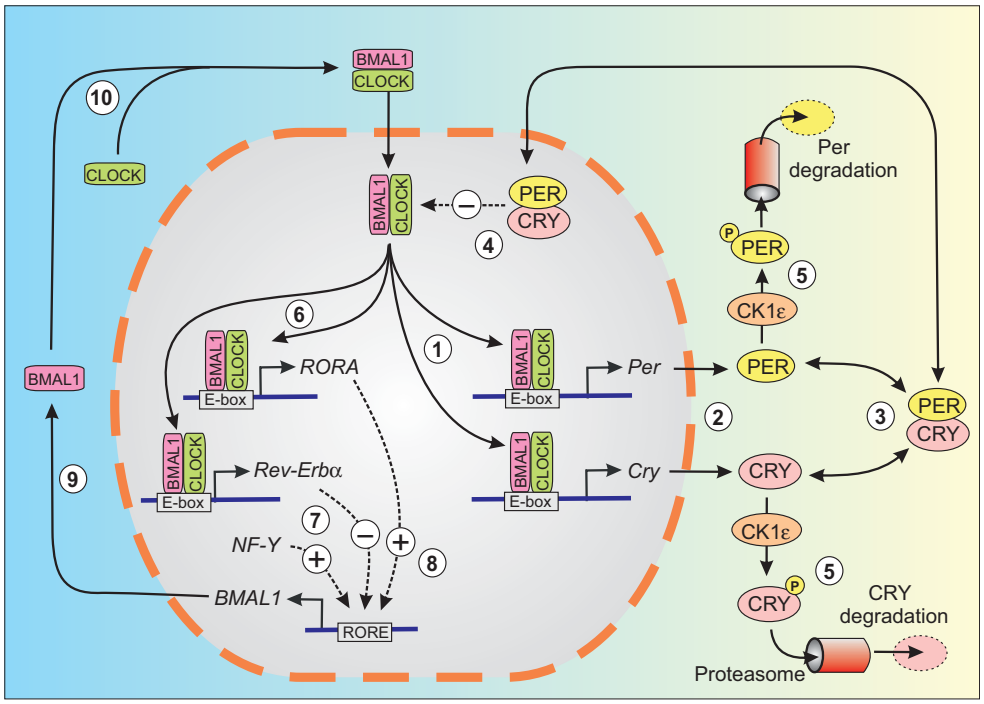
The circadian clock depends on the operation of clock genes (Module 6: Table circadian clock gene toolkit). The clock mechanism depends upon these clock genes being linked together through transcription/translation feedback loops, which have both positive and negative components (Module 6: Figure circadian clock molecular mechanism). In effect, there are two interacting feedback loops: the PER regulatory loop and the BMAL1 regulatory loop. These two loops are tied together because BMAL1 switches on PER, whereas PER switches off BMAL1. BMAL1 acts as a transcription factor, which functions in combination with CLOCK, which is constitutively expressed in cells. CLOCK has histone acetyltransferase (HAT) activity that not only enables it to carry out the protein acetylation of histones but it can also acetylate its partner BMAL1 on a highly conserved Lys-537. This acetyl group facilitates the ability of BMAL1 to interact with the repressor CRY (see Step 4 in Module 6: Figure circadian clock molecular mechanism). The nuclear receptor co-repressor 1 (N-CoR1), which associates with histone deacetylase 3 (HDAC3), also plays an important role in regulating the operation of the circadian clock.

Module 6: | Table circadian clock gene toolkit

Summary of the circadian clock genes that contribute to the molecular mechanism of the circadian oscillator.

Circadian clock gene	Comment
Per1	Period 1
Per2	Period 2
CRY1	Cryptochrome 1
CRY2	Cryptochrome 2
CLOCK	A basic helix–loop–helix factor that dimerizes with BMAL1
BMAL1	Brain and muscle Arnt-like protein 1; a basic helix–loop–helix factor that dimerizes with CLOCK
Rev-ERBα	?
RORA	Retinoic acid-related orphan receptor

Module 6: | Figure circadian clock molecular mechanism



Molecular mechanisms responsible for the driving the circadian clock.

