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Review

Calcium regulation of mitochondria motility and morphology

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ABSTRACT

In the Fifties, electron microscopy studies on neuronal cells showed that mitochondria typically cluster at synaptic terminals, thereby introducing the concept that proper mitochondria trafficking and partitioning inside the cell could provide functional support to the execution of key physiological processes. Today, the notion that a central event in the life of every eukaryotic cell is to configure, maintain, and reorganize the mitochondrial network at sites of high energy demand in response to environmental and cellular cues is well established, and the challenge ahead is to define the underlying molecular mechanisms and regulatory pathways. Recent pioneering studies have further contributed to place mitochondria at the center of the cell biology by showing that the machinery governing remodeling of mitochondria shape and structure regulates the functional output of the organelle as the powerhouse of the cell, the gateway to programmed cell death, and the platform for Ca^{2+} signaling. Thus, a raising issue is to identify the cues integrating mitochondria trafficking and dynamics into cell physiology and metabolism. Given the versatile function of calcium as a second messenger and of the role of mitochondria as a major calcium store, evidences are emerging linking Ca^{2+} transients to the modulation of mitochondrial activities. This review focuses on calcium as a switch controlling mitochondria motility and morphology in steady state, stressed, and pathological conditions.

1. Introduction

In any given eukaryotic cell, the architecture of the mitochondrial network is determined by the molecular machineries governing the trafficking and shape of the organelle. Its design reflects the physiological state of the cell in steady state, stressed, and pathological conditions because the mechanisms controlling mitochondrial dynamics also regulate the function of the organelle as the powerhouse of the cell, the gateway to programmed cell death, and the platform for Ca^{2+} signaling. In this process, calcium is emerging as a relay preposed to the intracellular distribution of the organelle at sites of high energy demand as well as a molecular switch for a number of mitochondrial membrane remodeling proteins. This review focuses on the aspects of mitochondria motility and dynamics that are directly or indirectly related to calcium, and it is intended to complement recent authoritative reviews on mitochondrial transport [1–5], dynamics [6,7], apoptosis [8–11], control of neuronal activities [12], and link to cell signaling [13]. We apologize in advance for omitting to quote every publication that has contributed to make emerge such exciting fields.

2. Distributing, positioning, anchoring, and regulating mitochondria activity at sites of high energy demand through calcium

The first observations describing differential positioning of mitochondria in cultured cells were reported nearly a century ago [14]. Forty years later, in the Fifties, electron microscopy studies showed that mitochondria typically cluster at synaptic terminals [15], eliminating the concept that the architecture of the mitochondrial network is the outcome of a random distribution process of the organelle, and introducing the possibility that proper mitochondria trafficking and partitioning inside the cell could provide functional support to the execution of key physiological processes [16–18]. Today, the notion that a central event in the life of every eukaryotic cell is to

Abbreviations: IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane; IMS, intermembrane space

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configure, maintain and reorganize the mitochondrial network at sites of high energy demand in response to environmental and physiological cues is well established [1], and the challenge ahead is to define the underlying molecular mechanisms and regulatory pathways.

2.1. Mitochondria anterograde and retrograde transport: raison d'être and calcium regulation

In vivo studies using time-lapse microscopy of fluorescently labeled mitochondria have shown that a sub-population of the organelle remains stationary, while another commutes, often pausing between movements. In polarized cells like budding yeast and neurons, two widely used models in mitochondria trafficking studies, the organelle movements along the major axis of the cell are prominent, and occur both away from the nucleus (anterograde transport) and toward the center of the cell and nucleus (retrograde transport). In *Drosophila* motor neuron axons, anterograde and retrograde movement velocities averaged 0.26 and 0.45 mm/s, respectively [19]. It should be noted, however, that observations of run length, pausing frequency, average velocity, and persistence of direction can significantly differ, possibly reflecting the diverse physiological states of the cell as well as tissue and experimental approaches used [2,20]. Interestingly, moving mitochondria that switch direction of movement are not observed [19], suggesting that individual organelle reprogramming is required to alternate between anterograde and retrograde trafficking.

Several pioneering laboratories have investigated the raison d'être of mitochondria bidirectional transport and found it to be linked to maintaining bioenergetically competent organelles at sites of high energy demand. In neurons, among the moving mitochondrial sub-population 90% of the organelles with high membrane potential move anterogradely and accumulate in the growth cone, where metabolic activities are intense. Conversely, 81% of mitochondria showing low membrane potential are transported in the opposite direction, toward the cell body [21], suggesting a role of retrograde transport in eliminating energetically compromised organelles. An alternative possibility would be that retrograde transport actively uncouples membrane potential, thereby working as an energy switch; however, this is unlikely because arresting mitochondria motility does not correlate to an overall increase in respiration. Therefore, while anterograde transport generates the network of stationary active organelles required at sites of high metabolic activities, retrograde motility maintains it by removing depolarized and damaged organelles. In line with this notion, elegant imaging and computational studies from the Sheetz's group have shown that, in the axons of neurons isolated from chicken embryos, stationary mitochondria originate from a fast moving, anterogradely transported pool of organelles [21]. In these cells, mitochondria are evenly distributed, a pattern that originates because the organelles are preferentially dispensed in the middle of the gaps existing between stationary mitochondria [21], suggesting that transport at these “drop site” must be inhibited. If so, a gradient of signaling factors such as Ca^{2+}, ADP, or a small mitochondrial G-protein like Rab32 [22], could play a role in mitochondria trafficking arrest. In this respect, inhibition of mitochondrial motility has been shown to correlate with altered Na^{+}, K^{+} or Ca^{2+} ionic balance, suggesting the possibility that calcium could be part of the mechanism that arrests mitochondrial trafficking [21,23]. Consistent with this concept, in neuronal axons, using a micropipette to locally manipulate ion balance and composition, iso-osmotic replacement of NaCl with mannitol, which does not change overall metabolic activity, increases intracellular Ca^{2+} levels and decreases anterograde and retrograde trafficking [21,24,25]. Further, in hippocampal rat neurons mitochondria motility is suppressed by calcium influx through L-type voltage-gated calcium channels and NMDA receptors. Conversely, reduced calcium-dependent synaptic activities increase overall mitochondrial trafficking [26].

