Module 5

OFF Mechanisms

Synopsis

Signalling pathways are composed of the ON mechanisms that generate internal signals and the OFF mechanism that remove these signals as cells recover from stimulation. Most attention will be focused on how second messengers and their downstream effectors are inactivated. The second messengers cyclic AMP and cyclic GMP are inactivated by phosphodiesterase (PDE). Inositol trisphosphate ($InsP_3$) metabolism is carried out by both inositol trisphosphatase and inositol phosphatases. Diacylglycerol (DAG) metabolism occurs through two enzyme systems, DAG kinase and DAG lipase.

In the case of Ca^{2+} signalling, recovery is carried out by the Ca^{2+} pumps and exchangers that remove Ca^{2+} from the cytoplasm. The mitochondria also play an important role in Ca^{2+} homoeostasis.

Many of these second messengers activate downstream effectors through protein phosphorylation, and these activation events are reversed by corresponding protein phosphatases.

Protein phosphatases

It has been estimated that the human genome encodes approximately 2000 protein kinases that phosphorylate an enormous number of intracellular proteins, many of which function in cell signalling. There is an equally impressive array of protein phosphatases that are responsible for removing these regulatory phospho groups. These protein phosphatases can be divided into two main groups: the protein tyrosine phosphatases (PTPs) and the protein serine/threonine phosphatases.

Protein tyrosine phosphatases (PTPs)

It has been estimated that tyrosine phosphorylation accounts for less than 0.1% of all the protein phosphorylation in cells. Nevertheless, this small amount of phosphorylation is critical because it is involved in some very important signalling systems, and particularly those concerned with regulating cell growth and development. The fact that the level of tyrosine phosphorylation increases 10–20-fold when cells are stimulated by growth factors or undergo oncogenic transformation highlights the importance of protein tyrosine phosphatases (PTPs) in signal transduction. Protein tyrosine phosphatase structure and function reveals that these enzymes belong to a large heterogeneous family that functions to dephosphorylate phosphotyrosine residues with a high degree of spatial and temporal precision. The PTP superfamily can be divided into classical protein tyrosine phosphatases and dual-specificity phosphatases (DSPs).

Protein tyrosine phosphatase structure and function

The protein tyrosine phosphatase (PTP) superfamily is a heterogeneous group of enzymes with widely divergent structures (Module 5: Figure tyrosine phosphatase superfamily). They can be divided into the classical PTPs and the dual-specificity phosphatases (DSPs). The former can be divided further into the non-transmembrane PTPs and the receptor-type PTPs. What all the phosphatases have in common is a signature motif (H-C-X-X-G-X-X-R) located in the PTP domain that is responsible for its catalytic activity. The different structural elements [e.g. Src homology 2 (SH2), PDZ and immunoglobulin-like domains] that flank this PTP domain function to regulate enzyme activity and to position the enzyme in the right location near its specific substrates. These structural elements are described in more detail when the individual enzymes are described.

All PTPs utilize the same catalytic mechanism during which the phosphate on the substrate is first transferred to the cysteine residue in the signature motif before being hydrolysed by water to release the phosphate anion (Module 5: Figure tyrosine phosphatase catalysis). This role of the cysteine residue in the phosphyltransfer reaction is an example of one of the oxidation-sensitive processes that is targeted by the redox signalling . Some of the reactive oxygen species (ROS) messenger functions depend upon this inhibition of PTPs (Module 2: Figure ROS formation and action).

These families of enzymes that hydrolyse phosphotyrosine residues have a critical cysteine residue in the active site, which is particularly susceptible to oxidation. During

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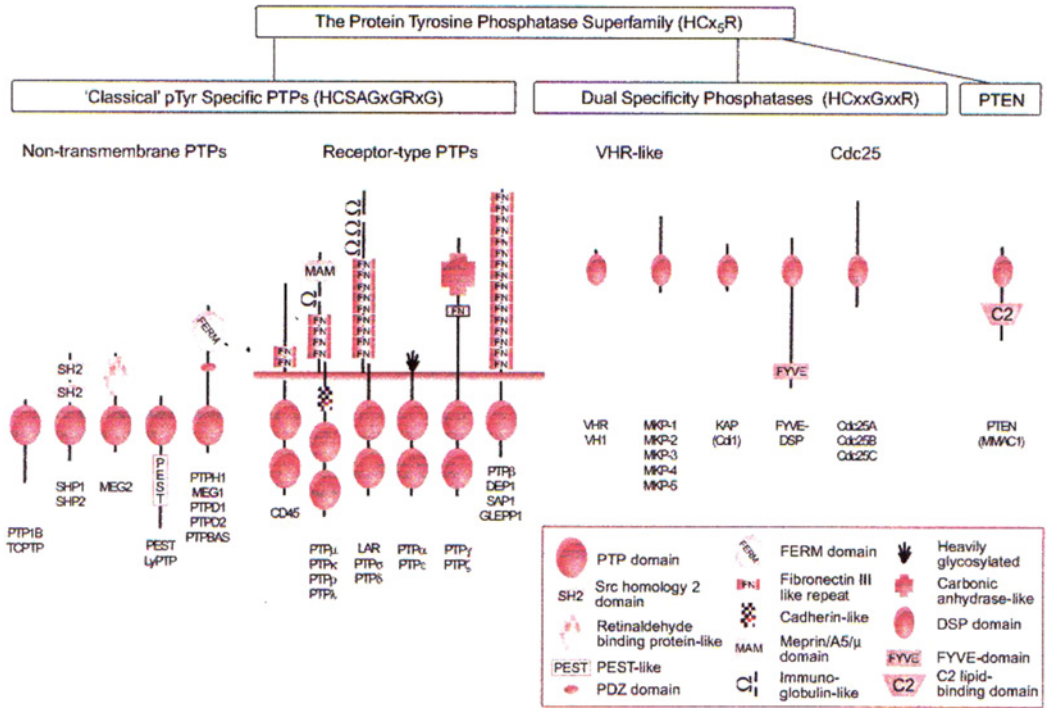
Module 5: | Figure tyrosine phosphatase superfamily



Summary of the protein tyrosine phosphatase (PTP) superfamily.
There is a very large family of protein tyrosine phosphatases (PTPs). Some are located in the cytoplasm, whereas others are so called receptor-type PTPs. See the text for further details. Reproduced from *Curr. Opin. Cell Biol.*, Vol. 13, Tonks, N.K. and Neel, B.G., Combinatorial control of the specificity of protein tyrosine phosphatases, pp. 182–195. Copyright (2001), with permission from Elsevier; see Tonks and Neel 2001.

redox signalling, this cysteine is oxidized, resulting in a decrease in the activity of the PTPs. Since the latter are normally expressed in great excess over the corresponding kinases, an oxidation-induced inhibition of phosphatase activity would greatly enhance the flow of information down those signalling cascades that rely on tyrosine phosphorylation, such as the MAP kinase signalling pathway and the Ca^{2+} signalling pathway. In the case of the latter, a positive-feedback mechanism operates between the ROS and Ca^{2+} signalling systems (Module 2: Figure ROS effects on Ca^{2+} signalling).

Classical protein tyrosine phosphatases

The classical protein tyrosine phosphatases are composed of two main groups, the non-transmembrane protein tyrosine phosphatases and the receptor-type protein tyrosine phosphatases (Module 5: Figure tyrosine phosphatase superfamily).

Non-transmembrane protein tyrosine phosphatases

The non-transmembrane protein tyrosine phosphatases (PTPs) are a heterogeneous family that share similar PTP domains, but have additional elements that determine both their location and their function within the cell. The following are some of the major members of the non-transmembrane PTPs:

- Protein tyrosine phosphatase 1B (PTP1B)
- T cell protein tyrosine phosphatase (TC-PTP)
- Src homology 2 (SH2) domain-containing protein tyrosine phosphatase-1 (SHP-1)
- Src homology 2 (SH2) domain-containing protein tyrosine phosphatase-2 (SHP-2)

Protein tyrosine phosphatase 1B (PTP1B)

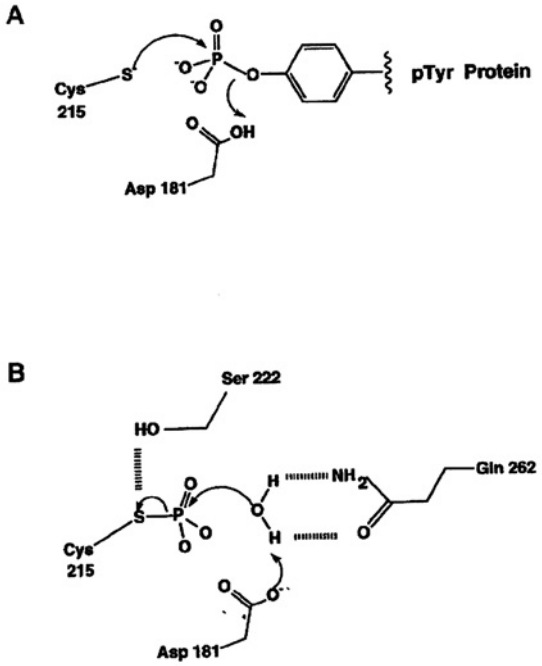
Protein tyrosine phosphatase 1B (PTP1B) has a typical protein tyrosine phosphatase (PTP) domain at the N-terminus and a regulatory region at the C-terminus. The latter contains a hydrophobic region that targets the enzyme to the cytoplasmic surface of the endoplasmic reticulum (ER). Despite this localization to the ER, some of the main substrates of PTP1B are the tyrosine kinase receptors, e.g. the epidermal growth factor (EGF) receptor and insulin receptor, and the non-receptor tyrosine kinase c-Src. PTP1B also acts on components of the JAK/STAT signalling pathway, such as STAT5a and STAT5b.

PTP1B plays a role in stabilizing cadherin complexes by dephosphorylating the phosphotyrosine residues on β-catenin. In order to bind to cadherin, PTP1B must be phosphorylated on Tyr-152 by the non-receptor protein tyrosine kinase Fer (Module 6: Figure classical cadherin signalling).

T cell protein tyrosine phosphatase (TC-PTP)

T cell protein tyrosine phosphatase (TC-PTP) has a similar structure to PTP1B, but operates on a different set of substrates. It exists as two alternatively spliced forms that differ with regard to the structure of the C-terminus. TC-48 has a hydrophobic domain resembling that of PTP1B

Module 5: | Figure tyrosine phosphatase catalysis



The catalytic mechanism of protein tyrosine phosphatases.
The signature motif, located at the bottom of a deep catalytic cleft, contains three important residues (cysteine, aspartate and glycine) that are necessary for the catalytic process. A. The peptide containing the phosphotyrosine (pTyr) residue enters the cleft where the cysteine residue initiates a nucleophilic attack. The aspartate residue has a critical role in protonating the phenolate leaving group in the substrate. B. Once the phosphate has been transferred to the cysteinyl group, the substrate leaves the enzyme, and the final step is to hydrolyse the phosphate. The co-ordination of a water molecule to the glycine residue favours the hydrolysis of the phosphoryl residue. The nucleophilicity of the water molecule is increased by the abstraction of a proton by the aspartate residue. Once the phosphate has been removed, the active site is ready to hydrolyse another phosphotyrosine residue. Reproduced from Pannifer, A.D.B., Flint, A.J., Tonks, N.K. and Barford, D. (1998) Visualisation of the cysteinyl-phosphate intermediate of a protein-tyrosine phosphatase by X-ray crystallography. J. Biol. Chem. 273:10454–10462, with permission from the American Society for Biochemistry and Molecular Biology; see Pannifer et al. 1998.

and is similarly located in the endoplasmic reticulum (ER). On the other hand, TC-45 lacks the hydrophobic residue but has a nuclear localization signal (NLS) that directs it into the nucleus. When cells are stimulated with epidermal growth factor (EGF), the TC-45 leaves the nucleus and interacts with the EGF receptor complex, where one of its targets appears to be Shc.

Src homology 2 (SH2) domain-containing protein tyrosine phosphatase-1 (SHP-1)

As their name implies, the Src homology 2 (SH2) domain-containing protein tyrosine phosphatases (SHPs) have two N-terminal SH2 domains (Module 6: Figure modular protein domains). There are two SHPs (SHP-1 and SHP-2), which have similar structures (Module 5: Figure structure of the SHPs). These SHPs must not be confused with the SH2 domain-containing inositol phosphatases (SHIPs), which form a subgroup of the Type II inositol polyphosphate 5-phosphatases, even though these two types of phosphatases often end up exerting very similar effects on cells.

Even though SHP-1 and SHP-2 are highly related structurally, they have very different functions. The primary function of SHP-1 is to inhibit signalling pathways that use tyrosine phosphorylation to transmit information. Many of its actions are directed against signalling systems in haematopoietic cells. It attaches itself to the signalling complexes via its SH2 domains, thereby enabling the protein tyrosine phosphatase (PTP) domain to dephosphorylate the phosphotyrosine residues involved in the process of signal transduction. Alternatively, SHP-1 is drawn into these signalling complexes through an attachment to various inhibitory receptors, particularly those that act to inhibit antigen and integrin receptor signalling. For example, SHP-1 is associated with the FcγRIII receptors that inhibit the FcεRI receptors in mast cells (Module 11: Figure mast cell inhibitory signalling).

SHP-1 participates in an important feedback loop that exists between the reactive oxygen species (ROS) and Ca^{2+} signalling pathways (Module 2: Figure ROS effects on Ca^{2+} signalling).

Src homology 2 (SH2) domain-containing protein tyrosine phosphatase-2 (SHP-2)

Even though Src homology 2 (SH2) domain-containing protein tyrosine phosphatase-2 (SHP-2) has a close structural resemblance to its related family member SHP-1, it has a very different function. Instead of exerting an inhibitory effect, it usually has a positive effect on the activity of various growth factor receptors such as the epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin, and perhaps also the platelet-derived growth factor (PDGF) and integrin receptors.

Receptor-type protein tyrosine phosphatases

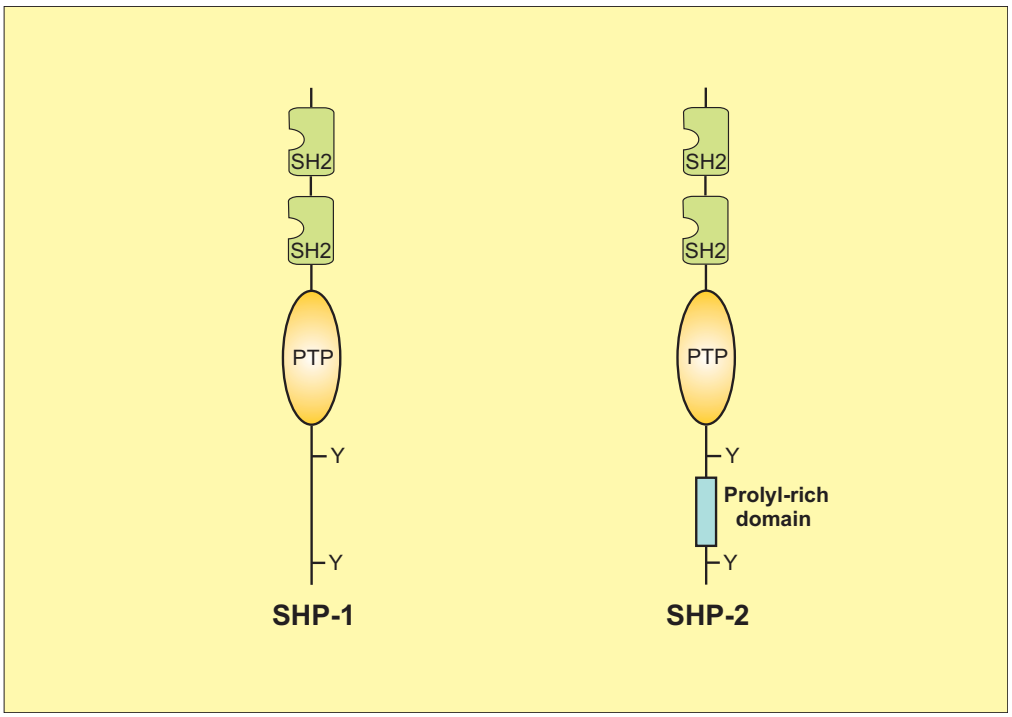
Receptor-type protein tyrosine phosphatase (RPTPs) have a transmembrane domain that retains them within the plasma membrane. Even though these enzymes are described as receptor-type, the nature of the ligand is poorly defined. Many of them have features of cell adhesion molecules and may thus be activated by cell-surface molecules embedded in neighbouring cells. This seems to be the case for RPTPμ and RPTPκ, which form homophilic interactions as they bind to identical molecules on opposing cells. The following are some of the major members of the RPTPs:

- CD45
- Protein tyrosine phosphatase α (PTPα)
- Leucocyte common antigen-related (LAR)

CD45

CD45 is a typical transmembrane protein tyrosine phosphatase (PTP) (Module 5: Figure tyrosine phosphatase superfamily). It has a highly glycosylated extracellular domain, and the cytoplasmic region has two PTP domains, but the second is catalytically inactive. CD45 has a critical function in T cell signalling, where it contributes early in the signalling cascade by activating Lck, which is a T-cell receptor transducer (Module 9: Figure TCR signalling).

Module 5: | Figure structure of the SHPs



Structural organization of the Src homology 2 (SH2)-domain-containing protein tyrosine phosphatases (SHP-1 and SHP-2).
The two mammalian Src homology 2 (SH2) domain-containing protein tyrosine phosphatases (SHPs) have very similar structures. The main features are the PTP domain and the two N-terminal SH2 domains. The C-terminal region has two tyrosine (Y) residues, which in the case of SHP-2 are separated by a prolyl-rich domain.

It acts by dephosphorylating the phosphate on Tyr-505, which opens up the molecular structure of Lck so that it can begin to phosphorylate ζ-associated protein of 70 kDa (ZAP-70). Similarly, CD45 functions in B-cell antigen receptor (BCR) activation by stimulating Lyn (Module 9: Figure B-cell activation).

Protein tyrosine phosphatase α (PTPα)
Protein tyrosine phosphatase α (PTPα) functions in the activation of the non-receptor Src family, where it removes the inhibitory phosphotyrosine residue.

Leucocyte common antigen-related (LAR)
The leucocyte common antigen-related (LAR) protein tyrosine phosphatase (PTP) has a number of specific developmental functions, such as a role in the terminal differentiation of alveoli in the mammary gland, as well as in development within the forebrain and hippocampus.

Dual-specificity phosphatases (DSPs)

As their name implies, these dual-specificity phosphatases (DSPs) are unusual in that they can dephosphorylate both phosphotyrosine (pTyr) and phosphoserine/phosphothreonine (pSer/pThr) residues. The following are some of the major members of the dual-specificity phosphatase family:

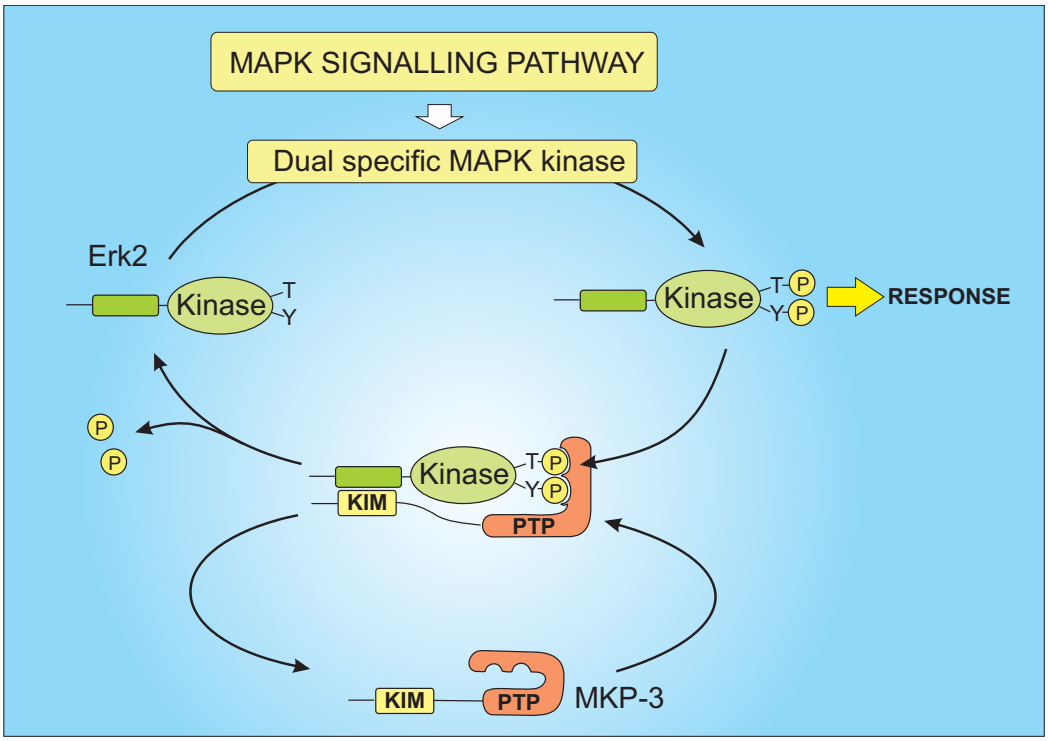
- Cdc25
- Mitogen-activated protein kinase (MAPK) phosphatases (MKPs)

Mitogen-activated protein kinase (MAPK) phosphatases (MKPs)
The family of mitogen-activated protein kinase (MAPK) phosphatases (MKPs) contains ten members (Module 2: Table MAPK signalling toolkit) that have specific functions in reversing the phosphorylation events responsible for the MAP kinase signalling pathway. One of the last events of this signalling cascade is the phosphorylation of the MAPKs by the dual-specificity MAPK kinases, which add phosphates to both tyrosine and threonine residues. During the recovery phase, these phosphates are removed by the MAPK phosphatases (Module 5: Figure dual-specificity MKP).

Some of the MAPK phosphatases are expressed constitutively, whereas others are actively induced when cells are stimulated, thus setting up a negative-feedback loop. An example of such a negative-feedback loop is evident for the extracellular-signal-regulated kinase (ERK) signalling pathway (Module 2: Figure ERK signalling). Another characteristic of these phosphatases is that they are often highly specific for particular targets. A good example of this specificity is illustrated by MAPK phosphatase-3 (MKP-3), which acts specifically to dephosphorylate ERK2.

Certain neurons, such as the medium spiny neurons in the striatum, express a striatal-enriched protein tyrosine phosphatase (STEP), which plays a highly specific role in regulating the neuronal MAPK signalling pathway (Module 10: Figure medium spiny neuron signalling).

Module 5: | Figure dual-specificity MKP



Mode of action of dual-specificity mitogen-activated protein kinase (MAPK) phosphatase.
Extracellular-signal-regulated kinase 2 (RK2), which is one of the main components of mitogen-activated protein kinase (MAPK) signalling, is phosphorylated on threonine (T) and tyrosine (Y) by MEK1/2, a dual-specificity MAPK kinase (Module 2: Figure ERK signalling). These phosphorylation events are reversed by MAPK phosphatase-3 (MKP-3). The specificity of the interaction between ERK2 and MKP-3 depends upon the latter having a kinase interaction motif (KIM) that binds to a specific site on ERK. This interaction enables the protein tyrosine phosphatase (PTP) domain to dephosphorylate the two phosphorylated residues on ERK2, thus curtailing its ability to stimulate downstream responses.

In response to *N*-methyl-aspartate (NMDA) stimulation, the increase in Ca^{2+} acts on calcineurin (CaN) to dephosphorylate and activate STEP, which then limits the duration of phospho-ERK signalling. By contrast, elevations in Ca^{2+} induced by voltage-operated channels (VOCs) or the release of internal Ca^{2+} have no effect, indicating a tight association between NMDA receptors and STEP.

Cdc25

The human genome contains three CDC25 dual-specificity enzymes (Cdc25A, Cdc25B and Cdc25C) (Module 9: Table cell cycle toolkit). This enzyme was first described as a regulator of the cell cycle in studies on yeast cells, and still retains its yeast nomenclature. The three human isoforms also act to regulate the cell cycle by controlling both the entry into S phase (Cdc25A) and the entry into mitosis (Cdc25B and C) (Module 9: Figure cell cycle signalling mechanisms). The level of Cdc25A increases in late G_1 and remains high throughout the rest of the cell cycle. The level of Cdc25B is increased during S phase to activate the entry into mitosis, and returns to a low level after mitosis is complete. The level of Cdc25C remains high throughout the cell cycle. All three isoforms have a similar C-terminal catalytic region, whereas the N-terminus, which has the regulatory regions, is somewhat variable. The activity of the Cdc25 isoforms is regulated by both activating and inhibitory phosphorylation. All three isoforms contain a phosphorylation site, which controls the binding of 14-3-3 protein that then inhibits the enzyme. This inhibitory site is phosphorylated by enzymes that are activated by cell stress, such as DNA damage. This stress-induced inhibition of the Cdc25 isoforms is thus an important mechanism for both G_1 and G_2/M cell cycle arrest.

