Minireview

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Conserved function, divergent evolution: mitochondrial outer membrane insertases across eukaryotes

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Abstract: Mitochondrial function relies heavily on the proper targeting and insertion of nuclear-encoded proteins into the outer mitochondrial membrane (OMM), a process mediated by specialised biogenesis factors known as insertases. These insertases are essential for the membrane integration of α-helical OMM proteins, which contain one or multiple hydrophobic transmembrane segments. While the general mechanisms of mitochondrial protein import are well established, recent research has shed light on the diversity and evolutionary conservation of OMM insertases across eukaryotic lineages. In *Saccharomyces cerevisiae*, the mitochondrial import (MIM) complex, composed of Mim1 and Mim2, facilitates the integration of various α-helical OMM proteins, often in cooperation with import receptors such as Tom20 and Tom70. In *Trypanosoma brucei*, the functional MIM counterpart pATOM36 performs a similar role despite lacking sequence and structural homology, reflecting a case of convergent evolution. In mammals, MTCH2 has emerged as the principal OMM insertase, with MTCH1 playing a secondary, partially redundant role. This review provides a comparative analysis of these insertases, emphasising their conserved functionality, species-specific adaptations, and mechanistic nuances.

Keywords: insertases; MIM complex; mitochondrial outer membrane; MTCH2; pATOM36

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1 Introduction

Under the endosymbiotic theory, mitochondria are proposed to have evolved from ancient, free-living prokaryotes that were engulfed by a larger cell, creating a symbiotic relationship. Supporting this hypothesis, mitochondrial DNA (mtDNA) exhibits a circular topology reminiscent of bacterial genomes, mitochondria similarly to Gram-negative bacteria are surrounded by a double membrane system, and mitochondria retain bacterial-like features in their metabolic enzymes and membrane transport systems (Kizmaz and Herrmann 2023; West et al. 2011). Among these similarities are the presence of β-barrel porins in the outer membrane, the occurrence of the Krebs cycle, and production of ATP via oxidative phosphorylation.

Biogenesis factors are crucial for mitochondrial performance since majority of mitochondrial proteins and all outer mitochondrial membrane (OMM) proteins are encoded by the nuclear genome and need to be synthesized in the cytosol and transported to the mitochondrial surface before being imported into the right organellar sub-compartment (Wiedemann and Pfanner 2017). The OMM, which hosts in fungi a few dozen proteins and in mammalian cells probably more, plays a key role in this process, hosting various proteins involved in import and sorting of substrate proteins (Papić et al. 2013). It also acts as a selective barrier, limiting access of proteins and large solutes to the inner compartments of the organelle (Heiden et al. 2000). The primary entry site for mitochondrial precursor proteins is the translocase of the outer mitochondrial membrane (TOM) complex, or its kinetoplastid equivalent, the archaic TOM (ATOM) in *Trypanosoma brucei*. For β-barrel proteins, the sorting and assembly machinery (SAM) complex, known also as the TOB complex, facilitates translocation and membrane integration (Kutik et al. 2008; Paschen et al. 2003). Among the OMM biogenesis factors are insertases that facilitate the membrane integration of α-helical transmembrane segments of OMM proteins (Dimmer et al. 2012; Papić et al. 2011).

The substrates of OMM insertases carry either a single membrane spanning α-helical segment or multiple

α-helical segments throughout the protein (Doan et al. 2020). The first OMM insertase to be identified was the MIM (Mitochondrial Import) complex in *Saccharomyces cerevisiae* (Figure 1) (Becker et al. 2011; Ishikawa et al. 2004; Papić et al. 2011; Waizenegger et al. 2005). Subsequently, pATOM36 was identified in *T. brucei* as a functional equivalent of the MIM complex (Pusnik et al. 2012; Vitali et al. 2018a). More recently, MTCH2 was defined as the principal insertase for α-helical OMM proteins in mammals, with MTCH1 playing a secondary, partially redundant role in facilitating the biogenesis of MTCH2 substrates (Figure 1) (Guna et al. 2022).

This review provides a comparative overview of mitochondrial outer membrane insertases across diverse eukaryotic lineages, highlighting both their conserved function, despite lineage-specific adaptations. By examining the diversification of these membrane integrating systems in protozoa, fungi, and mammals, we seek to elucidate how core principles of protein insertion have been preserved, while also accommodating the unique functional demands of different eukaryotic organisms.

