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## How mitochondrial dynamism orchestrates mitophagy

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### Abstract

Mitochondria are highly dynamic, except in adult cardiomyocytes. Yet, the fission and fusion-promoting proteins that mediate mitochondrial dynamism are highly expressed in, and essential to the normal functioning of, hearts. Here, we review accumulating evidence supporting important roles for mitochondrial fission and fusion in cardiac mitochondrial quality control, focusing on the PINK1-Parkin mitophagy pathway. Based in part on recent findings from *in vivo* mouse models in which mitofusin-mediated mitochondrial fusion or Drp1-mediated mitochondrial fission were conditionally interrupted in cardiac myocytes, we propose several new concepts that may provide insight into the cardiac mitochondrial dynamism-mitophagy interactome.

### Keywords

mitochondria; autophagy; mitochondrial fusion; mitochondrial fission; Parkinson's disease

### Introduction to mitochondrial dynamics

Mitochondrial dynamics refers to organelle fission, fusion, and subcellular translocation. In many cell types mitochondria exist as interconnected reticular networks; mitochondrial dynamism is frequent, perhaps continuous, in such cells. In addition to structural remodeling of mitochondrial networks, mitochondrial fission and fusion are implicated in homeostatic maintenance of mitochondrial DNA stability and respiratory function, as well as preventing or propagating programmed cell death<sup>1</sup>. Recent results of *in vivo* cardiomyocyte-specific genetic manipulation have revealed that mitochondrial dynamics factors function in cardiomyocytes in other ways. This functional distinctiveness can be attributed in part to absence of mitochondrial networks and unusually slow mitochondrial turnover in hearts (at least compared to liver)<sup>2</sup>. Further, as irreplaceable cardiomyocytes do not normally undergo programmed cell death, mitochondrial dynamics proteins are not needed as part of apoptosis<sup>3</sup>. Nevertheless, the mitochondrial fusion factors mitofusins (Mfn) 1 and 2 and optic atrophy (Opa) 1, and the mitochondrial fission factor dynamin-related protein (Drp) 1,

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are highly expressed in mammalian hearts, wherein their genetic ablation provokes striking cardiac dysfunction<sup>4, 5</sup>. Here, we consider evidence supporting important roles performed by outer mitochondrial membrane (OMM) fusion factors, Mfn 1 and Mfn2, and fission factor Drp1, in cardiac mitochondrial quality control. Other roles for mitochondrial dynamics factors that may impact cardiac health and disease, such as physical tethering between cardiac mitochondria and sarcoplasmic reticulum that facilitates mitochondria calcium import<sup>6-8</sup>, Opa1-mediated regulation of mitochondrial cristae structure<sup>9</sup>, and Mfn- or Opa1-mediated regulation of cardiomyocyte differentiation<sup>5, 10</sup>, have been reviewed elsewhere.

In the first part of this manuscript we examine current concepts of how mitochondrial fission and fusion are accomplished through highly ordered processes necessitated by the dual membrane architecture of these essential, but potentially highly toxic, organelles. We introduce the paradigm of asymmetrical mitochondrial fission as a central event in mitochondrial quality control, followed by a discussion of the best understood molecular pathway by which defective mitochondria are culled from cells, PTEN-induced putative kinase 1 (PINK1)-Parkin mediated mitophagy. These notions were developed, and the molecular mechanisms elucidated, largely through *in vitro* studies using cell-types other than cardiomyocytes. Thus, the second part of the review discusses the particular roles of Drp1, Mfn1, and Mfn2 in hearts, as revealed through *in vivo* genetic manipulation. Finally, we explore points of controversy within the area of cardiac mitochondrial dynamism, focusing on unexpected molecular functions of mitochondrial dynamics and mitophagy factors.

Prior to examining the molecular mechanisms that mediate mitochondrial fission and fusion it may be helpful to consider why mitochondria remodel their structures through the seemingly complicated and energy intensive process of breaking apart (fission) and then re-forming (fusion) organelles. One reason may be cellular context. For example, complete dissolution of mitochondrial networks precedes mitosis. Such network disassembly conceivably not only facilitates cytokinesis, but helps to deliver roughly equal proportions of the parental cell mitochondrial pool to each daughter cell<sup>1</sup>; mitochondrial networks subsequently have to be re-established in each daughter cell via generalized organelle fusion.

Dismantling and then reconstituting the cellular mitochondria network through sequential organelle fission, distribution, and re-fusion may be the most efficient means of partitioning mitochondria in mitosis. Limited mitochondrial structural remodeling also occurs between cell mitoses<sup>1</sup>, and one wonders about the biological advantages conferred by fusion-mediated incorporation of accessory organelles for focal network remodeling and growth, compared to (for example) organelle growth by enlargement and budding. We posit that the supramolecular structure of the mitochondrial respiratory apparatus lacks the plasticity necessary for existing organelles to morph into more complex structures. Thus, we discard the fluid-state model wherein mitochondrial respiratory chain complexes diffuse freely and inter-complex electron transfer occurs randomly<sup>11</sup> in favor of a solid state model in which respiratory complexes are organized into stable paracrystalline supercomplexes on the cristae<sup>12-14</sup>. Supporting this model, destabilizing respiratory supercomplexes (which promotes fluidity) diminishes mitochondrial respiratory function and increases oxidative

damage<sup>15, 16</sup>. It seems that major structural modifications of respiratory supercomplexes on paracrystalline cristal membranes would first require destabilizing the membrane, then incorporating additional individual protein components, and finally re-constructing the original highly organized structure. This is complicated and potentially disruptive. It would be easier to add pre-fabricated supercomplexes to pre-existing ones, as by fusing mitochondrial cristae.

In developing ideas about how fission and fusion of prefabricated mitochondria units is superior to fluidic mitochondrial remodeling we considered how military units are constituted and managed within an army's hierarchical organization structure. Here, each soldier represents an individual respiratory complex protein. Grouped together, these proteins form a squad (analogous to a respiratory complex). Squads are arranged into platoons, and ~6 platoons comprise a functional unit, the company (like one complete respiratory chain). Approximately ~6 companies form a battalion (similar perhaps to one lamelliform crista), 2–6 battalions form a brigade (like one mitochondrion), and so on, to divisions and corps that collectively comprise an army; this is analogous to an entire cellular mitochondrial collective. Acknowledging obvious differences in stoichiometric relationships between military units and mitochondrial respiratory complexes, understanding how the military manipulates its organization inspired thoughts as to how the rigid mitochondrial structure could be modified: During major re-organizations an army minimizes disruption by adding units rather than individual soldiers. To increase the size of a company, platoons are added. To increase the size of a battalion, companies are added, etc. Individual soldiers (proteins) are incorporated at a slow and steady pace to replace soldiers lost through retirement or attrition (representing mitochondrial protein biogenesis), but not to change the structure of the overall organization. By analogy, making a larger or different shaped mitochondrion through the wholesale incorporation of individual proteins (growing and budding) would interrupt ongoing organelle function. It would be better to add or subtract intact functional units in the form of respiratory supercomplexes, through mitochondrial fusion or fission.

## Molecular mechanisms and cell biology of mitochondrial dynamism

### Mitochondrial fission

As introduced above, mitochondria can undergo fission to remodel their physical structure. Additionally, they undergo symmetric fission to replicate and expand the cellular mitochondrial pool, and asymmetric fission as the initial step of a highly orchestrated process that maintains cell-wide mitochondrial fitness by sequestering and eliminating damaged organelle components. Replicative fission that facilitates distribution of mitochondria to daughter organelles has been reviewed elsewhere<sup>1, 5</sup>. Here we focus on asymmetric fission and targeted mitophagy as central steps in the overall mitochondrial quality control process.