The expression of Cdc25A is controlled by E2F. Once Cdc25A is expressed in the cytoplasm, it is available to activate cyclin-dependent kinase 2 (CDK2) to initiate the process of DNA synthesis. The activity of Cdc25A is very sensitive to DNA damage, which activates the checkpoint kinases 1 and 2 (CHK1 and CHK2) to phosphorylate Ser-123, which then promotes ubiquitination and rapid degradation. CHK1 is also responsible for phosphorylating Thr-507, which facilitates its interaction with 14-3-3 protein, which keeps the enzyme inactive until it is required.

Cdc25B, which plays an important role in the way cyclin B controls mitosis, is activated at the G_2/M transition (Module 9: Figure mitotic entry). Like the other Cdc25 isoforms, Cdc25B is kept quiescent by phosphorylating Ser-323 that provides a binding site for 14-3-3 protein. This site is phosphorylated by the p38 pathway and provides a mechanism whereby this component of the mitogen-activated protein kinase (MAPK) signalling pathway can arrest the cell cycle (Module 2: Figure MAPK signalling).

The Cdc25C enzyme is kept quiescent through phosphorylation of Ser-216, which provides a binding site for 14-3-3 protein. During entry into mitosis, this inhibitory phosphate is removed and this enables the Polo-like kinases (Plks) to phosphorylate other sites in the regulatory region that enables Cdc25C to begin to dephosphorylate CDK1-activating kinases (Module 9: Figure cell cycle signalling mechanisms).

Protein serine/threonine phosphatases

There are a very large number of kinases that contribute to the ON reactions of cell signalling by phosphorylating both serine and threonine residues on target proteins. By contrast, there is a relatively small group of protein serine/threonine phosphatases that remove these serine and threonine phosphates, thus reversing the activity of the kinases as part of the OFF reaction. The serine/threonine phosphatase classification reveals that most of these kinases belong to either the phosphoprotein phosphatase (PPP) family or the Mg^{2+}-dependent protein phosphatase (PPM) family.

Serine/threonine phosphatase classification

There are two major families of serine/threonine phosphatases (Module 5: Table serine/threonine phosphatases classification). With regard to signalling, the following members of the PPP family are particularly abundant and important with regard to cell signalling:

- Protein phosphatase 1 (PP1)
- Protein phosphatase 2A (PP2A)
- Protein phosphatase 2B (PP2B)
- Pleckstrin homology domain leucine-rich repeats protein phosphatase (PHLPP)

Module 5: | Table serine/threonine phosphatase classification
Classification of the protein serine/threonine phosphatases

Phosphatase	Comment
PPP family	
PP1 (protein phosphatase 1)	There are three PP1 genes that give rise to four isoforms; PP1 has multiple regulatory components Module 5: Table PP1 regulatory, targeting and inhibitory subunits and proteins
PP2A (protein phosphatase 2A)	An abundant and ubiquitous phosphatase that has multiple scaffolding and regulatory subunits (Module 5: Figure PP2A holoenzyme)
PP2B (calcineurin)	A Ca^{2+}-sensitive protein phosphatase (Module 4: Figure calcineurin)
PP4 (protein phosphatase 4)	May function in nuclear factor κB (NF-κB) signalling and histone deacetylase 3 (HDAC3) dephosphorylation
PP5 (protein phosphatase 5)	May function in control of cell growth
PP6 (protein phosphatase 6)	May function in G_1/S transition of cell cycle
PP7 (protein phosphatase 7)	Located in retinal and brain
PPM family	The PPM family contains approximately nine human genes; little is known about most of these enzymes except for PP2C and Ppm2
PP2C (Pmp1)	Prototypic member of the PPM family; implicated in dephosphorylation of cyclin-dependent kinases (CDKs), regulation of RNA splicing and control of p53 activity
Ppm2	Pyruvate dehydrogenase phosphatase
Pleckstrin homology domain leucine rich repeats protein phosphatase (PHLPP)	
PHLPP1	
PHLPP2	

The protein serine/threonine phosphatases are divided into two main families, the main phosphoprotein phosphatase (PPP) family and the smaller Mg^{2+}-dependent protein phosphatase (PPM) family.

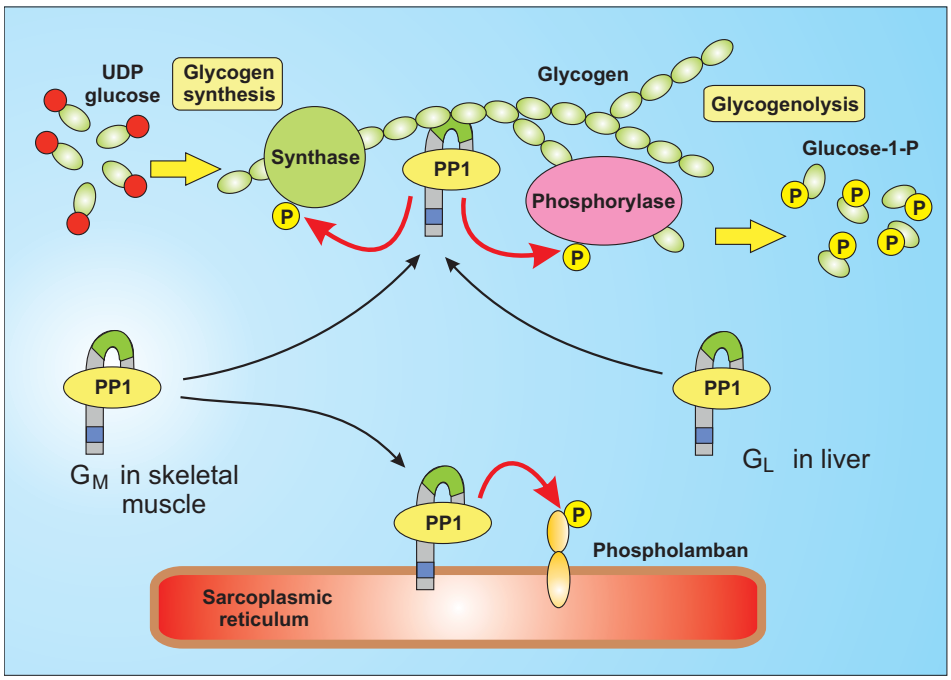
Protein phosphatase 1 (PP1)

Three genes code for the protein phosphatase 1 (PP1) catalytic subunit ($PP1_C$), which give rise to four isoforms (PP1α, PP1β, PP1γ1 and PP1γ2). Despite this limited number of catalytic subunits, PP1 performs a large number of functions operating in many different cellular locations. It owes this versatility to the fact that it can interact with a large number of regulatory and inhibitory proteins. The activity of PP1 is inhibited by inhibitor 1 (I-1) and by DARP-32. The function of the PP1 regulatory/targeting and inhibitory proteins are summarized in Module 5: Table PP1 regulatory and inhibitory subunits and proteins.

The regulatory subunits determine the substrate specificity and variable intracellular locations of PP1, which functions in the control of many cellular processes:

- The myosin phosphatase targeting subunit 1 (MYPT1) functions to localize PP1 to the myosin filaments in the contractile ring that controls cytokinesis during cell division (Module 9: Figure cytokinesis).
- PP1 plays an important role in the control of glycogen metabolism. In liver cells, G_L targets PP1 to glycogen, where it functions to dephosphorylate glycogen synthase and phosphorylase (Module 7: Figure glycogenolysis and gluconeogenesis). The glycogen-targeting subunit G_M in skeletal muscle also directs PP1 to the surface of glycogen granules, where it has a similar function. In skeletal muscle, it also targets PP1 to the sarcoplasmic reticulum (Module 5: Figure PP1 targeting to glycogen), where it functions to dephosphorylate the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump inhibitor phospholamban (Module 5: Figure phospholamban mode of action).
- PP1 controls smooth muscle relaxation (Module 7: Figure smooth muscle cell E-C coupling).
- Activity of the striatal-enriched phosphatase (STEP), which dephosphorylates ERK in neurons, is regulated by PP1 (Module 10: Figure medium spiny neuron signalling).
- PP1 contributes to the regulation of the Na^+-K^+-$2Cl^-$ cotransporter 1 (NKCC1) (Module 3: Figure cation chloride cotransporter).

Module 5: | Figure PP1 targeting to glycogen



Function of the regulatory proteins G_M and G_L in targeting protein phosphatase 1 (PP1) to both glycogen and the sarcoplasmic reticulum.
The regulatory protein G_M in skeletal muscle or G_L in liver has two targeting domains. One is located in the middle of the molecule (shown in green) that directs protein phosphatase 1 (PP1) to glycogen, where it can dephosphorylate the enzymes glycogen synthase and phosphorylase that control glycogen synthesis and glycogenolysis respectively. The other is a transmembrane region (shown in blue) in the C-terminal region that directs PP1 towards the sarcoplasmic reticulum, where it acts to dephosphorylate phospholamban, which functions to regulate the activity of the sarco/endo-plasmic reticulum Ca^{2+}-ATPase 2 (SERCA2a) pump (Module 5: Figure phospholamban mode of action).

- Cell volume regulation in response to hypotonicity depends upon the dephosphorylation of the K^+-Cl^- co-transporter 1 (KCC1) the by PP1 (Module 3: Figure cell volume regulation).
- The phosphorylation of AMPA receptors is regulated by PP1 and inhibitor 1 (I1) (Module 3: Figure AMPA receptor phosphorylation).
- PP1 dephosphorylats eIF2α to remove the inhibition of protein synthesis that is induced by PERK during endolpasmic reticulum (ER) stress signalling (Module 2: Figure ER stress signalling).

Dopamine and cyclic AMP-regulated phosphoprotein of apparent molecular mass 32 kDa (DARPP-32)

DARPP-32 is a dopamine and cAMP-regulated phosphoprotein of apparent molecular mass 32 kDa, which functions as a molecular switch to regulate the activity of protein phosphatase 1 (PP1). As its name implies, it is regulated by protein kinase A (PKA)-dependent phosphorylation and is localized in dopamine-sensitive neurons such as the medium spiny neurons found in the dorsal striatum and nucleus accumbens. DARPP-32 may function as a node to co-ordinate the activity of the dopamine and glutamate signalling pathways (Module 10: Figure medium spiny neuron signalling). This integration of two separate neural signalling pathways may underlie the neural plasticity that occurs during drug addiction.

In addition, DARPP-32 binds to Bcl-2 located on the inositol 1,4,5-trisphosphate receptor ($InsP_3R$) and is a key component of a negative-feedback loop that acts to regulate the release of Ca^{2+} from the endoplasmic reticulum (Module 3: Figure cyclic AMP modulation of the $InsP_3R$).

Protein phosphatase 2A (PP2A)

Protein phosphatase 2A (PP2A) is one of the most abundant of the serine/threonine protein phosphatases: it is estimated to make up about 0.3% of total cellular protein. It is a highly versatile enzyme in that it can operate in many different cellular regions. This versatility depends upon the protein phosphatase 2A (PP2A) holoenzyme organization, which is a trimeric structure consisting of the scaffolding protein A subunit of protein phosphatase 2A, a regulatory B subunit and a catalytic C subunit (Module 5: Figure PP2A holoenzyme). There are a large number of regulatory B subunits, which are responsible for directing the holoenzyme to different cellular locations. Protein phosphatase 2A (PP2A) function depends primarily on its role in reversing the phosphorylation events that are part of many signalling pathways, and particularly those controlling processes such as development, differentiation, morphogenesis and cell proliferation. Its inhibitory role in cell proliferation has led to its classification as a tumour suppressor.

A mutation arising from expansion of a CAG trinucleotide repeat of the Bβ gene (Module 5: Table PP2A subunits) is the cause of autosomal dominant spinocerebellar ataxia type 12 (SCA12). A role for PP2A in cancer has

Module 5: | Table PP1 regulatory, targeting and inhibitory subunits and proteins
The regulatory, targeting and inhibitory subunits and proteins of protein phosphatase 1 (PP1).

Subunit or protein	Cellular location and function
PP1 regulatory and targeting subunits and proteins	
Glycogen targeting	
G_M	Directs protein phosphatase 1 (PP1) to glycogen particles in skeletal and heart muscle; controls glycogen metabolism (Module 5: Figure PP1 targeting to glycogen)
G_L	Directs PP1 to glycogen particles in liver; controls glycogen metabolism; distributed widely, but high in liver and muscle
R5	Also known as protein targeting to glycogen (PTG) (Module 6: Figure glycogen scaffold).
Myosin/actin targeting	
MYPT1 (myosin phosphatase targeting subunit)	Directs PP1 to myofibrils in smooth muscle cells and non-muscle cells; also known as myosin-binding subunit (MBS); controls smooth muscle relaxation (Module 7: Figure smooth muscle cell E-C coupling)
MYPT2	Directs PP1 to myofibrils in skeletal muscle, where it controls contraction; also found in heart and brain.
Plasma membrane and cytoskeleton targeting	
Neurabin I	Neuronal plasma membrane and actin cytoskeleton; functions in neurite outgrowth and synapse morphology
Spinophilin (Neurabin II)	Widespread location on plasma membrane and actin; attaches PP1 to ryanodine receptors (Module 3: Figure ryanodine receptor structure)
A-kinase-anchoring protein 220 (AKAP220)	Brain and testis, where it is located on cytoskeleton to co-ordinate protein kinase A (PKA) and PP1 signalling.
Yotiao (a splice variant of AKAP350)	Located in the neuronal postsynaptic density (Module 10: Figure postsynaptic density), where it modulates synaptic transmission
PP1 inhibitory proteins	
I-1 (Inhibitor 1)	This inhibitor of PP1 is widely distributed
I-2 (Inhibitor 2)	
DARPP-32 (dopamine and cAMP-regulated phosphoprotein of apparent molecular mass 32 kDa)	This inhibitor of PP1 is found in brain and kidney

The function of PP1 is determined by its associated proteins that regulate its activity and are responsible for targeting it to its specific sites of action. (Information reproduced and adapted from Cohen 2002.)

Module 5: | Table PP2A subunits
Subunit composition of protein phosphatase 2A (PP2A).

PP2A subunits	Cellular location and function
PP2A scaffolding A subunits	
Aα	
Aβ	
PP2A regulatory B subunits	
B family	
Bα	Neuronal cell bodies and nucleus; linked to microtubules and is a tau phosphatase
Bβ	Abundant in brain and testis; in brain, it is in the cell body (excluding the nucleus) and extends into axons and dendrites; linked to microtubules and is a tau phosphatase; mutated in spinocerebellar ataxia type 12 (SCA12)
Bγ	Abundant in brain and testis
Bδ	
B' family	
B'α	Skeletal muscle and cardiac cells; targets PP2A to the apoptotic protein Bcl-2
B'β	Brain
B'γ	Skeletal muscle and cardiac cells; directs PP2A to L-type $Ca_V1.2$ channel to reverse protein kinase A (PKA)-dependent phosphorylation (Module 3: Figure $Ca_V1.2$ L-type channel)
B'δ	Brain
B'ε	Brain and testis
B'' family	
PR48	Located in the nucleus, where it interacts with Cdc6 in the pre-replication complexes during DNA synthesis
PR59	Interacts with p107, a retinoblastoma (Rb)-related protein that can arrest the cell cycle by dephosphorylating the transcription factor E2F
PR72	
PR130	Directs PP2A to the signalling complex assembled on A-kinase-anchoring protein 350 (AKAP350) localized on the centrosome; PR130 links PP2A to the ryanodine receptor (Module 3: Figure ryanodine receptor structure)
PP2A catalytic subunits	
Cα	
Cβ	

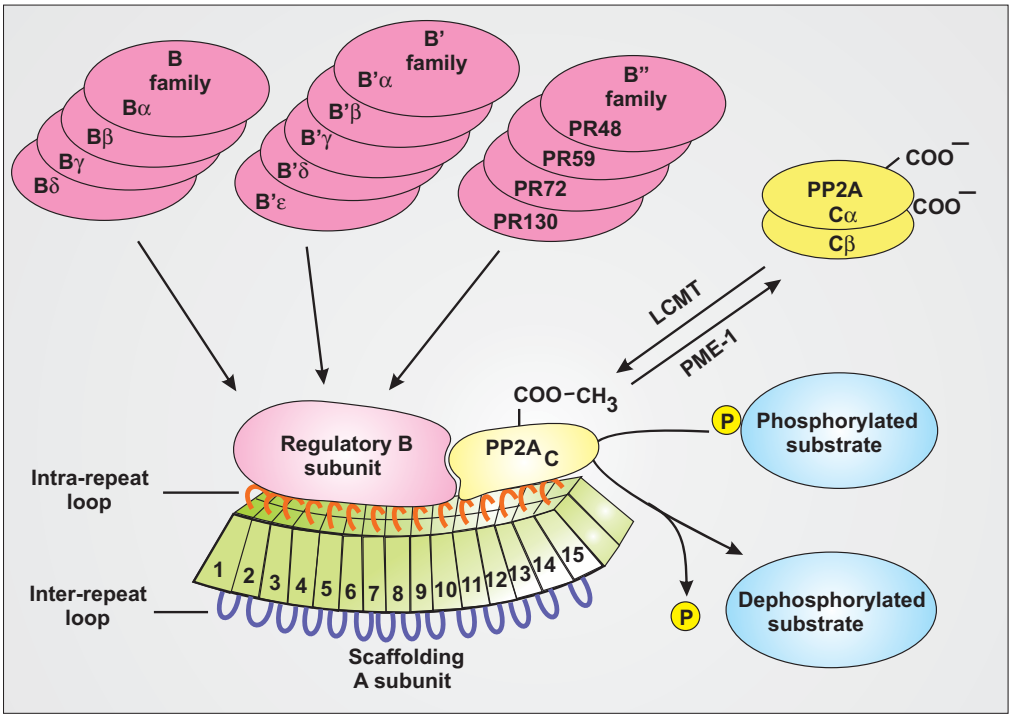
A large number of genes are used to encode the scaffolding, regulatory and catalytic subunits that are used to make up the diverse array of protein phosphatase 2A (PP2A) holoenzymes (Module 5: Figure PP2A holoenzyme).

emerged from the relationship between protein phosphatase 2A (PP2A) and tumour suppression.

Protein phosphatase 2A (PP2A) holoenzyme organization

Protein phosphatase 2A (PP2A) is a highly versatile enzyme that dephosphorylates a diverse array of proteins located in many different cellular locations. It owes this versatility to a large family of regulatory B proteins, which are part of the PP2A molecular toolkit (Module 5: Table PP2A subunits). The holoenzyme is a trimer composed of a PP2A scaffolding A subunit, which binds to a regulatory B subunit and a catalytic C subunit (Module 5: Figure PP2A holoenzyme). Given that there are two A subunits, two C subunits and at least 13 B subunits, many combinations are possible, resulting in multiple heterotrimeric holoenzymes. Much of the versatility of this enzyme

Module 5: | Figure PP2A holoenzyme



Assembly of the protein phosphatase 2A (PP2A) holoenzyme.

The protein phosphatase 2A (PP2A) holoenzyme is assembled from three subunits that have different functions. The molecular framework is provided by the scaffolding subunit (A), which is made up of 15 non-identical repeats, which are organized into a hook-shaped molecule. These repeats are connected by inter-repeat loops (shown in blue). Each repeat has two α-helices that are connected by intra-repeat loops (shown in orange), which line up to provide a cradle to bind the other subunits. Loops 1–10 are responsible for binding one of the regulatory B subunits, which belong to three families (B, B' and B''). There are two PP2A catalytic subunits (Cα and Cβ) and one of these attaches to loops 11–15. This recruitment of the catalytic subunit into the holoenzyme depends upon carboxymethylation of Leu-309 by leucine carboxmethyltransferase (LCMT) and is reversed by a phosphatase methylesterase (PME-1). Once assembled, the holoenzyme functions to dephosphorylate a wide range of phosphorylated substrates.

depends on the large number of B regulatory subunits that have subtly different properties, especially with regard to their ability to direct holoenzymes to different cellular regions and substrates. Some of the roles of the B subunit in determining PP2A function are summarized in Module 5: Table PP2A subunits, but many of the targeting functions are still being elucidated.

Protein phosphatase 2A (PP2A) function

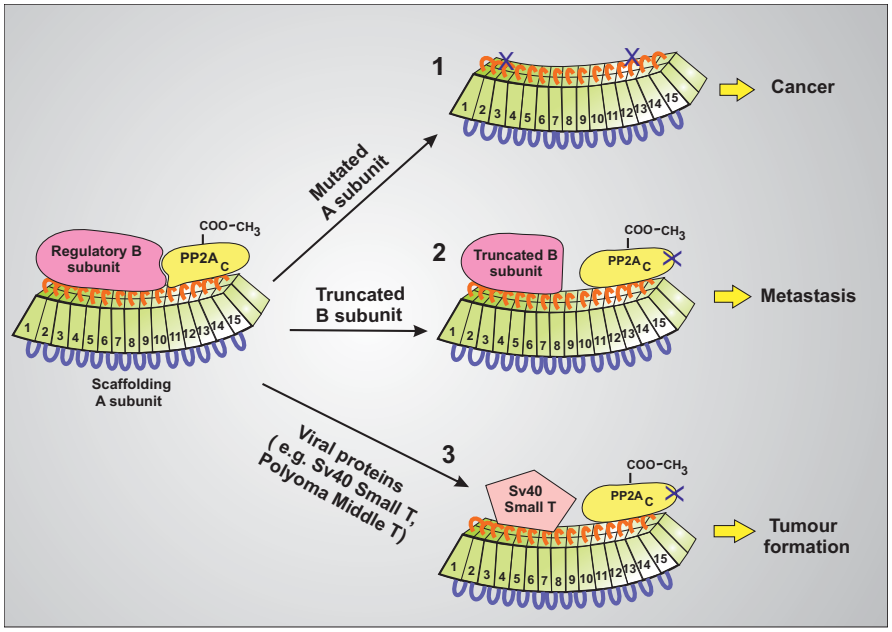
The primary role of protein phosphatase 2A (PP2A) is to dephosphorylate many of the phosphoproteins that function in cell signalling pathways:

- PP2A can modulate the MAP kinase signalling pathway both positively and negatively. With regard to the former, it can dephosphorylate some of the inhibitory sites on Raf-1. In addition, it can inhibit the signalling cascade by reversing some of the phosphorylation events downstream of Raf-1 (Module 2: Figure ERK signalling).
- Some of the key phosphorylation events of the canonical Wnt/β-catenin pathway are reversed by PP2A (Module 2: Figure Wnt canonical pathway).
- Protein kinase A (PKA)-dependent phosphorylation of the L-type $Ca_V1.2$ channel is reversed by PP2A (Module 3: Figure $Ca_V1.2$ L-type channel).
- PKA-dependent phosphorylation of the type 2 ryanodine receptor (RYR2) in cardiac cells is reversed by PP2A (Module 3: Figure ryanodine receptor structure).
- PP2A interacts with the scaffolding protein A-kinase-anchoring protein 79 (AKAP79), which is associated with synapse-associated protein 97 (SAP97), to come into close contact with the GluR1 subunit of the AMPA receptor (Module 10: Figure postsynaptic density).
- The phosphorylation status of the neuron-specific microtubule-associated protein tau, which has been implicated in Alzheimer's disease, is regulated by the PP2A holoenzyme carrying the Bβ regulatory subunit (Module 5: Table PP2A subunits).
- PP2A functions in Myc degradation.