The circadian clock depends upon a series of feedback loops that couple together the transcription and expression of the different clock genes. On the right is the regulatory feedback system that controls the expression and function of the *Per* and *Cry* genes (Steps 1–5). On the left are the regulatory loops that control the action of the *BMAL1* gene (Steps 5–10). The BMAL1/CLOCK heterodimer binds to the E-box enhancer sequence CACGTG. See the text for details of these two regulatory loops.

Another important component of the molecular mechanism is the transport of proteins in and out of the nucleus and their regulated degradation. The best way to understand the operation of the clock is to consider the operation of the two regulatory loops and how they are tied together through positive- and negative-feedback interactions.

PER regulatory loop

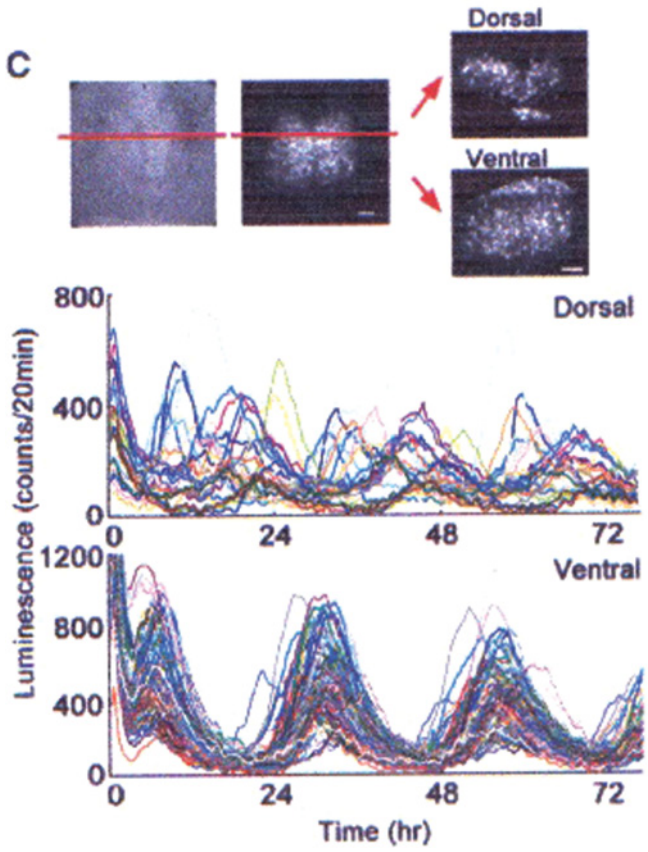
The PER regulatory loop consists of Steps 1–5 in Module 6: Figure circadian clock molecular mechanism:

1. The regulatory loop begins when the BMAL1/CLOCK heterodimer binds to the E-box sequences on the promoter regions of the *Per* and *Cry* genes to induce their transcription. This transcriptional activity depends on the histone acetyltransferase (HAT) activity of CLOCK, which acetylates both BMAL1 and histones H3 and H4.
2. Translation of the genes into PER and CRY result in an increase in their levels within the cytoplasm.
3. The two clock components combine to form a PER/CRY heterodimer, which translocates into the nucleus.
4. The PER/CRY heterodimer then inhibits the transcriptional activity of BMAL1, thus preventing further transcription of PER and CRY. This inhibition of transcription is enhanced by the histone deacetylase (HDAC) SIRT1.
5. These two clock components are removed by a degradation pathway that begins with their phosphorylation by casein kinase Iε (CKIε), which then marks them for ubiquitination and degradation by the proteasome. There is a rapid elevation of PER early in the light phase with a gradual decline to a low level that is maintained during the dark phase. This PER regulatory loop is driven by the BMAL1 regulatory loop.