2 OMM insertases in yeast – MIM complex

In *S. cerevisiae*, the MIM complex comprises two α-helical transmembrane proteins, Mim1 and Mim2. Although high-resolution structural insights are currently lacking, biochemical analyses indicate that the complex consists of multiple copies of Mim1 accompanied by one or two copies of Mim2. Further, it was demonstrated that Mim1 is crucial for complex formation; its absence abolishes complex assembly, whereas Mim2 deletion permits the formation of a Mim1-only complex (Dimmer et al. 2012). The transmembrane segment of Mim1 is both necessary and sufficient for oligomerization and function, whereas its cytosolic N- and C-terminal domains are dispensable (Popov-Čeleketić et al. 2008).

While deletion mutants lacking Mim1 are viable, they suffer from compromised cell growth, alterations in mitochondrial morphology, and reduced protein import efficiency (Hulett et al. 2008; Ishikawa et al. 2004; Waizenegger et al. 2005). Mitochondria lacking Mim1 exhibit reduced levels of Tom20, Tom70, and the small Tom proteins, indicating a dependency on Mim1 for their insertion. Although Tom40, a β-barrel protein, is not directly integrated by Mim1, its levels are also somewhat reduced in *mim1Δ* mutants, likely due to impaired assembly of the TOM complex as a whole (Becker et al. 2011; Vitali et al. 2018a). The effect of Mim1 on the functionality of the TOM complex led to its initial identification as a factor that generally influences the import of mitochondrial proteins (Ishikawa et al. 2004; Mnaimneh et al. 2004). This general effect became later clear when it was observed that the levels of the import receptor Tom20 are highly reduced in the absence of Mim1 and its assembly into the TOM complex is substantially dependent on the function of Mim1 (Becker et al. 2008; Hulett et al. 2008; Waizenegger et al. 2005). Specifically, the transmembrane domain of Tom20 contains critical residues essential for its correct docking within the TOM complex. This precise insertion and positioning are facilitated by Mim1. Accordingly, disrupting the interaction of Tom20 with Mim1 through either mutations in the transmembrane segment of Tom20 or the deletion of Mim1, impairs both the association of Tom20 with the TOM core complex and Tom20's function. Mim1 was thus identified as a unique assembly factor, catalyzing a docking reaction between an α-helical transmembrane segment and a membrane protein complex formed around a β-barrel structure (Becker et al. 2008; Hulett et al. 2008).

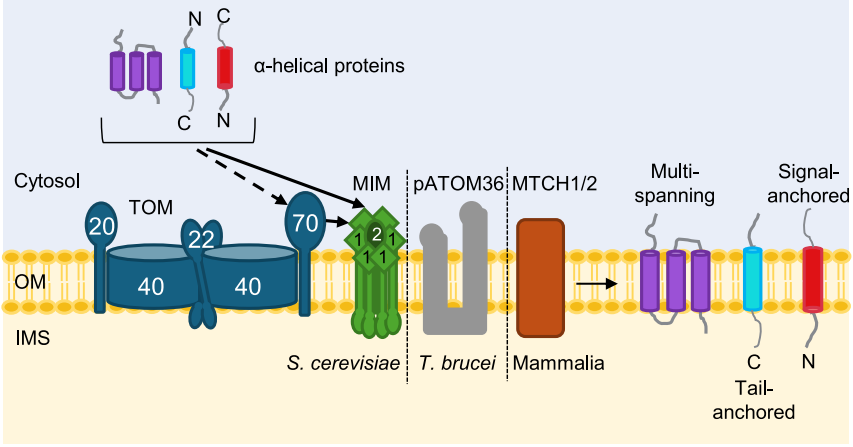

Figure 1: Overview of α-helical protein insertion into the mitochondrial outer membrane by insertases from different species. In *S. cerevisiae*, precursor proteins either get directly inserted into the OMM by the MIM complex or they initially interact with the Tom70 receptor and then are relayed to the MIM complex, which mediates their insertion into the membrane. IMS, intermembrane space; OM, outer membrane.