Protein phosphatase 2A (PP2A) and tumour suppression

One of the important actions of protein phosphatase 2A (PP2A) is to regulate cell proliferation, where it normally acts to reverse the protein phosphorylation of the proliferation signalling pathways driven by various growth factors (Module 9: Figure proliferation signalling network). For example, PP2A contributes to Myc degradation, which is an important regulator of cell proliferation and is often amplified in many human cancers. Modifications of PP2A either through mutation of its subunits or by interactions with viral proteins can cause cancer (Module 5: Figure PP2A modifications and cancer). The negative effects on

Module 5: | Figure PP2A modifications and cancer



Modifications of protein phosphatase 2A (PP2A) by mutations and interactions with viral proteins can cause cancer.
Protein phosphatase 2A (PP2A) is considered to be a tumour suppressor because cancers can develop or be exacerbated when the activity of this enzyme is reduced either by mutations of the subunits or by interactions with viral proteins:

1. Mutations of the scaffolding A subunit, which then fails to bind the B and C subunits, have been identified in a number of human cancers (breast, colon, lung and skin).
2. Truncation of the B subunit, which prevents it from interacting with the catalytic C subunit, has been implicated in metastasis.
3. Tumour-promoting viruses act by binding to the scaffolding A subunit to displace the regulatory B subunit.

cell growth have led to the concept of PP2A functioning as a tumour suppressor. Some of the most convincing evidence for this comes from the finding that simian virus 40 (SV40) small T antigen and polyoma virus small T and middle T antigens bind to the scaffolding A subunit, resulting in a decrease in phosphatase activity.

Protein phosphatase 2B (PP2B)

Protein phosphatase 2B (PP2B) is known more commonly as calcineurin (CaN), which is a Ca^{2+}-activated serine/threonine phosphatase (Module 4: Figure calcineurin).

Protein phosphatase 4 (PP4)

Not much is known about protein phosphatase 4 (PP4). Like the other serine/threonine phosphatases, PP4 is made up of a catalytic subunit (PP4C) that interacts with various regulatory subunits (R1, R2, R3 and α4). In addition, it can interact with signalling proteins such as nuclear factor κB (NF-κB) and histone deacetylase 3 (HDAC3). There is increasing evidence that PP4 may have a highly specific role in modulating a variety of signalling mechanisms. For example, it can activate NF-κB by dephosphorylating Thr-43. It may play a role in histone acetylation and chromatin remodelling by dephosphorylating HDAC3.

Pleckstrin homology domain leucine-rich repeats protein phosphatase (PHLPP)

There are two pleckstrin homology domain leucine-rich repeats protein phosphatases (PHLPP1 and PHLPP2) which are characterized by having an N-terminal PH domain followed by multiple leucine-rich repeats (LRR) and then a PP2C phosphatase domain. A splice variant isoform PHLPP1β, which is also known as suprachiasmatic nucleus circadian oscillatory protein (SCOP), has been implicated in long-term memory formation.

One of the main functions of PHLPP is to dephosphorylate the hydrophobic motif of protein kinase B (PKB) to inhibit the activity of this enzyme. The ability of PHLPP to inhibit PKB has been implicated in endocannabinoid-induced insulin resistance (Module 12: Figure insulin resistance).

PHLPP can also interact with K-Ras resulting in a decrease in the MAP kinase signalling pathway and this mechanism might play a role in the regulation of neuronal protein synthesis required for long-term memory formation.

Phosphodiesterase (PDE)

The OFF mechanism of the cyclic AMP signalling pathway and the cyclic GMP signalling pathway is carried out by phosphodiesterases (PDEs) that inactivate the two cyclic nucleotide second messengers (cyclic AMP and cyclic GMP). The PDEs belong to a large family comprising 11 PDE gene families (Module 5: Table PDE family properties). This extensive PDE family share one thing in common: they all hydrolyse cyclic nucleotide second messengers, but in other respects, they are very different

Module 5: | Table PDE family properties
Summary of the organization and properties of the 11 phosphodiesterase (PDE) families.

PDE family	Gene	Number of splice variants	Regulatory domain, role	Phosphorylation	Substrate(s)	Commonly used inhibitor
PDE1	1A, 1B, 1C	9	CaM, activation	PKA	cGMP, cAMP	KS-505
PDE2	2A	3	GAF, activation	Unknown	cAMP, cGMP	EHNA
PDE3	3A, 3B	1 each	Transmembrane domains, membrane targeting	PKB	cAMP	Milrinone
PDE4	4A, 4B, 4C, 4D	20	UCR1, UCR2, unclear	ERK, PKA	cAMP	Rolipram
PDE5	5A	3	GAF, unclear	PKA, PKG	cGMP	Sildenafil Dipyrimadole, Zaprinast
PDE6	6A, 6B, 6C	1 each	GAF, activation	PKC, PKA	cGMP	Dipyrimadole, Zaprinast
PDE7	7A, 7B	6	Unknown	Unknown	cAMP	None identified
PDE8	8A, 8B	6	PAS, unknown	Unknown	cAMP	None identified
PDE9	9A	4	Unknown	Unknown	cGMP	None identified
PDE10	10A	2	GAF, unknown	Unknown	cAMP, cGMP	None identified
PDE11	11A	4	GAF, unknown	Unknown	cAMP, cGMP	None identified

Some of the phosphodiesterase (PDE) families have more than one gene, and complexity is enhanced further by numerous splice variants. The different PDEs have variable substrate specificities: some hydrolyse either cyclic AMP or cyclic GMP, whereas others have dual specificity. Reproduced from *Handbook of Cell Signaling*, Vol. 2, Glick, J.L. and Beavo, J.A., Phosphodiesterase families, pp. 431–435. Copyright (2003), with permission from Elsevier; see Glick and Beavo 2003.

with regard to substrate specificity, kinetic properties, regulation and cellular distribution. Much of this variability resides in the N-terminal region, which has different domains that determine the unique characteristics of each family member (Module 5: Figure PDE domains). In the light of this enormous family diversity, it is difficult to make too many generalizations, so each family member is considered separately. Most information is available for PDE1–PDE6:

- PDE1 is a Ca^{2+}-sensitive cyclic AMP phosphodiesterase.
- PDE2 is a cyclic GMP-stimulated cyclic AMP phosphodiesterase.
- PDE3 is a cyclic GMP-inhibited cyclic AMP phosphodiesterase.
- PDE4 is a cyclic AMP phosphodiesterase.
- PDE5 is a cyclic GMP-specific phosphodiesterase sensitive toViagra.
- PDE6 is the cyclic GMP phosphodiesterase in photoreceptors.

PDE1

The characteristic feature of PDE1 is that it is activated by Ca^{2+}. This Ca^{2+} sensitivity depends on the Ca^{2+} sensor calmodulin (CaM), which binds to two CaM-binding domains located in the regulatory N-terminal region of PDE1 (Module 5: Figure PDE domains). The PDE1 family consists of three genes.

PDE1A

PDE1A, which has five splice variants, has a higher affinity for cyclic GMP (K_m approximately 5 μM) than cyclic AMP (K_m approximately 110 μM). Phosphorylation of PDE1A1 and PDE1A2 by protein kinase A (PKA) results in a decrease in its sensitivity to Ca^{2+} activation.

PDE1B

PDE1B, which has two splice variants, has a higher affinity for cyclic GMP (K_m approximately 2.7 μM) than for cyclic AMP (K_m approximately 24 μM). This isoform is strongly expressed in the brain. Phosphorylation of PDE1B by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) results in a decrease in its sensitivity to Ca^{2+} activation.

PDE1C

PDE1C, which has five splice variants, has a high affinity for both cyclic GMP and cyclic AMP (K_m approximately 1 μM). The PDE1C2 splice variant is located in olfactory sensory cilia, where it functions to regulate the role of cyclic AMP in transducing odorant stimuli (Module 10: Figure olfaction) It is also expressed in β-cells, where it functions to regulate glucose-induced insulin secretion.

PDE2

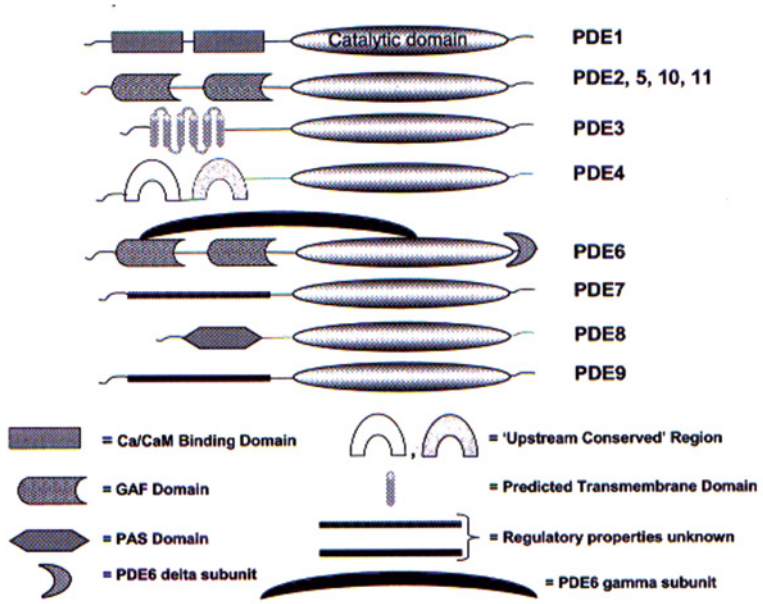
PDE2 is a cyclic AMP phosphodiesterase that can be stimulated by cyclic GMP. PDE2 exists as a single gene (PDE2A) that has three splice variants that determine its subcellular distribution, with PDE2A1 being soluble, whereas PDE2A2 and PDE2A3 are particulate. The membrane location of PDE2A2 may depend upon a transmembrane segment in the N-terminal region, whereas PDE2A3 appears to associate with membranes through an N-terminal myristoylation site.

PDE2 is strongly expressed in the brain and is also found in skeletal muscle, heart, liver, adrenal glomerulosa and pancreatic cells.

Although PDE2 is a dual-specificity enzyme capable of hydrolysing both cyclic AMP and cyclic GMP, the enzyme seems to favour cyclic AMP because cyclic GMP acts as an allosteric regulator that greatly enhances the ability of PDE2 to hydrolyse cyclic AMP. It is for this reason that this enzyme is referred to as a cyclic GMP-stimulated cyclic AMP PDE.

The ability of cyclic GMP to enhance the hydrolysis of cyclic AMP may account for the signalling cross-talk that occurs in some cells. For example, the nitric oxide (NO)/cyclic GMP-induced reduction in L-type Ca^{2+} channel activity in cardiac cells may depend upon cyclic GMP stimulating PDE2, thereby reducing the level of cyclic AMP that normally regulates these channels. Another example is found in zona glomerulosa cells, where atrial natriuretic factor (ANF) may inhibit the secretion of

Module 5: | Figure PDE domains



Domain structure of the phosphodiesterase (PDE) family that functions to hydrolyse and inactivate the second messenger cyclic AMP.
Many of the phosphodiesterase (PDE) family members are characterized by having paired regulatory domains in the N-terminal regulatory region. PDE1 has Ca^{2+}/calmodulin-binding domains; PDE2, PDE5, PDE6, PDE10 and PDE11 have cyclic GMP-binding GAF domains; PDE4 has upstream conserved regulatory regions 1 and 2 (UCR1 and UCR2). Reproduced from *Handbook of Cell Signaling*, Vol. 2, Glick, J.L. and Beavo, J.A., Phosphodiesterase families, pp. 431–435. Copyright (2003), with permission from Elsevier; see Glick and Beavo 2003.

aldosterone by using cyclic GMP to increase the activity of PDE2 to reduce the level of cyclic AMP, which drives the release of this steroid (Module 7: Figure glomerulosa cell signalling).

PDE3

There are two genes encoding PDE3, which is a cyclic GMP-inhibited cyclic AMP phosphodiesterase. They are characterized by having six putative transmembrane segments in the N-terminal region (Module 5: Figure PDE domains), which seem to be responsible for targeting this enzyme to cell membranes. These two family members (PDE3A and PDE3B) have different functions and cellular locations.

PDE3A

PDE3A is located in blood platelets, smooth muscle cells and cardiac myocytes.

PDE3B

PDE3B is found in brown and white fat cells, pancreatic β-cells and liver cells, all of which are cells that function in energy metabolism. This isoform is particularly important as an effector for the action of insulin in antagonizing the catecholamine-dependent lipolysis and release of fatty acids from white fat cells (Module 7: Figure lipolysis and lipogenesis). Insulin acts through the PtdIns 3-kinase signalling pathway to increase the enzymatic activity of PDE3B, and the resulting decline in the activity of cyclic AMP leads to a decrease in lipid hydrolysis. A similar mechanism operates in liver cells to carry out the anti-glycogenolytic action of insulin (Module 7: Figure liver cell signalling). Insulin-like growth factor I (IGF-I) and leptin may reduce insulin secretion in response to GLP-1 by stimulating the activity of phosphodiesterase PDE3B, thereby reducing the level of cyclic AMP (Module 7: Figure β-cell signalling).

Insulin resistance and obesity may arise from a reduced expression of PDE3B.

PDE4

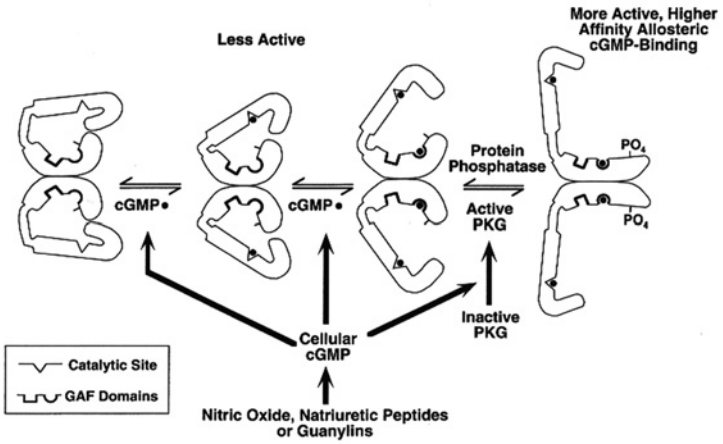
PDE4 functions only to hydrolyse cyclic AMP. It consists of four genes with approximately 20 splice variants, which fall into three main categories: long, short and super-short. Much of this variation depends upon the expression of their characteristic upstream conserved regions 1 and 2 (UCR1 and UCR2) in the N-terminal regulatory region (Module 5: Figure PDE domains). The long isoforms have both UCR1 and UCR2, the short isoforms lack UCR1, whereas the super-short isoforms lack UCR1 and have a truncated UCR2.

The activity of the various PDE4 isoforms can be regulated through a feedback loop operated through protein kinase A (PKA) and by inputs from other signalling pathways, such as MAP kinase signalling. The ability of PKA to modulate the activity of PDE4 is facilitated by the fact that they are both associated on the same scaffolding protein muscle A-kinase-anchoring protein (mAKAP).

PDE4A

PDE4A is located in the soma of olfactory neurons, in contrast with PDE1C2, which is in the cilium. PDE4A1

Module 5: | Figure PDE5 functional states



A model depicting different functional states of PDE5.

PDE5 functions as a dimer with the two subunits connected through a region that includes the allosteric cyclic GMP-binding GAF domains. During the course of enzyme activation, cyclic GMP appears to bind first to the catalytic site (K_m of 1–6 μM), which induces a conformational change that then enhances the affinity of cyclic GMP binding to the GAF domains. This binding to the regulatory region induces a further conformational change to expose the serine residue, which is then phosphorylated by cyclic GMP-dependent protein kinase (cGK). This phosphorylated state is the most active form of the enzyme. This thus represents a complex feedback loop whereby cyclic GMP promotes its own hydrolysis by binding allosterically to the enzyme and by promoting its phosphorylation by stimulating cGK. Reproduced from *Handbook of Cell Signaling*, Vol. 2, Francis, S.H. and Corbin, J.D., Phosphodiesterase-5, pp. 447–451. Copyright (2003), with permission from Elsevier; see Francis and Corbin 2003.

associates with membranes through a hydrophobic domain in the N-terminal region, whereas PDE4A5 is located at the plasma membrane, where it associates with proteins containing SH3 domains.

PDE4B

PDE4B plays an important role in inflammatory responses, because PDE4B$^{-/-}$ mice display a large decrease in their ability to release tumour necrosis factor α (TNFα) in response to lipopolysaccharide (LPS). The cyclic AMP signalling pathway functions in the modulation of inflammatory responses. It has an anti-inflammatory role in macrophages, and this inhibitory effect is usually dampened by the up-regulation of PDE4B (Module 11: Figure macrophage signalling). PDE4B has an important role in regulating the contractile activity of uterine smooth muscle cells. The antidepressant Rolipram inhibits PDE4B.

Mutations in PDE4B have been linked to schizophrenia.

PDE4D

PDE4D may play some role asthma. PDE4D$^{-/-}$ mice have been found to lack normal muscarinic responses, resulting in a loss of airway hyperreactivity.

PDE5

There is a single cyclic GMP-specific phosphodiesterase (PDE5) gene with three splice variants. It is a cyclic GMP-specific phosphodiesterase, which has a unique feature in that it is also regulated by cyclic GMP binding to the tandem GAF domains in the regulatory region (Module 5: Figure PDE domains). Binding of cyclic GMP to these GAF domains is necessary for protein kinase A (PKA) or cyclic GMP-dependent protein kinase (cGK) to phosphorylate a single site in the N-terminal region, which then results in an increase in both the rate of catalysis and cyclic GMP-binding affinity of the catalytic site. This complex combination of regulation through both the allosteric binding of cyclic GMP and phosphorylation by cGK can result in different functional states of the enzyme (Module 5: Figure PDE5 functional states).

PDE5 plays a major role in regulating the cyclic GMP signalling pathway in various cells, such as smooth muscle cells (Module 7: Figure smooth muscle cell cGMP signalling), blood platelets, renal tissue (proximal and collecting ducts), cerebellar Purkinje cells and pancreatic ducts.

In the case of corpus cavernosum smooth muscle cells, which regulate penile erection (Module 7: Figure corpus cavernosum), PDE5 is the target for Viagra , a drug used to treat male erectile dysfunction.

PDE6

PDE6 is a highly specialized enzyme that is the primary effector of visual transduction in vertebrate photoreceptors (Module 10: Figure phototransduction overview).

The stability of PDE6 is regulated by aryl hydrocarbon receptor-interacting protein-like 1 (AIPL1). Just how AIPL1 functions is not entirely clear, but it appears to function as a specific chaperone required for PDE6 biosynthesis and stability. Leber congenital amaurosis (LCA), which is an early onset human retinopathy, has been linked to mutations in the *AIPL1* gene.

Ca^{2+} pumps and exchangers

A variety of pumps and exchangers are responsible for removing Ca^{2+} from the cytoplasm (Module 5: Figure Ca^{2+} uptake and extrusion). The most obvious function of such pumps is therefore to enable cells to recover from

Ca^{2+}-induced signalling events. However, such pumps have two other important functions. Firstly, they ensure that the internal stores are kept loaded with signal Ca^{2+} by pumping Ca^{2+} into the sarcoplasmic reticulum (SR) of muscle cells or the endoplasmic reticulum (ER) of non-muscle cells. Pumps are also important for loading Ca^{2+} into the Golgi. Secondly, they maintain the resting level of Ca^{2+}. The constant leakage of Ca^{2+} into the cell down the very large concentration gradients facing the cytoplasm, both from the outside and from the internal stores, is expelled by pumps to ensure that the resting Ca^{2+} concentration is held constant at approximately 100 nM. A pump classification reveals that there are five different mechanisms responsible for carrying out these functions of recovery, maintaining the Ca^{2+} stores and the resting level of Ca^{2+}:

- Plasma membrane Ca^{2+}-ATPase (PMCA)
- Sodium/calcium exchangers (NCX and NCKX)
- Sarco/endo-plasmic reticulum Ca^{2+}-ATPase (SERCA)
- Mitochondrial uniporter
- Secretory-pathway Ca^{2+}-ATPase (SPCA)

Two of these pumps (PMCA and NCX) are located on the plasma membrane, whereas the others are located on internal organelles. The organization and distribution of Ca^{2+} pumps determines the properties of Ca^{2+} pumps, which are adapted to carry out different homoeostatic functions. The PMCA pump family consists of four genes with diversity enhanced by alternative splicing at two sites. The SERCA family has three genes, and alternative splicing gives at least six different isoforms. Likewise, the NCX has a family of three genes, and alternative splicing gives rise to numerous isoforms. The way in which alternative splicing can enhance diversity is illustrated for SERCA2a and its isoforms, which have not only different properties, but also different distributions. In summary, the molecular organization gives rise to a diverse repertoire of pumps from which cells can select out that combination of pumps that exactly meets their Ca^{2+} signalling requirements.

The molecular structure of the Ca^{2+} pumps is designed to transfer Ca^{2+} ions across membranes against very large electrochemical gradients. The exception to this is the mitochondrial uniporter, which is not a pump in the strict sense, but it is a channel that allows Ca^{2+} to flow from the cytoplasm into the mitochondrial matrix. The plasma membrane Ca^{2+}-ATPase (PMCA) molecular structure and that of the SERCA pump are very similar with regard to their main domains. They have ten transmembrane domains, with both the N-terminal and C-terminal ends facing the cytoplasm. The NCX and NCKX molecular structure consists of nine and 11 transmembrane domains respectively. They both have a large cytoplasmic loop connecting transmembrane domains 5 and 6 (Module 5: Figure sodium/calcium exchangers).

The different structural domains have specific functions, which have been well described for the sarco/endo-plasmic reticulum Ca^{2+}-ATPase (SERCA) pump structure and mechanism. There is less information on the NCX pump mechanism, where the energy to pump Ca^{2+} is derived from the flow of Na^+ down its electrochemical gradient.

Ca^{2+} pump regulation plays a critical role in enabling pumps to deal with large variations in the intracellular level of Ca^{2+}.

Alterations in the way cells pump Ca^{2+} have been linked to a variety of diseases. For example, Darier's disease is an autosomal skin disorder that results from a loss of one copy of the *SERCA2* gene. Brody disease results from a defect in the SERCA1a pump that is responsible for relaxing skeletal muscle. Hailey-Hailey disease is caused by an inactivating mutation of the secretory Ca^{2+}-ATPase.

Pump classification

Cells use different types of Ca^{2+} pumps, two types [plasma membrane Ca^{2+}-ATPase (PMCA) and Na^+/Ca^{2+} exchanger (NCX)] are located on the plasma membrane while the sarco/endo-plasmic reticulum Ca^{2+}-ATPase (SERCA) and the uniporter are located on internal organelles (Module 2: Figure Ca^{2+} signalling toolkit).

Plasma membrane pumps

The plasma membrane Ca^{2+}-ATPase (PMCA) located on the plasma membrane extrudes Ca^{2+} from the cell using energy derived from the hydrolysis of ATP.

The sodium/calcium exchangers (NCX and NCKX), which consist of two families, the Na^+/Ca^{2+} exchanger (NCX) and the $Na^+/Ca^{2+} - K^+$ exchanger (NCKX), are located on the plasma membrane and extrude Ca^{2+} in exchange for Na^+. The energy for Ca^{2+} extrusion is derived from the influx of Na^+, which enters the cell down its electrochemical gradient.

Organellar pumps

The sarco/endo-plasmic reticulum Ca^{2+}-ATPase (SERCA) located on either the sarcoplasmic reticulum (SR) of muscle cells or on the endoplasmic reticulum (ER) of non-muscle cells uses the energy derived from the hydrolysis of ATP to pump Ca^{2+} from the cytoplasm into the internal store.

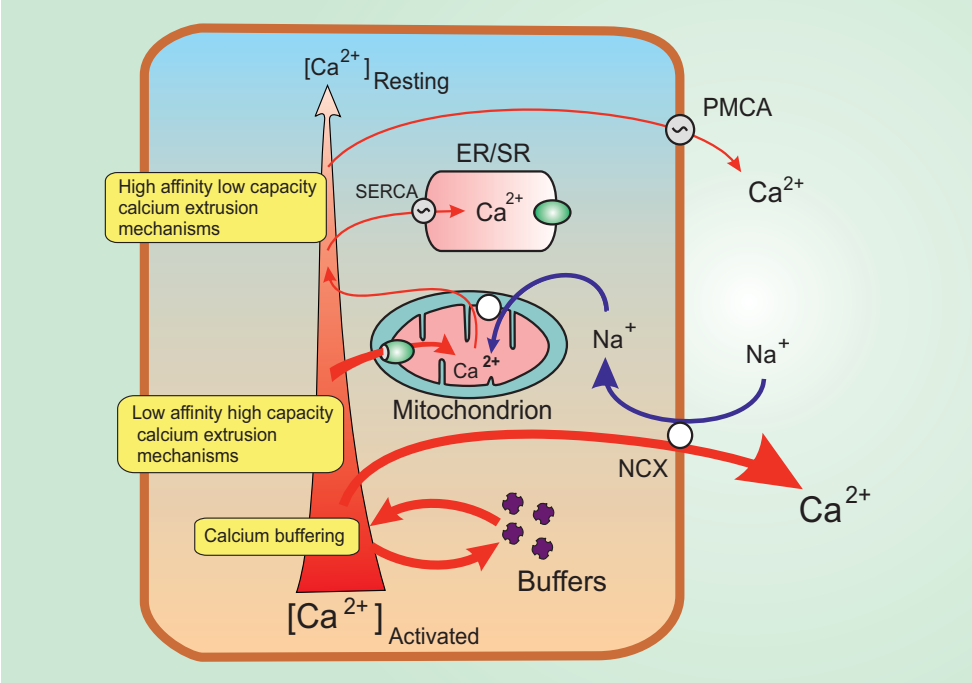
The secretory-pathway Ca^{2+}-ATPase (SPCA) is located on the Golgi, where it functions to maintain the level of Ca^{2+} within the lumen in order to maintain processes such as glycosylation, proteolytic processing and protein trafficking.