Mitochondrial oxidative phosphorylation uncoupling agents like CCCP also induce calcium transients and inhibit mitochondrial transport. However, this seems to be a non-specific effect, independent of mitochondrial depolarization and calcium release [27] because other uncouplers, such as DNP, FCCP and PCP, and complex III inhibitors like Antimycin A, increase or have no effect on mitochondrial transport [21,27,28]. Since CCCP is one of the most effective uncouplers [29] and can cause mitochondria to become consumers of ATP [25], its effect might occur because it lowers local ATP levels such that transport cannot be sustained [21]. More direct evidence of calcium participation to the organelle trafficking can be inferred from the identification of Miro, a mitochondrial outer membrane protein whose function in anterograde transport depends on intact EF-hand calcium binding domains [30] and, by implication, cytosolic calcium transients (discussed below).

2.2. Stationary mitochondria: regulating local power supply through calcium

Anterograde transport distributes functional organelles to sites of intense metabolic activities which, in neurons, include active synapses, nodes of Ranvier, myelination boundaries, axonal branches, and growth cones [1,31]. Mitochondria cluster at these locations, and their density changes in response to physiological cues. In rat hippocampal cultured neurons, the organelle dynamically redistributes into dendritic protrusions in response to synaptic excitation and their number increases during synaptogenesis and spine formation [26]. Conversely, preventing mitochondria to access synapses has profound deleterious effects on neuronal activity and synaptic plasticity [26,32]. In neurons, the pools of stationary mitochondria located at synaptic and dendritic terminals likely support bioenergetic and calcium buffering requirements for dendritic development and synaptic plasticity, two essential processes known to be regulated through Ca^{2+}-triggered transcriptional programs that drive the synthesis of the effector molecules required for long-term changes in neuronal function [33]. The mechanisms controlling mitochondrial docking within axons has just started to emerge. A recent study from the laboratory of Zu-Hang Sheng reported a role for axon-targeted syntaphilin (SNPH) in mitochondrial docking through its interaction with microtubules. Axonal mitochondria that contain exogenously or endogenously expressed SNPH lose mobility. Deletion of the mouse *snph* gene results in a substantially higher proportion of axonal mitochondria in the mobile state and reduces the density of mitochondria in axons, a phenotype that is fully rescued by reintroducing the *snph* gene into the mutant neurons. The *snph* mutant neurons exhibit enhanced short-term facilitation during prolonged stimulation, probably by affecting calcium signaling at presynaptic boutons [34].

The cellular strategy of crowding mitochondria to supply chemical energy at sites of intense metabolic activities is intrinsically limited by the typically modest amount of space available at sites like synaptic terminals, spines, and lamellipodia of the growth cone. Consistent with this concept, recent groundbreaking studies from Verburg and Hollenbeck have shown that the cell can also concentrate mitochondrial bioenergetic function by locally increasing respiration of mitochondria located at these sites. Using beads covalently coupled to Nerve Growth Factor (NGF) and Semaphorin to provide localized stimuli, this group showed that these survival and guidance cues could differentially affect mitochondria membrane potential. In areas of intense metabolic activities, organelles immediately adjacent to the site of NGF or Semaphorin stimulation showed a 40–50% increase in membrane potential, whereas mitochondria 10–50 μm away showed a smaller effect that declined to insignificance beyond 50 μm [35]. Inhibitors of PI3 kinase and MAP kinase abolished this effect, implicating these signal-transducing molecules in the upregulation of mitochondrial membrane potential and, by implication, ATP synthesis. Importantly, because simultaneous inhibition of these two

kinases does not produce an additive effect, their activity likely converges on one pathway before reaching an effector molecule on the mitochondria [35]. The identity of this pathway is unknown. However, calcium is known to activate MAPK via L-type voltage-gated calcium ion channel (L-VSCC) [36]. In this system, Ca^{2+} influx is sensed by calmodulin (CaM), which is bound to the L-VSCC carboxyl terminus situated at the mouth of the channel. Activated calmodulin initiates a cascade of events that leads to the activation of the Ras-MAPK pathway which, ultimately, is central to calcium-dependent gene transcription and control of synapse development and function [33,37,38]. Thus, dendritic development and synaptic plasticity could be linked to energetic metabolism through local Ca^{2+}-dependent activation of MAPK signaling that boost membrane potential of the stationary resident mitochondria.

2.3. Mechanisms of mitochondria transport in yeast and higher eukaryotes

The first clues revealing the *modus operandi* of mitochondrial transport came from the observation that, in neurons, the organelle is associated to central components of the trafficking machinery, microtubules [39–41]. Subsequent *in vivo* imaging studies investigating the velocity of mitochondria translocation provided further support to the notion that the organelle moves using these intracellular motor proteins. However, the mechanisms of mitochondria trafficking are significantly different in yeast, animals, and plants, perhaps reflecting the diverse functional specialization that the organelle acquired during evolution. As such, the identification of the regulatory mechanisms underpinning mitochondria motility in the major genetic systems are still the object of intense exploration [42] and exciting debates [2,43]. We here give an overview of the molecular processes implicated in mitochondria distribution, which have recently been authoritatively described in detail by others [2,3].