The exact route of α-helical precursor proteins from their synthesis in the cytosol to their integration into the OMM, remains only partially resolved. Recent research has provided insight into early cytosolic events during the targeting of signal-anchored (SA) α-helical OMM proteins. These precursors interact with cytosolic chaperones, including Hsp70, Hsp90, and Hsp40 family co-chaperones such as Ydj1 and Sis1, via their hydrophobic transmembrane segments (Drwesh et al. 2022). These (co)chaperone-substrate interactions facilitate recognition by the mitochondrial import receptors Tom70 and Tom20 and promote subsequent membrane integration (Becker et al. 2011). Whether similar chaperones interact directly with the MIM complex itself remains an open and intriguing question. In addition, Tom70 has been shown to act also as a cytosolic receptor for precursor proteins with multiple α-helical transmembrane domains, which are then transferred to the MIM complex for insertion into the OMM. On the other hand, MIM substrates like Atg32 (required for mitophagy) and Gem1 (a mitochondrial GTPase) were found to be only moderately dependent on Tom70 and instead, their targeting is facilitated by Tom20 (Eaglesfield and Tokatlidis 2021; Vitali et al. 2020). The MIM complex was also suggested to support the biogenesis of a separate class of α-helical proteins that are inserted into the OMM from the intermembrane space (IMS), such as Om45 and Mcp3 (Sinzel et al. 2017; Wenz et al. 2014).

Another distinct group of putative MIM substrates includes Fis1 and Mcr1, which, despite exhibiting characteristics typical of Mim1-dependent proteins, appear unaffected by Mim1 deletion (Vitali et al. 2020). Fis1 is a mitochondrial fission protein that localizes to both mitochondria and peroxisomes (Aravindan et al. 2025) and has been shown to insert into synthetic lipid vesicles in an unassisted, spontaneous manner (Kemper et al. 2008). Mcr1 exists in two isoforms: one anchored to the OMM and another localized within the intermembrane space (IMS). Although the OMM isoform of Mcr1 adopts a signal-anchored topology similar to canonical Mim1 substrates (like Tom20 or Tom70), its steady-state mitochondrial levels remain unchanged in *mim1Δ* cells (Vitali et al. 2020). Interestingly, in the absence of Mim1, a subpopulation of Fis1 and the OMM isoform of Mcr1 were observed to mislocalise to the endoplasmic reticulum (ER) (Doan et al. 2020). The basis for this mistargeting remains unclear; however, it has been proposed that the MIM complex may contribute to a quality control mechanism that prevents mistargeted proteins from aberrantly associating with the ER membrane (Vitali et al. 2020).

Although Mim2 is present at lower stoichiometry in the MIM complex (presumably one to two copies per complex), its deletion also results in reduced steady-state levels of α-helical OMM proteins, impaired complex assembly, growth defects, and altered mitochondrial morphology (Dimmer et al. 2012). Interestingly, overexpression of Mim2 can rescue the growth phenotype of *mim1Δ* cells (Dimmer et al. 2012; Dimogkioka et al. 2024), raising important questions regarding potential functional redundancy or compensatory mechanisms between the two subunits. Despite the prevailing view that Mim1 is the dominant and essential subunit for complex formation, this observation suggests a more nuanced interplay between Mim1 and Mim2.

While deletion of both Mim1 and Mim2 leads to a marked reduction in the abundance of α-helical mitochondrial outer membrane (MOM) proteins, direct evidence of substrate binding by either protein, under normal expression levels, is still lacking. Consequently, the precise molecular mechanism by which these insertases mediate membrane insertion remains unresolved. It is unclear whether Mim1 and Mim2 directly engage with precursor proteins to facilitate their integration into the lipid bilayer, or whether they function indirectly by perturbing the membrane to enable unassisted insertion of substrates.

Biophysical analyses have provided some insights into their potential mode of action. Purified Mim1 exhibited characteristic channel activity upon reconstitution into planar lipid bilayers, which was inhibited by Mim1-specific antibodies, suggesting a specific functional role. Co-reconstitution of Mim2 with Mim1 revealed that the Mim1–Mim2 complex forms a cation-selective channel, which could be conducive to the translocation of positively charged precursor segments (Krüger et al. 2017). Currently, it is unclear how the capacity of Mim1 to form an ion conducting channel is related to its function as an insertase.