The mitochondrial uniporter is not strictly a pump, but is included because it functions in mitochondrial Ca^{2+} uptake to remove Ca^{2+} from the cytoplasm. The uptake of Ca^{2+} through the uniporter is driven by the large transmembrane potential that is maintained across the inner mitochondrial membrane (Module 5: Figures mitochondrial Ca^{2+} signalling). Mitochondria modulate Ca^{2+} signalling by operating as a 'buffer' in that they rapidly sequester Ca^{2+} during the course of a response, and then return it to the cytoplasm through a mitochondrial Na^+/Ca^{2+} exchanger as the concentration of Ca^{2+} returns towards its resting level.

Properties of Ca^{2+} pumps

The different Ca^{2+} pumping mechanisms have very different properties with regard to their affinity for Ca^{2+} and

Module 5: | Figure Ca^{2+} uptake and extrusion



Functional organization of Ca^{2+} pumps.

The Na^+/Ca^{2+} exchanger (NCX) and the mitochondrial uniporter are particularly effective at pumping Ca^{2+} when the cytosolic Ca^{2+} concentration is high, in that they combine a low affinity for Ca^{2+} with a high capacity. The plasma membrane Ca^{2+}-ATPase (PMCA) and sarco/endo-plasmic reticulum Ca^{2+}-ATPase (SERCA) pumps have much lower capacities, but their much higher affinities enable them to reduce the level of Ca^{2+} back to its resting level. The different extrusion mechanisms thus co-operate with each other to regulate the level of Ca^{2+} over a large dynamic range.

the rate at which they can transport this ion across membranes (Module 5: Figure Ca^{2+} uptake and extrusion):

Low-affinity, high-capacity pumps

The Na^+/Ca^{2+} exchanger (NCX), the Na^+/Ca^{2+}–K^+ exchanger (NCKX) and the mitochondrial uniporter, for example, have low affinities for Ca^{2+}, but have very high capacities, and this enables them to function early in the recovery process, since they can rapidly remove the large quantities of Ca^{2+} that are released into the cytoplasm during signalling. The high capacity of NCX and NCKX is based on the rapid turnover rate of the exchanger, which can carry out 1000 to 5000 reactions/s.

High-affinity, low-capacity pumps

On the other hand, the plasma membrane Ca^{2+}-ATPase (PMCA), sarco/endo-plasmic reticulum Ca^{2+}-ATPase (SERCA) and secretory-pathway Ca^{2+}-ATPase (SPCA) pumps have lower capacities, but their higher affinities mean that they can continue to pump at lower Ca^{2+} levels, thus enabling them to maintain the internal stores and the resting level. The SPCA is unusual in that it can pump Mn^{2+} equally as well as Ca^{2+}. The PMCA and SERCA pumps have low capacities, because the ATP-dependent conformational process that occurs during the pumping mechanism occurs at a low rate (approximately 150 reactions/sec). These two pumps belong to the P_2 subfamily of P-type ion transport ATPases that are characterized by the formation of an aspartyl phosphate during the reaction cycle of the pump mechanism.

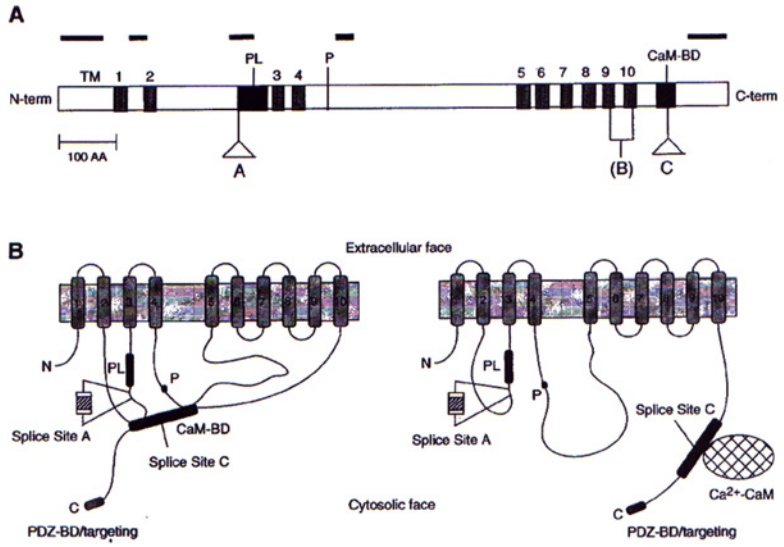
Organization and distribution of Ca^{2+} pumps

Ca^{2+} pumps have molecular structures designed to transfer Ca^{2+} ions across membranes against very large electrochemical gradients. This pumping problem has been solved in different ways. The diversity of Ca^{2+} pumps depends upon the existence of multigene families (Module 5: Table Ca^{2+} pumping toolkit) within which additional diversity is generated by alternative splicing. This diversity creates many isoforms with subtle variations, not only in their pumping properties but also in their Ca^{2+} pump regulation. An important consequence of all this diversity is that each cell has access to an enormous repertoire from which it can select out those pumps with properties exactly suited to their particular signalling requirements.

Plasma membrane Ca^{2+}-ATPase (PMCA)

The plasma membrane Ca^{2+}-ATPase (PMCA) gene family contains four closely related genes (PMCA1–PMCA4) with numerous alternatively spliced forms denoted by the lower-case letters of the alphabet (Module 5: Table Ca^{2+} pumping toolkit). Expression of these various splice isoforms is a regulated event in that they change in a consistent way during both development and differentiation. There are indications that changes in the level of Ca^{2+} may influence the expression of these splice isoforms. For example,

Module 5: | Figure PMCA domain structure



Domain structure of the plasma membrane Ca^{2+}-ATPase (PMCA).
A. The sites marked A and C are the main sites where alternative splicing occurs to create at least 20 different isoforms. These two splice sites occur in the two large cytoplasmic loops, and are thus likely to influence the way in which these two loops regulate pump activity. B. In the absence of calmodulin (CaM), the autoinhibitory C-terminal region is thought to bend around to inhibit enzymatic activity. In the presence of Ca^{2+}, CaM binds to the CaM-binding domain (CaMBD), and this regulatory chain is pulled away, resulting in an increase in pump activity. Reproduced from Strehler, E.E. and Zacharias, D.A. (2001) Role of alternative splicing in generating isoform diversity among plasma membrane calcium pumps. Physiol. Rev. 81:21–50; used with permission from The American Physiological Society; see Strehler and Zacharias 2001.

an elevation in the level of Ca^{2+} in cerebellar granule cells results in the up-regulation of PMCA1a, PMCA2 and PMCA3, but a down-regulation of PMCA4a. The main domain structure of the PMCAs reveals the presence of ten transmembrane (TM) domains with two large cytosolic loops between TM2 and TM3 and between TM4 and TM5 (Module 5: Figure PMCA domain structure). The latter is particularly significant, because it contains the aspartyl phosphorylation site (P). These two loops have important functions in Ca^{2+} pump regulation of PMCA activity. The PMCA isoforms 1 and 4 are widely expressed (Module 5: Table Ca^{2+} pumping toolkit), whereas isoforms 2 and 3 are mainly restricted to the brain and skeletal muscle. Within the brain, there are regional differences in the expression of these isoforms, e.g. PMCA2 is high in cerebellar Purkinje cells and cochlear hair cells, whereas PMCA3 is found mainly in the choroid plexus. A mutation in PMCA2 results in hearing loss.

One consequence of pump diversity is that cells have access to pumps that transport at different rates. Cells that have to generate rapid transients have the fastest pumps. For example, PMCA3f (skeletal muscle) and PMCA2a (stereocilia) are the fastest, whereas PMCA4b (Jurkat cells) is the slowest.

In kidney tubule cells, the PMCA1b plays an important role in the reabsorption of Ca^{2+} by the paracellular transport pathway (Module 7: Figure kidney Ca^{2+} reabsorption). The expression of genes that code for PMCA1b and PMCA2c are enhanced through the vitamin D control of Ca^{2+} homoeostasis.

Plasma membrane Ca^{2+}-ATPase (PMCA) molecular structure

Despite the large molecular diversity within the plasma membrane Ca^{2+}-ATPase (PMCA) family, the overall structure of all the members is very similar. They have ten transmembrane domains with both the N-terminal and C-terminal regions facing the cytosol (Module 5: Figure PMCA domain structure). Most of the extracellular and intracellular loops that link the transmembrane domains are relatively short, except for two of the four loops that face the cytosol. The largest cytoplasmic loop connecting TM4 and TM5 is of particular significance because it contains two important sites for the pump cycle. The first site is the nucleotide-binding domain, where the ATP binds to the pump molecule. The second site is the phosphorylation domain, which contains the invariant aspartate residue that is phosphorylated during the conformational changes that occur during each pump cycle (Module 5: Figure SERCA pump cycle).

The location of the PMCA pumps may be determined by binding to a family of PDZ domain-containing proteins. Such an interaction may occur through the PDZ interaction domains located in the C-terminal region.

Sarco/endo-plasmic reticulum Ca^{2+}-ATPase (SERCA)

The sarco/endo-plasmic reticulum Ca^{2+}-ATPase (SERCA) family of pumps contains three genes with numerous alternatively spliced isoforms (Module 5: Table Ca^{2+} pumping toolkit). The role of SERCA is to pump Ca^{2+} back into the endoplasmic reticulum

Module 5: | Table Ca^{2+} pumping toolkit
Summary of genomic organization, spliced isoform and distribution of Ca^{2+} pumps and exchangers.

Component	Spliced isoform	Distribution
Plasma membrane Ca^{2+}-ATPase (PMCA) pumps		
PMCA1 (the human gene is located on 12q21–q23)	PMCA1a	Excitable cells: brain, skeletal muscle, heart and kidney
	PMCA1b	Ubiquitous; a housekeeper pump
	PMCA1c	Skeletal muscle, heart
	PMCA1d	Skeletal muscle, heart
	PMCA1e	Brain
	PMCA1x	Ubiquitous; a housekeeper pump
PMCA2 (the human gene is located on 3p25–p26)	PMCA2a	Brain, heart, uterus
	PMCA2b	Widespread
	PMCA2c	Testis
	PMCA2w	Brain, kidney, uterus
	PMCA2x	Brain, heart
	PMCA2y	
	PMCA2z	Brain, heart
PMCA3 (the human gene is located on Xq28)	PMCA3a	Brain, spinal cord, testis
	PMCA3b	Adrenal, brain, skeletal muscle
	PMCA3c	
	PMCA3d	Brain
	PMCA3e	Skeletal muscle
	PMCA3f	Brain, skeletal muscle
	PMCA3x	
	PMCA3z	
PMCA4 (the human gene is located on 1q25–q32)	PMCA4a	Widespread
	PMCA4b	Ubiquitous; a housekeeper pump
	PMCA4x	Widespread
	PMCA4z	Heart, testis
Sarco/endo-plasmic reticulum Ca^{2+}-ATPase (SERCA) pumps		
SERCA1	SERCA1a	Fast twitch skeletal muscle
	SERCA1b	Fast twitch skeletal muscle
SERCA2	SERCA2a	Cardiac and slow twitch skeletal muscle
	SERCA2b	Ubiquitous; a housekeeper pump in smooth muscle and many other cells
	SERCA2c	Heart and skeletal muscle
SERCA3	SERCA3a	Mast cells, lymphocytes, platelets, monocytes, vascular endothelial cells and cerebellar Purkinje cells
	SERCA3b	Haematopoietic cells; blood platelets
	SERCA3c	Haematopoietic cells; Blood platelets
	SERCA3d	Heart and skeletal muscle
	SERCA3e	Pancreas and lung
	SERCA3f	Heart and skeletal muscle
Secretory-pathway Ca^{2+}-ATPase (SPCA) pumps		
SPCA1	SPCA1a–SPCA1d	Ubiquitous; located in the Golgi.
SPCA2		
Na^+/Ca^{2+} exchangers (NCXs)		
NCX1		Heart, kidney
NCX2		Neurons
NCX3		Neurons
Na^+/Ca^{2+}–K^+ exchangers (NCKXs)		
NCKX1		Rod photoreceptors (Module 10: Figure phototransduction) and platelets
NCKX2		Brain, cone photoreceptors
NCKX3		Brain, aorta, uterus and intestine
NCKX4		Brain, aorta, lung and thymus

With regard to the distribution, the list is not complete, but includes those tissues where the different isoforms are strongly expressed. (For a detailed description of the nomenclature and distribution of spliced variants, see Strehler and Zacharias 2001 for the PMCA pumps and Schnetkamp 2004 for NCKX.)

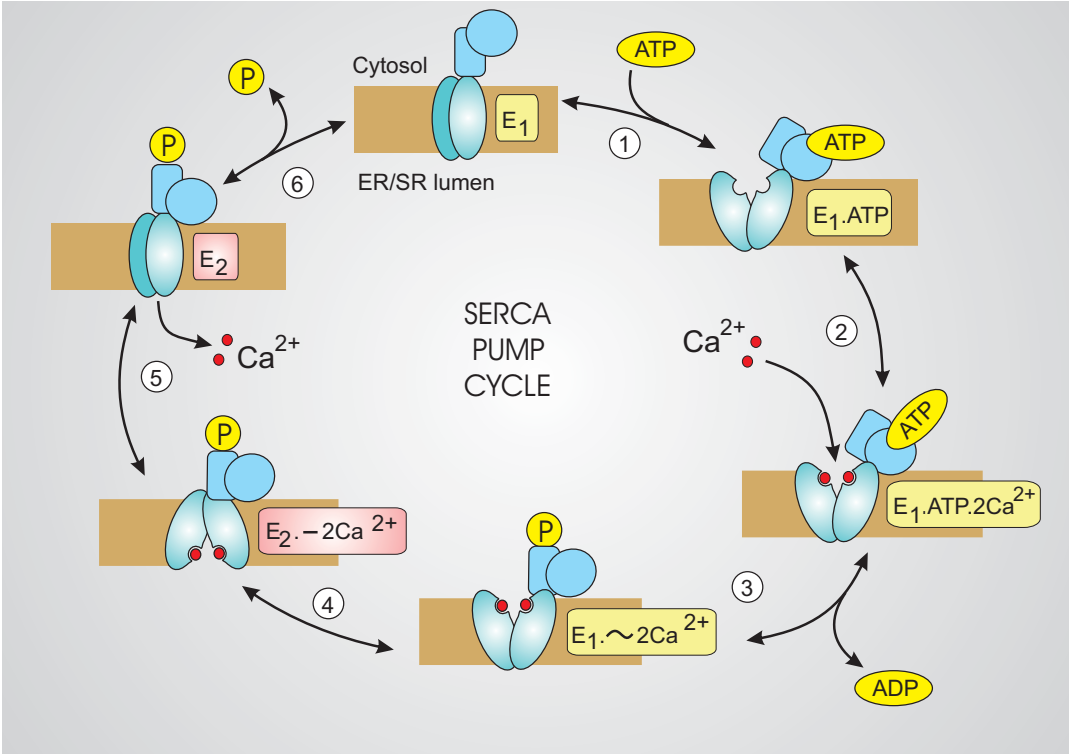
(ER)/sarcoplasmic reticulum (SR) (Module 5: Figure Ca^{2+} uptake and extrusion). There have been considerable advances in the understanding of the sarco/endo-plasmic reticulum Ca^{2+}-ATPase (SERCA) pump structure and mechanism of Ca^{2+} transfer across the ER/SR membrane.

Inactivating mutations of SERCA1 are the cause of Brody disease. Expression of SERCA2 is regulated by miR-152. A mutation of the SERCA2 pump causes Darier's disease. A decline in the activity of SERCA2a occurs in congestive heart failure (CHF) and may be associated with a decline in sumoylation. Addition of SUMO1 to SERCA2a markedly enhanced its stability. *SUMO-1* gene transfer has proved to be an effective therapy in animal models of CHF

Sarco/endo-plasmic reticulum Ca^{2+}-ATPase (SERCA) pump structure and mechanism

The resolution of the sarco/endo-plasmic Ca^{2+}-ATPase (SERCA) pump structure has provided a detailed scenario of how this pump might work. For the plasma membrane Ca^{2+}-ATPase (PMCA) and SERCA pumps, some of the transmembrane domains make up the Ca^{2+}-binding site

Module 5: | Figure SERCA pump cycle



The sarco/endo-plasmic reticulum Ca^{2+}-ATPase (SERCA) pump cycle.
The pump cycle consists of a series of biochemical reactions during which the pump switches between two major conformational states: an E_1 state when the Ca^{2+}-binding site faces the cytoplasm, and an E_2 state where the binding sites have switched to the opposite side and Ca^{2+} is released to the lumen. During each cycle, two Ca^{2+} ions are pumped for each ATP hydrolysed. This switch between the E_1 and E_2 states represents one cycle, and this occurs at a frequency of about 150 reactions/s. The way in which ATP powers each pump cycle is described in the text.

used to transfer Ca^{2+} across the membrane. ATP provides the energy to drive this transfer. Like the PMCA pump, the SERCA pump is a member of the P-type pumps, so called because the pump is energized by ATP phosphorylating an aspartate residue. This phosphorylation event induces the conformational change necessary to drive Ca^{2+} across the membrane. The following sequence of events occurs during the SERCA pump cycle (Module 5: Figure SERCA pump cycle):

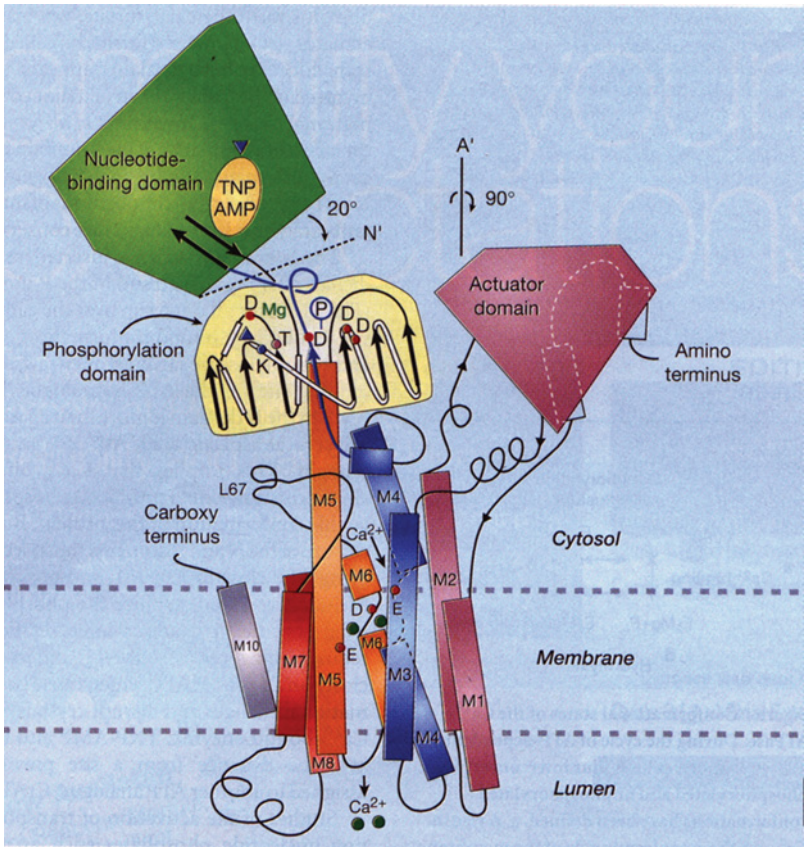
1. The resting E_1 state is energized by binding ATP to unveil two Ca^{2+}-binding sites that face the cytoplasm (E_1.ATP).
2. Ca^{2+} enters the two binding sites to form the E_1.ATP.2Ca^{2+} complex.
3. The binding of Ca^{2+} strongly activates the ATPase activity of the pump, resulting in the release of ADP and the transfer of phosphate to an aspartate residue to form a high-energy phosphorylated intermediate (E_1–P~2Ca^{2+}).
4. The energy stored in this phosphorylated intermediate is used to induce the conformational change to the E_2–P.2Ca^{2+} state during which Ca^{2+} moves across the bilayer.
5. In the lower-energy E_2–P.2Ca^{2+} state, the binding sites have a reduced affinity for Ca^{2+}, which is free to diffuse into the lumen.
6. Hydrolysis of the E_2–P phosphoenzyme enables the pump to return to the resting E_1 state, ready to begin another cycle.

The next question to consider is the molecular basis of this pump cycle. How are the individual steps of the pump cycle (Module 5: Figure SERCA pump cycle) related to the molecular structure of SERCA?A feature of SERCA structure is characterized by a number of distinct domains (Module 5: Figure SERCA1a pump), which have clearly defined roles at different stages of the pump cycle.

ATP binding and the loading of Ca^{2+} on to the binding site

The first steps in the pump cycle (Steps 1 and 2 in Module 5: Figure SERCA pump cycle) is ATP binding and the loading of Ca^{2+} on to the external binding sites. This ATP binds to the N domain, which forms a cap sitting over the P domain (Module 5: Figure SERCA1a pump). The resulting conformational change within the M (transmembrane) domains opens up two Ca^{2+}-binding sites. The first point to notice is that the N domain is somewhat removed from the transmembrane domains where Ca^{2+} translocates through the membrane. The transmission of such a conformational change has to be mediated through long-range allosteric interactions. The pathway for transmitting such molecular changes is still not clear, but there are several possibilities. One possibility is that the A domain (also referred to as the

Module 5: | Figure SERCA1a pump



Functional operation of the sarco/endo-plasmic reticulum Ca^{2+}-ATPase 1a (SERCA1a) pump.

The main domains of the sarco/endo-plasmic reticulum Ca^{2+}-ATPase 1a (SERCA1a) pump concerned with pumping are the nucleotide-binding domain (N), the phosphorylation domain (P), the actuator domain (A) and the transmembrane domains (M1–M10). The molecular events involving these domains are described in the text by reference to the transition states of the pump cycle. Reproduced by permission from Macmillan Publishers Ltd: *Nature*, MacLennan, D.H. and Green, N.M. (2000) Structural biology: pumping ions. 405:633–634. Copyright (2000); http://www.nature.com; see MacLennan and Green 2000.

transducer domain) may play a role. Another possibility is that information might be transmitted through the long rod-like central helix of M5 that extends from the inside of the membrane right up to the underside of the P domain. This seems to be a likely mechanism, because M5, together with M4 and M6, are the transmembrane domains that form the Ca^{2+}-binding pocket. Note that the N and P domains are formed from the loop that connects to M4 and M5. It is suggested that the conformational changes cause a disruption of M4 and M6 helices, and this opens up a pocket for the two Ca^{2+} ions to bind.

Phosphorylation of the aspartate residue and Ca^{2+} translocation through the membrane

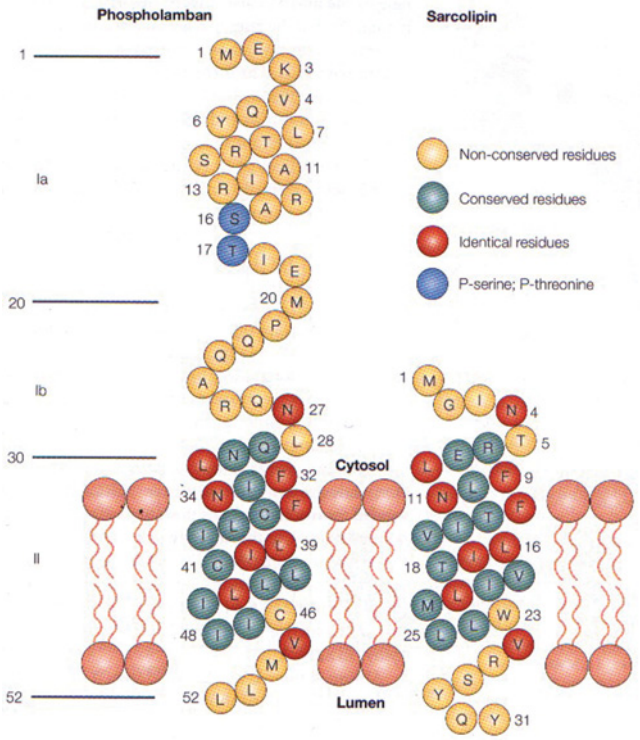
A critical phase in the transport process is the interaction between the N and P domains during which the terminal phosphate group of ATP is transferred to Asp-351. The problem here is that the ATP-binding site on the N domain appears from the structure to be located far away from this phosphorylation site. Somehow the two domains have to move in order for the two sites to approach close enough for the phosphorylation to occur. Once the energized $E_1{\sim}P$ state is formed, another conformational change transmitted through the mechanisms discussed earlier brings about the movement of the M domains so that the Ca^{2+}-binding pocket is altered to face the lumen to allow Ca^{2+} to enter the endoplasmic reticulum (ER). This remarkable molecular machine is beautifully designed to efficiently couple the site of energy conversion (the phosphorylation domain) to the translocation mechanism in the membrane.