In the budding yeast *Saccharomyces cerevisiae*, mitochondria predominantly interact with the actin cytoskeleton and use actin tracks for both anterograde and retrograde movements during cell division. Several lines of evidence support this notion. First, mutations in genes encoding actin or factors involved in actin filament dynamics lead to aberrant mitochondrial distribution and morphology [44–50]. Second, treatment of wild-type cells with actin filament-depolymerizing drugs produces defects in mitochondrial shape and transport [51]. Third, isolated mitochondria bind, in an ATP-sensitive manner, to actin filaments *in vitro* [45,46,51], and colocalize with and move along actin cables *in vivo* [44,45]. Currently, the model for retrograde transport in yeast supports a system where mitochondria bind to actin cables undergoing retrograde flow and use the associated forces for passive transport toward the mother cell tip [3,52]. Similar mechanisms are used for retrograde movement of endosomes in budding yeast and appear conserved [3]. However, it should be noted that in the fission yeast *Schizosaccharomyces pombe*, mitochondria distribution depends on microtubules [50]. In this organism, mitochondria colocalize with microtubules during interphase [53,54], and certain mutations in tubulin genes cause dramatic changes in mitochondrial distribution, with mitochondria becoming aggregated and asymmetrically positioned [54]. Thus, additional studies of the molecular basis for mitochondrial distribution are necessary to extend our understanding of this process in lower eukaryotes.

Studies in *Drosophila* and in the mammalian system have shown that mitochondria movement and distribution depend on the microtubule motor as well as on the actin cytoskeleton of the cell (reviewed in [1–3,55–57]). Retrograde transport is mediated by the minus end-directed microtubule motors, dyneins [19,58,59]. Elegant studies using *in vivo* imaging and loss-of-function mutants have shown that kinesin-1 is also critical for dynein-mediated retrograde movement [19], indicating that the two transport machineries are functionally coupled, and explaining why microtubule-depolymerizing agents, such as nocodazole, halt mitochondria motility altogether. In higher eukaryotes the mechanisms of mitochondria motility thus in part recapitulate those described in the budding and fission yeast. Nonetheless, the mechanisms of mitochondrial transport in higher eukaryotes are unique in few ways. For instance, in fission yeast mitochondria do not translocate along microtubules as they do in higher eukaryotic cells. Rather, the organelle binds to microtubules, and the polymerization and depolymerization of microtubules drive an extension and retraction, respectively, of mitochondrial tubules during interphase [50]. A possible reason for evolving new mechanisms of mitochondria trafficking in higher eukaryotes is to allow precise long-range distribution in cell types that, from nematodes to vertebrates, increased their longitudinal axis by few orders of magnitude (e.g. myocytes and neurons); or, in plants, to orchestrate light-dependent organelle positioning and orientation (reviewed in [60]).

2.4. Calcium and the regulation of the mitochondria transport machinery

Kinesins (KIFs) are the molecular motors conveying cargos along microtubules (for a recent review and nomenclature changes see [61,62]). In neurons, few members of this protein superfamily, including KIF5B, KIF5C (kinesin-1 family) and KIF1Bα (kinesin-3 family), transport mitochondria anterogradely [23,62–64]. KIF5s also transport lysosomes [65] and tubulin oligomers [66,67] and kinesin-1-mediated disruption of mitochondria motility impairs vesicular transport [68]. Further, the carboxyl-terminal region of KIF1Bα selectively interact with the PDZ domains of PSD-95, PSD-97, and S-SCAM, members of a family of synaptic vesicle-associated scaffolding proteins [69]. Taken together these data indicate that mitochondria do not utilize exclusive motor proteins to move inside the cell.

The regulated bidirectional transport to which mitochondria are subjected implies that this organelle has unique binding partners for the motor proteins that transport them. Consistent with this possibility, few mitochondrial proteins have been shown to anchor the organelle to the microtubule motor, including KBP, Syntabulin, Milton and Miro [68,70–72]. Syntabulin links mitochondria to motor protein KIF5B and participates in anterograde trafficking [71]. Knockdown of syntabulin expression with targeted small interfering RNA or interference with the syntabulin–kinesin interaction reduces mitochondrial density within axonal processes by impairing anterograde movement of the organelle [71]. Similarly, without Milton, mitochondria accumulate in neuronal cell bodies but not at synaptic terminals and axons. Further, transfected into HEK 293T cells, Milton induces a redistribution of mitochondria within the cell [72], supporting a role in organelle distribution in all cell types. It should be noted, however, that Milton does not appear to be conserved in yeast, perhaps reflecting the primary dependence of yeast mitochondria on the actin cytoskeleton rather than microtubules. In *Drosophila*, Milton and Miro directly interact with each other, to facilitate the recruitment of Milton to the mitochondria. Thus, these proteins are likely to form an essential protein complex that links kinesin-1 to mitochondria for anterograde transport [73]. Consistent with this possibility, Miro ablation in *Drosophila* also disrupts axonal and dendritic mitochondrial distribution, causing defective locomotion in the fly and premature death [68].

Miro (mitochondrial Rho) belongs to a family of highly conserved eukaryotic proteins required for mitochondrial distribution and morphology [30,74]. Whereas mammals have two Miro family members, Miro-1 and Miro-2 [74], yeast has only one, Gem1p [30]. Their structural organization is similar and includes two cytosolic GTPase domains separated by a linker region containing a pair of calcium-binding EF-hand folds and a carboxyl-terminal transmembrane domain for anchoring to the mitochondrial outer membrane [30,74,75]. In Gem1p, the CaM-like EF-hand domain consists of two α-helices that flank a 12 residue loop [30]. In CaM-like EF-hand proteins, carboxylated side chains in positions 1, 3, 5, 9, and 12 of the loop act as

