Active DNA demethylation: many roads lead to Rome

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Abstract | DNA methylation is one of the best-characterized epigenetic modifications and has been implicated in numerous biological processes, including transposable element silencing, genomic imprinting and X chromosome inactivation. Compared with other epigenetic modifications, DNA methylation is thought to be relatively stable. Despite its role in long-term silencing, DNA methylation is more dynamic than originally thought as active DNA demethylation has been observed during specific stages of development. In the past decade, many enzymes have been proposed to carry out active DNA demethylation and growing evidence suggests that, depending on the context, this process may be achieved by multiple mechanisms. Insight into how DNA methylation is dynamically regulated will broaden our understanding of epigenetic regulation and have great implications in somatic cell reprogramming and regenerative medicine.

Imprinted gene
A gene that is expressed in a parent-of-origin-specific manner.

Inactive X chromosome
The copy of X chromosome that is silenced in female chromosomes in order to equalize the expression of genes located in the X chromosome in males and females.

DNA methyltransferase
An enzyme that catalyses the addition of a methyl group to C or A.

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doi:10.1038/nrm2950
Published online 4 August 2010

Eukaryotic chromatin contains a wealth of information required for the growth and development of a multicellular organism. This information is not only stored genetically in the DNA sequence itself but also epigenetically through DNA methylation and post-translational modifications of histone proteins[1,2]. Although every nucleotide in the genome has the potential to be transcribed[3], the presence or absence of specific epigenetic marks influences gene expression, resulting in a transcriptional programme that specifies for a particular cell type. For example, in embryonic stem (ES) cells, active gene expression marks are found at pluripotent genes and repressive marks are found at lineage-specific genes. Thus, different cell types can be defined by their epigenetic and gene expression profiles.

During development, these transcriptional programmes undergo dynamic changes that ultimately lead to the production of distinct cell types and tissues that make up an organism. Accommodating such a transcriptional programme requires an epigenome that is both dynamic and flexible. Furthermore, the diversity of genetic material to be regulated necessitates the use of marks corresponding to short-term and long-term epigenetic memory, depending on the transcriptional requirements of the cell (as well as those of future generations). Developmental genes that are needed during the later stages of development are transiently held in a repressed state during early development. This is achieved through short-term epigenetic marks such as histone modifications, which can be removed before or within a few cell divisions.

By contrast, other regions of the genome are marked with epigenetic information that is stably maintained and heritable after many cell divisions. For example, imprinted genes, transposons and the inactive X chromosome require long-term silencing that is sustained throughout the development and lifespan of an organism. This is generally achieved by DNA methylation, an epigenetic mark that refers to the addition of a methyl group to the fifth carbon of base C. Because DNA methylation provides heritable, long-term silencing that is crucial for an organism, aberrant DNA methylation has been associated with cancer, imprinting-related diseases and psychiatric disorders[4–7].

In mammals, DNA methylation occurs predominantly in the context of CpG (C followed by G) dinucleotides, whereas DNA methylation in plants can occur at C bases in diverse sequence contexts[8]. The enzymes responsible for this modification, DNA methyltransferases (DNMTs), are well characterized and conserved in mammals and plants[8]. DNMTs fall under two categories: *de novo* and maintenance[9]. Patterns of DNA methylation are initially established by the *de novo* DNA methyltransferases DNMT3A and DNMT3B during the blastocyst stage of embryonic development[10,11] (FIG. 1). These methyl marks are then faithfully maintained during cell divisions through the action of the maintenance methyltransferase, DNMT1, which has a preference for hemi-methylated DNA[12–14]. Both the establishment and maintenance of DNA methylation patterns are crucial for development as mice deficient in DNMT3B or DNMT1 are embryonic lethal[11,15] and DNMT3A-null mice die by 4 weeks of age[11].

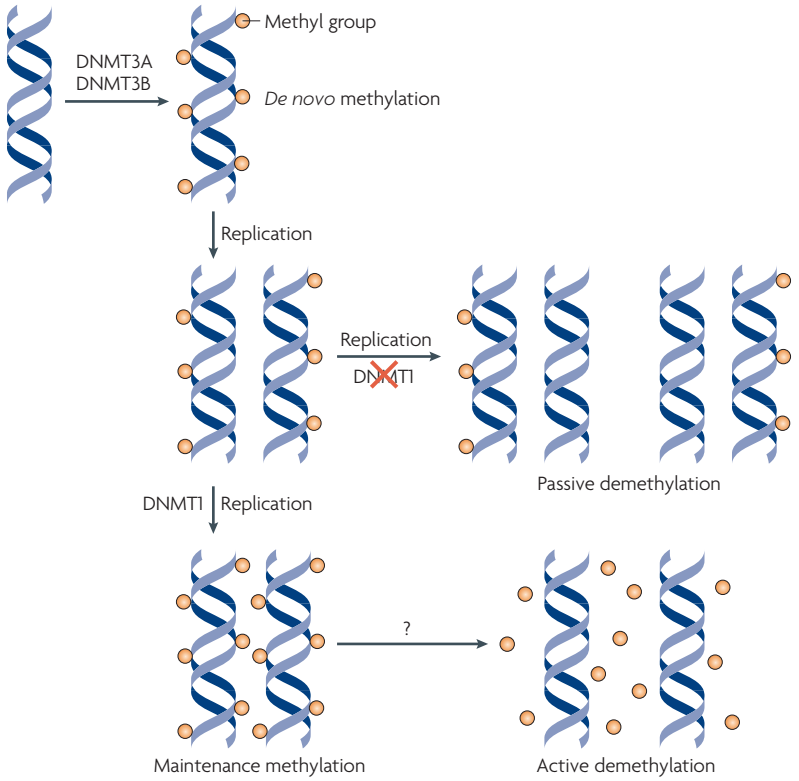

Figure 1 | **Mechanisms of DNA methylation and demethylation.** During early development, methylation patterns are initially established by the *de novo* DNA methyltransferases DNMT3A and DNMT3B. When DNA replication and cell division occur, these methyl marks are maintained in daughter cells by the maintenance methyltransferase, DNMT1, which has a preference for hemi-methylated DNA. If DNMT1 is inhibited or absent when the cell divides, the newly synthesized strand of DNA will not be methylated and successive rounds of cell division will result in passive demethylation. By contrast, active demethylation can occur through the enzymatic replacement of 5-methylcytosine (5meC) with C.

Hemi-methylated DNA
Duplex DNA in which only one of the two strands is methylated.

Zona pellucida
The glycoprotein coat that surrounds the oocytes and the early embryos of mammals.

Polar body
The structure that is extruded from the oocyte during meiosis and contains one haploid set of chromosomes.

Parthenogenesis
The production of a diploid offspring from two sets of haploid maternal gametes and no paternal contribution.

Gynogenesis
Parthenogenesis in which the embryo contains only maternal chromosomes owing to the failure of the sperm to fuse with the egg nucleus.

Although DNA methylation has been viewed as a stable epigenetic mark, studies in the past decade have revealed that this modification is not as static as once thought. In fact, loss of DNA methylation, or DNA demethylation, has been observed in specific contexts (see below) and can occur through active or passive mechanisms (FIG. 1). Active DNA demethylation is the enzymatic process that results in the removal of the methyl group from 5-methylcytosine (5meC) by breaking a carbon–carbon bond. By contrast, passive DNA demethylation refers to the loss of the methyl group from 5meC when DNMT1 is inhibited or absent during successive rounds of DNA replication. Whereas passive DNA demethylation is generally understood and accepted, the subject of active DNA demethylation has been controversial[16].

In this Review, we explore what is known about active DNA demethylation and the disputes that are embedded in this field. First, we describe the contexts in which DNA demethylation has been observed and discuss the evidence that supports an active mechanism. We then present the many enzymes that have been proposed to carry out active DNA demethylation. We conclude by discussing emerging themes and highlighting the remaining questions in this exciting field.

Evidence for active DNA demethylation

Even though DNA methylation contributes to stable, long-term and heritable silencing, it has become apparent that in some instances DNA methylation levels can rapidly change by mechanisms involving active DNA demethylation. Genome-wide and gene-specific demethylation events have both been observed, but current evidence suggests that the former only occurs at specific times during early development, whereas the latter occurs in somatic cells responding to specific signals.

Genome-wide DNA demethylation of paternal pronuclei. Prior to fertilization, mammalian gametes are at different stages of the cell cycle and their genomes are organized differently. The egg is meiotically arrested at metaphase II, resulting in a diploid genome that is packaged with histones. Mature sperm, however, have completed meiosis, but their haploid genomes are packaged with protamines instead of histones. When a sperm penetrates the zona pellucida to fertilize the egg, both gametes undergo rapid changes. The egg completes its second meiosis resulting in the extrusion of one copy of the genome as the polar body; the sperm reorganizes its genomic DNA by replacing protamines with histone proteins.

Shortly after the protamine–histone exchange, the sperm-derived paternal pronucleus undergoes genome-wide DNA demethylation[17,18], an event that occurs quite rapidly within 4–8 hours post-fertilization (FIG. 2a). Although there are some disputes regarding the timing and synchrony of DNA replication in the zygote[19–25], loss of DNA methylation is seen before the completion of the first cell division. Thus, it is unlikely that a passive demethylation mechanism is the cause for this observation. Furthermore, when zygotes were treated with the replication inhibitor aphidicolin, paternal genome demethylation was still detected[17,26], further supporting an active demethylation mechanism.

Paternal genome demethylation has been observed in many mammalian organisms, including human, mouse, rat, bovine and pig[17,18,27,28], but seems to be absent from others, such as sheep[29]. When sheep sperm are injected into mouse oocytes, demethylation is seen in the sheep-derived paternal genome[30], suggesting that the demethylating factor or factors are contributed by the oocyte. However, sheep oocytes injected with mouse sperm also resulted in demethylation of the mouse-derived paternal genome[30]. Although this occurs to a lesser extent compared to mouse oocytes, it is likely that factors present in the sperm or features unique to the paternal genome also contribute to demethylation. Consistent with this notion, mouse oocytes can demethylate multiple male pronuclei[31], but are incapable of demethylating the additional maternal genome in parthenogenetic, gynogenetic and digynic triploid zygotes[32].

Although immunostaining studies suggest that demethylation occurs globally, bisulphite sequencing indicates that some genomic regions are resistant to such a wave of demethylation. These genomic regions include imprinting control regions[33], intracisternal A-particle (IAP) retrotransposons[34] and centric and pericentric heterochromatin[31,35]. It is not clear why these genomic

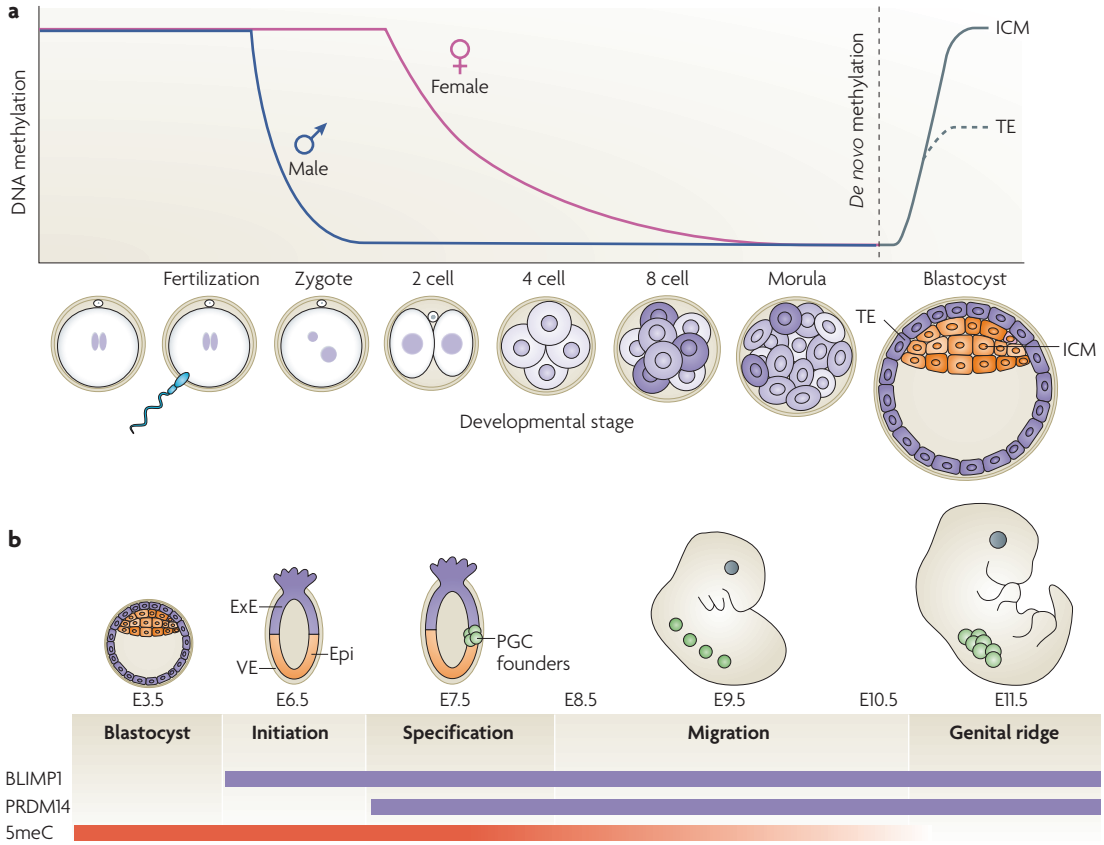

Figure 2 | **Dynamics of DNA methylation during development. a** | Active demethylation in the zygotic paternal genome. Shortly after a sperm fertilizes an egg, the paternal genome rapidly undergoes genome-wide active DNA demethylation and remains demethylated following multiple rounds of cell division. During this time, the maternal genome experiences gradual, passive demethylation. *De novo* methylation patterns are established by the DNA methyltransferases DNMT3A and DNMT3B during the development of the blastocyst. **b** | Active demethylation in primordial germ cells (PGCs). After implantation of the blastocyst at embryonic day 7.5 (E7.5), the extraembryonic ectoderm (ExE) and visceral endoderm (VE) produce signals that specify a subset of epiblast cells (Epi) to become PGCs. This process requires two key transcription factors, BLIMP1 (also known as PR domain zinc finger protein 1 (PRDM1)) and PDRM14, which are expressed during this stage of development. Following specification, PGC founder cells divide in the presence of the DNA methyltransferase DNMT1 and migrate towards the genital ridge. During this migration and on arrival at the genital ridge, 5-methylcytosine (5meC) is erased through an active mechanism. ICM, inner cell mass; TE, trophectoderm.

regions are resistant to this wave of DNA demethylation, but one possibility is that methylation of these regions may be required to ensure transcriptional repression and chromosomal stability. Additionally, the maternal genome remains methylated during this time even though it is exposed to the same cytoplasmic factors. Insight into how some regions in the paternal genome are targeted for DNA demethylation whereas other regions are resistant may also provide clues as to how the maternal genome is protected from active demethylation (BOX 1).

The significance of zygotic paternal genome DNA demethylation is unclear at present. Genome-wide demethylation may facilitate transcriptional activation of the paternal genome[36], which has been reported to occur before transcriptional activation of the maternal genome in some species[37]. Although some transposable elements and repeat sequences have been identified to be resistant to DNA demethylation, it is likely that others are still targets of DNA demethylation, given that these types of sequences account for half of the genome. Whether demethylation of transposable elements and repeat sequences results in their reactivation and, if so, what the significance of their reactivation is remains to be determined.

Genome-wide DNA demethylation of primordial germ cells. After fertilization, the one-cell zygote undergoes several cell divisions that ultimately lead to formation of the blastocyst. During this developmental period, the maternal genome undergoes passive DNA demethylation (FIG. 2a) — a gradual loss of DNA methylation occurs with each cell division[38] in a replication-dependent manner[39]. Consistent with this, maternally contributed DNMT1 (also known as DNMT1O) is excluded from the nucleus[40]. Although passive DNA demethylation seems to affect a large part of the genome, imprinted genes still retain their methylation marks. Recent genetic studies indicate that maternal and zygotic DNMT1 (REF. 41) and the zinc finger protein ZFP57 (REF. 42) are required to maintain the DNA methylation imprints during pre-implantation development.

Digynic triploid
An embryo that contains two maternal genomes and one paternal genome.

Bisulphite sequencing
A technique in which the treatment of DNA with bisulphite, which converts C to U but does not modify meC, is used to determine the DNA methylation pattern.

Blastocyst
An embryonic stage that is characterized by the formation of the first definitive lineages.

Box 1 | **Protection of the maternal genome from demethylation**

Whereas the paternal genome undergoes extensive demethylation, the maternal genome remains methylated even though it is exposed to the same cytoplasmic factors. This may be due to a mechanism that protects the maternal genome from this wave of demethylation or to a putative DNA demethylase that is specifically recruited to the paternal genome.

Sperm DNA is packaged with protamines, which are exchanged for canonical and noncanonical histones on fertilization. Interestingly, deposition of the histone variant H3.3 occurs asymmetrically, with a strong preference for the paternal pronucleus[158,159]. This raises the possibility that asymmetric H3.3 deposition may trigger the paternal genome-specific demethylation process. Asymmetric patterns of histone modifications have also been seen in the maternal and paternal pronuclei and may also contribute to the asymmetric demethylation process. For example, methylation, dimethylation and trimethylation at H3 Lys27 (H3K27me1, H3K27me2 and HsK27me3, respectively) and at Lys9 (H3K9me2 and H3K9me3) are clearly seen in the maternal pronucleus of zygotes, but are virtually undetectable in the paternal pronucleus[159–164]. Thus, the maternal genome may use a protective mechanism against demethylation by carrying specific histone variants or modifications.

Alternatively, a recent study has suggested that non-histone factors present in the oocyte might protect the maternal genome from demethylation[165]. Zygotes lacking stella (also known as *DPPA3* and *PGC7*), a maternal effect gene required for early development[166], exhibited demethylation of both pronuclei. Although stella can directly bind DNA *in vitro*, it seems to lack specificity for methylated DNA[165]. Therefore, how stella protects the maternal genome from demethylation remains to be determined.

At embryonic day 7.5 (E7.5), signals originating from the extraembryonic ectoderm and the visceral endoderm instruct a subset of posterior epiblast cells to become primordial germ cells (PGCs). Specification of PGCs involves the BMP4 and BMP8 signalling pathway and activation of transcription factors BLIMP1 (also known as PR domain zinc finger protein 1 (PRDM1)) and PRDM14 (REFS 43,44). These founder cells of the germ line begin to migrate at E8.5 and arrive at the genital ridge at E11.5. At the beginning of their specification and migration, PGCs are thought to have the same epigenetic marks as other epiblast cells. However, by the time they arrive at the genital ridge, many of these marks including DNA methylation have been erased[45–47] (FIG. 2b). Given that PGCs have undergone several cell cycles in the presence of DNMT1, this demethylation event is considered to be active. It is thought that global demethylation, including that of imprinted genes, takes place so that new DNA methylation patterns can be re-established, although experimental evidence supporting this remains to be shown.

Loci-specific active demethylation in somatic cells. Active DNA demethylation has also been reported in somatic cells, but only at specific genomic loci in response to certain signals. For example, within 20 minutes of stimulation, activated T lymphocytes undergo active demethylation at the interleukin-2 promoter-enhancer region in the absence of DNA replication[48]. Locus-specific demethylation has also been reported to occur at the promoter of brain-derived neurotrophic factor (*BDNF*)[49], the protein product of which is important for adult neural plasticity (FIG. 3a). In unstimulated neurons, the *BDNF* promoter is methylated, allowing for the recruitment of the repressive meC-binding protein, MeCP2. When depolarized with KCl, *BDNF* is upregulated, coinciding with the release of MeCP2 and demethylation of the promoter[49]. Because this event takes place in post-mitotic neurons, active demethylation is thought to be the underlying mechanism. In addition to T cells and neurons, active DNA demethylation has been reported to take place during nuclear hormone-regulated gene activation (FIG. 3b). For example, the *pS2* (also known as *TFF1*) promoter exhibits periodic methylation and demethylation that coincides with cyclical binding of oestrogen receptor-α (ERα) and expression of pS2 (REFS 50,51). Similarly, active DNA demethylation occurs at the cytochrome p450, subfamily 27B, polypeptide 1 (*CYP27B1*) promoter in response to parathyroid hormone (PTH)[52]. These studies suggest that DNA methylation may not function solely as a long-term silencing mark, but could also function in the dynamic regulation of genes that require rapid responses to specific stimuli.

Primordial germ cell
One of a population of embryonic cells from which germ cells are formed.

Mechanisms of active DNA demethylation

The importance of DNA methylation in diverse biological processes coupled with the observations of active DNA demethylation in embryonic development and somatic cells have led to extensive efforts in identifying DNA demethylases. DNA demethylase activity was first reported in murine erythroleukaemic nuclear extracts[53]. Although it was determined that 5meC was ultimately replaced by C in a replication-independent manner, this activity has not been further characterized. A DNA demethylase activity was also seen in rat myoblasts[54]. However, its sensitivities towards RNase and protease treatments were conflicting[55] and this activity was not pursued further.

Since then, several studies have led to the proposal of various mechanisms by which active DNA demethylation can occur. These include: enzymatic removal of the methyl group of 5meC, base excision repair (BER) through direct excision of 5meC, deamination of 5meC to T followed by BER of the T•G mismatch, nucleotide excision repair (NER), oxidative demethylation and radical *S*-adenosylmethionine (SAM)-based demethylation.

Enzymatic removal of the methyl group of 5meC. The simplest way to achieve DNA demethylation is through enzymatic removal of the methyl group of 5meC. This requires an enzyme with enormous catalytic power because of the strength of the carbon–carbon bond that needs to be broken. Methyl-CpG-binding domain protein 2 (MBD2) was the first reported protein to carry out this reaction. It did not require any specific cofactors, and was proposed to release methanol[56]. This thermodynamically unfavourable mechanism was hotly contested for several reasons. First, previous studies had shown that MBD2 can stably bind methylated DNA[57,58], making it unclear how binding could occur if MBD2 was so efficient at removing the methyl group. Further concerns were raised when MBD2-null mice were not only viable, but also exhibited normal methylation patterns[59]. Importantly, the paternal pronucleus of MBD2-null zygotes still exhibit normal demethylation[31]. These observations, coupled with the fact that no other laboratories could reproduce the reported enzymatic activity, have raised serious doubts on the capacity of MBD2 to serve as a DNA demethylase.

Regardless of the controversy surrounding MBD2, it is still conceivable that a *bona fide* DNA demethylation mechanism exists. In fact, numerous histone demethylases that can break a carbon–nitrogen bond have recently been discovered[60,61]. Although carbon–carbon bonds are inherently more difficult to break than carbon–nitrogen bonds, enzymes that have the capacity to do so have been reported in the thymidine salvage pathway[62] and the cholesterol synthesis pathway[63].

BER through direct excision of 5meC. It has been proposed for some time that DNA demethylation can be achieved through the BER DNA repair pathway (FIG. 4a). This type of repair involves a DNA glycosylase that removes the target base resulting in an abasic (apurinic and apyrimidinic (AP)) site. The DNA backbone is subsequently nicked by an AP lyase activity to generate a 5′ phosphomonoester and a 3′ sugar phosphate residue. An AP endonuclease then removes the 3′ sugar group leaving a single nucleotide gap that is ultimately filled in by DNA repair polymerases and ligases[64].

Active DNA demethylation can be accomplished by a DNA glycosylase that directly excises 5meC to initiate BER (FIG. 4a). Strong genetic and biochemical evidence supports the use of this mechanism in plants[65]. In *Arabidopsis thaliana*, DNA demethylation is mediated by the Demeter (Dme) family of DNA glycosylases, which consists of four members: DME, repressor of silencing 1 (ROS1; also known as DML1), DML2 and DML3 (REF. 65). The discovery that these DNA glycosylases suppress DNA methylation initially came from forward-genetic screens in *A. thaliana*. Whereas DME was discovered owing to the loss of expression of the imprinted gene *MEDEA* in a loss-of-function DME mutant[66], ROS1 was recovered in a genetic screen for mutants that confer promoter hypermethylation and transgene silencing defects[67].

DME, ROS1, DML2 and DML3 possess glycosylase activity against oligonucleotides containing 5meC[67–71]. In addition, all members of the Dme family possess AP lyase activity and are thus considered bifunctional glycosylases[69–71]. Besides CpG, DNA methylation in plants can occur in the context of CpNpG (where N is A, T or C) and CpNpN. All members of the Dme family have the capacity to recognize and remove meC bases from double-stranded DNA (dsDNA) oligonucleotides, irrespective of their sequence context *in vitro*[71]. However, attempts to determine the substrate specificity of these enzymes have resulted in conflicting reports owing to the use of different substrates and reaction conditions[68–71]. *In vivo* studies indicate that mutation of each of these genes results in hypermethylation in all sequence contexts but at distinct genomic loci[69,71–73], indicating that each of these enzymes has a unique *in vivo* function.

Although it is clear that plants use BER to achieve DNA demethylation, evidence supporting a similar mechanism in mammals has been less compelling. Despite the lack of an obvious mammalian orthologue of the ROS1 family, the first indication that a repair mechanism could contribute to DNA demethylation came from early studies in chicken embryo extracts[74], revealing 5meC glycosylase

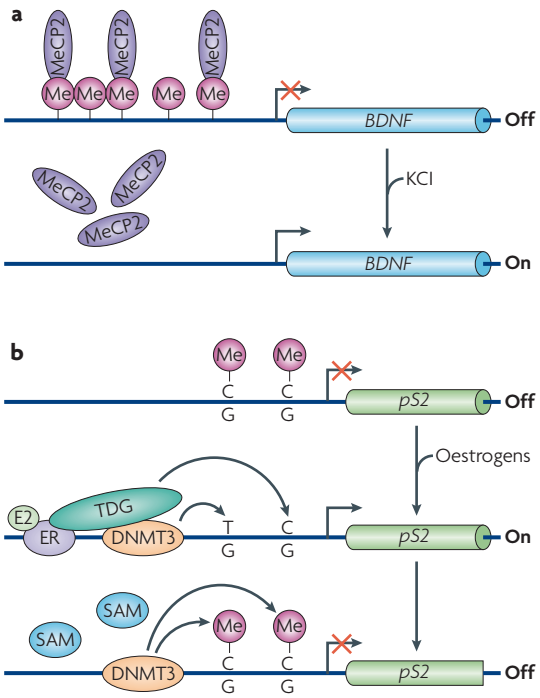

Figure 3 | **Locus-specific active DNA demethylation in somatic cells. a** | Active demethylation at the brain-derived neurotrophic factor (*BDNF*) promoter. In neurons, *BDNF* is maintained in a repressed state through DNA methylation and binding of the repressive methylcytosine (meC)-binding protein MeCP2. On depolarization with KCl, DNA methylation and MeCP2 binding are lost, concomitant with increased *BDNF* expression. This demethylation event is considered to be active because it occurs in post-mitotic neurons. **b** | Active demethylation at nuclear receptor target promoters. The promoter of the oestrogen receptor (ER) target gene *pS2* (also known as *TFF1*) undergoes cyclical rounds of methylation and demethylation that correspond to the repression and expression of the gene, respectively. Transcriptional activation of *pS2* occurs in the presence of oestrogens (E2) and coincides with demethylation of the promoter. This is achieved by deamination of 5meC by DNA methyltransferase 3 (DNMT3) followed by base excision repair (BER) of the T•G mismatch by T DNA glycosylase (TDG). To revert to repression, DNMT3 re-methylates the promoter. Although DNMT3 is involved in both methylation and demethylation, it is important to note that DNMT3 can only carry out the deamination step in the absence or at low concentrations of the methyl donor S-adenosylmethionine (SAM).

activity against hemi-methylated DNA[75]. Subsequent purification of this activity showed that it has three components: RNA, an RNA helicase related to the human p68 DEAD-box protein and a homologue of human T DNA glycosylase (TDG)[76–78]. Thus, 5meC glycosylase activity initially detected in chicken embryo extracts was attributed to TDG. However, its excision activity against 5meC was 30–40-fold lower compared with that against T[78]. Although TDG can flip C and C analogues into its active site, it does not possess the catalytic power to break the N-glycosidic bond[79]. It should be noted that the excision activity of TDG against 5meC is stimulated by the

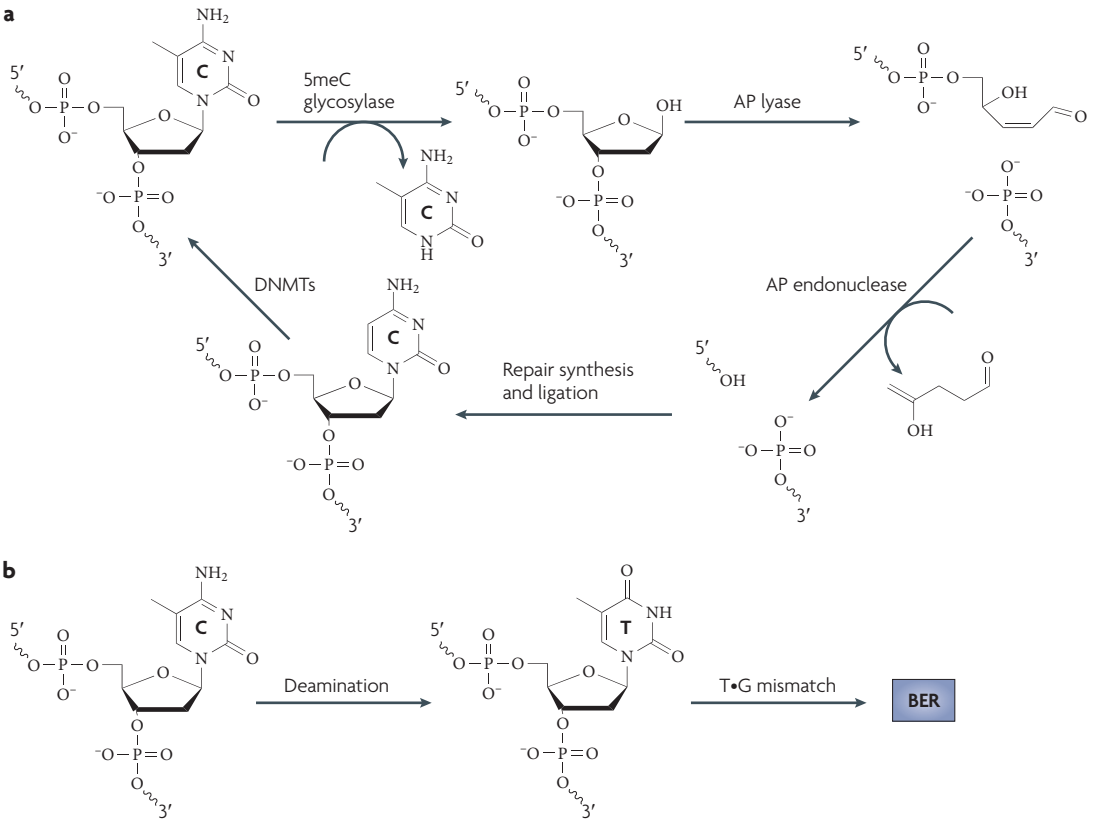

Figure 4 | **Base excision repair-based mechanisms for DNA demethylation. a** | Base excision repair (BER) through direct excision of 5-methylcytosine (5meC). Initiation of the BER pathway can be carried out by a glycosylase that directly excises 5meC to generate an abasic (apurinic and apyrimidinic (AP)) site. The DNA backbone is nicked by an AP lyase (or by the glycosylase itself if it is bifunctional). The 3′ sugar group is then cleaved by an AP endonuclease and the resulting single nucleotide gap is filled in with an unmethylated C by an unknown polymerase and ligase. It has been well established in plants that the demeter (Dme; also known as repressor of silencing 1 (ROS1)) family of enzymes can carry out the 5meC glycosylase reaction, but to date no mammalian enzymes have been reported to be capable of carrying out this step efficiently. **b** | Deamination of 5meC followed by BER. In contrast to direct excision of 5meC, deamination of 5meC produces T, which can be repaired by BER by a T•G mismatch glycosylase such as T DNA glycosylase (TDG) or methyl-CpG-binding domain protein 4 (MBD4) to regenerate an unmethylated C. DNMT, DNA methyltransferase.

presence of both RNA and the RNA helicase[78]. Similarly, both DNMT3A and DNMT3B have been reported to interact with and stimulate the enzymatic activity of TDG[80,81]. Future work should determine whether these interactions have an effect on substrate preference *in vitro* and whether loss of function of TDG has an effect on DNA methylation status *in vivo*.

In addition to TDG, the methyl-CpG-binding protein MBD4 has glycosylase activity against 5meC, but again this activity is 30–40-fold lower than its T•G mismatch glycosylase activity[82]. Not surprisingly, MBD4-null zygotes exhibit normal demethylation of the zygotic paternal pronucleus[83], and MBD4-null mice have an increased number of C to T mutations regardless of whether the C is methylated or not[84]. Despite its unfavourable biochemical properties, MBD4 was reported to carry out active DNA demethylation of the *CYP27B1* promoter in response to PTH[52]. Interestingly, phosphorylation by protein kinase C enhanced MBD4 glycosylase activity against 5meC[52], which may partially explain earlier enzymatic studies showing MBD4's preference for C over 5meC[85].

Deamination of 5meC to T followed by BER. DNA demethylation can also be achieved by deamination of 5meC to produce T, followed by BER to replace the mismatched T with unmethylated C (FIG. 4b). Both cytidine deaminases and DNMTs have been proposed to carry out the first step of this mechanism. On deamination of 5meC, T glycosylases such as TDG and MBD4 (see above) may function by repairing the mismatch.

Cytidine deaminases are important players in diverse biological processes such as the generation of antibody diversity, RNA editing and retroviral defence[86]. These processes require the production of mutations in DNA and RNA, which is achieved, in part, through the deamination of cytidine to uridine by the activation-induced deaminase (AID) and apolipoprotein B mRNA editing enzyme, catalytic polypeptide (APOBEC) family of proteins. APOBEC1, the founding member of this family, is involved in editing apolipoprotein B pre-mRNA[87,88]. The related deaminase AID was discovered to be essential for somatic hypermutation and class switch recombination of immunoglobulin genes in B cells[89,90]. Consistent with its role in the diversification of antibodies, AID-deficient

RNA editing
The post-transcriptional modification of RNA primary sequence by the insertion and/or deletion of specific bases, or the chemical modification of adenosine to inosine or cytidine to uridine.

Somatic hypermutation
The mutation of the immunoglobulin variable region in mature B cells during an immune response. It results in affinity maturation of the antibody response. Like class switch recombination, it requires activation-induced cytidine deaminase.

mice are viable and fertile and significant phenotypic abnormalities are seen only in B cells[89,90].

Despite the lack of developmental defects in AID-knockout mice, both AID and APOBEC1 have been shown *in vitro* and in an *E. coli* assay to have the capacity to deaminate 5meC to T in the context of single-stranded DNA[91]. AID and APOBEC1 are also expressed in mouse oocytes, ES cells and PGCs, which may be a consequence of their genomic location in a cluster of pluripotency genes that include nanog and stella (also known as *DPPA3* and *PGC7*)[91]. Nevertheless, expression of AID in PGCs and the early embryo points to a possible role in global DNA demethylation. Indeed, a recent large-scale bisulphite sequencing study indicates that DNA methylation levels of male and female PGCs derived from AID-null embryos increased about 4% (from 18% to 22%) and 13% (from 7% to 20%), respectively, when compared to their wild-type counterpart[92], suggesting that AID may contribute to PGC demethylation. However, because the DNA methylation levels in AID-null PGCs (~20%) are still relatively low compared with ES or somatic cells (70–80%), considerable demethylation still occurs in the absence of AID, indicating that other factors responsible for PGC demethylation remain to be identified.

Nevertheless, studies in zebrafish embryos have suggested that Aid, Mbd4 and the DNA repair protein Gadd45a (growth arrest and DNA-damage-inducible 45α) can cooperate in demethylating a methylated DNA duplex[93]. In this study, when a methylated linear dsDNA of ~740 bp was injected into a zebrafish embryo, demethylation of the injected DNA was seen when Aid and Mbd4 were co-expressed. The authors postulated that Aid deaminated 5meC, allowing Mbd4 to excise the T•G mismatch. Indeed, the T•G mismatch was detected using a PCR strategy, but only when Aid was expressed with a catalytic mutant of Mbd4 because the wild-type version excised the mismatch too quickly for it to be detected. Furthermore, when Aid and Mbd4 were titrated to levels that did not cause demethylation, the inclusion of Gadd45a elicited demethylation, indicating that these three proteins act cooperatively[93].

Although the above studies have provided some evidence that AID may contribute to mammalian DNA demethylation, decisive biochemical and genetic evidence supporting a major role in this process is still lacking. Biochemically, AID can act on 5meC in the context of single-stranded DNA but not dsDNA[91]. Genetically, AID-knockout mice exhibit the expected B cell and immunological defects[89,90], but no gross developmental or reproductive defects. Similarly, APOBEC1-knockout mice are also viable and fertile[94,95]. Although genetic redundancy may be a possible cause of the lack of expected developmental and reproductive phenotypes, such an explanation needs to be confirmed by generating combinational knockouts. Furthermore, because DNA methylation occurs symmetrically, deamination of both strands would give rise to a TG•GT double mismatch. There is no evidence indicating that either TDG or MBD4 can use a double mismatch as a substrate. Furthermore, processing of a double mismatch by the AP endonuclease would generate a DNA double-strand break. This would put tremendous pressure on the repair machinery if such a mechanism were used for global demethylation. However, for locus-specific DNA demethylation, such a mechanism would not present a big problem.

In addition to AID and APOBEC, DNMTs have recently been implicated in 5meC deamination, even though they are commonly known for their ability to catalyse DNA methylation. Evidence indicating their involvement in the deamination process initially came from studies in bacteria where the methyltransferases M. HpaII[96–98] and M. EcoRII[99,100] were shown to possess deaminase activities. Consistent with bacterial studies, the mammalian counterparts, DNMT3A and DNMT3B, have also been shown to possess deaminase activity *in vitro*[51]. As discussed above, the promoters of oestrogen-responsive genes undergo cyclical rounds of methylation and demethylation. Thus, the participation of DNMT3A and DNMT3B in both methylation and demethylation would facilitate rapid transcriptional cycling (FIG. 3b). Interestingly, ERα associates with and stimulates the activity of TDG[101,102], allowing for the repair of the T•G mismatch. DNMT3A and DNMT3B also associate with TDG and this interaction stimulates glycosylase activity[80,81]. Indeed, DNA demethylation was found to coincide with the recruitment of TDG and other BER enzymes[51].

However, it is surprising that DNMTs possess two opposing enzymatic activities. Although the methyltransferase activity of DNMT3A is inhibited by TDG[81], the 5meC deamination reaction can only occur under conditions where SAM concentrations are very low or nonexistent[51]. In order for DNMT3A to carry out efficient methylation and demethylation during transcriptional cycling, levels of SAM must fluctuate rapidly. Given that SAM is crucial for many essential biochemical and biological processes, it is difficult to imagine how this could be achieved without serious biological consequences.

Nucleotide excision repair. Another DNA repair pathway, NER, has also been proposed to carry out DNA demethylation. This type of repair is generally used to repair DNA containing bulky lesions, which form after exposure to chemicals or radiation. After damaged DNA is recognized, dual incisions flanking the lesion are made and a 24–32 nucleotide oligomer is released. The resulting gap is then filled in by repair polymerases and sealed by a ligase[64].

In an assay aimed at identifying proteins required for activation of a reporter that is silenced by DNA methylation, Niehrs and colleagues uncovered a novel function for GADD45A[103], which is encoded by a p53- and breast cancer type 1 susceptibility protein (BRCA1)-inducible gene and participates in diverse biological processes, including DNA damage response, cell cycle progression, apoptosis and NER[104]. Overexpression of GADD45A in mammalian cell lines leads to loci-specific and global demethylation, whereas knockdown results in DNA hypermethylation[103]. Because GADD45A had previously been implicated in NER[105,106], Barretto *et al.* explored the role of NER in DNA demethylation and found that loss of DNA methylation is accompanied by DNA synthesis and requires the NER endonuclease xeroderma pigmentosum

Class switch recombination
A mechanism that changes the class or isotype of antibody produced by an activated B cell. This does not change the affinity towards an antigen, but instead allows for interaction with different effector molecules.

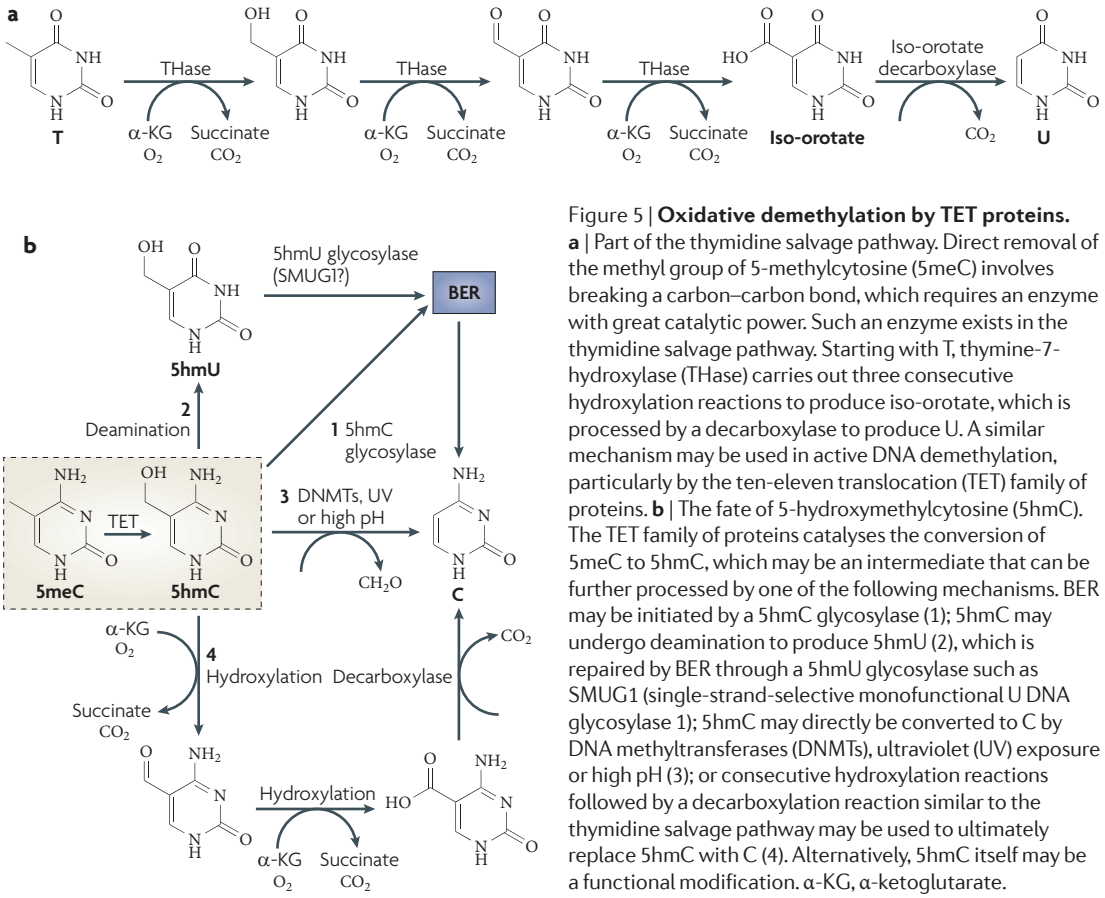

Figure 5 | **Oxidative demethylation by TET proteins.** **a** | Part of the thymidine salvage pathway. Direct removal of the methyl group of 5-methylcytosine (5meC) involves breaking a carbon–carbon bond, which requires an enzyme with great catalytic power. Such an enzyme exists in the thymidine salvage pathway. Starting with T, thymine-7-hydroxylase (THase) carries out three consecutive hydroxylation reactions to produce iso-orotate, which is processed by a decarboxylase to produce U. A similar mechanism may be used in active DNA demethylation, particularly by the ten-eleven translocation (TET) family of proteins. **b** | The fate of 5-hydroxymethylcytosine (5hmC). The TET family of proteins catalyses the conversion of 5meC to 5hmC, which may be an intermediate that can be further processed by one of the following mechanisms. BER may be initiated by a 5hmC glycosylase (1); 5hmC may undergo deamination to produce 5hmU (2), which is repaired by BER through a 5hmU glycosylase such as SMUG1 (single-strand-selective monofunctional U DNA glycosylase 1); 5hmC may directly be converted to C by DNA methyltransferases (DNMTs), ultraviolet (UV) exposure or high pH (3); or consecutive hydroxylation reactions followed by a decarboxylation reaction similar to the thymidine salvage pathway may be used to ultimately replace 5hmC with C (4). Alternatively, 5hmC itself may be a functional modification. α-KG, α-ketoglutarate.

group G-complementing protein (XPG), which interacts with GADD45A[103]. The recruitment of GADD45A and other components of the NER repair machinery to ribosomal RNA (rRNA) genes is facilitated by TBP-associated factor 12 (TAF12) and leads to DNA demethylation and rRNA gene activation[107]. However, it is not clear how the demethylation process is initiated and whether GADD45A is directly involved. More importantly, two independent studies have raised doubt on the role of GADD45A in the active DNA demethylation process. In the first study, the Pfeifer group carried out a series of experiments that were similar to those carried out by the Niehrs group, but obtained no evidence indicating that GADD45A had any effect on DNA methylation[108]. In the second study, analysis of the GADD45A-null mice indicated that loss of GADD45A function had neither loci-specific nor global effects on DNA methylation levels[109].