Ca^{2+} pump regulation

One of the hallmarks of Ca^{2+} pumps is their regulation, which enables them to adapt to changing circumstances. The most direct form of regulation is for Ca^{2+} to regulate its own activity, and this is particularly apparent for the plasma membrane Ca^{2+}-ATPase (PMCA) pump. Ca^{2+} acts through calmodulin (CaM) to stimulate the pump. When the pump is activated through the Ca^{2+}/CaM mechanism, the CaM remains bound for some time after Ca^{2+} signalling has ceased, thus allowing the pump to have a 'memory' so that it can respond more quickly to another Ca^{2+} transient.

There are various redox signalling effects on Ca^{2+} signalling and one of these is that an increase in reactive oxygen species (ROS) can influence Ca^{2+} homoeostasis by

Module 5: | Figure phospholamban and sarcolipin



The structural organization of the sarco/endo-plasmic reticulum Ca^{2+}-ATPase SERCA pump regulators phospholamban (PLN) and sarcolipin (SLN).
Phospholamban (PLN) has two main regions: a C-terminal α-helix that embeds the molecule in the sarcoplasmic reticulum (SR) membrane and an N-terminal cytoplasmic region. The latter has an α-helical region that is connected to the membrane region by a short β-turn. Sarcolipin (SLN) resembles PLN, except that it lacks most of the cytoplasmic domain. The high degree of homology, both conserved residues (grey/green) and identical residues (red), within the transmembrane domain indicates that this region is of particular significance in mediating the ability of these proteins to interact with, and inhibit, SERCA pumps. This inhibitory effect of PLN is reversed by protein kinase A (PKA) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) phosphorylating Ser-16 and Thr-17 respectively. Reproduced by permission from Macmillan Publishers Ltd: *Nat. Rev. Mol. Cell Biol.*, MacLennan, D.H. and Kranias, E.G. (2003) Phospholamban: a crucial regulator of cardiac contractility. 4:566–577. Copyright (2003); http://www.nature.com/nrm; see MacLennan and Kranias 2003.

oxidizing and inhibiting the PMCA, which will result in an elevation of the resting level of Ca^{2+}. Such a mechanism may be particularly relevant for the effect of inflammation in Alzheimer's disease (Module 12: Figure inflammation and Alzheimer's disease).

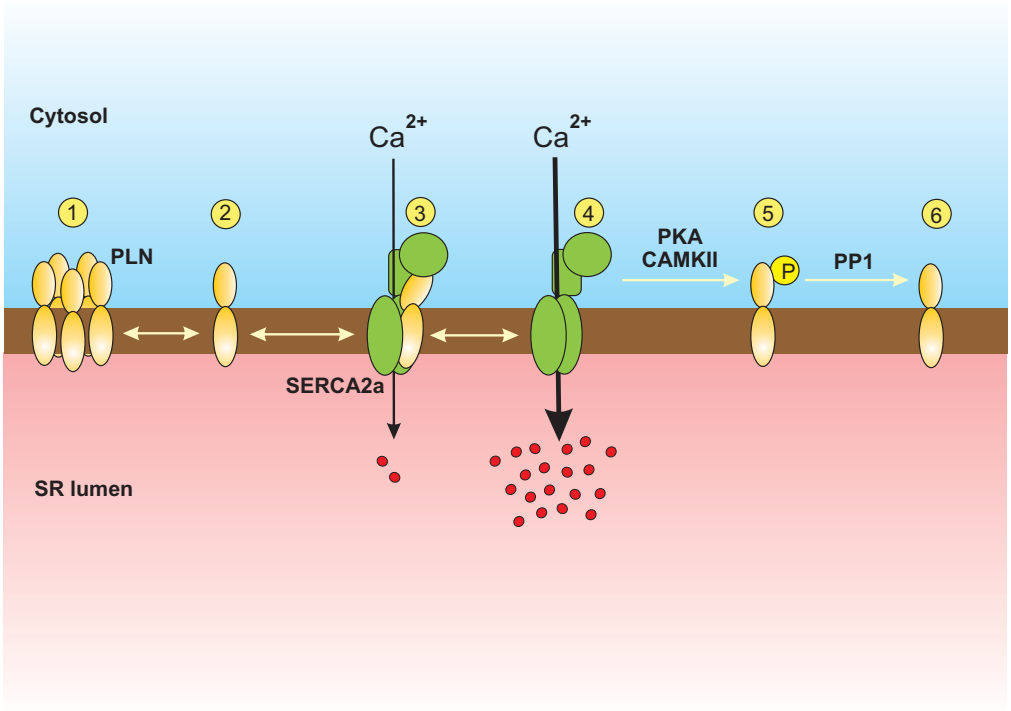
The Ca^{2+} pumps are sensitive to hormonal regulation, with control being exerted through various regulators such as phospholamban (PLN) and sarcolipin (SLN). PLN is particularly important, as it is sensitive to various signalling pathways operating through second messengers such as cyclic AMP and Ca^{2+} itself. Such regulation is critical for regulating cardiac contractility (Module 7: Figure ventricular Ca^{2+} signalling), where the strength of contraction is controlled by cyclic AMP, which acts by phosphorylating PLN to remove its inhibitor effect on the sarco/endo-plasmic reticulum Ca^{2+}-ATPase (SERCA) pump.

Phospholamban (PLN)

Phospholamban (PLN) is the primary regulator of the sarco/endo-plasmic reticulum Ca^{2+}-ATPase (SERCA) pump. It has an important function in cardiac cells, where it regulates the SERCA2a isoform (Step 7 in Module 7: Figure ventricular Ca^{2+} signalling), but it is also active in smooth muscle cells (Module 7: Figure smooth muscle cell spark). PLN is a transmembrane protein containing 52 amino acids (Module 5: Figure phospholamban and sarcolipin). It exists within the membrane in a number of states, with the unphosphorylated monomeric state being the one that binds to cardiac SERCA2a to inhibit its activity. PLN has a number of function states (Steps 1 to 6 in Module 5: Figure phospholamban mode of action):

1. A PLN pentamer, which forms when five PLN monomers come together, is stabilized by leucine–isoleucine zipper interactions.
2. Monomeric unphosphorylated PLN is the active form that binds to SERCA2a.
3. PLN binds in a groove running up both the transmembrane and cytosolic regions of SERCA2a (Module 5: Figure SERCA pump structure). PLN exerts its inhibitory action by regulating the Ca^{2+} affinity of the SERCA2a pump.

Module 5: | Figure phospholamban mode of action



Phospholamban (PLN) operates within the plane of the sarcoplasmic reticulum (SR) membrane to regulate the activity of the sarco/endo-plasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a) pump.

Phospholamban (PLN) operates in the plane of the sarcoplasmic reticulum, where it acts to regulate the activity of the sarco/endo-plasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a) pump. PLN has five distinct functional states, as described in the text.

4. The SERCA2a pump increases its Ca^{2+} pumping activity when the inhibitory effect of PLN is removed following its phosphorylation by protein kinase A (PKA) or Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII).
5. Inactive PLN is phosphorylated on either Ser-16 (by PKA) or Thr-17 (by CaMKII) (Module 5: Figure phospholamban and sarcolipin).
6. The phosphorylated PLN is converted back into its active inhibitory form following dephosphorylation by protein phosphatase PP1 (Module 5: Figure PP1 targeting to glycogen).

Studies on transgenic mice have established that PLN features significantly in the relationship between Ca^{2+} signalling and cardiac hypertrophy. There are also two examples of inherited human dilated cardiomyopathy that have been traced to mutations in PLN.

Sarcolipin (SLN)

Sarcolipin (SLN) resembles phospholamban (PLN) with regard to its transmembrane region, but its cytoplasmic region has been truncated (Module 5: Figure phosphalamban and sarcolipin). SLN is strongly expressed in fast-twitch skeletal muscle, but is low in heart. Like PLN, SLN functions to regulate sarco/endo-plasmic reticulum Ca^{2+}-ATPase (SERCA) activity and appears to bind to a similar groove in the SERCA molecule.

Secretory-pathway Ca^{2+}-ATPase (SPCA)

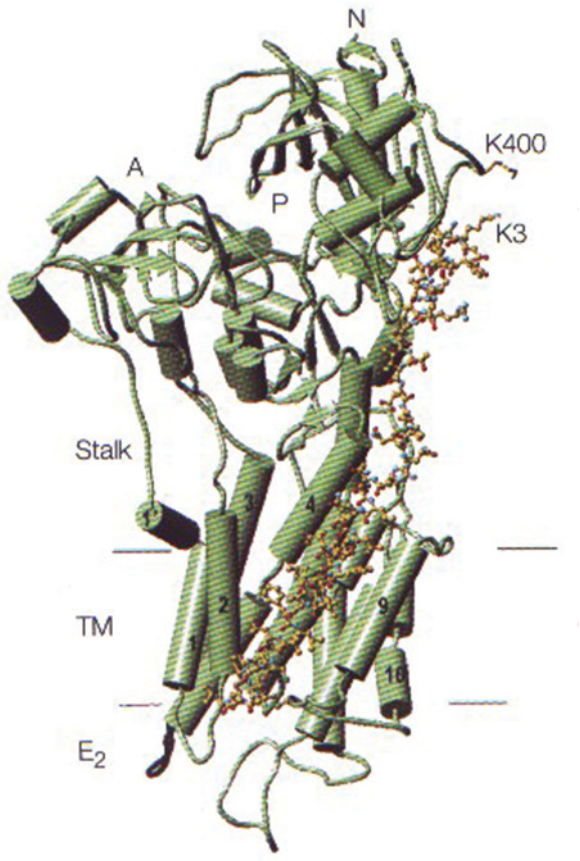
The Golgi contains both a sarco/endo-plasmic reticulum Ca^{2+}-ATPase (SERCA) pump and a secretory-pathway Ca^{2+}-ATPase (SPCA) that is responsible for pumping Ca^{2+} into the Golgi stacks. There is a variable distribution of these two pumps within the Golgi. The SERCA is found in the early parts of the Golgi, thus reflecting its origin from the endoplasmic reticulum (ER). The SPCA is restricted to the *trans*-Golgi region. The SPCA pumps Ca^{2+} into the Golgi using mechanisms similar to those described above for the SERCA pumps. Unlike the SERCA pumps, however, the SPCA is able to pump Mn^{2+} equally as well as Ca^{2+}.

There are two SPCA isoforms (SPCA1 and SPCA2). The *ATP2C1* gene that encodes SPCA1 is mutated in Hailey-Hailey disease.

Sodium/calcium exchangers (NCX and NCKX)

The Na^+/Ca^{2+} exchangers play a critical role in Ca^{2+} signalling because they provide a mechanism for rapidly extruding Ca^{2+} from cells (Module 5: Figure Ca^{2+} uptake and extrusion). These exchangers are particularly important in excitable cells such as cardiac cells, neurons and sensory neurons. However, they are also expressed on various non-excitable cells. There are two families of exchangers, the Na^+/Ca^{2+} exchanger (NCX) and the Na^+/Ca^{2+}–K^+ exchanger (NCKX) families (Module 5: Table Ca^{2+} pumping toolkit).

Module 5: | Figure SERCA pump structure



Interaction between phospholamban (PLN) and the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump.
Reproduced by permission from Macmillan Publishers Ltd: *Nat. Rev. Mol. Cell Biol.*, MacLennan, D.H. and Kranias, E.G. (2003) Phospholamban: a crucial regulator of cardiac contractility. 4:566–577. Copyright (2003); http://www.nature.com/nrm; see MacLennan and Kranias 2003.

Na^+/Ca^{2+} exchanger (NCX)

The Na^+/Ca^{2+} exchanger (NCX) was the first exchanger to be discovered. It functions to extrude Ca^{2+} from the cell in exchange for Na^+ (Module 5: Figure sodium/calcium exchangers). The energy from the Na^+ gradient across the plasma membrane is used to drive Ca^{2+} out of the cell against the large electrochemical gradient. There is no direct role for ATP, but it does have an indirect role because it powers the ouabain-sensitive Na^+ pump that establishes the Na^+ gradient. In kidney tubule cells, the NCX plays an important role in the reabsorption of Ca^{2+} by the paracellular transport pathway (Module 7: Figure kidney Ca^{2+} reabsorption).

The NCX has nine transmembrane (TM) segments with a large cytoplasmic loop connecting TM5 and TM6. The regions shaded in yellow in Module 5: Figure sodium/calcium exchangers contain α repeats (α1 and α2), which are highly homologous with two similar regions in the Na^+/Ca^{2+}–K^+ exchanger (NCKX). These are the only two regions that show close homology, and this would fit with the notion that α1 and α2 play a role in the binding and transport of cations.

NCX can operate in two modes, depending on the electrochemical potential, which determines the directionality of the Na^+ flux that drives the movement of Ca^{2+}. In the forward mode, Ca^{2+} is extruded from the cell, whereas in the reverse mode, Ca^{2+} is brought into the cell. This reverse mode may play an important role during excitation–contraction (E-C) coupling in heart cells, where the rapid build-up of Na^+ during the course of the action potential results in a local build-up of this ion, which then begins to flow out in exchange for Ca^{2+}. This influx of Ca^{2+} will contribute to the trigger Ca^{2+} entering through the L-type channel, and thus could facilitate the excitation processes. The NCX1 isoform has an important role in ventricular cell Ca^+ release (Module 7: Figure ventricular Ca^{2+} signalling).

There is some debate concerning the exact stoichiometry of NCX. Most measurements suggest that three Na^+ ions are transported for each Ca^{2+} ion, which means that the exchanger is electrogenic.

Na^+/Ca^{2+}–K^+ exchanger (NCKX)

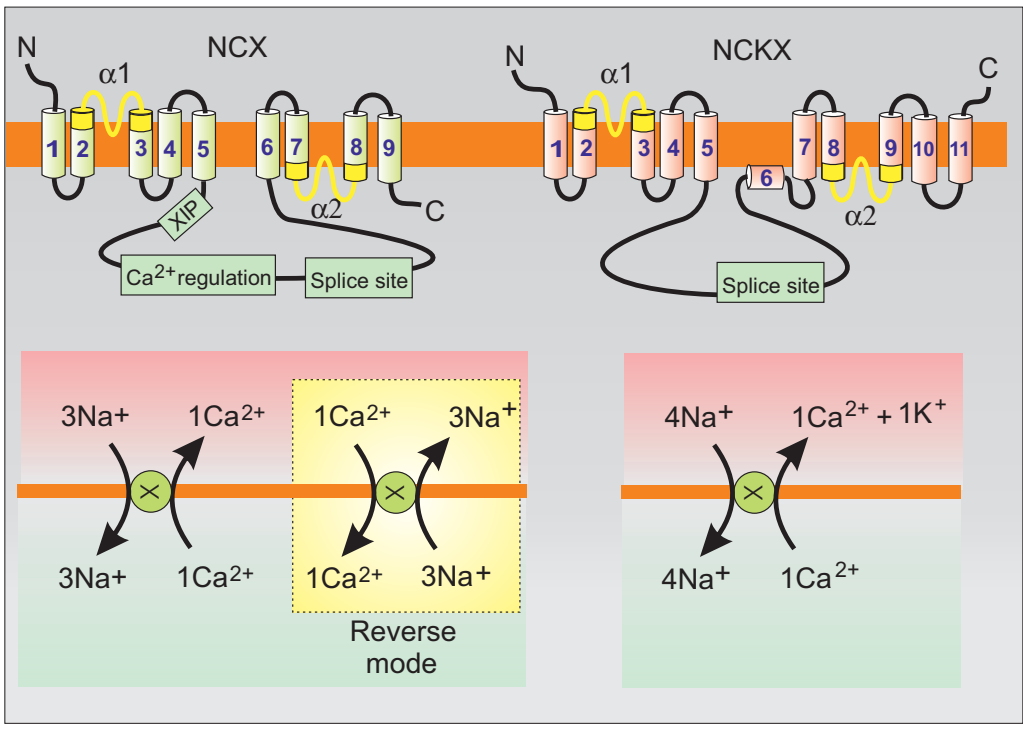
The Na^+/Ca^{2+}–K^+ exchangers (NCKXs) were first discovered in rod photoreceptors. There is a family of these exchangers with different distributions in both excitable and non-excitable cells (Module 5: Table Ca^{2+} pumping toolkit). They differ from the Na^+/Ca^{2+} exchangers (NCXs) in that they extrude both Ca^{2+} and K^+ in exchange for Na^+ (Module 5: Figure sodium/calcium exchangers). While some of their structural features resemble those found in NCX, there clearly are marked differences. The regions of closest homology are the two regions of α repeats (α1 and α2 shown in yellow), which play a role in binding cations during the exchange reaction.

The function of these exchangers has been defined best in photoreceptors, where they are the primary mechanism for extruding Ca^{2+} during the process of phototransduction (see Step 5 in Module 10: Figure phototransduction overview). The organization of the NCKX1 isoform in the photoreceptor is of interest because it appears to be complexed to the cyclic nucleotide-gated channel (CNGC) that is responsible for the cyclic GMP-dependent entry of Ca^{2+}. Furthermore, this entry channel/exchanger complex also appears to be linked to two proteins (peripherin and Rom-1) located in the rim of the intracellular disc. The functional significance of linking the plasma membrane to the internal disc through this protein complex is unknown.

Mitochondria

Mitochondria distributed throughout the cytoplasm have many functions. They generate ATP, they shape Ca^{2+} signals and respond to Ca^{2+} signals by increasing the production of ATP, they generate reactive oxygen species (ROS) and under extreme conditions, they release factors such as cytochrome *c* to induce apoptosis. Their primary function is the generation of ATP by oxidative phosphorylation. In addition, they also play a critical role in a number of other aspects of cell signalling, particularly Ca^{2+} signalling. Mitochondria contribute to

Module 5: | Figure sodium/calcium exchangers



Structure and function of sodium/calcium exchangers.

The organization and operation of the Na^+/Ca^{2+} exchanger (NCX) is shown on the left and the Na^+/Ca^{2+}–K^+ exchanger (NCKX) is shown on the right. There is a large central cytoplasmic loop, part of which has a site where alternative splicing occurs. NCX also has a Ca^{2+} regulatory site and a binding site for the exchanger inhibitory peptide (XIP). Both exchangers have two regions (α1 and α2, shown in yellow) containing α repeats that are highly homologous and are thought to be the sites where the cations bind to the exchangers. See the text for further details.

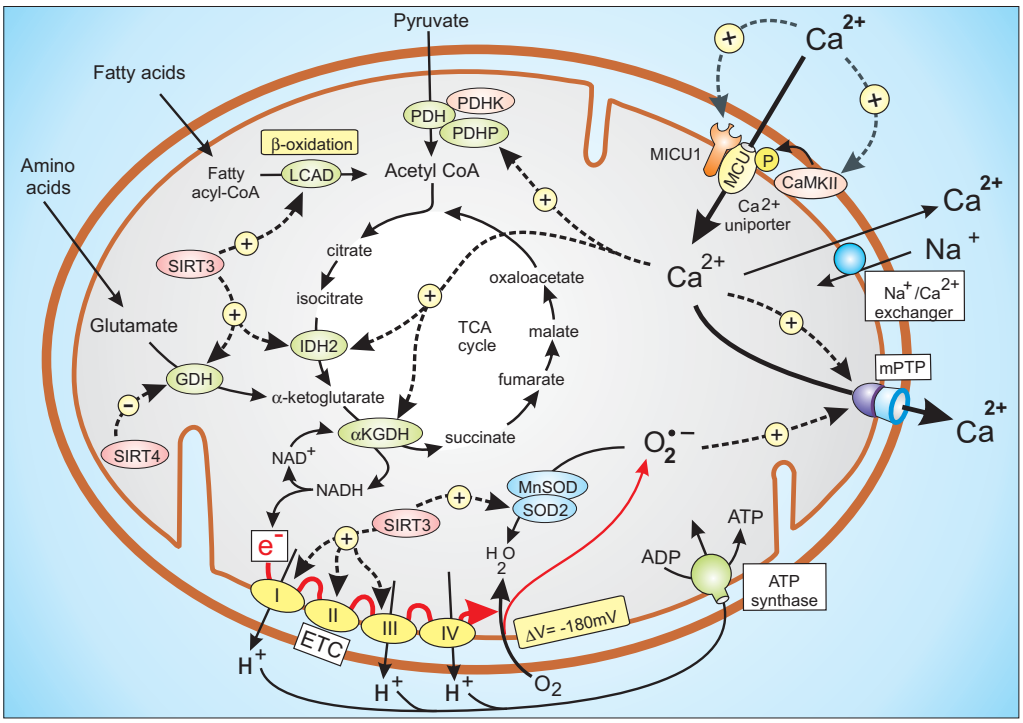
the dynamics of Ca^{2+} signalling (Module 5: Figure Ca^{2+} uptake and extrusion) by participating in the OFF reactions that remove Ca^{2+} from the cytoplasm during the recovery phase (Module 2: Figure Ca^{2+} transient mechanisms). Mitochondrial Ca^{2+} uptake through the mitochondrial Ca^{2+} uniporter (MCU) is responsible for the mitochondrial modulation of Ca^{2+} signals. Mitochondria function as Ca^{2+} buffers capable of shaping both the amplitude and the spatiotemporal profile of Ca^{2+} signals. Mitochondrial Ca^{2+} release mechanisms return Ca^{2+} back into the cytosol, where it can be sequestered by the endoplasmic reticulum (ER)/sarcoplasmic reticulum (SR). Indeed, there is a close functional relationship between the mitochondria and the ER/SR that occurs at a specialized contact zone known as the mitochondria-associated ER membranes (MAMs) (Module 5: Figure mitochondria-associated ER membrane).

An endoplasmic reticulum (ER)/mitochondrial Ca^{2+} shuttle, which is important for intracellular Ca^{2+} dynamics and cell signalling, can be both beneficial and deleterious. With regard to the former, an increased Ca^{2+} concentration within the mitochondrial matrix stimulates enzymes associated with the tricarboxylic acid (TCA) cycle, resulting in an increase in ATP production. Therefore there is a two-way relationship between cytosolic Ca^{2+} signals and mitochondrial function. In addition to the mitochondrial modulation of Ca^{2+} signals, mentioned above, there is a reciprocal Ca^{2+} modulation of mitochondrial function. For example, the uptake of Ca^{2+} acts to stimulate the oxidative processes that produce ATP. This increase in oxidation also enhances mitochondrial reactive oxygen species (ROS) formation (Module 2: Figure sites of ROS formation) that contributes to the redox signalling pathway. In addition, an alteration in the normal ebb and flow of Ca^{2+} through the mitochondria can be deleterious when an abnormally high load of Ca^{2+} is transferred from the ER/SR to the mitochondrion. This excessive uptake of Ca^{2+} into the mitochondria can activate the formation of the mitochondrial permeability transition pore (mPTP), which results in the release of proteins such as cytochrome *c*, which induce the caspase cascade that contributes to the apoptotic signalling network. Indeed, a large number of cell death signals appear to operate through the ER/mitochondrial Ca^{2+} shuttle.