electron donors for calcium coordination [76]. This differs from a second class of EF-hand motifs, where calcium is coordinated via backbone oxygen atoms. Thus, Miro proteins appear to have bona fide CaM calcium-binding domains, which are known to govern structural changes and protein activity by coordinated binding to Ca^{2+} [76]. Consistent with this notion, substitutions of conserved residues required for calcium coordination in this domain impair Gem1p activity [30]. Like other calcium binding proteins [77], mutagenesis of both EF-hands in Gem1p produces a stronger phenotype than mutagenesis of either domain alone [30]. Given the presence of two conserved GTPase domains, it appears likely that the EF-hands control their cycle of GTP hydrolysis. An alternative possibility would be a regulation of protein interaction with other Miro-binding partners. While this review was in press, Xinnan Wang and Thomas Schwarz (Harvard University) published a study in Cell showing that kinesin is present on all axonal mitochondria, including those that are stationary or moving retrograde. In this study the authors showed that the EF-hand motifs of Miro mediate Ca^{2+}-dependent arrest of mitochondria and elucidated the regulatory mechanism. Rather than dissociating kinesin-1 from mitochondria, Ca^{2+}-binding permits Miro to interact directly with the motor domain of kinesin-1, preventing motor/microtubule interactions. Thus, kinesin-1 switches from an active state in which it is bound to Miro only via Milton, to an inactive state in which direct binding to Miro prevents its interaction with microtubules. Disrupting Ca^{2+}-dependent regulation diminished neuronal resistance to excitotoxicity.

A number of studies have shown that mitochondrial movement requires signal-transducing proteins, including cyclin-dependent kinase 5 (CDK5), protein phosphatase-1 (PP1), and glycogen synthase kinase 3 beta (GSK3β) (reviewed in [13]), but their specific role in this process remains to be elucidated. Future exploration of the impact of these signaling pathways in regulating the molecular mechanisms that achieve specificity, directionality, and temporal control of mitochondrial trafficking in response to cellular cues is necessary as transport systems are known to be vulnerable to genetic and/or environmental insults and often result in human neurological or neurodegenerative diseases (discussed below).

An early event in T_h cell activation, which is central to the adaptive immune response, consists in the formation of an immunological synapse (IS) with an antigen presenting cell (the name IS was chosen in recognition of the structural and functional ways in which it resembles the synapses of the nervous system [78]). At IS, sustained calcium flowing into the T_h cell turns on transcription factors that ultimately activate the lymphocyte. Calcium enters in the IS through voltage-gated ion channels located in the plasma membrane of the IS. Therefore, when calcium levels rise in the IS, extracellular calcium influx in this structure stops. Thus, effective T_h cell activation requires that calcium IS channels stay open long enough to generate calcium transients capable to trigger downstream effector signaling cascades. Pioneering studies from the Hoth group have shown that when a T_h cell engages an antigen presenting cell, mitochondria rapidly move and position 200 nm from the IS. Such close proximity allow the organelle to soak up some of the Ca^{2+} influx in the IS, which in turn facilitates a larger and more sustained Ca^{2+}-transient in the IS and activation of the NFAT, AP1, and NF-kB pathways [79]. T_h cell mitochondria require microtubule tracks for their positioning in the IS. However, actin was also necessary in this process, perhaps reflecting the fact that motor complexes might be differently organized in non polarized cells [79]. Thus, mitochondrial re-localization during IS formation introduces the paradigm that mitochondria motility controls the Ca^{2+}-dependent activation of transcription factors.

Lymphocytes are able to sense extracellular directional chemoattractant gradients and to respond with asymmetric changes in cell morphology (polarization) and mobility (chemotaxis). Cell polarization and chemotaxis depend on the signaling of seven-transmembrane receptors coupled to heterotrimeric Gi proteins (G protein-coupled receptors). To achieve directed movement, cells organize and maintain spatial and functional asymmetry with a defined anterior (leading edge) and posterior (uropod). Elegant studies from the Viola group reported that mitochondria are transported to the uropod along microtubules during lymphocyte migration in a process requiring Gi protein signaling and mitochondrial fission. Classically, one of the initial events in chemoattractant receptor signaling is the physical association of heterotrimeric Gi proteins to the receptor, leading to the inhibition of adenylate cyclase and intracellular calcium mobilization. However, no inhibition of lymphocyte migration was observed in a Ca^{2+}-free medium, and no specific inhibition was observed when intracellular calcium was buffered. Further, DT40 (B lymphocyte cell line) triple inositol 1,4,5-trisphosphate receptor-knockout cells migrated as efficiently as wild-type cells, indicating that calcium signaling does not play a critical role during lymphocyte migration. However, by interfering with the expression of mitochondria-shaping proteins that regulate the dynamics of the organelles, it was shown that fusion–fission of the organelle constrains lymphocyte polarization and migration, suggesting that accumulation of mitochondria at the uropod of a migrating cell is required to regulate the cell motor of migrating lymphocytes in a calcium-independent manner [80].

2.5. Mitochondria motility and diseases

The energetic metabolism of the central nervous system (CNS) is unique because its rate is extraordinarily high and constant. Under resting conditions, CNS activities absorb 20% of the oxygen consumed by the human body, a staggering amount when considering that the CNS accounts for only 2% of the body weight. Mitochondria produce over 95% of ATP utilized by the brain, which is mostly (50–60%) used to maintain Na^+, K^+ and Ca^{2+} ion gradients across the plasma membrane (reviewed in [12]). Recent studies are beginning to indicate that mitochondria, in addition to serve as the powerhouse of the cell [81] and as crucial amplifiers of death signals [82–85], may also function as a unique and essential signaling platform (reviewed in [13]). It is not surprising, therefore, that mitochondrial dysfunctions impact neuronal survival and functions, and are central to the etiopathogenesis of a plethora of neurodegenerative diseases (reviewed in [9]) and neurological disorders (reviewed in [86]). This includes severe behavioral syndromes whose underpinnings are still largely unknown, like schizophrenia, bipolar disorder, and autism, and that recent studies have related to defective mechanisms of mitochondrial calcium uptake and energetic homeostasis [87–89].