GADD45B, another member of the GADD45 family, has also been implicated in active demethylation of genes that are crucial for adult neurogenesis[110]. Loss of GADD45B results in defects in neural progenitor proliferation and dendritic growth. This was attributed to promoter hypermethylation and the repression of *BDNF* and fibroblast growth factor 1 (*FGF1*), two genes crucial for neurogenesis[110]. However, GADD45B is not involved in zygotic DNA demethylation as GADD45B-null zygotes undergo normal paternal genome demethylation[111]. Because GADD45B has not been biochemically characterized, it is unknown whether it is directly involved in the active demethylation of neurogenesis genes.

Oxidative demethylation. Another possible mechanism by which DNA demethylation can be carried out is through oxidative demethylation. The *E. coli* enzyme AlkB is a member of the 2-oxoglutarate (2OG)-dependent dioxygenases and is involved in the bacterial response to alkylation damage to DNA. Using oxygen, iron and 2OG as cofactors, AlkB is able to carry out oxidative demethylation of 1-methyladenine and 3meC by releasing the methyl group as formaldehyde[112,113]. The same mechanism is used by the JmjC family of enzymes to demethylate histone substrates[60,114].

Although breakage of a carbon–carbon bond is energetically difficult, enzymes that catalyse such reactions do exist. As shown in FIG. 5a, thymine 7-hydroxylase can catalyse the conversion of T to iso-orotate through three consecutive oxidation reactions using oxygen, iron and 2OG as cofactors[115]. Iso-orotate can be further converted to C through a decarboxylation reaction. Although thymine 7-hydroxylase and iso-orotate decarboxylase have been isolated from fungi, such as *Rhodotorula glutinis, Neurospora crassa* and *Aspergillus nidulans*[62], no homologue of thymine 7-hydroxylase has been found in mammals. Interestingly, the trypanosome base J-binding proteins, JBP1 and JBP2, have properties similar to that of thymine 7-hydroxylase [116,117], prompting the Rao group

JmjC
(Jumonji C). An evolutionarily conserved motif. Proteins containing this domain are predicted to be protein hydroxylases or histone demethylases.

Base J-binding protein
A protein that binds to base J (β-D-glucosylhydroxymethyl-U), a modified T produced by hydroxylation and glucosylation of the methyl group of T.

to search for mammalian homologues with similarity to the dioxygenase domains of the JBP proteins. This effort led to the identification of the ten-eleven translocation (TET) family of proteins[118]. We have also independently characterized the mouse TET family[119].

TET1, the founding member of the TET family, was initially discovered in acute myeloid leukaemia (AML) as a fusion partner of the histone H3 Lys4 methyltransferase MLL[120,121]. Subsequent studies *in vitro* and in cultured cells showed that human TET1 is capable of hydrolysing 5meC to produce 5-hydroxymethylcytosine (5hmC) in DNA[118]. Similarly, all three members of the mouse TET family possess this enzymatic activity[119]. Consistent with the presence of a dioxygenase domain in the proteins and the predicted reaction mechanism, the putative iron-binding sites are required for their enzymatic activities[118,119]. Furthermore, TET1 is capable of acting on both fully methylated and hemi-methylated DNA[118].

Although 5hmC has previously been reported to exist in animal DNA[122], this modified base is not found in some cell types and tissues[118,123], thus raising the question of whether 5hmC is present in mammalian DNA at physiologically relevant levels. This issue was directly addressed in two cell types. In Purkinje neurons, 5hmC is ~40% as abundant as 5meC[124], whereas the frequency of 5hmC in ES cells was estimated to be approximately 1 in every 3,000 nucleotides[118]. Thus, it is evident that 5hmC constitutes a large fraction of mammalian DNA in some cell types.

The consequences of 5hmC in genomic DNA are currently unclear. Because 5hmC seems to be stable, it may function like other modifications by altering local chromatin structure or contributing to the recruitment or exclusion of other factors that influence transcription. For example, the transcriptional repressors MeCP2, MBD1, MBD2 and MBD4 bind to methylated DNA, but do not recognize 5hmC[125,126]. It is also possible that the TET proteins may facilitate passive demethylation in dividing cells such as ES cells as 5hmC is not recognized by DNMT1 (REF. 127); thus, newly replicated DNA would not maintain patterns of methylation. Alternatively, 5hmC may be an intermediate in an active demethylation pathway that ultimately leads to the replacement of 5meC with C (FIG. 5b). This could be achieved by several ways that include: BER by a 5hmC-specific DNA glycosylase (as such activity has been previously reported to exist in calf thymus extracts[128]), deamination of 5hmC to generate 5hmU followed by BER initiated by a 5hmU-specific glycosylase such as single-strand-selective monofunctional U DNA glycosylase 1 (SMUG1)[129], conversion of 5hmC to C through loss of formaldehyde on ultraviolet light exposure[130] or high pH[131] (or possibly carried out by DNMTs)[132], and two consecutive oxidation steps followed by decarboxylation similar to that used by the thymidine salvage pathway (FIG. 5a). It is not clear why TET proteins cannot catalyse consecutive reactions such as that of thymine 7-hydroxylase. Because all *in vitro* assays carried out so far used recombinant TET proteins alone, it is possible that association of TET proteins with their *in vivo* partners is necessary to confer such a capability. In this case, a decarboxylase may eventually remove the carboxyl group to complete the demethylation process.

Consistent with the relative enrichment of 5hmC in ES cells, recent studies have shed light on the role of TET1 in ES cell biology. During ES cell differentiation, *TET1* mRNA levels decline, coinciding with a decrease in 5hmC levels[118], which suggests that TET1 may be important for ES cell identity. Indeed, knockdown of TET1, but not TET2 or TET3, in mouse ES cells results in impairment of ES cell self-renewal and maintenance[119]. Analysis of the differentiated TET1-knockdown ES cells revealed a bias towards the trophoectoderm and primitive endoderm lineages. Furthermore, knockdown of TET1 at two-cell stage embryos followed by cell lineage tracing revealed that the knockdown cells are biased towards the trophoectoderm[119], indicating that TET1 is required for inner cell mass cell specification. Consistent with its role in ES cell self-renewal and maintenance, knockout of TET1 resulted in embryonic lethality (K. Hong and Y.Z., unpublished observations), making the evaluation of the role of TET1 on global demethylation of the paternal genome difficult.

With regard to the mechanism underlying the role of TET1 in ES cells, TET1 maintains nanog expression in ES cells by directly binding to the nanog promoter and protecting it from becoming hypermethylated, as knockdown of TET1 in ES cells resulted in downregulation of nanog expression concomitant with increased nanog promoter methylation[119]. Nanog seems to be one of the main TET1 targets as the phenotypes associated with TET1 knockdown can largely be rescued by ectopic expression of nanog[119].

Although TET2 is also expressed in ES cells, it seems that TET2 does not play a significant part in ES cell biology as knockdown of TET2 does not confer any obvious phenotype [119]. However, a flurry of recent studies have uncovered that dysfunction of human TET2 may be a key event in leukaemogenesis as human *TET2* is mutated in a range of human myeloid malignancies, including myelodysplastic syndromes (MDSs), myeloproliferative disorders (MPDs) and acute myeloid leukaemias (AMLs)[133–140]. Currently, *TET2* is the most frequently mutated gene that has been identified in patients with MDS and these mutations have been suggested to occur early during the pathogenesis of the disease[137]. Consistent with a role for TET2 in regulating DNA demethylation, aberrant DNA methylation is frequently found in patients with MDS[141]. Indeed, mutations of TET2 that mimic mutations identified in patients with MDS abolished the enzymatic activity of TET2 (A. C. D'Alessio and Y.Z., unpublished observations). Furthermore, the DNA methyltransferase inhibitor 5-azacytidine (5-azaC) has been shown to be an effective treatment for patients with high-risk MDS and secondary AML[142,143], indicating that aberrant DNA methylation plays a crucial part in MDS development and progression. The participation of TET2 in DNA demethylation may provide a molecular basis for the effectiveness of using methyltransferase inhibitors in the treatment of patients with MDS, thus setting the stage for understanding the molecular mechanism underlying the pathogenesis of leukaemias.

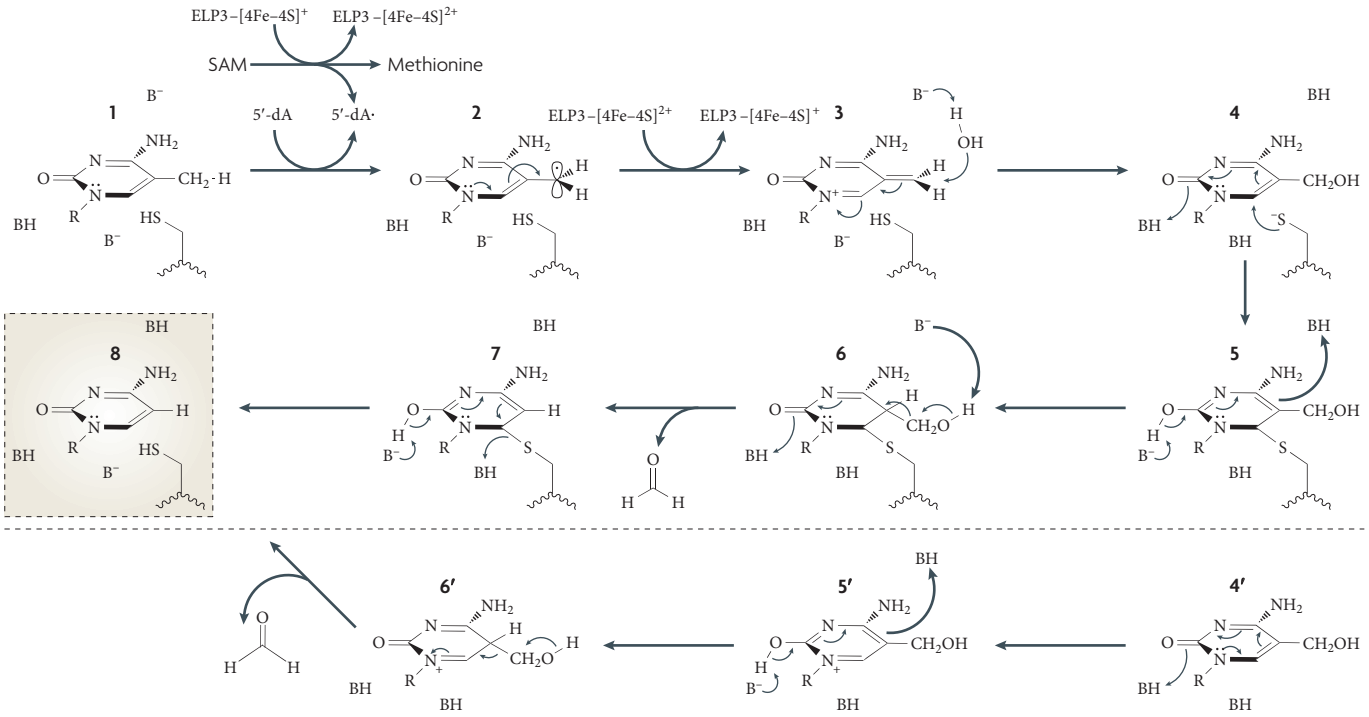

Figure 6 | **Proposed mechanism for ELP3-mediated DNA demethylation.** Mammalian elongator complex protein 3 (ELP3) contains an Fe–S radical S-adenosylmethionine (SAM) domain that is important for active DNA demethylation of the zygotic paternal genome. If ELP3 is indeed a functional radical SAM protein, it may directly carry out DNA demethylation through the following mechanism. First, ELP3 uses SAM to generate a 5′-deoxyadenosyl radical, which could extract a hydrogen atom from the 5-methyl group of 5-methylcytosine (5meC; 1) to form a 5meC radical (2). After an electron is donated back to the Fe–S to create the third intermediate (3), a water molecule would promote the formation of 5-hydroxymethylcytosine (5hmC) (4). A nucleophilic attack at carbon 6 can result in the carbon–carbon bond breaking to release formaldehyde (5–7). In the absence of an external nucleophile, an alternative pathway (4′–6′) that leads to the release of formaldehyde can also take place. Finally, an elimination step would produce an end product of C (8).

Radical SAM mechanism. Although many proteins have been proposed to carry out active DNA demethylation, none of the proteins discussed above have been shown to have a role in paternal genome demethylation in zygotes. To identify proteins involved in paternal genome demethylation, our laboratory used a candidate gene knockdown approach coupled with live-cell imaging. To facilitate a screen of candidate proteins, we developed a probe that consists of the Cys-X-X-Cys domain of MLL fused to enhanced green fluorescent protein (EGFP). Because the Cys-X-X-Cys domain has high affinity for unmethylated CpG[144], injection of mRNA encoding the probe into zygotes results in the accumulation of the probe at the demethylated paternal pronucleus[111], allowing live-cell imaging of the paternal genome demethylation process. Using this imaging system, we screened several candidate proteins by injecting small interfering RNAs (siRNAs) against each of the candidates into eggs before carrying out intracytoplasmic sperm injection (ICSI), and monitored the effect of the siRNA on the accumulation of the probe at the paternal pronucleus. This screen uncovered a role for elongator complex protein 3 (ELP3) in paternal genome demethylation. ELP3 knockdown prevented the accumulation of the probe in the paternal pronucleus at pronuclear stages 4 and 5 (REF. 111). In addition, immunostaining and bisulphite sequencing of selected retrotransposon elements further support a role for ELP3 in paternal genome demethylation[111].

ELP3 is a member of the core elongator complex (ELP1–ELP3), which combines with another subcomplex (ELP4–ELP6) to form the holo-elongator complex[145,146]. Because knockdown of the ELP1 and ELP4 components also impaired paternal genome demethylation, it is likely that the entire elongator complex may be involved in the demethylation process[111]. Interestingly, the Fe–S radical SAM domain of ELP3, but not the histone acetyltransferase (HAT) domain, is required for paternal genome demethylation[111]. Although this may provide a clue regarding the enzymatic mechanism of ELP3, recent studies in yeast suggest that the Cys-rich domain of Elp3 is required for the integrity of the elongator complex[147,148], raising the possibility that the Fe–S radical SAM motif may have a structural rather than an enzymatic role. Thus, direct biochemical evidence of the enzymatic activity of the elongator complex and genetic evidence using ELP3-null oocytes remain to be shown.

Interestingly, a recent study confirmed the presence of an Fe–S cluster in the bacteria *Methanocaldococcus jannaschii* Elp3 protein[149]. The assumption that mammalian ELP3 is a radical SAM protein has led to a potential mechanism for ELP3-catalysed DNA demethylation as outlined in FIG. 6 (S. J. Booker, personal communication).

Elongator complex
A protein complex originally identified in budding yeast to be associated with the elongating and hyperphosphorylated RNA polymerase II. It has also been implicated in tRNA modification, exocytosis and neuronal maturation.

SAM domain
A protein domain containing an Fe–S cluster that uses S-adenosylmethionine (SAM) to catalyse various radical reactions.

Box 2 | **Implications of active DNA demethylation in reprogramming**

Induced pluripotent stem (iPS) cells can be generated by introducing four transcription factors — octamer-binding protein 3 (OCT3; also known as OCT4 and POU5F1), SRY box-containing factor 2 (SOX2), krüppel-like factor 4 (KLF4) and MYC — into somatic cells[167,168]. Successful reprogramming requires the activation of endogenous *OCT4* and nanog genes, which are known to be silenced by DNA methylation in somatic cells[169–172]. Demethylation of the *OCT4* and nanog promoters is thus an integral event in iPS cell generation[173]. In fact, inefficient DNA demethylation is thought to be one of the causes of the low efficiency in iPS cell generation because the use of the DNA methylation inhibitor 5-azacytidine can increase the efficiency of iPS cell generation by converting partially reprogrammed cells to fully reprogrammed iPS cells[173].

Transcription factor-based iPS cell generation is a slow process compared to somatic cell nuclear transfer (SCNT) and cell fusion[174,175]. One possible explanation for this difference may be the mechanisms used to reactivate endogenous *OCT4* and nanog. Epigenetic reprogramming of somatic cells to pluripotent iPS cells may necessitate several cell divisions[176] owing to the absence of the DNA demethylase or demethylases required for demethylation of the *OCT4* and nanog promoters. By contrast, reactivation of *OCT4* and nanog can occur quickly during SCNT and cell fusion because the DNA demethylase or demethylases may already be present in eggs and embryonic stem (ES) cells. Consistent with this notion, reprogramming by cell fusion requires activation-induced deaminase (AID)-dependent demethylation and reactivation of *OCT4* and nanog[177]. Surprisingly, although AID was present at the *OCT4* and nanog promoters in fibroblasts, these promoters are methylated, suggesting that other factors or regulatory events are required for demethylation. Given that genetic evidence does not support an important role for AID in ES cells (see main text), it is unclear whether AID directly participates in promoter demethylation of these genes during somatic cell reprogramming. Regardless, it is evident that activation of pluripotent genes through DNA demethylation is an important step during the somatic cell reprogramming process. Identification and characterization of the enzymes involved should improve protocols of somatic cell reprogramming.

Like every radical SAM enzyme, the reaction is initiated by the generation of a powerful oxidizing agent, the 5′-deoxyadenosyl (5′-dA) radical, from SAM. The 5′-dA radical could extract a hydrogen atom from the 5-methyl group to generate a 5meC radical. In the next step, an electron is returned back to the Fe–S cluster to generate a third intermediate, which can be converted to the relatively stable 5hmC by the addition of a water molecule. In order to break the carbon–carbon bond, the next step requires the generation of an intermediate, the resulting carbanion of which would be stabilized. This can probably be achieved by a thymidylate synthase or methyltransferase type of mechanism, whereby a Cys residue carries out a nucleophilic attack at carbon 6, leading to the release of formaldehyde. In the absence of an external nucleophile, an alternative pathway leading to the release of formaldehyde can also take place. Finally, an elimination at the formaldehyde release step results in the final product of C.

Although future studies are required to validate or refute this proposed mechanism, we note that this work is not trivial for three reasons. First, the identities of mammalian ELP5 and ELP6 still need to be determined as an apparent orthologue of yeast Elp5 and Elp6 cannot be identified by sequence homology searches. Second, the radical SAM reaction occurs under anaerobic conditions and reconstitution of the elongator complex under anaerobic conditions is challenging. Finally, given that zygotic DNA demethylation occurs only on the paternal genome, some unique features of the paternal genome may be required in order for it to serve as a substrate. Despite these challenges, identification of the elongator complex as an important factor for paternal genome demethylation in zygotes allows for further studies towards understanding the molecular mechanisms of active DNA demethylation.

Concluding remarks

Observations of active DNA demethylation during embryonic development and in somatic cells have opened the door for many questions to be answered. In particular, how DNA demethylation is achieved in mammalian cells remains debatable as no single enzyme or mechanism has gained decisive biochemical and genetic support (see Supplementary information S1 (table)). It is possible that multiple mechanisms exist to carry out DNA demethylation and that the use of each one is dictated by the specific biological context.

Although repair-based mechanisms, particularly deamination of 5meC followed by BER, have offered an attractive mechanism for active DNA demethylation, genetic evidence is still lacking. Furthermore, the involvement of a repair-based mechanism in global DNA demethylation would put tremendous pressure on the repair machinery when considering that paternal pronucleus demethylation is completed within 4 hours[17,18]. Although AID seems to contribute to active demethylation in PGCs, it is only responsible for a small part of it as considerable demethylation still takes place in the AID-null PGCs[92]. Nevertheless, this mechanism does provide a reasonable explanation for loci-specific demethylation in response to gene-activation signals.

Although AID deficiency has some effect on PGC demethylation, there is no evidence that it affects paternal DNA demethylation in zygotes. Similarly, MBD4-null zygotes still experience paternal genome demethylation[83]. It seems that although repair-based mechanisms may be responsible for loci-specific DNA demethylation and partial demethylation in PGCs, their role in zygotic paternal genome demethylation is less likely. To date, the only factor shown to have a role in zygotic paternal genome demethylation is the elongator complex, although it is unclear whether its role is direct or indirect[111]. Future work should focus on gaining additional genetic evidence using elongator-null zygotes and elucidating its enzymatic activity. The recent demonstration that the TET family proteins are capable of catalysing the conversion of 5meC to 5hmC has raised the possibility that these proteins may have a role in active DNA demethylation[118,119]. We anticipate that work evaluating their role in demethylation of the zygotic paternal genome and PGCs is forthcoming. Furthermore, analysis of the fate and function of 5hmC will also attract a lot of attention.

In addition to determining the mechanism of active demethylation, one open question that remains is to what extent the paternal genome and PGCs are demethylated. Although this event is considered to be global, as determined by 5meC immunostaining, it is evident that some regions of the paternal genome are protected from this wave of demethylation. The advent of high-throughput analyses including chromatin immunoprecipitation-on-chip (ChIP-chip), ChIP sequencing (ChIP-Seq) and bisulphite sequencing (BS-Seq; bisulphite treatment

followed by high-throughput sequencing) has allowed for genome-wide profiling of epigenetic marks such as DNA methylation[92,150–153]. Using single-molecule, real-time sequencing, a recent study showed the feasibility of direct detection of modified nucleotides in DNA, including N6-meA, 5mC and 5hmC[154]. Future studies using these tools will undoubtedly determine precisely which genomic regions are demethylated and which regions are protected. However, improvements in the sensitivity of these technologies will be necessary for such experiments, given that paternal genomic DNA would need to be obtained from individual zygotes.

As well as being fundamental to our knowledge in epigenetics, a better understanding of how DNA demethylation occurs will allow for the development of techniques and approaches that will improve somatic cell reprogramming (BOX 2) and cancer treatment. Tumour suppressor gene silencing by promoter DNA methylation is thought to play an important part in cancer development[155]. Consistently, inhibitors of DNMTs have been used in the treatment of certain cancers[156]. Owing to the reversible nature of epigenetic modifications, developing drugs that target epigenetic factors is becoming one of the top priorities for many biotechnology and pharmaceutical companies[157]. It is anticipated that targeted demethylation of tumour suppressor genes may reactivate the silenced tumour suppressor genes, which can lead to cellular differentiation or halt uncontrolled cell proliferation.

The mechanism underlying the regulation of DNA methylation is a question that has elicited much attention and controversy over the past decade. Although recent studies have proposed numerous ideas as to how active DNA demethylation is carried out, many aspects are still contentious and a consensus has yet to be achieved. With the development of new technology and the studies described above, our continued and collective efforts in this field will hopefully provide clearer answers in the coming decade.

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Interspecies heterokaryon experiments reveal that AID is required for demethylation of the *OCT4* and nanog promoters during reprogramming.

Acknowledgements
We thank S. J. Booker for discussions regarding the radical SAM mechanism, and K. Hong and A. D'Alessio for critical comments on the manuscript. We apologize to colleagues whose work cannot be cited owing to space constraints. Work in the Zhang laboratory is supported by the National Institutes of Health (GM68804) and the Howard Hughes Medical Institute, of which Y.Z. is an investigator.

Competing interests statement
The authors declare no competing financial interests.

DATABASES
Entrez Gene: http://www.ncbi.nlm.nih.gov/gene
BDNF | *CYP27B1* | *FGF1* | *MEDEA* | nanog | *pS2* | stella
UniProtKB: http://www.uniprot.org
AID | AlkB | APOBEC1 | BLIMP1 | DME | DML2 | DML3 | DNMT1 | DNMT3A | DNMT3B | ELP3 | ERα | Gadd45a | GADD45B | JBP1 | JBP2 | MBD2 | MBD4 | MeCP2 | M.EcoRII | M.HpaII | MLL | PRDM1 | PRDM14 | PTH | ROS1 | SMUG1 | TAF12 | TDG | TET1 | XPG | ZFP57

FURTHER INFORMATION
Yi Zhang's homepage:
http://www.med.unc.edu/~zhangyi/lab.htm

SUPPLEMENTARY INFORMATION
See online article: S1 (table)

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Mitochondria and cell death: outer membrane permeabilization and beyond

Stephen W. G. Tait and Douglas R. Green

Abstract | Mitochondrial outer membrane permeabilization (MOMP) is often required for activation of the caspase proteases that cause apoptotic cell death. Various intermembrane space (IMS) proteins, such as cytochrome *c*, promote caspase activation following their mitochondrial release. As a consequence, mitochondrial outer membrane integrity is highly controlled, primarily through interactions between pro- and anti-apoptotic members of the B cell lymphoma 2 (BCL-2) protein family. Following MOMP by pro-apoptotic BCL-2-associated X protein (BAX) or BCL-2 antagonist or killer (BAK), additional regulatory mechanisms govern the mitochondrial release of IMS proteins and caspase activity. MOMP typically leads to cell death irrespective of caspase activity by causing a progressive decline in mitochondrial function, although cells can survive this under certain circumstances, which may have pathophysiological consequences.

Apoptosis is a genetically encoded programme leading to cell death that is involved in normal development and homeostasis throughout the animal kingdom. Deregulated apoptosis has been implicated in diverse pathologies, including cancer and neurodegenerative disease. The defining morphological characteristics of apoptosis include cell shrinkage, nuclear fragmentation, chromatin condensation and membrane blebbing, all of which are due to the proteolytic activity of the caspase proteases[1,2] (BOX 1; see Supplementary information S1 (movie)). Caspases orchestrate apoptosis through the cleavage of numerous proteins, ultimately leading to the phagocytic recognition and engulfment of the dying cell.

In vertebrate cells, apoptosis typically proceeds through one of two signalling cascades termed the intrinsic and extrinsic pathways, both of which converge on activating the executioner caspases, caspase 3 and caspase 7 (FIG. 1). In the intrinsic pathway, mitochondrial outer membrane permeabilization (MOMP), which leads to the release of pro-apoptotic proteins from the mitochondrial intermembrane space (IMS), is the crucial event driving initiator caspase activation and apoptosis (BOX 2). Following its release from mitochondria, cytochrome *c* binds apoptotic protease-activating factor 1 (APAF1), inducing its conformational change and oligomerization and leading to the formation of a caspase activation platform termed the apoptosome. The apoptosome recruits, dimerizes and activates an initiator caspase, caspase 9, which, in turn, cleaves and activates caspase 3 and caspase 7. Mitochondrial release of second mitochondria-derived activator of caspase (SMAC; also known as DIABLO) and OMI (also known as HTRA2) blocks X-linked inhibitor of apoptosis protein (XIAP)-mediated inhibition of caspase activity. MOMP is a highly regulated process, primarily controlled through interactions between pro- and anti-apoptotic members of the B cell lymphoma 2 (BCL-2) family (BOX 3).

In the extrinsic pathway, death receptor ligation causes the recruitment of adaptor molecules, such as FAS-associated death domain protein (FADD), that bind, dimerize and activate an initiator caspase, caspase 8. Active caspase 8 directly cleaves and activates the executioner caspases, caspase 3 and caspase 7. In so-called type I cells, caspase 8-mediated activation of the executioner caspases is sufficient to induce apoptosis in the absence of MOMP. Crosstalk between the extrinsic and intrinsic pathways occurs through caspase 8-mediated cleavage of BCL-2 homology 3 (BH3)-interacting domain death agonist (BID; a BH3 domain-only protein), leading to BID activation and MOMP. This step is crucial for death receptor-induced apoptosis in type II cells. The requirement for MOMP-induced XIAP antagonism discriminates between type I and type II cells in death receptor-mediated apoptosis. Hepatocytes are an *in vivo* type II cell; injection of wild-type mice with the FAS death receptor ligand, FASL,

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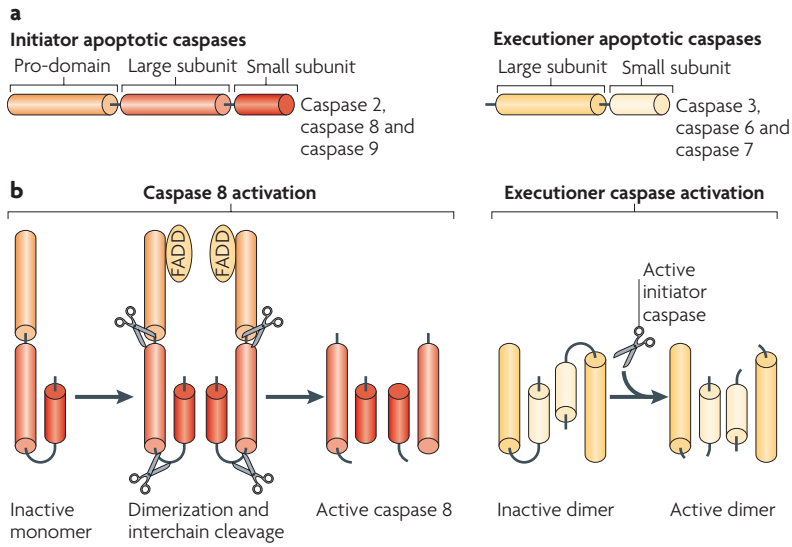
doi:10.1038/nrm2952
Published online 4 August 2010

Box 1 | **Caspase classification and activation**



Caspases (Cys Asp acid proteases) cleave substrates in a highly specific manner after the Asp residue in short tetrapeptide (X-X-X-Asp) motifs. Besides apoptotic roles, some caspase family members have non-apoptotic functions in processes such as cytokine maturation, inflammation and differentiation. Additionally, apoptotic caspases can have non-apoptotic roles in certain circumstances[128–130]. Apoptotic caspases can be divided into two classes: initiator and executioner caspases (see the figure, part **a**). Initiator caspases (caspase 2, caspase 8 and caspase 9) are the apical caspases in apoptosis signalling cascades and their activation is normally required for executioner caspase (caspase 3, caspase 6 and caspase 7) activation. The repertoire of initiator caspase substrates is limited and includes self-cleavage, BCL-2 homology 3 (BH3)-interacting domain death agonist (BID) and executioner caspases. By contrast, executioner caspases cleave hundreds of different substrates and are largely responsible for the phenotypic changes seen during apoptosis. Initiator caspase activation first involves dimerization of inactive caspase monomers (see the figure, part **b**). In the case of caspase 8, following death receptor ligation, dimers are formed by the recruitment of caspase 8 monomers through their pro-domains to the adaptor molecule FAS-associated death domain protein (FADD). Dimerization and interdomain cleavage are required for the activation and stabilization of mature caspase 8 (REFS 131–133). Although dimerization is required for caspase 9 activation and interdomain cleavage occurs, cleavage is involved in the attenuation rather than promotion of caspase 9 activity[89,134]. The activation mechanism of executioner caspases differs from that of initiator caspases (see the figure, part **b**). Executioner caspases are present as dimers in cells and are activated by cleavage, leading to intramolecular rearrangements and the formation of an enzymatically active dimer.

causes hepatocyte apoptosis that leads to rapid, fatal hepatitis, whereas BID-deficient mice are resistant to this[3]. Combined loss of BID and XIAP restores hepatocyte apoptotic sensitivity and hepatitis following FASL injection, providing genetic proof that MOMP-induced XIAP antagonism is required for FASL-induced apoptosis in hepatocytes[4].

In vertebrates, most apoptotic stimuli require MOMP for caspase activation and apoptosis. In contrast, MOMP is dispensable for apoptosis in the invertebrate organisms *Drosophila melanogaster* and *Caenorhabditis elegans*[5]. When MOMP has been detected in *D. melanogaster*, it seems to be a consequence rather than a cause of caspase activation[6]. Interestingly, although MOMP does not contribute to apoptosis, fission of the tubular mitochondrial network promotes apoptosis in both *C. elegans* and *D. melanogaster* through an as yet undefined mechanism[6,7].

Tubular mitochondrial network
Multiple fused mitochondria forming filamentous, elongated structures. These networks are highly dynamic owing to constant rounds of mitochondrial fission and fusion.

In most cases, MOMP is a point of no return for cell survival as cells die irrespective of caspase activity following MOMP[8]. Given this importance, addressing how the mitochondrial outer membrane is selectively breached, and why this causes cell death, remains an intense area of basic and translational research. Here, we focus on recent studies that provide new insight into how MOMP occurs, the nature of membrane permeabilization and how the release of IMS proteins can be regulated post-MOMP. We then discuss the regulation of caspase activity post-MOMP and how MOMP brings about cell death in either a caspase-dependent or caspase-independent manner. Finally, we review data showing that MOMP is not always an obligatory death sentence, as some cells can recover.

Pulling the trigger: activation of MOMP

BCL-2-mediated regulation of MOMP is discussed only briefly here, and the reader is referred to recent, extensive reviews for further details[9,10]. Activation of either BCL-2-associated X protein (BAX) or BCL-2 antagonist or killer (BAK) is essential for MOMP as cells lacking both proteins fail to undergo MOMP and apoptosis in response to diverse intrinsic stimuli[11]. BAX and BAK activity is largely controlled through interactions with other members of the BCL-2 family (BOX 3).

On activation, BAX and BAK undergo extensive conformational changes, leading to the mitochondrial targeting of BAX and the homo-oligomerization of BAK and BAX[12–14]. Oligomerization of BAX or BAK is likely to be required for MOMP as mutants of either protein that fail to form oligomers are unable to cause MOMP[15,16]. FRET-based analysis of BAX-mediated liposome permeabilization has provided compelling, real-time evidence for direct and dynamic interactions between truncated BID (tBID) and BAX, which precede BAX membrane insertion and liposome permeabilization[17]. This supports a model in which BAX (and by analogy BAK) activation requires interaction with BH3-only proteins. Structural analysis of BAX in complex with a chemically stapled BCL-2-interacting mediator of cell death (BIM; also known as BCL2L11) BH3 domain peptide termed BIM SAHB (stablized α-helices of BCL-2 domains) revealed a somewhat unexpected interaction site[18]. BIM SAHB does not bind in the BAX hydrophobic BH3-binding pocket (as occurs when the BID BH3 domain binds BAK[19]) but, instead, binds on the opposite side of BAX. Mutations in BAX that inhibit BIM SAHB binding attenuate BAX-induced MOMP, supporting a functional relevance for this interaction during BAX activation. However, it remains unclear whether direct activator proteins such as tBID and BIM interact with BAX in a similar manner to BIM SAHB, and whether BAK undergoes a similar activation mechanism by BIM SAHB.

A model for BAK activation and oligomerization, supported by biochemical data, has recently been proposed[16] (FIG. 2). In this model, BAK activation leads to exposure of its BH3 domain and its insertion into the hydrophobic groove of an adjacent, activated BAK molecule. The interaction is reciprocated, leading to the formation of a symmetrical BAK homodimer. Higher-order BAK oligomers are formed by dimer–dimer interactions mediated

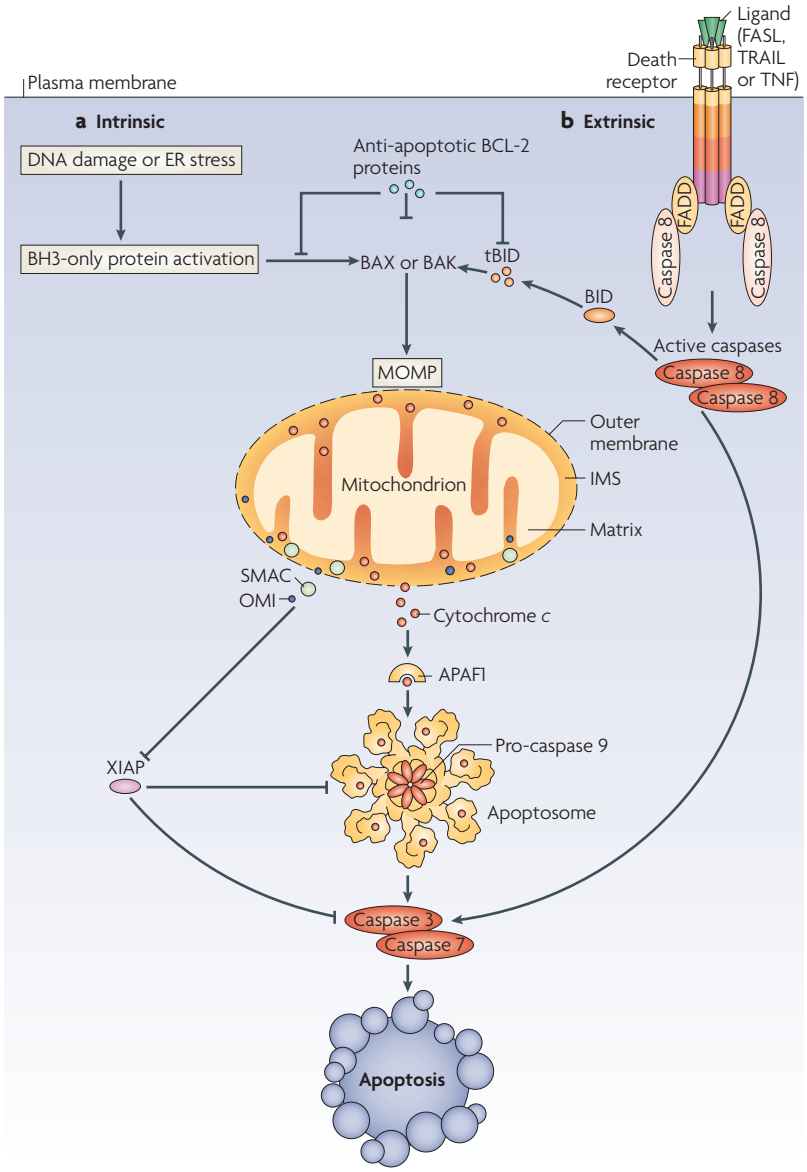


Figure 1 | **Intrinsic and extrinsic pathways of apoptosis. a** | Intrinsic apoptotic stimuli, such as DNA damage or endoplasmic reticulum (ER) stress, activate B cell lymphoma 2 (BCL-2) homology 3 (BH3)-only proteins leading to BCL-2-associated X protein (BAX) and BCL-2 antagonist or killer (BAK) activation and mitochondrial outer membrane permeabilization (MOMP). Anti-apoptotic BCL-2 proteins prevent MOMP by binding BH3-only proteins and activated BAX or BAK. Following MOMP, release of various proteins from the mitochondrial intermembrane space (IMS) promotes caspase activation and apoptosis. Cytochrome *c* binds apoptotic protease-activating factor 1 (APAF1), inducing its oligomerization and thereby forming a structure termed the apoptosome that recruits and activates an initiator caspase, caspase 9. Caspase 9 cleaves and activates executioner caspases, caspase 3 and caspase 7, leading to apoptosis. Mitochondrial release of second mitochondria-derived activator of caspase (SMAC; also known as DIABLO) and OMI (also known as HTRA2) neutralizes the caspase inhibitory function of X-linked inhibitor of apoptosis protein (XIAP). **b** | The extrinsic apoptotic pathway is initiated by the ligation of death receptors with their cognate ligands, leading to the recruitment of adaptor molecules such as FAS-associated death domain protein (FADD) and then caspase 8. This results in the dimerization and activation of caspase 8, which can then directly cleave and activate caspase 3 and caspase 7, leading to apoptosis. Crosstalk between the extrinsic and intrinsic pathways occurs through caspase 8 cleavage and activation of the BH3-only protein BH3-interacting domain death agonist (BID), the product of which (truncated BID; tBID) is required in some cell types for death receptor-induced apoptosis. FASL, FAS ligand; TNF, tumour necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand.

through a cryptic interface that is exposed following BAK activation[20]. Mutational analysis suggests that there is a similar mechanism for BAX homo-oligomerization[15].

Exactly how many molecules of BAX or BAK must oligomerize for MOMP to occur is unclear. One study found that four BAX molecules are sufficient to permeabilize an artificial membrane, whereas another study detected much larger BAX oligomers in apoptotic cells[21,22]. More recently, using single-cell imaging, the number of BAX molecules in a MOMP-inducing complex has been estimated at more than one hundred, although smaller complexes could not have been detected by this means owing to the limits of optical resolution[23]. Accurately determining the amount of active BAX and BAK molecules required for MOMP will provide insight into how MOMP occurs. However, this remains challenging owing largely to the small amounts of activated BAX and BAK that are required for MOMP and because both proteins continue to homo-oligomerize post-MOMP[24].

Mechanisms of MOMP

Although the requirement for activated BAX and BAK to induce MOMP is not debated, the means by which they carry out this task is. Here, we discuss the biophysical characteristics of MOMP, its kinetics and whether it allows for selective or non-selective release of IMS proteins. We then review how active BAX and BAK are thought to permeabilize the mitochondrial outer membrane.

