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Review

The mitochondrial permeability transition pore (PTP) — An example of multiple molecular exaptation?

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ABSTRACT

The mitochondrial permeability transition (PT) is a well-recognized phenomenon that allows mitochondria to undergo a sudden increase of permeability to solutes with molecular mass ≤1500 Da, leading to organelle swelling and structural modifications. The relevance of PT relies on its master role in the manifestation of programmed cell death (PCD). This function is performed by a mega-channel (in some cases inhibited by cyclosporin A) named permeability transition pore (PTP), whose function could derive from the assembly of different mitochondrial proteins.

In this paper we examine the distribution and characteristics of PTP in mitochondria of eukaryotic organisms so far investigated in order to draw a hypothesis on the mechanism of its evolution. As a result, we suggest that PTP may have arisen as a new function linked to a multiple molecular exaptation of different mitochondrial proteins, even though they could nevertheless still play their original role.

Furthermore, we suggest that the early appearance of PTP could have had a crucial role in the establishment of endosymbiosis in eukaryotic cells, by the coordinated balancing of ATP production by glycolysis (performed by the primary phagocyte) and oxidative phosphorylation (accomplished by the endosymbiont). Indeed, we argue on the possibility that this new energetic equilibrium could have opened the way to the subsequent evolution toward metazoans.

1. Introduction

1.1. The mitochondrial permeability transition pore (PTP) — a master of cell fate

The occurrence of swelling in isolated mammalian mitochondria has been recognized since 1950s by several authors, who showed that this phenomenon was induced by Ca^{2+}, phosphate (P_i) and free fatty acids (FFA), while was inhibited by adenine nucleotides, Mg^{2+} and acidic pH [1–5]. Only during the 1970s Hunter and Haworth named this phenomenon permeability transition (PT) [6–8] and, in addition, associated it to the presence of pores with an estimated diameter of ca. 3 nm [9]. When PT happens, mitochondria undergo a sudden increase of permeability to solutes with molecular mass ≤1500 Da, a process that is detectable after Ca^{2+} accumulation [10,11]. PT is selectively inhibited by cyclosporin A (CsA) [12–15], a drug able to inhibit also a mitochondrial mega-channel [16]. The latter is a large-conductance channel (up ca. 1.3 nS in symmetrical KCl, with a pore diameter of ca. 30 Å), which thus appears to be responsible for the manifestation of PT [17,18]. Therefore, PT has been associated to a pore, named permeability transition pore (PTP) (Fig. 1), a structure that results to be highly regulated by an array of modulators, i.e. transmembrane electrical potential, matrix pH, divalent cations and adenine nucleotides [19–21].

The first and still debated function related to PT is the release of Ca^{2+} from the mitochondrial matrix [20]. Since Ca^{2+} is the most important second messenger in all eukaryotic cells, the control of its cytoplasmic concentration is crucial to regulate many enzymatic activities and thus several vital processes such as gene expression, metabolic control, cell plasticity, motility, proliferation and cell death [22]. In particular, cell death may be distinguished into two types, named necrotic and

Abbreviations: ANT, adenine nucleotide translocator; TSPO, outer membrane translocator protein of 18 kDa (formerly known as the peripheral benzodiazepine receptor); CsA, cyclosporin A; CyP-D, cyclophilin-D; DhMUC, *Debaryomyces hansenii* mitochondrial unspecific channel; EMAP II, endothelial monocyte-activating polypeptide II; FFA, free fatty acids; IMM, inner mitochondrial membrane; MARCKS, myristoilated alanine-rich C kinase; substrate mito BK_{Ca}, large conductance, calcium-activated K^+ channel; mito K^+_{ATP}, ATP-regulated K^+ channel; mito Kv1.3, voltage-regulated K^+ channel; NEM, N-ethylmaleimide; OMM, outer mitochondrial membrane; PCD, programmed cell death; PiC, phosphate carrier; PT, permeability transition; PTP, permeability transition pore; ROS, reactive oxygen species; ScMUC, *Saccharomyces cerevisiae* mitochondrial unspecific channel; VDAC, voltage-dependent anion channel; YMUC, yeast mitochondrial unspecific channel

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programmed cell death (PCD). In turn, PCD can then be subdivided into autophagic (or autophagic-like in plants), apoptotic (or apoptotic-like in plants) and lysosomal death [23]. Although the mechanisms leading to cell death may be diverse, a mitochondrial pathway has been documented in animal [19,24,25], plant [26,27] and yeast [28] cells undergoing PCD. In all cases, a central role is played by PT, which is responsible for mitochondrial swelling with the consequent release of pro-apoptotic factors (i.e. cytochrome *c*, AIF, Smac-Diablo). This phenomenon could be limited to a small number of mitochondria and, in this case, autophagy is the most probable outcome [29,30]. When a large population of mitochondria is involved, these organelles are subjected to swelling, which determines the release of the apoptotic factors [31]. If the ATP drops to low levels (less than 50%), the cell itself undergoes necrosis [32], but apoptotic death is likely to occur when this level is maintained relatively high [33].

1.2. Understanding PTP by studying its evolution

The pore involved in PT seems to be the result of the assembly of proteins, which are still hypothetical and known to carry out *per se* completely different functions. Thus, PTP may be considered one of the most obscure issues of biology. In any case, PTP seems to imply the cooperation of different proteins. The major putative components, hitherto described, include: the voltage-dependent anion channel (VDAC) or porin, localized in the outer mitochondrial membrane (OMM); the adenine nucleotide translocator (ANT) in the inner mitochondrial membrane (IMM); the peripheral benzodiazepine receptor and the Bcl-2 family proteins; the hexokinase bound to porin; the cyclophilin-D (CyP-D), a matrix peptidyl–prolyl cis–trans isomerase and the intermembrane space creatine kinase [19]. In addition, other components have been successively added: the protein import machinery (TIM and TOM), as well as the respiratory complex I [34]. These components have been substantially found in all mitochondria in which PTP has been detected, except for creatine kinase that seems to be absent in plant cells. However, besides CyP-D, a precise structural/functional role for these proteins has to be still firmly established.

Two of the major candidates believed to perform the channel activity (i.e. VDAC and ANT) appear now to be modulators rather than constituents. Indeed, mitochondria lacking all isoforms of VDAC maintain normal pore activity [35,36] along with sensitivity to CsA. Similarly, on the basis of knock-out mutant studies, ANT revealed not to be essential for the activity of the channel [35]. Accordingly, polyclonal antibodies, used to identify a CyP-D-binding protein as ANT, cross-react with the phosphate carrier (PiC) [37]. Therefore, the latter has been proposed as a component of PTP [37], a conclusion that has been further confirmed by recent results using siRNA in HeLa cells to reduce PiC expression [38]. Nevertheless, genetic ablation of *Ppif* gene, encoding CyP-D, or CyP-D inhibition by CsA unmask the inhibitory site for P_i, which thus appears to be the actual PTP desensitizer agent, a result that, however, does not necessarily implicate the involvement of the PiC [39]. Hence, P_i inhibition reveals to be the most widespread feature of PTP among the mitochondria so far examined in the eukaryotic domain.

Even PTP localization on both OMM and IMM is now questionable in the light of the finding that PT has been very recently described in mitoplasts, thus showing that PTP is an inner membrane event regulated by the outer membrane through specific interactions with the peripheral outer membrane translocator protein of 18 kDa (TSPO, formerly known as the peripheral benzodiazepine receptor)[40].

First identified in mammals, now PTP has been found in mitochondria of other clades of Eukarya. This evidence has led to compare PTP structure, function and regulation in mitochondria from different species [34,41]. However, in such works, the possible evolutionary mechanisms, by which PTP could be arisen, have not been considered. Hence, in this paper, we attempt to address this matter by describing the appearance of PTP via a mechanism of cooption/exaptation, described for the first time in a different context in 1982 [42]. The terms exaptation and cooption are currently used as synonyms, albeit with the former an old trait (if any) is compared with a new one, while the latter implies that both traits have to be present at the beginning, but then one of these has been later coopted to perform its current function. The PTP interpretation in this frame fits better that of exaptation, a phenomenon that would give scientists a deeper insight of the multiple roles of mitochondria in cell biology. Understanding PT in terms of exaptation implies an enormous epistemological potential: how many bizarre results have been left unreported because in apparent contrast with the adaptationist "dogma" of evolutionary biology? Yet, many data might be re-evaluated in the light of a more open interpretation of the structure/activity relationships displayed by macromolecular or supra-molecular entities.