Aberrant mitochondrial transport has been recognized to be implicated in the development of major pathologies of the CNS, including Alzheimer's (AD), Parkinson's, Huntington's (HD), Lou Gehrig's, and amyotrophic lateral sclerosis (ALS). However, given the broad impact of organelle positioning on cell physiology and survival [68,72,73], the actual contribution of defective mitochondrial transport in the progression of these diseases remains unclear and is likely indirect. Consistent with this notion, axonal transport defects caused by mutations in a number of kinesin and dynein motor proteins have been implicated in neurodegeneration [90–92].

In HD, the pathological relevance of disturbed mitochondrial transport is illustrated by the extensive axonal clogging that are caused by HD mutant forms of huntingtin [93–95], suggesting that steric blockade of mitochondria transport at these clogged sites could be an etiological agent of the disease. However, this concept should be accepted with caution as other findings challenge it. For instance, *in vivo* analysis of "organelle clogs" caused by kinesin and dynein mutations showed that mitochondria could still move vigorously within and pass through the clogs, raising the possibility that axonal jamming may instead reflect sites of autophagocytosis of senescent mitochondria that are stranded in axons by retrograde transport failure, possibly a protective process aimed at suppressing cell death signals and neurodegeneration [19]. Nonetheless, the notion that

sustained altered distribution of mitochondria can be sensed by the cell and trigger an apoptotic elimination process is supported by recent findings showing that buildup of mitochondria in neurites and dysregulation of mitochondrial motor proteins caused by an AD-associated mutant form of ubiquitin is associated with activation of both the mitochondrial stress and p53 cell death pathways, thereby supporting the hypothesis that neuritic clogging of mitochondria triggers a cascade of events characterized by local activation of mitochondrial stress followed by global cell death [96].

In ALS, motor nerve specimens from affected patients show decreased retrograde axonal transport of organelles, including mitochondria [97,98]. However, since reduced retrograde transport of cargos such as neurotrophic factors, mitochondria, and membrane vesicles could damage motor neurons by multiple mechanisms, at this time the pathogenic contribution of impaired mitochondria retrograde transport remains speculative [99]. Interestingly, hereditary spastic paraplegia (HSP) caused by mutations in a subunit of the m-AAA protease paraplegin has also been associated to impaired mitochondria axonal transport [100]. Paraplegin presides to protein quality control of the organelle, suggesting that HSP-related defects in mitochondrial transport and axonal degeneration may result from the accumulation of non-degraded, misfolded inner membrane proteins or impaired regulatory steps during mitochondrial biogenesis, or both [101]. Thus, the failure to regulate central mitochondrial activities can phenotypically converge in compromised organelle motility and contribute to the pathological development of diverse neurodegenerative diseases.

3. Regulating mitochondrial membrane fusion and fission through calcium

The eukaryotic cell evolved between two and three billion years ago through the acquisition of an alpha-proteobacteria endosymbiont, which later became the mitochondria. This ancestry resulted in the formation of a double-layered organelle that is organized in four distinct subcompartments: the outer mitochondrial membrane (OMM), the intermembrane space (IMS), the inner mitochondrial membrane (IMM), and the matrix. The IMM can be further subdivided in an inner boundary membrane and in the cristae compartment, bag-like folds of the IMM connected to it via narrow tubular junctions which are known to change structure depending on the energetic state of the organelle [102]. Mitochondria morphology can assume spherical, rod-like, or tubular shapes, and depends on membrane-fusion and -fission activities. The equilibrium between these two opposing actions determines the shape of the mitochondrial network in steady state. Typically, suppression of membrane fusion or dominance of membrane fission causes mitochondria to fragment into short rods or spheres; conversely, disruption of membranes fission or prevalence of fusion generates interconnected tubules. In certain cell types mitochondria are mainly elongated, while in others they are fragmented. However, the mitochondrial architecture is typically dynamic, as it reflects the physiological state of the cell in steady state, stressed, and pathological conditions. Importantly, the functional versatility of the organelle depends on maintaining a highly dynamic mitochondrial reticulum, a paradigm demonstrated by the fact that loss of membrane fusion or fission is lethal and that a number of human diseases are caused by disruption of these activities (reviewed in [9]). Pioneering studies in *S. cerevisiae* have shown that mitochondria fusion and fission are governed by a small but growing set of conserved proteins (reviewed in [103]). Here, we will discuss recent findings supporting calcium participation in cellular control of these “mitochondria shaping” proteins.

3.1. Calcium and the mechanisms of mitochondria fission

A central effector molecule in mitochondria fission is the mammalian dynamin-related protein 1 (Drp1 [104]), Dnm1 in *S. cerevisiae* [105]. Drp1 and Dnm1 are large cytosolic GTPases that, upon fission triggering, are recruited to the mitochondrial outer membrane at punctate foci. At these sites, the dynamin-like protein self-assembles into a functional scission complex consisting of oligomers that form ring-like structures that, through a GTP-dependent mechanism, constrict the organelle, thereby providing the force required to produce a local membrane scission event. *In vitro*, Dnm1 forms spirals whose internal diameter matches that of mitochondrial constriction sites (111 nm [106]). The mechanism underpinning the initial recruitment of Drp1 at punctate locations of the mitochondrial OMM is a rate-limiting step of mitochondria fission [106]. Thus, Drp1 inhibition, or its down-regulation, results in an overall dominant effect of membrane fusion activities, causing mitochondria to be fused and interconnected.