The major protein effector of mitochondrial fission is Drp1. Structurally, Drp1 possesses an amino terminal GTPase domain, a middle domain, and a carboxyl terminal GTPase effector domain (Figure 1). Under normal cellular conditions, 97% of Drp1 is cytosolic<sup>17</sup>; it therefore must be recruited to the OMM to promote fission. Analogous to the actions of

dynamins in endocytic vesicle fission<sup>18</sup>, OMM-localized Drp1 self-assembles into spirals and then constricts in a GTP-dependent manner, ligating and separating the inner and outer mitochondrial membranes<sup>19, 20</sup> (think of Drp1 as forming individual sausage links from a single long sausage casing; Figure 1). The particular factors that stimulate Drp1 translocation from the cytosol differ in apoptosis, mitosis, mitophagy, and mitochondrial remodeling. For example, during cell division Drp1 is phosphorylated by mitotic kinase cyclin B-CDK1 complex<sup>21</sup> and then recruited to the mitochondria through the actions of a phosphorylated small Ras-like GTPase called RALA and its effector RALBP1<sup>22</sup>. On the other hand, during apoptosis Drp1 interacts with Bax and contributes to cytochrome c release<sup>23</sup>. Accordingly, genetic or chemical inhibition of Drp1 protect from some, but not all, forms of programmed cell death<sup>23–26</sup>.

Drp1 constriction sites are often marked by endoplasmic reticulum (ER); the ER-mitochondria contact is prior to, and independent of, Drp1 recruitment<sup>20</sup>. As a consequence of impaired mitochondrial fission, fibroblasts derived from Drp1-deficient mice are hyper-elongated and partially resistant to mitochondrial fragmentation induced by pharmacological uncoupling agents<sup>27</sup>. Nevertheless, the filamentous mitochondria of Drp1 null murine embryonic fibroblasts (MEFs) undergo fragmentation during mitosis, revealing the existence of one or more mechanisms capable of promoting mitochondrial fission independent of Drp1<sup>27</sup>.

Because Drp1 lacks a clear hydrophobic transmembrane domain it is thought to bind to the OMM via resident receptor proteins. Fission 1 (Fis1) is a major Drp1 receptor protein in yeast, but Fis1 deficiency does not disrupt Drp1 association with mitochondria or affect mitochondrial morphology in colon carcinoma cells<sup>28</sup>. On the other hand, Mitochondrial fission factor (Mff) colocalizes with Drp1 on mammalian mitochondria, and RNAi-mediated Mff knockdown both reduces Drp1 association and induces mitochondrial elongation; Mff overexpression promotes mitochondrial fragmentation<sup>29</sup>. Other proteins that may help recruit Drp1 to OMM are mitochondrial dynamics protein of 49 and 51 kDa (MiD49 and MiD51)<sup>30, 31</sup>. Finally, there is intriguing evidence that Drp1 can bind to mitofusins and evoking a change in Mfn structure from a fusion-incompetent closed conformation to a fusion-competent open configuration<sup>32</sup> (see Figure 2). This putative Drp1-Mfn interaction would have the effect of inverting the canonical fission-promoting function of Drp1 by promoting Mfn-mediated fusion. Thus, Drp1 is functionally inert when sequestered in the cytosol. Drp1 provokes mitochondrial constriction and fission after binding to Mff and possibly other proteins at sites of ER contact. And, mitochondria-localized but non-oligomerized Drp1 may promote mitochondrial fusion by binding to and dis-inhibiting mitofusins. How the various effects of Drp1 may be regulated through phosphorylation, S-nitrosylation, ubiquitination and SUMOylation (Figure 1) is under investigation<sup>33</sup>.

### Mitochondrial fusion

As an engineering problem, mitochondrial fission is straightforward: ligate and separate (like making sausage links). The double membrane/double space structure of mitochondria is no impediment to Drp1-mediated ligation. The sausage metaphor is imperfect, however, as mitochondria are constructed more like a turducken than a brautwurst. Turducken

(popularized by the great NFL coach and sports announcer John Madden during Thanksgiving Day football broadcasts of the 1980s and 1990s<sup>34</sup>) is a chicken stuffed inside a duck stuffed inside a turkey. This creates concentric layers of poultry, resembling the central compartment of mitochondrial matrix, enclosed by the crista/IMM, which is separated from the OMM by the inter-membrane space (Figure 2). This double membrane, double space architecture is critical for proper respiratory function and the integrity of the compartments must be maintained throughout fusion. Thus (like making a large turducken from two turkeys, two ducks, and two chickens), mitochondrial fusion occurs layer by layer. The first step involves physical tethering of two mitochondria via trans interactions between the carboxyl terminal domains of OMM mitofusins of two organelles. Like Drp1, Mfn1 and Mfn2 are dynamin family GTPases. Both Mfn isoforms have cytosolic N-terminal GTPase domains, two cytosolic hydrophobic heptad repeat coiled-coil domains (HR1 and HR2), and a small hydrophobic transmembrane domain (Figure 2). Consequently, mitofusins insert into the OMM much like a safety pin in cloth, with the vast majority of the protein being exposed to the cytosol and available to interact with cytosolic factors (Figure 2). Because Mfn1 and Mfn2 are similar, inter-molecular interactions between the cytosolic carboxyl-terminal HR2 domains of mitofusins located on different organelles can occur in either a homotypic (Mfn1-Mfn1 and Mfn2-Mfn2) or heterotypic (Mfn1-Mfn2) manner<sup>35</sup> with the heterotypic shown to be more efficient and to yield a higher rate of successful fusion events<sup>36</sup>. Additionally, Mfn2 (but not Mfn1) localizes to endo/sarcoplasmic (ER/SR) reticulum as well as mitochondria. For this reason, Mfn2 contributes to mitochondria-ER/SR calcium signaling, regulating acute metabolic demand and pathological ER stress<sup>37-39</sup>. Mitofusin-mediated physical tethering of mitochondria is GTP-independent and fully reversible, whereas GTP hydrolysis is essential to irreversible OMM fusion.

As noted, mitofusins are essential for the first two stages of mitochondrial fusion (tethering followed by OMM fusion). Accordingly, genetic deletion or RNAi-mediated suppression of mitofusins in cultured cells and *Drosophila* or mammalian cardiomyocytes produces abnormally small organelles, commonly referred to as “fragmented mitochondria” (although mechanistically they do not actively fragment, but are simply unable to undergo normal fusion)<sup>35, 40-43</sup>. The location of Mfn1 and Mfn2 on the OMM, which is the physical interface between mitochondria and cytosol, optimally positions them to participate in information exchange between the mitochondrion and its host cell. As discussed below, the pathophysiological implications of mitofusin expression, regulation, and dysfunction therefore extend far beyond simply modulating mitochondrial morphology.

Membrane-by-membrane mitochondrial fusion maintains the structural integrity of the IMM and matrix, thus preserving oxidative phosphorylation and avoiding formation (or at least maintaining the physical sequestration) of cytotoxic oxidizing molecules generated upon interruption of the electron transport chain (Figure 2). Accordingly, after OMM fusion a related dynamin family GTPase, Opa1, promotes IMM fusion<sup>44,45</sup>. The consequences of Opa1 loss of function in cultured cells and *in vivo* models are both phenotypically and mechanistically complex. Mitochondrial tethering and OMM fusion still occur (through the actions of Mfn1 and Mfn2), but absence of Opa1-mediated IMM fusion produces mitochondria that are not only structurally heterogeneous, but that exhibit

generalized dissipation of the normal IMM electrochemical potential and profoundly impaired cellular respiration<sup>46</sup>. These results point to a major role for Opa1 in maintaining normal crista morphology that is essential for proper assembly and functioning of electron transport chain supercomplexes<sup>47, 48</sup>. The differing roles played by OMM and IMM fusion factors have been demonstrated in *Drosophila* heart tubes: interrupting Mfn-mediated OMM fusion evoked a cardiomyocyte ER stress response that could be rescued by enhancing mitochondrial processing of unfolded proteins, whereas interrupting Opa1-mediated IMM fusion compromised mitochondrial function that was rescued by reactive oxygen species scavenging<sup>49</sup>.