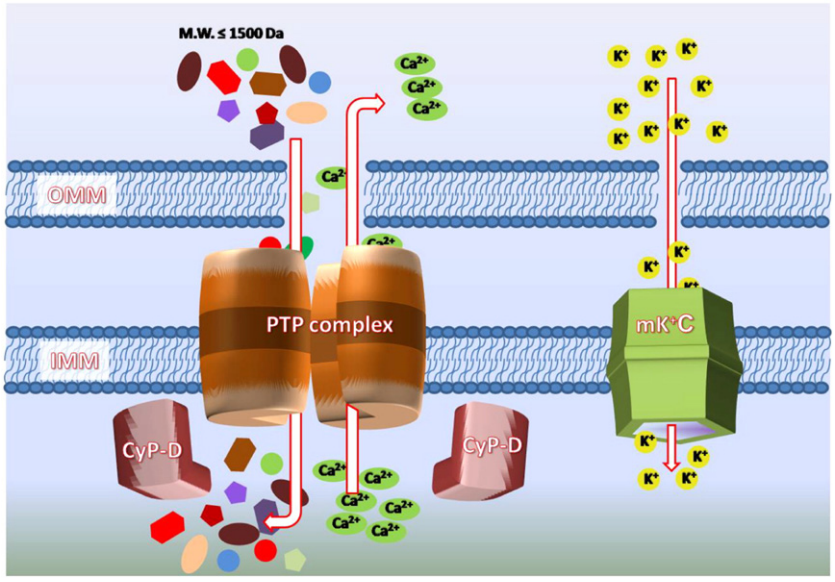

Fig. 1. Simplified representation of the two major devices responsible for the permeability transition in mitochondria. i) PTP complex; CyP-D, cyclophilin-D; ii) mK^+C, K^+ channels. The direction of arrows describes the solute fluxes during the opening of the channels.

2. The PTP in the major *taxa* of living organisms

2.1. Chordata (mammals, fish and amphibians)

2.1.1. Mammals

In mammals the study of PTP activity has been supported by the prospect to better understand pathophysiological mechanisms underlying various human diseases, in particular as a potential executioner of cell death [22,43] and as a target for cardio-protection [44]. Therefore, a great deal of efforts has been done to define two major PTP roles, i.e. those in calcium-mediated signaling and in pathology [45], which appear to be related to its switching from low- to high-conductance states [46].

The PTP opening can be either transient or permanent. The transient opening leads to different consequences, such as loss of the electrochemical potential, uncoupling of oxidative phosphorylation, ATP decline [37] and release of matrix solutes (determining Ca^{2+} depletion) [43]. The re-establishment of the cellular homeostasis of ATP and/or cytosolic Ca^{2+} presumably brings about closing of the pore. However, when homeostasis is severely compromised, these changes, though transient, can induce cell necrosis due to the activation of phospholipases, nucleases and proteases [47]. By contrast, long-lasting opening could determine permanent membrane depolarization followed by *cristae* unfolding, matrix swelling, release of cytochrome *c* and apoptogenic factors, which lead towards PCD [48]. The equilibrium between two possible fates, i.e. necrotic or apoptotic cell death, depends on the intracellular ATP level [49]. However, the regulatory machinery of the PTP function is extremely complex, as revealed by the numerous positive or negative effectors acting on PTP opening [50,51]; the major of them are reported in Table 1.

2.1.2. Regulation by Ca^{2+}

In this scenario, cytosolic Ca^{2+} concentration, which is involved in cell signaling [52], plays a pivotal role [43,53]. Its level is controlled by plasma membrane Ca^{2+} channels, or via release from intracellular stores, such as endoplasmic and sarcoplasmic reticulum [54]. Mitochondria cooperate in Ca^{2+} homeostasis and signaling by smoothing the cytosolic waves [55] and, in this context, PTP works as a Ca^{2+} channel by promoting Ca^{2+} release. PTP allows transient openings [56,57], a process that is switched on by mitochondrial matrix Ca^{2+}. The specificity for this cation is absolute, while other divalent cations, such as Sr^{2+}, Mn^{2+}, Ba^{2+} and Mg^{2+}, act as inhibitors [6,58]. In energized mitochondria, the Ca^{2+} uptake is accomplished by the ruthenium red-sensitive mitochondrial Ca^{2+} uniporter [59,60], whose structure and gene have been now identified [61,62]. This Ca^{2+} uptake is balanced by the Na^{+}/Ca^{2+} antiporter and a Ca^{2+} efflux pathway, both sensitive to membrane depolarization [22]. This interplay between Ca^{2+} efflux and influx tunes the PTP open-closed transition. Although more complex effectors must be in action, the PTP opening during *in vivo* ischemia–reperfusion seems to be mainly mediated by oxidative stress rather than Ca^{2+} changes [63].

In concert with Ca^{2+}, the mitochondrial matrix accumulates P_i, which has long been known as an activator of mitochondrial PT [13]. Inorganic P_i has been considered as a fine regulator of mammalian PTP, since it forms an amorphous (non crystalline) matrix precipitate with Ca^{2+}, so lowering the PTP opening probability [64]. Conversely, it may be considered a stimulator, being a chemical buffer able to maintain pH at optimal levels for PTP opening [65]. New findings on P_i inhibitory effect by binding on P_iC [39] have been mentioned above in Section 1.2.

2.1.3. Regulation by ROS

Oxidative stress is known to induce PTP opening in mammalian mitochondria [66]. Reactive oxygen species (ROS) modulate PTP opening by oxidizing different sites, two of which are thiol groups (SH), located in the matrix site. The first, suggested to correspond to Cys_{160} of adenine nucleotide translocase (ANT) [44,67], is regulated by glutathione oxidation and is protected by low concentration of N-ethylmaleimide (NEM) or monobromobimane [68]. The second, identified as Cys_{56} of ANT [44,67], is sensitive to the redox state of the matricial pyridine nucleotides, perhaps with the mediation of thioredoxin or lipoamide [69,70]; this site is protected by NEM, but not by monobromobimane. A third sensor is an external SH, promoting PTP opening by reaction with NEM or copper-*ortho*-phenantroline [71]. It must be considered that ROS act in synergy with Ca^{2+} [53]. Indeed, ROS increase the Ca^{2+} cell concentration that favors ATP production and, in turn, Ca^{2+} promotes ROS generation during oxidative phosphorylation. Then, beyond a threshold level, Ca^{2+} triggers PTP transient openings, leading to ROS production at mitochondrial level, which determines inhibition of complex I and IV, displacement of cytochrome *c*, inhibition of electron flow and release of glutathione. This opening, involving nearby mitochondria via the released factors, could start a cascade of events triggering PCD [43]. Another opposite effect is the inhibition of PTP, induced by singlet oxygen generated by UV radiation [72]. This effect, mediated by porphyrins in mitochondrial domains, involves, in particular, His and Cys residues on the PTP, and an 18 kDa translocation protein in the outer membrane, previously identified as the peripheral TSPO [45]. Thus, ROS, being both inducers and inhibitors depending on the metabolic context, might be the molecular effectors of transient and, presumably, recurrent PTP openings.

2.1.4. Regulation by pH and membrane voltage

The pH and voltage dependence of PTP opening has been recognized since 1992 [58]. The PTP is closed by high, inside negative, electrical potentials. The matrix pH optimum for pore opening is 7.4 [73], while its opening probability decreases by both increasing (by an unknown mechanism) or decreasing this pH value through reversible protonation of hystidyl residues [65]. When pH is above 7.0, the inhibition could be due to proton competition with Ca^{2+} for its binding at the regulation site [73,74]. It has been suggested that a hypothetical sensor could regulate the PTP opening by receiving (and translating) signals from both the transmembrane and the surface potentials [22].

2.1.5. Inhibition by CsA

The main accepted feature, associated to PTP activity, is the inhibition caused by CsA [75]. This inhibition is used as a key to understand

Table 1
Regulatory characteristics of PTP in mitochondria from different taxa of eukaryotic cells. The induction of opening (+) or closure (−) of PTP by different regulators is indicated. Contrasting effects depending on the regulator amount are labelled as +/−. Unknown effects are labelled as ? and n.d. means not detected.

		Chordata			Arthropoda		Fungi	Plantæ
		Mammal	Fish	Amphibian	Insect	Crustacean	Yeast	Angiosperm
Regulators	Ca^{2+}	+	+	+	+	−	+/−	+
	P_i	+/−	+	+	+	−	−	+
	$\Delta\psi$ loss	+	?	?	+	−	?	+
	pH	+/−	?	?	?	?	?	+/−
	CsA	−	+	+	−	−	No	−
	ROS	+	+	+	+	−	+	+
	AN	−	?	?	?	?	ATP+/ADP−	−
PCD induction		Yes	Yes	Yes	Yes	n.d.	Yes	Yes

the role of PTP in several models of human diseases [76]. The effect of CsA on PTP has been proved by several indirect findings. Few years ago, working with mice knock-out mutants, it has been demonstrated that the target for CsA is CyP-D [76–79], which is inhibited by the drug at the same concentration inhibiting the PTP [80]. However, the PTP can be assembled and function even in the absence of CyP-D, which modulates the Ca^{2+}-dependent PTP opening, with no consequences on the PTP regulation [76].

