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*Perspectives*

## The Syntrophy hypothesis for the origin of eukaryotes revisited

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20 The discovery of Asgard archaea, phylogenetically closer to eukaryotes than other archaea, together with  
21 improved knowledge of microbial ecology impose new constraints on emerging models for the origin of the  
22 eukaryotic cell (eukaryogenesis). Long-held views are metamorphosing in favor of symbiogenetic models  
23 based on metabolic interactions between archaea and bacteria. These include the classical Searcy's and  
24 hydrogen hypothesis, and the more recent Reverse Flow and Entangle-Engulf-Enslave (E<sup>3</sup>) models. Two  
25 decades ago, we put forward the Syntrophy hypothesis for the origin of eukaryotes based on a tripartite  
26 metabolic symbiosis involving a methanogenic archaeon (future nucleus), a fermentative myxobacterial-like  
27 deltaproteobacterium (future eukaryotic cytoplasm) and a metabolically versatile methanotrophic  
28 alphaproteobacterium (future mitochondrion). A refined version later proposed the evolution of the  
29 endomembrane and nuclear membrane system by invagination of the deltaproteobacterial membrane.  
30 Here, we adapt the Syntrophy hypothesis to contemporary knowledge, shifting from the original hydrogen  
31 and methane-transfer-based symbiosis (HM-Syntrophy) to a tripartite hydrogen and sulfur-transfer-based  
32 model (HS-Syntrophy). We propose a sensible ecological scenario for eukaryogenesis in which eukaryotes  
33 originated in early Proterozoic microbial mats from the endosymbiosis of a hydrogen-producing Asgard  
34 archaeon within a complex sulfate-reducing deltaproteobacterium. Mitochondria evolved from versatile,  
35 facultatively aerobic, sulfide-oxidizing and, potentially, anoxygenic photosynthesizing, alphaproteobacterial  
36 endosymbionts that recycled sulfur in the consortium. The HS-Syntrophy hypothesis accounts for  
37 (endo)membrane, nucleus and metabolic evolution in a realistic ecological context. We compare and  
38 contrast the HS-Syntrophy hypothesis to other models of eukaryogenesis, notably in terms of the mode and  
39 tempo of eukaryotic trait evolution, and discuss several model predictions and how these can be tested.  
40

41 Eukaryogenesis was a unique major evolutionary transition resulting in significant average cell complexity  
42 increase. This foundational event led to an impressive radiation of morphologically diverse phyla, most of  
43 them unicellular (protists) but many including multicellular taxa such as animals, fungi, kelp and land  
44 plants<sup>1</sup>. Elusive for a long time, reconstructing a mechanistically plausible and ecologically realistic model  
45 for the origin of eukaryotes appears now within reach thanks to recent advances in molecular  
46 phylogenomic tools, genome-binning from metagenomes and a better knowledge of microbial diversity  
47 and function in natural ecosystems. Until recently, notwithstanding the generally accepted endosymbiotic  
48 origin of mitochondria and chloroplasts, models proposing the symbiotic origin of eukaryotes directly  
49 from bacterial and archaeal ancestors were largely dismissed<sup>2,3</sup>. The prevailing view stated that an  
50 independent proto-eukaryotic lineage sister to archaea evolved most eukaryotic features (complex  
51 cytoskeleton, endomembranes, nucleus, phagocytosis, etc.) before it engulfed the mitochondrial  
52 alphaproteobacterial ancestor<sup>4-6</sup>. This view started to vacillate with the realization that truly primary  
53 amitochondriate eukaryotes were not known<sup>7</sup> and additionally deteriorated with phylogenomic trees  
54 where eukaryotes branched within archaea, albeit without clear sister groups<sup>8</sup>. The discovery of Asgard  
55 archaea, a phylogenetically deep-branching lineage sharing more and more similar genes with eukaryotes  
56 than other archaea<sup>9,10</sup>, has further fostered this paradigm shift on eukaryogenesis. Eukaryotes are no  
57 longer on the same footing as archaea and bacteria as one of the original primary domains of life<sup>11</sup>; they  
58 are a third, but secondary, domain resulting from the evolutionary merging of specific archaeal and  
59 bacterial lineages<sup>2,6,12-14</sup>. Moreover, current knowledge about Asgard general metabolic potential and  
60 preferred biotopes (mostly sediments and microbial mats, where intimate metabolic interactions are the  
61 rule<sup>3,15</sup>), realistically favor symbiogenetic models based on metabolic symbioses or syntrophies<sup>16,17</sup>. This is  
62 further supported by the syntrophic nature of the first cultured Asgard member, *Candidatus*  
63 *Prometheoarchaeum syntrophicum*, an anaerobic organism able to grow in symbiosis with a sulfate-  
64 reducing deltaproteobacterium, a methanogenic archaeon or both<sup>18</sup>. Collectively, this strongly supports  
65 cooperative models for the origin of the eukaryotic cell<sup>2,3,6,17</sup> whereby higher complexity evolved from the  
66 physical integration of prokaryotic cells combined with extensive gene and genome shuffling<sup>12,19-21</sup>.