### Dys-dynamism; what happens when mitochondrial fission and fusion go wrong

The biological importance of mitochondrial fission and fusion is unambiguously revealed by embryonic lethality in homozygous mouse knockouts of the genes encoding Mfn1, Mfn2, Opa1 or Drp1<sup>27, 40, 50</sup>. Loss of function genetic mutations linked to clinical disease underscore the importance of intact fission and fusion in the human condition: Mfn2 mutations cause Charcot-Marie-Tooth syndrome type 2A, an autosomal dominant axonal peripheral neuropathy<sup>51</sup>, Opa1 mutations cause autosomal-dominant optic atrophy<sup>52</sup>, and a Drp1<sup>A395D</sup> mutation identified in an infant with multisystem failure impairs Drp1 assembly and induced a mitochondrial fission defect<sup>53</sup>.

Altering the normal balance between mitochondrial fusion and fission in cells with interconnected mitochondrial networks has predictable effects on mitochondrial morphometry. A shift toward increased fusion and/or decreased fission produces elongated organelles with greater interconnectivity. Conversely, decreased fusion and/or increased fission produces shorter organelles having poorly connected networks, the so-called fragmented mitochondria phenotype<sup>27, 40, 46, 54</sup>. Conventional wisdom holds that more connectivity (and by inference more fusion or less fission) is a healthier situation, and that less connectivity (or fragmentation) is deleterious. These pathophysiological inferences make sense given that mitochondrial networks disintegrate early in cells undergoing apoptosis<sup>55</sup>, and that fusion between healthy and damaged mitochondria has the potential to dilute out damaged components in the latter, referred to as repair via complementation<sup>56</sup>. Indeed, pharmacological inhibition of Drp1-induced mitochondrial fission is protective in a number of different tissue injury models<sup>57–61</sup>. However, the notion that small or fragmented mitochondria are necessarily bad (especially in cardiac myocytes wherein the mitochondria are already “fragmented” in comparison with most other cell types) is not supported by a number of observations: 1. Highly fused or interconnected mitochondria are more, not less, susceptible to staurosporine-stimulated apoptosis<sup>62</sup>; 2. Cells with defective mitochondrial fusion caused by Mfn2 deficiency are protected against programmed cell death<sup>63</sup>; 3. Forced Drp1 expression in *Drosophila* cardiomyocytes provokes mitochondrial fragmentation without any apparent adverse consequences<sup>49</sup>; and 4. Activation of nutrient oxidation by adrenergic stimulation of the brown adipose tissue is accompanied by mitochondrial fragmentation that is essential for increasing energy expenditure in this tissue<sup>64</sup>. Thus, as recently opined by Sun and Finkel<sup>65</sup> it seems unreasonable to conclude that mitochondrial dysmorphology is the primary or only cause of organelle, cellular, or tissue dysfunction reported after genetic mitofusin ablation *in vitro*<sup>66</sup> or *in vivo*<sup>67–69</sup>. Finally, a bidirectional

exchange of mitochondrial components between healthy and damaged mitochondria has the potential to harm the healthy organelle as well as to help the damaged one. We refer to fusion-mediated contamination of good mitochondria by bad as “mitochondrial contagion”, which occurs in mitophagically impaired Parkin-deficient *Drosophila* hearts and can be prevented by suppressing mitofusins<sup>70</sup>. In the following sections we explore the inextricable interplay between mitophagy and mitochondrial dynamism.

## How mitochondrial dynamism impacts mitochondrial quality control

The word “mitophagy” is a contraction of mitochondria and autophagy, and refers to the process by which cells eat their own mitochondria. Mitophagic engulfment of mitochondria by autophagosomes and subsequent transfer to degradative lysosomes can occur during generalized macroautophagy as during nutrient deprivation, or as a highly selective process that targets dysfunctional mitochondria<sup>71</sup>. Selective mitophagy as a means of mitochondrial quality control is the current focus.

There is a tendency to conceive of mitochondria as living linear lives. Mitochondria are “born” or assembled through biogenesis<sup>72</sup>, they function for a time, and after an inevitable age-related decline in function are either repaired (through fusion-mediated complementation)<sup>56, 73</sup> or transported, via mitophagy, to the figurative mitochondrial junkyard. As comfortable as this anthropomorphic construct is, mitochondria are not people. Rather, the life cycle of mitochondria resembles that of the primordial bacteria from which they are descended<sup>74, 75</sup> (Figure 3). Mitochondria replicate through *symmetric fission*: a healthy parent organelle produces two healthy daughters that grow by adding new components through mitochondrial biogenesis (i.e. induction of nuclear-encoded mitochondrial genes and incorporation of their protein products into pre-existing mitochondria) and by fusing with other healthy mitochondria. Over time or as a consequence of extrinsic stress a given mitochondrion will sustain damage too severe for correction through biogenic or fusion-mediated repair and is removed via mitophagy.

To understand the importance of mitophagic quality control it is worth emphasizing that healthy mitochondria employ oxidative phosphorylation to produce energy in the form of ATP that drives virtually all biological processes, but damaged organelles transform into cytotoxic factories for reactive oxygen species (ROS). Thus to paraphrase Erasmus of Rotterdam: “Mitochondria – can’t live with them, nor without”. Mitochondrial ATP synthesis depends upon an electrochemical gradient generated across the inner mitochondrial membrane through a series of redox reactions (see Figure 2): the transfer of electrons between complexes of the electron transport chain is coupled to extrusion of protons (hydrogen ions) across the inner mitochondrial membrane and into the mitochondrial intermembrane space. Reversal of this proton flow powers the mitochondrial ATP synthase, a molecular rotor<sup>76</sup>. The terminal electron acceptor is molecular oxygen, forming superoxide anion ( $O_2^-$ ) that is normally reduced through the sequential actions of superoxide dismutase (to form hydrogen peroxide;  $O_2^- + 2H \rightarrow H_2O_2$ ) and catalase (to form water;  $2 H_2O_2 \rightarrow 2 H_2O + O_2$ ). When electrons prematurely leak from complexes I or III of the electron transport chain<sup>77, 78</sup>, or when specific endogenous mitochondrial enzymes such as Romo1 (Reactive oxygen species modulator 1) are activated<sup>79</sup>, damaging superoxide

radicals and/or hydrogen peroxide can accumulate within or escape from mitochondria and attack critical proteins, lipids, and mitochondrial or nuclear DNA. Because production of ROS in damaging amounts does not normally occur, substantial ROS production is a marker of mitochondrial dysfunction. As protection against injury from mitochondrial-derived ROS that overwhelm endogenous protective mechanisms (catalase and superoxide dismutase<sup>80</sup>) cells employ sophisticated surveillance and elimination mechanisms to identify and remove dysfunctional mitochondria.

The cellular decision to remove a dysfunctional mitochondrion is necessarily dichotomous; either the organelle is retained or it is targeted for autophagy. Thus, a continuum of mitochondrial fitness ranging from the perfectly healthy ATP generator at one extreme to the completely dysfunctional toxic ROS generator at the other has triggered a categorical decision regarding mitochondrial fate. A decision to reserve mitophagy for severely damaged mitochondria places the cell at risk from toxic effects of dysfunctional organelles that have not achieved the high threshold of dysfunction necessary to trigger their removal. If, however, the cell lowers its threshold for mitochondrial removal and targets less severely impaired organelles, it will inevitably eliminate mitochondria still functioning at some level, thus depriving itself of their ATP. Furthermore, the cell will have to expend resources to replace the recycled organelles. Nature has addressed this quandary by integrating mitochondrial dynamism with mitophagy in the form of asymmetric mitochondrial fission<sup>81</sup>.