BMAL1 regulatory loop

The BMAL1 regulatory loop consists of Steps 6–10 in Module 6: Figure circadian clock molecular mechanism:

Module 6: | Figure SCN clock synchronization



Synchronization of the circadian clocks in suprachiasmatic nucleus (SCN) dorsal and ventral neurons.
The expression of the *Per* gene was studied in both the dorsal and ventral region that were separated by a surgical incision. Cells in the ventral region retained a remarkable degree of synchronicity, whereas this was lost in the dorsal cells. Reproduced with permission from Yamaguchi, S., Isejima, H., Matsuo, T., Okura, R., Yagita, K., Kobayashi, M. and Okamura, H. (2003) Synchronization of cellular clocks in the suprachiasmatic nucleus. Science 302:1408–1412. Copyright (2003) American Association for the Advancement of Science; see Yamaguchi et al. 2003.

6. The increase in the BMAL1/CLOCK heterodimer, which initiates the PER regulatory loop (Step 1) also activates other E-box genes such as RORA and Rev-ERBα that play a critical role in regulating the expression of BMAL1.
7. The Rev-ERBα is produced quickly and acts to inhibit the transcription of BMAL, which will effectively reduce the formation of BMAL1 and thus curtail its transcription during the latter parts of the light phase. The Rev-ERBα is removed during the dark phase, thus enabling the cycle to start again. This increased activation of BMAL1 is enhanced by the nuclear transcription factor Y (NF-Y).
8. The RORA gene product is longer-lived than Rev-ERBα, which means that when the latter is degraded during the dark phase, the RORA can begin to activate the expression of BMAL1 towards the end of the light phase.
9. The level of BMAL1 begins to rise during the transition from the dark to light phase.
10. BMAL1 interacts with CLOCK to form the BMAL1/CLOCK heterodimer, which starts the whole process off again (i.e. it once again initiates Step 1 of the PER regulatory cycle). The acetylation of BMAL1 by CLOCK facilitates its interaction with CRY.

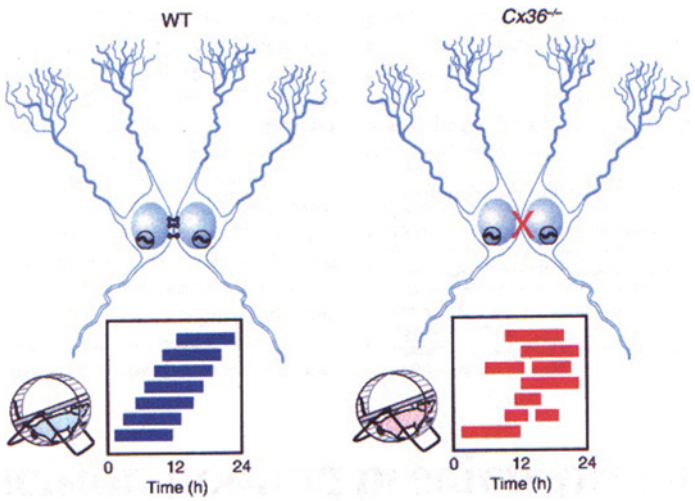
Mutation of casein kinase Iε (CKIε), which results in a decrease in the ability of this kinase to phosphorylate the PER proteins of the circadian clock, is responsible for familial advanced sleep phase syndrome (FASPS).

An important feature of the clock mechanism is the circadian clock synchronization and entrainment process that ensures that all the individual clocks in each suprachiasmatic nucleus (SCN) neuron operate in phase with each other.