Biophysical characteristics of MOMP. Live-cell imaging of MOMP, by monitoring the release of green fluorescent protein (GFP)–cytochrome *c* from mitochondria during apoptosis, demonstrated that, although the onset of MOMP is highly variable, most mitochondria undergo MOMP within 5 minutes of initiation[25]. More recently, single-cell imaging at high temporal resolution has shown that MOMP can initiate from a defined point or points in a cell and proceed in a wave-like manner across all mitochondria in the cell. Although the mechanism remains unclear, inhibitor studies implicate roles for protein phosphorylation and endoplasmic reticulum Ca^{2+} pumps in wave propagation[26–28]. Potentially, caspase activity may contribute to intracellular MOMP waves through cleavage and activation of BID. However, somewhat at odds with these findings is the observation that the length of time between the first and last mitochondrion in a cell to undergo MOMP is unaffected by lowering temperature, which argues against an enzymatic component to the process[25]. This discordance may be due to the higher temporal resolution achieved in more recent studies, or, perhaps rather than contributing to wave propagation, enzymatic processes may lower the initial threshold for individual mitochondria to undergo MOMP, thereby indirectly affecting release kinetics.

Whether MOMP displays any selectivity for the release of different IMS proteins has been subject to much scrutiny. BAX-mediated liposome permeabilization *in vitro* leads to the equally efficient release of 10 kDa and 2 MDa dextrans, suggesting that MOMP displays no selectivity for protein size[29]. In cells, proteins larger than 100 kDa (the predicted size of soluble SMAC–GFP dimers) are released following MOMP. In contrast, a tetrameric SMAC–dsRed

Box 2 | **Apoptogenic IMS proteins**

Cytochrome *c*.
Although cytochrome *c* is primarily recognized as a key component of electron transport during oxidative phosphorylation, it is also absolutely required for caspase activation following mitochondrial outer membrane permeabilization (MOMP). Cells lacking cytochrome *c* fail to activate caspases and are resistant to intrinsic apoptosis[135]. Moreover, knock-in mice expressing cytochrome *c* Lys27Ala, which retains respiratory chain function but cannot bind apoptotic protease-activating factor 1 (APAF1), display similar neurological phenotypes to APAF1- and caspase 9-null mice, and cytochrome *c* Lys72Ala knock-in cells fail to activate caspases and undergo apoptosis following pro-apoptotic stimuli[136]. Cytochrome *c* Lys72Ala-expressing thymocytes display apoptotic sensitivity, in contrast to the resistance seen in APAF1-null and BAX–BAK double knockout thymocytes[137], suggesting that other MOMP-dependent mediators of APAF1 activation might exist, although cytochrome *c* Lys72Ala might also retain a residual capacity to activate APAF1 (REF. 138).

SMAC and OMI
X-linked inhibitor of apoptosis protein (XIAP) inhibits caspase activity by directly binding active caspases, caspase 9, caspase 3 and caspase 7 (REF. 139). MOMP counteracts this through the release of second mitochondria-derived activator of caspase (SMAC; also known as DIABLO) and OMI (also known as HTRA2), two intermembrane space (IMS) proteins that directly bind XIAP and antagonize its ability to inhibit caspases[140–142]. Loss of SMAC or OMI, either alone or in combination, does not result in resistance to cell death. In fact, paradoxically, OMI-deficient cells are more sensitive to many intrinsic apoptotic stimuli, which may be due to the loss of OMI's mitochondria chaperone function[143]. The recent discovery that IAP antagonistic drugs induce degradation of IAPs, thereby deregulating nuclear factor κB (NF-κB) signalling and causing tumour necrosis factor (TNF)-dependent death, raises the interesting possibility that SMAC and OMI may also modulate IAP levels and NF-κB signalling following their release[144–146].

Others
The role of apoptosis-inducing factor (AIF) in promoting cell death is unclear. Following MOMP, AIF release is either slow or requires caspases and therefore probably does not contribute greatly to apoptotic cell death[31,147]. However, mitochondrial release of AIF (for example, by calpain[51]) may contribute to cell death in cell types such as neurons when caspase function downstream of MOMP is inhibited, leading to caspase-independent cell death (CICD). Endonuclease G is a mitochondrial IMS protein that can also be released following MOMP, whereby it contributes to apoptosis and CICD through cleavage of nuclear DNA[148]. However, endonuclease G deficiency has no effect on apoptotic DNA fragmentation or CICD[149].

fusion protein (with a predicted size of 190 kDa) failed to undergo release on MOMP, suggesting that MOMP may have size limitations *in vivo*[30]. Potentially, this retention may also be due to physicochemical properties such as enhanced membrane binding of the tetrameric molecule. Live single-cell imaging of multiple IMS proteins showed identical kinetics of IMS protein release from mitochondria following MOMP, irrespective of size[31]. However, in another study it was found that the duration of MOMP was slightly longer in the case of SMAC–mCherry (with a predicted dimeric size of 100 kDa) relative to cytochrome *c*–GFP (with a predicted size of 42 kDa)[28]. Importantly, in both studies the onset of SMAC and cytochrome *c* release was simultaneous, indicating that they exit mitochondria by a similar mechanism. Selective release of IMS proteins following MOMP has been seen in cells deficient for dystrophin-related protein 1 (DRP1; also known as UTRN), a dynamin-like protein that is required for mitochondrial fission. In DRP1-deficient cells, SMAC readily undergoes mitochondrial release but most cytochrome *c* is retained in mitochondria following MOMP[32–34]. However, the means by which DRP1 promotes mitochondrial cytochrome *c* release following MOMP remain unclear.

Proteinaceous channels. BCL-2 proteins such as BAX and BCL-XL (also known as BCL2L1) display structural similarities with bacterial pore-forming toxins, leading to the hypothesis that BAX and BAK themselves might directly form pores in the mitochondrial outer membrane[35,36] (FIG. 2). Along these lines, several studies have found that BAX forms ion channels or membrane pores in artificial membranes; however, anti-apoptotic BCL-2 proteins can also form membrane channels *in vitro*[37]. More recent evidence for MOMP occurring through a BAX or BAK pore has emerged from patch-clamping analysis of mitochondria undergoing MOMP. Using tBID to trigger MOMP, a mitochondrial outer membrane channel is formed that increases in conductance (and therefore size) over time, with similar kinetics to MOMP, implicating the channel (termed by the authors as the mitochondrial apoptosis-induced channel (MAC)) as the cause of MOMP[38]. The step-wise growth of these channels suggests that sequential recruitment of activated, membrane-bound BAX and BAK dimers to a small pore results in a channel of increasing size, which ultimately allows cytochrome *c* to be released from the mitochondrial IMS. Inhibitors that block MAC formation *in vitro* inhibit MOMP and apoptosis in cells, in support of MAC as the relevant MOMP-inducing mechanism, although it remains possible that these inhibitors may directly block BAX and BAK activation[39]. One caveat concerning the BAX or BAK pore model is that most studies have described channels that are only large enough to accommodate cytochrome *c*, but MOMP clearly allows for the release of much larger proteins, as noted above.

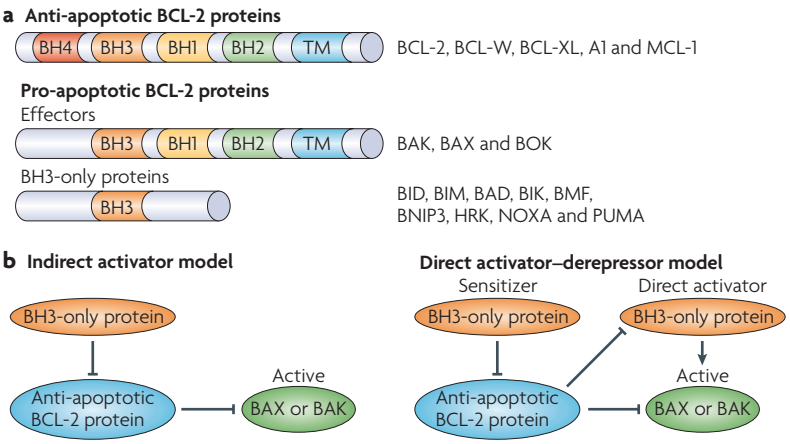
Rather than BAX and BAK forming pores themselves, it has been proposed that the modulation of existing mitochondrial channels such as the mitochondrial permeability transition pore complex (PTPC) — a multiprotein complex built up at the contact site between the inner and outer mitochondrial membranes — may have a causal role in MOMP. Cells lacking cyclophilin D, an essential component of the PTPC, display normal apoptotic sensitivity to a range of stimuli, effectively ruling out any role for the PTPC in MOMP[40–42]. Alternatively, MOMP has been postulated to require BAK and BAX modulation of voltage-dependent anion channel (VDAC) function. VDACs are the main pathway for metabolite diffusion across the mitochondria. However, loss of all three VDAC isoforms imparts no resistance of cells or isolated mitochondria to either MOMP or apoptosis[43]. Other studies have found that VDAC2 interactions with BAK are required to hold BAK inactive or for its mitochondrial localization[44,45].

Lipidic pores. An alternative model suggests that interaction of activated BAX and BAK with outer membrane lipids leads to membrane bending and, ultimately, formation of transient lipid pores or inverted micelles, thereby allowing IMS protein release[46–48] (FIG. 2). Lipid pores would account for several key aspects of MOMP, including the release of large IMS proteins and the difficulties in visualizing proteinaceous pores in the mitochondrial outer membrane. Accordingly, activated BAX can induce liposome permeabilization *in vitro*, leading to the release of encapsulated protein or dextrans in a size-independent manner[17,29]. Recently, cryo-electron microscopy (EM)

FRET
(Förster resonance energy transfer). The non-radiative transfer of energy from a donor fluorophore to an acceptor fluorophore that is typically <80 Å away. FRET will only occur between fluorophores in which the emission spectrum of the donor has a significant overlap with the excitation of the acceptor.

Liposome
A vesicle made of lipid bilayer in an aqueous environment. Membrane proteins can be incorporated in the bilayer.

Box 3 | **Regulation of MOMP by the BCL-2 family**



The B cell lymphoma 2 (BCL-2) family of proteins is divided into three groups based on their BCL-2 homology (BH) domain organization (see the figure, part **a**). Pro-apoptotic BCL-2 proteins can be sub-divided into effectors (the proteins that actually cause mitochondrial outer membrane permeabilization (MOMP)) or BH3 only (the proteins that relay the apoptotic signal to the effectors). Although BCL-2-related ovarian killer protein (BOK) displays similar domain architecture to BCL-2-associated X protein (BAX) and BCL-2 antagonist or killer (BAK), there is little evidence that it is a functional effector. Two prominent models of BAX and BAK activation have been proposed, termed the indirect activator (or neutralization) and direct activator–derepressor models of activation[150,151] (see the figure, part **b**). The indirect activator model asserts that BAX and BAK are bound in a constitutively active state by anti-apoptotic BCL-2 proteins and that competitive interactions of BH3-only proteins with anti-apoptotic BCL-2 family members is sufficient to release activated BAX and BAK. In the direct activator–derepressor model, BAX and BAK are activated following interaction with a subset of BH3-only proteins known as direct activators, and anti-apoptotic BCL-2 proteins prevent MOMP either by sequestering the activating BH3-only proteins or by inhibiting activated BAX and BAK. A second subset of BH3-only proteins, termed sensitizers, cannot directly activate BAX and BAK but neutralize anti-apoptotic BCL-2 proteins. Definitive proof for either model has proved challenging; it is likely that aspects of both models are correct. BAD, BCL-2 antagonist of cell death; BID, BH3-interacting domain death agonist; BIK, BCL-2-interacting killer; BIM, BCL-2-interacting mediator of cell death; BMF, BCL-2-modifying factor; BNIP3, BCL-2 and adenovirus E1B 19 kDa protein-interacting protein 3; HRK, harakiri; PUMA, p53 upregulated modulator of apoptosis; TM, transmembrane.

analysis of BAX-permeabilized liposomes revealed openings of varying size (25–100 nm) that appeared concurrently with permeabilization in a manner that was BCL-XL inhibitable[49]. The diameter of these openings is consistent with the ability of BAX to induce the size-independent release of dextrans. Supporting a lipidic pore model, the edges of these BAX-induced pores are smooth and devoid of proteinaceous material. In contrast, protein pores formed by the toxin pneumolysin, as analysed by cryo-EM, are uniform in nature and decorated around the edges with toxin molecules[50]. However, similar pore-like structures have yet to be found on mitochondria during MOMP.

Patch clamping
An electrophysiological technique used for measuring ion channel activity over membranes. Typically, a small diameter (1 μm) micropipette serves as the electrode and is applied to a small area of membrane (the 'patch'), allowing the activity of one or a few ion channels to be measured.

Micelle
An aggregate (typically spherical) of varying size comprised of lipids. In aqueous environments, the hydrophobic lipid tails orientate to the centre of the micelle and the hydrophilic head groups are on the surface.

Post-MOMP regulation of IMS proteins

Although MOMP itself provides little specificity as to which IMS proteins are released, studies suggest that release of different IMS proteins can be selective. This may be through the regulation of IMS protein interaction with mitochondrial membranes or by mitochondrial inner membrane remodelling.

IMS protein membrane attachment. Apoptosis inducing factor (AIF) is an IMS protein that is anchored on the mitochondrial inner membrane and displays much slower release kinetics than cytochrome *c* following MOMP[31]. This protracted release may be owing to the requirement for AIF cleavage post-MOMP by cytosolic proteases to liberate AIF from the mitochondrial inner membrane. One candidate protease is cytosolic calpain I, which can cleave AIF *in vitro* to promote its release from permeabilized mitochondria[51].

Electrostatic interactions between cytochrome *c* and the mitochondrial lipid cardiolipin have been proposed to regulate its release[52]. However, it might be expected that the ionic strength of the cytosol should suffice to disrupt these interactions[53].

Post-MOMP mitochondrial inner membrane remodelling. Other mechanisms may control IMS protein release following MOMP. Mitochondrial cristae are involutions of the mitochondrial inner membrane that greatly increase the mitochondrial surface area available for oxidative phosphorylation and ATP synthesis. Cristae are dynamic structures and their accessibility to the IMS is largely dictated through regulation of cristae junction size. As most cytochrome *c* resides in mitochondrial cristae, several studies have addressed whether cristae remodelling provides an additional means of regulating cytochrome *c* release following MOMP. Various BH3-only proteins, including BID, BIM, BNIP3 (BCL-2 and adenovirus E1B 19 kDa protein-interacting protein 3) and BCL-2-interacting killer (BIK) have been found to promote mitochondrial cristae remodelling[54–57]. Treatment of mitochondria *in vitro* with the BH3 protein tBID induced dramatic inner membrane remodelling, leading to interconnected cristae with widened junctions and cytochrome *c* mobilization into the IMS[55]. Two IMS proteins, optic atrophy protein 1 (OPA1; a dynamin-like GTPase) and presenilins-associated rhomboid-like protein (PARL; a rhomboid protease) have been found to regulate cristae remodelling during apoptosis[58,59]. Following MOMP, disassembly of OPA1 hetero-oligomers is required for the widening of cristae junctions, whereas PARL cleavage of OPA1 generates an OPA1 cleavage product that maintains tight cristae junctions that prevent cytochrome *c* release. Functionally, PARL loss renders cells more susceptible to apoptosis induced by intrinsic stimuli, whereas OPA1 overexpression is protective[58,59].

Although cristae remodelling requires activated BAX or BAK, it can occur in the absence of MOMP because pharmacological inhibitors of MOMP still allow remodelling to occur[54]. Remodelling was associated with the mobilization of cytochrome *c* to the IMS and, like previous studies, disassembly of OPA1 was required for remodelling to occur. In this study, however, gross changes in mitochondrial morphology were not apparent. Instead, a subtle narrowing, rather than widening, of cristae junctions occurred. Similarly, correlative light microscopy and EM of apoptotic cells revealed that gross alterations in mitochondrial structure were detected only after MOMP and caspase activation had occurred, arguing against a causal role for large mitochondrial structural changes

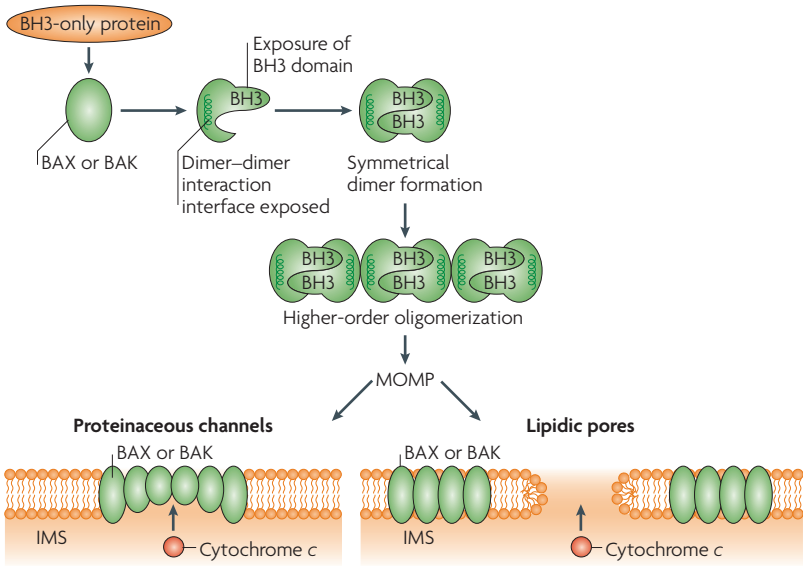


Figure 2 | **BAX and BAK activation and pore formation.** The binding of B cell lymphoma 2 (BCL-2) homology 3 domain (BH3)-only proteins to BCL-2-associated X protein (BAX) and BCL-2 antagonist or killer (BAK) leads to extensive conformational changes during their activation. The BH3 domain and hydrophobic cleft are exposed, allowing symmetrical BAX or BAK dimers to form through reciprocal BH3 domain–cleft interactions. During activation, a dimer–dimer interaction surface is also exposed, allowing higher-order oligomers to form. Higher-order oligomers promote mitochondrial outer membrane permeabilization (MOMP) by unclear means, perhaps through forming proteinaceous channels or by destabilizing lipid membranes and forming lipidic pores. IMS, intermembrane space.

in promoting IMS protein release[60]. Given that even in a closed conformation cristae junction width should easily accommodate cytochrome *c* exit, it is unlikely that alterations in cristae width *per se* regulate cytochrome *c* release. One study has found that cytochrome *c* resides either in the IMS or in the mitochondrial cristae and these pools do not readily interchange[61]. By contrast, the basal diffusability of cytochrome *c* in mitochondria has recently been shown to account for its rapid and complete release on MOMP[62]. Some studies have found that partial release of cytochrome *c* is sufficient to drive apoptosis, albeit at slower kinetics, whereas others have found apoptosis is blocked under these conditions[32–34,54,55]. These contrasting results may simply reflect varying thresholds for cytochrome *c*-induced caspase activation in different cell types[63].

Cristae junction
A connection between the mitochondrial cristae and the mitochondrial IMS. The diameter of cristae junctions can be altered, thereby regulating the accessibility of mitochondrial cristae to the IMS.

Nitrosylation
A post-translational protein modification involving the addition of a nitrosyl group to the Cys residue of a target protein, potentially altering target protein function.

Post-MOMP regulation of caspase activity

In addition to inhibition by XIAP, various mechanisms curtail caspase activity following MOMP. Under healthy conditions, these inhibitory mechanisms may exist to preserve cell viability should accidental MOMP occur in a limited number of mitochondria, but they are overwhelmed when MOMP occurs in most mitochondria, such as during apoptosis. Caspase activation following MOMP in a minority of mitochondria has been proposed to initiate a MOMP amplification loop through the executioner caspase-mediated cleavage and activation of proteins such as BID and BCL-2 (REFS 64,65). Post-MOMP regulation of caspase activity centres around the regulation of caspase 9 activity, either directly or indirectly, through effects that occur on cytochrome *c*-induced APAF1 apoptosome formation (FIG. 3).

Regulation of apoptosome assembly. Following mitochondrial release, cytochrome *c* promotes APAF1 conformational changes, leading to APAF1 oligomerization and assembly into a heptameric, wheel-like structure (the apoptosome) that recruits pro-caspase 9, promoting its dimerization and activation. Apoptosome formation requires APAF1-mediated dATP binding[66]. Paradoxically, physiological levels of nucleotides inhibit apoptosis by directly binding cytochrome *c*, preventing APAF1–cytochrome *c* interactions and apoptosome formation[67]. Along similar lines, transfer RNA (tRNA) binds cytochrome *c* and inhibits apoptosome formation by blocking the interaction of cytochrome *c* with APAF1 (REF. 68).

The pro-apoptotic activity of cytochrome *c* may also be regulated by redox, whereby cytochrome *c* oxidation promotes its pro-apoptotic activity and reduction inhibits it[69,70]. Mechanistically, the means by which the redox status affects the pro-apoptotic function of cytochrome *c* is unknown and other studies have found that reduced cytochrome *c* is still proficient at activating caspases *in vitro*[71,72]. The addition of a haem moiety to cytochrome *c* occurs in the mitochondrial IMS and is required for its ability to promote caspase activity following MOMP[73]. Interestingly, nitrosylation of the cytochrome *c* haem moiety occurs under apoptotic conditions[74]. Modelling the effects of nitrative stress by disruption of the cytochrome *c* Met80–haem interaction promotes cytochrome *c* nuclear translocation in non-apoptotic cells, leading to the upregulation of a protective stress response. This suggests that nitrosylation can impart novel non-apoptotic roles on cytochrome *c*[75].

Normal intracellular levels of potassium also inhibit apoptosome assembly. This inhibition can be overcome by increased concentrations of cytochrome *c*, suggesting that extensive MOMP, as seen during apoptosis, is required for caspase activation and apoptosis[76]. Intracellular levels of Ca^{2+} have also been found to inhibit apoptosome activity by blocking nucleotide exchange on monomeric APAF1, thereby inhibiting apoptosome formation[77]. Various proteins, including heat shock proteins such as HSP70 and HSP90, have been shown to negatively influence apoptosome function, either by inhibiting its formation or by preventing the recruitment of pro-caspase 9 (REFS 78–81).

Apoptosome activity can also be positively modulated, thereby enhancing caspase 9 activity. Putative HLADR-associated protein I (PHAPI; also known as pp32) stimulates apoptosome activity and caspase activation by preventing APAF1 aggregation and promoting nucleotide exchange on APAF1 (REFS 82,83). Reduced expression of PHAPI imparts apoptotic resistance to cells, enabling clonogenic survival that may be relevant during tumorigenesis[84]. Interestingly, APAF1 also has a non-apoptotic role in regulating DNA damage-induced cell cycle arrest, raising the possibility that modulators of APAF1 apoptotic function can also alter its cell cycle checkpoint functions[85].

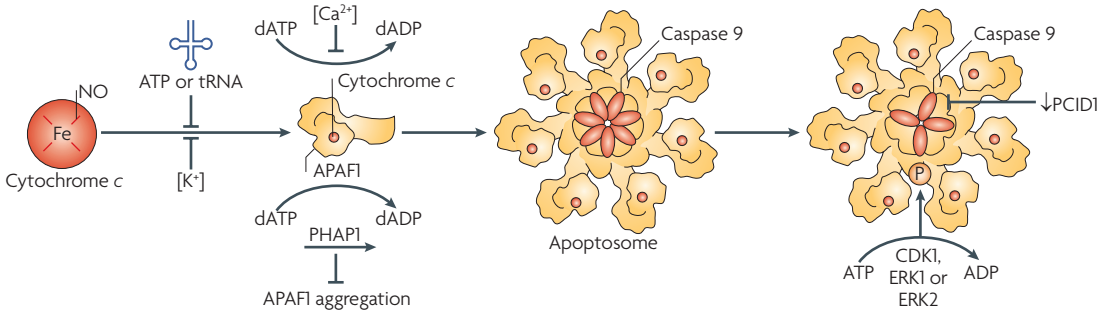


Figure 3 | **Post-MOMP regulation of caspase activity.** Cytochrome *c* binds an apoptotic protease-activating factor 1 (APAF1) monomer, leading to its oligomerization into a heptameric wheel-like structure called the apoptosome that recruits and activates caspase 9. Physiologic levels of nucleotides such as ATP or transfer RNA (tRNA) can block cytochrome *c* binding to APAF1 and inhibit apoptosome formation by directly binding cytochrome *c*. Cytochrome *c* requires its haem moiety, which is acquired in the mitochondrial intermembrane space, in order to bind and activate APAF1. Nitrosative stress can modify the haem moiety and attenuate the pro-apoptotic function of cytochrome *c*. Intracellular levels of potassium inhibit apoptosome formation, at least in part, by competing with cytochrome *c* for APAF1 binding, but high levels of cytochrome *c* can overcome this inhibition. Binding of cytochrome *c* to APAF1 stimulates APAF1-dependent dATP hydrolysis, driving apoptosome formation. Putative HLADR-associated protein I (PHAPI; also known as pp32) enhances this process by both promoting nucleotide exchange on APAF1 and inhibiting aggregation of the APAF1 monomer. Intracellular Ca^{2+} can inhibit nucleotide exchange, thereby blocking apoptosome formation. Direct phosphorylation of caspase 9 by kinases, including cyclin-dependent kinase 1 (CDK1)–cyclin B1, extracellular signal-regulated kinase 1 (ERK1; also known as MAPK3) and ERK2 (also known as MAPK1), inhibits caspase 9 activity by unknown means. Finally, downregulation of PCI domain-containing protein 1 (PCID1; also known as EIF3M) can negatively regulate caspase 9 levels, thereby effecting caspase activation following mitochondrial outer membrane permeabilization (MOMP). NO, nitric oxide.

Regulation of caspase 9 activation. Several kinases have been shown to phosphorylate caspase 9, inhibiting its enzymatic activity[86]. In human caspase 9, Thr125 is the main inhibitory phosphorylation site and is targeted by several kinases, including extracellular signal-regulated kinase 1 (ERK1; also known as MAPK3), ERK2 (also known as MAPK1) and cyclin-dependent kinase 1 (CDK1)–cyclin B1 (REFS 87,88). Phosphorylation of Thr125 or mutation to a phosphomimetic residue impairs the ability of cytochrome *c* to induce caspase activity *in vitro*[88]. During mitosis, CDK1–cyclin B1-mediated phosophorylation of caspase 9 on Thr125 attenuates its activity. Prolonged mitotic arrest, induced by microtubule stabilizing agents such as taxol, leads to caspase 9-dependent death that can be enhanced by inhibition of caspase 9 phosphorylation at Thr125 (REF. 87). Although it is clear that phosphorylation can negatively affect caspase 9 activity, the means by which this occurs is unclear as it does not affect the recruitment of caspase 9 to the apoptosome[88]. Whether phosphorylation inhibits other aspects of caspase 9 activation, such as its ability to dimerize, remains to be tested.

Apoptosome-mediated activation of caspase 9 leads to caspase 9 auto-processing, which greatly reduces its affinity for the apoptosome and results in the loss of caspase 9 activity[89]. Therefore, apoptosome-mediated caspase 9 activation is a 'molecular timer', the activity of which is largely due to intracellular caspase 9 levels. Accordingly, regulation of caspase 9 expression also controls caspase activity post-MOMP. PCI domain-containing protein 1 (PCID1; also known as EIF3M) is the human orthologue of Tango 7, a pro-apoptotic effector that regulates expression of the *D. melanogaster* initiator caspase pro-Dronc[90]. Interestingly, downregulation of PCID1 decreases expression of pro-caspase 9, leading to the reduction of caspase activity during apoptosis. Although it is not known how PCID1 regulates pro-caspase 9 levels, the finding that PCID1 is commonly downregulated in pancreatic cancer suggests that it may be clinically important[91].

The end game: how MOMP kills cells

MOMP leads to the rapid activation of caspases and apoptosis. However, in the absence of caspase activity (for example in APAF1- and caspase 9-deficient backgrounds[92–94]), cells undergo caspase-independent cell death (CICD), which thereby defines MOMP as a point of no return (see Supplementary information S2 (movie)). Although cell death is the usual outcome following MOMP, the mechanisms and kinetics by which cells die differ greatly depending on caspase activity. Here, we review what happens after MOMP and how these events bring about cellular demise through caspase-dependent and caspase-independent means (FIG. 4).

Cellular effects of MOMP. Following MOMP, caspase activation ensues and results in the cleavage of hundreds of proteins and, ultimately, apoptosis. Caspase cleavage of any given protein substrate can activate or inhibit its function and, although hundreds of caspase substrates have been identified, many are probably innocent bystanders that play no actual part in apoptosis[2]. In the absence of caspase activity, cell death normally occurs following MOMP, albeit with much slower kinetics than apoptosis. MOMP has been proposed to cause CICD either by the release of IMS proteins, such as AIF and endonuclease G, or through a progressive decline in mitochondrial function leading, among other effects, to ATP depletion[95]. Cells can continue to undergo cell division following MOMP provided that caspase activity is inhibited[96].

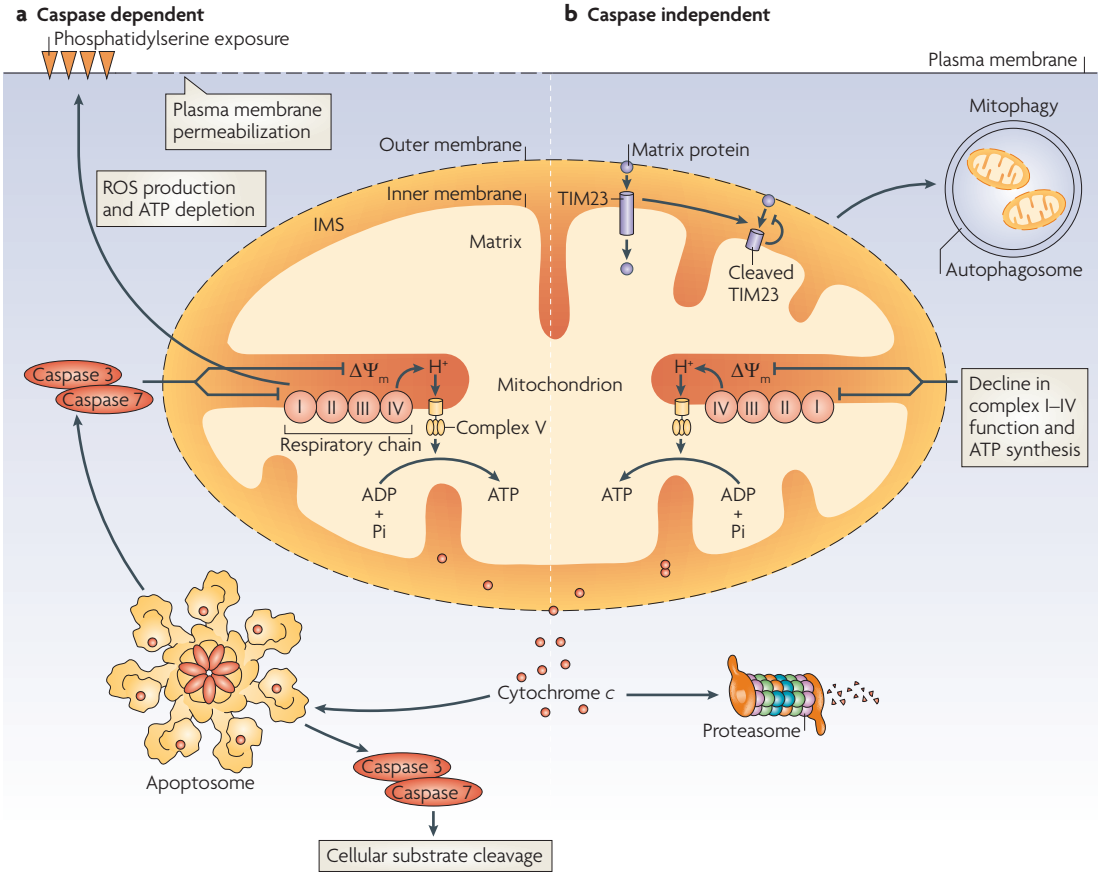


Figure 4 | **Cellular effects of MOMP. a** | Caspase-dependent effects. Mitochondrial outer membrane permeabilization (MOMP) leads to the release of cytochrome *c* from mitochondria, which activates caspases to cleave numerous cellular substrates, causing apoptosis. Respiratory chain complexes I–IV generate the proton gradient over the mitochondrial inner membrane that drives ATP generation by ATP synthase (complex V). Executioner caspases (caspase 3 and caspase 7) enter the mitochondrial intermembrane space (IMS) following MOMP, disrupting complex I and complex II activity. In the case of complex I, this occurs partly through cleavage of an essential complex I subunit, NADH–ubiquinone oxidoreductase 75 kDa subunit (NDUFS1). Collectively, these caspase-dependent effects lead to a loss of transmembrane potential ($\Delta\Psi_m$) and ATP synthesis, and an increase in reactive oxygen species (ROS) production. These effects of mitochondrial dysfunction contribute to the exposure of phosphatidylserine on the outer leaflet of the plasma membrane and its permeabilization, which occurs during apoptosis. **b** | Caspase-independent effects. Following MOMP, initial levels of cytochrome *c* in the cytoplasm are sufficient to support respiration in the permeabilized mitochondria. In the long-term, levels of cytochrome *c* might be rate limiting owing to proteasome-dependent degradation. Even in the absence of caspase activity, respiratory chain complex I – IV activity drops over time, leading to a gradual loss in $\Delta\Psi_m$ and ATP synthesis, which effectively starves the cell. TIM23 is an essential component of the inner membrane protein translocase. Following MOMP, TIM23 undergoes inactivation through cleavage by an unknown intramitochondrial protease, effectively blocking new protein import into the mitochondrial matrix. Finally, MOMP triggers the removal of permeabilized mitochondria by the autophagic machinery, a process termed mitophagy.

In this study, CICD correlated with a progressive decline in the mitochondrial function and ATP generation that preceded the mitochondrial release of AIF and endonuclease G, suggesting that MOMP contributes to CICD primarily through loss of mitochondrial function.

Mitochondrial effects of MOMP. Mitochondria are dynamic organelles that constantly undergo cycles of fission and fusion with one another. Wide-scale mitochondrial fission occurs at or around the point of MOMP, irrespective of caspase activity but dependent on DRP1 (REF. 97). The role of mitochondrial fission during apoptosis in mammalian cells is unclear and, although fission occurs after MOMP, it may not depend on it[28,98,99]. MOMP and fission can be dissociated as fission occurs in situations where MOMP is blocked following an apoptotic stimulus, for example when BCL-XL is overexpressed[98]. Indeed, other studies have found that BCL-2 family members regulate mitochondrial morphology in healthy cells[100,101].

Although fission is not required for MOMP, DRP1 may contribute to this process. Pharmacological inhibitors of DRP1 block MOMP *in vitro*, a setting in which mitochondrial fission does not occur, thereby implying that DRP1 contributes to BAX- or BAK-induced MOMP independently of mitochondrial fission[102]. However, cells lacking DRP1 undergo MOMP, ruling out an absolute requirement for DRP1 for this event[33,103].

The maintenance of mitochondrial transmembrane potential ($\Delta\Psi_m$) is crucial for many mitochondrial functions, including ATP synthesis, ion homeostasis and protein import into the mitochondrial matrix. Following MOMP, $\Delta\Psi_m$ is dissipated through caspase-dependent and caspase-independent means[104,105]. Caspase-dependent dissipation of $\Delta\Psi_m$ is mediated, at least in part, through caspase cleavage of NADH–ubiquinone oxidoreductase 75 kDa subunit (NDUFS1), an essential component of respiratory chain complex I[106]. In permeabilized mitochondria, executioner caspases gain entry to the IMS and cleave NDUFS1, which leads to a sequential reduction in complex I activity, a drop in $\Delta\Psi_m$, a rapid reduction in ATP synthesis and an increase in reactive oxygen species (ROS). Interestingly, expression of a non-cleavable form of NDUFS1 delays the kinetics of $\Delta\Psi_m$ loss and phosphatidylserine exposure on the outer leaflet of the plasma membrane (a key feature of apoptosis that contributes to the phagocytosis of the dying cell) following MOMP. This indicates a direct role for caspase-induced mitochondrial dysfunction in mediating this process. Furthermore, modulation of mitochondrial dysfunction during apoptosis by the expression of non-cleavable NDUFS1 alters how the immune system responds to a dying cell[107]. Caspase-dependent mitochondrial ROS production oxidizes the immunostimulatory protein high mobility group protein B1 (HMGB1) in dying cells, thereby promoting immune tolerance, whereas expression of non-cleavable NDUFS1 reduces ROS levels, which blocks HMGB1 oxidation and leads instead to an immunostimulatory response. There are probably other mitochondrial caspase targets as non-cleavable NDUFS1 only partially rescues the caspase-dependent loss in $\Delta\Psi_m$, and respiratory chain complex II activity is also inhibited following MOMP in a caspase-dependent manner[106,108].

Mitochondrial function deteriorates even in the absence of caspase activity, leading to a progressive loss in $\Delta\Psi_m$ and ATP production, although how this occurs remains unclear[96,109]. Analysis of cells undergoing CICD has shown that respiratory complexes I and IV are lost in the absence of caspase activity at later time points following MOMP. One obvious reason for the loss of respiratory function might be cytochrome *c* release. However, following MOMP, cytochrome *c* remains at sufficiently high levels in the mitochondrial IMS to allow respiration[105]. At later time points, proteasome-dependent degradation of cytochrome *c* may promote respiratory dysfunction[110]. Alternatively, access of cytosolic enzymes to the mitochondrial IMS following MOMP may lead to inactivating post-translational modifications such as cleavage of crucial mitochondrial proteins. TIM23, an essential component of the inner membrane protein translocase complex, undergoes proteolytic inactivation following MOMP[111]. Cleavage of TIM23 was found to require an intramitochondrial protease and was associated with reduced cell viability following MOMP. Dysfunctional mitochondria can be specifically targeted for autophagic degradation through a process termed mitophagy. Mitophagy triggers include the loss of mitochondrial membrane potential and membrane permeability transition[112–114]. Interestingly, MOMP has also been shown to promote mitophagy[109,115]. Although mitophagy is primarily a homeostatic mechanism to ensure damaged mitochondria are removed, taken to its extreme, mitophagy can remove all mitochondria from a cell, effectively committing that cell to death[115]. However, one recent study showed that complete removal of mitochondria by enhanced mitophagy did not result in cell death for at least 4 days, suggesting that this is not a mechanism of cell death in short time frames[114]. The finding that cells can survive, at least in the short term, without mitochondria also suggests that CICD is not due solely to the loss of mitochondrial function, but may also involve an active role for permeabilized mitochondria.

Cellular recovery post-MOMP

The prevailing view that MOMP is a point of no return for cell survival is likely to be true in most, but importantly not all, situations. Recovery from MOMP probably has important pathophysiological consequences, enabling long-term survival of post-mitotic cells and promoting tumour cell survival[95]. Here, we review the mechanisms that govern cell survival following MOMP.

Survival following 'accidental' MOMP. Typically, MOMP causes the permeabilization of most mitochondria, leading to lethal caspase activation. However, studies have shown that there is a threshold for cytochrome *c*-mediated caspase activation that is influenced by many factors such as nucleotide and XIAP levels, as we have already discussed. This raises the possibility that in a minority of mitochondria MOMP might be insufficient to trigger apoptosis. Laser irradiation of neuronal mitochondria, leading to permeabilization of 15% of the mitochondrial population, was insufficient to trigger apoptosis[116]. However, whether accidental MOMP occurs in a few mitochondria in the absence of apoptosis remains an open question.

Post-mitotic cellular recovery. Sympathetic neurons and cardiomyocytes can survive following MOMP, perhaps necessitated by the life-long requirement for these post-mitotic cells[117,118]. Following terminal differentiation, both cell types express low levels of APAF1 and are unresponsive to microinjection of cytochrome *c*[119,120]. Apoptotic sensitivity is restored following upregulation of APAF1, addition of recombinant SMAC or deletion of XIAP, implicating an important role for XIAP-mediated caspase inhibition in regulating cell death. Both cell types survive following MOMP, suggesting that endogenous SMAC and OMI are at insufficient levels to neutralize XIAP activity[121,122]. In the case of neurons, prolonged apoptotic signalling following nerve growth factor (NGF) withdrawal induces a so-called 'competence to die' owing to down-regulation of XIAP levels[121]. The redox status of cytochrome *c* influences its pro-apoptotic activity following MOMP in neurons[123] (see above). Neurons display high levels of glycolysis, which, besides producing ATP, raises the intracellular levels of glutathione synthase (GSH) though the pentose–phosphate shunt. Following MOMP, cytochrome *c* is reduced and held inactive by GSH, which inhibits caspase activation, whereas oxidation of cytochrome *c* promotes its activity. Tumour cells, similar to

Transmembrane potential
The voltage (or electrical potential) difference between one side of a membrane and the other.

Post-mitotic cell
A cell that is neither preparing to nor undergoing cell division.

Pentose–phosphate shunt
A metabolic pathway that generates NADPH and pentose sugars from glucose-6-phosphate. Indirectly, NADPH serves as an important antioxidant by reducing glutathione.

neuronal cells, are typically glycolytic and therefore may also inhibit the pro-apoptotic activity of cytochrome *c* in a similar manner.

Recovery in proliferating cells. Proliferating cells can also recover from MOMP provided caspase activity is inhibited. This may have important implications for oncogenesis and cancer therapy because tumour cells often display defects in caspase activation downstream of MOMP, through diverse mechanisms such as reduction in APAF1 expression or upregulation of XIAP[109,124,125]. Using a retroviral cDNA screen, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was found to protect cells from death downstream of MOMP, provided that caspase activity was inhibited, owing to its well-characterized role in glycolysis and a novel role in autophagy induction, partly through the upregulation of autophagy-related protein 12 (ATG12)[109]. The anticancer drug imatinib (Gleevec; Novartis), inhibits breakpoint cluster region protein (BCR)–abelson (ABL) kinase function and promotes both apoptosis and CICD. Interestingly, in some imatinib-resistant BCR–ABL-expressing cells, higher levels of GAPDH produce resistance to CICD that can be reverted by limited small interfering RNA knockdown of GAPDH, suggesting that protection from CICD by GAPDH may be therapeutically relevant[126].

One intriguing aspect concerning cellular recovery following MOMP is how the crucial process of cellular repopulation with intact mitochondria occurs. Addressing this issue, a recent study has found that MOMP can be incomplete, such that some mitochondria fail to undergo MOMP following an apoptotic stimulus and remain intact[127]. Increased levels of anti-apoptotic BCL-2 proteins on specific mitochondria probably account for their resistance to MOMP, supported by a lack of BAX or BAK activation on these mitochondria and reversion to complete MOMP after treatment with the BCL-2 antagonist, ABT-737. Importantly, the presence of intact mitochondria strongly correlates with cellular recovery under conditions of MOMP, suggesting that these are the 'seed' mitochondria that can repopulate the cell. In healthy neurons, MOMP leading to caspase 3 activation is required for effective AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptor internalization at postsynaptic junctions[128]. Incomplete MOMP probably plays an important role in this process by promoting receptor internalization while preserving cell viability.