Pulse chase experiments show that in the absence of a general signal for the reduction in mitochondrial mass, mitochondria that are targeted for mitophagy have a relatively depolarized membrane potential before being targeted for autophagy<sup>81</sup>. This subpopulation of mitochondria also have reduced likelihood of being involved in fusion events as both mitofusions and OPA1 fusion proteins are either cleaved or degraded, a process induced by either inner membrane depolarization or reduced mitochondrial ATP production<sup>82</sup>. Thus, mitochondria targeted for autophagy are characterized by being relatively depolarized and remaining solitary. The time between mitochondrial depolarization and autophagosomal engulfment varies from less than 1 hour to ~3 hours, suggesting the existence of a transient population of pre-autophagic mitochondria<sup>81, 83</sup>. The existence of a pre-autophagic pool may explain the heterogeneity of mitochondrial membrane potential in most cell types<sup>84</sup>. The process that feeds mitochondria into the pre-autophagic pool is therefore expected to play a key role in determining the rate of removal of mitochondria. The development of a technology to label individual mitochondria and track their membrane potential using membrane potential dyes allowed for the identification of the event at which depolarized mitochondria are produced<sup>85</sup>. A photo-activatable form of green fluorescent protein (PA-GFP) has been targeted to the matrix of mitochondria. Laser mediated photo-conversion of the PA-GFP in one mitochondrion allowed its tagging and tracking, and staining with positively charged dyes such as TMRE provided information on membrane potential. This kind of analysis showed that fission events produce functionally dissimilar daughter mitochondria; in other words, asymmetric mitochondrial fission. While one daughter leaves the fission event with membrane potential that is similar or higher compared to the mother mitochondrion, the other daughter may have depolarized membrane potential that may recover or persist. The latter daughter has reduced chance of being involved in a fusion event and if membrane potential is not recovered, it is becoming part of the pre-autophagic



pool. Evidence supports that asymmetric fission is the result of uneven distribution of dysfunctional mitochondrial components including oxidized or older proteins<sup>81, 86</sup>. This model explains the role of fusion in mitochondria quality control as it may allow for the redistribution of damaged components while fission and mitophagy are responsible for the elimination. Since fusion and mitophagy are two competing fates of the post fission daughter mitochondria, giving preference to the daughter with the higher membrane potential generates a sorting mechanism where damaged material is segregated before being eliminated. The selective nature of fusion is therefore key to the efficiency of the quality control mechanism. The selectivity is dependent on transitioning of dysfunctional daughter mitochondria from being fusion competent to permanently solitary mitochondria, a process that may be mediated by coupling the degradation or post-translational modification of fusion proteins to the respective bioenergetic functioning of the healthy and impaired daughter mitochondria.

The mechanism by which sub-mitochondrial partitioning of damaged from healthy components is achieved prior to asymmetric fission is not known, but similar asymmetrical fission quality control processes are observed in budding yeast<sup>87–89</sup>. The paracrystalline structure of IMM mitochondrial respiratory supercomplexes suggests an intriguing possibility<sup>12–14</sup>: because misfolded and damaged proteins are physically incompatible with the supermolecular structure of IMM respiratory complexes they are passively excluded. To illustrate this point, consider (instead of soldiers and army units) respiratory proteins of the IMM to be Lego blocks, each fitting with the next to form respiratory complexes that fit together to form supermolecules, which in turn fit together to form supercomplexes (Figure 3). Normal Lego proteins can be readily inserted (like biogenesis) or entire units added (like fusion). But misfolded proteins, represented here by Legos melted in the microwave (likely by some toddling future cardiovascular scientist), cannot be integrated into the highly ordered structure. Because they do not fit they segregate and accumulate (Figure 3). The same process may apply to proteins of the IMM. After partitioning, physically separating normal from abnormal Lego proteins (as by mitochondrial fission), placing the normal Legos back in the box with other functional Legos for future use (as with mitochondrial fusion), and disposing of damaged components in the trash (as by mitophagy) follows naturally.

### **Mitochondrial dynamism-mitophagy cross talk in the heart**

If the concept of asymmetric mitochondrial fission in mitophagy is correct, then interruption of mitochondrial fission or fusion should impact not only mitochondrial shape, but mitochondrial quality. Several recent reports suggest that this is, in fact, the case.

The first suggestion that genetic perturbation of cardiomyocyte mitochondrial dynamics factors would compromise mitochondrial quality control mechanisms was the observation that unusually small, degenerated mitochondria accumulated in adult mouse hearts after conditional combined ablation of the mitochondrial fusion factors Mfn1 and Mfn2<sup>43</sup>. In that study an increase in physically abnormal mitochondria was associated with impaired cardiomyocyte respiration, but not with measurable alterations in substrate-stimulated oxygen consumption by isolated cardiac mitochondria. This internal inconsistency was

subsequently resolved when it was discovered that standard isolation procedures did not efficiently capture the fragmented mitochondria produced by interrupting mitochondrial fusion. After the procedure was appropriately modified not only was it clear that Mfn1/Mfn2 double deficient mitochondria are functionally impaired, but the smallest mitochondria exhibit the greatest dysfunction<sup>54</sup>. So, why weren't these impaired mitochondria eliminated by the mitophagy quality control apparatus? Simply interrupting fusion should not directly interrupt mitophagy (see Figure 3). Indeed, individual cardiac deletion of Mfn1 had little effect on cardiomyocyte mitochondria, whereas deletion of Mfn2 provoked a chronically progressive cardiomyopathy associated with accumulation of enlarged (not fragmented) mitochondria<sup>90</sup>. This result suggested a role for Mfn2, but not Mfn1, in mitochondrial quality control, prompting the discovery that PINK1-phosphorylated Mfn2 is a mitochondrial binding partner for Parkin. Studies of Mfn2-deficient hearts also led to detection of a Parkin-independent, ROS-dependent alternate pathway for mitochondrial quality control<sup>91</sup>. Thus, the mitochondrial fusion protein Mfn2 is essential to Parkin-mediated mitophagy, at least for mouse cardiomyocytes and neurons<sup>90, 92</sup>.

Three recent papers have also implicated the mitochondrial fission factor Drp1 in cardiac mitophagy. Cardiac-specific ablation of Drp1 in the early postnatal period<sup>54, 93</sup> or in adult mice<sup>54, 94</sup> induced cardiomyopathies and perturbed cardiac macroautophagy or mitochondrial autophagy; the exact nature of the abnormality in mitophagy/autophagy attributed to Drp1 deficiency differed between studies. It is worth considering that if asymmetric mitochondrial fission normally precedes mitophagy as depicted in Figure 3, then chronic suppression of fission by ablating Drp1 would be predicted to have different consequences on mitophagy, depending upon whether mitophagy is assayed early or late after Drp1 deletion<sup>4</sup>. Shortly after loss of Drp1-mediated fission, mitophagy should be suppressed because the mechanism by which damaged and dysfunctional components are segregated into the depolarized daughter organelle is lost. With time, however, as the overall health of the cellular mitochondrial population deteriorates due to interruption of the asymmetric fission/mitophagy process, the parent organelles will themselves become sufficiently dysfunctional to trigger mitophagy; when this occurs cell-wide, mitophagy will be seen to be increased. Indeed, if mitochondrial biogenesis is inadequate to replace mitochondria in sufficient numbers, then accelerated mitophagy late after Drp1 ablation will consume a significant portion of the mitochondrial pool, resulting in mitochondrial insufficiency. The aggregate data to date support this time-dependent evolution in phenotype<sup>4, 54, 94</sup>, but more definitive studies using better genetic models are necessary to concretely establish a role for Parkin-mediated mitophagy in the cardiomyopathy that develops after cardiac Drp1 ablation. In the following section we examine our current understanding of the molecular events underlying mitophagy, and explore the implications of functional cross-talk between mitophagy and mitochondrial dynamism mediated by the mitochondrial fusion factor, Mfn2.