Circadian clock synchronization and entrainment

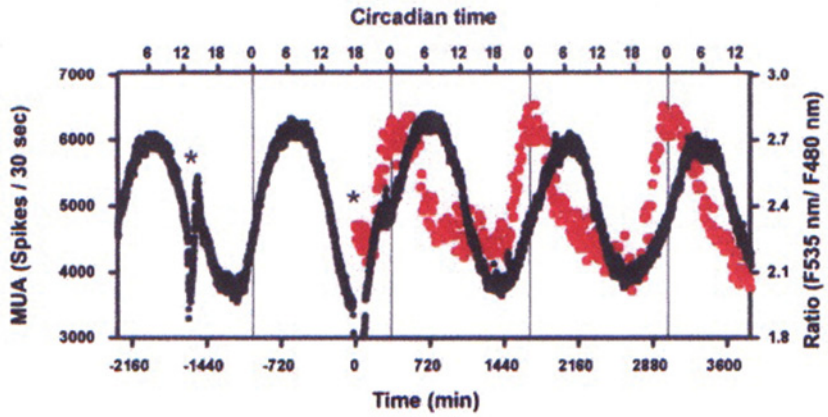
Synchronization of the individual clocks in the suprachiasmatic nucleus seems to be carried out by two separate intercellular mechanisms: one based on chemical synaptic transmission operating mainly through γ-aminobutyric acid (GABA), the other through direct communication through gap junctions (Module 6: Figure circadian clock location). The role of synaptic transmission as a synchronization mechanism varies with the two regions of

Module 6: | Figure activity rhythms in Cx36$^{-/-}$ mice



Suprachiasmatic nucleus (SCN) neuronal synchronization is lost in mice deficient in the gap junction component Cx36.
Coupling between wild-type (WT) neurons maintains a regular diurnal rhythm, as measured by wheel-running activity as shown in blue at the bottom. Each bar represents the activity over a 24 h period, with each plotted from the top to the bottom. In the Cx36$^{-/-}$ mice, neuronal coupling is lost and the diurnal rhythm (shown in red) becomes much more chaotic. Reproduced by permission from Macmillan Publishers Ltd: *Nat. Neurosci.*, Colwell, C.S. (2005) Bridging the gap: coupling single-cell oscillators in the suprachiasmatic nucleus. 8:10–12. Copyright (2005); http://www.nature.com/neuro; see Colwell 2005.