Concluding remarks

Considerable progress has been made in recent years addressing the regulation of MOMP, how it occurs and why it brings about cell death. However, many outstanding questions remain. Although we are beginning to understand how BAX and BAK become activated, the means by which they permeabilize the mitochondrial outer membrane remain elusive. Cell survival is possible following MOMP but it remains unclear whether MOMP in a minority of mitochondria actually occurs under healthy conditions, an event that would explain why cells can regulate caspase activity post-MOMP.

How MOMP initiates caspase activation leading to apoptosis is relatively well elucidated. In contrast, beyond 'mitochondrial catastrophe', we have very little mechanistic insight into how MOMP contributes to cell death in a caspase-independent manner. Following from this, the manner by which a cell dies following MOMP, either by caspase-dependent or caspase-independent means, may have profound effects on how the immune system and neighbouring cells react to it, but this is under-studied. Finally, as we have discussed, MOMP need not always lead to a dead end: both post-mitotic and mitotic cells can recover from MOMP, although the detailed mechanisms governing cell survival remain scarce. In summary, many fundamental questions remain about the process of MOMP and how it controls life and death, and we anticipate that future findings will greatly facilitate the manipulation of this process for therapeutic purposes.

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Acknowledgements
We thank A. Oberst, F. Llambi and J. Tait-Mulder for critical reading of the manuscript.

Competing interests statement
The authors declare no competing financial interests.

DATABASES

UniProtKB: http://www.uniprot.org
AIF | APAF1 | BAK | BAX | BCL-XL | BID | BIK | BIM | BNIP3 | caspase 3 | caspase 7 | caspase 8 | caspase 9 | DRP1 | FADD | FASL | NDUFS1 | OMI | OPA1 | PARL | PCID1 | PHAPI | SMAC | TIM23 | XIAP

FURTHER INFORMATION

Douglas R. Green's homepage: http://www.stjude.org/green
Deathbase: http://deathbase.org/

SUPPLEMENTARY INFORMATION

See online article: S1 (movie) | S2 (movie)

ALL LINKS ARE ACTIVE IN THE ONLINE PDF

Cell adhesion: integrating cytoskeletal dynamics and cellular tension

J. Thomas Parsons, Alan Rick Horwitz‡ and Martin A. Schwartz**

Abstract | Cell migration affects all morphogenetic processes and contributes to numerous diseases, including cancer and cardiovascular disease. For most cells in most environments, movement begins with protrusion of the cell membrane followed by the formation of new adhesions at the cell front that link the actin cytoskeleton to the substratum, generation of traction forces that move the cell forwards and disassembly of adhesions at the cell rear. Adhesion formation and disassembly drive the migration cycle by activating Rho GTPases, which in turn regulate actin polymerization and myosin II activity, and therefore adhesion dynamics.

Blebbing cell
A cell that extends a round, dynamic process from the its membrane.

Substrate compliance
A measure of the elasticity of the material to which cells adhere and is related to the distance a material deforms under force. It is the inverse of stiffness and is given in units of 1 per Pascal.

Extracellular matrix
The fibrillar material made of collagens, laminin, fibronectin or other glycoproteins, and proteoglycans, which forms a solid substratum under or around cells *in vivo* and in culture.

Lamellipodium
A broad, flat protrusion at the leading edge of a cell that moves owing to actin polymerization that is generally induced by Rac activation.

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doi:10.1038/nrm2957

The morphological features of migrating cells can vary considerably. Round, highly protrusive or blebbing cells (for example, lymphocytes and cancer cells in some environments) are at one extreme and seem to migrate using weak adhesions. Highly spread cells (for example, fibroblasts and endothelial cells) are at the other extreme and have many large adhesions; their migration is often referred to as being mesenchymal. In reality, there is a continuum of migration modes that seem to be determined by several factors, among the most important being substrate compliance (and perhaps dimensionality) and the intrinsic contractility of the cells.

Directional migration is initiated by extracellular cues such as a gradient of growth factors or chemokines. However, directional cues can also include mechanical forces (for example, cell stretching or fluid flow), extracellular matrix (ECM) proteins (for example, collagen and fibronectin), the topography and mechanics of the ECM[3–6] and electrochemical gradients[7]. Cells initiate the migration cycle by polarizing and extending protrusions of the cell membrane towards the cue[11]. These protrusions comprise large, broad lamellipodia, spike-like filopodia or both and are driven by the polymerization of actin filaments[12]. Protrusions are then stabilized by adhesions that link the actin cytoskeleton to the underlying ECM proteins, and actomyosin contraction generates traction forces on the substratum. Contractility also promotes the disassembly of adhesions at the cell rear to allow the cell to move forwards[18]. Signals from both newly formed and more stable adhesions influence cytoskeletal organization and actin polymerization, and cytoskeletal structures in turn influence the formation and disassembly of the adhesions[18]. These bidirectional interactions coordinate adhesion, signalling, mechanical stresses and the spatial dynamics of cytoskeletal organization, leading to directional cell movement. The spatially segregated migration machinery and the signalling processes that regulate them are integrated by the cytoskeleton and vesicle trafficking, which span the entire cell.

Although cells express various cell surface adhesion receptors (including integrins, syndecans and other proteoglycans, cadherins and cell adhesion molecules), the integrin family of transmembrane heterodimeric receptors is the best studied and plays a prominent part in cell migration[20]. Integrin extracellular domains bind to specific sequence motifs present in proteins such as fibronectin, collagen and other ECM proteins. The binding of integrins to their extracellular ligands induces a conformational change that unmasks their short cytoplasmic tails, which promotes their linkage to the actin cytoskeleton through multiprotein complexes[4,5,20]. The integrin–actin linkage is mediated by several proteins, some of which bind directly to actin (BOX 1). The best studied are talin, which transitions integrins to an active state by binding to their cytoplasmic domain through its 'head domain' and to filamentous actin (F-actin) and vinculin through sites in the 'tail domain'[1], vinculin, which also binds F-actin directly, and the actin cross-linking protein α-actinin[10,14]. Although the linkage of integrins to actin has been recognized for many years, the hierarchal structure of the linkage is probably complex. The network of protein interactions that potentially link integrins to the actin cytoskeleton has been intensely studied and globally organized into a structure termed the adhesome[21]. The most recent version of the adhesome includes 180 protein–protein interaction nodes, defining a network that is rich in complexity and connectivity[22].

Box 1 | **Key proteins linking integrins to actin**

Talin
Talin is an actin-binding protein that forms antiparallel homodimers. The amino-terminal FERM (protein 4.1, ezrin, radixin and moesin) domain binds β-integrin tails and is sufficient to activate integrins. The carboxy-terminal rod domain interacts with vinculin and filamentous actin[1].

Vinculin
Vinculin is an actin-binding protein that is associated with cell–cell and cell–extracellular matrix junctions. It is comprised of a globular head domain linked to a tail domain by a short Pro-rich sequence. The intramolecular interaction between the head and tail masks binding sites for talin, actin and other effectors[10].

α-actinin
α-actinin is an actin cross-linking protein that belongs to the spectrin superfamily. It forms antiparallel homodimers in a rod-like structure, with one actin-binding domain on each side of the rod. It can therefore cross link two filaments of actin[14].

Kindlins
The kindlins are members of a family of conserved FERM domain–containing proteins named after the gene mutated in Kindler syndrome, a rare skin blistering disease. Although it is not clear exactly how kindlins activate integrins, they seem to act synergistically with talins to do so[16,17].

The integrins also recruit, indirectly, scaffold and signalling proteins such as paxillin[23] and the protein Tyr kinase focal adhesion kinase (FAK)[24], respectively, which in turn associate with additional molecules that regulate signalling to Rho GTPases. The Rho GTPases act as a regulatory convergence node that dictates cytoskeletal and adhesion assembly and organization. Importantly, integrin signalling networks regulate the activation state of the Rho-family small GTPases — Rac, Rho and CDC42 — by recruiting guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) to adhesion complexes. In turn, Rho GTPases regulate adhesion assembly and disassembly by activating pathways that lead to contraction and actin polymerization.

The main objective of this Review is to describe the importance of the interplay between actin, contraction and adhesion dynamics (the formation and disassembly of adhesions), and how the dynamics of this process orchestrate the reiterative cycle of membrane protrusion, cell adhesion, forward movement and rear retraction — the canonical steps in cell migration. We review the evidence linking adhesion assembly and disassembly to the process of integrin binding to extracellular ligands and how adhesion dynamics are coupled to actin polymerization and myosin II-generated tension — processes that are in turn regulated by the activation of Rho GTPases and protein Tyr phosphorylation.

Adhesion: a dynamic structural continuum

Historically, integrin-dependent adhesions have been classified based on size, stability and location in the cell. However, the relative cellular distribution of the different types of adhesions is in fact dependent on the cell type and the composition and mechanical properties of the ECM substrate[5,18] (FIG. 1; see Supplementary information S1 (movie)). Indeed, as we describe below, adhesion formation, maturation and disassembly is a continuous process driven by the balance of actin polymerization and actomyosin contraction.

Focal complexes, focal adhesions and fibrillar adhesions. Fibroblasts migrating on fibronectin- or collagen-coated surfaces exhibit small, short-lived adhesions (hereafter referred to as nascent adhesions) in the lamellipodium, which form immediately behind the leading edge. Nascent adhesions (which are optimally visualized using TIRF microscopy) can either turn over rapidly, in ~60 seconds, or mature to larger, dot-like adhesions referred to as focal complexes. Focal complexes reside slightly further back from the leading edge, at the lamellipodium–lamellum interface, are slightly larger in size (approximately 1 μm in diameter) and persist for several minutes (FIG. 1). As the migration cycle continues, focal complexes can continue to mature into larger, elongated focal adhesions, which are typically 2 μm wide and 3–10 μm long and reside at the ends of large actin bundles or stress fibres[25] that extend from near the front of the cell along the sides to the cell centre or the rear. As traction forces move the cell forwards, focal adhesions at the rear of the cell disassemble. Fibroblasts grown in fibronectin-rich environments for extended times form fibrillar adhesions, which are characterized by long lifetimes and a highly elongated structure. These specialized adhesions are involved in fibronectin matrix assembly and reorganization of the ECM and are not prominent in rapidly migrating cells.

Although focal complexes, focal adhesions and fibrillar adhesions show quantitative differences in the levels of protein components such as phosphotyrosine, zyxin and tensin[26], they seem to be in a continuum of structures rather than distinct classes. Furthermore, not all cells exhibit the full range of adhesion structures. For example, cells of the myeloid lineage, such as neutrophils and macrophages, have small, highly dynamic adhesions (nascent adhesions and focal complexes) that facilitate their rapid movement on ECM substrates, whereas more contractile cells, such as migrating fibroblasts, endothelial and smooth muscle cells, have more prominent, stable adhesions (focal complexes and focal adhesions).

The contractile nature of many cells combined with the mechanical properties of the matrix (such as compliance, dimensionality and fibre orientation) plays an important part in determining the nature of the adhesions[3,27]. For example, fibroblasts or epithelial cells can be grown in or on materials of variable mechanical stiffness[28,29]. Cells propagated on softer substrates contain smaller and more dynamic adhesions, whereas cells on stiffer substrates exhibit larger and more stable adhesions that are typical of cells on matrix-coated glass or plastic. Thus, adhesion size and distribution reflect the contractile state of the cell, which emphasizes the importance of the interaction between pliability and contraction in shaping adhesion dynamics. The size of adhesions in cells in three-dimensional matrices resembles those formed by cells on more pliable substrates; however, it is likely that the dimensionality of the matrix also contributes to adhesion organization. The adhesions of cells attached to large collagen fibres are large and oriented along the fibres in a two-dimensional (2D)-like organization[30]. These observations underscore how the composition, organization and mechanical properties of the matrix combine to regulate adhesion.

Filopodium
A long, thin protrusion at the periphery of cells and growth cones. Filopodia are composed of F-actin bundles and are often induced by the activation of CDC42.

Actomyosin
A complex of myosin and actin filaments. Activation of the myosin motor leads to shortening of the filaments and subsequent cellular movements.

Guanine nucleotide exchange factor
A protein that activates specific small GTPases by catalysing the exchange of bound GDP for GTP.

GTPase-activating protein
A protein that inactivates small GTP-binding proteins, including Ras and Rho family members, by increasing their rate of GTP hydrolysis.

TIRF
(Total internal reflection fluorescence). A microscope exploiting evanescent wave excitation of the thin region (~100 nm) at the contact area between a specimen and the glass coverslip (of a distinct refractive index). It provides improved signal to noise ratios for the observation of events near the coverslip–water interface.

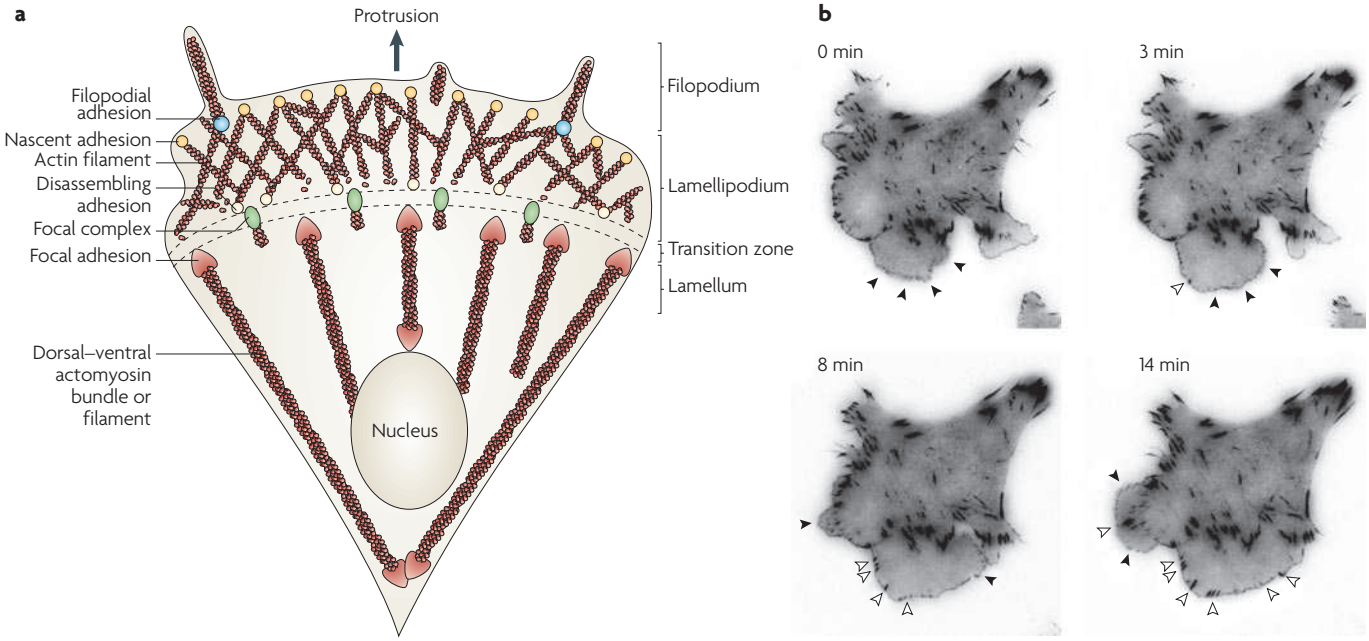

Figure 1 | **Structural elements of a migrating cell. a** | Adhesion is closely coupled with the protrusions of the leading edge of the cell (filopodia and lamellipodia). Adhesions (nascent adhesions) initially form in the lamellipodium (although adhesions may also be associated with filopodia) and the rate of nascent adhesion assembly correlates with the rate of protrusion. Nascent adhesions either disassemble or elongate at the convergence of the lamellipodium and lamellum (the transition zone). Adhesion maturation to focal complexes and focal adhesions is accompanied by the bundling and cross-bridging of actin filaments, and actomyosin-induced contractility stabilizes adhesion formation and increases adhesion size. **b** | TIRF micrographs of a Chinese hamster ovary (CHO) cell expressing paxillin–mEGFP (monomeric enhanced green fluorescent protein) on glass coated with fibronectin (5 μg ml^{-1}). Images were acquired every 5 seconds, and representative images from 0, 3, 8 and 14 minutes are shown (see REF. 49). Closed arrow heads denote nascent adhesions assembling and turning over in protrusions. Open arrow heads indicate maturing adhesions that begin to elongate centripetally (that is, towards the cell centre) when protrusion pauses or halts. For a movie of this experiment see supplementary information S1 (movie).

Lamellum
A distinct region of dense actin behind the lamellipodium.

Three-dimensional matrix
(3D matrix). Cells that migrate on top of a thin layer of ECM are considered to be in 2D, whereas cells that are inside and surrounded by ECM on all sides are considered to be in 3D.

Osteoclast
A mesenchymal cell with the capacity to differentiate into bone tissue.

ARP2/3
A complex consisting of seven subunits, including the actin-related proteins ARP2 and ARP3, that, on activation by WASP-family proteins, binds to the sides of existing actin filaments and nucleates the growth of new filaments to form a dendritic network.

Podosomes and invadopodia. Podosomes and invadopodia are yet another class of adhesions and arecharacteristically found in leukocytes of the monocytic lineage, endothelial and smooth muscle cells, and in tumour cells, respectively[31,32]. Podosomes are small, circular, highly dynamic adhesions comprised of a central actin core, with integrins and other adhesion-associated proteins arranged in a ring around the centre. In osteoclasts, and sometimes in other cells, podosomes reside in clusters that form circular rings at the cell periphery. Although each podosome is highly transient, with a typical lifetime of 2–10 minutes, the rings can be quite stable[33]. Invadapodia resemble podosomes, but they do not arrange into rings, are much more stable and can protrude further into the ECM[34]. Both podosomes and invadapodia contact the substratum and function as sites of localized protease secretion and ECM degradation[35]. Localized ECM degradation is thought to contribute to the invasiveness of normal leukocytes and cancer cells, as well as to bone resorption[31]. Although it is likely that the formation and disassembly of podosomes and invadapodia share many features with the other classes of integrin-dependent adhesions described above, we will not consider them further in this Review (for recent reviews see REFS 32,35,36).

Processes coupled to adhesion dynamics

The formation and disassembly of the different types of adhesions have been studied mainly in 2D culture systems using spreading or migrating fibroblasts or fibroblast-like cells plated on fibronectin, collagen or other purified ECM proteins[37,38]. The mechanistic insights derived from these studies, however, seem to apply to a wide range of other cell types including mesenchymal, epithelial and endothelial cells, as well as leukocytes and neuronal cells. There is abundant evidence that adhesion assembly and disassembly is closely coupled with two fundamental cellular processes: actin polymerization and myosin II-generated tension.

Actin polymerization in adhesion dynamics. The initial step in the migration cycle, protrusion of a leading edge, is driven by actin polymerization in filaments organized into two distinct zones, the lamellipodium and the lamellum[39]. In the lamellipodium, actin is arranged in a dendritic, or branched, structure that is localized beneath the membrane[12]. Polymerization of lamellipodial actin is catalysed by the ARP2/3 complex, the activity of which is regulated by the Rho GTPases Rac and CDC42 through downstream effectors belonging to the Wiskott–Aldrich syndrome protein (WASP) and

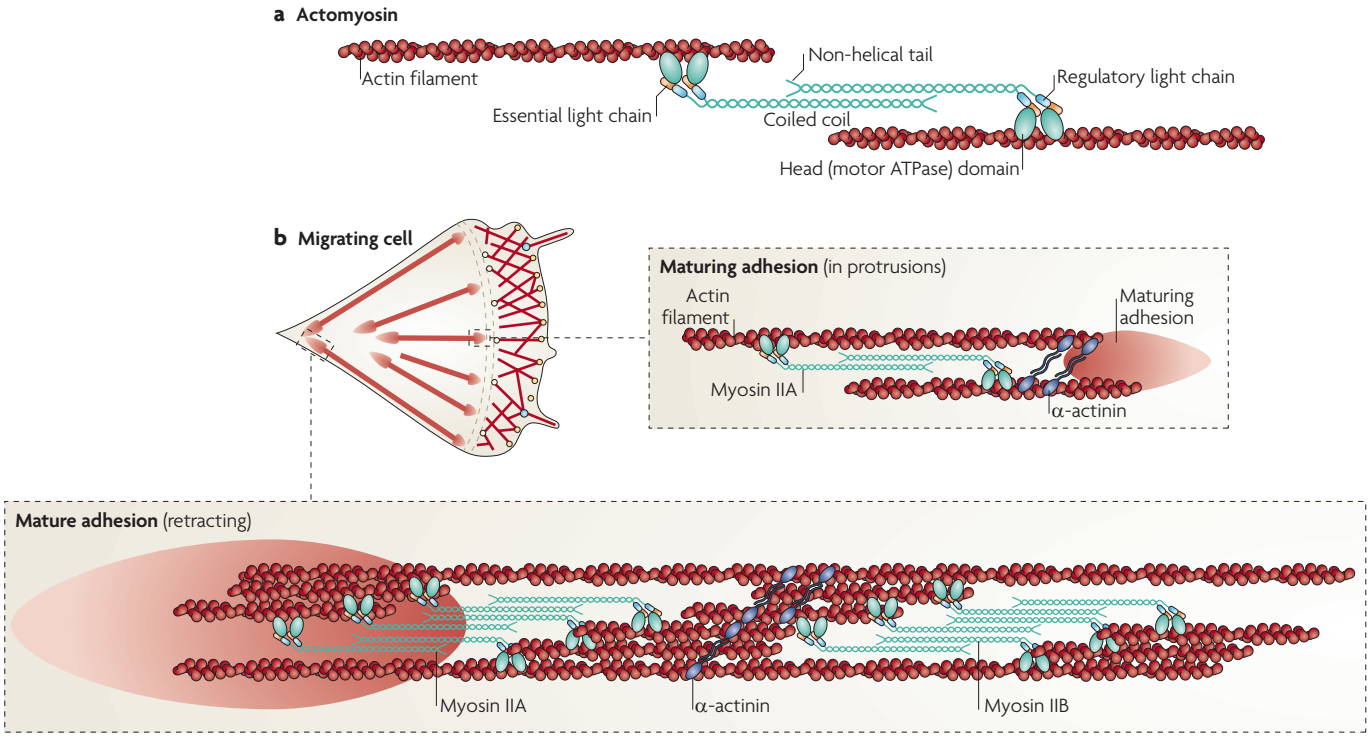

Figure 2 | **Myosin II and adhesion maturation and turnover. a** | Adhesions elongate along actin filaments that contain myosin IIA, which cross links the actin filaments and exerts tension on them. This leads to tension on the conformational sensitivity, and clustering of, adhesion molecules that are directly or indirectly associated with actin. Myosin II activity is regulated by phosphorylation on the regulatory light chain at Thr18 and Ser19, although other regulatory sites in the heavy chain are also implicated in its activities. For a more complete discussion of myosin II structure and function see REF. 8. **b** | In a migrating cell, myosin IIA acts at a distance to regulate adhesion maturation and turnover as it is juxtaposed to, but not directly associated with, the maturing adhesion at the cell front. α-actinin cross links actin filaments. Adhesions at the rear are associated with large actin filament bundles that contain both myosin IIA and myosin IIB. Their activity mediates rear retraction and adhesion disassembly.

WASP-family verprolin homologue (WAVE; also known as SCAR) families of proteins[12,40]. Lamellipodial actin undergoes rapid retrograde flow driven by the resistance of the membrane to actin polymerization at the leading edge[41]. In the lamellum, actin filaments reside in parallel bundles that undergo a slower retrograde movement largely owing to myosin II contraction (see below). In the region of convergence between the lamellipodium and the lamellum, known as the transition zone, dendritic actin depolymerizes and reorganizes into bundles[42–44].

Actin filaments in the central and rear regions of migrating cells are often organized into thick bundles called stress fibres[45] (FIG. 1). Dorsal stress fibres connect to the substrate through focal adhesions at one end. Transverse arcs, which are not directly anchored to the substrate, are generated by the annealing of myosin-II–actin bundles and ARP2/3-nucleated actin bundles at the lamella. Finally, ventral stress fibres arise from dorsal stress fibres and transverse arcs and are anchored to focal adhesions at both ends[44]. Each of these structures depend on the activity of Rho and its effectors Rho-associated protein kinase (ROCK) and the formin mouse diaphanous 1 (mDia1), contain myosin II and α-actinin and are contractile[18,44,46].

Myosin II-generated tension in adhesion dynamics. Contraction of actin stress fibres is mediated by myosin II, which moves antiparallel actin filaments past each other and thereby provides the force that rearranges the actin cytoskeleton (FIG. 2). Myosin II also bundles actin filaments owing to its oligomeric nature and actin-binding properties[8,47]. Myosin II is comprised of two heavy chains, two regulatory light chains (RLCs) and two essential light chains and has three isoforms (myosin IIA, myosin IIB and myosin IIC), which are specified by the different heavy chains that they contain. Myosin IIA and myosin IIB are present in most cells, whereas myosin IIC is not widely expressed and may have a role in cancer[8]. Myosin II activity (ATP hydrolysis and actin filament formation) is regulated by the reversible phosphorylation of Thr18 and Ser19 of the RLC of the myosin II molecule. This phosphorylation is controlled by several protein kinases and phosphatases, many of which are regulated by Rho GTPases. Although myosin II is not present in the lamellipodium, its activity influences membrane protrusion at the leading edge. For example, knockdown of myosin II with small interfering RNAs or treatment of cells with blebbistatin (a small molecule inhibitor of myosin II) reduces actin bundling in the protrusion, increases the

Retrograde flow
The movement of actin filaments or other cell components from the cell edge towards the centre, generally driven by actin polymerization at the leading edge.

Transverse arc
A bundle of actin filaments that forms parallel to the leading edge and undergoes retrograde movement towards the cell centre.

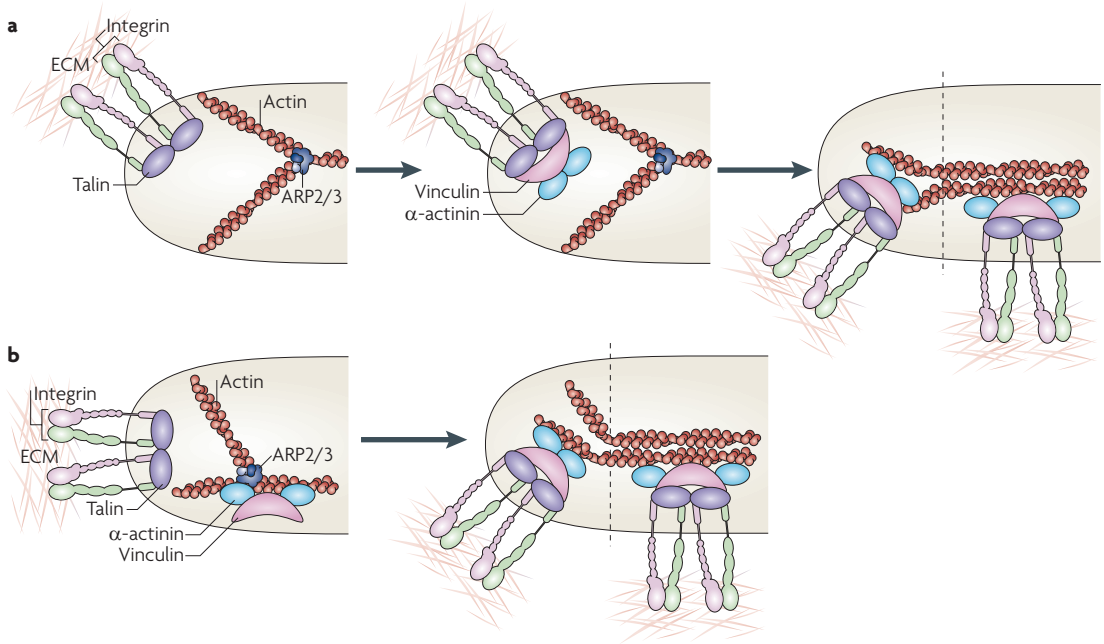

Figure 3 | **Models for the assembly of nascent adhesions. a** | In one model, adhesion nucleation is initiated by the binding of integrins to extracellular matrix (ECM) proteins, their ligand-mediated clustering and the coordinate assembly of new adhesion complexes on the clustered integrin cytoplasmic domains, which are depicted here as a complex with talin, vinculin, α-actinin and dendritic actin (middle panel). Maturation of the adhesions is mediated by increased tension on them and the bundling and cross-bridging of the actin filaments (right panel). **b** | A second model posits that adhesion formation is coupled to actin polymerization and that vinculin (and perhaps focal adhesion kinase (FAK)) bind directly to actin-related protein 2/3 (ARP2/3) complexes and colocalize before adhesion formation (left panel). These complexes then bind integrins (depicted here in association with talin), stabilizing the nascent adhesion (right panel). As in part **a**, maturation of the adhesions is mediated by increased tension on the adhesions and the bundling and cross-bridging of the actin filaments.

protrusion rate and decreases the size of adhesions[43,47], underscoring the requirement for myosin II activity in maintaining these structures.

Adhesion dynamics

In motile cells, the earliest detectable adhesions (nascent adhesions) form in the lamellipodium just behind the leading edge (FIG. 1). Their assembly is independent of myosin II activity but is proportional to the protrusion rate of the leading edge and requires ARP2/3 complex-mediated actin polymerization[48,49]. These adhesions contain integrins, talin, vinculin, α-actinin, paxillin and FAK, among other proteins, and are enriched in phosphotyrosine[49], an indication that these adhesions are active signalling complexes (see below). As the leading edge of a migrating cell moves forwards, nascent adhesions either elongate and grow or disassemble, depending on the cell type. Disassembly occurs when the nascent adhesions encounter the zone of depolymerizing actin at the juncture of the lamellipodium and lamellum. Adhesion turnover in this region is therefore coincident with actin severing and the disassembly of branched actin structures. Nascent adhesions can also mature into focal complexes coincident with periodic or occasional pauses of the forward movement of the leading edge. These pauses correlate with, and depend on, myosin II-dependent contractile events[47–49]. The actin cross-linking protein α-actinin has also been implicated as a crucial component of adhesion maturation as it is the earliest component detected in maturing adhesions and it accumulates with actin filaments before other adhesion components[49]. Either new actin polymerization or the reorganization of existing actin filaments at the junction of the lamellipodium and lamellum creates templates for maturation[49]. The time of appearance and spatial organization of α-actinin suggests that it is crucial for orienting these actin templates and linking the actin filaments to the adhesions.

Although tension is clearly important for adhesion maturation, different adhesion components show differential sensitivity to tension[50]. For example, the incorporation of paxillin, talin and integrin are independent of myosin II activity, whereas FAK, zyxin and α-actinin are dependent on it. Paxillin phosphorylation seems to be tension-sensitive and a key regulator of maturation, in part through its effect on vinculin binding[50].

Models of nascent adhesion nucleation. The mechanisms by which nascent adhesions are nucleated, elongate and disassemble are not yet clear. Two possible models for nascent adhesion nucleation have been proposed (FIG. 3). In the first model, nucleation of adhesions is initiated by the binding of integrins to ECM proteins, their ligand-mediated clustering and the subsequent assembly of new adhesion complexes on their clustered cytoplasmic domains (FIG. 3a). In the second model, the assembly is initiated by actin polymerization and uses dendritic actin

as a template for the nucleation of adhesion complexes (FIG. 3b). Evidence for the first model comes from the presence of activated integrins near the leading edge of protrusions in migrating cells and the juxtaposition of nascent adhesions forming beneath the lamellipodium in contact with the ECM. Ligand-bound, clustered integrins would then form a 'multivalent' scaffold that binds other adhesion components (such as vinculin and talin) and recruits additional integrins, all of which ultimately link to actin filaments. This general model also gains strong support from studies using ligand or anti-integrin antibodies coupled to beads, which induce the clustering of adhesion components around the bead[51]. The second model is suggested by evidence that adhesion formation is coupled to actin polymerization, and that vinculin and FAK bind directly to ARP2/3 complexes and colocalize with ARP2/3 before adhesion formation[47–49]. These complexes could therefore nucleate integrin-containing complexes before integrin binds to the ECM. In reality, these models are not mutually exclusive; there is likely to be some degree of integrin clustering before ligation, with ligation increasing the clustering and signalling. Detailed molecular studies are needed to fully understand the possible mechanisms.

Myosin II promotes adhesion maturation and stability. The activity of myosin II and the resulting tension exerted on adhesions seem to be important factors in determining the balance between adhesion disassembly and maturation[3,4,47,52]. In Chinese hamster ovary (CHO) cells, in which myosin II activity is low, nascent adhesions are readily seen in the lamellipodium, whereas in more contractile cells, nascent adhesions are scarce and most rapidly mature to focal complexes[47,49]. Inhibiting myosin II with blebbistatin prevents adhesion maturation and greatly increases nascent adhesions. Conversely, myosin IIA overexpression in CHO cells inhibits leading edge protrusion and increases nascent adhesion maturation to focal complexes[47]. Thus, initial adhesion assembly is mechanistically and kinetically linked to actin polymerization in the lamellipodium, whereas myosin II activity and tension exerted on actin in the lamellum contribute to the maturation of newly formed adhesions to focal complexes and focal adhesions.

How does myosin II promote adhesion maturation and stability? One way is through the generation of tension, which directly perturbs the conformation of proteins in the adhesion complex. For example, the application of forces *in vitro* to single talin rods exposes cryptic binding sites for vinculin. Because the talin head domain interacts with integrins while its tail binds actin filaments, talin bears the force transmitted from the actin cytoskeleton to the matrix. Thus, actomyosin contraction would trigger force-dependent talin unfolding and increase talin–vinculin binding to reinforce the adhesion[53]. The vinculin tail domain also provides a linkage to actin[54]. In cells, recruitment of vinculin to adhesions is driven by changes in tension[55]. This recruitment is probably controlled at the molecular level, at least in part, by tension-induced conformation changes that result in the perturbation of the interaction between its amino- and carboxy-terminal domains[10]. Other adhesion-associated molecules, such as paxillin and CRK-associated SRC substrate (CAS; also known as p130cas), may also change conformation under tension (or tension-induced signals) to reveal new protein-binding and/or phosphorylation sites[21,56,57]. The ensuing protein–protein interactions and/or phosphorylation would activate these scaffold proteins to recruit additional signalling proteins (see below). Recently, α5β1 integrin was reported to undergo a conformational change in response to myosin II-generated cytoskeletal force, suggesting that this force, combined with ECM stiffness, triggers an integrin switch that is required to generate signals through the adhesion complex[58].

A second action of myosin II on adhesion maturation occurs through its cross-linking properties. Phosphorylation of myosin II RLCs increases myosin II's assembly into bipolar myosin filaments, which bundle actin. Indeed, myosin II mutants that assemble into filaments and bind actin but lack the motor activity required to produce tension, still induce focal adhesions[47], indicating that both contractility and actin bundling probably contribute to the maturation and stabilization of adhesions.

Adhesion linkages: the clutch. The tension exerted on adhesions depends on the efficiency of the linkage between actin and the ECM, namely the efficiency of the adhesion 'clutch'[5,59–61]. Actin in the lamellipodium undergoes retrograde flow from two sources. One is the force from membrane resistance at the leading edge, which is created by actin polymerization itself and causes rearward actin flow. The other is from myosin II-mediated contraction of actin filaments in the lamellum. Thus, the net rate of forward protrusion of the leading edge is determined by the rate of actin polymerization minus these rearward forces. Adhesions function as traction points that resist the force arising from the rearward flow of actin filaments and shunt the force to the substratum, resulting in increased protrusion. However, the efficiency of this shunting, or resistance, seems to be variable, as some adhesion components move in a retrograde direction with the actin but not at the same rate, pointing to a 'slippage' in the actin–adhesion linkage. This has led to the idea that the link between adhesions and actin is regulated by a clutch-like mechanism. When the clutch between adhesions and rearward flowing actin is engaged, rates of forward protrusion of the leading edge increase while the adhesions undergo force-dependent maturation[61,62]. The efficiency of this clutch seems to differ among cells, suggesting that it is regulated; this idea has important implications for the efficiency of tension-induced adhesive signalling[59,60]. Interestingly, myosin II localizes on actin filaments several micrometres away from the adhesions, suggesting that the contractile forces generated by myosin II are transmitted down the filament to the adhesions; that is, myosin II acts at a distance[47].

Adhesion disassembly at the cell front and rear. Finally, tension and other factors contribute to adhesion disassembly at both the front and the rear of the cell[63]. At the front, disassembly occurs most prominently at the lamellum–lamellipodium interface, presumably owing to

Box 2 | **Key regulators of adhesion dynamics: Rho GEFs and Rho GAPs**

PIX proteins
PAK-interacting exchange factor (PIX) proteins were originally identified as binding partners for the CDC42 and Rac target and effector, p21-activated kinase (PAK). PIX proteins (PIXα and PIXβ) contain a DBL homology (DH) domain, but only PIXα has significant guanine nucleotide exchange factor (GEF) activity for Rac, which is under tight control through intramolecular interactions involving several binding partners[2].

DOCK180
180 kDa protein downstream of CRK (DOCK180; also known as DOCK1) is a GEF that, following integrin receptor activation, forms a complex with CRK-associated SRC substrate (CAS; also known as p130cas) and CRK which is targeted to focal adhesions. DOCK180 interacts with the small GTPase RAC1, but not with Rho or CDC42, and functions as a GEF to activate Rac. The CRKII–DOCK180–Rac cascade promotes the reorganization of the actin network, membrane ruffling, lamellipodial protrusion and phagocytosis of apoptotic cells[9].

GIT
G protein-coupled receptor (GPCR) kinase-interacting protein (GIT) is a member of a family of ADP-ribosylation factor GTPase activating proteins (ARFGAPs). Members of this family share common binding partners, including paxillin, PIX, GPCR kinase (GRK) and focal adhesion kinase (FAK)[2]. The role of their association with focal adhesion proteins is still poorly understood but it may be a point of convergence for ARF and integrin signalling.

ARHGAP22
ARHGAP22 (also known as RHOGAP2) is a Rho GAP that converts RAC1 to an inactive GDP-bound state. Expression of ARHGAP22 inhibits RAC1-dependent lamellipodium formation[13].

p190RhoGEF
p190RhoGEF (also known as RGNEF) is a brain-enriched, RHOA-specific GEF, the highly interactive carboxy-terminal domain of which provides potential linkage to multiple pathways in a cell[15].

p190RhoGAPs
p190RhoGAPs exist in two isoforms, A and B. p190RhoGAPs regulate actin cytoskeleton dynamics, membrane ruffling, neurite retraction, smooth muscle contraction, cytokinesis, cellular morphology, cellular motility and invasion, embryonic neuronal development and vascular permeability[19].

actin depolymerization and reorganization. Disassembly also occurs in regions undergoing retraction at both the cell front (as a part of the extension and retraction cycle of a protrusion) and the rear. Disassembly associated with retraction is usually accompanied by an apparent 'sliding' of adhesions, which accompanies the inward movement of the cell edge, and then the 'dispersal' of adhesion structures. Although not fully understood, adhesion sliding seems to be a Rho GTPase- and myosin II-dependent form of treadmilling, in which the peripheral edge of the adhesion disassembles while the central edge assembles[64,65]. Thus, although the whole adhesion moves, individual components exchange in and out of it but otherwise remain stationary. Interestingly, integrins, but not the cytoplasmic components of adhesions, are sometimes seen on the substratum behind migrating cells, indicating a severing between integrin and the cytoplasmic components of the adhesion during release[66]. This effect is blocked by a myosin II inhibitor[67], suggesting that it is also tension-dependent.

The Ca^{2+}-activated protease calpain has also emerged as an important mediator of adhesion disassembly in retracting regions[68]. Calpain inhibition by chemical inhibitors, biological agents (such as calpastatin) and genetic deletion block disassembly. Both talin 1 and the integrin β3 cytoplasmic domain have been identified as key calpain substrates in adhesion disassembly, although there are many others with a functional significance that is less well investigated[69–71].

Barbed end
The fast-polymerizing end of an actin filament, which is defined by the arrowhead-shaped decoration of actin filaments with myosin fragments.

Regulation of adhesion dynamics

The Rho GTPases Rac, Rho and CDC42 together regulate adhesion by directly controlling the balance between actin-mediated protrusion and myosin II-mediated contraction[72–75].

Rac, Rho and CDC42 activity in adhesion dynamics. As expected, Rac and CDC42 are activated at the front of migrating cells, but with distinct spatial and temporal characteristics[76]. RHOA is prominently activated at the cell rear and also, unexpectedly, at the front[77–80]. Current evidence indicates that Rac and CDC42 probably have partially overlapping functions in mediating the formation of actin-rich protrusions at the leading edge[81]. Whereas the expression of activated CDC42 alone produces filopodia, and expression of activated Rac stimulates broad lamellipodia, leading-edge protrusion in most cells probably involves both. The activation of Rho at the leading edge was surprising, as Rho was thought mainly to activate myosin II in the rear of the cell[80]. However, colocalization data suggest that Rho in this region couples selectively to the formin mDia1 (REFS 77–79), which binds actin barbed ends, and promotes polymerization[82] through mDia1 rather than through myosin II activation.

Rac and CDC42 induce protrusions in most cells by activating the WASP homologue (WH) domain-containing proteins neural WASP (NWASP) and WAVE, which in turn induce actin polymerization by directly activating the ARP2/3 complex. Rac and CDC42 also bind and activate the PAK Ser/Thr kinases (PAK1, PAK2 and PAK3). PAKs have multiple cytoskeletal targets, including LIM kinase, which is activated by PAK and enhances actin polymerization by inactivating cofilin — a protein that disassembles actin filaments[83,84]. PAK also activates myosin II by phosphorylating its RLCs. RHOA activation leads to the maturation of focal adhesions through its ability to activate myosin II, which promotes adhesion maturation and stability, as discussed above. Rho activates myosin through ROCK1 and ROCK2, which act mainly by inactivating a subunit of myosin phosphatases (myosin phosphatase-targeting subunit 1 (MYPT1; also known as PPP1R12A)), thus sustaining myosin II RLC phosphorylation. As mentioned above, Rho also activates the formin mDia1 to promote actin polymerization. Both of these effector pathways contribute to actin polymerization, bundling and adhesion formation[46].

Adhesion dynamics are regulated by complex feedback loops with the Rho proteins and a poorly understood reciprocity between Rac and Rho activation that is presumably mediated through the action of GEFs and GAPs (BOX 2). Recently, it was shown that the activation of a novel photoactivatable RAC1 (PA-RAC1) was sufficient to produce cell motility and control the direction of cell movement. Importantly, local activation of PA-RAC1 inhibited RHOA activation in protrusions of migrating fibroblasts[85].

The molecular basis for the reciprocal regulation of Rac and Rho is not understood. On the one hand, Rac can inhibit Rho through the activation of p190RhoGAP (also known as GRLF1) to adhesions[86], which can reduce tension at the leading edge to allow more continuous forward protrusion[87]. On the other hand, maturation of focal complexes into focal adhesions involves activation of Rho downstream of Rac, perhaps through the recruitment of a Rho GEF to adhesions[88]. Conversely, Rho can inhibit Rac through a pathway that involves ROCK, possibly through mechanical tension stimulating a Rac GAP such as ARHGAP22 (REFS 13,89). Understanding this reciprocity and these feedback loops is an important issue that needs to be addressed.