2.1.6. Regulation by nucleotides

The mitochondrial PT regulation by adenine nucleotides is known since 1979 [7], but has been well elucidated only in the early 1990s [15,81]. The evidence that the PTP opening is inhibited by ATP and ADP, but not by their Mg^{2+} complexes or by other nucleotides (AMP, GDP, GTP) [82,83], suggests that the proper target of PTP inhibition by adenine nucleotides is ANT [44], because also bongkrekic acid (inhibitor) and carboxyatractyloside (stimulator) could set the ANT in an opposite conformation [84], affecting the pore in reverse directions. Following the Halestrap's hypothesis [44], this inhibition by nucleotides could be due to a regulation of PTP opening promoted by the membrane potential-dependent binding site for ADP on ANT.

2.1.7. Fish and amphibians

In the past, the occurrence of a PT in fish has been demonstrated only in the great green goby (*Zosterisessor ophiocephalus*) [85] and in the rainbow trout (*Oncorhynchus mykiss*) [86]. In the first case, the PT from liver mitochondria revealed to be similar to that described in rat liver mitochondria. The main difference concerns the higher concentration of Ca^{2+} required to induce the phenomenon. This difference could be ascribed to a lower binding affinity for Ca^{2+} at the level of the critical site present in the pore-forming structures in these mitochondria. This feature has been interpreted as a defence mechanism for fish living in polluted water [85]. In the second case, the different induction of PT could be related to an adaptation of the great green goby in particularly harsh environments, as it displays a high tolerance and resistance to various pollutants. The hypothetical role of PTP has been also investigated as a potential mechanism involved in the occurrence of cell death (apoptotic or necrotic), caused by copper exposure in rainbow trout hepatocytes. In particular, in Cu-exposed trout hepatocytes, apoptotic cell death appears related to the onset of the PTP, as CsA fully inhibits apoptotic cell death, whereas necrotic death occurs independently from it [86].

More recently [41], the PT has been described in zebrafish (*Danio rerio*). This PT shows features similar to those of the mammalian pore, like desensitization by CsA, appropriate responses to the key modulators of the mammalian PTP (voltage-dependence, pH, dithiol oxidants, etc.). Moreover, the role of PTP has been also examined in the Baltic lamprey (*Lampetra fluviatilis*) liver, where its bioenergetic parameters have been associated to seasonal variations. In winter the suppression of energy metabolism in mitochondria from lamprey liver causes the opening of PTP in its low-conductance state. These responses (e.g. leaky mitochondrial membranes, low concentrations of mitochondrial adenine nucleotides, etc.) exhibit analogies with some mitochondrial features of patients showing important mitochondrial pathologies [87]. However, differently from what observed in human mitochondrial diseases, mitochondria from lamprey liver are able to overcome the energetic fall. In spring, prior to spawning and death, the lamprey exhibits a sharp activation of its metabolism, which is associated to the activation of the mitochondrial substrate oxidation. The latter is accompanied by closing of the PTP, since the inner mitochondrial membrane is not permeable to protons in this period [87].

Occurrence of the PTP has been verified also in amphibians, since the mitochondrial PT plays an important role in 3,5,3′-triiodothyronine (T3)-induced apoptosis in the tadpole (*Rana rugosa*) tail, resulting in tail shortening [88]. Indeed, T3 and Ca^{2+} induce a large amplitude swelling in mitochondria isolated from the liver of this species, while CsA suppresses this effect. Regarding *Rana temporaria*, it has been suggested that during all seasons (differently from lamprey) PTP is probably closed in liver mitochondria [87].

Although the above evidence is only circumstantial and fragmentary, so it is possible to conclude that, in non-mammalian vertebrates (fish and amphibians), the PTP shows similarities to the mammalian one. Hence, the mammalian PT model seems to be largely shared among the species, belonging to Chordata, hitherto studied (Table 1). Thus, it appears that mammals have inherited a “perfect” function from more ancient vertebrates. Its uniformity in Chordata suggests that PT, and its underlying molecular machinery, fulfils a vital role.

2.2. Arthropoda (insects and crustaceans) and Nematoda (Caenorhabditis elegans)

Little is known about PTP in Arthropoda. Differently from the mammalian PTP, mitochondria from *Artemia franciscana*, a crustacean that can tolerate anoxia for years, is able to actively load extramitochondrial calcium up to 1 mM without undergoing a PT [89]. In addition, PTP does not open in response to a variety of inducers of the mammalian PTP. Nevertheless, the mitochondria show a high concentration mercury-induced PT, which is CsA-insensitive. It appears, therefore, that a PT, whether regulated or unregulated, may be an essential function for the survival of such an organism. The apparent lack of a PT has been confirmed in the ghost shrimp *Lepidophthalmus louisianensis*, a known prolonged-anoxia tolerant species [90]. Even in this case, it has been shown that Ca^{2+} plus P_i do not trigger the opening of a regulated PTP in mitochondria, as it does in the mammalian ones. Therefore, it could be stated that in crustaceans the lack of a typical mammalian PT could be an adaptive mechanism to tolerate an extended period of anoxia.

Among arthropods, an important model organism (*Drosophila melanogaster*) has been recently investigated to examine the properties of Ca^{2+} transport in mitochondria [91]. The mentioned paper shows that *D. melanogaster* mitochondria possess Ca^{2+} transport systems that are very close to those of mammals. In particular, their mitochondria show a ruthenium red-sensitive Ca^{2+} uptake. Nevertheless, Ca^{2+} release, mediated by PTP, is CsA-insensitive and is inhibited by P_i. For this reason, it has been suggested that *D. melanogaster* mitochondria seem to have a PTP with intermediate features between yeast mitochondria (see Section 2.3) and those of mammals (see Section 2.1 and Table 1).

Summarizing, it is possible only to affirm that crustaceans do not exhibit a canonical PT, since this transition is a Ca^{2+}-insensitive PTP, while *D. melanogaster* mitochondria shows a typical Ca^{2+} transport, but a PTP with intermediate features.

Regarding Nematoda, a recent paper shed new light about the role of ANT as an important regulator of PCD in *Caenorhabditis elegans* [92]. This protein plays a similar function to its mammalian counterpart (the exchange of cytosolic ADP with mitochondrial ATP, its role in apoptosis). Despite its evolutionary conserved functions, there is no direct evidence showing that *C. elegans* mitochondria possess a mammalian-like PTP complex. Possibly, PT should be investigated by testing potential effectors related to *C. elegans* metabolism.

2.3. Fungi (yeasts)

Yeast mitochondria possess an inner membrane large-conductance unselective channel that has a similar exclusion size to that of the mammalian counterpart. As proposed by Manon and co-workers [93], this pore has been named yeast mitochondrial unspecific channel (YMUC), since it has been characterized in two different yeast species, specifically indicated as ScMUC (in *Saccharomyces cerevisiae*) and DhMUC (in *Debaryomyces hansenii*). Apparently, *Pichia pastoris*, *Yarrowia lipolytica*

and *Endomyces magnusii*, non-conventional yeasts, lack such an unspecific channel [34].

In *S. cerevisiae*, as well as in *D. hansenii*, the complete structural characteristics of MUC have not yet been defined. Orthologues of the main supposed components of the mammalian PTP have been identified in *S. cerevisiae*, namely: i) AAC1, AAC2, AAC3 orthologous of ANT; ii) VDAC related genes, POR1, coding for the major isoform VDAC1, and POR2, although VDAC2 is probably not a channel also in yeast [94]; iii) Cpr3p, the mitochondrial cyclophilin involved in the process of protein folding [95]. Nevertheless, their direct role in pore formation has been questioned, since PT is still detected in mitochondria from a yeast strain lacking, respectively, of ANT [35] and VDAC1 [96]. Therefore, it has been proposed that both ANT and VDAC could play a role in the regulation of the pore [34]. Recently, the involvement of the mitochondrial P_iC as a constituent and sensor of P_i in ScMUC has also been described [97].

In spite of the structural similarity reported between the putative main components of ScMUC and those of mammalian PTP, the characteristics of the former are different from those showed by the mammalian counterpart [30,41,98]. In particular, ATP, GTP, dATP, dGTP, and GDP are inducers of ScMUC opening, while ADP or high P_i concentrations close the pore [99]. Furthermore, ScMUC is not sensitive to CsA, although the latter is able to bind and inhibit Cpr3p [41,95]. Therefore, it has been suggested that Cpr3p would not interact with ScMUC opening, thus explaining why the inhibitory effect of P_i is observed in yeast, while in mammals the association of CyP-D with PTP would hinder the P_i inhibitory site [41]. Recently [97], it has been observed that in yeast mitochondria, depleted of P_iC, the sensitivity of ScMUC to P_i is lower, but the permeability transition is still induced by ATP, although the open channel results smaller. Therefore, it has been proposed that P_iC is not essential for the pore activity. Nevertheless, it represents an important constituent of ScMUC as P_i sensor [97].

Regarding the effects of Ca^{2+} on PT in yeast, conflicting results have been reported. In particular, as a further remarkable difference between yeast and mammalian PT, cytoplasmic Ca^{2+} is able to induce the closure of ScMUC by interacting with VDAC, even though mitochondria from modified yeast strains, lacking this protein, still show PT [100]. Conversely, since yeast mitochondria lack an endogenous Ca^{2+} uniport [30,101], ScMUC is unaffected by exogenous Ca^{2+}, even in the presence of Ca^{2+} ionophore ETH129 [41,102]. Indeed, it has been recently reported that, even when yeast mitochondria are permeabilized to Ca^{2+}, the induction of PT is established under optimized experimental conditions, just when mitochondria are incubated with low P_i concentration (e.g. 2 mM) [103]. Nevertheless, yeast *E. magnusii* mitochondria possess a system for Ca^{2+} influx and efflux, but no induction of PT has been observed so far [30,104].