Module 6: | Figure SCN cytosolic Ca^{2+} oscillation

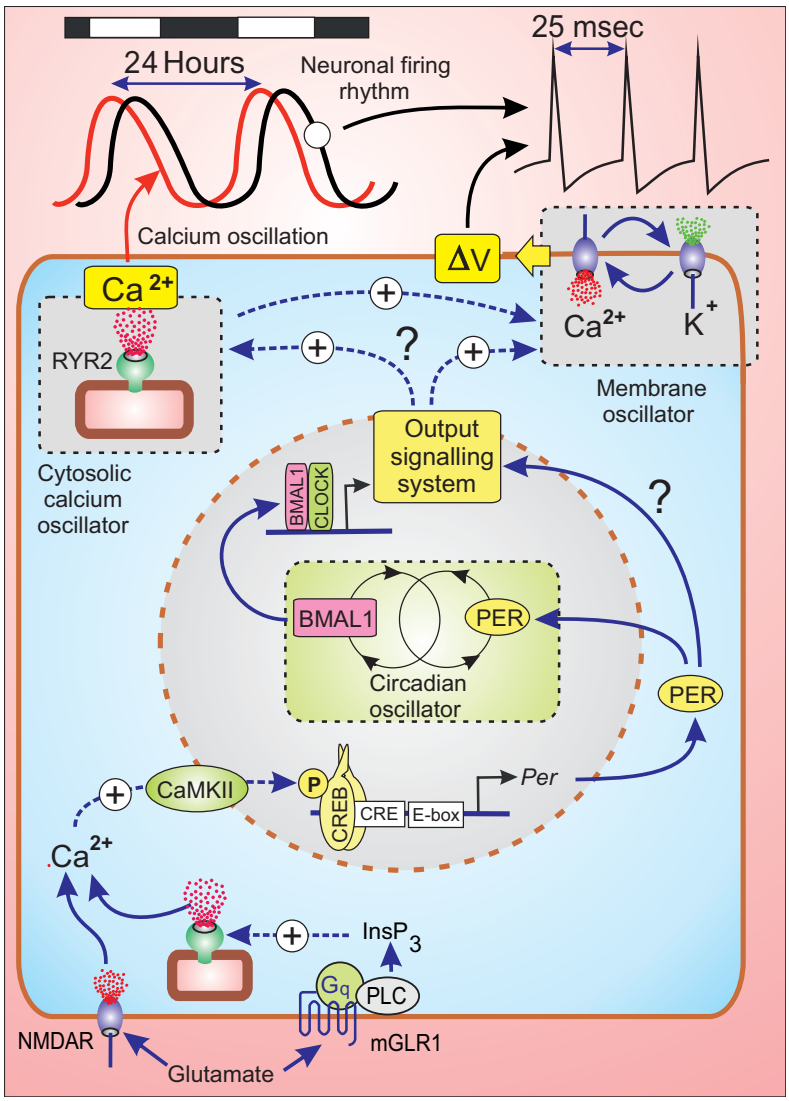


Simultaneous recording of Ca^{2+} oscillations and neural electrical activity rhythms in suprachiasmatic nucleus (SCN) neurons.
A Ca^{2+}-sensitive fluorescent protein was used to monitor the intracellular level of Ca^{2+} (red dots), while electrical activity was recorded using a microelectrode array (black trace). The two responses had a similar wave form, but the Ca^{2+} trace was advanced by about 4 h. Reproduced from *Neuron*, Vol. 38, Ikeda, M., Sugiyama, T., Wallace, C.S., Gomp, H.S., Yoshioka, T., Miyawaki, A. and Allen, C.N., Circadian dynamics of cytosolic and nuclear Ca^{2+} in single suprachiasmatic nucleus neurons, pp. 253–263. Copyright (2003), with permission from Elsevier; see Ikeda et al. 2003.

the suprachiasmatic nucleus (SCN). The ventral core SCN neurons are synchronized/entrained by the photic stimuli coming from the retinal along the retinohypothalamic tract (RHT). An extreme form of entrainment occurs during phase resetting when a period of light is given during the dark phase. This pulse of light produces a rapid increase in the expression of *Per*, which is important in resetting the clock. The question that therefore emerges is how does the photic signal produce the sudden expression of clock genes such as *Per*?One suggestion is that the RHT input releases neurotransmitters such as glutamate that act on the ventral neurons to stimulate signalling pathways such as an increase in Ca^{2+}, which then activates transcription factors such as activating protein 1 (AP-1) and cyclic AMP response element-binding protein (CREB) to initiate the expression of the clock genes (Module 6: Figure circadian clock input–output signals).

Light induces phase delays of the circadian clock, which may be regulated by release of Ca^{2+} from ryanodine receptors (RYRs). On the other hand, release of Ca^{2+} by inositol 1,4,5-trisphosphate receptors ($InsP_3Rs$) has been implicated in glutamate-induced phase delay. Therefore it seems that Ca^{2+} release from internal stores can play some role in the entrainment of the circadian clock. The ability of Ca^{2+} to phase-shift the clock depends on Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII),

Module 6: | Figure circadian clock input–output signals



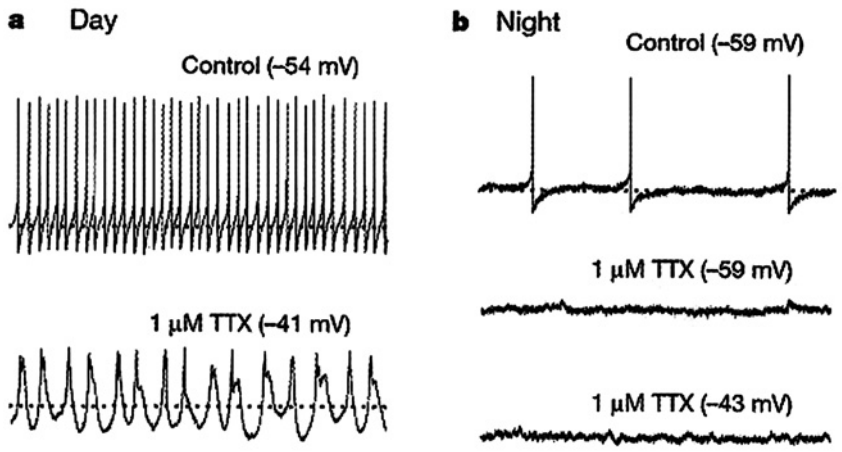
The proposed function of cell signalling pathways in mediating the input and output signals of the circadian clock.
The nucleus contains the circadian oscillator, which sets up fluctuations in the level of the clock components PER and BMAL1 (see Module 6: Figure circadian clock molecular mechanism for details. The activity of this circadian oscillator can be modulated by various input signals, such as the neurotransmitter glutamate, which can generate Ca^{2+} signals to activate *Per* transcription using the transcription factor cyclic AMP response element-binding protein (CREB). In addition, the circadian oscillator must communicate with the outside world. How this is done is still a mystery. Components of the clock, such as PER and BMAL1, may activate various output signals that may act by switching on various oscillatory systems, such as cytosolic Ca^{2+} oscillator that produces a Ca^{2+} oscillation with a period of 24 h (red trace). In addition, there is a much faster membrane oscillator that initiates the action potentials that make up the neuronal firing rhythm (black trace) that lags behind the Ca^{2+} oscillation by about 4 h.