Generation of ATP

The mitochondrion is often referred to as the 'powerhouse' of the cell because of its ability to generate ATP. However, this role in energy transformation is intimately connected with its other role, which is to modulate Ca^{2+} signalling (Module 5: Figure mitochondrial Ca^{2+} signalling). Mitochondria can metabolize a number of carbon sources such as pyruvate, fatty acids and amino acids (e.g.glutamine). Pyruvate is converted into acetyl-CoA by pyruvate dehydrogenase (PDH) that is fed into the tricarboxylic acid (TCA) cycle. The activity of PDH

Module 5: | Figure mitochondrial Ca^{2+} signalling



Relationship between mitochondrial metabolism and Ca^{2+} signalling.
Mitochondria are energized by pyruvate, which enters the mitochondrion where it is metabolized by the tricarboxylic acid (TCA) cycle (or citric acid cycle) to produce the reducing equivalents that are transferred to the electron transport chain located in the inner mitochondrial membrane. The subsequent extrusion of protons creates a large (−150 to −180 mV) negative membrane potential across this inner membrane. This large proton gradient is used to energize both ATP synthesis and the uptake of Ca^{2+}. Protons flowing back through the oligomycin-sensitive ATP synthase drive the synthesis of ATP.

is regulated by reversible phosphorylation: PDH kinase (PDHK) phosphorylates and inactivates PDH, whereas a PDH phosphatase (PDHP) removes this phosphorylation resulting in PDH activation. This PDHP is activated by Ca^{2+}. Fatty acids enter the mitochondrion and are then converted into acetyl-CoA through a process of β-oxidation. One of the key enzymes of β-oxidation is long-chain acyl CoA dehydrogenase (LCAD), which is activated by SIRT3. Amino acids can also be used to fuel the TCA cycle. For example, after entering the mitochondrion, glutamine is converted into glutamate that is then converted into α-ketoglutarate by glutamate dehydrogenase (GDH). The activity is regulated by both SIRT3 and SIRT4. The subsequent conversion of α-ketoglutarate dehydrogenase (αKGDH) is accompanied by the reduction of NAD^+ to NADH, which then functions to feed an electron (e^-) into the electron transport chain (ETC). Some of the enzymes of the TCA, such as PDHP, αKGDH and isocitrate dehydrogenase 2 (IDH2), are activated by Ca^{2+} as part of Ca^{2+} modulation of mitochondrial function (Module 5: Figure mitochondrial Ca^{2+} signalling). The mitochondrial sirtuins also play an important role in regulating this metabolic activity,

The electrons derived from TCA then travel down the electron transport chain (ETC), which is composed of the respiratory complexes I–IV, during which protons are ejected into the cytoplasm. Oxygen is the final electron acceptor, but approximately 2-5% of the oxygen consumed is incompletely reduced and appears as superoxide ($O_2^{-}\bullet$), and this mitochondrial reactive oxygen species (ROS) formation can contribute to the redox signalling pathway. Efficient operation of the ETC is very dependent on cardiolipin (CL), which provides a lipid environment to maximize the flow of electrons along the various carriers (Module 5: Figure cardiolipin).

The removal of H^+ creates the large membrane potential of –180 mV, which is used to energize both ATP synthesis by the ATP synthase and the uptake of Ca^{2+} by the uniporter. This uptake of Ca^{2+} has a maximum velocity that is very much larger than the Ca^{2+} exchanger that returns Ca^{2+} to the cytoplasm, which means that mitochondria can rapidly accumulate large amounts of Ca^{2+}, much of which is bound to mitochondrial buffers or it precipitates as crystals of calcium phosphate. These buffers ensure that the concentration of Ca^{2+} within the matrix does not rise much above 1 μM. During the recovery phase of Ca^{2+} signals, the accumulated Ca^{2+} is returned to the cytoplasm by the mitochondrial Na^+/Ca^{2+} exchanger. Under exceptional circumstances, when Ca^{2+} overwhelms the mitochondria, the mitochondrial permeability transition pore (mPTP) is activated to speed up the release of Ca^{2+}. Ca^{2+} has two main signalling functions within the mitochondrial matrix: it activates the mPTP and it also stimulates the TCA cycle to enhance the formation of ATP. One of

the consequences of the latter is an increase in the production of $O_2^{-\bullet}$, which acts synergistically with Ca^{2+} to activate the mPTP.

Cardiolipin (CL)

Cardiolipin (CL) is a diphosphatidyl glycerolphospholipid located primarily in the inner mitochondrial membrane (IMM) where it plays an important role in creating a membrane environment to maximize the operation of the electron transport chain (ETC). CL consists of two phosphatidic acid (PA) molecules that are connected together by a glycerol bridge (Module 5: Figure cardiolipin) and is an example of a non-bilayer-forming lipid because it has a conical shape with a small negatively charged hydrophilic head and a larger hydrophobic domain. The biosynthesis of CL occurs mainly in the mitochondrion, but it depends on the ER for the provision of precursors that are passed to the mitochondrion through distinct contact sites. The newly synthesized CL usually has four saturated acyl chains and this nascent CL is then remodelled through a transacylation reaction resulting in the incorporation of more unsaturated fatty acid acyl chains that are highly symmetrical. In heart mitochondria, the main acyl chain is linoleic acid (C18:2), whereas lymphoblasts have oleyl chains ($C_{18:1}$). A key enzyme in this lipid remodelling is taffazin (Taz1), a product of the *TAZ1* gene which is mutated in Barth syndrome.

The CL within the IMM clump together to form unique microdomains that provide a lipid scaffold by creating environments that maximizes electron fluxes through components of the electron transport chain (ETC) and facilitates the operation of metabolic carriers, such as the ATP/ADP carrier (AAC). This supporting role for CL is essential to maintain normal rates of oxidative phosphorylation. Another important scaffolding role for CL is to provide a lipid anchor that attaches cytochrome c to the outer surface of the IMM (Module 5: Figure cardiolipin). The mitochondrial flippase called phospholipid scramblase 3 (PLS3) can transport CL formed in the IMM across to the outer mitochondrial membrane (OMM) where it can perform various functions particularly during apoptosis (see below).

One of the problems with CL is that it is prone to peroxidation of the acyl chains, which completely alters its role as a scaffold supporting the processes of oxidative phosphorylation. CL is particularly vulnerable because it is clustered around the very components that generate reactive oxygen species (ROS) such as superoxide ($O_2^{-\bullet}$) (Module 5: Figure mitochondrial Ca^{2+} signalling). The oxidation of CL can also play an important role in facilitating the onset of apoptosis, which occurs in two steps. Firstly, the oxidized CL is no longer capable of tethering cytochrome c, which is released into the space between the IMM and the outer mitochondrial membrane (OMM). Secondly, oxidized CL is transported across to the OMM where it functions to activate and oligomerize the Bax and Bak that form the large channels that enables cytochrome c to leak out into the cytoplasm where it initiates apoptosis (Module 5: Figure cardiolipin). Melatonin, which is concentrated within the mitochondria, functions as an antioxidant that scavengers the peroxyl radical responsible for the peroxidation of CL (Module 5: Figure cardiolipin).

Deterioration in the function of CL is thought to contribute to the link between mitochondrial dysfunction and ageing.

Mitochondrial sirtuins

Many of the metabolic process responsible for the generation of ATP are regulated by the sirtuin family members SIRT3, SIRT4 and SIRT5, which are located primarily within the mitochondrion (Module 5: Figure mitochondrial Ca^{2+} signalling).

SIRT3 has a pervasive effect on mitochondrial metabolism by enhancing the activity of the tricarboxylic acid (TCA) cycle, the electron transport chain (ETC) responsible for oxidative phosphorylation and a reduction in mitochondrial reactive oxygen species (ROS) formation (Module 2: Figure sites of ROS formation). It deacylates and activates long-chain acyl CoA dehydrogenase (LCAD) and isocitrate dehydrogenase 2 (IDH2) thus enhancing β-oxidation and the TCA cycle respectively. It also activates glutamate dehydrogenase (GDH) to enhance the use of amino acids as an energy source for the TCA cycle. The operation of the ETC is also regulated by SIRT3 that deacylates complexes I, II and III. SIRT3 can also reduce the deleterious effects of the ROS that are generated during the operation of the ETC by activating superoxide dismutase 2 (SOD2).

SIRT4 seems to act on glutamate dehydrogenase (GDH). Instead of activating it, as SIRT3 does, it inhibits GDH. Unlike the other sirtuins that deacylate proteins, SIRT4 acts through an ADP ribosylation reaction.

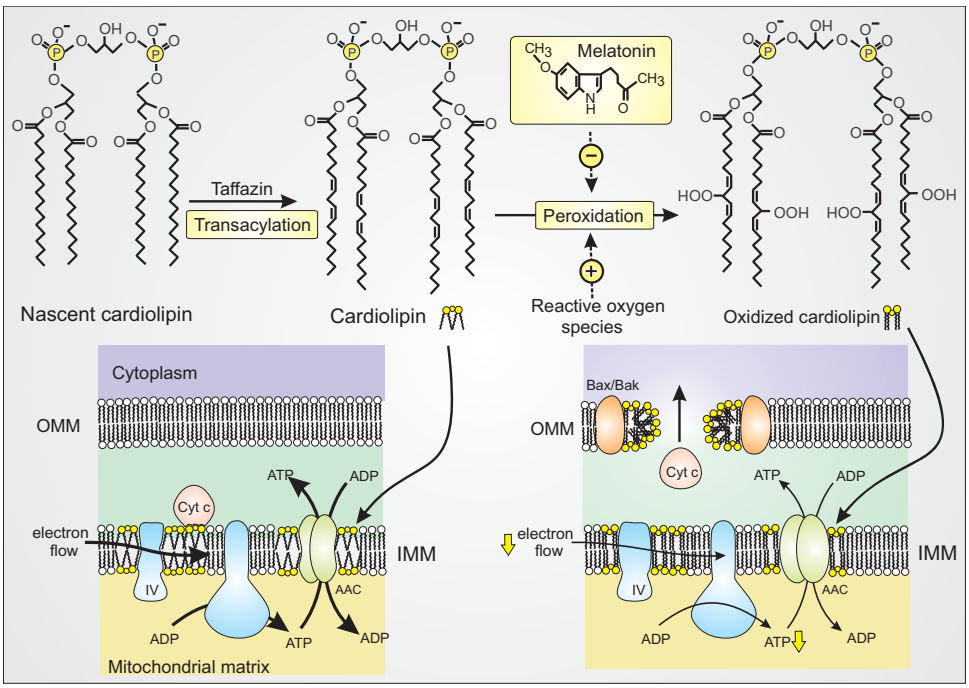
Mitochondrial Ca^{2+} uptake

In resting cells, the concentration of Ca^{2+} within the mitochondrial matrix is 80–200 nM, which is close to the level in the cytoplasm. When the cytosolic level of Ca^{2+} begins to rise, Ca^{2+} enters the mitochondrion through a mitochondrial Ca^{2+} uniporter (MCU) driven by the same proton gradient that is used to power ATP synthesis (Module 5: Figure mitochondrial Ca^{2+} signalling). A mitochondrial Ca^{2+} release mechanism then returns Ca^{2+} from the mitochondrial matrix back into the cytoplasm. This ebb and flow of Ca^{2+} through the mitochondrion is dramatically demonstrated when cells are generating repetitive Ca^{2+} spikes (Module 5: Figure mitochondrial Ca^{2+} oscillations).

Mitochondrial Ca^{2+} uniporter (MCU)

A mitochondrial Ca^{2+} uniporter (MCU) located in the inner mitochondrial membrane is responsible for taking up Ca^{2+} from the cytoplasm (Module 5: Figure mitochondrial Ca^{2+} signalling). Since the uniporter functions as a channel, it is able to take up Ca^{2+} over a wide range of concentrations. It can take up Ca^{2+} slowly at the normal global levels of Ca^{2+} (approximately 500 nM). As the concentration continues to rise, uptake increases in a steeply Ca^{2+} concentration-dependent manner, with half-maximal activation occurring at around 15 μM.

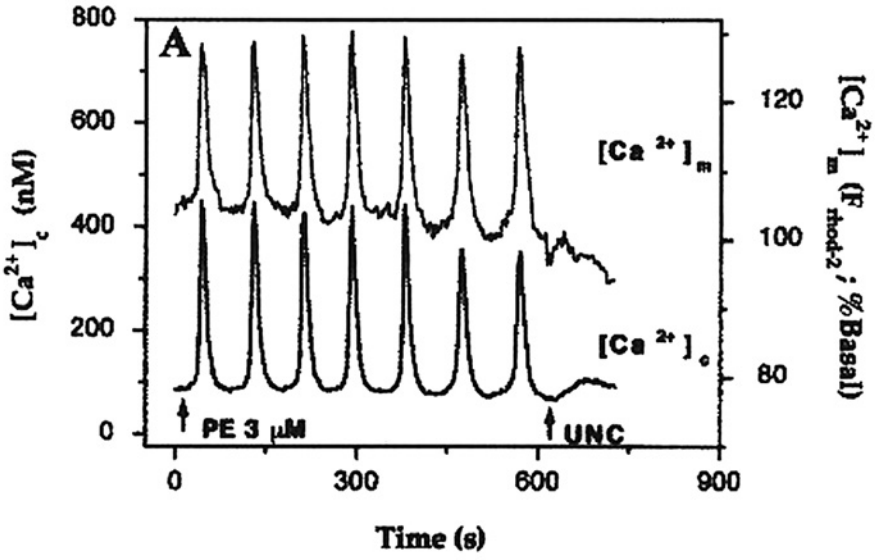
Module 5: | Figure cardiolipin



Cardiolipin formation and oxidation.

Following its synthesis at the inner mitochondrial membrane (IMM), the nascent cardiolipin (CL) is remodelling by the enzyme taffazin, which exchanges the saturated fatty acids with unsaturated fatty acids. The latter are particularly susceptible to peroxidation by reactive oxygen species. This oxidation can be protected by melatonin. The CL provides a membrane niche that facilitates electron flow, it tethers cytochrome c to the IMM and it facilitates the ATP/ADP carrier (AAC). Peroxidation of CL greatly reduces these key mitochondrial processes. Oxidized CL also moves to the OMM where it interacts with Bak and Bax to form pores to allow cytochrome c to escape into the cytoplasm. The Information for this Figure was taken from Osman et al. (2011).

Module 5: | Figure mitochondrial Ca^{2+} oscillations



Mitochondrial tracking of cytosolic Ca^{2+} transients in hepatocytes.

Hepatocytes stimulated with phenylephrine (PE) generate repetitive Ca^{2+} transients (lower trace, $[Ca^{2+}]_c$). The Ca^{2+} concentration in the mitochondria (upper trace, $[Ca^{2+}]_m$) closely tracked the cytosolic oscillation. Reproduced from *Biochim. Biophys. Acta*, Vol. 1366, Robb-Gaspers, L.D., Rutter, G.A., Burnett, P., Hajnóczky, G., Denton, R.M. and Thomas, A.P. (1998) Coupling between cytosolic and mitochondrial calcium oscillations: role in the regulation of hepatic metabolism, pp. 17–32. Copyright (1998), with permission from Elsevier; see Robb-Gaspers et al. 1998.

Mitochondria can accumulate as much as 25–50% of the Ca^{2+} released from the endoplasmic reticulum (ER). This ability to sequester Ca^{2+} quickly at such high concentrations means that the mitochondria are particularly effective at accumulating Ca^{2+} when they lie close to Ca^{2+} channels, where the elementary events generate very high local concentrations of Ca^{2+}. Under these conditions, the uptake of Ca^{2+} is so fast that it can temporarily collapse the mitochondrial membrane potential, and such depolarizations have been recorded in neurons following Ca^{2+} entry through voltage-operated channels (VOCs).

The uptake of Ca^{2+} through the MCU appears to be regulated by Ca^{2+} acting through two mechanisms. Firstly, the mitochondrial calcium uptake 1 (MICU1) protein, which has two E-F hands, may function to monitor the external level of Ca^{2+} and increase entry through the mitochondrial Ca^{2+} uniporter (MCU). Secondly, Ca^{2+} can also enhance entry by stimulating phosphorylation of the MCU by CaMKII (Module 5: Figure mitochondrial Ca^{2+} signalling).

Voltage-dependent anion channel (VDAC)

The voltage-dependent ion channel (VDAC) is located in the outer mitochondrial membrane (OMM) where it facilitates the movement of ions, such as Ca^{2+}, and various metabolites (ATP, ADP, malate and pyruvate). The VDAC plays an important role in the transfer of Ca^{2+} from the ER to the mitochondrion during the operation of the endoplasmic reticulum (ER)/mitochondrial Ca^{2+} shuttle (Module 5: Figure ER/mitochondrial shuttle).

Mitochondrial Ca^{2+} release

The Ca^{2+} that is taken up by mitochondria during signalling is released back to the cytoplasm by various efflux pathways:

Na^+-dependent Ca^{2+} efflux

Like so many functions in the mitochondrion, this mode of efflux is driven by the negative membrane potential. Ca^{2+} is extruded from the mitochondrion by means of a Na^+/Ca^{2+} exchanger, which has a stoichiometry of three Na^+ ions for one Ca^{2+} ion. The Na^+ that enters down the electrochemical gradient is exchanged for Ca^{2+} (Module 5: Figure mitochondrial Ca^{2+} signalling). Pharmacological agents such as amiloride, diltiazem, bepridil and CGP37157 inhibit the exchanger.

Na^+-independent Ca^{2+} efflux

Mitochondria appear to have a Na^+-independent Ca^{2+} release mechanism that is rather slow and may play a role in extruding Ca^{2+} under resting conditions. Extrusion is dependent on the transmembrane potential, and may depend upon an H^+/Ca^{2+} antiporter.

Mitochondria-associated ER membranes (MAMs)

The mitochondrial-associated ER membranes (MAMs) are specialized functional zones where regions of the endoplasmic reticulum (ER) come into close contact with the mitochondria (Module 5: Figure mitochondrial-associated ER membranes). Since the gap between the ER and mitochondria can be very narrow (i.e.10–20 nM), proteins on the two membranes can interact with each other. The close apposition between the ER and the outer mitochondrial membrane (OMM) is maintained by the mitofusins. The MFN2 forms a dimeric anti-parallel complex with either MFN2 or MFN1 located in the OMM. A phosphofurin acid cluster sorting protein 2 (PACS2) may function to maintain the stability of the MAMs. The MAMs contain enzymes such as long-chain fatty acid-CoA ligase type 4 (FACL4) and phosphatidylserine synthase-1 (PSS-1) that function in the lipid synthesis and trafficking between the two organelles.

Rab32 is localized to the MAMs where it acts as an anchor for protein kinase A (PKA) that contributes to the regulation of Ca^{2+} signalling by phosphorylating the $InsP_3Rs$.

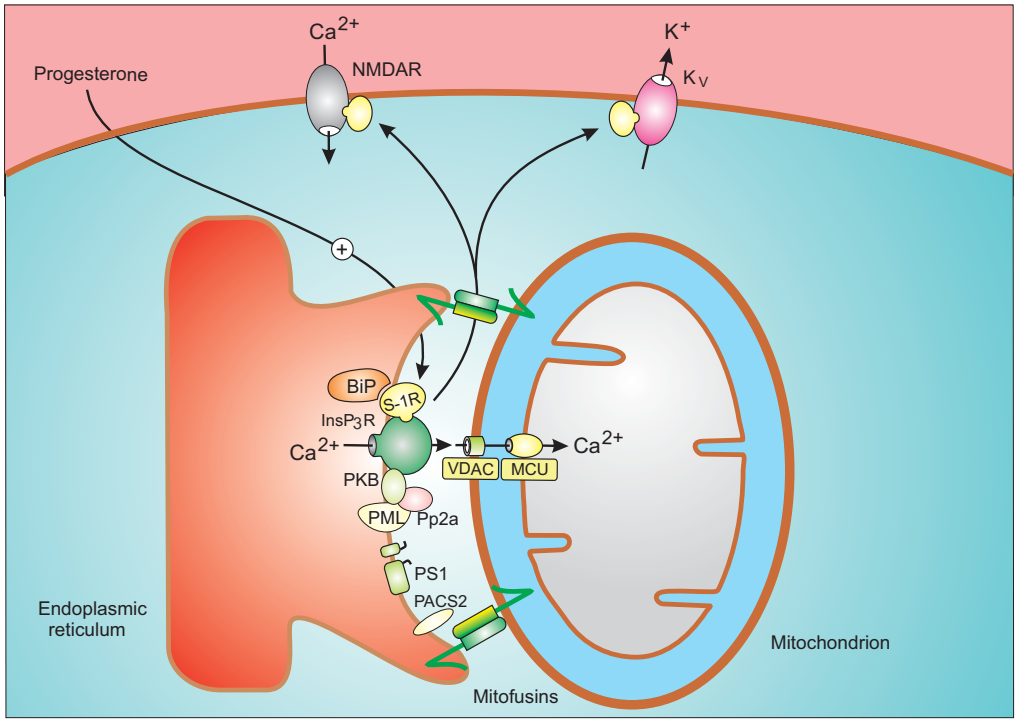
A number of important functions are located within this contact zone. One of these functions is the endoplasmic reticulum (ER)/mitochondrial Ca^{2+} shuttle whereby Ca^{2+} stored within the ER lumen is rapidly transferred to the mitochondrion through a sequence of channels: the inositol 1,4,5-trisphosphate receptors ($InsP_3Rs$) on the ER membrane, the voltage-dependent ion channel (VDAC) on the outer mitochondrial membrane and the mitochondrial Ca^{2+} uniporter (MCU) on the inner mitochondrial membrane. The interaction between the $InsP_3R$ and VDAC is facilitated by the mitochondrial chaperone glucose-regulated protein 75 (Grp75).

The activity of the $InsP_3R$ within the MAM is regulated by a number of proteins (Module 5: Figure mitochondrial-associated ER membranes). For example, a multiprotein complex consisting of the tumour suppressor promyelocytic leukaemia (PML) protein, protein phosphatase 2A (PP2a) and protein kinase B (PKB) functions to regulate $InsP_3R$ activity. The anti-apoptotic action of PKB is mediated, at least in part, by phosphorylation of the $InsP_3R$ to markedly reduce its ability to release Ca^{2+}. This inhibition is reversed following its dephosphorylation of the $InsP_3R$ by PP2A. Another important component of the MAM are the sigma-1 receptors (Sig-1Rs), which are ER chaperones associated with both the $InsP_3Rs$ and the Ca^{2+}-sensitive chaperone protein BiP, which is located within the ER lumen. When the luminal level of Ca^{2+} declines, the Sig-1R dissociates from BiP and then acts as a chaperone to stabilize the $InsP_3Rs$.

MAMs and Alzheimer's disease

The activity of the mitochondrial-associated ER membranes (MAMs), which are specialized functional zones where regions of the endoplasmic reticulum (ER) come into close contact with the mitochondria (Module 5: Figure mitochondrial-associated ER membranes), are markedly increased in Alzheimer's disease (AD). Both the degree of communication and the functionality of the MAMs are enhanced in AD, which is consistent with the calcium hypothesis of Alzheimer's disease. The MAMs are one of the main locations of the presenilins (PS1 and PS2) and may thus be a primary site where the β-amyloid precursor protein (APP) is hydrolysed to release the β-amyloid

Module 5: | Figure mitochondria-associated ER membrane



MAM organization and function.
The endoplasmic reticulum and the mitochondria are functionally linked together at mitochondria-associated ER membrane (MAM) junctions that are held together by the mitofusins. A large number of proteins are located within the MAM such as the inositol 1,4,5-trisphosphate receptors ($InsP_3Rs$), sigma-1 receptor (S-1R), phosphofurin acid cluster sorting protein 2 (PACS2), presenilin-1 (PS1), promyelocytic leukaemia (PML), protein phosphatase PP2a and protein kinase B (PKB)

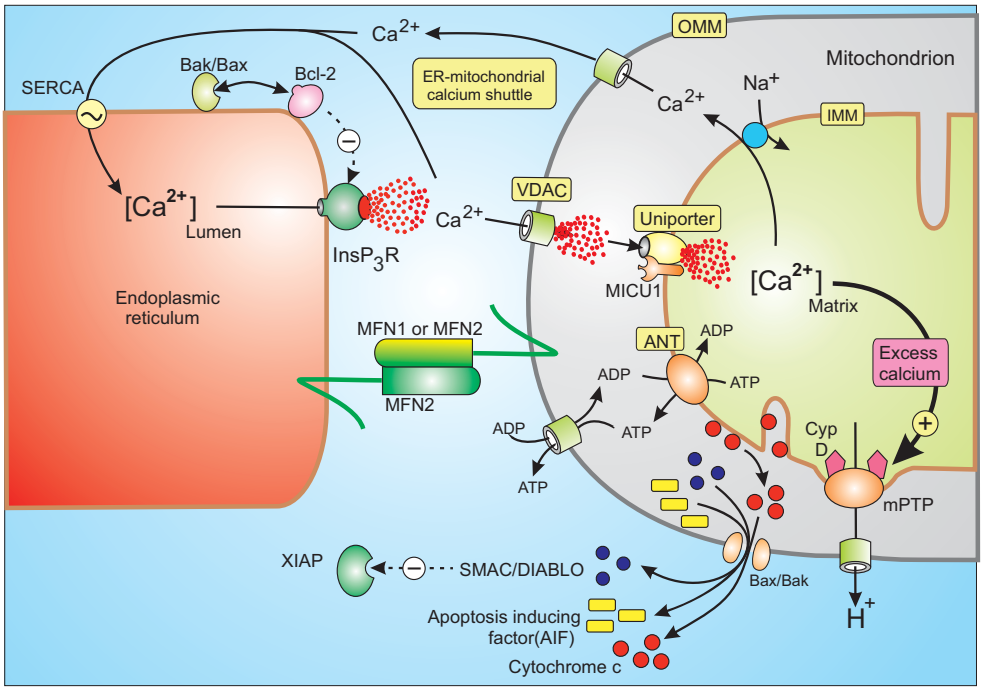
responsible for inducing the onset of AD (Module 12: Figure APP processing). An increase in the extent and activity of the MAMs may contribute to neurodegeneration by enhancing the transfer of Ca^{2+} from the ER to the mitochondria resulting in the onset of both memory loss and neuronal cell death that characterizes Alzheimer's disease (AD) (Module 12: Figure amyloids and Ca^{2+} signalling).