In the halophilic yeast *D. hansenii*, a marine organism, it has been observed that PTP is completely blocked by high P_i concentrations and is closed by Ca^{2+} and Mg^{2+}. Furthermore, in the presence of low concentration of P_i (e.g. 0.4 mM), when DhMUC is open, both Na^+ and K^+ are able to induce the closure of the pore [105], a behavior that has been interpreted as a specific adaptive feature to marine environment [34,105].

The comparison between the yeast pore and its mammalian counterpart indicates that the regulation of the former is simpler and the differences might be explained on the basis of the different environments faced by yeast and mammalian cells [41]. Even though the physiological role(s) played by ScMUC is still under debate, some functions have been proposed for such a pore: i) regulation of the volume of the mitochondrial matrix [93]; ii) dissipation of the proton gradient through a system that would be active when energy consumption is low (e.g. when redox balance must be maintained by eliminating the excess NAD(P)H in oxidative processes); iii) protein import into the mitochondria [106]; iv) triggering of apoptosis in yeast, since the expression of mammalian pro-apoptotic proteins could induce or prevent yeast cell death [102].

2.4. Plantæ (Tracheophytes)

The evidence for the presence of a PT in plants has been obtained for the first time in pea mitochondria, where it has been shown that CsA is able to delay the collapse of the transmembrane electrical potential induced by Ca^{2+}, ADP and P_i addition [107]. Such phenomenon is detectable only in the presence of dithioerythritol, indicating the requirement for an adequate redox state. These results have been confirmed and further extended in potato tuber by Arpagaus and co-workers [108], who showed that also in this species mitochondria undergo a swelling, dependent again on the presence of a reducing agent. In addition, a CsA-insensitive, Ca^{2+}-stimulated PT has been detected in potato tuber [109] and in wheat [110] mitochondria. Therefore, it seems that both CsA-sensitive and CsA-insensitive PT may coexist in plant mitochondria (Table 1).

In plants, particular attention has been paid to the effect of CsA, whose inhibitory effect on PT has been often used as a diagnostic tool to recognize the involvement of mitochondrial PTP in plant cell death [111]. However, as seen above in potato mitochondria, the CsA-dependent PT inhibition is not a common feature in plant kingdom. This is only the case of PT induced by biotic stress [112], as well as by oxidative and anoxic stress [109,110]. In all these cases, Ca^{2+} is a necessary requirement, although not "permissive". These experimental findings suggest a possible double regulation of PT as found in mammalian mitochondria [41]. According to this model, low- and high-amplitude openings are distinguished, although such an evidence is still lacking in plant mitochondria. The first mode involves a transient depolarization due to short time opening, rearrangement of the *cristae*, release of cytochrome *c*, without the rupture of the OMM. The second way involves long lasting depolarization, which is a more dramatic phenomenon, leading to loss of ionic homeostasis, depletion of matrix pyridine nucleotides, matrix swelling, OMM rupture and, at last, triggering the mitochondrial pathway towards apoptosis. Under these conditions, a striking ATP depletion occurs, leading to energy shortage. Therefore, the time of the opening could represent a crucial factor, decisive to determine which kind of death program the cell has to address [111].

Similarly to mammalian cells, PT is ascribed to the presence of a pore, which hypothetically involves ANT, VDAC [113,114] and, probably, CyP-D [115]. Evidence for the presence of CyP-D in plant mitochondria is only circumstantial [116,117]. It is possible to speculate that this protein may be present in mitochondria exhibiting a PT CsA-sensitive, while it could be absent in those CsA-insensitive. Therefore only in the first case it is possible to hypothesize a regulation based on CyP-D. Among the other hypothesized components, usually having other physiological roles and proposed to regulate such a pore, there is also the PiC [34], but its precise role has not been fully elucidated yet. In addition, the involvement in PTP formation of hexokinase, whose binding to VDAC could interfere in the interaction between the anion channel and Bax, has been shown only in *Nicotiana benthamiana* [118].

As suggested [119], activation of the PTP appears to be related to biotic and abiotic stresses [110,120], in which the pore could be interpreted as a perceiving structure. Several factors are able to regulate PTP opening, including ROS, Ca^{2+} reservoir stored in the mitochondrial matrix, electrochemical potential across the mitochondrial membrane, free fatty acids, pH, P_i and ATP. Secondary messengers, such as hydrogen peroxide and Ca^{2+}, induce an increase in ROS production by the mitochondrial electron transport chain [121]. Such impairment of the respiratory chain is responsible for the decrease of the trans-membrane potential leading to PTP opening. The consequent structural alteration of the *cristae* and, definitely, of mitochondrial morphology, provokes cytochrome *c* release with interruption of the electron transport chain and ATP depletion, resulting in cell death. These phenomena could be

prevented by adding bongkrekic acid that inhibits ANT, or CsA, which displaces CyP-D binding to ANT [122–124].

From these observations, both Ca^{2+} and P_i appear to be the only inducing factors shared by all the PTP structures up to date evidenced in plants [34,41]. Besides these ubiquitous modulators, a wide range of other molecules have been found to influence PT manifestation and, because of their wide presence, they could be defined as tissue and/or species-dependent. These compounds may act both as a positive modulators, as in the case of ROS [125,126] and catalase [107], or as a negative effectors, in the case of spermine [112], ADP [109], thiol redox agents [108], La^{3+} [127] and ruthenium red [109].

2.5. Other clades of Eukarya

Despite the numerous papers concerning the activity of a PTP in animals, fungi and plants, to our knowledge, no direct evidence is currently available on the presence of high- and low-amplitude PT in other organisms such as Amoebozoa (in Unikonta), Chromalveolates, Rhizaria, Excavata, red and green algae (in Bikonta) (Fig. 2).

Some of the putative protein components of the mammalian PTP such as ANT and VDAC, are ubiquitous in different taxa of Eukarya, but they do not seem to perform PTP-related functions. Cyclophilins [128,129], mitochondrial VDAC [130] and ANT have been found in mitochondria of numerous organisms ranging from Gram-negative bacteria to different eukaryotes, but this evidence does not suffice to claim that these proteins function as a PTP. To this regard, quite interesting is the finding that the eukaryotic unicellular *Acanthamoeba castellanii* possesses both a TSPO receptor, identified as VDAC, in the OMM, and ANT in the inner membrane [131], which, by analogy, could perform a regulatory role, similar to that known in animals and recently updated in ref. [40].

Moreover, the most important indirect evidence, leading to consider the presence of PTP also in these eukaryotes, is the observation that PCD occurs in different organisms [132], including unicellular ones [133,134]. Clearly, PCD cannot occur via a mitochondrial pathway in parasitic protozoa lacking mitochondria, but it is reasonable to assume as a basic biological principle that mitochondria do endow unicellular eukaryotes with a mitochondrial pathway to PCD [135]. Indeed a mitochondrial response to oxidative stress that implicates membrane permeability and release of cytochrome *c* has been observed in *A. castellanii* [136].

2.6. PT and mitochondrial K^+ channels

In mammalian mitochondria, a K^+ cycle is also involved in the regulation of mitochondrial metabolism by changes in matrix volume [137], linked to mitochondrial PT, induced by K^+ channel-regulated fluxes [138] (Fig. 1). In human tissues, three different K^+ channels have been detected: i) the ATP-regulated K^+ channel (mito K^+_{ATP}) [139–141]; ii) the mitochondrial large conductance calcium-activated K^+ channel (mito BK_{Ca}) [142]; the voltage-regulated K^+ channel (mito Kv1.3) [143]. These K^+ channels are potentially involved in the prevention of ischemic heart disease [144,145], in cytoprotection [146] and, at least for mito K^+_{ATP} [147,148] and mito Kv1.3 [143], in PCD signal transduction. These effects, similarly to those observed in Tracheophytes, Amoebozoa and Euglenozoa (see Sections 2.4 and 2.5), might be associated to PT, linked to matrix swelling and moderate membrane depolarization [148]. On the other hand, it has been shown that the target for cardio-protection is the low Ca^{2+} matrix influx associated to mito K^+_{ATP} activity [149] and mito BK_{Ca} [150]. This suggests a correlation between the activity of the mito K^+_{ATP} channel and the probability of PTP opening [151,152]. Following this insight, it has been proposed that the mito K^+_{ATP} channel inhibition may exert a PTP-activating effect [153,154].