The spatial and temporal activation of Rac, Rho and CDC42 at the leading edge of migrating cells has been examined recently using the simultaneous visualization of two GTPase biosensors paired with computational multiplexing approaches[80]. Surprisingly, RHOA is activated near the cell edge concomitant with leading-edge advancement. In contrast, CDC42 and RAC1 are activated distal to the leading edge with a delay of ~ 40 seconds. Thus, both the timing and spatial characteristics of RAC1, CDC42 and RHOA activation are distinct. The spatial localization of RAC1 and CDC42 activation are consistent with the fact that these GTPases stimulate dendritic actin polymerization, which is essential for the leading edge. The role of RHOA is less clear but, as mentioned above, mDia1 activation is an attractive pathway as it promotes polymerization of the initial actin filaments needed for ARP2/3-mediated dendritic polymerization. mDia1 also attaches an actin barbed end to the membrane and allows the insertion of actin monomers at the end of the filament. However, active Rac or CDC42 can induce protrusions when RHOA is inhibited; thus, cooperation between these GTPases is not essential. Overall, much remains to be learned about how these proteins regulate dynamics at the leading edge.

Regulation of Rho GTPases in adhesion dynamics. Various scaffold proteins organize signalling complexes that regulate Rho GTPases. Protein Tyr kinases (PTKs), such as SRC, FAK, Abelson kinase 1 (ABL1) and ABL2, and their adhesion-associated substrates, function as scaffolds to differentially organize the regulatory proteins that control the activity of the Rho GTPases and, therefore, actin and adhesion dynamics and organization[24,88,90,91]. For example, PTKs phosphorylate adhesion proteins such as paxillin and CAS, which then bind and localize activated forms of GEFs and GAPs for Rho GTPases, as well as SH2-containing adaptor proteins such as CRK and NCK[92]. These SH2-containing adaptor proteins recruit additional regulators of downstream kinases, including extracellular signal-regulated kinases (ERKs) and PAKs[92–94]. Thus, paxillin, FAK and CAS are examples of adhesion-associated proteins that function as 'switchable' scaffolds, in which phosphorylation of their Tyr residues leads to the recruitment of functional regulators of Rho GTPases and other signalling proteins. Other adhesion proteins such as zyxin and tensin may also be switchable scaffolds, although their role in regulating adhesion dynamics is less clear[95–97]

Adhesion assembly seems to be a key regulator of scaffold phosphorylation. For example, the catalytic activity of both FAK and SRC is stimulated by recruitment to newly formed adhesions[98]. Adhesion-dependent autophosphorylation of FAK leads to the recruitment and activation of SRC, which mediates Tyr phosphorylation of FAK itself, paxillin and other adhesion molecules[99,100]. Notably, paxillin and CAS undergo conformational changes concomitant with phosphorylation on their Tyr residues and recruitment into adhesion structures[23,56]. The importance of Tyr phosphorylation in the activation of the paxillin scaffold was revealed by the observations that phosphorylation of paxillin on Tyr31 and Tyr118 regulates the coordinated formation of lamellipodia or the induction of myosin II-dependent contraction[21]. Overexpression of phosphomimetic paxillin (Tyr31Glu and Tyr118Glu) enhances lamellipodial protrusion and the formation of nascent adhesions, whereas overexpression of non-phosphorylatable paxillin (Tyr31Phe and Tyr118Phe) induces large focal adhesions, prominent fibrillar adhesions and fibronectin fibrillogenesis, which are characteristic of highly contractile cells. These observations are consistent with Tyr-phosphorylated paxillin being a scaffold for the recruitment of positive regulators of Rac and CDC42. Similarly, Tyr phosphorylation of CAS recruits the SH2-containing adaptor protein CRK, which in turn recruits or activates the Rac GEF DOCK180 (180 kDa protein downstream of CRK; also known as DOCK1) and the RAP1 GEF C3G (also known as RAPGEF1)[92]. Ser phosphorylation of paxillin has also been reported to regulate adhesion turnover and protrusion dynamics in migrating cells[101].

The scaffold functions of FAK seem to be important in the recruitment of Rho GAPs and Rho GEFs[15,88]. The association of FAK with p190RhoGAP seems to be important for RHOA inhibition during fibronectin-stimulated cell spreading, which facilitates lamellipodial protrusion[19,102]. Less clearly understood is the interaction of FAK with p190RhoGEF (also known as RGNEF). In cells plated on fibronectin for long periods of time, FAK seems to selectively associate with p190RhoGEF, suggesting that as cells become more contractile, positive regulation of RHOA is the dominant activity in more mature adhesions[88]. Additional scaffold proteins, other kinases and protein phosphatases (for example, tensin, zxyin, integrin-linked kinase (ILK), particularly interesting new Cys-His protein 1 (PINCH; also known as LIMS1), parvin, Abl, FYN and SH2 domain-containing Tyr phosphatase 2) are reported to associate with focal adhesions[91,95,96,103–106]. Numerous studies have shown that knock down, knock out or overexpression of these proteins modulate adhesion structures and dynamics in complex ways; however, the mechanisms and regulation are poorly understood.

Unifying the principles of adhesion

As discussed above, the adhesive steps in the migration cycle — assembly, maturation and disassembly — are tightly coupled to actin polymerization and organization and to actin–myosin contraction, which are in turn regulated by Rho GTPases and PTKs[5] (FIG. 4). The first step

Computational multiplexing
A mathematical method to correlate multiple time-dependent variables obtained during time-lapse imaging of cells.

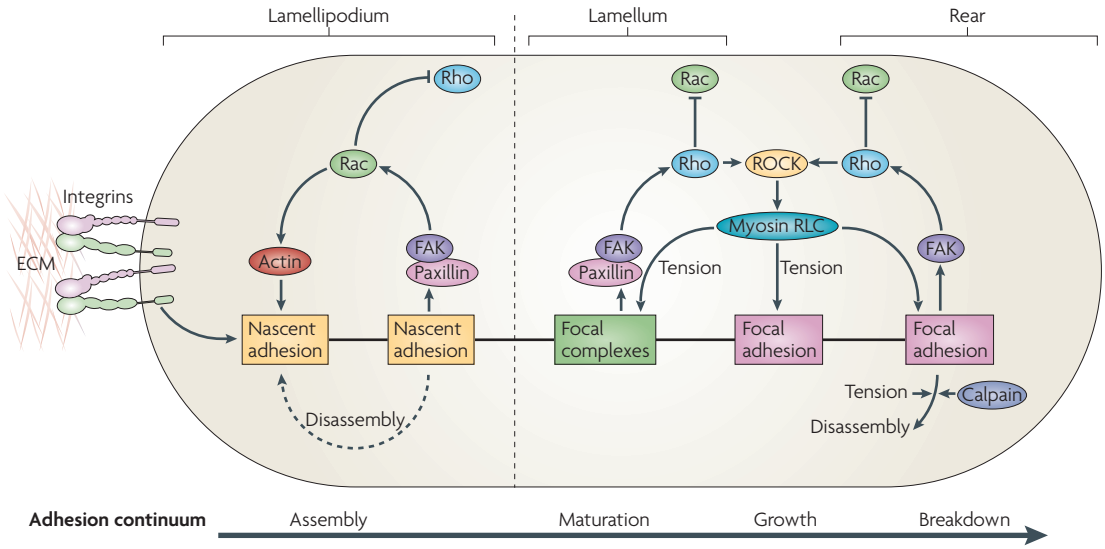

Figure 4 | **Adhesion maturation and Rho GTPase activation.** Nascent adhesion formation and disassembly are coupled with the forward movement of the lamellipodium. Maturation of adhesions is dependent on actomyosin in the lamellum, where adhesions become larger. Adhesion formation and disassembly in the lamellipodium is driven by the activation of Rac (and perhaps the localized suppression of Rho activity), which involves activation of the Tyr-phosphorylated scaffolds, paxillin and focal adhesion kinase (FAK). In the lamellum, adhesion maturation is accompanied by localized activation of Rho, perhaps through FAK-dependent recruitment of Rho guanine nucleotide exchange factors (GEFs) and Rho GTPase-activating proteins (GAPs). Rho activation sustains the activation of myosin II through the action of Rho-associated protein kinase (ROCK), which controls the kinases and phosphatases that regulate its regulatory light chain (RLC) phosphorylation. Myosin II-generated tension sustains adhesion maturation by cross linking- and tension-induced conformational changes in various adhesion proteins (see main text). Disassembly of adhesions at the cell rear is Rho GTPase- and myosin II-dependent, and may also involve the action of proteases, such as calpains, on adhesion-linked proteins. ECM, extracellular matrix.

in the cycle is formation of nascent adhesions beneath the lamellipodium near the leading edge. These adhesions not only stabilize the leading edge through contact of the cell with the ECM, but their formation leads to the generation of signals that activate Rac and CDC42, reinforcing the actin polymerization at the leading edge and subsequent membrane protrusion.

Nascent adhesions disassemble as the lamellipodium moves forwards, unless they connect with actomyosin in the lamellum, in which case they mature and become larger. Adhesion maturation is probably accompanied by localized activation of Rho, perhaps through FAK-dependent recruitment of Rho GEFs. Rho activation sustains the activation of myosin II through the action of ROCK on the kinases and phosphatases that regulate myosin II RLC phosphorylation. Myosin II-generated tension sustains adhesion maturation through cross-linking and tension-induced conformational changes in various adhesion proteins. Although myosin II controls adhesion maturation and disassembly, the extent to which these processes occur probably reflects the efficiency of the linkage between actin and the ECM by the adhesion clutch and/or pliability of the matrix, two factors that contribute to myosin II activity and intracellular tension. Cells on rigid surfaces coated with high densities of ECM proteins exhibit large, myosin II-dependent focal adhesions, whereas cells on pliable substrates coated with low densities of adhesion molecules tend to have smaller adhesions[3]. Indeed, artificially increasing integrin clustering can make cells behave on soft substrates as if adhered to rigid ones[107]. These data suggest that cells sense the mechanical properties of the substratum and subsequently modulate myosin II activity, integrin clustering, adhesion size and composition, and downstream signalling. However, the model also implies that myosin II is regulated through changes in integrin signalling. Indeed, the feedback loop that connects adhesion, contractility and signalling almost certainly involves Rho GTPases and/or the regulation of myosin II activity and actomyosin tension.

Cell adhesion, contractility and signalling play central parts in the front–back polarization of migrating cells and hence in regulating directional motility. In fibroblasts, adhesions at the leading edge generate signals that activate Rac, which in turn leads to dendritic actin polymerization and establishment of the cell front. Conversely, actomyosin bundles and stable adhesions are crucial for generating the cell rear[108,109]. An attractive hypothesis is that the cross-linking and bundling by myosin IIB generates large, stable actin filaments and adhesions, which inhibits adhesion signalling to Rac (FIG. 3). Recent studies have shown that actin filament bundles in the cell rear contain activated myosin IIB, which is crucial for the formation and stabilization of the rear[47,110]. The partitioning defective 3 (PAR3)– or PAR6–protein kinase Cα (PKCα) complex is also implicated in cell polarity[111]; however, the role of adhesion in this process remains to be clarified.

Migration in disease

Migration is a prominent feature of many diseases, including cancer and chronic inflammation. It is also important in stem cell transplantation strategies, where injected cells may need to migrate into target tissues, and in wound repair, where enhanced cell migration contributes to wound closure. Although adhesion receptors and ECM ligands have been studied as potential targets for therapeutic strategies, differences in adhesion dynamics and maturation may also play a part in disease and therefore offer targets for intervention and diagnosis. For example, myosin II activity regulates migration through its effects on adhesion maturation and signalling. Thus, strategies directed at specific regulators of myosin II, such as Rho kinase and myosin light chain kinase (MLCK), provide another route to the regulation of migration. In addition, the activation (phosphorylation) status of key effectors of the Rho kinases might be a parameter for predicting invasive potential; that is, whether the cells are primed for migration. The feasibility of this strategy derives from the relatively small number of molecules downstream of Rac and Rho that regulate adhesion dynamics and signalling for migration. Thus, there are provocative new opportunities for both therapeutic and diagnostic techniques that may play an important part in clinical medicine.

Some remaining questions

The model for adhesion dynamics described here supports contemporary views of directed cell migration[18], in which the compartmentalization of Rac and/or CDC42 and Rho activity maintains the direction of cell movement[109]. The balance of actin polymerization and myosin II-generated contractility provide both feed-forward and feedback loops that regulate adhesion formation and disassembly. In the framework of this model, several important questions remain. What regulates the efficiency of coupling between adhesions and rearward flowing actin? What determines whether an adhesion strengthens under force, as occurs in the front of migrating cells, versus disassembles, as occurs in the rear? How do cells sense the rigidity of the ECM to control myosin II activation and how does this feed back to regulate signalling by adhesions? Do different adhesions generate distinct signals and, if so, how, when and where? What is the role of Rho at the leading edge? How do changes in adhesion and migration pathways underlie immune disorders, developmental defects and cancer cell invasion and metastasis? Clearly, understanding the fundamental mechanisms that govern adhesion signalling offers unique opportunities to design and implement therapeutic interventions that may have a considerable impact on the treatment of human disease.

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Acknowledgements
The authors wish to acknowledge the help of M. Vicente-Manzanares and C. Choi in the preparation of the figures and C. Choi for making the supplementary movie. The authors acknowledge support from the Cell Migration Consortium (U54 GM64346), NCI CA40042 (to J.T.P), NIGMS-GM23244 (to A.R.H) and GM47214 (to M.A.S).

Competing interests statement
The authors declare no competing financial interests.

DATABASES
UniProtKB: http://www.uniprot.org
CAS | CDC42 | FAK | mDia1 | paxillin | talin 1

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Molecular mechanisms of organelle inheritance: lessons from peroxisomes in yeast

Andrei Fagarasanu, Fred D. Mast, Barbara Knoblach and Richard A. Rachubinski

Abstract | Preserving a functional set of cytoplasmic organelles in a eukaryotic cell requires a process of accurate organelle inheritance at cell division. Studies of peroxisome inheritance in yeast have revealed that polarized transport of a subset of peroxisomes to the emergent daughter cell is balanced by retention mechanisms operating in both mother cell and bud to achieve an equitable distribution of peroxisomes between them. It is becoming apparent that some common mechanistic principles apply to the inheritance of all organelles, but at the same time, inheritance factors specific for each organelle type allow the cell to differentially and specifically control the inheritance of its different organelle populations.

Formin
One of a group of conserved proteins that nucleate actin assembly by promoting the incorporation of new actin monomers into the growing plus end of an actin filament, with which they remain associated.

Actin monomer
A monomer of actin (also known as globular actin (G-actin)) that polymerizes into helical actin filaments called filamentous actin (F-actin) or microfilaments.

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doi:10.1038/nrm2960
Published online
18 August 2010

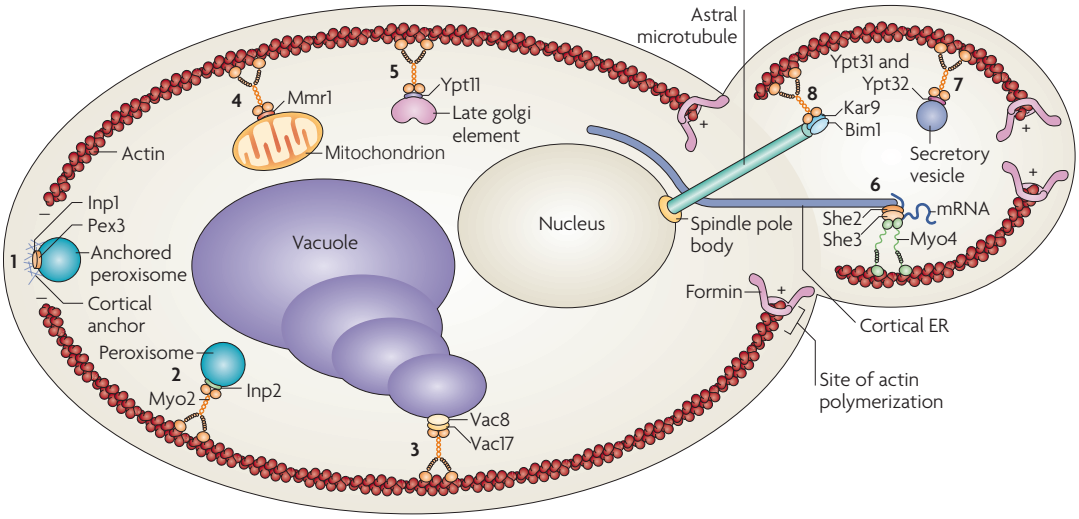
The cytoplasm of every eukaryotic cell is elaborately subdivided into discrete, specialized, membrane-bound structures called organelles. Each organelle has a characteristic morphology and is equipped with a specific set of proteins and lipids to create a microenvironment that is elegantly suited to carry out its defined functions in a cell[1–4]. The different organelles communicate through a constant exchange of material that, although enhancing the metabolic efficiency of cells, does not compromise each organelle's steady-state composition and identity[5]. The complexity in both the architecture and function of organelles makes it energetically unfavourable for cells to rapidly manufacture organelles *de novo*, even in the case of organelles that originate from other intracellular compartments[3,6]. Rather, a template-based biogenesis mechanism involving the growth and division of pre-existing organelles is the preferred method of maintaining organelle populations during cell proliferation. With each round of cell division, cells duplicate and apportion their various organelles to the two resulting cells with high accuracy, a process called organelle inheritance[1].

In the past decade, considerable advances have been made in understanding the molecular mechanisms of organelle inheritance using the budding yeast *Saccharomyces cerevisiae*. *S. cerevisiae* has facilitated the study of organelle inheritance because its growth is highly polarized, with a mother cell forming a bud that is initially much smaller than itself. At first glance it would seem that cells that divide by median fission (for example, mammalian cells) need only to disperse their organelles randomly in the cytoplasm to achieve organelle inheritance on cytokinesis; however, organelle partitioning in these cells has also been shown to be an ordered process involving the cytoskeleton and motor proteins[7–12]. This Review highlights the recent progress made in uncovering the molecular basis of peroxisome inheritance in yeast; however, we do not hesitate to diverge from the field of peroxisome inheritance to draw the reader's attention to fascinating complementary findings arising from studies of inheritance of other organelles. One emergent theme is that, although each organelle uses specific molecular components to ensure its inheritance by future generations of cells, a set of fundamental rules applies to the mechanisms of inheritance of all organelles. The timing of this Review coincides with an unprecedented understanding of these common denominators, leading to the formulation of unifying themes and testable general paradigms for the partitioning of all organelles.

Organelle inheritance in budding yeast

S. cerevisiae multiplies by a repetitive pattern of growth and division termed budding. At the beginning of each cell cycle, cells select a site for bud emergence based on physical cues from previous cell cycles[13,14]. Among the signalling molecules and polarity-establishing factors attracted to this future bud site is a conserved class of proteins called formins[14,15]. Formins function in assembling unbranched actin filaments by holding on to the plus end of an actin filament while catalysing the incorporation of new actin monomers[16–19]. As formins are strategically positioned at the future bud site, they

Box 1 | **Class V myosin-driven transport in *S. cerevisiae***



Most organelles in *Saccharomyces cerevisiae* engage a class V myosin motor to travel to the bud. The class V myosins myosin 2 (Myo2) and myosin 4 (Myo4) adhere to various organelles through specific receptor or adaptor molecules. Whereas Myo2, like all other class V myosins, forms a homodimer, several monomeric Myo4 motors function as a multi-motor complex[81,82].

The nature of the receptor and adaptor molecules on the different myosin cargoes has been elucidated (see the figure). Peroxisomes are anchored at the cell periphery by the peroxisomal membrane proteins inheritance of peroxisomes protein 1 (Inp1) and peroxin 3 (Pex3) (see the figure; 1). Myo2 attaches to peroxisomes through its interaction with Inp2, an integral membrane protein of peroxisomes[32], and carries them to the bud (see the figure; 2). Myo2 associates with the vacuole through its interaction with the receptor vacuole-related protein 17 (Vac17), which docks at the vacuole membrane through its interaction with Vac8, a vacuolar membrane protein (see the figure; 3). The Myo2–Vac17–Vac8 transport complex drives the bud-directed movement of the vacuole segregation structure, a finger-like projection of the vacuolar membrane destined for the daughter cell[30,31]. Growing evidence suggests that mitochondrial Myo2 receptor-related protein 1 (Mmr1) functions as the Myo2 receptor on mitochondria[34,35] (see the figure; 4). Late compartments of the Golgi apparatus are carried by Myo2 to the bud through its interaction with the Rab GTPase Ypt11 (REF. 68) (see the figure; 5). Interestingly, the association of Ypt11 with Golgi membranes is mediated by Ret2, a coat component of COPI vesicles. As COPI vesicles also function in the retrieval of escaped endoplasmic reticulum (ER) components, the Myo2–Ypt11–Ret2 complexes are involved in the bud-directed transport not only of Ret2-containing Golgi membranes but also of COPI recycling vesicles containing ER-resident proteins, thereby probably contributing to the inheritance of both Golgi and ER elements[45]. Myo4 powers the co-migration of cortical ER structures and specific mRNA molecules to the growing bud through the adaptor proteins, She3 and She2 (REFS 26,27,83) (see the figure; 6). How the She3–She2 complex adheres to the ER membrane is not known. Myo2 carries secretory vesicles to sites of growth in the bud through its interaction with the Rab GTPases Ypt31 and Ypt32 (REF. 69) (see the figure; 7). Myo2 also assists in the initial orientation of the nucleus by directing the plus ends of astral microtubules to the bud via the Kar9–Bim1 complex[39,40] (see the figure; 8). By contrast, the nucleus, together with the perinuclear ER, is partitioned to the bud by a microtubule-based mechanism.

GTPase
A regulatory protein that binds and hydrolyses GTP. GTPases act as molecular switches by alternating between active (usually GTP-bound) and inactive (usually GDP-bound) forms.

COPI vesicle
(Coatomer protein complex I vesicle). A membrane-bound vesicle that buds from Golgi compartments and functions as a carrier in both intra-Golgi transport and Golgi-to-ER retrograde transport.

Actin cable
A long bundle of actin filaments in yeast that can span the entire cell.

Post-Golgi secretory vesicle
A secretory, membrane-bound vesicle that buds from late compartments of the Golgi and is transported along cytoskeletal elements to the plasma membrane.

Cell wall
The rigid, outermost layer of plant cells and some yeasts and bacteria. The yeast cell wall consists almost entirely of homopolysaccharides of glucose, mannose, and *N*-acetylglucosamine.

Class V myosin
An actin-based molecular motor specialized in the intracellular transport of various cargoes, including membrane-bound organelles.

assemble an axis cytoskeleton made up of many actin cables that extend into the mother cell and converge at the site of bud emergence. These actin cables guide the delivery of post-Golgi secretory vesicles containing cell wall and plasma membrane components and enzymes for cell wall synthesis to the bud site[13,20]. Controlled dissolution of the yeast cell wall at the selected bud site, coupled to the fusion of secretory vesicles with the plasma membrane in this region, results in localized expansion of the cell surface into a growing bud.

Throughout bud growth, a subset of formins remains stably anchored at the mother cell–bud neck region to facilitate secretory vesicle entry into the bud (BOX 1). By contrast, formins inside the bud change their location according to the stages of the cell cycle, resulting in the targeting of secretory vesicles, and thus growth, to different locations in the bud[21]. Formins initially are localized at the bud tip (apical growth), then uniformly distribute over the entire bud cortex (isotropic growth) and, at the end of the cell cycle, relocate to the mother cell–bud neck for deposition of the septum that separates mother and daughter cells[14].

The same actin cables that underlie polarized secretion form the tracks that organelles use to travel to the growing bud for their inheritance (BOX 1). The bud-directed transport of organelles is mediated by class V myosins, which capture various cargoes in the mother cell and use the actin tracks for their movement towards the formin-rich regions in the bud that are the sites of active growth. The amino termini of class V myosins constitute the 'head', which contains the motor domain and hydrolyses ATP to advance towards the plus end

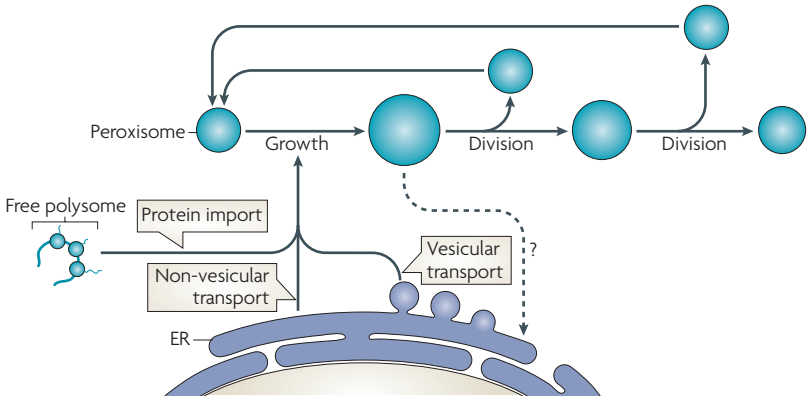


Figure 1 | **The growth and division cycle of peroxisomes.** Peroxisome membrane expansion is achieved mainly by material coming from the endoplasmic reticulum (ER), presumably through both vesicular[1,54,56–59] and non-vesicular pathways[60]. A retrograde pathway, yet to be demonstrated, could retrieve escaped ER proteins and recycle factors involved in the assembly of vesicles destined for the peroxisome[1,56,57,102] (dashed arrow). In contrast, all matrix proteins and some peroxisomal membrane proteins are acquired by the peroxisome by post-translational import from the cytosol after synthesis on free polysomes. The division of mature peroxisomes is needed to maintain peroxisome numbers during cell proliferation[1]. In contrast to the prevailing view that peroxisome division is the simultaneous severing of peroxisomal tubules at multiple regular intervals[1,59,63,85,103], it is more likely that peroxisomes divide asymmetrically, with small daughter peroxisomes pinching off from larger parental ones[32,104,105] (see BOX 2 for further details on peroxisome division).

of actin filaments, and their carboxyl termini form a globular domain called the 'tail', which is specialized in binding cargo through specific adaptor proteins[22–25] (BOX 1). *S. cerevisiae* ensures the delivery of its organelles to daughter cells through an apparently unequal division of labour between its two class V myosins, myosin 4 (Myo4) and myosin 2 (Myo2). Myo4 is involved in the movement of cortical endoplasmic reticulum (ER)[26] and specific mRNA molecules[27], whereas Myo2 powers the bud-directed movement of most membrane-bound organelles, including late Golgi elements[28], a portion of the vacuole[25,29–31], peroxisomes[32,33] and mitochondria[34,35]. Myo2 also drives the transport of post-Golgi secretory vesicles, which underlies polarized cell growth[36–38], and carries the plus ends of cytoplasmic microtubules into the bud for orientation of the nucleus[39,40] (BOX 1).

To achieve faithful organelle inheritance, organelle delivery to the bud must be accompanied by the retention of a subset of organelles in the mother cell. Organelles must also be retained in the bud after transfer to prevent their diffusion back to the mother cell. Interestingly, Myo2 is not released immediately from segregating organelles after reaching its destination, suggesting that Myo2 itself is initially responsible for preventing the diffusion back to the mother cell of newly inherited organelles[28,32,41,42]. Later, organelles are transferred from Myo2 to anchoring devices that are likely to be the same in both the mother cell and bud[43–45].

Studies of peroxisome dynamics have been instrumental to understanding how the processes of organelle transport and organelle retention in both mother and daughter cells are regulated and coordinated to result in a harmonious distribution of organelles at cytokinesis.

Cortical endoplasmic reticulum
Tubular–reticular elements of the yeast ER that line the cell periphery.

Late Golgi element
A Golgi structure (or compartment) that is involved in the final stages of protein sorting.

Vacuole
An essential yeast organelle involved in the detoxification, storage and turnover of proteins.

Cytokinesis
The final stage of the cell cycle, when the cytoplasm is divided. In yeast, cytokinesis leads to the separation of mother and daughter cells.

β-oxidation of fatty acids
The process by which a fatty acid in its acyl-CoA-activated form is broken down to generate multiple molecules of acetyl-CoA, which enter the citric acid cycle. In yeast, fatty acid β-oxidation is restricted to peroxisomes.

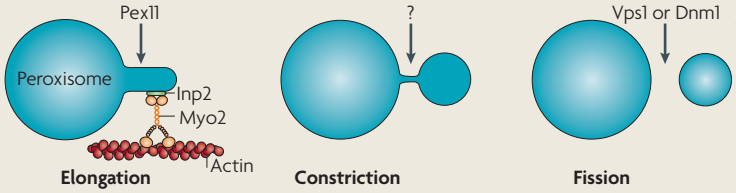
Peroxisome dynamics

Peroxisomes are ubiquitous organelles that contain enzymes responsible for multiple biochemical pathways, notably the β-oxidation of fatty acids and the metabolism of hydrogen peroxide[1,46]. Even though peroxisomes seem to be discrete entities in the cell, multiple lines of evidence suggest that they derive from the ER[47–56]. Many peroxisomal membrane proteins (PMPs) sample the ER membrane *en route* to mature peroxisomes[48–50,52,53,55]. The transfer of proteins and lipids from the ER to peroxisomes is thought to occur both by vesicles that bud from specialized regions of the ER[1,54,56–59] and by a non-vesicular pathway acting principally in the transfer of lipids[60]. However, all matrix proteins, together with some PMPs, are imported into peroxisomes directly after being synthesized in the cytosol (FIG. 1).

Even though peroxisomes can form *de novo* from the ER, this does not occur in yeast unless cells are devoid of peroxisomes; for example, because of a segregation defect occurring during cell division[61]. Rather, the ER-to-peroxisome pathway normally functions to supply existing peroxisomes with membrane components to sustain their multiple rounds of growth and division[1,59,62] (FIG. 1). Peroxisome division therefore needs to be tightly coordinated with the cell cycle to maintain peroxisome numbers in a growing cell population[63] (see BOX 2 for the mechanism of peroxisome division).

Coordination with the cell cycle. With each round of cell division, peroxisomes follow a defined sequence of events that results in their equitable distribution between mother and daughter cells at cytokinesis[32,33,43]. In unbudded cells, peroxisomes are static and scattered over the entire cell cortex. As soon as a bud emerges from the mother cell, peroxisomes start detaching one by one from their cortical positions and travel towards the nascent bud in a Myo2-dependent manner[33]. Recruitment of peroxisomes from the mother cell cortex to the bud continues until the peroxisome populations in the mother cell and bud are approximately equal. Peroxisome division, which is needed to maintain peroxisome numbers on cell division, accompanies the cell cycle-coordinated dynamics of peroxisomes. Small peroxisomes detach from larger, cortically anchored peroxisomes in the mother cell and migrate to the bud. Peroxisome division is not confined to the mother cell cortex, as migrating peroxisomes have been seen to divide both on the way to the bud and inside the bud[32]. When peroxisomes reach the bud, they continue to follow Myo2 to sites of polarized growth, clustering initially at the growing bud tip and later distributing over the entire bud cortex. Only a few peroxisomes in the bud and mother cell are relocated by Myo2 to the mother cell–bud neck region before cytokinesis; the rest remain anchored at the bud and mother cell cortices. These observations point to a tightly regulated interplay between peroxisome retention and motility in controlling the segregation of peroxisomes during cell division[1,6,44].

Box 2 | **Molecular mechanism of peroxisome division**



In all cell types studied to date, the division of peroxisomes was shown to be mediated by dynamin-related proteins (DRPs)[1,59,84,85]. These large GTPases assemble on and deform intracellular membranes to mediate their fission[86]. Vacuolar protein sorting-associated protein 1 (Vps1) and, to a lesser extent, Dnm1 are the DRPs implicated in peroxisome division in *Saccharomyces cerevisiae*[33,71]. Because DRPs, unlike classical dynamins, lack lipid-binding pleckstrin homology domains, they associate with their target membranes through specific receptors[86]. For example, a complex consisting of the tail-anchored protein Fis1 and the peripheral membrane proteins mitochondrial division protein 1 (Mdv1) and Ccr4-associated factor 4 (Caf4) recruits Dnm1 to the peroxisomal membrane[87]. How Vps1 attaches to peroxisomes is unclear. Yeast cells lacking peroxisomal DRPs contain only one or two tubular peroxisomes with a segmented morphology resembling beads on a string[33,61,71]. This observation suggests that other factors acting upstream of DRPs normally prepare the peroxisomal membrane for the DRP-catalysed final scission event by elongating and constricting it. Peroxisome division has therefore been viewed as consisting of three steps: the elongation or tubulation of peroxisomes, the constriction of the peroxisomal membrane and the fission of peroxisomes[1,59,84,85,88] (see the figure). The mechanisms underlying peroxisomal membrane elongation and constriction are poorly understood. Morphological observations, not yet supported by mechanistic evidence, have implicated the peroxisomal membrane protein peroxin 11 (Pex11) in peroxisome elongation. Interestingly, the molecular machinery that propels the bud-directed movement of peroxisomes might also be involved in peroxisome tubulation, as the inability of the myosin 2 (Myo2) motor to associate with the peroxisomal membrane, caused by either a point mutation in Myo2 (REF. 66) or the absence of the Myo2 receptor on the peroxisomal membrane[61], precludes the elongation of peroxisomes in cells lacking peroxisomal DRPs.

Peroxisome retention

A subset of peroxisomes assume static positions at the mother cell periphery throughout bud growth. Interestingly, when the cell cycle of *S. cerevisiae* is artificially prolonged, approximately half of the peroxisomes remain immobilized in the mother cell[43], indicating that the faithful partitioning of peroxisomes between mother cell and bud requires, in addition to a translocation machinery that drives the bud-directed movement of peroxisomes, the presence of anchoring structures that actively retain a specific subset of peroxisomes in the mother cell[43,44]. Although such anchoring structures have long been proposed, their components and the mechanisms involved in immobilizing organelles have remained elusive.

Inp1 attaches peroxisomes to the cell cortex. Inheritance of peroxisomes protein 1 (Inp1) is a peripheral membrane protein of peroxisomes with an essential role in immobilizing peroxisomes at the cell cortex[43]. The main features of cells lacking Inp1 are the presence of a large proportion of mother cells that are devoid of peroxisomes and buds that contain the entire peroxisome population. *In vivo* video microscopy showed that peroxisomes in unbudded cells lacking Inp1 are highly mobile and display chaotic movements instead of being static and cortically localized. During budding, all peroxisomes concentrate at the sites of polarized growth, which results eventually in the complete depletion of peroxisomes from the mother cell, a situation that is never seen in wild-type cells[43]. By contrast, overproduction of Inp1 causes all peroxisomes to maintain fixed cortical positions in the mother cell, thus preventing their normal delivery to the bud[43,44]. Interestingly, whereas Inp1 normally resides exclusively on peroxisomes, overproduced Inp1 decorates both peroxisomes and the cell cortex, suggesting that Inp1 has an intrinsic affinity for a structure that lines the cell periphery. Therefore, Inp1 probably resides on the cytosolic face of the peroxisomal membrane and functions in peroxisome retention by linking peroxisomes to an as yet uncharacterized cortical anchor[6,43,44]. Recently, peroxin 3 (Pex3), a protein regulating the ER-to-peroxisome biogenic pathway, was shown to dock Inp1 at the peroxisomal membrane[64] (BOX 1).

Inp1 probably also mediates the cortical retention of peroxisomes after they are transferred to the bud, as judged by the high frequency of peroxisomes that aberrantly return to mother cells that lack Inp1 (REF. 43). The cortical retention of peroxisomes in the bud is also required to prepare the bud for the next cell cycle, when, as a mother cell, it will have to retain its equal share of the peroxisome population[6,44].

To what cortical structure do peroxisomes adhere? The anchoring device must be an extensive structure as peroxisomes do not display any preference for a specific location at the mother cell cortex but seem instead to be scattered over the entire cell periphery[43,44]. Studies of mitochondrial dynamics suggest a possible role for the cortical ER in anchoring peroxisomes (BOX 3). Another candidate for the peroxisome anchor at the cell cortex is the plasma membrane itself.

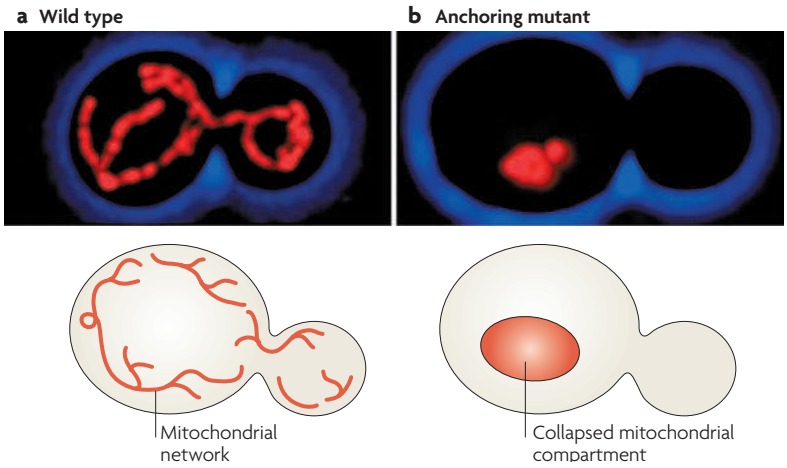
Inp1 also functions in peroxisome division. In addition to having defects in peroxisome inheritance, cells lacking Inp1 have fewer and larger peroxisomes than wild-type cells, pointing to an additional role for Inp1 in peroxisome division. Conceptually, peroxisome anchoring and division can be thought of as inextricably linked processes because the immobilization of organelles might be a prerequisite for their efficient division, especially if the division involves forces that pull on the organelle membrane (see the discussion of Inp2 below, and BOX 2). There are other known instances in which the anchoring of organelles seems to be required for their ability to undergo fission. For example, mutations in maintenance of mitochondrial morphology protein 1 (Mmm1), mitochondrial distribution and morphology protein 10 (Mdm10) and Mdm12, proteins that function primarily in connecting mitochondria to the cortical ER (BOX 3), result in the formation of giant, spherical mitochondria, reflecting their inability to undergo fission. Also, it was recently shown that the detachment of Woronin bodies from their mother peroxisomes in the filamentous fungus *Neurospora crassa* fails in the absence of leashin 1 — a gene that encodes LAH1, which mediates peroxisome adherence to the cell cortex. This suggests that the cortical association of peroxisomes precedes and promotes their division[65].

Pleckstrin homology domain
A sequence ~100 amino acids in length that binds a special class of lipids called phosphoinositides.

Tail-anchored protein
An integral membrane protein that is post-translationally sorted to organelles, is anchored to the phospholipid bilayer by a single stretch of hydrophobic amino acids close to its C termini and has its N termini exposed to the cytosol.

Woronin body
An organelle that is derived from a peroxisome and is found in filamentous fungi only. Woronin bodies occlude the septal pores between cells in response to wounding, thereby restricting the loss of cytoplasm at sites of injury.

Box 3 | **A possible role for the cortical ER in anchoring mitochondria**



The mitochondrial compartment forms a branched tubular network distributed throughout the periphery of a yeast cell[89,90]. During bud growth, the mitochondrial network gradually invades the bud, a process requiring the actin cytoskeleton and myosin 2 (Myo2)[34]. Extension of the mitochondrial network into the bud is balanced by anchoring portions of the mitochondrial tubules at the mother cell cortex, preferentially at the pole opposite the site of bud emergence[91] (see the figure; part **a**). Two integral membrane proteins, maintenance of mitochondrial morphology protein 1 (Mmm1) and mitochondrial distribution and morphology protein 10 (Mdm10), initially thought to reside in the outer mitochondrial membrane[92–96], were shown to be required for the immobilization of mitochondria in the mother cell[91,95]. Mutations in either Mmm1 or Mdm10 led to the collapse of the entire mitochondrial network into a giant sphere that lost its association with the mother cell cortex (see the figure; part **b**). Therefore, Mmm1 and Mdm10 were surmised to function in attaching mitochondrial tubules to a structure lining the cell cortex, presumably actin[91], similarly to how Inp1 functions in attaching peroxisomes to the cell periphery. However, it was recently unequivocally shown that Mmm1 is not mitochondrial but actually an endoplasmic reticulum (ER)-resident membrane protein that interacts in *trans* with the outer mitochondrial membrane protein Mdm10 as part of a larger complex termed the ER–mitochondrion encounter structure (ERMES), which tethers the two organelles[97]. The other two proteins in this complex, Mdm12 and Mdm34, are important mediators of the Mmm1–Mdm10 interaction. Interestingly, on deletion of Mdm12 or Mdm34, mitochondrial tubules coalesce into large spherical structures similar to those seen in cells lacking either Mmm1 or Mdm10 (REFS 92,97,98). Collectively, these findings point to an unexpected function of the cortical ER in anchoring mitochondria to the cell periphery. Further support for a role of the ER in immobilizing mitochondria came from studies showing that Ypt11, a protein involved in the transfer of both ER and late Golgi elements to the bud[45,68,99] (BOX 1), is required for the retention of newly inherited mitochondria in buds[41,100]. This observation suggests that ER elements in the bud can capture mitochondrial tubules and prevent their return to the mother cell[6,45]. It would be interesting to determine whether cortical ER also contributes the docking sites onto which peroxisomes adhere in an Inp1-dependent manner.

Filamentous fungus
A fungus that grows from its tip by the extension of elongated, thread-like structures called hyphae. Hyphae are usually divided into cellular units by incomplete septa that are perforated with pores large enough to allow organelles to pass through.

Irrespective of the influence of peroxisome anchoring on the efficiency of peroxisome division, the interaction of Inp1 with the dynamin-related protein, vacuolar protein sorting-associated protein 1 (Vps1), and the Pex11-like protein Pex25 (REF. 43), supports a direct role for Inp1 in peroxisome division[44]. It will be important to determine whether the two functions of Inp1 (that is, peroxisome immobilization and peroxisome division) are genetically dissectible in an Inp1 molecule[1,44].

Collectively, these findings show that Inp1 is a peroxisomal protein that is involved primarily in mediating the adherence of peroxisomes to cortical structures. However, as all peroxisomes contain Inp1 in roughly similar amounts[43], it was unclear how cells ensure that half of their peroxisomes are delivered to the bud during cell division while the other half remain anchored at the mother cell cortex. An answer to this question came from studies on the regulation of peroxisome motility[66].

Bud-directed motility of peroxisomes

While a subset of peroxisomes remains static at the mother cell cortex throughout bud growth, the remaining peroxisomes detach one by one from their anchoring sites and move rapidly and vectorially to the daughter cell. Peroxisome inheritance can then be viewed essentially as a balance between processes that retain some portion of the peroxisome population in the mother cell while transferring the rest of the population to the daughter cell. Cells must coordinate this organellar tug of war to achieve effective peroxisome inheritance.