Endoplasmic reticulum (ER)/mitochondrial Ca^{2+} shuttle

The function of the endoplasmic reticulum (ER) is intimately connected with that of the mitochondria. These mitochondria-associated ER membranes (MAMs) play an important role in lipid synthesis, apoptosis and Ca^{2+} homeostasis. With regard to the latter, the concept of an ER/mitochondrial Ca^{2+} shuttle operating within a MAM has emerged from the fact that the ER and the mitochondria form a highly dynamic interconnected network that functions both to generate and to modulate Ca^{2+} signals (Module 5: Figure ER/mitochondrial shuttle). The close association between these two organelles is maintained by the mitofusins (MFNs). The Ca^{2+} stored within the ER lumen is released into the cytoplasm by inositol 1,4,5-trisphosphate receptors ($InsP_3Rs$) and ryanodine receptors (RYRs) to provide the cytosolic Ca^{2+} signal to activate many cellular processes. During the recovery phase, this cytosolic Ca^{2+} can be dealt with in different ways. It can be returned directly to the ER by the sarco/endo-plasmic reticulum Ca^{2+}-ATPase (SERCA) pump. Alternatively, Ca^{2+} is taken up by the mitochondrion and then returned to the ER through the ER/mitochondrial shuttle. The mitochondria assist with recovery phase by rapidly sequestering some of the released Ca^{2+} and then later returning it to the ER. During normal signalling, there is therefore a continuous ebb and flow of Ca^{2+} between these two organelles. The normal situation is for most of the Ca^{2+} to reside within the lumen of the ER, except during Ca^{2+} signalling, when a variable proportion passes through the mitochondria. At equilibrium, the bulk of internal Ca^{2+} is therefore in the ER, where it not only functions as a reservoir of signal Ca^{2+}, but also plays an essential role in protein processing within the ER.

During various forms of stress, the normal distribution of Ca^{2+} is altered and can result in stress signalling and apoptosis. For example, a decrease in the ER content of Ca^{2+} can initiate the endoplasmic reticulum (ER) stress signalling pathway (Module 2: Figure ER stress signalling). If the Ca^{2+} that is lost from the ER is taken up by the mitochondria, it can result in opening of the mitochondrial permeability transition pore (MTP), collapse of the mitochondrial membrane potential and the release of factors [e.g. cytochrome *c*, apoptosis-inducing factors (AIFs) and second mitochondrial-derived activator of caspases (SMAC), which is also known as the direct IAP-binding protein with low pI (DIABLO)] that activate the caspase cascade responsible for apoptosis (Module 5: Figure ER/mitochondrial shuttle). SMAC/DIABLO

Module 5: | Figure ER/mitochondrial shuttle



The endoplasmic reticulum (ER)/mitochondrial Ca^{2+} shuttle.

The endoplasmic reticulum (ER) and the mitochondria are functionally linked through their participation in Ca^{2+} signalling. During Ca^{2+} signalling, a small bolus of Ca^{2+} is periodically released to the cytoplasm by the inositol 1,4,5-trisphosphate receptors ($InsP_3Rs$). While most of the Ca^{2+} is returned to the ER by the sarco/endo-plasmic reticulum Ca^{2+}-ATPase (SERCA) pump, a proportion enters the mitochondria through the voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane (OMM), and then through the uniporter in the inner mitochondrial membrane (IMM). Mitochondrial calcium uptake 1 (MICU1) may regulate the uniporter. The Ca^{2+} in the mitochondrial matrix is then returned to the ER through a series of exchangers, channels and pumps. Firstly, Ca^{2+} leaves the matrix through a Na^+/Ca^{2+} exchanger and then enters the cytoplasm through the VDAC. Once in the cytoplasm, it can be taken back into the ER by the SERCA pumps.

functions to inhibit X-chromosome-linked inhibitor of apoptosis protein (XIAP), which is a potent inhibitor of caspases 3, 7 and 9. These factors cross the OMM though large pores created by Bak and Bax through a process that is facilitated by cardiolipin (Module 5: Figure cardiolipin). A build-up of matrix Ca^{2+} will also increase the production of reactive oxygen species (ROS), which contribute to the activation of the MTP that is responsible for Ca^{2+}-induced apoptosis. The Bcl-2 superfamily control of Ca^{2+} signalling might depend upon an alteration of this ER/mitochondrial shuttle. This control seems to be exerted through Bcl-2 that binds to the $InsP_3R$ to reduce the release of Ca^{2+} (Module 5: Figure ER/mitochondrial shuttle). This protective effect of Bcl-2 is neutralized by Bak and Bax through their ability to bind Bcl-2, thus pulling it away from the $InsP_3R$.

Glucose-regulated protein 75 (Grp75)

The glucose-regulated protein 75 (Grp75), which is also known as mortalin, belongs to the Hsp 70 family of chaperone proteins. While it is found in the ER, plasma membrane and cytoplasmic vesicles, its primary location is at the mitochondrion where it functions to transfer cytoplasmic proteins to the mitochondria. It also is located in the mitochondria-associated ER membranes (MAMs) where it helps to connect the inositol 1,4,5-trisphosphate receptors ($InsP_3Rs$) on the ER membrane to the voltage-dependent ion channel (VDAC) on the outer mitochondrial membrane (Module 5: Figure mitochondria-associated ER membrane).

Mortalin has been linked to neurodegeneration in both Alzheimer's disease (AD) and Parkinson's disease (PD). In PD, the level of mortalin is reduced in the affected regions of the brain in PD patients. Mortalin also interacts with DJ-1 protein that has an important role in PD. In AD patients, there is a marked reduction in the expression of mortalin and this seems to be associated with mitochondrial dysfunction and an increase in amyloid-induced toxicity.

Wolframin

Wolframin, which is sometimes referred to as WFS1, is a membrane protein that has nine transmembrane regions that is located within the endoplasmic reticulum. The N-terminal hydrophilic region extends into the cytoplasm whereas the C-terminal hydrophilic region extends into the ER lumen. One of the functions of wolframin is to provide a scaffold to regulate the transcription factor ATF6, which is part of the endoplasmic reticulum (ER) stress signalling pathway (for details see step 3 in Module 2: Figure ER stress signalling).

One form of Wolfram syndrome (WFS1) is caused by mutations in the Wolfram syndrome 1 (*WFS1*) gene.

CDGSH iron sulfur domain 2 (CISD2)

CDGSH iron sulfur domain-containing protein 2 (CISD2), which is also known as nutrient-deprivation autophagy factor-1 (NAF-1), ERIS and Noxp70, is a transmembrane protein whose location is somewhat uncertain. There are descriptions of its presence in the outer mitochondrial membrane (OMM) and in the endoplasmic reticulum (ER). Some of the confusion may arise because these membranes are often closely linked together at MAMs during the operation of the ER/mitochondrial Ca^{2+} shuttle (Module 5: Figure ER/mitochondrial shuttle). Most information is available on its role in the ER where it seems to function in Ca^{2+} signalling and autophagy (Module 11: Figure autophagy). CISD2 binds to Bcl-2 and this CISD2–Bcl-2 complex is closely associated with the inositol 1,4,5-trisphosphate receptor ($InsP_3R$) (Module 3: Figure $InsP_3R$ regulation). The CISD2 seems to function as a co-factor to regulate the role of Bcl-2 in determining both the release of Ca^{2+} by the $InsP_3R$ and it contributes to the way Bcl-2 regulates the activity of Beclin-1 that controls autophagy.

CISD2 also plays an important role in ageing. A deficiency in CISD2 causes an acceleration of ageing whereas an enhanced expression increases longevity. In studies on mice where CISD2 has been knocked out, there is an increase in luminal Ca^{2+} levels and this may trigger larger global Ca^{2+} signals that induce autophagy causing increased signs of ageing. There is degeneration of skeletal muscle and a skeletal myofibre conversion with a shift towards type I muscle fibres, which is consistent with the phenotypic reprogramming of skeletal muscle that occurs during ageing.

One form of Wolfram syndrome (WFS) is caused by mutations in CDGSH iron sulfur domain-containing protein 2 (CISD2).

Mitochondrial permeability transition pore (mPTP)

Another important mechanism for releasing Ca^{2+} from the mitochondrion is the mitochondrial permeability transition pore (mPTP), which can have both physiological and pathological consequences (Module 5: Figure mitochondrial Ca^{2+} signalling).

The mPTP has a number of components and there is still some uncertainty as to which of these constitutes the pore in the inner mitochondrial membrane (IMM). One of the candidates is the adenine nucleotide translocase (ANT) that normally functions as a gated pore mediating the entry of ADP and the release of ATP. Under certain conditions, especially a high level of Ca^{2+} within the matrix, the translocase opens up to form the non-selective pore. Bongkrekic acid, which binds to ANT, is a potent inhibitor of apoptosis. This ANT may also be associated with the voltage-dependent anion channel (VDAC), which normally functions to enhance the permeability of the outer membrane. Another pore candidate is the inner membrane anion channel (IMAC).

Pore opening requires cyclophilin-D (CyP-D), which might act to control the assembly and opening of the mPTP. CyP-D is a mitochondrial isoform of a family of cyclophilins that are sensitive to cyclosporin A (CsA), which not only functions as an immunosuppressant, but also is a potent inhibitor of apoptosis.

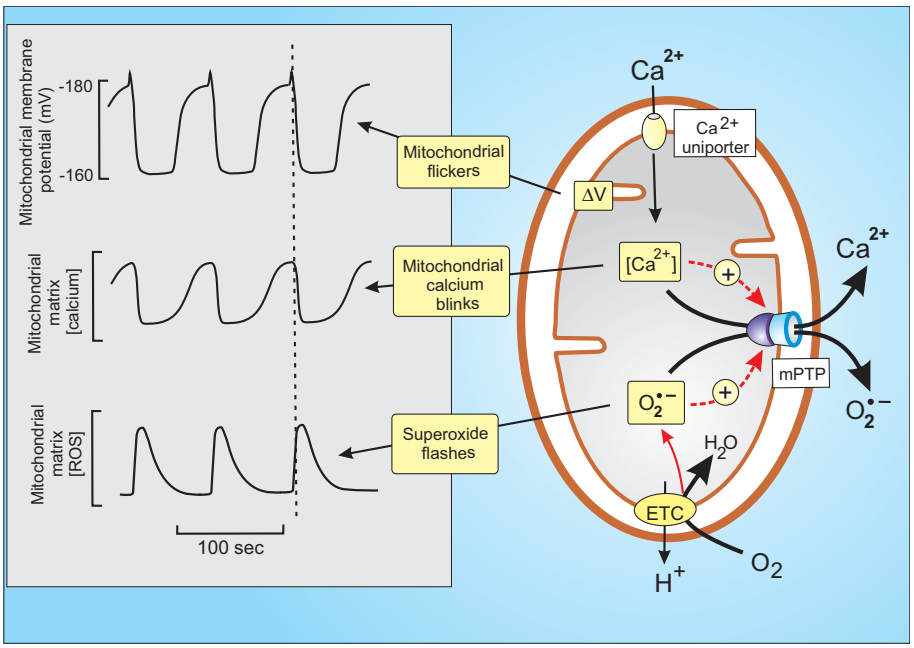
The mPTP is a non-selective channel with a very high conductance (pore radius 1–1.3 nm) capable of releasing both metabolites and ions. A large number of factors control the opening of the mPTP. Two key factors are an increase in matrix Ca^{2+} concentration and the ROS-dependent oxidation of dithiols located on ANT. Opening of the mPTP seems to require both oxidative stress and an increase in Ca^{2+} (Module 5: Figure mitochondrial Ca^{2+} signalling). It seems that overloading of the mitochondria is not in itself deleterious, unless it occurs in the presence of other factors, such as a change in the redox state or a decline in the level of ATP. Mitochondrial reactive oxygen species (ROS) formation, which is a by-product of the flow of electrons down the electron transport chain, is responsible for opening the mPTP. The superoxide radical ($O_2^{-\bullet}$) oxidize the *vic*-thiols on ANT, and possibly also on CyP-D, to facilitate the conformational change that opens the pore (Module 5: Figure mitochondrial Ca^{2+} signalling). This oxidative mechanism is normally prevented by the highly reduced state within the mitochondrial matrix that is maintained by high levels of glutathione (GSH).

Opening of the mPTP can have both physiological (mPTP and mitochondrial Ca^{2+} homoeostasis) and pathological (mPTP and apoptosis) consequences.

mPTP and mitochondrial Ca^{2+} homoeostasis

Overloading the mitochondrion with Ca^{2+} can result in Ca^{2+}- induced apoptosis through a prolonged activation of the mPTP channel. However, there are indications that this channel may have a physiological role to protect mitochondria by functioning as a safety valve to release excess Ca^{2+}. Such a mechanism is of particular importance in those cells that function by generating repetitive pulses of Ca^{2+} as occurs in cardiac cells, neurons such as the dopaminergic substantia pars compacta neurons and in liver cells (Module 5: Figure mitochondrial Ca^{2+} oscillations). During each cytosolic Ca^{2+} transient, some of the Ca^{2+} floods into the mitochondrion through the mitochondrial Ca^{2+} uptake mechanism to generate a corresponding mitochondrial Ca^{2+} transient. An example of this process has been described in liver cells (Module 5: Figure mitochondrial Ca^{2+} oscillations). In order to maintain Ca^{2+} homoeostasis, the amount of Ca^{2+} entering during the rising phase has to leave during the recovery phase before the onset of the next transient as seems to be the case in liver cells. In other cells such as cardiac cells and neurons where Ca^{2+} oscillations are more frequent, the recovery processes are not always sufficiently active to remove all the Ca^{2+} following each transient and this can result in a gradual elevation in the baseline level of Ca^{2+}. In resting cardiac cells, for example, the level of Ca^{2+} within the matrix is thought to be 0.1–0.2μM when cells are at rest, but this can rise to 0.7 μM when the heart is beating normally or to 1.1 μM when the cytosolic transients are elevated following treatment with isoproterenol. This elevation has an important physiological role in the Ca^{2+} modulation of mitochondrial function in that it is a catabolic signal that

Module 5: | Figure mitochondrial flickers



Mitochondrial flickers.
Brief openings of the mitochondrial permeability transition pore (mPTP) result in periodic depolarizations of the inner mitochondrial membrane (IMM ΔV) known as mitochondrial flickers. The onset of each flicker coincides with a superoxide flash and may also result in a temporary reduction in the concentration of Ca^{2+} within the mitochondrial matrix. These putative mitochondrial calcium blinks have yet to be described. If such blinks exist, they could play an important role in preventing the matrix from being overloaded with Ca^{2+} that will result in apoptosis. The dashed line represents the temporal relationship between the different events. The information used to construct this Figure was taken from Guzman et al. (2010); and Wang et al. (2008).

activates key regulatory enzymes of the tricarboxylic acid (TCA) cycle such as pyruvate dehydrogenase, oxoglutarate dehydrogenase and isocitrate dehydrogenase (Module 5: Figure mitochondrial Ca^{2+} signalling).

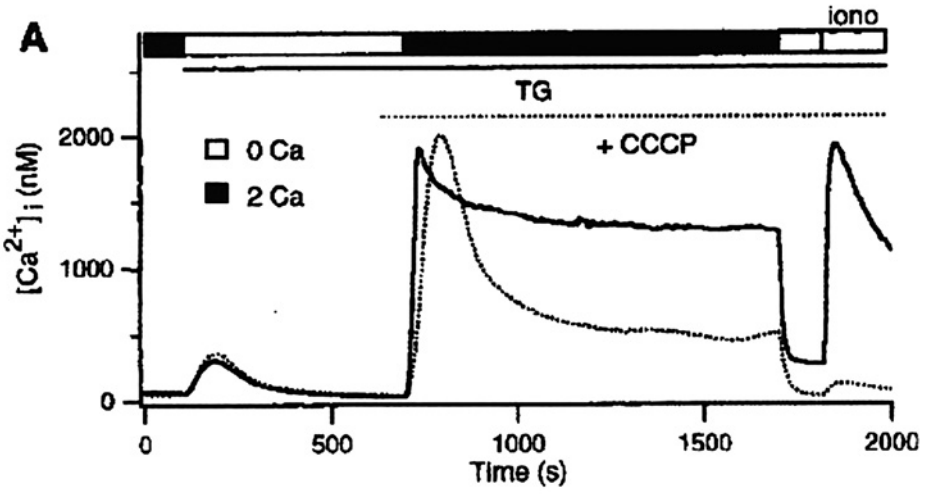
While an elevation in the level of Ca^{2+} within the mitochondrion can have such beneficial effects, a Ca^{2+} overload will trigger Ca^{2+}-induced apoptosis through a prolonged opening of the mPTP. However it seems that brief openings of the mPTP can function as a Ca^{2+} leak pathway to guard against the deleterious effects of excess elevations in the level of mitochondrial Ca^{2+}. The existence of brief mPTP openings is probably responsible for the periodic fluctuations in mitochondrial membrane potential, which are known as mitochondrial flickers (Module 5: Figure mitochondrial flickers). At the onset of each flicker, there often is a brief membrane hyperpolarization preceding the sudden fall in membrane potential that usually declines by about 20mV from its normal level of −180mVs. The onset of each flicker, which results from the temporary opening of the mPTP, may result in a temporary reduction in the concentration of Ca^{2+} within the mitochondrial matrix. These putative mitochondrial blinks, which have yet to be described, may result from the sudden efflux of Ca^{2+} through the mPTP. If such blinks exist, they could play an important role in preventing the matrix from being overloaded with Ca^{2+} that will result in apoptosis. Such a mechanism may be particularly important in substantia nigra pars compacta (SNc) dopaminergic neurons that experience regular pulses of Ca^{2+} every few seconds (Module 10: Figure tonic oscillation in DA neurons) that increase the vulnerability of the mitochondria and this is likely to be the cause of Parkinson's disease.

The onset of each flicker also coincides with a superoxide flash resulting from a brief increase in the rate of superoxide ($O_2^{-\bullet}$) formation, which might be a direct consequence of the membrane depolarization. When the membrane depolarizes, there will be a temporary decline in oxidative phosphorylation and the electrons that are flowing down the electron transport chain will be diverted to ROS formation (Module 5: Figure mitochondrial flickers). This brief increase in ROS soon declines because some of the excess $O_2^{-\bullet}$ will escape through the mPTP.

mPTP and apoptosis

The best known function of the mPTP is Ca^{2+}-induced apoptosis. The mPTP is the focal point of many apoptotic signals, including Ca^{2+}, reactive oxygen species (ROS), and possibly also members of the Bcl-2 superfamily. The sudden release of protons results in collapse of the mitochondrial membrane potential with immediate cessation of most mitochondrial functions, together with a catastrophic release of essential components such as cytochrome *c* and apoptosis-inducing factors (AIFs) that then go on to activate apoptosis (Module 5: Figure ER/mitochondrial shuttle). Mitochondria thus play a pivotal role in regulating apoptosis because they lie at the centre of a complex

Module 5: | Figure mitochondria and Ca^{2+} entry



Mitochondrial modification of Ca^{2+} entry.

In this study on Jurkat leukaemic T cells, thapsigargin (TG) added in a Ca^{2+}-free solution (0 Ca) emptied the internal Ca^{2+} store by blocking the sarco/endo-plasmic reticulum Ca^{2+}-ATPase (SERCA) pumps. This empty store then activated a store-operated channel (SOC), as evident by the marked increase in Ca^{2+} that occurred when 2 mM Ca^{2+} was added back to the bathing medium (black bar). In cells that had been treated with carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) to inhibit the mitochondria (dotted line), the initial peak was the same, indicating that the entry channel was fully activated, but this response then declined to a much lower level. This lower plateau probably results from the fact that the mitochondria lying close to the entry channels remove Ca^{2+}, thereby negating its inhibitory effect on entry (Module 9: Figure T-cell Ca^{2+} signalling). Reproduced from *The Journal of Cell Biology*, 1997, vol. 137, pp. 633–648 by copyright permission of The Rockefeller University Press; see Hoth et al. 1997.

web of interactions that link apoptotic signals to the caspase cascade (Module 11: Figure apoptosis). Given this central role in the process of apoptosis, there is much interest concerning the nature of the mPTP channel and how it is activated.

Mitochondrial modulation of Ca^{2+} signals

Mitochondria can take up large quantities of Ca^{2+} very rapidly and thus can modulate various aspects of Ca^{2+} signalling. In addition to functioning as a Ca^{2+} buffer, mitochondria can also modulate the flow of Ca^{2+} through both the entry and release channels.

Mitochondria function as immobile buffers. The Ca^{2+} that is taken up during the course of a Ca^{2+} signal is then released back into the cytoplasm, where it is either returned to the endoplasmic reticulum (ER) or pumped out of the cell. The level of Ca^{2+} within the mitochondrial matrix is held constant by means of buffers and by formation of a calcium phosphate precipitate (Module 5: Figure mitochondrial Ca^{2+} signalling). During prolonged periods of stimulation, large amounts of Ca^{2+} are taken up by the mitochondrion, and this is then gradually unloaded during periods of rest. In the case of nerve terminals, for example, it can take up to 10 min for the mitochondrial level of Ca^{2+} to return to its resting level following a period of intense stimulation.

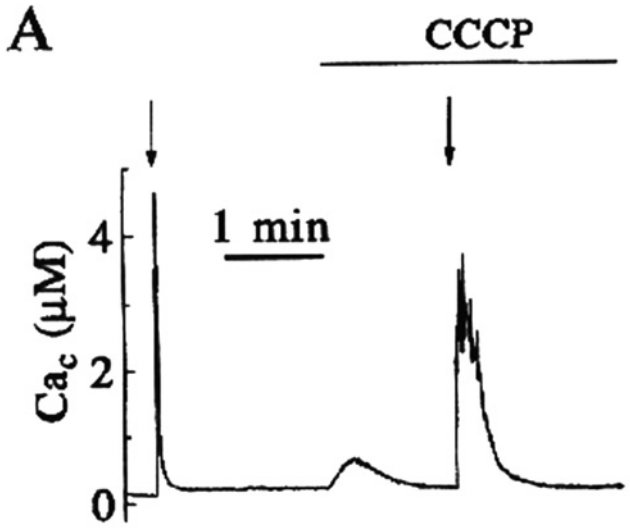
This ability of Ca^{2+} to sequester large amounts of Ca^{2+} can markedly modify both the shape and the amplitude of cytosolic Ca^{2+} signals. An example of the former is the ability of mitochondria to enhance Ca^{2+} signals by dampening out the negative feedback effects that normally limit the activity of Ca^{2+} channels, as occurs in T cells (Module 9: Figure T-cell Ca^{2+} signalling). When mitochondria are inhibited, the entry of external Ca^{2+} is markedly reduced (Module 5: Figure mitochondria and Ca^{2+} entry).

Mitochondria can also modify the shape of Ca^{2+} transients, which depend upon the sequential activation of ON and OFF reactions (Module 2: Figure Ca^{2+} transient mechanisms). The sharpness of the transients depends not only on how quickly Ca^{2+} is introduced into the cytoplasm, but also on how quickly it is removed by the various OFF reactions. The mitochondria play an important role in the kinetics of the recovery phase because this becomes considerably prolonged when their activity is inhibited (Module 5: Figure chromaffin cell Ca^{2+} transients).