67 The first symbiogenetic models date back to more than 20 years ago. Among them, the more detailed  
68 were the Serial Endosymbiosis Theory<sup>22-24</sup>, the Hydrogen hypothesis<sup>25</sup> and the Syntrophy hypothesis<sup>26,27</sup>.  
69 In the original Syntrophy hypothesis, we proposed that eukaryotes evolved from a tripartite metabolic  
70 symbiosis based on i) interspecies hydrogen transfer from a fermenting deltaproteobacterial host to an  
71 endosymbiotic methanogenic archaeon and ii) methane recycling by a versatile methanotrophic,  
72 facultative aerobic alphaproteobacterium<sup>26</sup> (Hydrogen-Methane –HM– Syntrophy). From an ecological  
73 perspective, these metabolic interactions were reasonable, being widespread in anoxic and redox-  
74 transition settings<sup>2</sup>. However, knowledge about archaeal diversity and metabolism was then much more  
75 fragmentary than today, and the metabolic potential of uncultured lineages remained inaccessible. The  
76 probable involvement of an Asgard archaeal relative in eukaryogenesis imposes new constraints, such  
77 that realistic models need to take into account their metabolic potential and ecology. Accordingly, several  
78 symbiogenetic models are currently being put forward. They differ on the metabolic interactions  
79 proposed (**Box 1**) and, importantly, the tempo and mode of evolution of key eukaryotic traits (**Box 2**).  
80 Here, we present an updated version of the Syntrophy hypothesis based on a tripartite metabolic  
81 symbiosis involving interspecific hydrogen and sulfur-transfer (HS-Syntrophy) occurring in redox-  
82 transition ecosystems: a complex sulfate-reducing deltaproteobacterium (host), an endosymbiotic

83 hydrogen-producing Asgard-like archaeon (future nucleus) and a metabolically versatile, facultatively  
84 aerobic, sulfide-oxidizing and potentially anoxygenic photosynthesizing, alphaproteobacterium (future  
85 mitochondrion). We briefly discuss the evolution of the endomembrane system, the nucleus and the  
86 genome<sup>19</sup> in the framework of the HS-Syntrophy hypothesis. This model makes several predictions that  
87 differentiate it from alternative scenarios including, notably, the two-step origin of the nucleus (first, as  
88 distinct metabolic compartment before its consecration as major genetic reservoir and expression center)  
89 and the bacterial origin of eukaryotic membranes and cytoplasm. We propose ways to specifically test  
90 some aspects of different eukaryogenesis models and offer suggestions for future avenues of research.