Inp2 functions as the peroxisomal receptor for Myo2. As already mentioned, Myo2 is the actin-based motor responsible for moving most organelles, including peroxisomes, to the bud. Given the diversity of Myo2 cargoes and the deleterious effects of competition among them for access to Myo2, Myo2's attachment to and detachment from different organelles must be tightly controlled. Importantly, despite most organelles being carried by the same molecular motor, the times in the cell cycle during which they exhibit actin-based motility, although similar, are not identical[44,67]. Interestingly, the surface of the Myo2 tail was shown to contain distinct, although in some cases overlapping, regions for specific interaction with different organelles[66,67] (FIG. 2). Therefore, each organelle was proposed to have its own Myo2 receptor or adaptor that recruits Myo2 to its membrane. Receptor proteins that physically connect Myo2 to its cargoes have been shown to indeed be different and specific for each type of organelle[30,32,35,39,68,69] (BOX 1). The availability of Myo2 receptors on the surface of organelles probably dictates the timing of Myo2 recruitment to organelles and thereby organelle motility. Thus, to ensure the correct segregation of organelles, cells have to impose a tight temporal regulation on the levels and activities of the various organelle-specific receptors for Myo2 during the cell cycle.

Multiple lines of evidence indicate that Inp2 functions as the peroxisome-specific receptor for Myo2. Inp2 is a peroxisomal membrane protein required for the localization of peroxisomes to buds[32]. In cells lacking Inp2, peroxisome segregation to buds is drastically compromised, but the segregation of other organelles is unimpaired. Inp2 interacts directly with the Myo2 globular tail, and overproduction of Inp2 results in delivery of the entire peroxisome population to the bud[32]. Also, point mutations in the Myo2 tail resulting in a specific defect in interacting with Inp2, disrupt peroxisome distribution to buds[66]. Moreover, a strong correlation has been shown between the strength of the Myo2–Inp2 interaction and the efficiency of peroxisome inheritance[66]. All these observations clearly show that Inp2 is the adaptor molecule that connects Myo2 to the peroxisomal membrane.

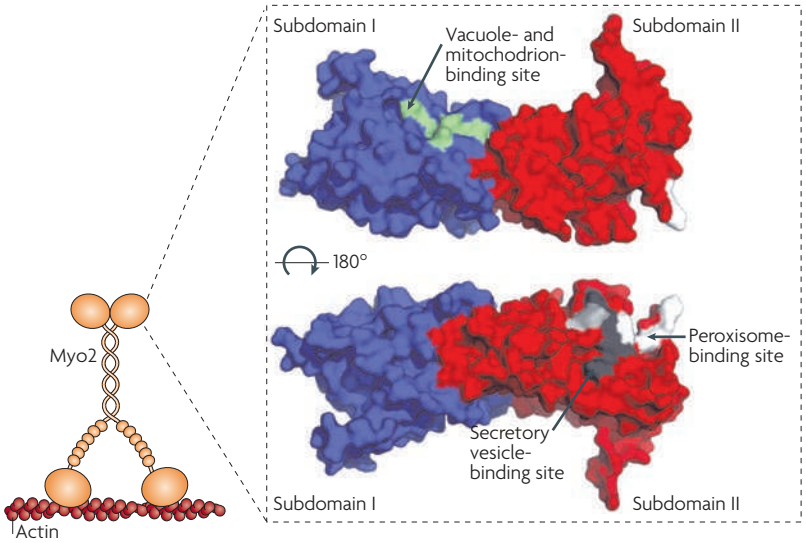


Figure 2 | **Organelle binding to the surface of the Myo2 tail.** The globular tail of myosin 2 (Myo2) is composed of subdomain I (blue) and subdomain II (red). Regions on the surface of the Myo2 tail that bind different organelles are shown. Whereas the vacuole and mitochondrion seem to bind to the same region (green) in subdomain I[25,34,66,67,106], the regions that bind peroxisomes (white) and secretory vesicles (black) are located in subdomain II[66,67] and partially overlap (grey). These regions were identified by assessing the ability of Myo2 with various point mutations to carry the different organelles to the bud. Confirmation that these surface patches on the Myo2 tail represent *bona fide* sites for binding peroxisomes, vacuoles and secretory vesicles has come from studies showing that they are the sites at which the corresponding organelle-specific receptors (that is, inheritance of peroxisomes protein 2 (Inp2), vacuole-related protein 17 (Vac17) and Ypt31 or Ypt32, respectively) adhere to the myosin motor[30,66,69]. Mitochondrial Myo2 receptor-related protein 1 (Mmr1), the Myo2 receptor on mitochondria, has not yet been shown to interact with the putative mitochondrion-binding region on the Myo2 surface. Also, whether the mitochondrion-binding region overlaps exactly with the vacuole-binding site or extends beyond it remains to be established.

Intriguingly, occasional bud-directed movements of peroxisomes have been seen in cells lacking Inp2 (REF. 32). One possible explanation for this is that connections between peroxisomes and organelles such as mitochondria or the ER enable peroxisomes to sometimes co-migrate with these compartments to the growing bud. Interestingly, it has been shown that in the unicellular red alga *Cyanidioschyzon merolae,* peroxisome inheritance is achieved solely through a piggyback mechanism involving their attachment to segregating mitochondria[70].

Inp2 responds to organelle and cell cycle cues. Inp2 levels fluctuate during the cell cycle in a pattern that correlates with peroxisome dynamics, suggesting that oscillations in Inp2 levels result in the assembly and disassembly of Inp2–Myo2 transport complexes at the peroxisomal membrane[1,32,44]. Inp2 levels are low during early budding, when few peroxisomes display bud-directed motility, and peak in medium-sized budded cells, when most directional migrations of peroxisomes to daughter cells occur. Later in the cell cycle, when about half of the peroxisomes have been delivered to the bud, Inp2 levels start to decrease and return to basal values before cytokinesis[32,44].

Interestingly, the amounts of Inp2 on individual peroxisomes vary dramatically, with only a subset of peroxisomes displaying detectable Inp2 levels[32]. A strong correlation exists between the levels of Inp2 on different peroxisomes and their segregation fates as Inp2 is present in detectable amounts only on peroxisomes that are delivered to the bud in a Myo2-dependent manner[44]. The establishment of this Inp2 gradient along the cell division axis offers a possible explanation for the equitable distribution of peroxisomes during normal cell division. When Inp2 is synthesized, it gets loaded onto only a select set of peroxisomes, which in turn results in the preferential recruitment of Myo2 to their membranes[1,32]. Therefore, the Inp2 gradient along the mother cell–bud axis would be generated by both the ability of a subset of peroxisomes to accumulate Inp2 and the selectivity of Myo2 in transporting only these Inp2-containing peroxisomes into the bud. By extracting about half of the initial peroxisomes from the mother cell, Myo2 would help distribute the peroxisome population equitably between mother and daughter cells[44]. At the end of the cell cycle, Inp2, now concentrated in the bud, is degraded so that it can undergo another round of synthesis and degradation in the ensuing cell cycle. However, if different peroxisomes have different affinities for Inp2, a paradoxical implication of this model is that the bud would be enriched for peroxisomes with higher affinities for Inp2. In the next cell cycle, when the bud becomes a mother cell, how would it be able to again send only half of its peroxisomes to its newly forming bud?

An answer to this conundrum came from an analysis of Inp2 dynamics in cells that express forms of Myo2 that are specifically defective in transporting peroxisomes but which retain the ability to carry other organelles[66]. Inp2 accumulated compensatorily on all peroxisomes in the mother cell when peroxisomes failed to be delivered to the bud (FIG. 3a). This indicated that all peroxisomes have the intrinsic ability to recruit Inp2 but this ability is influenced by peroxisome placement in the budded cell. These findings are consistent with a model in which peroxisomes, when delivered to the bud, initiate a negative feedback signal that is relayed to the mother cell to trigger degradation of Inp2 on peroxisomes that have not yet left for the bud (FIG. 3b). The more peroxisomes are sent to the bud, the stronger the signal to degrade Inp2 on peroxisomes in the mother cell and the lower the likelihood that additional peroxisomes are inserted into the bud. Therefore, correct segregation of peroxisomes in wild-type cells is not dependent on an *a priori* selection of peroxisomes that will be sent to the bud. Rather, all peroxisomes have Inp2 and thus a chance to migrate to the bud, but the cell gradually ends peroxisome transfer by degrading Inp2 on mother cell peroxisomes in response to peroxisome insertions into the daughter cell. This mechanism requires that the machinery responsible for Inp2 turnover is not confined to the bud. The degradation machinery remains unknown, but a likely candidate is the ubiquitin–proteasome system. Further evidence that all peroxisomes can acquire Inp2 comes from studies of cells lacking Inp1.

Dynamin
One of a group of large GTPases required for the mechanochemical scission of newly formed vesicles in endocytosis, the division of organelles and the regulation of cytokinesis.

Ubiquitin–proteasome system
Essential intracellular machinery for protein degradation, whereby proteins are tagged by the covalent attachment of multiple ubiquitin monomers and then transferred to a large, cytoplasmic, barrel-like protein complex called the proteasome for degradation.

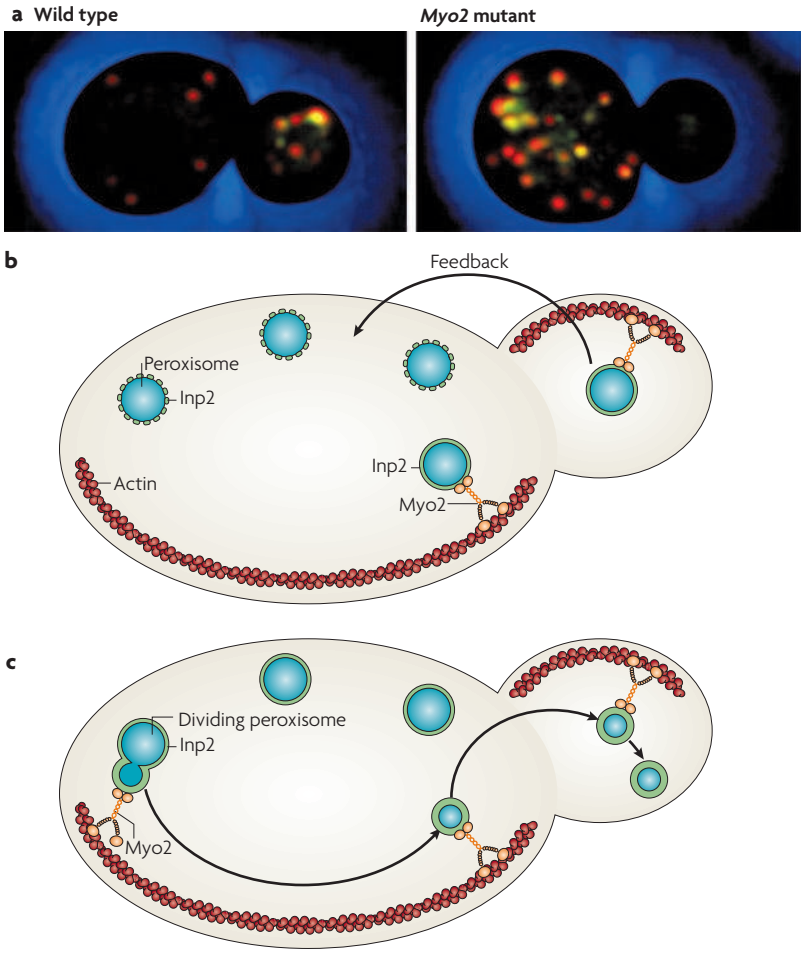


Figure 3 | **Mechanism for Inp2 polarization across the mother cell–bud axis.** **a** | Inheritance of peroxisomes protein 2 (Inp2; green) is polarized on successful peroxisome (red) inheritance. Inp2 decorates only peroxisomes transferred to the bud in wild-type cells[66]. When peroxisome transport is blocked, such as in cells with a myosin 2 (Myo2) mutant unable to bind Inp2, Inp2 is found in detectable amounts on every peroxisome in the mother cell, indicating that Inp2 can be acquired by all peroxisomes[66]. As an Inp2 gradient across the division axis accompanies successful peroxisome inheritance, understanding how the Inp2 gradient is established is key to unraveling the strategies used by cells to partition their peroxisomes equitably. **b** | Two mechanisms are proposed to act together to account for the polarization of Inp2. First, peroxisomes with greater amounts of Inp2 recruit sufficient Myo2 molecules to dislodge them from their fixed cortical positions and carry them to the bud. The presence of peroxisomes in the bud initiates a feedback signal that leads to Inp2 degradation on peroxisomes in the mother cell. **c** | Second, the involvement of Myo2 in the process of peroxisome division allows Myo2 to recruit Inp2-enriched peroxisomes produced by peroxisome division and carry them to the bud. An uneven segregation of Inp2 molecules on peroxisome division would be facilitated if Inp2 molecules were already polarized in the peroxisomal membrane before the recruitment of Myo2. For example, Inp2 might be excluded from the part of the peroxisomal membrane engaged in peroxisome–cortical connections and thus found preferentially on the portion of the peroxisome facing the cytosol. This second mechanism for Inp2 polarization provides an explanation for how the processes of peroxisome inheritance and peroxisome division are coordinated. Both mechanisms can account for the observation that a Myo2 mutant unable to bind Inp2 leads to a depolarization in the localization of Inp2; that is, Inp2 decorates all peroxisomes in the mother cell. The image in part **a** is reproduced, with permission, from REF. 66 © 2009 The Rockefeller University Press.

Peroxisomes in these cells have lost their affinity for the cell cortex and the entire peroxisome population is eventually transferred to the daughter cells, presumably in an Inp2- and Myo2-dependent manner.

At the end of the cell cycle, Inp2 is degraded irrespectively of the outcome of peroxisome inheritance as Inp2 turnover occurs in both wild-type cells and cells with forms of Myo2 specifically defective in transporting peroxisomes[32,66]. Therefore, the degradation of Inp2 later in the cell cycle seems to respond to cell cycle cues rather than to peroxisome positioning cues. In wild-type cells, degradation of Inp2 late in the cell cycle allows peroxisomes in the bud to be released from the grip of Myo2 and captured by cortical structures in an Inp1-dependent manner. The transfer of peroxisomes from their translocation machinery to their cortical anchoring devices in the bud, the opposite process to the dislodgement of peroxisomes from the mother cell cortex, completes the cycle of peroxisome inheritance.

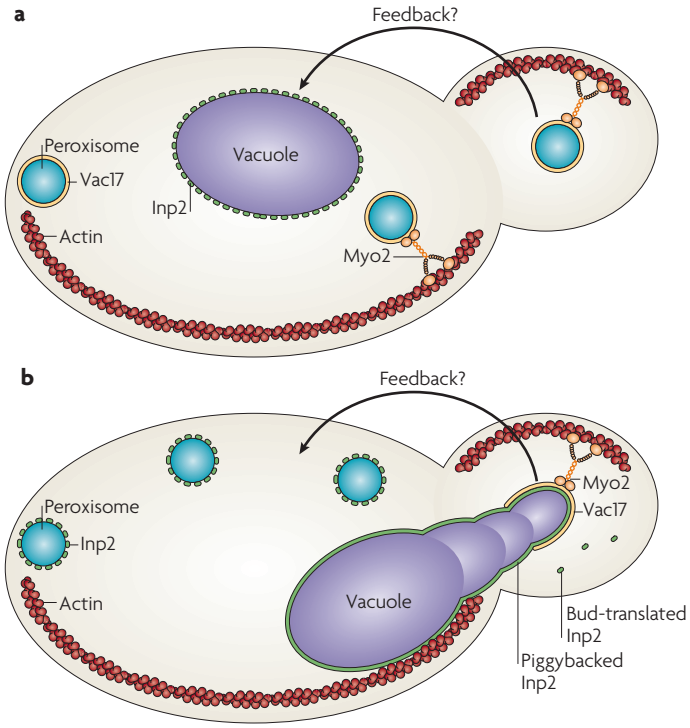
The mechanism of peroxisome division may also offer some insight into how an Inp2 gradient across the mother cell–bud axis is achieved in wild-type cells. During the constitutive division of peroxisomes, a process required to maintain peroxisome number during cell proliferation, new peroxisomes arise from older, parental peroxisomes (BOX 2). Dynamin-related proteins Vps1 and Dnm1 execute the final scission step in this process, allowing detachment of daughter peroxisomes from parental ones[33,71]. As a result, cells lacking both Vps1 and Dnm1 usually contain only one giant peroxisome. However, the single peroxisome present in cells lacking Vps1 and Dnm1 is stretched into a tubule that bridges the mother and daughter cells and divides with each cell cycle. Interestingly, it was recently shown that peroxisomes failed to divide altogether in cells that lack Vps1, Dnm1 and Inp2 (REF. 61). This finding implicated Inp2, and consequently Myo2, in the apparently dynamin-independent fission of peroxisomes seen in cells lacking Vps1 and Dnm1. It is likely that pulling forces exerted by the Myo2 motor at the peroxisomal membrane cooperate with retention mechanisms that anchor the single enlarged peroxisome in the mother cell to stretch the peroxisomal membrane, eventually tearing it apart[1]. This might explain in part the contribution of Inp1 to the process of peroxisome division (see above). It will be important to establish whether Myo2 and Inp2 also play a similar part in wild-type cells (BOX 2). If this is indeed the case, Myo2 could be envisaged to promote the sequestration of Inp2 into bud-destined, peroxisomal vesicles that would pinch off from cortically anchored parental peroxisomes and then migrate to the bud (FIG. 3c). In support of this idea, peroxisomal vesicles have been seen to detach from cortical peroxisomes and then rapidly migrate to the bud (see above). However, it is not known whether these daughter peroxisomes are actually enriched for Inp2. Most peroxisomes that travel to the bud are dislodged completely from the mother cell cortex, so the relative contribution of Inp2 polarization during peroxisome division to Inp2 polarization in cells remains to be established. Immunoelectron microscopy studies would best assess whether Inp2 is indeed asymmetrically distributed in the membrane of dividing peroxisomes.

Dynamics of other Myo2 receptors. Interestingly, the dynamics of other Myo2 receptors in *S. cerevisiae* is strikingly similar to the dynamics of Inp2. For example, vacuole-related protein 17 (Vac17) and mitochondrial Myo2 receptor-related protein 1 (Mmr1), the Myo2 receptors on the vacuole and mitochondrion, respectively (BOX 1), are normally found exclusively on the portions of organelles that enter the bud[31,35]. Also, their levels are increased when vacuolar and mitochondrial inheritance is compromised[31,35]. This was interpreted as showing that Myo2 receptor degradation is confined to the bud[31]. However, Vac17 and Mmr1 were also found to cover the entire surface of the vacuole and mitochondrion when their inheritance was disrupted[35,72], akin to the situation in which Inp2 decorates all peroxisomes when peroxisome inheritance is disrupted[66]. Taken together, these observations fit better with a model in which Myo2 receptors are initially protected in the bud and degraded in the mother cell on organelle inheritance. It is therefore likely that feedback signals relayed from the bud to the mother cell, similar to the one regulating Inp2 levels, operate to control the levels of Myo2 receptors on other organelles (BOX 4).

Kar9 is the Myo2 receptor found on astral microtubules and is involved in orienting the mitotic spindle (BOX 1). Myo2 carries the plus ends of astral microtubules emanating from the older spindle pole body (that is, the spindle pole body inherited from the previous cell division), thus aligning the mitotic spindle with the cell division axis. Kar9 is polarized in its distribution, being associated exclusively with the microtubule ends that were carried by Myo2 to the bud. It was proposed that Kar9 is normally loaded onto the older spindle pole body only, from where it is transferred to the pole-associated astral microtubules to later recruit Myo2 for bud-directed transport[73]. However, Kar9 has been seen to associate initially with both spindle poles and then later with the bud-directed pole only[74]. Interestingly, recent evidence has shown that the asymmetry of Kar9 localization is disrupted on inefficient transfer of microtubule ends into the bud. In cells harbouring Myo2 mutants that are unable to transport astral microtubule plus ends to the bud, Kar9 decorates microtubules arising from both spindle pole bodies[75]. A feedback mechanism could account for the normal asymmetric distribution of Kar9 to ensure that ultimately one spindle pole only is transmitted to the bud for proper alignment of the mitotic spindle with the cell division axis[75]. Similarly, surveillance mechanisms that monitor and influence the inheritance of ER elements according to a cell's needs have been recently reported[76].

Pex3 proteins are implicated in peroxisome motility. Pex3B, one of two Pex3 protein family members in the yeast *Yarrowia lipolytica*, was recently shown to promote peroxisome motility through its interaction with the myosin V motor that carries peroxisomes to the bud[77]. As deletion of Pex3 genes had previously always led to the absence of peroxisomes in cells, the presence of two Pex3 proteins in *Y. lipolytica* offered an 'evolutionary' window of opportunity to observe a function for Pex3 proteins in peroxisome motility[77]. Because Pex3 proteins are highly conserved across organisms, these findings suggested a potential role for Pex3 in peroxisome motility in all organisms, in addition to their well-established role in peroxisome biogenesis at the ER. It would be interesting to test whether *S. cerevisiae* Pex3 collaborates with Inp2 in attaching peroxisomes to Myo2. Given its role in recruiting Inp1

Box 4 | **Defining the feedback mechanisms regulating Myo2 receptor levels**



The feedback from bud to mother cell that adjusts the levels of myosin 2 (Myo2) receptor in each organelle could be triggered by the arrival of components of the organelle membrane or the Myo2 receptors themselves in the bud.

To test the first possibility, one can construct cells in which peroxisomes, the vacuole or mitochondria are delivered to the bud without their normal Myo2 receptors (see the figure, part **a**). One way to do this is to swap Myo2 receptors between organelles. For example, vacuole-related protein 17 (Vac17) can be relocated to peroxisomes and inheritance of peroxisomes protein 2 (Inp2) to the vacuole. These cells should aberrantly deliver vacuoles by Inp2 and peroxisomes by Vac17 to the bud. By replacing endogenous Myo2 with a mutant Myo2 that cannot bind Inp2, vacuoles will be retained in the mother cell, whereas peroxisomes, now denuded of Inp2, will reach the bud by Myo2–Vac17 interactions. If peroxisome delivery to the bud results in decreased levels of Inp2 on vacuoles in the mother cell, then a peroxisomal component other than Inp2 initiates the feedback that results in Inp2 degradation in the mother cell.

To test directly the second possibility that the presence of Myo2 receptors in the bud is sufficient to initiate their degradation in the mother cell, buds have to be populated with receptors in the absence of their corresponding organelles (see the figure, part **b**). For Inp2, one can use the above described Myo2 mutant cells that do not inherit peroxisomes and therefore accumulate Inp2–green fluorescent protein (GFP) on peroxisomes in the mother cell[66] and express additional Inp2 molecules that target to the bud independently of peroxisomes. Inp2 can be targeted independently to the bud by piggybacking it on the vacuole or mitochondria, as described above, or by expressing a chimeric transcript consisting of the *INP2* open reading frame and the 3′ untranslated region of *ASH1* mRNA, which should be translated exclusively in the bud[101]. The level of Inp2–GFP, which is restricted to peroxisomes in the mother cell of these Myo2 mutants, would show whether the non-peroxisomal Inp2 molecules targeted to the bud can initiate feedback leading to Inp2 degradation in the mother cell. Similar experiments with the vacuolar and mitochondrial Myo2 receptors would test the generality of the feedback process from bud to mother cell in adjusting Myo2 receptor levels.

to the peroxisomal membrane[64], *S. cerevisiae* Pex3 would be ideally positioned to act as a molecular switch between peroxisome anchoring and motility.

Post-translational regulation

How do the antagonistic factors Inp1 and Inp2 wage their tug of war to retain some peroxisomes and reposition others so that the entire peroxisome population is distributed evenly between mother and daughter cells in successive rounds of cell division? As discussed above, cells have evolved compensatory mechanisms that can adjust the level and distribution of Inp2 according to peroxisome placement in the dividing cell[66]. In addition, however, post-translational modification probably fine-tunes the activities of these factors and ensures tight coordination of peroxisome inheritance with the cell cycle.

It is unknown whether the cortical anchoring of peroxisomes or other cellular organelles is controlled directly by cell signalling events, but several lines of evidence indirectly support this hypothesis. First, as peroxisomes are scattered throughout the cell periphery rather than being localized to distinct sites in interphase cells, the hypothetical peroxisome anchor seems to be an abundant cortical structure. Furthermore, even though Inp1 levels oscillate slightly with the cell cycle, a considerable amount of Inp1 is present throughout the cycle, decorating all peroxisomes[43]. As the ability of peroxisomes to attach to the cell cortex does not seem to be limited by the availability of either the anchor or its peroxisomal receptor, Inp1, transient modification of the anchor and/or Inp1 may account for the controlled release of peroxisomes from the mother cell cortex and reattachment of transferred peroxisomes to the bud cell cortex.

Such an additional layer of regulation by post-translational modification has been shown for Inp2 (REF. 66). Inp2 is synthesized and degraded with each cell cycle. Additionally, it is reversibly phosphorylated at the beginning and end of the cell cycle. Notably, although position-dependent cues resulting from peroxisome placement in the cell affect total Inp2 levels, they do not influence the timing of Inp2 phosphorylation, which is thus coupled to progression of the cell cycle[66]. However, the relative amounts of the different phosphorylated forms of Inp2 differ in wild-type cells and cells defective in peroxisome delivery to buds, suggesting that some phosphorylation events may occur preferentially in the mother cell and others in the bud. Vac17 was also shown to fluctuate in total abundance and phosphorylation with the cell cycle[31,72], although with slightly different dynamics compared to Inp2. As different organelles display Myo2-dependent motility at different times of the cell cycle[78], we may anticipate temporally distinct activation and inactivation patterns for individual organelle receptors. Interestingly, Vac17 has been shown to be phosphorylated by the cyclin-dependent kinase Cdk1 (also known as Cdc28), which leads to an increased affinity of Vac17 for Myo2 and thus seems to control the timing of vacuole movement[72]. Moreover, the p21-activated kinases (PAKs) Ste20 and Cla4 were shown to be required for Vac17 degradation and termination of vacuole inheritance[79]. PAKs might phosphorylate Vac17 to prime it for destruction in the bud[79], but it remains unknown whether phosphorylation of Vac17 is required for its degradation or whether Vac17 is a direct target of PAKs. Whether the two observed phosphorylation events of Inp2 prepare it for engagement by the myosin motor and subsequent degradation is also currently unknown. However, the presence of several Cdk1 phosphorylation sites in the primary sequence of Inp2 does suggest that common regulators coordinate organelle motility with stages of the cell cycle.

Concluding remarks

During each cell cycle, cells have to control tightly the placement and thus the inheritance of all their different organelles. This task is made more difficult because cells have a limited arsenal of cytoskeletal elements at their disposal to mediate organelle transport. With the notable exception of the nucleus, all yeast organelles have to share the same actin cables and myosin motors to travel to the bud. However, the factors used by organelles to hold on to the myosin motors are different for each type of organelle. By using organelle-specific factors as regulatory targets for organelle motility, cells are able to differentially control the delivery of each organelle to the growing bud.

The dynamics and regulation of the different organellar myosin receptors seem to obey common principles. For example, organelle-specific factors display cell cycle-coordinated synthesis and turnover, which mark the start and end of organelle motility, respectively. Growing evidence indicates that between these two events, cells monitor the extent of organelle transfer to the daughter cell and influence the levels of each organelle receptor for molecular motors accordingly. Also, an additional level of regulation of the activity of the various myosin receptors on different organelles is provided by the regulated phosphorylation and dephosphorylation of the receptors. Retention of a subset of organelles in the mother cell and capture of the delivered organelles in the bud, both of which are important to achieve correct organelle inheritance, are also mediated by organelle-specific factors. This again allows regulatory pathways controlling organelle placement to act discriminately on different organelle types.

Although we have advanced greatly our understanding of organelle inheritance, some aspects of the process, especially how it relates to overall cell physiology, remain less clear. For example, it remains to be determined how organelle inheritance is temporally coordinated with the processes of organelle growth and division. Also, the connections between organelle inheritance and events of the cell cycle, although beginning to come to light, are far from clear. Interestingly, a cell cycle checkpoint that delays cytokinesis in response to defects in mitochondrial inheritance in budding yeast has recently been reported[80]. Given that common principles govern the inheritance of all organelles, similar checkpoints may function in the partitioning of other cytoplasmic organelles. Unravelling the mechanisms of organelle inheritance will therefore surely have profound implications for understanding the eukaryotic cell cycle in general.

ASH1
(Asymmetric synthesis of HO). A gene encoding a repressor that inhibits the transcription of homothallic switching endonuclease (HO) —an endonuclease that causes mating-type switching in *S. cerevisiae*. *ASH1* mRNA is transported before translation to the bud, where Ash1 prevents the daughter cell from switching its mating type on cell division.

Astral microtubule
(Also called cytoplasmic microtubule). A microtubule that radiates outwards from a centrosome (or spindle body in yeast). Astral microtubules are important for positioning the mitotic spindle during cell division.

Spindle pole body
A multilayered, cylindrical structure embedded in the nuclear envelope that functions as the microtubule-organizing centre in yeast in a manner similar to centrosomes in higher eukaryotes.

Cyclin-dependent kinase
One of a group of Ser/Thr kinases involved in regulating the cell cycle. They are activated by association with a class of proteins called cyclins, the concentration of which varies in a cyclical manner during the cell cycle.

p21-activated kinase
One of a group of evolutionarily conserved Ser/Thr kinases involved in the regulation of actin cytoskeleton dynamics.

Cell cycle checkpoint
A control mechanism that prevents a cell from progressing to the next phase of the cell cycle before the preceding phase has been accurately completed.

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Acknowledgements
A.F. is the recipient of a Ralph Steinhauer Award of Distinction from the Government of Alberta. F.D.M. is a Vanier Scholar of the Canadian Institutes of Health Research and the recipient of a Studentship from the Alberta Heritage Foundation for Medical Research. R.A.R. is an International Research Scholar of the Howard Hughes Medical Institute. Research in the Rachubinski laboratory is supported by grants 9208, 15131 and 53326 from the Canadian Institutes of Health Research. The authors thank R. Edwards from the Department of Biochemistry, University of Alberta, for help in rendering the Myo2 structure in figure 2.

Competing interests statement
The authors declare no competing financial interests.

DATABASES
UniProtKB: http://www.uniprot.org
Dnm1 | Inp1 | Inp2 | Kar9 | Mdm10 | Mdm12 | Mmm1 | Mmr1 | Myo2 | Myo4 | Pex3 | Vac17 | Vps1

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Mitochondrial protein import: from proteomics to functional mechanisms

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Abstract | Mitochondria contain ~1,000 different proteins, most of which are imported from the cytosol. Two import pathways that direct proteins into the mitochondrial inner membrane and matrix have been known for many years. The identification of numerous new transport components in recent proteomic studies has led to novel mechanistic insight into these pathways and the discovery of new import pathways into the outer membrane and intermembrane space. Protein translocases do not function as independent units but are integrated into dynamic networks and are connected to machineries that function in bioenergetics, mitochondrial morphology and coupling to the endoplasmic reticulum.

α-proteobacterium
A Gram-negative (outer membrane-containing) bacterium, such as *Rickettsia* spp., that is probably the closest living bacterial relative of mitochondria.

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doi:10.1038/nrm2959

According to the endosymbiont hypothesis, mitochondria are derived from an ancient α-proteobacterium that was taken up by a primordial eukaryotic cell ~1.5–2 billion years ago[1]. Mitochondria have a central role in energy conversion and are thus termed the powerhouses of eukaryotic cells. The complexes of the respiratory chain in the mitochondrial inner membrane use energy, which is gained from the oxidation of food molecules, to pump protons across the membrane and generate a membrane potential ($\Delta\psi$). The proton gradient is then used to drive the mitochondrial ATP synthase that produces the bulk of ATP for the cell. Additionally, however, eukaryotic cells have intimately integrated mitochondria into multiple metabolic and signalling pathways[2–4]. Mitochondria have crucial roles in the metabolism of amino acids and lipids and the biosynthesis of haem and iron–sulphur clusters. Studies in recent years identified an unexpectedly large number of signalling molecules located in or at mitochondria and revealed a central function of mitochondria in pathways leading to programmed cell death.

Like Gram-negative bacteria, mitochondria contain two membranes (an outer membrane and an inner membrane) and two aqueous spaces (the intermembrane space and the matrix) (FIG. 1). Mitochondria have retained a complete genetic system in the matrix; however, only ~1% of mitochondrial proteins are encoded by the mitochondrial genome and synthesized in the matrix. Mitochondrion-encoded proteins form a few subunits of the respiratory chain complexes and are typically inserted into the inner membrane by an export and assembly machinery, called the oxidase assembly (OXA) machinery[1,5].

99% of mitochondrial proteins are encoded by nuclear genes. These include genes that were transferred from the endosymbiont to the nucleus and genes for new mitochondrial proteins that arose during eukaryotic evolution. In present-day organisms, the nuclear genome codes for all mitochondrial outer membrane and intermembrane space proteins, as well as for most inner membrane and matrix proteins[1,2,5].

Nucleus-encoded mitochondrial proteins are synthesized as precursor proteins on cytosolic ribosomes and imported into the organelle[1,5]. Until 2003, only two main protein import pathways into the inner membrane and matrix were known. Since then, the studies on mitochondrial protein biogenesis have received a major boost with the identification of numerous new import components and import pathways to the intermembrane space and outer membrane. The rapid development of the mitochondrial protein import field is an excellent example of how a systematic proteomic analysis can provide novel insight into a complex cellular process when combined with genetics and functional biochemical studies. In this Review, we first give an overview of the protein import pathways and summarize which approaches led to the discovery of new mitochondrial import components and entire import pathways. Then we discuss the functional implications for the mechanisms of protein sorting and the integration of protein translocases into a dynamic network of interactions.

Overview of mitochondrial protein import

Nucleus-encoded mitochondrial precursor proteins possess targeting signals that are recognized by receptors on the mitochondrial surface. The targeting signals then direct the precursors to their functional destination in the mitochondrial subcompartments. Two main groups of targeting signals can be distinguished[1,5]. The first group is the amino-terminal extensions of precursors,

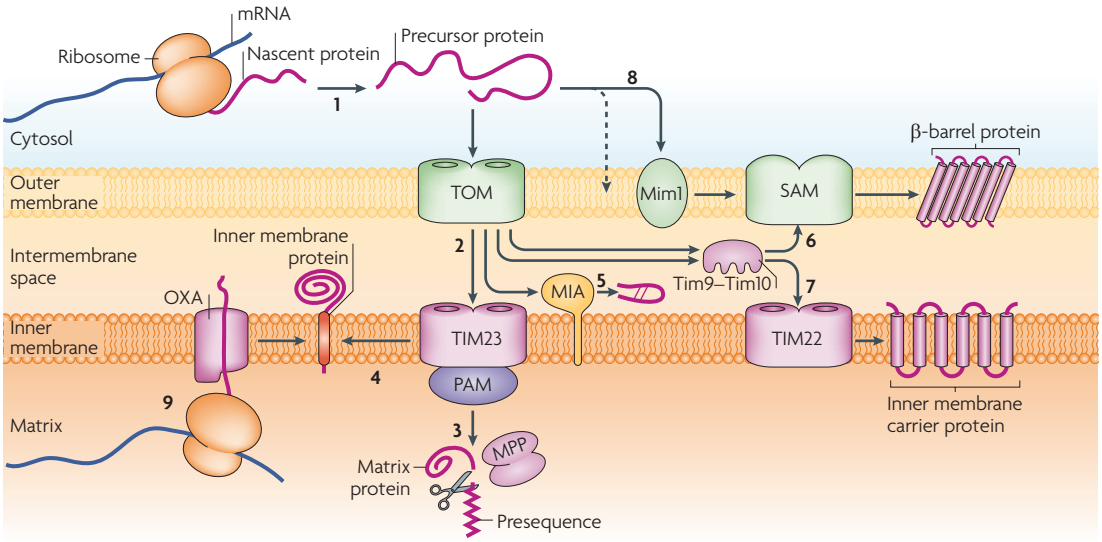


Figure 1 | **Biogenesis pathways of mitochondrial proteins.** Most mitochondrial proteins are synthesized on cytosolic ribosomes and imported through the translocase of the outer mitochondrial membrane (TOM) complex (1). After passage through the TOM channel, the precursor proteins can use different sorting machineries (2). Presequence-carrying proteins destined for the matrix are imported by the presequence translocase of the inner membrane (TIM23) complex and presequence translocase-associated motor (PAM). Mitochondrial processing peptidase (MPP) removes the presequences (3). Some proteins are laterally released from the TIM23 complex into the lipid phase of the inner membrane (4). The mitochondrial intermembrane space assembly (MIA) machinery drives the import and oxidative folding of many intermembrane space proteins (5). The Tim9–Tim10 chaperone complex transfers hydrophobic precursor proteins through the intermembrane space to either the sorting and assembly machinery (SAM) complex, in the case of β-barrel proteins of the outer membrane (6), or through the carrier pathway to the TIM22 complex (7). The precursors of α-helical outer membrane proteins do not use the TOM channel but are inserted into the membrane by different pathways, some involving mitochondrial import 1 (Mim1; 8). A few proteins are synthesized on matrix ribosomes and are exported into the inner membrane by the oxidase assembly (OXA) machinery (9).

which are the classical mitochondrion-targeting signals. These presequences are usually proteolytically removed after import into mitochondria. Second, many precursor proteins are not synthesized with cleavable extensions but contain internal targeting signals that remain part of the mature protein. This second group includes different types of precursor proteins and targeting signals.

Currently, at least five main classes of precursor proteins are known, each class following a different import route into mitochondria (FIG. 1). The common entry gate for most precursors is formed by the translocase of the outer membrane (TOM) complex[1,5–8], but after passing through the TOM complex, the precursors use different pathways to the mitochondrial subcompartments. The four main pathways are described below, but the insertion of α-helical proteins into the outer membrane probably involves several other pathways that have only been partly characterized.

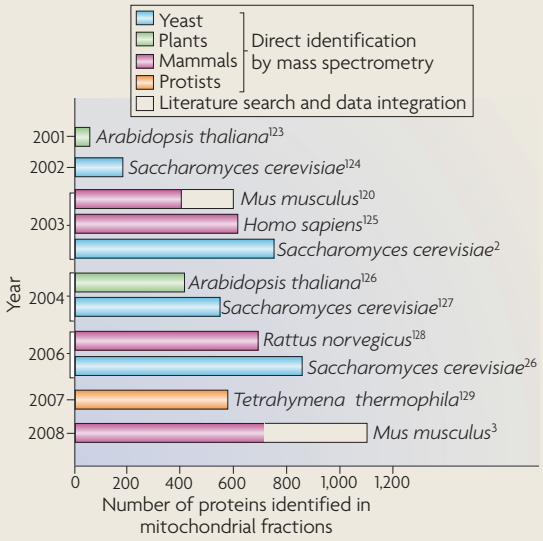
Presequence pathway to the matrix and inner membrane. Cleavable precursors (also termed preproteins) are transferred from the TOM complex to the presequence translocase of the inner membrane (TIM23) complex[1,5,7]. From here they are either laterally released into the inner membrane or are completely imported into the matrix with the help of presequence translocase-associated motor (PAM). Most matrix proteins, including many metabolic enzymes, are synthesized with cleavable presequences.

Carrier pathway to the inner membrane. Many inner membrane proteins that contain multiple transmembrane segments are synthesized with internal targeting signals. The main group is formed by metabolite carriers such as the ADP and ATP carrier. These hydrophobic proteins are imported through the TOM complex, chaperone complexes of the intermembrane space and the carrier translocase, the TIM22 complex[9–11].

Oxidative folding pathway of the intermembrane space. Many proteins of the intermembrane space contain internal targeting signals and characteristic Cys motifs. The precursors are imported by the TOM complex and the mitochondrial intermembrane space assembly (MIA) machinery that oxidizes the Cys residues[12–14].

Transport pathways of outer membrane proteins. All proteins of the outer mitochondrial membrane are synthesized as non-cleavable precursors carrying internal targeting signals. The outer membrane possesses two types of membrane-integrated proteins: α-helical proteins (for example receptors), which are anchored in the outer membrane by one or more transmembrane α-helical segments, and pore-forming β-barrel proteins that consist of multiple transmembrane β-strands. β-barrel membrane proteins have been found only in the outer membranes of bacteria, mitochondria and chloroplasts; all other membranes, including the mitochondrial inner membrane,

Box 1 | **New import components and the mitochondrial proteome**



Three lines of research were mainly responsible for the identification of new components of the mitochondrial protein import machinery. First, translocase complexes were isolated under mild conditions such that loosely associated subunits remained associated. Sensitive mass spectrometry techniques together with the availability of completely sequenced genomes then led to the direct identification of new subunits, in particular of the presequence translocase of the inner membrane complex, TIM23, and presequence translocase-associated motor (PAM)[19–21]. Second, native gel techniques such as blue native electrophoresis[22] are powerful tools for the efficient separation of intact membrane protein complexes. Import intermediates of precursor proteins could thereby be visualized, leading to the identification of the β-barrel pathway[15] and the dynamic composition of the TIM23 complex[23–25]. Third, the large-scale identification of the mitochondrial proteome yielded many proteins of unknown function that were analysed by a combination of yeast genetics and functional biochemical assays. Purified mitochondria proved to be an excellent substrate for a systematic mass spectrometry analysis. Within a few years, most *Saccharomyces cerevisiae* and several mammalian mitochondrial proteins were identified. Although previous studies had the problem that proteins of low abundance and membrane proteins were underrepresented, a systematic analysis of the most comprehensive proteome of *S. cerevisiae* mitochondria with ~850 identified proteins revealed that the use of different separation techniques (such as chromatographic steps, and one- and two-dimensional gels) and the high sensitivity of mass spectrometry overcame the bias against those proteins, and all classes of proteins analysed were successfully identified[26]. In addition, some proteomic studies included detailed literature searches and integration of data from different sources to maximize the number of identified proteins[3,120] (see the figure).

The next level of proteomic analysis of mitochondria includes several approaches, such as the analysis of mitochondrial subcompartments (such as the outer membrane proteome)[109,121], the determination of protein modifications (for example, the mitochondrial phosphoproteome)[118,119] and the identification of the amino termini of proteins[28,122], and thus a large-scale determination of presequences (the mitochondrial N-proteome)[28].

contain α-helical membrane proteins only. The mitochondrial import pathway for β-barrel proteins has been identified and involves the TOM complex, intermembrane space chaperones and the sorting and assembly machinery (SAM) complex of the outer membrane[15–18]. The β-barrel pathway thus constitutes the fourth protein import pathway into mitochondria.

Functional proteomics of mitochondria

How were so many new mitochondrial import components identified in recent years? A combination of several approaches, including sensitive mass spectrometry, yeast genetics, isolation of translocase complexes under mild conditions and analysis of import intermediates by native gel systems, have yielded most identifications[19–26]. Mitochondria are the first cell organelle for which a comprehensive proteomic analysis has been achieved[27] (BOX 1). Currently, ~85% of the ~1,000 yeast mitochondrial proteins[26] and 70–75% of the ~1,500 mammalian mitochondrial proteins have been identified[3]. The functional classification of the mitochondrial proteome and the remarkable variety of functions carried out by mitochondria are discussed in BOX 2.