In the case of Tracheophytes, as above seen, evidence for the presence of PTP is still limited and circumstantial. As a consequence, it cannot be firmly stated to date whether the occurrence of PT is a common feature of these plants or, instead, it is species- or organ-specific phenomenon. Another possibility is that its activity may be masked (or

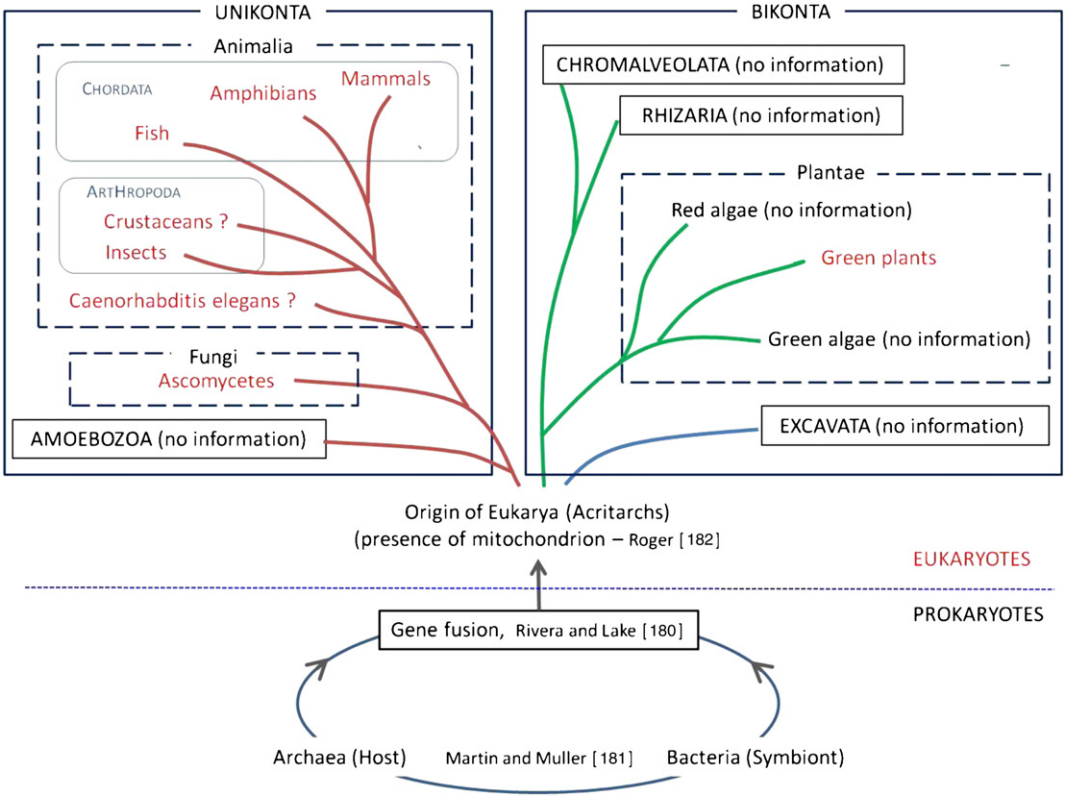

Fig. 2. Origin of the eukaryotic cell (lower part) and phylogenetic tree (upper part) showing the major "supergroups" of Eukarya. In red the clades in which mitochondrial PTP has been described [180–182].

even replaced) by the functioning of other channels or pores, also present in the IMM. In agreement with this assumption, some of the well-known modulators of PTP, such as ATP, CsA, P_i or ROS, have been shown to effectively regulate, sometimes in an opposite way, the activity of a mitochondrial K^+-selective channel, sensitive to ATP, named mito K^+_{ATP} channel [155]. Similarly to the animal counterpart, this channel has also been demonstrated to be functionally present in various plant tissues and species, such as pea and soybean in dicotyledons [156,157], wheat and *Arum* spp. in monocotyledons [158–160], spruce and fir in gymnosperms [155,161–163].

These channels share some common biochemical similarities, such as ATP inhibition, GTP and diazoxide activation, voltage-dependence, modulation by the mitochondrial redox state and by FFA [164], as well as insensitivity to Mg^{2+} and selectivity for K^+ and Rb^+ [155]. On the other hand, only the mito K^+_{ATP} channel observed in some angiosperms [160,165] and gymnosperms [162,163] is specifically activated by CsA, prevented by high voltage values of the electrical potential [157] and modulated by nitric oxide [166]. Recently, the electrophysiological characterization of the mito K^+_{ATP} channel in durum wheat has been fully elucidated [167]. More significantly, in this work, it is shown that plant mito K^+_{ATP} channel, differently from the mammalian counterpart, displays a significant conductance of 150 pS in 150 mM K^+, able to effectively decrease the membrane potential. This feature implies that additional roles may be accomplished by these channels in plant organelles.

Indeed, the K^+ uptake mediated by this channel, working together with an electroneutral K^+/H^+ exchanger, plays multiple functional roles, affecting the proton motive force, controlling organelle bioenergetics, and inducing a mild uncoupling. This energy-dissipating activity has been shown to be involved in: i) prevention of ROS production and oxidative stress [156,158]; ii) the response to hyperosmotic drought and salinity stress, as well as other environmental factors [159,168,169]; iii) thermogenesis in tubers and floral organs [163,170]. Additionally, the K^+ flux is considered to be large enough to cause mitochondrial swelling, by ultimately affecting the fate of the mitochondrion/cell itself. In plants, the K^+_{ATP} channel is involved in a low-amplitude PT, able to release pro-apoptotic cytochrome *c* and to induce the onset of PCD [162,165,166,171]. In agreement, it is noteworthy to consider that the affinity of the plant channel towards ATP (values of K_i around 0.3–0.5 mM) is lower than that of mammalian mitochondria, suggesting that, in plants, a decrease in ATP synthesis may considerably affect and modulate the *in vivo* opening and closure of this channel [167].

Moreover, it has to be mentioned that also a highly active, ATP-insensitive, Ca^{2+}-dependent K^+ uptake pathway has been identified in tomato, potato and maize mitochondria [170]. Its activity is suggested to be dependent on either a distinct large-conductance Ca^{2+}-activated mito BK_{Ca} channel, recently observed by the group of Jarmuszkiewicz [161,172] or, alternatively, on the mito K^+_{ATP} channel itself, depending on the modulation or association/dissociation of its regulatory subunits [167]. Recently, a large-conductance Ca^{2+}-insensitive and iberiotoxin-sensitive channel has been also discovered in potato tuber mitochondria [173]. Hence, it is more correct, as suggested by Pastore and co-workers [155], to refer to various K^+ channels in the plant IMM, like in their animal counterparts.

Finally, with regard to the K^+-driven low-amplitude PT, the activity of a mito K^+_{ATP} channel has also been shown in mitochondria of *A. castellanii* [174] and mitoplasts of two Euglenozoa [175].

3. The evolutionary context where PTP arose

3.1. The origin of the eukaryotic cell and the phylogenetic tree of eukaryotes

In this paragraph the origin of the first eukaryotic cell and phylogenetic tree of Eukarya are outlined to the aim of defining the context in which PTP evolved.

Living organisms are currently subdivided into three domains, named Bacteria, Archaea and Eukarya [176]. The first two are prokaryotes, while the third includes all eukaryotic organisms. The fossil record of eukaryotic cells (protists) is known from a relatively small number of Proterozoic rocks of approx. 1.8–1.3 Gya [177]. However, in the light of more recent, intriguing evidence [178], acritarchs (a lineage of protists) could have appeared very early (approx. 3.2 Gya ago).

The complexity of the eukaryotic cell, with respect to the prokaryotic cell, depends mainly on the presence of a system of endomembranes and organelles that were shaped during evolution through different steps. Mitochondria and chloroplasts, the major organelles, are the "power houses" of the cells, although now they have been recognized to accomplish several other major functions in cell physiology.

Between the end of the 19th and the beginning of the 20th century, some scientists (A.F.W. Schimper, A. Meyer and C. Mereschkowsky) speculated on the evolutionary origin of what is known today as the eukaryotic cell, but the first complete theory was proposed only at the end of sixties of the last century by Lynn Margulis [179]. According to the so-called endosymbiotic theory, mitochondria and chloroplasts have originated, most probably, by two distinct endosymbiotic events. Both organelles have evolutionary ancestors in Gram-negative prokaryotes — mitochondria arose from an alpha-proteobacterium, chloroplasts from an ancient cyanobacterium — which were engulfed by the primary phagocyte (probably an archeal eocyte, provided of a cytoskeleton).

However, the origin of eukaryotic cells is complex and still remains enigmatic. In the light of other evidence, eukaryotic cells could derive from a genome fusion [180]. According to the latter authors, the eukaryotic genome resulted from a fusion of two diverse prokaryotic genomes and, therefore, the first branches of the tree of life should be actually described as a "ring of life" (Fig. 2, lower part). In particular, eukaryotic cells seem to be the result of a fusion between eubacterial and archaebacterial genomes, where the operational genes were primarily supplied from Eubacteria, whereas informational genes derived from Archaebacteria [180–182].