Furthermore, it remains to be determined whether insertion occurs co-translationally, coupled to ribosomal synthesis, or post-translationally, following complete cytosolic synthesis. Structural elucidation of the MIM complex and *in vitro* interaction studies with purified components will be critical for advancing our understanding of its role in α-helical protein insertion and the potential interplay with cytosolic chaperone systems.

3 OMM insertase in *Trypanosoma* – pATOM36

The OMM insertase pATOM36 was identified in *T. brucei* as a novel component associated with the ATOM complex, the primary translocase responsible for mitochondrial protein import in trypanosomes. Initially, pATOM36 was implicated in facilitating the import of a subset of mitochondrial matrix

proteins. Subsequent studies revealed that it also plays a role in the assembly and/or membrane integration of a specific group of OMM proteins, including subunits of the ATOM complex itself (Pusnik et al. 2012).

Interestingly, pATOM36 exhibits dual localization: aside from its mitochondrial function, a fraction of the protein associates with the tripartite attachment complex (TAC), a specialized structure that connects the basal body of the flagellum to the kinetoplast DNA (kDNA). Depletion of pATOM36 results in kDNA missegregation, highlighting its role in both mitochondrial protein biogenesis and organelle inheritance (Käser et al. 2016). Although pATOM36 exhibits dual functionality, no evidence suggests a role for Mim1 or Mim2 in mitochondrial genome inheritance.

Functionally, the role of pATOM36 in ATOM complex assembly is reminiscent of the contribution of Mim1 and Mim2 to TOM complex biogenesis in yeast. However, despite these functional parallels, pATOM36 shares neither sequence nor structural homology with Mim1 or Mim2. While the Mim proteins are each predicted to contain a single transmembrane domain, pATOM36 is predicted to span the membrane at least twice. Nevertheless, given that the structures of the MIM complex and pATOM36 remain unresolved, a definitive comparison of topology and architecture cannot yet be made. At the sequence level, a single conserved structural motif may suggest a shared mechanistic feature; all three proteins contain a GxxxG(A) motif, mediating transmembrane helix–helix interactions. In pATOM36, this motif is located in only one of its transmembrane domains, and deletion of the C-terminal 75 amino acids, including the GxxxG(A) motif, completely abolishes its role in ATOM complex assembly (Käser et al. 2016; Vitali et al. 2018a). In yeast, mutating this motif resulted in compromised oligomerization of Mim1 and delayed growth (Popov-Čeleketić et al. 2008). These findings suggest that, although structurally divergent, both the MIM complex and pATOM36 may rely on similar helix-helix interactions to perform their insertase function.

Despite the lack of sequence or structural homology, functional complementation studies have demonstrated that pATOM36 is a functional equivalent of the yeast MIM complex. Expression of pATOM36 in *S. cerevisiae* cells lacking Mim1, Mim2, or both, successfully rescued the growth defect to a degree comparable to that observed upon re-introducing Mim1. Moreover, pATOM36 restored the steady-state levels of canonical MIM substrates such as Tom20 and Tom70, as well as indirectly affected proteins like Tom40, whose assembly depends on proper TOM complex biogenesis. Interestingly, Ugo1, another α-helical outer membrane protein, was not restored to normal levels, suggesting substrate selectivity in pATOM36-mediated insertion (Vitali et al. 2018a).

In vitro import assays using mitochondria isolated from MIM complex deletion cells confirmed that pATOM36 could partially rescue the import defect of Tom20, but not of Ugo1, reinforcing its substrate specificity. Furthermore, pATOM36 expression corrected the aberrant mitochondrial morphology observed in MIM-deficient cells, restoring the typical wild-type tubular network (Vitali et al. 2018a). These findings highlight the conserved functional role of pATOM36 in OMM protein biogenesis, despite evolutionary divergence in sequence and structure, and point to intriguing mechanistic differences in substrate recognition between insertases across species.

The next logical question was whether the MIM complex could reciprocally take over the function of pATOM36. In line with the ability of pATOM36 to complement the absence of the MIM complex in yeast, it was shown that co-expression of Mim1 and Mim2 can rescue the protein biogenesis defects observed in pATOM36-deficient *T. brucei*. Of note, expression of Mim1 or Mim2 alone was insufficient to restore function, highlighting the necessity for both components (Vitali et al. 2018a). Importantly, successful complementation required that Mim1 and Mim2 be expressed at similar stoichiometric levels, suggesting a cooperative mechanism between the two proteins in facilitating OMM protein biogenesis.