The mitochondrial proteome includes several proteins of unknown function. How is it possible to find potential new components of the protein import machinery among them? The genetic possibilities of yeast proved to be of great value. After sequencing the genome of *Saccharomyces cerevisiae* as the first eukaryotic genome, a joint effort of yeast laboratories yielded a large collection of gene deletion mutants with phenotypic analyses. Organisms like yeast that are capable of fermentative growth can live without a respiratory chain, but the mitochondrial protein import machinery is essential for cell viability[4,5]. Thus, a particular focus was put on the mitochondrial proteins of unknown function that are encoded by genes essential for life. Conditional mutants of the candidates were analysed for protein import into mitochondria and several new import components were identified, including SAM and PAM subunits[16–21]. A major finding of these functional proteomic analyses was the identification of the MIA pathway of the intermembrane space[12–14].

Presequence pathway and processing

The presequence pathway is responsible for the import of nearly all mitochondrial matrix proteins and a considerable fraction of inner membrane proteins. The presequences are located at the N termini of preproteins and typically consist of ~15–50 amino acids (FIG. 2a). Presequences are positively charged and form amphipathic α-helices that are recognized by TOM and TIM23 complexes in a sequential manner[1,5–7]. Thus, presequences direct the preproteins to mitochondria and across outer and inner membranes into the matrix. Preproteins destined for the inner membrane

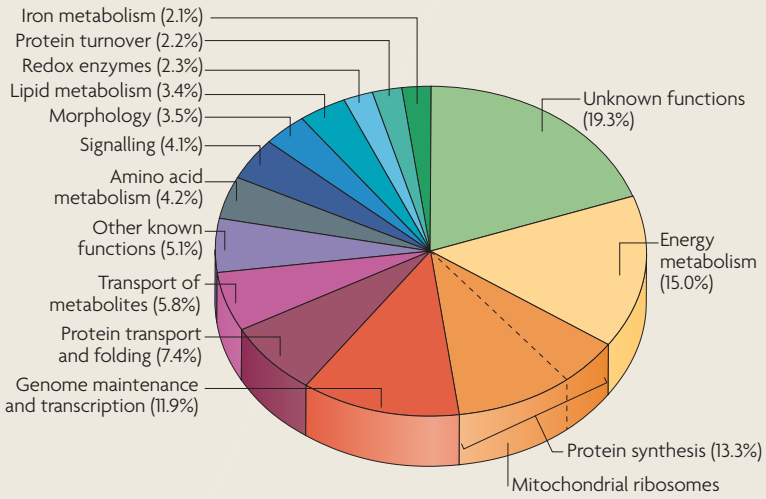
Box 2 | Functional classification of the mitochondrial proteome

The presumed functions of the ~850 proteins of the yeast mitochondrial proteome[26] are assigned according to the *Saccharomyces* genome database (SGD; January 2010)[130] (see the figure, part **a**). Remarkably, the classical textbook function of mitochondria in energy metabolism, including the respiratory chain complexes, mitochondrial ATP synthase and citric acid cycle (Krebs cycle), is carried out by only ~15% of the different proteins. Mitochondria carry out various functions, from many metabolic processes to protein transport and turnover, maintenance of membrane morphology, signalling and redox processes. For more than 160 proteins (19%), no reliable information on their function is available.

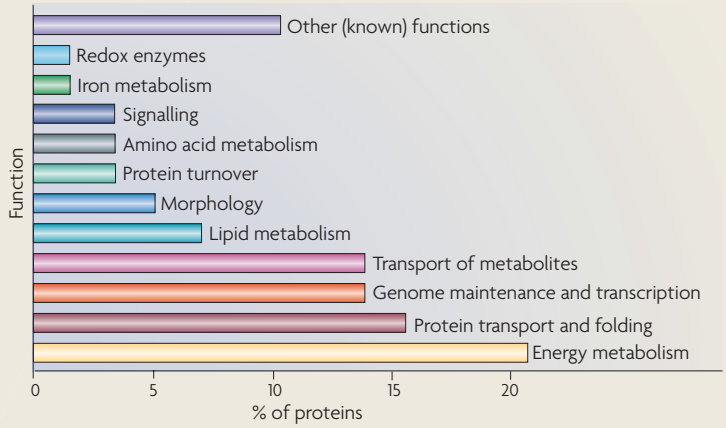
A comparison of the functional classification of ~750 mitochondrial proteins in 2003 (REF. 2) to the ~850 proteins classified in 2010 shows the areas of active research on new mitochondrial proteins. From 2003–2010, functions could be assigned to ~60 mitochondrial proteins (see the figure, part **b**). Areas of particularly high activity were energy metabolism, protein transport and folding, genome maintenance and transport of metabolites.

Because of the rapid identification of new mitochondrial import components in several laboratories, some of the components were given more than one name. Examples are topogenesis of outer membrane β-barrel proteins (TOBs) for the sorting and assembly machinery (SAM) complex components and translocase of the inner membrane 14 (Tim14)–Tim16 for the presequence translocase-associated motor 18 (Pam18)–Pam16 module of the import motor PAM. The components were typically identified in *Saccharomyces cerevisiae*, and the SGD[130] provides an excellent platform for the exact assignment and curation of yeast genes and proteins, including all alias names. To avoid any confusion, we use the SGD standard nomenclature in this Review. The SGD names also serve as an unambiguous basis for the standard nomenclature of mitochondrial import components in other organisms.

a ***Saccharomyces cerevisiae*** **mitochondrial proteome (2010)**



b **Novel functions of** ***Saccharomyces cerevisiae*** **mitochondrial proteins (2003–2010)**



additionally contain a hydrophobic sorting signal that arrests translocation in the inner membrane. A large-scale determination of the N termini of mature mitochondrial proteins (the N-proteome) suggested that 60% or more of all mitochondrial proteins may be synthesized with N-terminal extensions[28].

Import by TOM and TIM23 complexes. The TOM complex comprises the central component Tom40, three preprotein receptors, Tom20, Tom22 and Tom70, and several small TOM proteins[5–7,29] (FIG. 2a). The initial recognition of presequences on the mitochondrial surface occurs by the receptors Tom20 and Tom22. Whereas Tom20 binds to the hydrophobic surface of the presequence, Tom22 recognizes the positively charged surface. The preproteins are translocated through the import channel formed by the β-barrel protein Tom40. Preproteins using the presequence import pathway cross the membranes as linear polypeptide chains that may adopt an α-helical and/or extended conformation. On the intermembrane space side of the outer membrane, the preproteins interact with the intermembrane space tail of the receptor Tom22.

Translocation across the inner mitochondrial membrane is mediated by the presequence translocase, the TIM23 complex. Whereas the channel-forming Tim23 and associated partner Tim17 have been known to be a part of this complex for over 15 years, the identification of Tim50 and Tim21 by proteomic approaches provided important insight into the cooperation of the TIM23 and TOM complexes during preprotein transfer. Tim21, Tim50 and Tim23 expose domains to the intermembrane space that transiently connect the TOM and TIM23 complexes[19,30–32] (FIG. 2a).

Tim50 is the first component of the inner membrane that binds to the preprotein emerging in the intermembrane space. Tim50 is tightly associated with the intermembrane space domain of Tim23 (REFS 31,32) and thus the information about the entry of a preprotein into the intermembrane space can be directly transferred to the import channel. Moreover, Tim50 also plays an important part in the absence of preproteins[33]. The channel formed by Tim23 is large enough to permit the translocation of a polypeptide chain in an α-helical conformation[34]. In the absence of a preprotein in transit, an open Tim23 channel would lead to a major leakage of ions across the inner membrane and thus to a dissipation of the $\Delta\psi$. Therefore, the opening and closing of the Tim23 channel has to be tightly regulated. The intermembrane space domain of Tim50 induces channel closure[33]. Tim50 releases the channel block only on arrival of a presequence allowing preproteins to pass through the channel. When the polypeptide chain has traversed the channel, Tim50 induces its closing and the next round of translocation will be initiated when a new preprotein emerges in the intermembrane space[31–35].

Tim21 binds to the intermembrane-space tail of Tom22 and thus, like Tim50 and Tim23, contributes to the direct but transient connection between the translocases of the outer and inner membranes[23,31,36,37]. The currently available evidence suggests that Tim21 does not bind preproteins but rather plays a regulatory part in preprotein

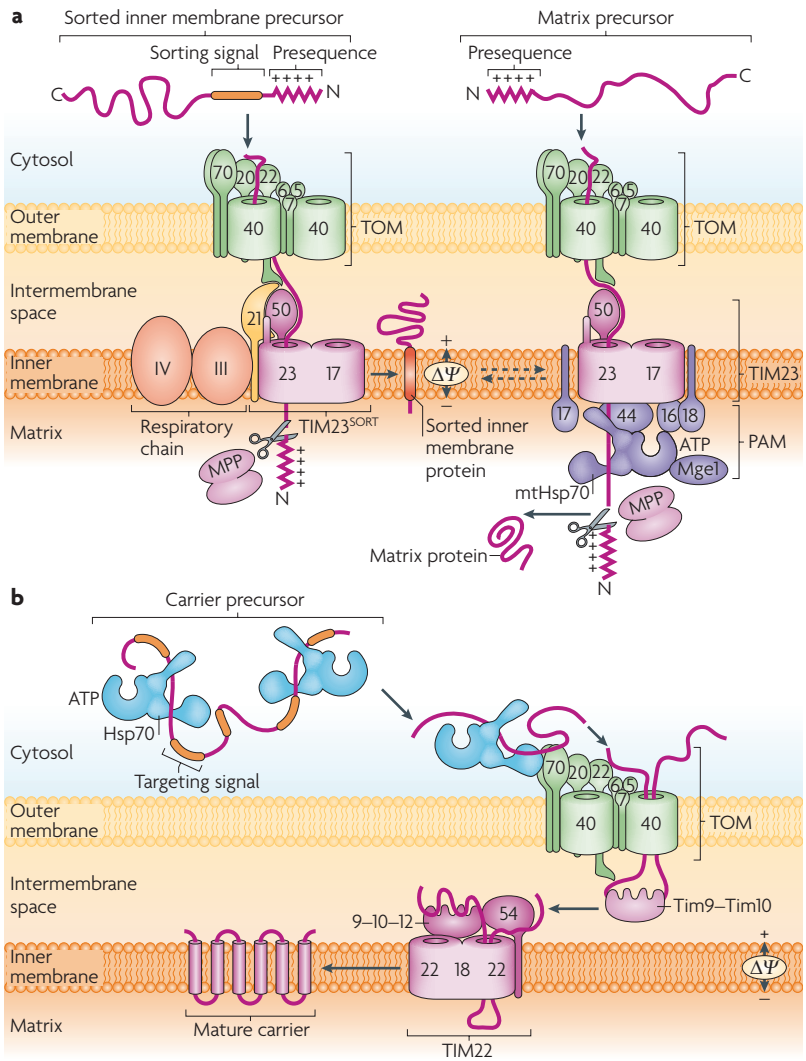


Figure 2 | **Classical routes of protein import: the presequence pathway and the carrier pathway. a** | The presequence pathway. The precursor proteins are imported by the translocase of the outer membrane (TOM) complex, which involves recognition by the receptors Tom20 and Tom22 and translocation through the Tom40 channel. The presequence translocase of the inner membrane complex, TIM23, which comprises Tim23, Tim17 and Tim50, functions in two modular forms. TIM23^{SORT} additionally contains Tim21, which transiently binds to the TOM complex and a supercomplex of the respiratory chain (left). TIM23^{SORT} mediates the lateral release of preproteins with hydrophobic sorting signals into the inner membrane. The other TIM23 form lacks Tim21 but interacts with presequence translocase-associated motor (PAM) and transports proteins into the matrix (right). This form is called TIM23–PAM. The membrane potential ($\Delta\psi$) drives translocation of the presequence through the TIM23 complex and ATP powers the chaperone mtHsp70 (matrix 70 kDa heat shock protein) of PAM. Mitochondrial processing peptidase (MPP) removes the presequences. **b** | The carrier pathway. The precursors of metabolite carriers contain internal targeting signals. Cytosolic chaperones (such as Hsp70) prevent aggregation of the precursors. The receptor Tom70 possesses binding sites for precursors and chaperones. After translocation through Tom40, the precursors are transferred by the Tim9–Tim10 chaperone through the intermembrane space. The carrier translocase of the inner membrane, the TIM22 complex, drives the membrane insertion of imported proteins in a $\Delta\psi$-dependent reaction.

transfer from the outer membrane to the inner membrane. Tim21 competes with presequences for binding to Tom22. Thus, the binding of Tim21 to Tom22 leads to a release of the preprotein and promotes its further transfer towards the Tim23 channel.

The $\Delta\psi$ across the inner membrane is crucial for translocation of the presequences through the Tim23 channel. Two roles have been assigned to $\Delta\psi$: activation of Tim23 and an electrophoretic effect that drives the import of presequences ($\Delta\psi$ is negative on the matrix side of the inner membrane and presequences are positively charged)[7,33,34].

Coupling of TIM23 to motor and respiratory chains. Whereas it has been shown that the purified and reconstituted TIM23 complex itself can carry out the basic function of protein membrane insertion[38], the complex cooperates with three further machineries *in organello*. In addition to the transient interaction with the TOM complex, the TIM23 complex cooperates with the import motor PAM and respiratory chain complexes[7,24,25].

On translocation of the presequence across the inner membrane, preproteins can follow one of two routes. They are either laterally released into the inner membrane or completely imported into the matrix[5,7]. Whereas the matrix-targeted preproteins are hydrophilic and the N-terminal presequence usually contains the necessary information for import (FIG. 2a; right), inner membrane-sorted preproteins possess at least one hydrophobic transmembrane segment (FIG. 2a; left). The hydrophobic segment functions as a sorting signal that arrests translocation in the TIM23 complex (stop–transfer) and induces lateral release of the preprotein into the lipid phase of the membrane.

Two forms of the TIM23 complex have been identified. One form, termed TIM23^{SORT}, contains Tim21 in addition to the three core components, Tim50, Tim23 and Tim17. TIM23^{SORT} is involved in the early stage of preprotein transfer from the TOM to the TIM23 complex, and in the lateral release of preproteins with an inner membrane-sorting signal[23,38,39] (FIG. 2a; left). The other form, termed TIM23–PAM, functions in the translocation of preproteins into the matrix. This form is associated with the subunits of PAM, but lacks Tim21 (REFS 23,39) (FIG. 2a; right).

Tim21 has a dual role[23,36,39,40]. It not only binds to the TOM complex but is also involved in the interaction of the TIM23^{SORT} complex with the respiratory chain (FIG. 2a; left). Tim21 alternates between binding Tom22 and a supercomplex of the respiratory chain that contains two proton-pumping complexes, complex III (also called the cytochrome bc_1 complex) and complex IV (also called cytochrome oxidase)[40]. The exact molecular function of TIM23^{SORT}–respiratory chain coupling has not been elucidated, but the available results suggest that the close vicinity of TIM23^{SORT} to proton-pumping complexes ensures an efficient use of the electrochemical proton gradient for preprotein translocation[24,25,40].

For translocation of hydrophilic preproteins into the matrix, Tim21 (and the respiratory chain complexes) is released from the TIM23 complex, whereas the motor PAM associates with the translocase. The core of PAM is formed by the molecular chaperone mtHsp70 (matrix 70 kDa heat shock protein), which binds the unfolded polypeptide chain and drives its translocation into the matrix in an ATP-powered manner[5,7]. mtHsp70 cooperates with several

co-chaperones. For a long time, only two co-chaperones of mtHsp70 were known: the nucleotide exchange factor Mge1 (also known as mitochondrial GrpE), which stimulates the release of ADP from mtHsp70; and Tim44, which is a docking site for mtHsp70 at the TIM23 complex (FIG. 2a; right). Based on this limited set of motor components, two models for the mechanism of the import motor were proposed and discussed in a controversial manner: active pulling of the preprotein by conformational changes of Tim44-associated mtHsp70, and passive trapping of the preprotein simply by the binding of mtHsp70 molecules (and thus preventing a back-sliding of the polypeptide chain). Subsequently, proteomic studies led to the identification of three new membrane-associated co-chaperones, Pam16, Pam17 and Pam18, that are required to coordinate the function of mtHsp70 at the translocase of the inner membrane. Pam18 is a J-type co-chaperone that stimulates the ATPase activity of mtHsp70. The J-related Pam16 forms a complex with Pam18 and functions as a negative regulator[20,21,41–43]. Pam17 is involved in the organization of the TIM23–PAM interaction[44,45]. Thus, the mitochondrial HSP70 system is regulated by five different co-chaperones, revealing a much higher complexity of PAM function than anticipated. A combination of both mechanisms, trapping and pulling, may best explain the dynamic activity of this multisubunit motor.

The existence of two forms of the TIM23 complex has been discussed and an alternative model of a single-entity translocase in which the TIM23 complex and PAM are permanently associated was proposed[45]. The analysis of the reaction cycle of the TIM23 complex in cooperation with TOM, the respiratory chain and PAM shows that neither a single-entity translocase nor two permanently separated translocase forms are sufficient to describe the reaction mechanism, but that the different TIM23 forms are in dynamic exchange with each other[23,24,39] (FIG. 2a). For example, matrix-targeted preproteins initially use the $TIM23^{SORT}$ complex for transfer from the TOM to the TIM23 complex and then the TIM23–PAM machinery for translocation into the matrix[39].

Processing enzymes and protein stability. On arrival in the matrix, most presequences are proteolytically removed by a heterodimeric enzyme, mitochondrial processing peptidase (MPP)[46]. Both subunits of MPP are essential for cell viability, underscoring the crucial function of MPP in mitochondrial biogenesis. Several preproteins are processed a second time by inner membrane-bound enzymes or matrix-located enzymes (BOX 3).

Some preproteins that are sorted to the intermembrane space carry a bipartite presequence, consisting of a positively charged matrix-targeting signal and a hydrophobic sorting signal that arrests translocation in the inner membrane. The matrix-targeting signal is removed by MPP, but the sorting signal is cleaved off by inner membrane peptidase (IMP), which has its active centre on the intermembrane-space side of the inner membrane, and thus the mature protein is released into the intermembrane space. In a few cases, the sorting signal is not processed by IMP but by the rhomboid protease processing of cytochrome *c* peroxidase 1 (Pcp1), which cleaves in the membrane[47–50] (BOX 3). Cleavage by Pcp1 is of particular importance for the formation of an isoform of the inner membrane fusion protein, mitochondrial genome maintenance 1 (Mgm1)[51].

Two processing enzymes can carry out a second cleavage step in the matrix. The mitochondrial intermediate peptidase Oct1 typically removes an octapeptide after cleavage by MPP[47,50], but it is unknown why this second cleavage occurs. Recently, a mitochondrial aminopeptidase was identified[28,52] — the intermediate cleaving peptidase Icp55 — that typically removes a single amino acid residue after processing by MPP. Icp55 was discovered in a systematic analysis of the N termini of mitochondrial proteins (the N-proteome), revealing a difference of one amino acid for numerous mitochondrial proteins when the mature N terminus and the MPP-cleaved product were compared[28] (BOX 3). Why should one amino acid be removed when the preceding cleavage by MPP leads to the removal of ~15–50 amino acids? It turned out that Icp55 plays an important part in the stabilization of mitochondrial proteins, leading to the discovery of an 'N-end rule pathway' of protein turnover in mitochondria. The N-end rule has been established for protein turnover in bacteria and the eukaryotic cytosol; it connects the N-terminal amino-acid residue of proteins with their half-life[53,54]. Certain large amino-acid residues at the N terminus of a protein favour its degradation by the N-end rule pathway (destabilizing residues), and other amino acids at the N terminus indicate a longer half-life (stabilizing residues). In cases where the cleavage by MPP generates an N terminus with a destabilizing amino acid, Icp55 removes this residue. Thereby, an N terminus carrying a stabilizing residue is typically generated[28]. The activity of Icp55 thus favours the stabilization of the mitochondrial proteome.

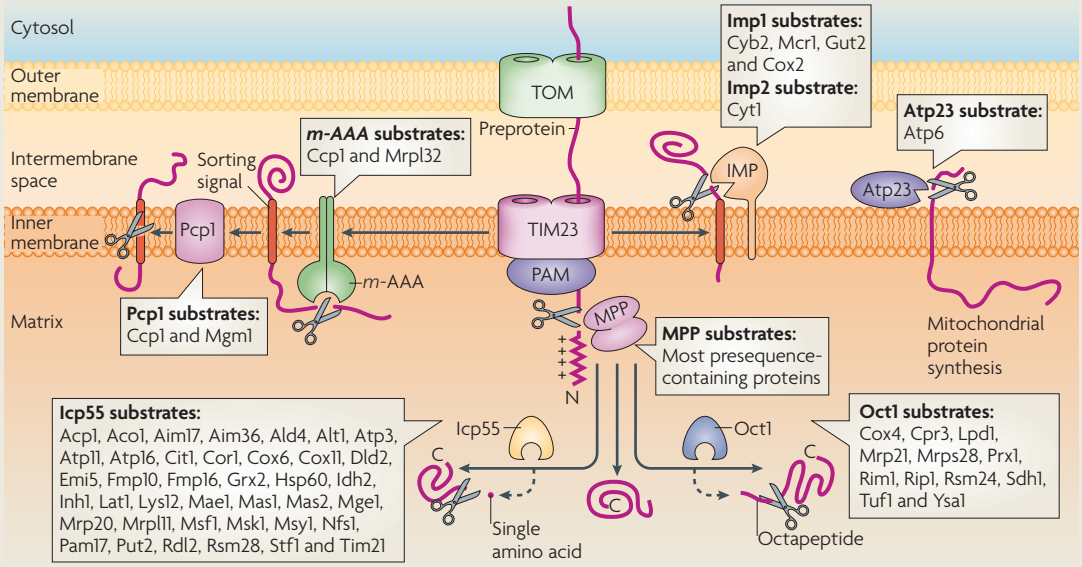
Carrier import pathway

The carrier pathway transports the hydrophobic precursors of non-cleavable proteins of the mitochondrial inner membrane. These proteins are integrated into the membrane by multiple α-helical transmembrane segments. Main representatives of these proteins are the metabolite carriers such as the ADP and ATP carrier, and the phosphate carrier, which belong to a large family of inner membrane carrier proteins that mediate the translocation of metabolites between the matrix and intermembrane space. Further examples of multi-spanning inner membrane proteins that are imported by the carrier pathway include the core subunits Tim23, Tim17 and Tim22 of the inner membrane translocases. Proteins using the carrier pathway are synthesized without cleavable presequences but contain internal segments that target the hydrophobic precursors to mitochondria and into the inner membrane[5,7]. A carrier protein contains approximately three to six discontinuous internal targeting elements that have only been partly characterized.

Chaperone-guided transport of carrier precursors. The carrier import pathway uses the same mitochondrial entry gate, the TOM complex, as the presequence pathway. However, the mechanisms of translocation differ greatly.

70 kDa heat shock protein
An ATP-dependent molecular chaperone that is essential in unstressed and stressed cells. The chaperones bind hydrophobic segments of unfolded proteins, preventing protein aggregation and promoting protein transport and folding.

Box 3 | **Mitochondrial processing enzymes and protein stability**



Presequence-carrying proteins are proteolytically cleaved by matrix processing peptidase (MPP), which removes the positively charged matrix-targeting sequences of most proteins (see the figure; precursor cleavage is indicated by scissors). Several proteases, such as the membrane-bound inner membrane peptidase (IMP) and the rhomboid intramembrane protease processing of cytochrome *c* peroxidase 1 (Pcp1), can carry out a second processing step (see the figure). These proteases cleave in or after hydrophobic sorting signals. The official names from the *Saccharomyces* genome database (SGD) have been used here for the known substrates of the proteases (see the figure).

In the matrix, a second processing step can be carried out by the intermediate cleaving peptidase Icp55, which removes single amino-acid residues and the matrix intermediate peptidase Oct1, which removes segments of approximately eight amino acids. Although Icp55 is the most recently identified mitochondrial peptidase, the long list of substrates indicates its importance in mitochondrial biogenesis[28]. Indeed, the aminopeptidase Icp55 removes destabilizing amino acids according to the 'N-end rule pathway' of protein degradation[53,54] and thus typically leads to the mature protein carrying a stabilizing amino acid at its amino terminus. Icp55 is peripherally associated with the inner mitochondrial membrane.

MPP does not remove all presequences as at least two preproteins are cleaved by the ATP-dependent *m*-AAA protease of the inner membrane[48,131]. Although preprotein processing by the *m*-AAA protease is not required for cell viability, it is important for mitochondrial function as one of its substrates is a subunit of the mitochondrial ribosome. Inactivation of the protease impairs ribosome assembly and leads to the human neurodegenerative disorder hereditary spastic paraplegia[131].

Proteolytic processing has also been shown for a few proteins that are encoded by the mitochondrial genome and inserted into the inner membrane: ATP synthase subunit 6 (Atp6), which is processed by the intermembrane space-exposed protease Atp23 (REFS 132,133), and cytochrome *c* oxidase subunit 2 (Cox2), which is processed by Imp1 (REF. 134) (see the figure).

Acp1, acyl carrier protein 1; Aco1, aconitate hydratase 1; Alt1, alanine aminotransferase 1; Ccp1, cytochrome *c* peroxidase 1; Cit1, citrate synthase 1; Cor1, cytochrome *b*-c1 complex subunit 1; Cyb2, cytochrome *b*2; Cyt1, cytochrome *c*1; Dld2, D-lactate dehydrogenase 2; Emi5, early meiotic induction protein 5; Fmp, found in mitochondrial proteome; Grx2, glutaredoxin 2; Hsp60, 60 kDa heat shock protein; Idh2, isocitrate dehydrogenase subunit 2; Inh1, ATPase inhibitor 1; Mae1, NAD-dependent malic enzyme 1; Mrp, mitochondrial ribosomal protein; PAM, presequence translocase-associated motor; Rsm24, mitochondrial ribosomal small subunit protein 24; Sdh1, succinate dehydrogenase flavoprotein subunit 1; Stf1, stabilizing factor 1; TOM, translocase of the outer membrane; TIM, translocase of the inner membrane.

Deafness dystonia syndrome
An X chromosome-linked neurodegenerative disease that includes deafness, cortical blindness and dystonia. It was the first human disease caused by a defect in the mitochondrial protein import machinery (specifically, in Tim8 (known as TIMM8A in humans)). It is also called Mohr–Tranebjaerg syndrome.

Molecular chaperones in the cytosol and intermembrane space are crucial to prevent aggregation of the hydrophobic carrier precursors in the aqueous environment. Chaperones of the HSP70 and HSP90 classes bind to the precursors upon their synthesis on cytosolic ribosomes. The chaperones not only guide the precursors to mitochondria but also specifically bind to the receptor Tom70 and thus ensure a direct transfer of the precursors to the import machinery of mitochondria[29,55,56] (FIG. 2b). Tom70 possesses binding sites for precursor proteins and chaperones[29,55]. ATP is needed to release the precursor proteins from the chaperones. With the help of the receptors Tom20 and Tom22, the precursor is inserted into the Tom40 channel. In contrast to presequence-carrying preproteins, carrier precursors are not translocated as linear polypeptide chains but traverse the outer membrane in a loop structure[57]. Hexameric chaperone complexes in the intermembrane space — Tim9–Tim10 and the homologous Tim8–Tim13 — bind to the precursor proteins and transfer them through the aqueous intermembrane space to the inner membrane[10,58,59]. Defects in human TIM8 lead to deafness dystonia syndrome[60].

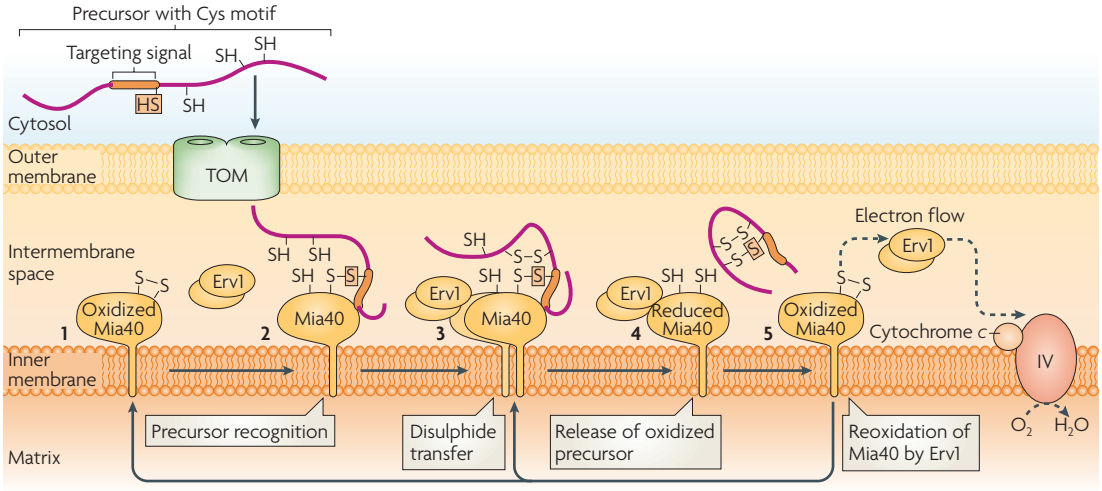


Figure 3 | **Oxidative import and folding in the intermembrane space.** The mitochondrial intermembrane space assembly (MIA) machinery is responsible for the import of small intermembrane-space proteins that contain characteristic Cys motifs (with sulphhydryl groups (SH)). MIA includes the protein disulphide carrier Mia40 and the sulphhydryl oxidase Erv1 (essential for respiration and viability 1). Erv1 oxidizes Mia40 by transferring a disulphide bond (S–S) to it (1). Oxidized Mia40 functions as a receptor for the incoming precursor protein and binds its targeting signal, forming a transient disulphide bond (2). Mia40 transfers the disulphide bond to the precursor (3). Thus, Mia40 becomes reduced (4) and is re-oxidized by Erv1 (5). For transferring several disulphide bonds to the precursor, Mia40 may shuttle between Erv1 and the precursor (known as a disulphide relay), or Erv1, Mia40 and the precursor protein may be associated in a transient ternary complex (in a process known as disulphide channelling and probably involving several molecules of Erv1 and Mia40). Protein oxidation (the formation of disulphide bonds) is coupled to the removal of electrons that flow from the precursor via Mia40 to Erv1 and from here, via cytochrome *c*, to the respiratory chain.

Protein insertion into the inner membrane. The carrier translocase of the inner membrane, the TIM22 complex, forms a twin-pore translocase[9] (FIG. 2b). A modified form of the intermembrane space chaperone, comprising Tim9, Tim10 and the related protein Tim12, docks onto the TIM22 complex[5,11]. The translocation channel is formed by the integral membrane protein Tim22. Two further integral membrane proteins, Tim54 and Tim18, are associated with Tim22. Tim54 exposes a large domain to the intermembrane space and probably serves as a docking point for the Tim9–Tim10–Tim12 complex[61]. Tim18 is involved in the assembly of the TIM22 complex[61].

Precursor proteins interact with the TIM22 complex in two stages[9]. They are first bound to the Tim9–Tim10–Tim12 chaperone complex on the surface of the translocase. Activation of the Tim22 channel involves the internal targeting signals of the precursors and the $\Delta\psi$. The precursors are probably inserted into the translocase in a loop structure. Finally, the proteins are laterally released into the lipid phase of the inner membrane, although the mechanism of release has not yet been clarified. Tim22 is homologous to Tim23 and Tim17 of the presequence translocase, indicating that these core components of the inner membrane translocases were derived from a primordial machinery by gene duplication[62].

Intermembrane space assembly

The identification of the MIA machinery completely changed the view of how the mitochondrial intermembrane space is assembled. For a long time, it was assumed that, like the cytosol, the intermembrane space possesses a reducing environment and thus the oxidation of proteins (the formation of disulphide bonds) would be disfavoured. A systematic analysis of new proteins that were found in the mitochondrial proteome led to the identification of Mia40 (REFS 12–14). This protein is essential for cell viability and contains characteristic Cys motifs. Mia40 became the core component of a new machinery that inserts disulphide bonds into proteins imported into the intermembrane space, leading to the unexpected finding that many intermembrane space proteins contain disulphides; that is, they are in an oxidized state.

Mia40 as a receptor and protein disulphide carrier. Most intermembrane space proteins are small proteins that are synthesized without cleavable presequences but contain Cys motifs[63,64]. The precursors of these proteins are synthesized on cytosolic ribosomes and translocated across the outer membrane in a reduced, unfolded conformation (FIG. 3). Mia40 functions as a receptor on the intermembrane space side of the outer membrane. It recognizes an internal signal of the precursor proteins that includes a conserved hydrophobic residue and a Cys residue[65,66]. Mia40 binds the precursor in a hydrophobic cleft and by the formation of a transient disulphide bond[67,68]. Mia40 then acts as a protein disulphide carrier that transfers disulphide bonds to the imported proteins and thus promotes their oxidation to mature forms.

Disulphide relay and channelling. Mia40 does not form disulphide bonds *de novo* but functions as an oxidoreductase that is part of a disulphide-transferring reaction chain (the disulphide relay)[69–72]. The sulphhydryl oxidase essential for respiration and viability 1 (Erv1)

generates disulphide bonds and transfers them to Mia40 by the formation of transient intermolecular disulphide bonds. Mia40 then transfers the disulphides onto the substrate; that is, the imported protein. As the formation of disulphides is coupled to the removal of electrons, electrons flow from the imported proteins via Mia40 to Erv1, and from here via cytochrome *c* to the respiratory chain[71,73,74] (FIG. 3).

The substrates of Mia40 typically receive more than one disulphide bond. Different models are conceivable for how multiple disulphides are transferred. On the one hand, the protein disulphide carrier Mia40 may function in an alternating relay by shuttling between binding to the oxidase Erv1 and the substrate, and thus the transfer of two or more disulphides will require several cycles of binding and release[69]. On the other hand, it has been reported that Erv1, Mia40 and the substrate are physically associated in a transient ternary complex[75]. In the case of mitochondria, the interactions between disulphide carrier and substrate, as well as between disulphide carrier and oxidase, are much more stable and long-lived than the transient interactions in the disulphide relays of the endoplasmic reticulum (ER) and bacterial periplasm. Although the redox conditions of the mitochondrial intermembrane space are more oxidizing than in the cytosol, they are probably less oxidizing than in the ER and periplasm. In an alternating relay, partially oxidized substrates are released and thus the conditions in the intermembrane space may favour a reduction of the substrates. In a ternary complex containing all three partners, disulphides can be directly transferred without the intermittent release of substrate (disulphide channelling)[75] (FIG. 3). The exact molecular mechanism of disulphide transfer and the role of individual Cys residues involved are only partly understood. It is likely that more than one molecule of Erv1 and Mia40 participate in the formation of the ternary complex and that non-covalent interactions between the partner proteins will be important to stabilize such a complex and for the formation of transient disulphide linkages[72,75].

Protein sorting to the outer membrane

All proteins of the mitochondrial outer membrane are imported from the cytosol. The membrane contains two different types of integral proteins, α-helical proteins and β-barrel proteins. These proteins are not simply inserted into the membrane by the TOM complex. A combination of proteomic studies and functional analysis of transport intermediates have led to the identification of at least two different sorting pathways of outer membrane proteins.

β-barrel pathway through TOM and SAM. The precursors of β-barrel proteins are initially transported across the outer membrane by the TOM complex and are inserted into the outer membrane from the intermembrane-space side by the SAM complex[15] (FIG. 4a). The channel-forming core of the SAM complex, Sam50, is homologous to BamA (also known as Omp85) of the bacterial β-barrel assembly machinery (BAM)[16–18]. The basic mechanism of β-barrel insertion into the outer membrane has thus been conserved from Gram-negative bacteria to mitochondria, although the other components of SAM and BAM are not related to each other[76]. The β-barrel precursors are not directly transferred from the TOM complex to the SAM complex, but the intermembrane space chaperones Tim9–Tim10 and Tim8–Tim13 participate in the transport of the precursors[77,78]. A sorting signal that corresponds to the most C-terminal β-strand of the precursor directs insertion into the SAM complex and binds Sam35, a partner protein of Sam50 (REF. 79). Sam37, the third subunit of the core SAM complex, promotes the release of precursors into the lipid phase of the outer membrane[80]. The exact mechanism of precursor insertion and release is not yet known. It is unknown whether the precursor proteins are translocated through a channel formed by monomeric Sam50 or whether a channel is located between several Sam50 molecules. A third possibility is that precursor proteins may be inserted at the SAM–lipid interface.

The insertion of typical β-barrel proteins such as the main outer membrane protein porin is mediated by this core SAM complex (FIG. 4a). A more complicated pathway is used by the precursor of Tom40. This central TOM subunit not only has to be inserted into the outer membrane but also has to assemble with the other TOM subunits to the oligomeric TOM complex. The SAM complex provides a modular assembly platform for the TOM machinery. A fraction of SAM complexes contain a fourth subunit, mitochondrial distribution and morphology 10 (Mdm10), which promotes the association of Tom40 with α-helical TOM proteins such as the precursor of Tom22 (REFS 81–84). Interestingly, Mdm10 is not only located in the SAM complex but is also a subunit of the MDM complex and thus connects protein assembly with mitochondrial distribution and morphology, as discussed below.

Outer membrane insertion of α-helical proteins. The outer membrane contains different types of α-helical proteins that can be distinguished by the location of their transmembrane segments in N-terminal, middle or C-terminal protein regions (FIG. 4b). The hydrophobic segments typically function as targeting signals of the proteins. The receptors Tom20 and Tom70 are representatives of N-terminally anchored proteins. They use a further outer membrane protein, mitochondrial import 1 (Mim1), for membrane insertion[85–87]. Mim1 was identified in a genome-wide screen in yeast[88] and transiently interacts with the SAM complex[86]. The insertion pathway for proteins with internal signals has only been analysed for a few proteins. The precursor of Tom22 uses TOM receptors for targeting to mitochondria and then the SAM complex for membrane insertion[89] (FIG. 4b). Precursors with multiple transmembrane segments were shown to use Tom70 and intermembrane-space components but not other TOM components for insertion into the outer membrane[90]. Tom70 and the intermembrane-space components may carry out chaperoning functions for the hydrophobic precursors. An insertase (proteinaceous machinery that mediates membrane insertion of the precursors) has not been identified so far. Different pathways have been suggested for proteins with a C-terminal membrane anchor (C-tail). The precursors of small TOM proteins were found to depend on Mim1 for membrane insertion[82].

Porin
A pore-forming protein of the mitochondrial outer membrane that is permeable for many metabolites. It is the most abundant outer membrane protein and is also called voltage-dependent anion channel (VDAC).

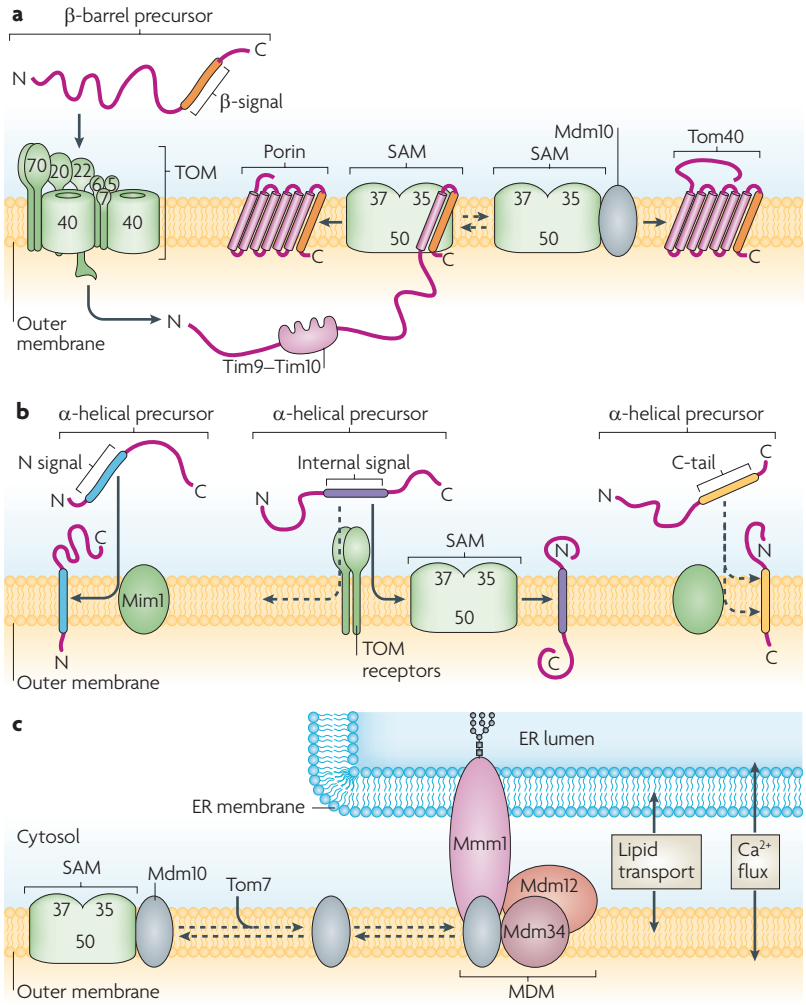


Figure 4 | **Protein sorting to the outer mitochondrial membrane and connection to the ER. a** | β-barrel proteins are imported by the translocase of the outer membrane (TOM) complex and bind to the translocase of the inner membrane 9 (Tim9)–Tim10 chaperone on the intermembrane-space side of the outer membrane. The β-sorting signal initiates the insertion of the β-barrel precursors into the sorting and assembly machinery (SAM) complex, which comprises Sam35, Sam37 and Sam50. β-barrel proteins such as porin (a voltage-dependent anion channel) are then inserted into the lipid phase. The β-barrel precursor of Tom40 assembles with α-helical TOM proteins, which involves a larger SAM complex that includes mitochondrial distribution and morphology 10 (Mdm10). **b** | α-helical outer membrane proteins are imported by different routes that have only been partly characterized. The membrane anchor sequences typically function as targeting signals. Precursors with amino-terminal signals can use mitochondrial import 1 (Mim1) for membrane insertion. Other precursors can use TOM receptors, the SAM complex or unknown mechanisms. **c** | Mdm10 is located in two different complexes: the SAM complex and the MDM complex, which comprises Mdm34, Mdm12 and maintenance of mitochondrial morphology 1 (Mmm1). Mmm1 is anchored in the endoplasmic reticulum (ER) membrane. The ER–mitochondrion junction formed by the MDM complex may be involved in the transport of lipids and Ca^{2+}. The small TOM subunit, Tom7, favours the dissociation of Mdm10 from the SAM complex and thus negatively regulates the assembly of the TOM complex[96].

Mitofusin
A mitochondrial outer membrane protein required for fusion of mitochondria and maintenance of mitochondrial morphology.

It was reported that other C-tail proteins do not require any of the known outer membrane machineries, but the lipid composition of the membrane is important for their efficient insertion[91,92]. These proteins may use an unknown insertase or be directly inserted into the lipid phase of the outer membrane.