It is possible that mitochondria evolved from a planktonic marine alpha-proteobacterial lineage (clade SAR11) that participated in multiple interspecific cell colonization events. Sometimes, this resulted in a parasitic relationship, but at least in one case this led to symbiosis [183]. For the two above-mentioned hypotheses, the important question is about the motility of the free-living mitochondrial ancestor and its ability to survive in hypoxic conditions. Recent results, obtained by sequencing the genome of *Candidatus Midichloria mitochondrii*, belonging to Rickettsiales, show that this species has 25 genes associated to flagellar assembly and cbb_3-type cytochrome oxidase (a terminal oxidase), which enables it to survive at low levels of oxygen, an environment that probably characterized the early atmosphere of the Earth [184]. Therefore, the mitochondrial ancestor could have played a more active and parasitic role in eukaryogenesis.

For a long time the evolutionary relationships among eukaryotic organisms (mainly protists: protozoans and algae) were inferred from morphological and biochemical data, which permitted to classify the diversity of these organisms (mainly microbial) into a large number of distinct lineages. This picture was partially modified by studies of comparative genomics. Recent advances in this field converge towards a phylogenetic tree composed of five large hypothetical "supergroups" (Fig. 2, upper part), describing eukaryotic diversity, although the order of divergence among these groups remains uncertain [185,186]. The first supergroup, named Unikonta, is a controversial taxon that includes also Amoebozoa and Opisthokontes (including animals and fungi) [187]. The second (Excavates) includes numerous protists, many of which are anaerobic and/or parasitic organisms. The third (Rhizaria) is a supergroup, recently recognized [187], very widespread in nature, although numerous species are less known. The diversity of algae and other protists is accounted by the fourth supergroup (Chromalveolata) that includes Stramenopiles and Alveolates. Finally, members of Plantae (fifth supergroup)

are characterized by the presence of plastids originated by a process of primary endosymbiosis. Phylogenies of many plastid genes support the view that this supergroup has to be considered as a whole [188]. These supergroups have been later, in part, reshuffled, because Chromalveolata (Stramenopiles-Alveolates) and Rhizaria have been assembled in a larger supergroup identified by the acronym SAR [189].

In spite of this complex scenario, from available evidence it is possible to infer that the mitochondrial endosymbiosis could have happened prior the divergence of extant eukaryotes [182]. As seen in Section 2, mitochondrial PT has been detected in several eukaryotic organisms that are considered as "model organisms" [190]. Unfortunately, no information is available about sponges and cnidarians, as well as for Chromalveolata, Rhizaria and Excavates (Fig. 2, upper part).

3.2. Exaptation in the evolution of living organisms

Since the publication of Darwin's *On the Origin of Species by Natural Selection*, the Darwinian theory still provides the most compelling explanation of the evolution of living organisms. However, in the past, some aspects have been inadequately examined, such as the presence and distribution of alternative phenotypes toward which existing living organisms may evolve [191]. There are apparently forbidden phenotypes that cannot be explained by an apparently adaptive disadvantage, while, on the contrary, "monstrous" phenotypes are generated even by point mutations. Therefore, some evolutionary biologists prefer to adopt a pluralistic approach, which allows to consider also other forces acting in evolution — e.g. symbiosis, contingency, niche constraints, structural constraints, etc. [192,193]. The recent birth of Evo-Devo (*Evolutionary Developmental Biology*) is perhaps the most significant field opened by this new perspective [194–196].

In this novel scenario, new emphasis has also acquired the concept of exaptation, introduced since 1982 by Stephen J. Gould and Elisabeth S. Vrba [42], who re-evaluated and extended the concept of preadaptation already conceived by Charles R. Darwin. Meanwhile, this evolutionary strategy became an "exaptation program", which has been also subjected to an open peer commentary [197].

As it is known, the core of the Darwinian theory is represented by an explanation of adaptation, i.e. how a character was shaped for its current use. However, as argued by Gould and Vrba [42], a trait previously selected to accomplish a specific function, may be subsequently exapted (coopted) for a new role (*type one exaptation*). In the extreme case, a character without a function, whose origin cannot be ascribed to natural selection (non-adaptation), may also be exapted to perform its current function (*type two exaptation*). The phenomenon of exaptation — exemplified by Gould and Lewontin [198] with the metaphor of the spandrels in the dome of the Basilica of St. Mark in Venice — is now widely recognized in very different evolutionary contexts, ranging from the molecular level to species, including human beings [193]. On the basis of recent suggestions, human behavior, symbolic cognition [197,199] and altruism [200] would have evolved through exaptations. But even in unicellular organisms, the active cell death, although explained as a maladaptive trait maintained as a by-product of selection or pro-survival functions, may be interpreted as an altruistic trait (exapted), under conditions in which kin/group selection can act [133].

Another aspect has to be pointed out. In the Darwinian theory, the evolutionary changes are considered to be the product of sorting, a term by which the differential birth and death among individuals within a population is described. Natural selection, acting on organisms in the 'struggle for existence', supplies a determinant of sorting, albeit the latter may be also triggered by other causes, such as genetic drift. When we try to interpret a biological phenomenon, in the light of this distinction, several types of evolutionary targets (genome, metabolism, cell, organ, organism, species), at ascending levels of inclusion (hierarchical perspective), have to be considered [201].

In this new conceptual framework, exaptation may thus display its distinctiveness and function at the different levels of complexity at which evolution may act. Indeed, the role of exaptation at the genomic level has been stressed [202]. This view is partially in agreement with a recent definition of gene, suggested as the result of the ENCODE project [203]. These authors describe the history of this elusive concept, from gene as a discrete unit of inheritance to gene as ORF (open reading frame). Their conclusion is that "a gene is a union of genomic sequences encoding a coherent set of potentially overlapping functional products." This definition highlights "how integral the concept of biological function is in defining genes," which, in this context, might be a source of type one exaptations.

This picture may be completed by another paper, also resulting from the ENCODE project, in which the functional behavior of 1% of the human genome was studied [204]. The latter authors stated that: "Surprisingly many functional elements are seemingly unconstrained across mammalian evolution. This suggests the possibility of a large pool of neutral elements that are biochemically active but provide no specific benefit to the organisms." This statement was interpreted as a form of store of genes, conceived as a "warehouse material for natural selection." Less restrictedly, we suggest these genes may constitute row matter for evolution, which can also experience forms of type two exaptations. This suggestion is in line with the "genomenclature" proposed some years ago [205], which allows to recognize pseudogenes and "junk DNA" as parts of a vast repertoire of sequences able to shape an organism during evolution. This genetic potential is termed "*pot*onous" or "*pot*ogenes" and its products may be exapted for novel functions.

From the above considerations, we can infer that exaptation may involve only the proteomic/functional level, without any determination at the genome level, except for the (protein) factors controlling its transcription. This concept, already put forward by Weiner and Maizels, is still neglected in biochemistry [206]. Conversely, we think that exaptation can also display its extraordinary potentialities at this molecular/functional level. Therefore, we assert that new functions may also arise from exaptation of pre-existing proteins/activities to form new complexes and functions.

Probably the first example of molecular/functional exaptation, also reported by Gould and Vrba in their pivotal paper [42], is α-lactalbumin, a milk protein of unknown function, which has an amino acid sequence similar to that of lysozyme. Although α-lactalbumin by itself does not display any enzymatic activity, it is a component of the two-protein lactose synthase [207]. A similar example is offered by the β-subunit of the plant mitochondrial ATP synthase, which exhibits PP_iase activity [208]. Thus it seems that a protein with a PP_iase activity (PP_i could be an ancient reservoir of energy) has been exapted and brought together with other proteins to form the ATP synthase complex. Other suggestive examples were reported and critically examined [193]. Therefore, we summarize here only the most relevant.

The human tyrosyl-transfer RNA synthase catalyses the covalent attachment of the amino acid tyrosine to the corresponding molecule of tRNA. This enzyme has high sequence similarity (49% identity) with the endothelial monocyte-activating polypeptide II (EMAP II) [209,210], a cytokine that activates endothelial monocytes during inflammation [211] and stimulates phagocytic cells, triggering apoptosis. Tyrosyl-tRNA may be secreted when cells undergo apoptosis to be then cleaved into two cytokines behaving as EMAP II [212]. The synthetic activity of tyrosyl-tRNA synthase has to be considered primary, while its opposite role in cell death (as EMAP II) is considered to be secondary, because it appears the result of a gene duplication. The new function (apoptosis) performed by this cytokine has been interpreted as an exaptation, occurring only after this second gene (the new isoform of tyrosyl-tRNA) has been expressed and its product released outside the cell [211]. The versatility of tyrosyl-tRNA synthase is very surprising, because it may function also as a cofactor for self-splicing of Group I intron in *Neurospora* mitochondrial RNA [213].

However, the most prominent example of multiple exaptation is offered by crystallines of the eye lens. Crystallines are the most abundant proteins in the eye lens of vertebrates, but have been found also in invertebrates, such as the marine sponge *Geodia cydonium* [214]. They are numerous and diverse, but can be subdivided into two groups [215]: structural tension proteins (e.g. α-, β- and γ-crystallines), widespread the eye in lens of many vertebrates; taxon specific crystallines, found in more specific evolutionary lineages. The first are the result of exaptations that have involved small proteins (heat shock proteins), while the second were exapted starting from very different enzymes (e.g. alcohol dehydrogenase, α-enolase, glutathione *S* transferase, transketolase, aconitase, etc.). Unlike tyrosyl-tRNA synthase, crystallines are an example of acquisition of a new function, without gene duplication [216]. In other words, crystallines are an extraordinary example of multifunctionality and creativity of the molecules of life [217].