4 OMM insertase in mammals – MTCH1 and MTCH2

The identification of the OMM insertase in mammalian cells was a long-standing question that was only recently resolved. MTCH2, a member of the SLC25 carrier protein family, which was previously implicated in lipid metabolism, mitochondrial fusion, and apoptosis (Labbé et al. 2021; Zaltsman et al. 2010), has now been established as the principal insertase for α-helical OMM proteins (Guna et al. 2022). Although extensively studied, its role in mitochondrial protein biogenesis remained elusive until depletion studies and usage of a split-GFP assay revealed that MTCH2 loss led to reduced steady-state levels of several α-helical OMM proteins, including known mammalian homologues of substrates of the yeast MIM complex such as Tom20 and Tom70 (Guna et al. 2022).

Interestingly, MTCH1, a paralogue of MTCH2 and also a member of the SLC25 family, was shown to have a complementary, albeit less pronounced, role. Unlike most SLC25 members, MTCH1 and MTCH2 do not localize to the inner mitochondrial membrane and lack a yeast homologue. Co-depletion of MTCH1 and MTCH2 resulted in an

additive reduction in the biogenesis of α-helical OMM proteins, suggesting partial redundancy or cooperation between the two proteins (Guna et al. 2022). However, a subsequent study examined MTCH1 independently and demonstrated that its depletion alone did not affect the levels of specific OMM proteins, indicating a more supplementary role in membrane insertion (Muthukumar et al. 2024). Both MTCH1 and MTCH2 are predicted to contain six transmembrane α-helices. Even so, MTCH1 consists of 389 amino acids, compared to 303 residues in MTCH2, with the difference primarily attributed to an extended N-terminal domain in MTCH1, which AlphaFold predicts to be disordered.

As observed with pATOM36, MTCH1 and MTCH2 exhibit no structural or sequence homology with neither the yeast Mim proteins nor with the *T. brucei* pATOM36. Furthermore, although the GxxxG(A) motif is present in the sequences of MTCH1 and MTCH2, it does not appear within the α-helical segments of the proteins in the predicted structures by AlphaFold (Jumper et al. 2021). To investigate whether these mammalian proteins could functionally compensate for the loss of the MIM complex, MTCH2 and MTCH1 were expressed in *S. cerevisiae* strains lacking Mim1, Mim2, or both. Surprisingly, MTCH1, rather than MTCH2, was able to rescue the growth defect associated with MIM complex deletion. In contrast, expression of MTCH2 not only failed to complement the growth phenotype but also appeared to exert a mildly deleterious effect on yeast growth. Functionally, MTCH1 restored steady-state levels of known MIM substrates, improved mitochondrial protein import capacity, and reinstated TOM complex assembly and mitochondrial morphology to near wild-type conditions (Dimogkioka et al. 2024). Given this unexpected functional divergence, it will be of interest to study the structural features of MTCH1 and MTCH2.

The existence of both MTCH1 and MTCH2 raises intriguing questions about their necessity and potential functional divergence. Expression data from the Human Protein Atlas (www.proteinatlas.org) reveals that MTCH1 is consistently expressed across a wide range of tissues, whereas MTCH2 displays more variable and generally lower expression levels. Notably, MTCH2 is more abundant in respiratory tissues and male-specific organs such as the testis. This differential expression suggests a potential tissue-specific requirement or regulation, likely hinting at functional specialization. However, further studies are required to clarify whether the two proteins can functionally compensate for one another in mammalian systems. For example, testing whether MTCH1 overexpression can rescue MTCH2 deficiency, and vice versa.

5 General characteristics of OMM insertases

The diversity of insertases across species, ranging from the MIM complex in yeast, to pATOM36 in *T. brucei*, and MTCH1/2 in mammals, leads to a fundamental question: what defines an insertase? Despite their similar function in facilitating the membrane integration of α-helical OMM proteins, these insertases share no obvious sequence or structural homology. Furthermore, yeast employs a complex of two subunits (Mim1 and Mim2), while trypanosomes and mammals appear to utilise a single protein to fulfil this role. The mechanistic details of how these insertases function remain unclear. To date, direct interaction between these insertases and their substrates has not been conclusively demonstrated.