Thus, our current knowledge on the sorting and insertion of α-helical outer membrane proteins is only fragmentary. It is clear that the proteins do not follow the classical route through the Tom40 import channel, in contrast to most mitochondrial proteins, and so the mechanisms and components of α-helical insertion pathways await further analysis.

Outer membrane assembly and ER–mitochondrion junctions. Mdm10 was identified in a genetic screen for components that affect mitochondrial distribution and morphology[93]. It is present in at least two different protein complexes: the SAM complex and the MDM complex[81,83,84,94–96] (FIG. 4c). The identification of Mdm10 as a subunit of SAM unexpectedly connected the fields of protein assembly and mitochondrial morphology, which had been considered as independent fields before.

A further surprise came when the MDM complex was characterized. In addition to Mdm10, the complex includes other so-called morphology components: maintenance of mitochondrial morphology 1 (Mmm1), Mdm12 and Mdm34 (also known as Mmm2) (REFS 95,97,98). Mmm1 was found to be inserted into the membrane of the ER and to be glycosylated[99] (FIG. 4b). Thus, the MDM complex connects part of the ER membrane with the mitochondrial outer membrane and was also termed the ER–mitochondrion encounter structure (ERMES). Junctions between the ER and mitochondria have been known for many years[100], but their molecular identity remained unknown. An important role of the MDM complex is in the formation of ER–mitochondria junctions. A study in mammalian cells indicated that another protein, mitofusin, is also involved in this process[101]. The current evidence suggests that ER–mitochondrion junctions are important for the transfer of lipids and Ca^{2+} between both organelles.

The exact molecular function of the MDM complex is not known. It may function as an organizing centre that provides a platform for the connection of mitochondria with the ER and the transfer of ions, lipids and possibly other molecules[102]. The dual localization of Mdm10 in MDM and SAM complexes suggests that the protein assembly machinery is connected to the organizing centre, and indeed an involvement of the MDM complex in protein assembly has been reported[84,94]. A close functional connection between SAM and MDM complexes is also suggested from the analysis of mitochondrial morphology in yeast cells. Whereas wild-type mitochondria form a tubular network, mutant cells of MDM components contain condensed mitochondria (so-called giant mitochondria)[93] and SAM mutants show similar changes in mitochondrial shape[81,94,96]. These morphological alterations may result from defects in outer membrane assembly and defects in ER–mitochondrion junctions.

Perspectives

The large-scale identification of the mitochondrial proteome and its systematic analysis by biochemical and genetic approaches provided invaluable information for the identification of new mitochondrial import components and pathways, including the oxidative folding

Cardiolipin
A large, dimeric phospholipid that is a characteristic of mitochondria and consists of two phosphatidyl moieties linked by glycerol.

Liposome
An artificial lipid vesicle that is typically formed by a phospholipid bilayer (membrane).

pathway of the intermembrane space and the β-barrel pathway of the outer membrane. Protein translocases do not function as independent units but are integrated into dynamic networks. The TIM23 complex, which interacts with complexes in three different mitochondrial compartments (the TOM complex, the respiratory chain and the matrix motor), serves as a paradigm. The dynamic association of Mdm10 with different outer membrane complexes indicates that the translocases are also connected to machineries that function in ER–mitochondrion junctions, lipid transport and maintenance of mitochondrial morphology.

Seeing the rapid increase in the number of identified mitochondrial proteins (BOX 1), the question of whether, and how, it may be possible to achieve a complete coverage of the mitochondrial proteome arises. As not all mitochondrial proteins are expressed under the same growth conditions or in all organs of multicellular organisms, proteomic analyses of cells from different growth phases and media will be required for single-cell organisms, whereas the analysis of multiple organs and different developmental stages will be required for animal or plant mitochondria. It is likely that the search for new mitochondrial import components is not yet finished as the mitochondrial proteome still contains many proteins with unknown function (BOX 2) and several pathways of protein import are only understood in part, such as the insertion of α-helical precursors into the outer membrane. Additionally, the mitochondrial inner membrane may contain further insertases. Although the presequence pathway and the carrier pathway strictly require a $\Delta\psi$, $\Delta\psi$-independent import has been observed for a few precursor proteins[103,104]. These proteins may be imported by an alternative machinery that can operate in the absence (or at low levels) of a $\Delta\psi$.

There are different views about whether and how protein synthesis at ribosomes and translocation into mitochondria are coupled. As the *in vitro* import assays with isolated mitochondria work efficiently in a post-translational manner (that is, with fully synthesized precursor proteins), it has been assumed that a coupling of ribosomes to mitochondria is not involved. However, most precursor proteins were not analysed in the *in vitro* assays and several proteins tested could not be imported[105]. The presence of ribosomes on the mitochondrial surface has been known for many years[106]. More recently, mRNAs for mitochondrial proteins were found to be enriched at mitochondrion-associated ribosomes[107,108] and the corresponding precursor proteins were found to accumulate at the outer membrane[109]. Future studies have to consider the possibility that co-translational targeting of mitochondrial proteins may be much more important than anticipated.

The role of the lipid environment of the membranes has been underestimated for a long time. In addition to the studies on outer membrane protein insertion that indicate the importance of the lipid composition[92,102], the analysis of the mitochondrial signature lipid, the dimeric phospholipid cardiolipin, provided important information. The efficient integration of purified TIM23 complexes into liposomes requires cardiolipin[38], and the lack of cardiolipin influences the organization of the TIM23–PAM complex[110–112]. Whereas cardiolipin is enriched in the mitochondrial inner membrane, a small but significant fraction of cardiolipin was found in the outer membrane and shown to influence the activity of the TOM and SAM complexes[113]. Lipids may affect protein biogenesis by influencing the activity of translocase complexes but may also directly participate in the process of membrane insertion.

The structural analysis of mitochondrial import components is an important field of research. In addition to single-particle electron microscopic analysis of purified translocase complexes[9,114,115], high resolution structures have been reported for receptor domains, mitochondrial chaperones and the processing peptidase MPP[6,10,29,37,42,46,116]. To understand the molecular mechanisms of membrane translocation, it will be crucial to obtain high resolution structures of the membrane-integrated translocation channels, including that of preproteins in transit.

As recent studies revealed that mitochondria are involved in numerous human diseases[117], a systematic analysis of the mitochondrial proteome will serve as a basis to define their molecular mechanisms. Little is known about the regulation of the mitochondrial protein import machinery. The mitochondrial proteome led to the identification of several protein kinases, phosphatases and GTPases[2,3]. A first analysis of the mitochondrial phosphoproteome suggested that many more proteins than assumed are phosphorylated[118,119]. We expect that a systematic analysis of the phosphorylation of mitochondrial proteins and the assignment of the responsible kinases and phosphatases, as well as the analysis of GTPases, will provide a wealth of information of how mitochondria are embedded into cellular signalling networks and will help us understand the molecular basis of mitochondrial diseases.

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Acknowledgements
The authors' research is supported by the Deutsche Forschungsgemeinschaft, Excellence Initiative of the German Federal and State Governments (EXC 294 BIOSS; GSC-4 Spemann Graduate School), Trinationales Graduiertenkolleg GRK 1478, Bundesministerium für Bildung und Forschung, Sonderforschungsbereich 746, Gottfried Wilhelm Leibniz Program, Landesforschungspreis Baden-Württemberg and Fonds der Chemischen Industrie.

Competing interests statement
The authors declare no competing financial interests.

DATABASES
UniProtKB: http://www.uniprot.org
Erv1 | Mdm10 | Mdm12 | Mdm34 | Mia40 | Mim1 | Mge1 | Mmm1 | Pam16 | Pam17 | Pam18 | Sam35 | Sam37 | Sam50 | Tim8 | Tim9 | Tim10 | Tim12 | Tim13 | Tim17 | Tim18 | Tim21 | Tim22 | Tim23 | Tim44 | Tim50 | Tim54 | Tom7 | Tom20 | Tom22 | Tom40 | Tom70

FURTHER INFORMATION
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SGD: www.yeastgenome.org
ALL LINKS ARE ACTIVE IN THE ONLINE PDF

OPINION

New functions of aminoacyl-tRNA synthetases beyond translation

Min Guo, Xiang-Lei Yang and Paul Schimmel

Abstract | Over the course of evolution, eukaryotic aminoacyl-tRNA synthetases (aaRSs) progressively incorporated domains and motifs that have no essential connection to aminoacylation reactions. Their accretive addition to virtually all aaRSs correlates with the progressive evolution and complexity of eukaryotes. Based on recent experimental findings focused on a few of these additions and analysis of the aaRS proteome, we propose that they are markers for aaRS-associated functions beyond translation.

The aminoacylation reaction, which is catalysed by aminoacyl-tRNA synthetases (aaRSs), fuses each amino acid to its cognate tRNA. This reaction requires amino acid activation through condensation of the amino acid with ATP to form an aminoacyl adenylate. The activated amino acid is then transferred to the 3′ end of the cognate tRNA (BOX 1). Because the cognate tRNAs harbour the anticodon triplets of the genetic code, the specific aminoacylations catalysed by the aaRSs establish the rules of the universal genetic code. Thus, aaRSs arose early in evolution, perhaps replacing the activities of ribozymes, the first catalysts of aminoacylations, as the transition was made from the RNA to the protein world.

All aaRSs have an aminoacylation domain, which encodes the active site that recognizes the specific amino acid, ATP and the 3′ end of the bound tRNA. On the basis of the architecture of this domain, the enzymes are split into two classes (each comprising ten enzymes): class I, in which the domain has a Rossmann nucleotide-binding fold, and class II, in which the domain is a seven-stranded β-sheet with flanking α-helices[1,2]. These two architectures are thought to have arisen from opposite (that is, complementary) strands of RNA genomes that may have existed in the RNA world and that encoded a class I (strand 1) and a sister class II (complementary strand 2) aaRS[3,4]. The complementary sister aaRSs can be modelled to bind simultaneously to opposite sides of the tRNA acceptor stems, thereby covering much of the tRNA[5,6]. They could have served, among other possibilities, as chaperones to protect the tRNA substrate from destruction by nucleases and phosphate bond-cleaving metal ions. Apart from the well-conserved catalytic units, many aaRSs made later additions of less-conserved anticodon-binding domains to more efficiently recognize tRNAs.

In addition to the aminoacylation functions, about half of the aaRSs added an editing function, which enables removal of the wrong amino acid from its cognate tRNA[7]. These aaRSs face a greater challenge to differentiate the cognate verses the non-cognate amino acid (for example, Ile from Val) than the others.

This editing function is an important mechanism to prevent mistranslation, during which the wrong amino acid is inserted at a specific codon. For life to thrive, the challenge of preventing mistranslation through the mischarging of tRNA had to be overcome. For this reason, the addition of an editing domain to an aminoacylation domain happened before the time that the three kingdoms of life diverged from the last universal common ancestor (LUCA), with strong selective pressure ever since to keep both domains throughout evolution[8].

Throughout evolution, aaRSs have also incorporated domains with no apparent connection to their aminoacylation reactions. In this Opinion, we investigate the general logic and purpose of these new domains, especially in eukaryotes. As described below, our analysis shows that these domain additions were accretive and progressive, following the increasing complexity of eukaryotic organisms. We also find that this ensemble and pattern of domain additions was specific to aaRSs. Importantly, a close inspection of the pattern of domain additions shows that the new functions that have been identified for some of these domains were introduced at precise times in evolution and were associated with the appearance of a new biological function (such as a circulatory system). This observation raises the possibility that the domain additions played an important part in expanding the complexity and sophistication of newly emerging organisms.

Box 1 | **Basic function of aaRSs**

Aminoacyl-tRNA synthetases (aaRSs) provide the first resource for the production of proteins. The algorithm of the genetic code is established in this first reaction of protein synthesis. In this reaction, aaRSs catalyse the attachment of amino acids to their cognate tRNAs that bear the triplet anticodons of the code. These enzymes catalyse the attachment of amino acids in a two-step reaction. The amino acid (aa) is first condensed with ATP to form a tightly bound aminoacyl adenylate (aa–AMP), and inorganic pyrophosphate (PP_i) is released:

$aa + ATP + aaRS \rightarrow aaRS(aa–AMP) + PP_i$

The activated aa–AMP is then transferred from the adenylate to the 3′ end of the tRNA to form aa–tRNA. This also releases AMP and the aaRS:

$aaRS(aa–AMP) + tRNA \rightarrow aa–tRNA + AMP + aaRS$

Because of their essential role in protein synthesis, genes encoding aaRSs appeared when life began[1]. As a family of 20 enzymes in general (one for each amino acid), aaRSs are constrained by evolutionary pressure to preserve this essential activity, but they still managed to develop additional functions during evolution.

Table 1 | **Summary of domains added to aaRSs***

aaRS	*Saccharomyces cerevisiae*	*Caenorhabditis elegans*	*Drosophila melanogaster*	*Danio rerio*	*Homo sapiens*	Position in human aaRSs
CysRS	UNE-C_1 and UNE-C_2	UNE-C_1 and UNE-C_2	UNE-C_1 and UNE-C_2	UNE-C_1 and UNE-C_2	UNE-C_1, UNE-C_2 and GST	201–297, 664–831 and 1–82
GlnRS	UNE-Q	UNE-Q	UNE-Q	UNE-Q	UNE-Q	1–260
ArgRS		LZ	LZ	LZ	LZ	5–32 and 41–64
MetRS‡	GST	GST, LZ and EMAPII	GST and WHEP§	GST and WHEP	GST and WHEP	8–218 and 844–897
ValRS				GST	GST	2–218
LeuRS		UNE-L	UNE-L	UNE-L	UNE-L	1064–1176
IleRS				UNE-I	UNE-I	1065–1266
TrpRS				WHEP	WHEP	8–64
TyrRS			ELR and EMAPII	ELR and EMAPII	ELR and EMAPII	91–93 and 360–524
GluRS	GST	GST and WHEP	GST and WHEPs‖	GST and WHEPs‖	GST and WHEPs‖	1–178 and 749–805 or 822–878 or 900–956
ProRS		WHEP				
HisRS		WHEP	WHEP	WHEP	WHEP	3–43
PheRS	UNE-F	UNE-F	UNE-F	UNE-F	UNE-F	6–100
AspRS	N-helix	N-helix	N-helix	N-helix	N-helix	6–24
LysRS	N-helix	N-helix	N-helix	N-helix	N-helix	20–40
AsnRS	UNE-N	UNE-N	UNE-N	UNE-N	UNE-N	1–99
ThrRS	UNE-T	UNE-T	UNE-T	UNE-T	UNE-T	1–80
AlaRS	None	None	None	None	None	NA
SerRS				UNE-S	UNE-S	461–514
GlyRS		WHEP	WHEP	WHEP	WHEP	2–61
MSC p18¶			GST	GST	GST	1–154
MSC p38¶			LZ and GST	LZ and GST	LZ and GST	50–80 and 149–319
MSC p43¶			LZ and EMAPII	LZ and EMAPII	LZ and EMAPII	8–28, 38–72 and 150–311

GST, glutathione S-transferase domain; LZ, Leu-zipper motif; MSC, multi-aaRS complex; NA, not applicable. *Each added sequence was checked by iterative searching of the non-redundant database of NCBI using PSIBLAST. Unique, unfamiliar domains (termed UNEs) are defined when no homologous sequences (E value < 0.005) were found in other aminoacyl-tRNA synthetases (aaRSs) after the PSIBLAST converged. Also, with the exception of PheRS, the eight UNEs are not similar to any in sequence databases of all bacterial or archaeal tRNA synthetases. Furthermore, apart from PheRS and GlnRS, no UNE bears similarity (E value < 0.005) to sequences found in non-synthetase proteins. ‡The only aaRS to not irreversibly retain domains. §*D. melanogaster* MetRS has three WHEP domains at the carboxy terminus (InterPro: A1ZBE9), which were not found in the previous reports. ‖The WHEP domain links GluRS and ProRS as a fusion enzyme. ¶First appears in *D. melanogaster*.

Annotation of new domains and motifs

With a clear understanding of the essential role and logic of the aminoacylation and editing domains, we were surprised to find that, as the tree of life ascended, aaRSs progressively added domains and sequence motifs that are connected to neither aminoacylation nor editing. TABLE 1 displays an annotation of shared domains or motifs (those in more than one synthetase) and unique domains or motifs (those in a specific synthetase) found in the eukaryotic aaRS proteome. The five shared domains or motifs are structural modules that are similar to those seen in other proteins, such as a specialized version of the helix-turn-helix motif known as the WHEP domain, the oligonucleotide binding fold-containing EMAPII domain that is also found in p43 (an auxiliary factor found in the multi-aaRS complex (MSC) of eukaryotes), the Leu-zipper motif, the glutathione *S*-transferase (GST) domain and a specialized amino-terminal helix (N-helix)[9]. These domains were added to specific aaRSs as the tree of life ascended from lower to higher eukaryotes. Because the Leu-zipper motif and the WHEP and GST domains are used for forming complexes with other proteins[10], and because the EMAPII domain can function as a cytokine when cleaved from the aaRS or p43 by binding to a cell surface receptor[11], these domain additions are strongly suggestive of an interaction with a partner protein[12–15].

Unlike the shared domains, the eight unique sequence motifs are specific to only one synthetase. With the exception of PheRS, the eight unique sequence motifs are not similar to any that we could find from searching sequence databases of all bacterial or archaeal aaRSs. In addition, apart from PheRS and GlnRS, their sequence is not similar to those of non-synthetase proteins. We designate these unique sequences as UNE-L, UNE-S and so on, in which the single letter designates the amino acid type of the synthetase that harbours the specific unique domain (so UNE-L is associated with LeuRS and UNE-S with SerRS). These unique domains and motifs were added to specific aaRSs at distinct points in evolution. Similarly to the shared domains and motifs, UNE domains were grafted onto the canonical aaRS long after the aminoacylation function was established. Importantly, with the exception of MetRS, these shared and unique domains or motifs were irreversibly retained by the respective aaRS until humans evolved.

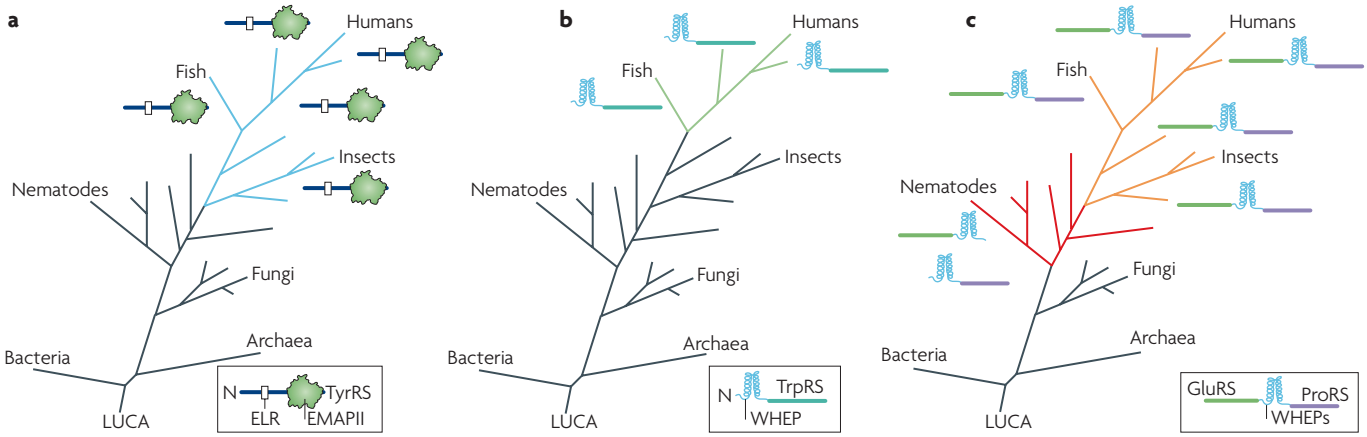

Figure 1 | **Domain additions to specific higher eukaryote aminoacyl-tRNA synthetases. a** | The temporal appearance of the tripeptide ELR (Glu-Leu-Arg) motif and EMAPII domain, which confer and regulate the cytokine activities of TyrRSs. ELR and EMAPII were added simultaneously to TyrRSs, starting from insects. **b** | The temporal appearance of the WHEP domain in TrpRSs. The WHEP domain was found to regulate the angiostatic function of human TrpRS. **c** | The temporal appearance of the WHEP domain in GluRS–ProRSs. Initially separated, GluRS and ProRS gained WHEP domains in nematodes and fused into one protein that is linked by WHEP domains in higher eukaryotes. LUCA, last universal common ancestor.

Connecting new domains to new functions

Novel functions that are distinct from their role in translation have been identified for a few human aaRSs. Three examples of these functions, which depend on the addition of one or more new domains or motifs, are given below. Many additional examples are anticipated to emerge in the future as more and more of these new structural units are investigated.

TyrRS. FIGURE 1a traces the addition of two new sequence and structural elements to TyrRS in evolution. The tripeptide ELR (Glu-Leu-Arg) and the EMAPII domain were added at the stage of insects and have been retained ever since. Neither ELR nor EMAPII is found in lower eukaryotic, archaeal or bacterial TyrRSs. Although EMAPII is fused to the carboxy terminus of the catalytic domain of TyrRS, ELR is in the catalytic domain itself. However, mutations in ELR or ablation of EMAPII had little effect on the aminoacylation activity[16–18]. These observations suggest that the additions of ELR and EMAPII to higher eukaryotic TyrRSs are associated with new functions that are not related to aminoacylation. Furthermore, because the two additions occurred simultaneously, each might be needed for the development of the same new function.

Indeed, both additions are known to work in concert in receptor-mediated signalling pathways that are associated with angiogenesis[19]. ELR is the signature sequence of CXC chemokines that have angiogenic activity by binding CXC-chemokine receptor 1 (CXCR1) or CXCR2 (REF. 20). EMAPII binds α5β1 integrin receptor on the surface of endothelial cells and inhibits integrin-dependent cell adhesion and spreading. Furthermore, following internalization by endothelial cells, EMAPII inhibits hypoxia-inducible factor 1α (HIF1α)-regulated angiogenesis[21].

Two high resolution crystal structures of human TyrRS suggested an autoinhibitory structure in which the EMAPII domain of TyrRS blocks ELR, making it inaccessible to its receptors on the cell surface and thereby shielding or muting the embedded angiogenic activity of TyrRS[22,23]. After secretion of TyrRS, removal of EMAPII by proteolytic cleavage unmasks the ELR tripeptide motif. Thus, the two additions — a new domain (EMAPII) and a new tripeptide motif (ELR) — to TyrRS are connected to the same function.

TrpRS. FIGURE 1b traces the addition of the WHEP domain to TrpRS. The new domain was added at the stage of chordates and has been retained ever since. This domain has been found to have a regulatory function, as it inhibits the activation of the angiostatic activity of TrpRS that evolved in higher eukaryotes. TrpRS is strongly upregulated by interferon-γ (IFNγ), along with other angiostatic factors such as MIG (also known as CXCL9) and 10 kDa IFNγ-induced protein (IP10; also known as CXCL10)[24]. Indeed, removal of WHEP (by alternative splicing or proteolysis) activates TrpRS as a potent angiostatic factor[25–27], which then binds to vascular endothelial cadherin (VE-cadherin; a protein involved in endothelial cell adhesion and consequently angiogenesis)[28]. Strikingly, two protruding Trp side chains near the N terminus of VE-cadherin fit into the adenylate pocket (made up of Trp and AMP subpockets) of TrpRS, thereby inducing VE-cadherin–TrpRS complex formation[29]. High-resolution crystal structural analysis of human TrpRS showed that the position of the WHEP domain makes the subpockets sterically inaccessible to VE-cadherin while still allowing entry of the small molecule substrates (Trp and AMP) so as not to interfere with aminoacylation[30]. Similarly to TyrRS, at least one role for the newly added domain is to regulate the accessibility of a signalling motif in TrpRS that interacts with its cognate receptor.

GluRS–ProRS. A third example, which also involves a WHEP domain, is the unusual GluRS–ProRS (EPRS) fusion enzyme. In this case, the WHEP domain serves as a linker to join the two aaRSs. The WHEP domain first appeared in GluRS and ProRS in nematodes and was retained (in EPRS) ever since[31] (FIG. 1c). EPRS is part of the MSC found in higher eukaryotes[32]. Earlier work showed that, after stimulation with IFNγ, EPRS is phosphorylated and released from the MSC and then becomes part of the IFNγ-activated inhibitor of translation (GAIT) complex. This complex subsequently silences translation by binding (mediated by WHEP) to a stem loop structure (known as the GAIT element) in the 3′ untranslated region of one or more mRNAs that function in pathways for inflammation and iron homeostasis[33]. Thus, EPRS is linked to the inflammatory

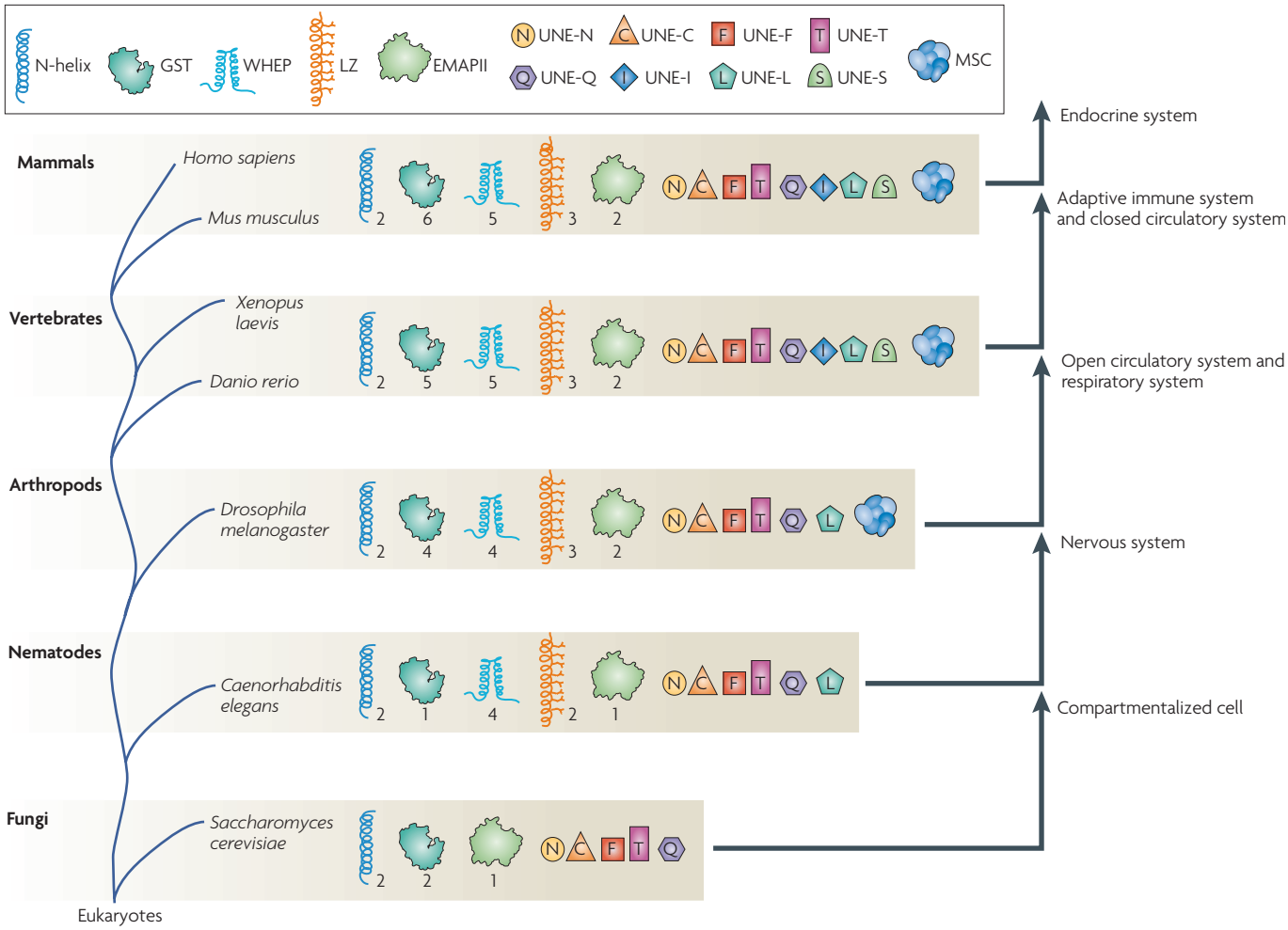

Figure 2 | **Temporal elaboration of new domains for all aaRSs and the increasing complexity of organisms.** The appearance of new domains that have been joined to eukaryotic aminoacyl-tRNA synthetases (aaRSs) is shown for specific clades, as increasingly complex organisms are presented in evolution. Each of the clades is represented by a model species for which sequence databases were complete for the aaRSs. These model species are: *Homo sapiens*, *Danio rerio*, *Drosophila melanogaster*, *Caenorhabditis elegans* and *Saccharomyces cerevisiae*. As a result of being limited to using model species for each clade, some domains not seen in the model species may be present in other species of the same clade as the various databases are expanded. The ensembles of all of the domain additions are clearly indicated by the increasing numbers of each new domain in aaRSs and of the multi-aaRS complex (MSC)-associated proteins that first appeared in arthropods. We designate the unique motifs as UNE-L, UNE-S and so on, in which the single letter designates the amino acid type of the aaRS that harbours the specific unique domain (so UNE-L is associated with LeuRS and UNE-S with SerRS). Note that, once a new domain is joined to an aaRS, it is irreversibly retained as the tree of life ascends. GST, glutathione S-transferase domain; LZ, Leu-zipper motif.

response. EPRS is also thought to repress the translation of proteins involved in other signalling pathways that are sensitive to signalling from IFNγ and hypoxia, including vascular endothelial growth factor (VEGF). This silencing is enabled by the RNA and protein interactions of the WHEP domains[34,35].

New domains and evolution

The three examples of TyrRS, TrpRS and EPRS are consistent with the idea that new domains fused to aaRSs are markers of new functions for these proteins. Other added domains have been identified, and their appearance correlates with the emergence of new biological functions.

More general appearance of new domains. To investigate the full extent of the elaboration of these markers, FIG. 2 shows the appearance at specific points in evolution of all of the new domains and motifs that have been joined to eukaryotic aaRSs. Three points are clear. First, the time of initial acquisition of a eukaryote-specific domain is specific to the aaRS. The idiosyncratic nature of the initial acquisition, together with the observation that most domains and motifs are found in only one or a few aaRSs, could suggest that each aaRS has a different expanded function (or functions)[36–38].

Second, the number of aaRSs that harbour additions grows progressively as the tree of life ascends, raising the possibility that the additions themselves are part of building the complexity of higher organisms. Indeed, 19 out of 20 human aaRSs (AlaRS being the exception) have added one or more new domains or motifs. For example, the number of aaRSs harbouring the GST domain increases from two in fungi to four in insects, to five in fish and six in humans.

Third, and most striking, after a new domain has been added to an aaRS at a specific point in evolution, it is conserved from then on as an integral part of the enzyme. Thus, progressive and accretive additions of new domains or motifs to aaRSs during evolution may reflect a role in building the progressive complexity of organisms.

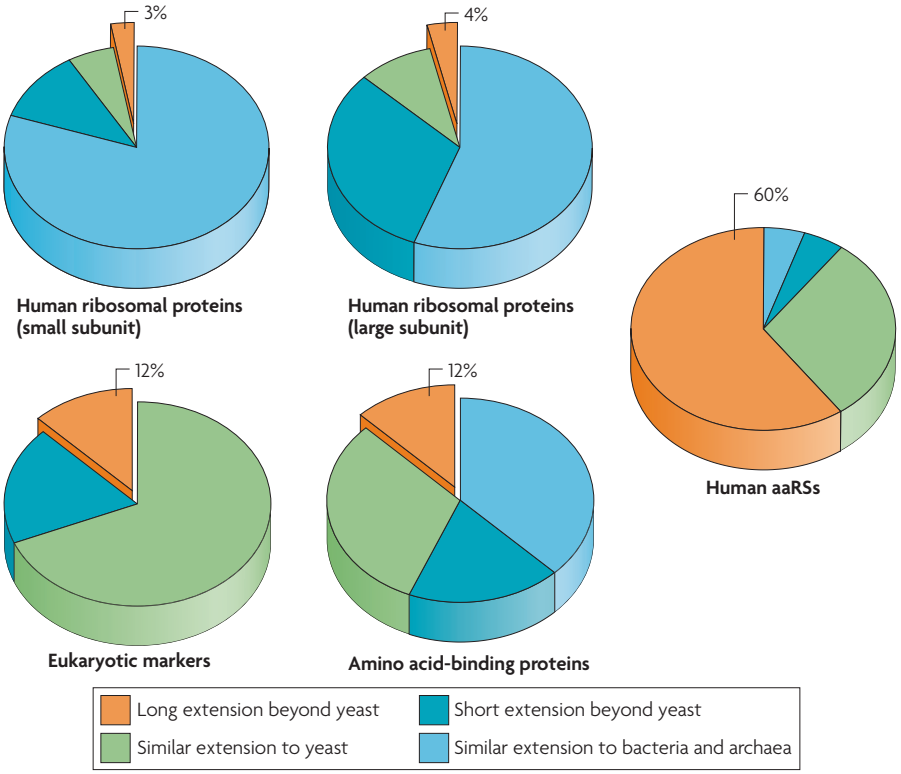

Figure 3 | **Sequence extensions of human ribosomal proteins, eukaryotic markers, amino acid-binding proteins and aaRSs.** Sequences of each of 79 human ribosomal proteins were analysed for appended sequences (domains or short peptide motifs) in a similar manner to that for aminoacyl-tRNA synthetases (aaRSs). The sequences were organized into four groups: similar to bacterial and archaeal orthologues (0–25 amino acids); short eukaryote-specific extensions beyond yeast (25–80 amino acids); lower eukaryote-specific extensions (found, for example, in yeast) that are longer than 80 amino acids but are not further extended in species higher than yeast; and higher eukaryote-specific extensions that were added only in species higher than yeast and that are longer than 80 amino acids. Percentages for the long extension beyond yeast group are shown. A similar analysis was done on two more protein groups. One was a group of 16 of the 17 recently identified protein markers that can be used to assemble the eukaryotic tree of life (one protein was left out because its sequence is incomplete); most members of this group have no bacterial or archaeal orthologue. The other was a group annotated as amino acid-binding proteins (Gene Ontology Database term 0016597). After removing duplicated genes, incomplete sequences and aaRSs, 16 proteins remained and were analysed.

New biology correlates with new functions. To elaborate further on the possibility that these domains have roles in building the progressive complexity of organisms, we considered the development and expansion of the vascular system and the associated angiogenesis signalling pathways. If specific new domains or motifs were important for developing (or for being part of) new vascular biology associated with higher organisms, then the appearance of these domains or motifs in evolution should coincide with the emergence of the new biology. In this regard, it is interesting to note that the ELR motif and the EMAPII domain of TyrRS and the WHEP domain of TrpRS (which have roles in vascular biology (see above)) are absent in *Caenorhabditis elegans*, which has no circulatory system. By contrast, the addition of ELR and EMAPII to TyrRS first appeared with the emergence of insects, which have a primitive open circulatory system. As blood circulation further developed into the advanced closed circulatory system of vertebrates, more regulators of angiogenesis were needed. Extra domains (or genes) added during this transition could potentially serve such a function. Interestingly, a WHEP domain was added to TrpRS at this step. In addition, a unique sequence motif, UNE-S, emerged in the C terminus of SerRS of all vertebrates, from fish to humans.

Independently, three forward-genetics mutational studies in zebrafish suggested a role for SerRS in vasculature development, which is not related to the enzymatic function of the aaRS[39–41]. Two studies suggested a role for zebrafish SerRS in regulating VEGF-directed angiogenesis[40]. Specifically, they found that two of three SerRS mutations that caused defective vasculature resulted in premature stop codons that deleted the UNE-S domain[40,41]. Thus, we raise the possibility that the function of UNE-S is related to the development of a closed vascular system. Similarly, the biological function of the other UNEs is of great research interest. It will be important to examine the correlation of domain additions with the emergence of other biological functions.

Markers are unique to aaRSs

The biological complexity of an organism is facilitated by alternative splicing of pre-mRNAs[42], post-translational modifications, paralogous gene duplications[43] and the adaptation of protein multifunctionality[44,45]. Among these possibilities, multifunctionality of a single protein is considered a more efficient way to coordinate the organization and maintenance of a global system[46], especially for solving the discrepancy of species complexity with the limited number of host genes. Over the years, numerous proteins have been found to have many distinct functions that are achieved by domain combinations or by the modification of unused surface areas[47–50]. However, these multifunctional proteins belong to different protein families and their many distinct functions are specific to them. They therefore lack the coherence at the family level that is seen with aaRSs.

Among multifunctional proteins, ribosomal proteins are an interesting protein family[51]. Many ribosomal proteins have multiple functions in bacteria, archaea and eukaryotes[52,53], and several of these functions are related to ribosomal biogenesis, such as surveillance of ribosome biosynthesis (for example, by the binding of ribosomal proteins to their own mRNAs or rRNAs). In addition, numerous ribosomal proteins have non-ribosomal functions: many bind and activate p53-related E3 ubiquitin ligase MDM2, 40S ribosomal protein S3 binds damaged DNA in *D. melanogaster* and nicks DNA at abasic sites in mammalian cells[51], and 60S ribosomal protein L13a participates (together with EPRS) in the GAIT complex-mediated regulation of translation of mRNAs that are associated with inflammatory pathways[54,55].

We were interested to see whether the expanded functions of the ribosomal proteins are also associated with new domain additions. For this purpose, a similar analysis to that described above was carried out with the ~80 cytoplasmic ribosomal proteins. In contrast to the aaRSs, more than half of the ribosomal proteins in eukaryotes

are almost identical in length to their archaeal homologues (FIG. 3). Most of the rest have fewer than 25 amino acids as extensions at the N or C terminus, and just 7 have extensions of longer than 80 amino acids. But strikingly, no stepwise, progressive additions occurred during the long evolution of eukaryotes: most of the longer extensions of human ribosomal proteins are also seen in yeast. However, with aaRSs, progressive domain additions clearly correlate with the increasing complexity of organisms (FIG. 2) and impart functions that are unrelated to aminoacylation.

Concerned about the unusual conservation of scaffolding ribosomal proteins, we also looked at a new set of eukaryotic molecular markers[56]. These are a group of functionally different proteins, the sequences of which can be used to construct a phylogenetic tree of eukaryote evolution. These markers include the α-subunit of the transport protein Sec61, ubiquitin-activating enzyme E1 (UBA1), the spliceosome subunit U5 small nuclear ribonucleoprotein and the RNA polymerase II initiation factor TFIIH. The length of these proteins varies from 300 to ~2,000 amino acids. Again, aaRSs showed a much higher portion (60%) of domain additions in higher eukaryotes than these eukaryotic markers (12%) (FIG. 3). A second analysis was carried out on human amino acid-binding proteins (as labelled in the Gene Ontology Database) that are shared across different species, yielding similar results.

Perspective

Our analysis indicates that aaRSs are unique in their acquiring of new activities through the addition of new domains that correlate with the progressive complexity of eukaryotes. It is important to note that some appended domains improve the canonical function. For example, the WHEP domain of human MetRS has a tRNA-sequestering function[57], and the Leu-zipper motif in ArgRS is important for the formation of the MSC, which can enhance channelling of tRNA to the protein synthesis machinery[58]. It is therefore possible that the added domains or motifs may have later adopted new functions beyond translation.

New domains are essential for each of the three orthogonal (that is, beyond translation) functions of the human aaRSs discussed above. We speculate that other examples will also be shown to require one or more new domains or motifs for elaboration and regulation of the orthogonal activity. Two instances, among others, are human LysRS and GlnRS, which have developed functions in the immune response and cell death. LysRS is phosphorylated through the mitogen-activated protein kinase pathway in stimulated mast cells[38]. It is then released from the cytoplasmic MSC and translocated to the nucleus, where it forms a complex with the transcription factor microphthalmia-associated transcription factor (MITF) to enable the expression of genes that regulate the immune response. GlnRS interacts with apoptosis signal-regulating kinase 1 (ASK1; also known as MAP3K5) and inhibits cell death induced by ASK1 in a Glu-dependent manner through its catalytic domain[59]. Although their functions have not been defined, domains not directly associated with aminoacylation have been added to each of these aaRSs (FIG. 2).

The aaRSs are ancient and were perhaps the first proteins to develop sites for binding specific amino acids. Thus, they were in an ideal spot to develop new functions. By using a pre-existing amino acid- and AMP-binding pocket, as was done for the interaction of TrpRS with the protruding side chains of VE-cadherin, nature avoided the re-invention of another amino acid side chain-binding site[29]. Therefore, in at least some instances, the new, expanded functions of these enzymes may have been initiated through random interactions with protruding side chains on other proteins. After an initial contact, an interaction may have been further developed and refined with sophisticated domain additions to the aaRS.

If the amino acid-binding pocket played a key part as an evolutionary force to develop new protein–protein interactions, other amino acid-binding proteins might also show a progressive addition of new domains. However, our analysis showed that aaRSs have a much higher percentage of acquired sequence extensions than these amino acid-binding proteins. This result further highlights the uniqueness of aaRSs, and also shows that amino acid-binding pockets are not sufficient to explain the robust development of new domains and functions associated with aaRSs.

We raise the possibility that the domain additions in aaRSs were needed, at least in part, for the development of the complexity of organisms, by connecting aaRSs to pathways of angiogenesis[16], the immune response[38], inflammation[33], apoptosis[59] and neural development[15,60]. Because the aminoacylation activities of aaRSs are not dispensable, the new functions had to evolve in the tight constraints of an essential genetic environment. Therefore, disease-causing mutations associated with aaRSs can occur only if aminoacylation activity is kept sufficient. It is perhaps for this reason that there are many diseases associated with aaRSs[61,62], which in some instances do not involve the aminoacylation function (such as some of the dominant mutations in the genes encoding TyrRS or GlyRSs that cause the peripheral neuropathy Charcot–Marie–Tooth disease[63–65]). It will be important to investigate (in appropriate model organisms) the effects of point mutations and deletions in newly added domains on organismal development and homeostasis. It is these kinds of experiments that can test more rigorously whether these accretive domain additions were essential for building the increasing complexity of the tree of life. However, even without these data being available at this time, the existing work suggests that the new domain additions can act as starting points for discovering more functions of eukaryotic aaRSs beyond translation and for understanding some of the many disease associations.

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doi:10.1038/nrm2956
Published online 11 August 2010

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Acknowledgements
This work was supported by grants GM 15539, GM 23562 and U54RR025204 from the National Institutes of Health, grant CA92577 from the National Cancer Institute and a fellowship from the National Foundation for Cancer Research.

Competing interests statement
The authors declare no competing financial interests.

DATABASES
UniProtKB: http://www.uniprot.org
ASK1 | CXCR1 | CXCR2 | EPRS | HIF1α | IFNγ | IP10 | MDM2 | MIG | MITF | UBA1

FURTHER INFORMATION
Paul Schimmel's homepage: www.scripps.edu/mb/schimmel-yang

ALL LINKS ARE ACTIVE IN THE ONLINE PDF