## Mfn2 and PINK1-Parkin mitophagy signaling

The most thoroughly explored mechanism for homeostatic mitochondrial quality control is PINK1-Parkin mediated mitophagy. PINK1 (PTEN-induced putative kinase) and Parkin are prototypical Parkinson's disease factors; mutations in their genes were the first identified

causative events linked to hereditary (autosomal recessive) early onset Parkinson's disease<sup>95, 96</sup>. The foundation for our current understanding of mitophagy was discovering how the mitochondrial localized kinase PINK1 interacts functionally with the cytosolic E3 ubiquitin ligase Parkin, which was defined in *Drosophila*<sup>97, 98</sup> and extended to mammalian systems<sup>99</sup>. A detailed discussion of the individual fruit fly studies that linked Parkin and PINK1 to mitochondrial fitness, and eventually proved that PINK1 is upstream of Parkin in a single mitochondrial quality control pathway, is beyond the scope of this review, but it is a fascinating tale of how genetic manipulation in flies provided fundamental biological insights into the human condition<sup>100</sup>.

### **PINK1, the ignition switch for Parkin-mediated mitophagy**

If mitochondrial depolarization, ROS, or protein misfolding are the input variables that activate mitophagy<sup>101, 102, 103</sup>, then PINK1 senses these inputs and initiates the appropriate response. Mitochondrial damage stimulates PINK1 kinase activity, which turns on Parkin-mediated mitophagy. The molecular mechanisms by which this happens were elucidated by Youle and colleagues<sup>101</sup>. For those interested in a detailed history of these discoveries and the neurosciences perspective, Youle recently authored a detailed overview of the fundamentals of PINK1 – Parkin biology in the context of Parkinson's disease<sup>104</sup>.

The revelation that PINK1 is central to mitochondrial quality control was unexpected because PINK1 protein levels are so low in normal mitochondria as to be virtually undetectable<sup>101</sup>; normal mitochondria are therefore PINK1-deficient. The key to unlocking mitophagy signaling was the observation that PINK1 is readily detected in damaged mitochondria, appearing after mitochondrial depolarization<sup>101</sup>. This area of investigation has seen rapid advances over the past few years; here and in Figure 4 we summarize current concepts. PINK1 kinase is encoded by the *PINK1* (previously called *Park6*) gene, which like all but the 13 proteins encoded by mitochondrial DNA is part of the nuclear genome. PINK1 protein is therefore translated by the ribosomal apparatus and imported into mitochondria. In normal mitochondria, as fast as PINK1 is imported it is proteolytically degraded. Consequently, normal mitochondria have very little PINK1 protein or PINK1 kinase activity<sup>101</sup>. Upon mitochondrial depolarization however, this import-and-immediately-degrade PINK1 process is interrupted, causing PINK1 to accumulate and phosphorylate its substrate proteins. Conceptually, this has been likened to a mitochondrial “dead-man switch”<sup>5</sup>, in which healthy mitochondria expend effort to actively degrade PINK1, thus staying alive by avoiding mitophagic destruction. In damaged mitochondria that no longer support PINK1 degradation, passive accumulation of PINK1 triggers the organelle's mitophagic destruction.

The specifics of mitochondrial PINK1 import and degradation are inextricably linked to Parkin-mediated mitophagy. (How mitochondria generally import nuclear-encoded proteins via membrane translocases for the OMM and IMM [called TOM and TIM, respectively] has been recently reviewed<sup>105</sup>.) Unprocessed 63 kDa PINK1 protein is transported across OMM by the TOM complex, delivered to the IMM translocase, TIM, and proteolytically processed in a manner typical for imported mitochondrial matrix proteins<sup>106</sup>. In healthy mitochondria, processed PINK1 is rapidly cleaved by PARL (presenilin-associated rhomboid-like

protein)<sup>107, 108</sup>, generating a 52 kDa PINK1 fragment that escapes into the cytosol and undergoes proteasomal degradation directed by an N-Degron<sup>109</sup>. Thus, mitochondrial PINK1 levels are suppressed. Mitochondrial dysfunction impairs TIM-mediated PINK1 translocation across the IMM, protecting it from degradation by PARL and maintaining its physical association with TOM on the OMM<sup>110</sup>. From here PINK1 phosphorylates available substrates<sup>111</sup>.

### **Mfn2 is a phosphorylation substrate of PINK1 and a receptor for Parkin**

To promote mitophagy, PINK1 that accumulates on damaged mitochondria must induce cytosolic Parkin to translocate to, and ubiquitinate OMM proteins on, the organelle. Simultaneously, to prevent contamination of the healthy mitochondrial pool by the damaged organelle, PINK1 inhibits fusion of the damaged mitochondrion. There are differing views regarding the biochemical events that induce mitochondrial Parkin localization and that interrupt mitochondrial fusion. Indeed, it is almost certain that signaling redundancy is built into this important mechanism of mitochondrial quality control and that multiple pathways involved.

PINK1 phosphorylates Parkin on Ser65<sup>112–116</sup>, and Parkin poly-ubiquitinates mitofusins<sup>82, 117–120</sup>. The former observation implicates PINK1-mediated phosphorylation in Parkin recruitment to damaged mitochondria, whereas the latter observation suggests that selective Mfn degradation can suppress mitochondrial fusion in organelles targeted for mitophagic destruction<sup>121–124</sup>. But as Sherlock Homes declared (in Sir Arthur Conan Doyle's *The Bascombe Valley Mystery*) "There is nothing more deceptive than an obvious fact". Indeed, although PINK1 does phosphorylate Parkin (on Ser65 within the Parkin ubiquitin-like domain), recent studies from multiple laboratories have revealed that it is PINK1-phosphorylated ubiquitin, and not PINK1-mediated phosphorylation of Parkin itself, which is essential to enable Parkin as an E3 ubiquitin ligase<sup>125–128</sup> (Figure 4).

The observation that PINK1 can phosphorylate ubiquitin complexes on OMM proteins<sup>128</sup>, and not just free ubiquitin<sup>125, 126</sup>, has suggested that non-specific anchoring of Parkin to phospho-ubiquitinated OMM proteins is a mechanism for its PINK1-mediated recruitment to mitochondria<sup>104, 129</sup>. This is consistent with the idea that Parkin binding to phospho-ubiquitin can accelerate Parkin-mediated OMM protein ubiquitination as a positive feedback amplification loop<sup>128</sup>. But this is a loop after all, and it seems inefficient to have the primary event of Parkin binding, which is a prerequisite for OMM protein ubiquitination, to rely upon pre-existing ubiquitinated OMM proteins. Chen and Dorn reported that PINK1-mediated phosphorylation of Mfn2 on Thr111 and Ser442 conferred Parkin binding activity to Mfn2<sup>90</sup>. Functional ablation of these phosphorylation sites by their mutational substitution with Ala abrogated Mfn2-Parkin binding, whereas mimicking Mfn2 phosphorylation by mutational substitution with Glu conferred constitutive Parkin binding. According to this mechanism, stabilized PINK1 located on the OMM phosphorylates Mfn2, transforming it into a receptor to which Parkin can bind, thereby bringing it into physical proximity of its many mitochondrial ubiquitination substrates<sup>90</sup>. PINK1-mediated phosphorylation of free ubiquitin activates Parkin's E3 ubiquitin ligase activity<sup>125, 126</sup>, and

its phosphorylation of ubiquitinated OMM proteins amplifies mitophagy signaling<sup>128</sup> (Figure 4).

The proposed role for PINK-phosphorylated Mfn2 as a Parkin receptor<sup>90</sup> is viewed as controversial by some. The most common concern is reflected in comments by Pickrell and Youle<sup>104</sup> that “Parkin translocates constitutively to mitochondria in Mfn1/Mfn2-KO cells, arguing that Mfn2 is not involved in translocation.” This may over-interpret the results of experiments performed using fibroblasts derived from germ-line knockout mice. While Parkin can indeed translocate to mitochondria of Mfn2-deficient murine embryonic fibroblasts (MEFs), it should be noted that mitophagy (measured as lysosomal incorporation of mitochondria after pharmacological uncoupling of respiration from ATP synthase) takes place in MEFs lacking any of the three postulated mitophagy effectors, PINK1, Mfn2, and Parkin (G Dorn, unpublished results). Results such as these<sup>130</sup> do not demonstrate that Mfn2 (or PINK or Parkin) are unimportant in mitophagy. Rather, they support the existence of secondary compensatory mitophagy mechanisms<sup>131, 132</sup> that are induced when primary pathway are interrupted. Indeed, both cardiomyocyte-specific and neuronal-specific deletion of Mfn2 in mice induce distinctive defects in Parkin localization to depolarized mitochondria<sup>90, 92</sup>. Absence of Parkin translocation in Mfn2 deficient neurons suggests that another concern, that Mfn2 may act as a Parkin receptor only in hearts<sup>133</sup>, is also overstated.