There are at least four other examples that can be interpreted as exaptations. MARCKS (myristoilated alanine-rich C kinase substrate) and its cognate MARCKS-related protein (MRP) are abundant, widely distributed proteins that in solution are labile but essential for controlling cell shape changes [218]. There is little evidence on their secondary structure, because they are able to switch among different conformations — presumably functional — a trait interpreted as a putative example of exaptation. Class I ligases are a group of artificial ribozymes, originally selected from random pools of mRNA sequences, whose catalytic activity depends on metals. Magnesium is the preferred, although these ligases are active with other divalent cations (e.g. Ca^{2+}, Sr^{2+} and Mn^{2+}). They exhibit also the ability to accumulate metals, an activity that was interpreted as an exaptation [219]. The protein avidin, found in white egg, is normally able to bind biotin to form a stable homotetramer. However, this protein is also exapted to be oligomerized with the extracellular mosaic protein fibropellin, thus changing function [220]. Finally, porin or VDAC, a putative component of PTP, is another example of molecular exaptation. These channels are widespread in Gram-negative bacteria, chloroplasts and, obviously, mitochondria [221,222], but they are also expressed in plasma membranes, where VDAC1 exhibits a trans-plasma membrane NADH-ferricyanide reductase activity [223].

In our opinion, molecular exaptation could be described by other examples, which have been, however, interpreted in a different manner. We refer to many enzymes, defined "moonlighting", with reference to the capability of performing, besides their enzymatic activity, other non-enzymatic functions (e.g. structural or regulatory). In addition, other enzymes, qualified as "catalytically promiscuous", are capable of catalysing secondary reactions [224]. Among moonlighting proteins there are pephyrin, phosphoglucose isomerase and the above mentioned tyrosyl-tRNA synthase of *Neurospora crassa*, while among proteins with catalytic promiscuity we mention aminoglycoside kinase, tetrachlorohydroquinone dehydrogenase and aldolase antibody 38C2. Hence, we may conclude that molecular (functional) exaptation is a very common phenomenon, which shaped the metabolism of eukaryotic cells, a domain that Gould defined "a kingdom extremely fertile."

4. The origin of PTP by multiple molecular exaptation

From the above described evidence, it is clear that PT is not a function linked to a specific protein(s) expressed by a gene(s). On the other hand, PT cannot be ascribed to the long list of functions classified as "orphan" metabolic activities (corresponding to 30–40%), for which the relative genes have to be still discovered [225]. Rather, PTP seems to emerge as an assembly of several proteins, whose transport function is highly regulated. In any case, PTP appears to be the result of the cooperation of several mitochondrial proteins probably localized in the OMM and IMM, as well as in the matrix. Hence, we suggest that such a function, the PT, could be derived from exaptation of different pre-existing functional proteins (channels, enzymes or other) that still perform their specific activity. In other words, we propose that a multiple exaptation of type one led to a highly regulated functional complex, which could have arisen early during eukaryote evolution. Indeed, the main characteristics of the PT are highly conserved, having been identified, with minor differences, in evolutionary divergent organisms [34,41] (Fig. 2, upper part). Although a typical PTP has not been shown in anoxia-tolerant crustaceans [89,90], it has been identified in several members of Animalia (mammals, amphibians, fish and insects) but also in Ascomycetes (yeasts). These different organisms seem to possess mitochondria with the basic putative components of PTP that, in several cases (i.e. fish, insects and yeasts), results to be CsA-insensitive. However, this discrepancy appears to be taken over by the observation that P_i is the actual inhibitor, common to the various expressions of PT. Thus, it seems that the coordination of the symbiotic relationship between the cell and mitochondria is dictated by P_i, an indicator of the energy status of the cell [39]. Since in the presence of CyP-D this inhibition is lacking, it would be useful to examine P_i effect in mitochondria showing a CsA-insensitive PTP. Unfortunately, no information is available on PT in mitochondria from Amaebozoas, Excavates, Chromoalveolates and green algal cells, the latter being crucial organisms in the evolution of plants. This information would be very useful to reconstruct the phylogeny of this function.

The evolution of PTP by a mechanism of exaptation might also help us to understand some differences exhibited by PT in the different taxa examined. Being the result of an assembly of different proteins, this aggregation could have happened in a very flexible (and variable) way to better face the internal (cellular) and the external (environmental) contexts in which PTP arose. The consequent versatility of PTP is exemplified by its ability to switch from low- to high-conductance states [46], functions that can overlap those accomplished by the mito K^+_{ATP} channels, commonly recognized in mammalian mitochondria [19]. As seen above, some species of flowering plants have mitochondria exhibiting a high-amplitude PT, which is either CsA-sensitive [107,108] or CsA-insensitive [109,110]. Similarly to animals, plants also show a low-amplitude PT mediated by mito K^+_{ATP} channels [155,161] that, in some cases, are stimulated by CsA in mitochondria isolated from both angiosperms [160,165] and gymnosperms [162,163]. The molecular identity of the plant K^+ channel(s) has not been conclusively elucidated yet; nevertheless, several distinct putative candidates have been proposed [155,161]. On the basis of functional similarities, it has been suggested that the mito K^+_{ATP} channel would consist of Kir and SUR subunits, similar to those forming the tetramer of the well-known surface K^+_{ATP} channels [226–228]. However, on the basis of a new, suggestive hypothesis, plant mito K^+_{ATP} channel may be ascribed to a multi-functional complex, resulting from the interaction among mitochondrial proteins (e.g. ATP-binding cassette, P_iC, ANT, ATP synthase and succinate dehydrogenase), some of which are also involved in the formation of PTP [144,167,229]. Hence, even the mito K^+_{ATP} channel, performing functions similar to PTP, might be the result of exaptation.

5. When did PT appear during evolution?

It is possible to hypothesize that the relationship between the endosymbiont and the host was depending, at the beginning, on transporters and channels present in the outer and inner membranes inherited from the free-living ancestor, a Gram-negative bacterium. The evolution of a primitive endosymbiotic relationship to a more advanced one, in which the host masters and exploits the guest, appears to be determined by the formation of more complete protein-import machinery and by the insertion into the inner membrane of protein carriers for better obtaining energy from the host [230]. This process could have been driven by the host cell (outside view), thus its proteins were "imposed" on the ancestral endosymbiont, but also the endosymbiont could have had an active role in establishing key elements of the protein import pathways (inside view),

in particular for both TOM complex in the OMM and TIM complex in the IMM of the ancestral bacterial proteins [231]. Although the prokaryotic ancestor of TOM40 remains to be determined, it is possible to predict that this translocase subunit could have been the site where additional subunits (e.g. TOM7 and TOM22) aggregated, so enhancing the function of this primitive translocase [232]. In other words, we can suggest that also this event could be due to exaptation.

The main question now is: when did PT appear during evolution? Unfortunately, it is difficult to reconstruct a cladogram, based on mitochondrial PT, for the organisms exhibiting it, since this structure/function is, as hypothesized, the result of an assembly of different proteins, whose composition is not still completely elucidated. A tentative answer could, however, be provided by searching genomic databases for the presence in the model species of at least some of the PT's putative molecular components. A PTP component, appearing to be shared among Eukarya, is represented by cyclophilins for their crucial regulatory role. Therefore, we performed a phylogenetic analysis by comparing protein sequences of mitochondrial cyclophilin/peptidyl–prolyl isomerase, found in the main model organisms exhibiting PT (Fig. 3). From this comparison, it also appears that a homologous protein is present in Gram-negative bacteria (*Legionella* sp.) and that its basic structure is phylogenetically linked to those recognized in eukaryotic organisms known to accomplish PT. The targeting of these eukaryotic proteins to mitochondria is demonstrated by the high score exhibited by the prediction analysis of their N-terminal amino acid sequences. In addition, this cladogram shows a pattern that only partially overlaps to that of the major phylogenetic trees described for the evolution of these organisms. In particular, the position of fungi is questionable, because these organisms are closer to animals. This partial discrepancy might depend on the limited number (only cyclophilins) of proteins compared and organisms examined. In any case, it is possible to infer that cyclophilins and, consequently, PTP may have not been arisen by convergence phenomena. Very interesting in this frame is the presence in *Legionella pneumophila*, in addition to a cyclophilin polypeptide (lpg1982), of two others polypeptides (lpg0211, lpg1974) corresponding, respectively, to the peripheral TSPO (PBR) and VDAC [233]. Therefore, the latter authors argue that these proteins may be recruited in a multiprotein complex, similar to PTP, which could regulate intracellular survival and/or proliferation.