A similar phenotype is caused by down-regulation of another central component of the mitochondrial fission program, the mammalian Fission protein 1 (hFis1 [107]), Fis1p in *S. cerevisiae* [108]. hFis1 and Fis1p are two 16 kDa conserved integral proteins of the OMM, containing a single transmembrane domain and a tetratricopeptide repeat domain facing the cytosol (TPR, involved in protein–protein interaction [107]). Fis1p is required for yeast Dnm1 recruitment and oligomerization on the OMM [108]. Fis1p engages Dnm1 through one of two adaptor proteins, Mdv1 or Caf4 [108–110]. However, homologs of these yeast proteins have not been identified in mammals, and in this system hFis1 is not essential for Drp1 recruitment [111,112], suggesting that during metazoan evolution the mechanism of Drp1 activation on the OMM might have lost the requirement for adaptor proteins. Nonetheless, cross-linking studies and fluorescence resonance energy transfer experiments indicate that hFis1 is a Drp1 receptor on the OMM as these proteins bind to each other via hFis1 TRP domain [113]. However, it should be noted that in mammalian cells actin filaments and microtubules also appear to function in recruiting Drp1 to the organelle, thereby providing support to alternative mechanisms of Drp1 activation [114,115]. The central role of Drp1 and hFis1 in mitochondrial fission is compellingly supported by the notion that their level of expression is proteolytically controlled by the opposing actions of sumoylation and ubiquitination [116–118]. Recently, calcium-dependent regulation of sumoylation activities have been reported [119,120], and examples of calcium regulation of ubiquitin ligases exist [121,122]. Evidence for the involvement of calcium signaling in sumoylation and ubiquitination of mitochondrial proteins is lacking, but their potential impact on mitochondrial dynamics warrants future investigations in this area.

Endophilin B1, a member of the endophilin family of fatty acid acyl transferases that participate in endocytosis, is another molecule implicated in mitochondrial fission [123]. Endophilin B1 cycles between the cytosol and the OMM, where it binds through the concave face of a highly conserved banana-shaped fold (BAR domain) that senses the curvature of the lipid bilayer and facilitates membrane pinching during fission [124]. Downregulation of Endophilin B1 results in the formation of a net of long tubules of OMM enveloping several distinct inner mitochondrial membrane-bound matrix compartments, indicating IMM scission continuing in the absence of OMM scission. The mechanism of Endophilin B1 in OMM fission is still elusive, although it might be similar to that of Endophilin-1, a key regulator of clathrin-mediated synaptic vesicle endocytosis. During endocytosis, Endophilin-1 builds complexes with Dynamin I, the fission GTPase responsible for severing the neck of the nascent endocytic vesicle [125]. Endophilin-1 interacts directly with voltage-gated Ca^{2+} channels, and the formation of this complex is Ca^{2+} dependent [126]. In hippocampal neurons, expression of a dominant-negative Endophilin-1 mutant that constitutively binds to Ca^{2+} channels inhibits endocytosis. The primary structural determinant implicated in this process resides within amino acid residues 200–219 of Endophilin-1. This motif is conserved among Endophilin-1 isoforms, but not in Endophilin B1. However, this small motif is not a Ca^{2+}-binding domain per se but, rather, a structural regulatory determinant

for Endophilin-1 Ca^{2+}-regulated activity. Therefore, sequence conservation is not required, and Endophilin B1 regulation through Ca^{2+} is conceivable. Consistent with this possibility, recent studies have linked Endophilin B1 to Ca^{2+}-regulated autophagy. Endophilin B1 binds Beclin-1, a Bcl-2-interacting protein with autophagy-promoting activity [127–130], and colocalizes within punctate foci with autophagy related proteins during nutrient depletion [131]. Autophagy is an evolutionarily conserved lysosomal pathway involved in the starvation-dependent turnover of cellular macromolecules and organelles. This process is potently induced by a Beclin-1-dependent rise in free cytosolic calcium [132,133], suggesting that further investigation of Endophilin B1 regulation through calcium might help elucidating how mitochondria fission intersects this cellular pathway.

MTP18 (mitochondria protein, 18 kDa) is an intermembrane space protein anchored to the IMM. Knockdown experiments showed that MTP18 is essential for cell viability as it causes cytochrome *c* release and sensitizes cells to proapoptotic stimuli. Reduction of MTP18 also impairs mitochondrial morphology, resulting in the formation of a highly interconnected mitochondrial reticulum. Conversely, overexpression of MTP18 induces Drp1-dependent mitochondria fragmentation, suggesting a functional role of MTP18 in maintaining the integrity of the mitochondrial reticulum [134,135]. Given its localization at the interface between the IMM and OMM, it has been hypothesized that MTP18 could control the concerted fission of the two mitochondrial membranes [13]. MTP18 is also a downstream effector of phosphatidylinositol 3 kinase (PI3K) signaling pathway, which plays a crucial role in cell growth and survival by controlling the magnitude of Ca^{2+} influx from voltage-gated calcium channels opening upon membrane depolarization [136]. Thus, this protein could modulate mitochondrial dynamics in response to physiological changes in intracellular Ca^{2+} concentrations.

GDAP1 (ganglioside-induced differentiation associated protein 1) is a mammalian mitochondrial outer membrane protein that is mutated in Charcot–Marie–Tooth disease type 4A (CMT-4A) [137,138]. Whereas its overexpression causes fragmentation of mitochondria with no effect on cell death, down-regulation of GDAP1 or the expression of certain patient mutant forms which are truncated and no longer localize to mitochondria tend to elongate the organelle indicating that GDAP1 activity impact the mitochondrial network architecture by promoting mitochondrial fission [137–139]. The available information about the biology of GDAP1 is scarce. Some hints about the molecular function of this protein have been provided by bioinformatic analyses [140]. These studies showed that GDAP1 and related proteins in invertebrates and vertebrates contain characteristic glutathione S-transferase domains (GST). Based on its particular domain features, GDAP1 was proposed as the founder of a new GST family. Members of this family are characterized by an enlarged interdomain located between the GST-N and GST-C domains, and contain COOH-terminal hydrophobic helices that could serve as transmembrane domains. It has been speculated that GDAP1 could be part of a mechanism regulating mitochondrial fission by acting as a sensor of the mitochondrial redox (NAD^+/NADH) state [138], a process that, in turn, depends on cytosolic [Ca^{2+}] oscillations [141]. All together, these data suggest the possibility that GDAP, rather than a bona fide fission protein, could be part of the molecular mechanism integrating fission into cell physiology through calcium.