which activates CREB to control the transcription of *Per1* and/or *Per2*. Just how the increase in PER induces a phase change is still somewhat of a mystery. The ventral cells can respond very quickly to such photic stimuli and they then have to synchronize the dorsal neurons.

These dorsal neurons are synchronized by an intercellular communication network based on various neurotransmitters, such as GABA and vasoactive intestinal peptide (VIP). When the ventral core neurons become active during the light phase, they begin to release GABA and VIP to entrain the activity of the dorsal neurons, thus synchronizing the activity of both sets of neurons. When the dorsal region was severed from the ventral region, the latter was able to maintain its synchronicity, but this was lost in the dorsal region (Module 6: Figure SCN clock synchronization).

The other synchronization mechanism seems to depend on having intact gap junctions to provide a direct avenue of communication between the SCN neurons (Module 6: Figure circadian clock location). The nature of the information that is passed from cell to cell is likely to be the passage of electrical current, but it is possible that low-molecular-mass messengers such as cyclic AMP or $InsP_3$ may also be passed from cell to cell to synchronize their rhythms. It is apparent that the permeability of the gap junctions may vary during the light/dark cycle. They are maximally open during the peak of the light response, when neural activity is at its peak. The VIP released by the ventral core neur-

Module 6: | Figure firing rates of SCN neurons



Firing rate of suprachiasmatic nucleus (SCN) neurons recorded during either the day or night.
Suprachiasmatic nucleus (SCN) neurons prepared from rats during the day had a lower membrane potential than that found for neurons during the night. The former also had a much higher frequency of spontaneous action potentials (4 Hz) than those recorded during the night phase (0.4 Hz). When cells were treated with tetrodotoxin (TTX), action potentials disappeared in both cases. However, the day cells were left with a slow oscillation of membrane potential which had a frequency similar to that recorded for the action potentials. The nature of this membrane oscillator may provide clues about how the circadian oscillator communicates with the plasma membrane, as discussed in the text. Reproduced by permission from Macmillan Publishers Ltd: *Nature*, Pennartz, C.M.A., de Jou, M.T.G., Bos, N.P.A., Schaap, J. and Geurtsen, A.M.S. (2002). Diurnal modulation of pacemaker potentials and calcium current in the mammalian circadian clock. 416:286–290. Copyright (2002); http://www.nature.com; see Pennartz et al. 2002.

ons seems to be critical for opening up the gap junctions during this activity phase.

The importance of such intercellular communication through gap junctions is evident in transgenic mice where Cx36 has been deleted (Module 6: Figure activity rhythms in $Cx36^{-/-}$ mice).

The critical function of this circadian clock synchronization is that all the clocks function together to produce the distinct circadian clock output signals at the same time during each light/dark cycle.