A mechanistic link between PINK1, Mfn2, and Parkin is attractive because it can explain how the PINK1-Parkin pathway simultaneously initiates mitophagy and shuts down fusion, as required to preclude mitochondrial contagion<sup>70</sup>. It is possible that PINK1-mediated phosphorylation may instantaneously convert Mfn2 from a fusion protein to a Parkin binding protein. This mechanism of modulating mitochondrial fusion in mitophagy would have the advantage of being more rapid and direct than Parkin-mediated Mfn2 ubiquitination, extraction, and proteasomal degradation<sup>82, 117–120</sup>. Indeed, the importance of Parkin-mediated ubiquitination of specific proteins, leading to their selective elimination from soon-to-be autophagocytized organelles, is unclear. Parkin-mediated ubiquitination of Mfn1 and Mfn2 might deplete these fusion promoting proteins by targeting them for proteasomal degradation, thus interrupting mitochondrial fusion and placing the organelle in quarantine until it can be mitophagically eliminated<sup>121, 122</sup>. Parkin does not selectively ubiquitinate pro-fusion proteins; it also ubiquitinates the pro-fission protein, Drp1<sup>134</sup>, which would move the fission/fusion equilibrium in the opposite way toward mitochondrial fusion. Indeed, Parkin promiscuously ubiquitinates a hundred or more OMM proteins<sup>135, 136</sup>, essentially painting the organelle with a coat of ubiquitin that attracts autophagosomes<sup>137</sup>. Thus, during mitophagy at least, Parkin-mediated OMM ubiquitination does not seem to fine tune OMM protein expression of organelles that, in any case, are shortly headed to the graveyard. (A more surgical role for Parkin in mitochondrial protein turnover via vesicular export is described below.) Furthermore, there is little delay between Parkin localization to mitochondria and their engulfment by autophagosomes. Live cell microscopy of cultured cells revealed that Parkin localization, focal protein ubiquitination, and regional mitochondrial fragmentation with autophagosomal engulfment all occur within minutes of mitochondrial injury<sup>83</sup>. This provides little time for selective extraction and proteasomal degradation of mitofusins.

As noted, this area of investigation is rapidly advancing. Uncertainties about the mechanism for Parkin translocation (are there chaperones?) and the identity of putative Parkin receptors require additional experimentation. For example, one can conceive of studies using Mfn2PINK1 phosphorylation site mutants having constitutive, or that completely lack, Parkin binding activity to interrogate PINK1-Parkin signaling *in vivo*.

### In vivo studies of PINK1-Parkin signaling

The roles of PINK1 in Parkin-mediated mitophagy, as a *proximal effector* that accumulates only in depolarized mitochondria, as an *inducer* (via phosphorylation of Mfn2) of Parkin translocation to mitochondria, and as a *Parkin activator* through phosphorylation of ubiquitin, support its central role in this form of mitochondrial quality control. Given the nearly unassailable evidence that loss-of-function mutations in the PINK1 gene cause hereditary Parkinson's disease in humans<sup>96, 138, 139</sup>, one might anticipate that PINK1 gene deletions engineered into mice would recapitulate the human neuropathology. That has not been the case. Indeed, multiple efforts to ablate PINK1 or Parkin in mice by genetically deleting exons encoding critical protein domains have produced a “negligible neurodegenerative pattern in the (dopaminergic neuron-rich) substantia nigra pars compacta, a region clearly affected in Parkinson's disease patients”<sup>140</sup>. Even combined deletion of the mouse genes for PINK1, Parkin, and DJ-1 (another gene in which loss-of-function mutations have been causally linked to early Parkinson's disease) has failed to recapitulate the hallmark loss of dopaminergic neurons in the brain<sup>141</sup>. Perhaps not surprisingly, baseline cardiac phenotypes in PINK1 and Parkin knockout mice are also modest, although interruption of PINK1-Parkin mediated mitophagy increases susceptibility to myocardial damage in conditions such as advancing age and ischemic injury wherein chronic mitochondrial impairment likely requires aggressive mitophagic culling<sup>142–146</sup>. The failure of germ-line PINK1 or Parkin gene deletion in mice to recapitulate human disease phenotypes may be attributable to developmental plasticity that promotes induction of compensatory pathways. Favoring this notion is transcriptional upregulation of other E3 ubiquitin ligases in germ-line Parkin-knockout mouse hearts<sup>70</sup>.

### What the future may hold...

One of the questions raised by apparent compensatory induction of alternate mitochondrial quality control pathways in mice (and possibly humans<sup>147</sup>) having genetic loss of PINK1 or Parkin function is “What is the nature of these other pathways?”. This area of research is active and our understanding is both incomplete and evolving. Conventional wisdom has been that mitochondrial depolarization or a downstream reduction in ATP synthesis may constitute central stimuli for mitophagy. However, depolarization may be a late and final result of a decompensating organelle. In the sections above we considered how it could be detrimental for cells to wait until mitochondria are completely depolarized before triggering their sequestration and removal. New work has identified other, potentially earlier stimuli for mitophagy. In one example, induction of the mitochondrial unfolded protein response prompted PINK1 accumulation and Parkin recruitment; these mitochondria were subsequently eliminated despite maintaining a healthy polarization status<sup>102</sup>. Likewise, increased mitochondrial release of ROS can stimulate alternate mechanisms of

mitochondrial quality control in hearts having a primary impairment of Parkin recruitment<sup>91</sup>. It seems that different triggers exist for mitophagy that provoke a quality control reaction proportional to organelle damage. Here, we describe a variation on this theme, PINK1-Parkin involvement in preserving mitochondrial fitness via export of damaged proteins in mitochondrial-derived vesicles.

It may be helpful to consider PINK1-Parkin dependent mitophagy and mitochondria vesicle formation in the broader context of mitochondrial quality control mechanisms by introducing a new metaphor, your car. A new car (like a healthy mitochondrion) will, over time or because of untoward circumstances, run down and/or sustain damage. In this circumstance mitophagy is analogous to waiting until the car no longer runs, and then trading it in on a new model. Although necessary every few years, this is inconvenient and expensive. It is preferable to delay purchasing a new car by properly maintaining it and performing necessary repairs; our cells seem to take a similar approach to their mitochondria.

Functionality of cars (and mitochondria) can be optimized through preventative maintenance. Routine car maintenance includes exchanging used engine oil and filter, replacing worn tires and belts, and restoring any parts that unexpectedly fail; individual components are removed and replaced as needed. Mitochondria may accomplish the same thing, protein by protein, using endogenous proteases and the ubiquitin/proteasome pathway (Figure 5). Thus, unfolded or oxidized internal mitochondrial proteins (i.e. those that are located within the matrix or intermembrane space) are proteolytically degraded and replaced<sup>148</sup>. Mitochondrial outer membrane proteins that are not readily accessible to these proteases are subject to degradation via the standard ubiquitin/proteasome pathway after ubiquitination in a Parkin-independent manner, as by Mule/ARF-Bp1 mediated ubiquitination and removal of the anti-apoptotic factor Mcl-1<sup>149</sup>. Both of these mechanisms promote turnover of damaged or undesirable mitochondrial proteins in a manner that maintains basal function.