3.2. Calcium and the mechanisms of mitochondria fusion

In mammals, the central components of the mitochondrial fusion machinery include mitofusin-1 (Mfn1), mitofusin-2 (Mfn2), optic atrophy 1 (Opa1), and presenilin-associated rhomboid-like (Parl) (for recent reviews see [6,9,10,13,142,143]). Whereas deletion of Mfn1, Mfn2, and Opa1 impairs embryonic development in the mouse at different stages [144–146], ablation of Parl is lethal from the fourth postnatal week due to cachectic death [147]. Unlike the fission protein Drp1, the regulation of the proteins governing the fusion program of the organelle has not yet been directly linked to calcium. Further, mitochondrial fusion reaction *in vitro* does not require presence of Ca^{2+} [148,149].

Mitofusins possess a cytosolic N-terminal GTPase domain, two TM domains spanning the OMM and two cytosolic C-terminal domains that are crucial for protein–protein interaction [150,151]. To promote fusion, Mfn1 docks two juxtaposed mitochondria via its second coiled coil domain, forming homo- and hetero-oligomeric complexes [152]. The role of the two mitofusins in fusion seems to be different. Mfn1 has higher GTPase activity and induces fusion more efficiently than Mfn2 [153]. Mfn2 is also localized to the ER [154], and a role of this protein in the formation of the juxtaposition between endoplasmic reticulum (ER) and mitochondria that form the structural basis for interorganellar Ca^{2+} signaling has been proposed and recently elegantly demonstrated by the group of Luca Scorrano [155,156].

Opa1, mutated in dominant optic atrophy, the most common cause of inherited optic neuropathy, promotes fusion from the inside of the organelle as it is anchored to the IMM, with the GTPase domain facing the IMS [148,157]. Opa1 and Mfn1 are on the same genetic pathway as Opa1 requires Mfn1, but not Mfn2, to mediate fusion [157]. Opa1 exists in eight different splice variants [158] and its function in cristae morphogenesis and apoptosis [147,159] is proteolytically regulated by the mitochondrial rhomboid protease Parl [147] and by members of the mitochondrial AAA metalloprotease family via a prohibitin pathway [160–165].

Mammalian Parl proptotypes a eukaryotic subfamily of the rhomboid protease family (PARL subfamily) [166]. Its members are mitochondrial and possess, as a unique structural signature, a transmembrane helix (TMH) located upstream of the six TMHs that form a catalytically active rhomboid domain [166–174]. Although initially described as a molecule implicated in PS1 biology, a possibility now no longer valid, Parl discovery first introduced the concept that rhomboids are intramembrane-cleaving proteases [175], a notion subsequently validated in rhomboid proteins from bacteria to mammals(reviewed in [176]). Three lines of evidence indicate that calcium is unlikely implicated in the regulation of members of the PARL family: (i) rhomboid activity *in vitro* does not require calcium [177]; (ii) crystallographic data of the rhomboid domain do not show any possible structural or functional role for this element [167–170]; (iii) non-mitochondrial rhomboid with EF-hand calcium-binding domains have been described in *Drosophila* and humans (Rhomboid-2 and -4 [166]), but no calcium-related domains are present in members of the PARL subfamily.

Parl is embedded in the IMM, with the N-terminus, termed Pβ domain [178], exposed to the matrix, and the C-terminus to the IMS [179]. The Pβ domain emerged during vertebrate evolution and it is strongly conserved in mammals as 58 of its 62 residues are invariant, and there are no insertions or deletions [178]. This indicates that this domain was subjected to strong purifying selection, which can only be explained by functional constraints because in unconstrained sequences evolving neutrally, very few, if any, invariant residues would be expected to survive the 100 Ma of evolution separating mammalian orders [180,181]. Parl N-terminal Pβ domain is constitutively phosphorylated at Ser-65, Thr-69, and Ser-70 [179]. Functionally, this hyper-phosphorylation blocks a proximal cleavage event (Ser-77↓Ala-78; β-cleavage [178]) that induces mitochondria fragmentation [179] through a block of mitochondrial fusion (Pellegrini and McBride, unpublished observations). The identity of the protease and of the kinase/phosphatase switch that executes β-cleavage is unknown, thereby leaving open the possibility that the β-cleavage-dependent role of Parl in mitochondria dynamics could be ultimately regulated through calcium.

3.3. Calcium as a molecule integrating mitochondrial dynamics to cell physiology

The overall configuration of the mitochondrial architecture is determined by the integration of organellar and cytosolic cues. This is

essential to coordinate, for instance, mitochondria shape remodeling during cell cycle. In HeLa cells, the interconnected mitochondrial network observed in interphase becomes fragmented in the prophase, and the organelle stays organized in small spherical units during metaphase and anaphase, possibly to secure minimal interference with mitotic spindle assembly and proper stochastic mitochondria distribution into the daughter cells; restoration of filamentous mitochondria begins in the late mitotic phase, to be completed in the daughter cells [182]. Mitochondria fragmentation at the prophase depends on the activation of the pro-fission molecule Drp1 through phosphorylation of its Ser-585 residue by Cdk1/cyclin B, a universal cell cycle kinase required for mitotic/meiotic cell cycle entry. Cdk1/cyclin B activity needs to be eliminated for mitotic/meiotic exit. This process is triggered by sustained cytosolic Ca^{2+} concentration rise [183–185], suggesting that Drp1 activity during cell cycle is regulated by calcium transients. However, the Drp1 phosphorylation consensus motif (PA<u>S</u>PQK) is conserved in mammals and, to a less extent, in *Caenorhabditis elegans* (KT<u>S</u>PQE), but not in yeast, consistent with the notion that in *S. cerevisiae* the filamentous mitochondrial network is maintained throughout mitosis [103]. Thus, the possible role of calcium in the regulation of Drp1-mediated mitochondrial fission during cell cycle would be a process acquired during metazoan evolution.