Ca^{2+} modulation of mitochondrial function

Aerobic generation of ATP by the mitochondria is tightly regulated. There is a direct control mechanism exercised through the ATP/ADP ratio that automatically increases metabolism when the level of ATP declines. In addition, Ca^{2+} functions as a catabolic signal in that it activates key regulatory enzymes of the tricarboxylic acid (TCA) cycle responsible for fuelling the generation of ATP, such as pyruvate dehydrogenase phosphatase (PDHP), isocitrate dehydrogenase 2 (IDH2) and α-ketoglutarate dehydrogenase (αKGDH) (Module 5: Figure mitochondrial Ca^{2+} signalling). When Ca^{2+} builds up within the mitochondrion, it activates the TCA cycle, that then increases the supply of reducing equivalents and hence an increase in

Module 5: | Figure chromaffin cell Ca^{2+} transients



Mitochondrial modulation of the shape of Ca^{2+} transients in adrenal chromaffin cells.

In response to a 500 ms depolarization (first arrow) to activate Ca^{2+} entry, there was a rapid increase in Ca^{2+} concentration that recovered rapidly to give a very sharp transient (first arrow). When this stimulus was repeated in the presence of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) to inhibit the mitochondria, the transient had a much smaller amplitude and a much longer recovery period. This experiment demonstrates how mitochondria accelerate the recovery phase by soaking up Ca^{2+}. Reproduced from *The Journal of Cell Biology*, 1997, vol. 136, pp. 833–844 by copyright permission of The Rockefeller University Press; see Babcock et al. 1997.

ATP formation (Module 5: Figure cytosolic and mitochondrial Ca^{2+} transients). This feedback mechanism is an example of the interaction between metabolic messengers and cell signalling pathways (Module 2: Figure metabolic signalling). Such an interaction may explain how pyruvate can markedly enhance cardiac cell Ca^{2+} signalling (Module 2: Figure pyruvate and Ca^{2+} signalling).

The increase in mitochondrial metabolism will also enhance the formation of the superoxide radical ($O_2^{-\bullet}$), and this mitochondrial reactive oxygen species (ROS) formation can contribute to redox signalling. In addition, $O_2^{-\bullet}$ formation within the mitochondria can act synergistically with Ca^{2+} to open the mitochondrial permeability transition pore (mPTP).

Mitochondrial motility

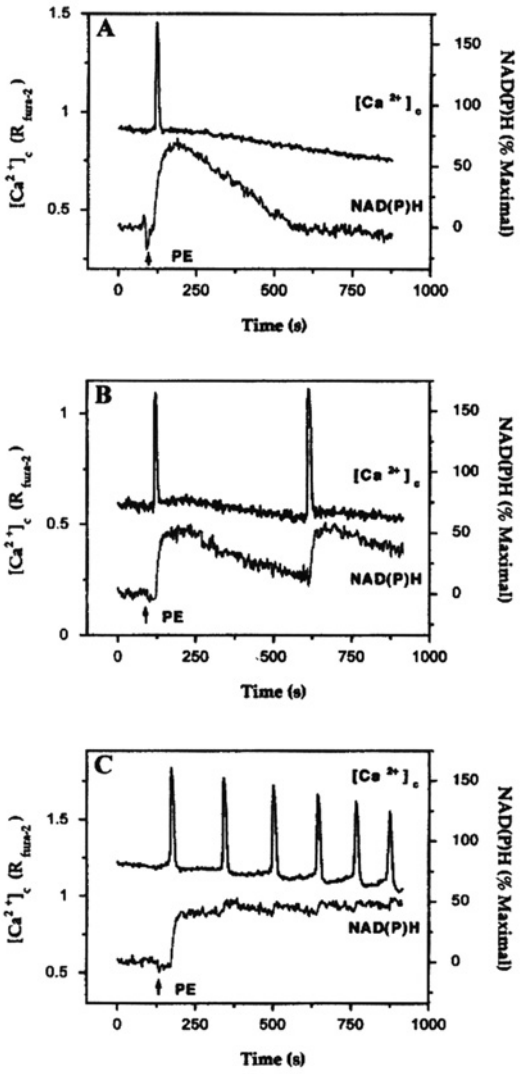
Mitochondria are not static in cells. They move around to cellular regions where metabolic demands are high. This movement is particularly evident in neurons, where their energy demands are widely dispersed because of their complex morphology (Module 10: Figure neuronal morphology). For example, during gene transcription, energy is required at the soma, but when information is being processed at the synapses on the spines and dendrites, energy demand will shift from the cell body to the periphery.

Mitochondria travel around the cell attached to the microtubules (MTs) and are propelled by plus end-directed kinesin and minus end-directed dynein motor proteins (Module 5: Figure mitochondrial motility). The way in which these motors are controlled to direct mitochondria to different regions in the cell is still somewhat of a mystery. However, there is clear experimental evidence that the movement of mitochondria is rapidly inhibited by increases in intracellular Ca^{2+}. One way in which Ca^{2+} inhibits mitochondrial movement depends on the mitochondrial Rho-GTPase (Miro) protein family (Miro 1 and Miro 2). These Rho-GTPases have two EF-hand Ca^{2+}-binding domains. A current hypothesis is that Miro and associated proteins, such as Milton, might be part of a complex that attaches the mitochondria to the kinesin motor, which is the primary anterograde mitochondrial motor (Module 4: Figure kinesin cargo transport in neurons). In regions of high Ca^{2+}, Miro functions as the sensor responsible for detecting Ca^{2+} and this results in the motor detaching from the microtubule (Module 5: Figure mitochondrial motility). The motor domain of kinesin interacts with Miro when the latter is bound to Ca^{2+}. Such a mechanism could explain how mitochondria accumulate in regions where there is intense activity, since this is also likely to be where there are microdomains of Ca^{2+}.

Mitochondrial Rho-GTPase (Miro)

The mitochondrial Rho-GTPase (Miro) protein family has two members (Miro 1 and Miro 2). They are GTPase-activating proteins (GAPs) that have two EF-hand Ca^{2+}-binding domains. They function together with Milton to form a complex that attaches the mitochondria to the kinesin motor such as kinesin-3 (Module 5: Figure mitochondrial motility).

Module 5: | Figure cytosolic and mitochondrial Ca^{2+} transients



Relationship between cytosolic Ca^{2+} transients and mitochondrial metabolism in hepatocytes.

Cytosolic Ca^{2+} oscillations ($[Ca^{2+}]_c$), which were induced by phenylephrine, markedly enhanced mitochondrial metabolism, as monitored by measuring the level of NAD(P)H. The three cells depicted here had very different spike frequencies. Cell A had a low frequency and illustrates how a single spike resulted in a marked increase in NAD(P)H, which then recovered very much more slowly than the Ca^{2+} transient. In cells B and C, which were spiking at higher frequencies, the repetitive Ca^{2+} spikes occurred before recovery was complete, thus giving either a sawtooth profile (cell B) or a maintained elevation of mitochondrial metabolism (cell C). Reproduced from *Biochim. Biophys. Acta*, Vol. 1366, Robb-Gaspers, L.D., Rutter, G.A., Burnett, P., Hajnóczky, G., Denton, R.M. and Thomas, A.P. (1998) Coupling between cytosolic and mitochondrial calcium oscillations: role in the regulation of hepatic metabolism, pp. 17–32. Copyright (1998), with permission from Elsevier; see Robb-Gaspers et al. 1998.

Mitochondrial fission and fusion

There are a family of mitochondrial-shaping proteins that regulate mitochondrial morphology. The tethering and fusion of mitochondria are controlled by the dynamin-related mitofusins (MFNs). Mitofusin 1 (MFN-1) is located in the outer mitochondrial membrane (OMM) where it acts to tether mitochondria to each other or to the ER, whereas the MFN2 seems to have a regulatory role. The optic atrophy 1 (OPA1) protein co-operates with MFN1 in driving mitochondrial fusion.

Mitochondrial fission is regulated by proteins such as dynamin-related protein (DRP-1), which is a cytoplasmic protein. During fission, DRP-1 attaches to the OMM by binding to its adaptor hFis1 and seems to function to sever both the OMM and the IMM.

Mitofusins (MFNs)

The mitofusins (MFNs) are dynamin-like GTPases that function in mitochondrial fusion. They also have an additional function in holding together the mitochondria and the endoplasmic reticulum, which is of critical importance for the operation of the endoplasmic reticulum (ER)/mitochondrial Ca^{2+} shuttle (Module 5: Figure ER/mitochondrial shuttle). The ER membrane has MFN2 that forms dimeric anti-parallel complexes with either MFN2 or MFN1 located in the outer membrane of the mitochondrion.

Charcot-Marie-Tooth disease 2A is caused by mutations in MFN2.

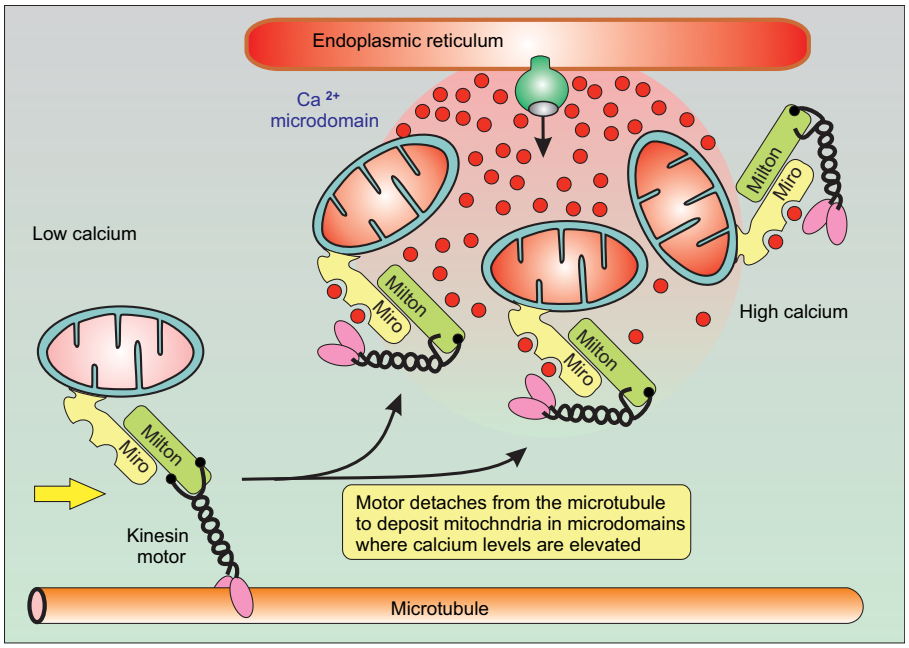
Optic atrophy 1 (OPA1)

Optic atrophy 1 (OPA1), which is one of the nuclear-encoded mitochondrial proteins that resembles dynamin-related GTPases, has two main functions. Firstly, OPA1 co-operates with mitofusin 1 (MFN-1) to drive mitochondrial fusion. The MFN-1 may drive fusion of the OMMs whereas OPA1 may function to induce IMM fusion. Secondly, it can close off the openings of the cristae junctions to form a diffusion barrier that has important physiological consequences particularly with regard to the regulation of apoptosis (Module 5: Figure OPA1 and mitochondrial cristae remodeling).

There are two forms of OPA1: a soluble form and an integral membrane form. The soluble form exists in the intermembrane space between the OMM and the IMM. A presenilin-associated rhomboid-like (PARL) protease located on the IMM is thought to be responsible for forming this soluble OPA1. The integral membrane form has a transmembrane domain that anchors OPA1 in the membrane of the cristae. The soluble form oligomerizes with the two membrane forms to staple together the two opposing cristae membranes to form a barrier. One of the primary functions of this barrier is to maintain cytochrome c within the cristae, which is where most of the mitochondrial respiration takes place. By restricting cytochrome c within the cristae, the OPA1 barrier effectively prevents apoptosis.

There are various ways in which this barrier can be broken down. Apoptotic signals such as tBid may stimulate proteases to disassemble the oligomeric OPA1 complex that forms the barrier. Formation of reactive oxygen species (ROS) following oxidative stress may also increase the release of cytochrome c from the space between the cristae through two mechanisms (Module 5: Figure OPA1 and mitochondrial cristae remodeling). Localization of cytochrome c is also facilitated by its association with cardiolipin (Module 5: Figure cardiolipin).

Module 5: | Figure mitochondrial motility



Control of mitochondrial motility by Ca^{2+}: a working hypothesis.

When Ca^{2+} concentrations are low, mitochondria are pulled along microtubules by kinesin motors. Proteins such as Milton and Miro attach mitochondria to the kinesin motor. The latter is important for Ca^{2+} control, because it contains two EF-hands. The microdomains of Ca^{2+} formed around channels like the inositol 1,4,5-trisphosphate receptor ($InsP_3R$) or the ryanodine receptor (RYR) activate Miro, which then induces the motors to detach from the microtubules thus depositing the microchondria in regions of high Ca^{2+} concentration.

However, an increase in ROS results in peroxidation of cardiolipin and this releases the cytochrome c, which is now free to diffuse out of the space between the cristae, and this egress is enhanced by a ROS-dependent dismantling of the OPA1 barrier.

Expression of OPA1 is regulated through the genotoxic stress activation of the NF-κB signalling mechanism (Module 2: Figure NF-κB activation). Mutations in the *OPA1* gene are responsible for optic atrophy 1.

Presenilin-associated rhomboid-like (PARL) protease

The presenilin-associated rhomboid-like (PARL) protease is a mitochondrial integral membrane protein that has seven transmembrane domains with the N-terminal facing the matrix (Module 5: Figure OPA1 and mitochondrial cristae remodelling). Once it is inserted into the inner mitochondrial membrane it undergoes proteolytic processing that releases a 25-amino-acid peptide called Pβ, which has a nuclear-targeting sequence that can translocate to the nucleus and may function in mitochondria-to-nucleus signalling,

The active form located in the IMM acts as a protease to process various mitochondrial proteins. For example, it can cleave integral optic atrophy 1 (OPA1) to liberate soluble OPA1 that can then oligomerize with integral OPA1 to form a barrier across the opening of the cristae (Module 5: Figure OPA1 and mitochondrial cristae remodelling).

An increased risk for type 2 diabetes has been associated with variations in the *PARL* gene. Also, a missense mutation in the *PARL* gene has been identified in some Parkinson's disease patients.

Parkin

Parkin is a redox-sensitive ubiquitin E3 ligase that can mono- and polyubiquitinate residues at both lysine-48 and lysine-63. One of its numerous substrates is Parkin interacting substrate (PARIS). Parkin can also contribute to a stress-protective pathway through a genotoxic stress activation of NF-κB signalling mechanism that results in an increase in the expression of optic atrophy 1 (OPA1), which is critical for maintaining the cristae in mitochondria and thereby prevents apoptosis (Module 5: Figure OPA1 and mitochondrial cristae remodelling).

Mutations in *PARK2* (the gene for Parkin) has been linked to Parkinson's disease (PD).

PTEN-induced putative kinase 1 (PINK1)

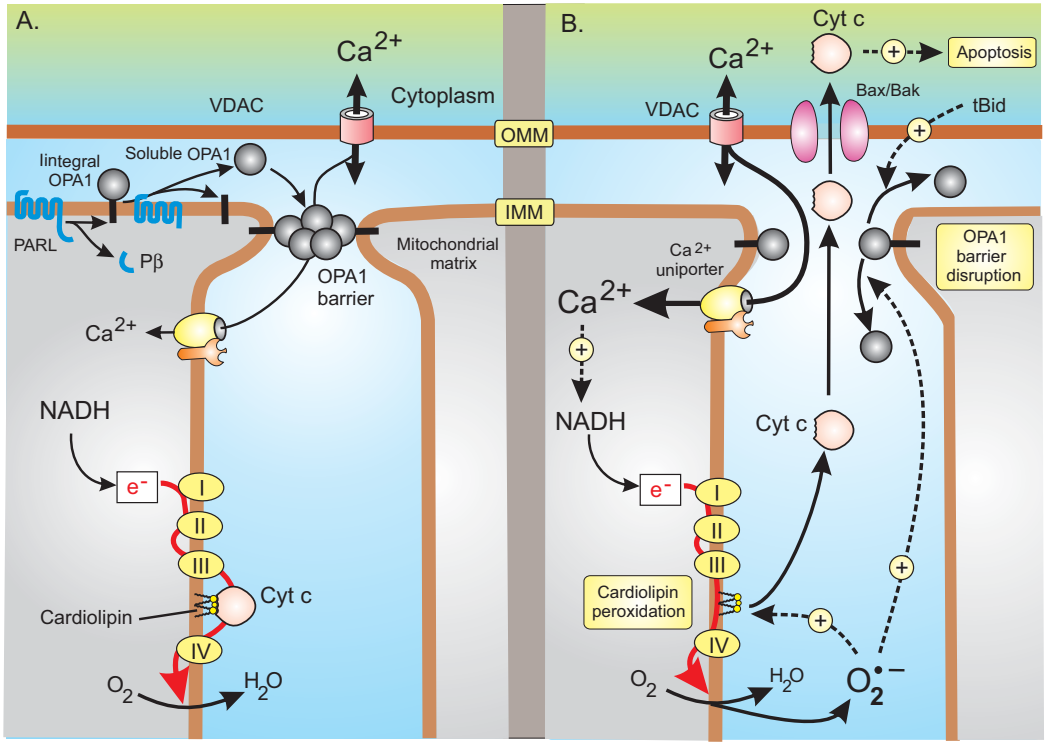
PTEN-induced putative kinase 1 (PINK1) is a mitochondrial protein kinase that is linked functionally to Parkin to regulate mitochondrial fission and fusion. PINK1, which associates with dysfunctional mitochondria that have low membrane potentials, recruits Parkin that marks out these impaired mitochondria for removal by mitophagy.

The *PINK1* gene that codes for PINK1 is one of the autosomal recessive genes that has been implicated in familial Parkinson's disease (PD).

Mitochondrial biogenesis

Mitochondrial biogenesis and maintenance is controlled by many factors such as exercise, cold and hormones (insulin, glucagon and thyroid hormone). Also, there is an age-related decline of mitochondrial functions such as oxidative phosphorylation that have been implicated in a

Module 5: | Figure OPA1 and mitochondrial cristae remodelling



OPA1 remodelling of mitochondrial cristae.
An oligomeric complex between integral membrane OPA1 and soluble OPA1, which is formed by presenilin-associated rhomboid-like (PARL) protease, forms a barrier across the opening of the cristae. Following redox stress, the increase in reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot-}$), has two effects; it causes peroxidation of cardiolipin to release cytochrome c (Module 5: Figure cardiolipin) and it remodels the organization of the cristae by disrupting the OPA1 oligomeric barrier to allow cytochrome c to diffuse through the Bax/Bak channel where it can trigger apoptosis. Opening up the inside of the cristae also allows Ca^{2+} greater access to the Ca^{2+} uniporters and the resulting increase in its concentration in the matrix enhances mitochondrial metabolism and this will increase ROS formation causing further mitochondrial damage.

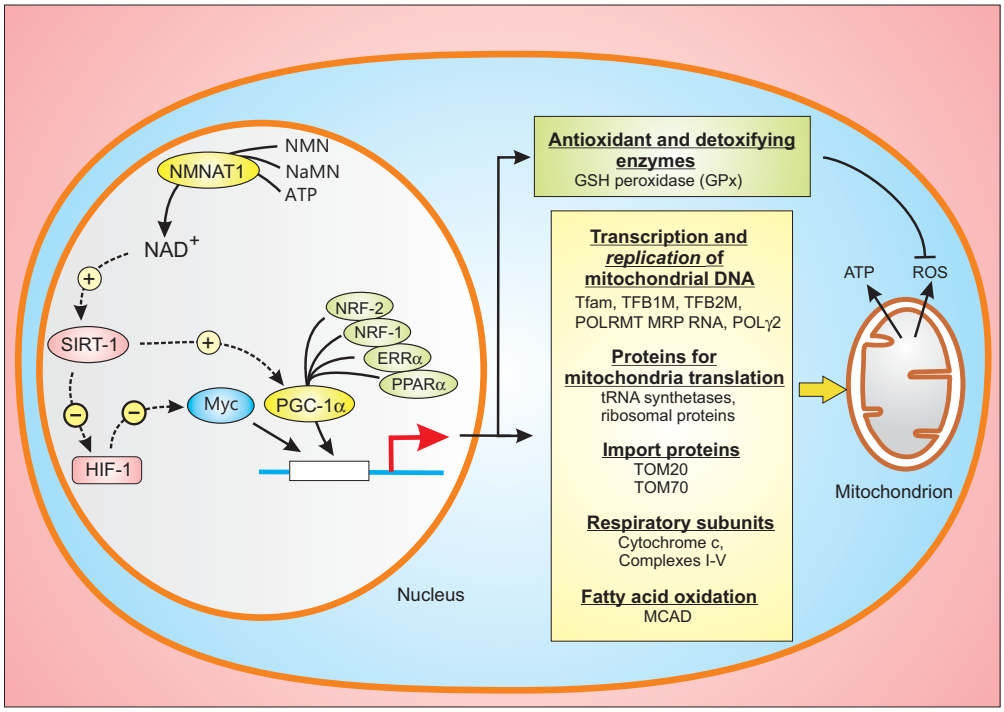
number of neurodegenerative diseases so there is a strong imperative to understand just how mitochondrial function is maintained. Mitochondrial components are under bigenomic control in that they are encoded by both nuclear and mitochondrial genes.

There are thirteen genes located on the small circular mitochondrial DNA. Some of these genes encode subunits for the respiratory complexes I, III and V of the electron transport system. It also encodes the transfer RNAs and two ribosomal RNAs that are used to translate the proteins used in the electron transport system. All the other mitochondrial components are produced by nuclear genes.

Expression of the mitochondrial nuclear genes is regulated by a number of transcriptional cascades. A number of these are orchestrated by the peroxisome-proliferator-activated receptor γ (PPARγ) coactivator-1α (PGC-1α) (Module 4: Figure *PGC-1α* gene activation). The PGC-1α then co-ordinates the activity of the transcription factors such as nuclear respiratory factor-1 (NRF-1), NRF-2, oestrogen receptor α (ERRα) and peroxisome-proliferator-activated receptor α (PPARα) that control the expression of the numerous components necessary for mitochondrial biogenesis (Module 5: Figure mitochondrial biogenesis). The NAD^+ signalling pathway located in the nucleus also plays a role through its regulation of Myc. The nicotinamide mononucleotide adenylyltransferase 1 (NMNAT1) located in the nucleus uses nicotinamide mononucleotide (NMN) or nicotinic acid mononucleotide (NaMN) that interacts with ATP to form nicotinamide–adenine dinucleotide (NAD^+). The NAD^+ then acts on SIRT1 that deacylates HIF-1 that normally acts to inhibit mitochondrial genes that function in oxidative phosphorylation. In addition, SIRT1 acts to stimulate the activity of PGC-1α. High levels of NAD^+ are thus essential for maintaining the transcription of the essential components of mitochondrial metabolism. A decline in nuclear NAD^+ levels may be a contributory factor for mitochondrial dysfunction and ageing.

The NRF-1 is responsible for the expression of the mitochondrial transcription factor (Tfam), transcription factor B1 mitochondrial (TFB1M) and transcription factor B2 mitochondrial (TFB2M), which interact with each other to form a transcriptional complex that controls expression of the mitochondrial genes described above. NRF-1 is also responsible for driving the expression of the translocase of outer mitochondrial membrane 20 (TOM20), which is part of the mechanism responsible for importing proteins into the mitochondrial matrix from the cytosol. The TOM20 plays a role in initiating the transport process by recognizing the precursor proteins that are destined to function

Module 5: | Figure mitochondrial biogenesis



Mitochondrial biogenesis.
The peroxisome-proliferator-activated receptor γ (PPARγ) coactivator-1α (PGC-1α) plays a central role in co-ordinating the activity of the transcription factors such as NRF-1, NRF-2, ERRα and PPARα that control the expression of the numerous components necessary for mitochondrial biogenesis. Nicotinamide–adenine dinucleotide (NAD^+) acts to regulate the activity of the transcription factor Myc that also functions in regulating mitochondrial biogenesis. The idea and information for this Figure was taken from Figures 5 and 9 in Scarpulla (2008).

within the mitochondrion. In addition, NRF-1 acts to increase the expression of antioxidant and detoxifying enzymes responsible for reducing the formation of reactive oxygen species (ROS).

The NRF-2 transcription factor activates all 10 of the cytochrome oxidase subunits such as cytochrome oxidase IV and Vb.

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