A prevailing model proposes that insertases function by modifying the lipid environment, similar to scramblases; proteins that facilitate the redistribution of phospholipids between bilayer leaflets. Recent computational and experimental work showed that MTCH2 contains a hydrophilic groove spanning the membrane, reminiscent of known scramblases such as VDAC, which is known to form a channel for the transport of ions and small metabolites (Bartoš et al. 2024). Molecular dynamics simulations confirmed that MTCH2 can lower the energetic barrier for lipid flip-flop across the bilayer, demonstrating lipid scrambling activity. The proposed scramblase capacity of MTCH2 might be related to additional putative functions of the protein as regulator of metabolism and lipid homeostasis (Chourasia et al. 2025; Labbé et al. 2021). Interestingly, this scrambling activity appears to be a shared property of many insertases and both MTCH1 and MTCH2 were proposed to exhibit such capacity. This suggests that the presence of a hydrophilic groove may be a defining structural feature of insertases. Similar architectures are found in other membrane insertases, such as YidC/Oxa1 and the ER membrane complex (EMC) (Li et al. 2024; McDowell et al. 2021).

A common trait among insertases is their involvement in the biogenesis of the TOM/ATOM complex and other α-helical OMM proteins. However, there is a degree of substrate specificity that differs between species. For instance, Ugo1 is unaffected by pATOM36, and similarly, Fis1 and Mcr1 can integrate into the OMM even in the absence of the MIM complex. Moreover, although MTCH1 can rescue the growth defect in MIM-deficient yeast, it does not appear to significantly affect the levels of canonical MTCH2 substrates when it individually knocked down in mammalian cells. These findings suggest that, despite

functional convergence, insertases have diverged evolutionarily, likely adapting to species-specific requirements.

6 Insertase biogenesis – chicken or the egg?

An open question is how insertases themselves are integrated into the mitochondrial outer membrane. This presents a classic "chicken-and-egg" dilemma: if insertases are required for membrane insertion, how are the first insertase molecules embedded in the bilayer in the first place? One possibility is that a small number of insertase molecules can spontaneously integrate into the lipid bilayer. Supporting this idea, the observation that re-expression of Mim1 or heterologous insertases (such as MTCH1 or pATOM36) can rescue the growth defects of Mim1 deletion strains implies that some initial membrane integration can occur independently of pre-existing MIM complexes (Dimogkioka et al. 2024; Vitali et al. 2018a). This suggests that the biogenesis of re-introduced insertases may begin through a low-efficiency, spontaneous insertion process, which is later amplified as newly inserted insertase molecules facilitate the membrane integration of subsequent ones.

Given the existence of insertase-like proteins in other organelles, such as the ER, an important question is what ensures the organelle-specific localisation of mitochondrial insertases. It is still unclear whether the transmembrane domain alone determines their targeting to mitochondria or whether additional signals or cytosolic factors are required. For example, when Mim1 was fused to GFP at its N-terminus and overexpressed, it showed some mislocalisation to the ER (Vitali et al. 2018b). This mislocalisation was dramatically enhanced in the absence of Djp1, a J-protein co-chaperone implicated in Mim1 targeting (Papić et al. 2013). This observation suggests that specific targeting of insertases may rely on chaperone-mediated guidance or other cytosolic factors. Another possibility is that Mim1 mislocalisation to the ER is influenced by Spf1, an ER P5-type ATPase (Cronin et al. 2002). Spf1 plays a critical role in maintaining the lipid and sterol composition of intracellular membranes (Cronin et al. 2002) and its deletion promoted mistargeting of OMM tail-anchored proteins (like Gem1 and Fis1) to the ER (Krumpe et al. 2012). Later studies clearly demonstrated that Spf1 extracts mistargeted proteins from the ER membrane (McKenna et al. 2020). Thus, it is likely that the correct localisation of Mim1 to the OMM depends on Spf1 activity. Lastly, Mim1 mislocalisation might occur under stress conditions or during interactions with other organelles, such as the ER or lipid droplets. Understanding the determinants of mitochondrial specificity for insertases like Mim1, MTCH1/2, and pATOM36 will be essential to fully comprehend their regulation and function.

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