Sometimes a car requires more than routine maintenance, but is not a total loss, such as needing new brakes or a water pump. In mitochondria this seems to be accomplished through PINK1 and Parkin-mediated formation of mitochondrial vesicles and their export to lysosomes<sup>150</sup>. These mitochondrial vesicles contain oxidized proteins generated during times of oxidant stress<sup>151, 152</sup>. Heidi McBride has proposed that focal oxidative damage to mitochondrial proteins provokes local accumulation of PINK1 kinase and limited recruitment of Parkin, promoting incorporation of a “patch” of damaged proteins into a newly-generated vesicle that separates from the mitochondrion and travels to degradatory lysosomes<sup>153</sup>. The specific mechanisms for cargo segregation, vesicle formation, and lysosomal targeting are poorly understood, but a role for Parkin in this process is consistent with previous evidence for its involvement in endocytic vesicle formation at cell membranes<sup>154, 155</sup>.

Finally, there are circumstances in which major damage has occurred but the car is still useful after major repairs, such as replacing the engine or transmission or some other integrated sub-system. We envision that this is the function of Parkin-mediated bit-by-bit autophagy<sup>83</sup> (Figure 5). Here, substantial but still localized damage is excised from the

parent mitochondrion via spatially restricted fragmentation, and the damaged fragment is removed via the usual mitophagy apparatus, thus both repairing and sparing the parent. It should be noted that categorization of different forms of PINK1-Parkin mediated mitochondrial protein turnover mechanisms on the basis of scale, i.e. vesicles, bit-by-bit, and mitophagy, may be artificial. Given the mechanistic commonalities, the labels may be misapplied to what is actually a continuum of response that is variably invoked according to need.

## Summary

Mitophagy and mitochondrial dynamism are highly integrated at the functional level. Molecular crosstalk between the primary effectors of each pathway evokes complex regulatory and counter-regulatory mechanisms that exclusively eliminate damaged mitochondria, thus preserving fitness of the overall cellular mitochondrial collective. The newly described role of Mfn2 as mitochondrial fusion factor when not acted upon by PINK1, and Parkin receptor when it is, suggests that mitochondrial fusion and mitophagy are contextual and mutually exclusive, which protects healthy mitochondria from fusion-mediated contamination by dysfunctional organelles. Likewise, parallel involvement in PINK1 and Parkin in multiple mitochondrial quality control mechanisms points to functional redundancies in this crucial activity, revealing how deterioration in mitochondrial fitness has become an emerging theme in chronic degenerative disease, and uncovering multiple opportunities for therapeutic intervention.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

<b>Drp1</b>	Dynamin-related protein 1
<b>Fis1</b>	Fission 1
<b>HR</b>	heptad repeat
<b>IMM</b>	Inner mitochondrial membrane
<b>MEF</b>	murine embryonic fibroblast
<b>Mff</b>	Mitochondrial fission factor
<b>Mfn</b>	Mitofusin



<b>Opa1</b>	Optic atrophy 1
<b>OMM</b>	Outer mitochondrial membrane
<b>PA-GFP</b>	Photo-activatable green fluorescent protein
<b>PARL</b>	presenilin-associated rhomboid-like protein
<b>PINK1</b>	PTEN-induced putative kinase 1
<b>ROS</b>	Reactive oxygen species
<b>TIM</b>	Translocase of the inner mitochondrial membrane
<b>TOM</b>	Translocase of the outer mitochondrial membrane

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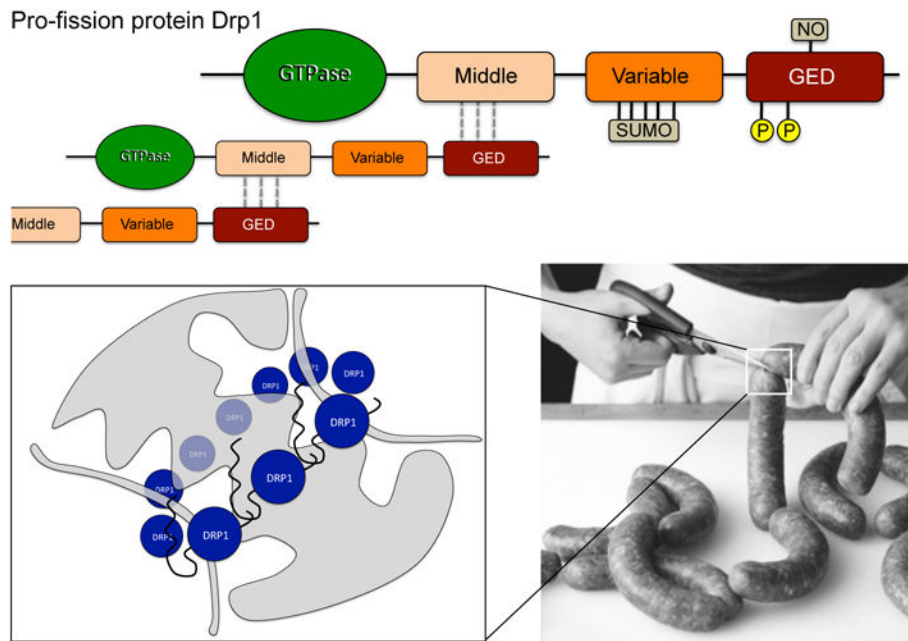
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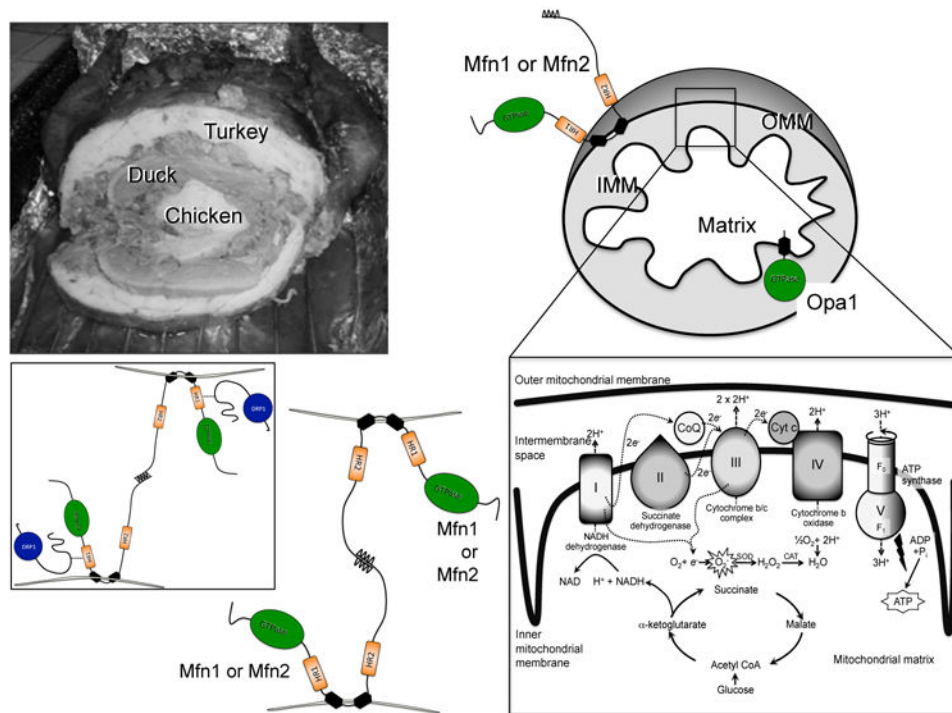
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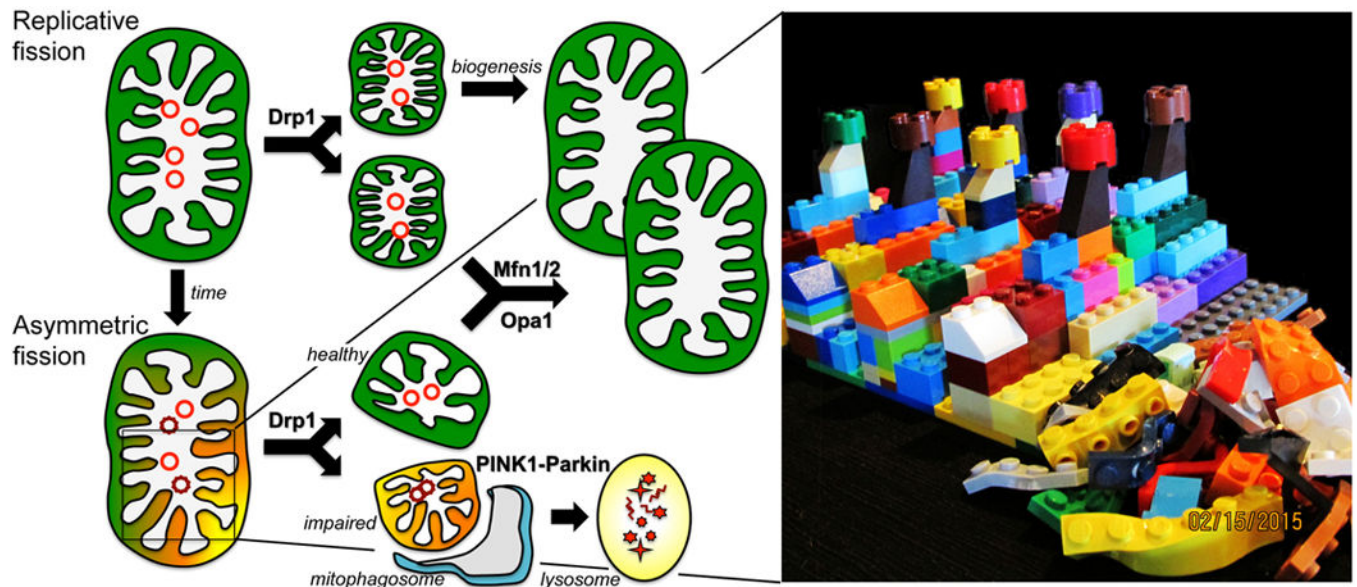