Circadian clock output signals

In order for the circadian clock in the suprachiasmatic nucleus (SCN) to orchestrate processes in the rest of the brain and periphery it has to transmit output signals. Most of these come from the dorsal shell neurons (red arrow in Module 6: Figure circadian clock location). These output signals seem to depend upon an increase in the electrical activity of the SCN neurons as they begin to increase the frequency of their action potentials during the light phase. The problem to try to understand is how the circadian oscillator communicates with the plasma membrane to alter its properties to begin to generate action potentials during the day. This is a difficult problem that has still not been properly solved. A working hypothesis is developed in Module 6: Figure circadian clock input–output signals that attempts to pull together some recent observations. The first point to make is that the output signals may be linked to an suprachiasmatic nucleus (SCN) cytosolic Ca^{2+} oscillator and an suprachiasmatic nucleus (SCN) membrane oscillator. The hypothesis is that these two oscillators are activated in some way by the circadian oscillator. The most likely possibility is that components of the clock mechanism that appear in the cytoplasm, such as PER and BMAL1, are responsible for stimulating an output signalling system that results in the activation of these two oscillators (Module 6: Figure circadian clock input–output signals).

Suprachiasmatic nucleus (SCN) cytosolic Ca^{2+} oscillator

A cytosolic Ca^{2+} oscillation has been recorded in mouse suprachiasmatic nucleus (SCN) neurons that have a frequency of 24 h. The neural activity recorded by microelectrode arrays had a very similar wave form, but appeared about 4 h after the Ca^{2+} oscillation (Module 6: Figure SCN cytosolic Ca^{2+} oscillation). This Ca^{2+} oscillation was not altered by tetrodotoxin (TTX) or nimodipine, which completely suppress the rhythm of electrical activity. Since it was suppressed by ryanodine, it is suggested that the Ca^{2+} oscillation may be caused by the periodic release of Ca^{2+} by ryanodine receptors (RYRs) on the internal stores. Since the Ca^{2+} oscillation precedes the electrical rhythm, it is possible that it may alter some parameter in the membrane that is responsible for driving the suprachiasmatic nucleus (SCN) membrane oscillator.

Suprachiasmatic nucleus (SCN) membrane oscillator

A characteristic feature of the suprachiasmatic nucleus (SCN) neurons is that they display a diurnal neuronal firing rhythm of electrical activity (the black curve in Module 6: Figure SCN cytosolic Ca^{2+} oscillation). The increase in electrical activity that is seen during the light phase results from a change in membrane properties that sets up a fast membrane oscillator that is responsible for driving the spontaneous action potentials that appear with

a periodicity of about 25 ms (Module 6: Figure circadian clock input–output signals). An example of this high-frequency discharge is shown in Module 6: Figure firing rates of SCN neurons). SCN neurons recorded during the day had a much higher frequency than those recorded at night. When the action potentials were blocked by tetrodotoxin (TTX), the day neurons retained a regular train of membrane potential oscillations that had a similar frequency to the action potentials, so it seems likely that this represents the presence of a typical membrane oscillator that is switched on in the SCN neurons during the light phase. If this is the case, then the ionic basis of this oscillation may help explain how the circadian clock communicates with the plasma membrane.

These membrane oscillations were blocked by the removal of external Ca^{2+} or by the addition of nimodipine, which inhibits the Ca_V1 family of L-type channels, indicating that voltage-operated channels (VOCs) are one component of the membrane oscillator (Module 6: Figure circadian clock input–output signals). Another important component is likely to be a K^+ channel that is responsible for the repolarization of the action potential. The difference in the firing rate between the day and night neurons seems to depend on a change in the property of the membrane in the day neurons, which not only have a lower membrane potential, but also have a higher input resistance. The change in membrane potential does not seem to be the critical factor because oscillations failed to appear in the night neurons, even when they were depolarized to the same degree. There is something about the change in input resistance that results in the regular membrane potential oscillations that are responsible for firing the action potentials. Further information is necessary to determine how this membrane oscillator is switched on by the circadian oscillator (Module 6: Figure circadian clock input–output signals).

It is important to establish how this membrane oscillator is activated, because it drives the neuronal firing rhythm that is responsible for the output of electrical and hormonal signals produced by the circadian clock in the SCN.

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