In the light of this evidence, PTP seems to be co-evolved with other mitochondrial metabolite transport systems to regulate the endosymbiont-host relationship. Nevertheless, we hypothesize that PT acquired its master function in connection with the advantage of regulating the exchanges of molecules between the host and the endosymbiont and, consequently, the energetic state of the proto-eukaryotic cell.

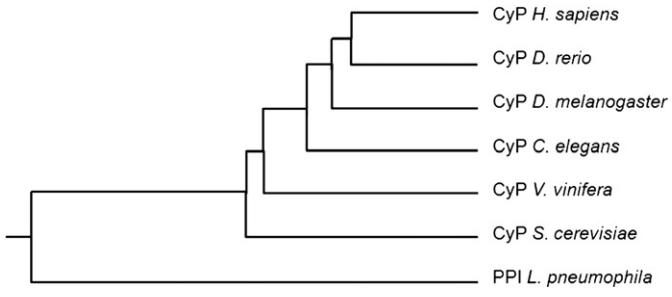

Fig. 3. Cladogram of homologous cyclophilins/peptidyl–prolyl cis–trans isomerases from different taxa. For the eukaryotic sequences, the presence of the mitochondrial target peptide was predicted by TargetP program (http://www.cbs.dtu.dk/services/TargetP). In brackets are indicated the NCBI Reference Sequence accession numbers and the prediction scores for the mitochondrial target peptide [257]. The following sequences were used: CyP *Homo sapiens* (P30405.1, 0.948); CyP *Danio rerio* (NP_001032199.1, 0.587); CyP *Drosophila melanogaster* (NP_523366.2; 0.858); CyP *Caenorhabditis elegans* (NP_506561.1, 0.820); CyP *Saccharomyces cerevisiae* (NP_013633.1, 0.819); CyP *Vitis vinifera* (XP_003633689.1, 0.932); PPI *Legionella pneumophila* (YP_001250736.1, n.a.). Sequences were aligned by ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2) and the cladogram was obtained by Phylip 3.67 package program (http://mobyle.pasteur.fr/cgi-bin/portal.py?#forms::protpars) by ProtDist and Fitch-Margoliash methods.

PTP could have appeared when the early aerobic parasite/endosymbiont experienced an increase of O_2, which caused a corresponding increase of oxidative phosphorylation. In parallel, the host (probably) had the capacity of performing an efficient glycolysis. Thus, the energetic situation of the early aerobic proto-eukaryotic cell might have been similar to that observed in cancer cells (Warburg effect) [234], where glycolysis accounts for 50 to 70% ATP produced [235], and to that detected in yeast grown aerobically with glucose [236]. These assumptions are in agreement with a very recent hypothesis, suggesting that cancer cells can be assimilated to a protozoan-like cell. Therefore, life of protists during the Pre-Cambrian period [237] could have been featured by a cancer-like type of energetic metabolism [238]. However, this would have hindered the successive evolution of multicellular organisms, obviously relying on more efficient mechanisms of energy production. The extraordinarily flexible function performed by PTP could have offered a finely tuneable mechanism, allowing a rapid shift from oxidative phosphorylation to glycolysis and viceversa, so avoiding a simultaneous aerobic and anaerobic ATP synthesis. Indeed, Warburg metabolism may arise from a tubulin-dependent closure of VDAC, which results in inhibition of mitochondrial function [238]. Why is ATP excess harmful for the cell? Because it is closely connected to ROS production, which is increased when ATP is high and the electron transport flux is not down-regulated accordingly. The opening of PTP possibly caused an uncoupling that would have decreased this risk, lowering both ATP and ROS levels and thus ameliorating the endosymbiotic relationship.

The role played by PTP in optimising both ATP synthesis and ROS production is even more stringent, as suggested by the above cited model of P_i as a PTP energy sensor [39], corroborated by plentiful observations done in living organisms where just a moderate decrease of ATP level almost immediately results in apoptotic cell death. This death turns to necrotic death if ATP reaches low levels, through a process characterized by formation of cells with intermediate hallmarks, named necrapoptotic cells [239]. To understand properly this key role of PTP, it could be useful to integrate the cell-centered perspective by adopting an ecological one: PT-dependent apoptosis appears to be a process antagonizing the anaerobic metabolism (production of ATP), which would result in a "selfish" dissipation of the free energy available in oxidizable substrates. Thus, PT seems to assure that only the cells with the best performances in terms of ATP yield can survive, while the others will undergo apoptosis. It can be speculated that this bioenergetic coordination might have been tested in unicellular species long before the appearance of multicellular organisms, being an advantage for the survival of the community of cells living in the same niche. Still from the beginning, this advantage could be arisen because bacterial cells were a population forming a near-isogenic clone. Thus, from an evolutionary point of view, the survival of a sister cell, in terms of gene propagation, represented an advantage also for the dead cell (altruistic behavior). Later on, this model could have offered the possibility of the evolution of multicellular organisms, in which, again, survival would depend on finely tuned bioenergetics. The consequences of energetically selfish behaviors in complex organisms is clearly seen in cancer, whose cells grow to the detriment of the host organism [238].

Mammalian apoptosis is under control of pro- and anti-apoptotic proteins of the Bcl-2 family. These proteins are usually subdivided into three classes on the basis of their pro- or anti-apoptotic role and their Bcl-2 Homology (BH) domains [240]. Although their regulatory mechanism is still largely unknown, it is useful to speculate if and how these proteins may have contributed to the regulation of PT, allowing cells with low performances in terms of ATP yield, to undergo apoptosis.

The pro- and anti-apoptotic functions of Bcl-2 family members seem to be a reminiscence of the mechanism performed by colonial prokaryotes, which appears to be mediated by modules of toxin-antitoxin systems, encoded by a pair of genes [241–243]. In a very recent paper [244], it has been shown that this type of PCD is performed in *E. coli* by the *mazEF* module encoding a stable toxin (Mazf) and a labile antitoxin (MazE). *mazEF*-dependent cell death is a population phenomenon that requires a quorum-sensing pentapeptide named Extracellular Death Factor (EDF). This process overlaps with a second death pathway, named apoptotic-like death (ALD), which is mediated by *recA* and *lexA* genes. Under conditions of severe DNA damage, *mazEF* genes trigger a cell death that inhibits ALD. The suicide of the cells by the first mechanism, releasing substrates, is interpreted as an altruistic behavior to favor the survival of the remaining cells. Although apoptotic proteins in eukaryotes have homologs in prokaryotes [245], their presence seems to be sporadic and does not allow to conclude that the pro- and anti-apoptotic proteins of prokaryotes are conserved in eukaryotes. In particular, the members of the Bcl-2 family do not have homologs in eubacteria, fungi and tracheophytes. The only example of a shared anti-apoptotic protein is represented by BAX inhibitor-1 [246].

Protists also exhibit forms of PCD, suggesting an ancient evolutionary origin of this regulated process, although the identification of the molecular components of their cell death machinery is still scarce [134]. Nevertheless, mammalian Bcl-2 family members are cross-functional in plant cell death regulation [247–250], as their heterologous expression permits to regulate PCD in yeast and plants. Hence, we may speculate that the mechanism of regulation, based on Bcl-2 family members, might have arisen during evolution after the appearance of PTP, thus reflecting a historical separation of the two processes in question [251].

Several members of the Bcl-2 family (e.g. Bcl-2, Bcl-xL, Bax and Bak) can regulate PTP. In addition, other Bcl-2 proteins may *per se* form autonomous channels that contribute to amplify the depolarizing effect induced by PT. This led to hypothesize two mechanisms, involving the opening of two mitochondrial channels, the PTP in the IMM and the mitochondrial apoptosis-induced channel (MAC) in the OMM [252]. Activation of MAC would be regulated by Bcl-2 proteins. According to Forte and Bernardi [251], it is also reasonable to suggest an integrated action between PTP and Bcl-2 proteins, occurring at the level of transient activation of the PTP. In any case, the anti-apoptotic effect of these proteins, in particular Bcl-2, neither requires the maintenance of the mitochondrial transmembrane electrical potential nor prevents or delays the decrease of cellular ATP level [253–255], which, as above seen, is crucial to define the fate of the cells (apoptotic, necroapoptotic, or necrotic death).

On the basis of the above considerations, we may infer that cell death, linked to loss of ATP, can constitute a form of primordial death, which allows to eliminate cells with low energetic performances, in a way that can be assimilated to an altruistic behavior. But with the increase of the complexity of the organisms and the related diversified functions, other mechanisms of regulation, such as that based on Bcl-2 family proteins, might have arisen during the course of evolution, favoring the development of metazoans. This view may also include the possibility that some components of the Bcl-2 family (Noxa and Mcl-1) could become the executioners of apoptosis in T cells subjected to glucose starvation [256].

The complex architecture of PTP, acquired by exaptation, rendered it more prone to regulation in a changing environment, permitting it to accomplish such a function. Therefore, it is possible to infer that PTP could have contributed, during evolution, to assure both energetic and regulatory functions, particularly in metazoans. Hence, we may conclude that exaptation can supply a fertile conceptual framework to interpret and re-interpret biochemical functions and structures of elusive evolutionary origin.

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