A step forward in our understanding of calcium as a cellular cue governing mitochondrial shape remodeling came with the discovery from the Strack and Blackstone labs that Drp1-dependent mitochondrial fragmentation is controlled by phosphorylation at a conserved site by Cdk1 and protein kinase A (PKA) [186,187]. These groups showed that phosphorylation of the Ser residue in the consensus sequence RKL<u>S</u>$_{637}$ARE of Drp1, which is conserved in all splice variants of the human, rat, and mouse protein, inhibits fission activity. Conversely, dephosphorylation of Ser-637 by the Ca^{2+}-dependent phosphatase calcineurin promotes mitochondrial fission and is involved in the propagation of apoptosis [186]. Recent studies from the Scorrano's group have shown that inducers of mitochondrial depolarization also trigger Ca^{2+}, calcineurin-dependent dephosphorylation of Drp1 on Ser-637, driving its translocation to mitochondria and causing fission of the organelle [188]. Calcineurin is a heterodimeric protein composed of a catalytic subunit (CnA) that binds calmodulin and a regulatory subunit (CnB) that binds Ca^{2+}. It is involved in the transduction of Ca^{2+}-mediated signals in diverse cellular programs, including proliferation, death, and vesicles secretion. Calcineurin is activated in a Ca^{2+}-calmodulin dependent fashion [189]. *In vitro* assays using mutant *Drp1* carrying the phosphomimetic Ser → Asp substitution (S637D), suggest that phosphorylation of this residue impairs intramolecular association of Drp1 into large fission-related complexes, and attenuates the dynamin's GTPase activity [187]. However, this latter observation is controversial [186] and inconsistent with the recent finding that mutant *Drp1* S637D is retained in the cytosol [188]. It has been proposed that the opposing effects of PKA and calcineurin on the same site serve as a switch to translate signals associated with Ca^{2+} changes of different strength, duration, and tone into different mitochondrial morphologies. During physiological (i.e., agonist evoked) Ca^{2+} signaling, activation of PKA can prevail over calcineurin mediated dephosphorylation of Drp1. Moreover, compartmentalization of both Ca^{2+} and cAMP signals could play a role in the local regulation of mitochondrial shape. Conversely, long lasting Ca^{2+} plateaus in the cytosol linked to full activation of calcineurin and to generalized fragmentation could for example account for the apoptotic mitochondrial fission [188].

Data recently reported from the Matsushita group show a different scenario of how Drp1 activity and mitochondrial fission could be regulated through calcium transients-dependent phosphorylation of this protein. In hippocampal neurons isolated from rat embryos and grown in culture for 9–12 days, treatment with 45 mM K^+ for 15 min produce an immediate and partially reversible arrest in mitochondria motility. The organelle then fragments and looses its cristae organization without, however, inducing apoptosis. This stimulation triggers a rapid increase in intracellular Ca^{2+}, possibly through N and P/Q types Ca^{2+} channels (VDCC). VDCC-associated Ca^{2+} signaling stimulates phosphorylation of Drp1 at Ser-637 via activation of Ca^{2+}/calmodulin-dependent protein kinase I (CaMKIα) (note that in this study Ser-637 corresponds to Ser-600, due to the rat Drp1 splice variant used). In neurons and HeLa cells, phosphorylation of Ser-637 activates Drp1 translocation to the mitochondria, thereby inducing fission of the organelle. *In vitro* studies show that Ser-637 phosphorylation of wild-type Drp1 by CaMKIα substantially increases the amount of Drp1 pulled down by GST-hFis1, suggesting that phosphorylation increases Drp1 binding affinity for hFis1 and, by implication, its recruitment to the mitochondrial surface. The molecular and cellular bases explaining the reason why in this study Drp1 phosphorylation activates fission are unclear, particularly because CaMKIα, PKA, Cdk1, and calcineurin are all widely expressed proteins, and no cell type-specific mechanisms can thus be envisaged. It has been suggested that phosphorylation of Drp1 at Ser-637, although necessary for regulation of mitochondrial morphology, is not sufficient on its own and that activation of CaMKIα has additional roles in regulation of Drp1 interaction with mitochondria [190]. Additional investigations on whether Ca^{2+}- and cAMP-dependent signaling pathways can act synergistically to regulate mitochondrial dynamics are thus required.

The endoplasmic reticulum can elicit proapoptotic signaling that results in transmission of Ca^{2+} to the mitochondria, which in turn activates Drp1 recruitment to the OMM to drive mitochondrial fragmentation and apoptosis. An additional role for Drp1 in this process consists in opening the cristae, where the major stores of cytochrome *c* reside, suggesting the possibility that Drp1 participation in this pathway may require still undiscovered postranslational modifications of the GTPase protein [191].

4. Conclusions

The contribution of calcium in the regulation and execution of mitochondria motility and morphology in steady state and pathological conditions has just started to emerge. Being mostly indirect, the actual function of calcium in these processes might appear minor. However, there are convincing evidences suggesting that this second messenger might play a much bigger role in orchestrating these activities, thereby warranting future research in this field.

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