**Figure 1. Sausage link model of mitochondrial fission by Drp1**

Top, schematic depiction of Drp1 molecular structure. The GTPase domain (green) is at the amino terminus and the GTPase-effector domain (GED; rust) is at the carboxyl terminus. The GED interacts with the middle domain of adjacent molecules (dashed lines) to promote head-to-toe oligomerization. GTP-mediated constriction of oligomeric ring structures constricts and severs the mitochondrion like forming sausage links (bottom). Sites for some post-translational modifications are represented on the uppermost structure; P is phosphorylation, NO is S-nitrosylation, SUMO is sumoylation.

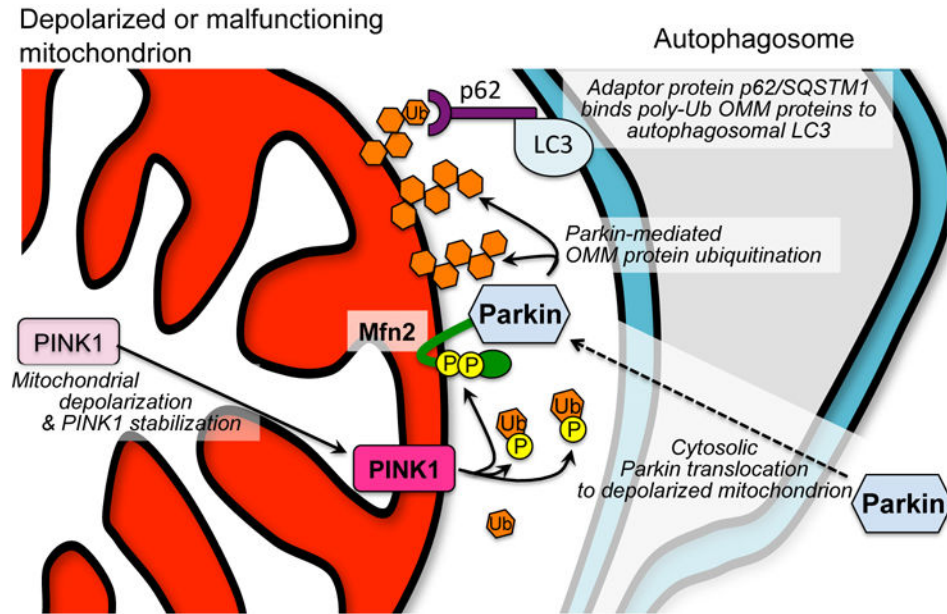


**Figure 2. Turducken model of mitochondrial structure and fusion by Mfn1, Mfn2, and Opa1**  
 Top, multi-compartment structure of turducken (left) and cartoon mitochondrion (right). Outer mitochondrial membrane (OMM) fusion protein mitofusins (Mfn) 1 or 2 and inner mitochondrial membrane (IMM) fusion protein Opa1 are shown with GTPase domains in green. Exploded view of electron transport chain and associated pathways is bottom right. Bottom left, schematic depiction of Mfn-Mfn binding in trans, tethering two mitochondria; inset is authors' conception of how pro-fission protein Drp1 (blue) may facilitate Mfn-mediated fusion.

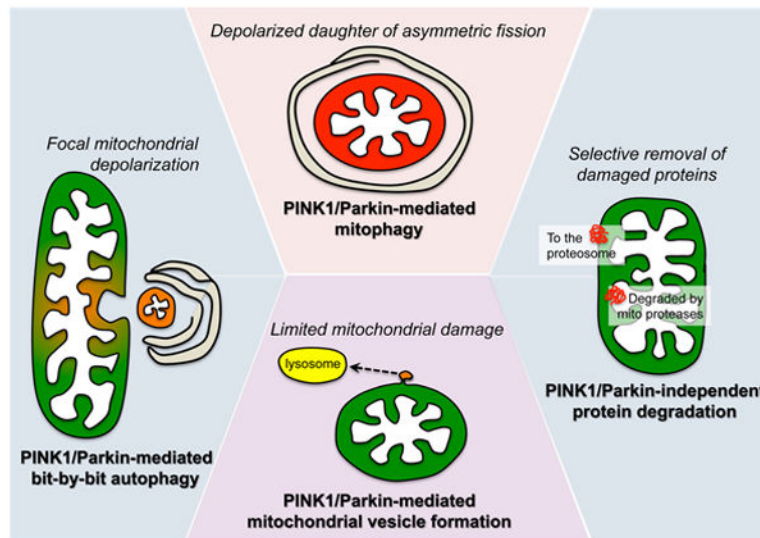


**Figure 3. Replicative and asymmetric fission and intra-organelle partitioning of damaged components**

Left; diagram of symmetric replicative fission (top) and asymmetric fission leading to selective mitophagy of the impaired daughter organelle (bottom). Right; illustration with Lego blocks of how misfolded proteins (front right) might passively segregate from functioning respiratory supercomplexes (rear left).



**Figure 4. Molecular events leading to selective mitophagy of dysfunctional mitochondria** Left; mitochondrial depolarization or other impairment interrupts normal proteolytic processing of PINK1 kinase. PINK1 accumulates on the OMM and phosphorylates Mfn2, promoting its recruitment of Parkin, and ubiquitin (Ub), enabling its utilization by Parkin. Poly-ubiquitinated OMM proteins bind p62 linked to autophagosomal LC3, thus targeting the mitochondrion for mitophagy.



**Figure 5. Mechanisms of mitochondrial quality improvement, from macro to micro**  
 At the top is PINK1/Parkin-mediated mitophagy of an intact organelle, as with the depolarized daughter of an asymmetric fission event (see Figure 3). To the left is bit-by-bit autophagy of localized mitochondrial damage, which is excised and engulfed by an autophagosome via Parkin activity. At the bottom is PINK1/Parkin-mediated formation of mitochondria-derived vesicle, incorporating damaged components into a vesicle that transports them to lysosomes. On the right is shown PINK1-Parkin independent proteasomal removal of outer mitochondrial membrane proteins, and protease-dependent degradation of internal mitochondrial proteins, the most selective of the quality improvement processes.