91

## 92 **The ecological context of the eukaryogenetic symbiosis**

93 Despite the challenges associated to the interpretation of the earliest life traces, the oldest reliable  
94 eukaryotic fossils can be dated back to at least 1.65 Ga<sup>28</sup>. This imposes a minimal age for the origin of  
95 eukaryotes that roughly agrees with the oldest boundaries of recent molecular dating estimates for the  
96 last eukaryotic common ancestor (LECA; 1.0-1.6 Ga)<sup>29</sup> and the eukaryotic radiation (<1.84 Ga)<sup>30</sup>. At the  
97 same time, LECA was complex, being endowed with mitochondria and resembling modern protists<sup>20,21</sup>.  
98 Although the alphaproteobacterial lineage that gave rise to the mitochondrion remains to be precisely  
99 identified<sup>31</sup>, it is clear that the mitochondrial ancestor was aerobic<sup>32</sup>, but likely also possessed anaerobic  
100 respiratory capacities (as in many modern protists). This implies that i) aerobic respiration had already  
101 evolved in bacteria when the mitochondrial endosymbiosis occurred and ii) oxic or microaerophilic  
102 conditions existed in the environment where the mitochondrial endosymbiosis took place or in its  
103 immediate vicinity. Aerobic respiration possibly evolved (almost) in parallel to cyanobacterial oxygenic  
104 photosynthesis, which led to the oxygenation of the atmosphere, the Great Oxidation Event (GOE), some  
105 2.4 Ga ago at the beginning of the Proterozoic (2.5-0.5 Ga)<sup>33,34</sup>. Therefore, eukaryogenesis took place  
106 between the GOE and the minimum age of the oldest unambiguous eukaryotic fossils<sup>28</sup>. If some older,  
107 more difficult to affiliate, fossils<sup>35</sup> are indeed eukaryotic, eukaryogenesis might have occurred during the  
108 first three to five hundred million years after the GOE.

109 What did the Earth look like at that time? Before the GOE, the atmosphere and oceans were  
110 essentially anoxic, which constrained existing biogeochemical cycles. The atmosphere rapidly oxygenated  
111 from 2.33 Ga but sulfate levels in oceans increased slower<sup>33</sup>, limiting the biological S cycle<sup>36,37</sup>. This means  
112 that oceans were oxygen-poor during the early Proterozoic, when eukaryotes evolved; the deep ocean  
113 remained anoxic until the beginning of the Phanerozoic (500 Ma)<sup>38-40</sup>. If an aerobic mitochondrial  
114 ancestor suggests oxygen availability at or near the environment where eukaryotes finally evolved,  
115 current knowledge on Asgard archaea ecology and metabolism strongly suggests that the archaeon  
116 involved in eukaryogenesis, and hence the first eukaryogenetic steps, were strictly anaerobic. Asgard  
117 archaea are mostly found in deep-sea sediments<sup>9,10,18,41</sup> and microbial mats<sup>10</sup>, including thermophilic  
118 ones<sup>10,42</sup>. Thus, with the exception of some derived planktonic Heimdallarchaeota, which more recently  
119 acquired the capacity to oxidize organics using nitrate or oxygen as terminal electron acceptors<sup>16,43</sup>, the  
120 vast majority of Asgard archaea thrive in anoxic environments, as their ancestors did, degrading organics  
121 and producing or consuming hydrogen<sup>16</sup>. These observations argue in favor of redox transition  
122 environments, where anoxic and oxic/microoxic zones are in close proximity, as favored ecosystems for  
123 eukaryogenesis. Furthermore, since the early Proterozoic deep ocean was anoxic, it is more likely that  
124 eukaryotes evolved in shallow sediments or microbial mats, where redox gradients established, like

125 today, from the oxygen-enriched surface where cyanobacterial oxygenic photosynthesis took place to the  
126 increasingly anoxic layers below.

127 Phototrophic microbial mats are particularly interesting potential eukaryogenesis cradles. They were  
128 the Proterozoic ‘forests’, dominating shallow aquatic and terrestrial habitats, as abundant fossil  
129 stromatolites (lithified microbial mats) show<sup>34,44,45</sup>. These light- and redox-stratified microbial  
130 communities are phylogenetically and metabolically diverse<sup>42,46,47</sup>. Although microorganisms in modern  
131 mats are different from their Proterozoic counterparts, core metabolic functions have been mostly  
132 preserved across phyla<sup>48</sup> and at the ecosystem level, suggesting that functional shifts observed in mats  
133 across redox gradients today reflect early metabolic transitions<sup>49</sup>. Most primary production occurs in  
134 upper layers, where light can penetrate, via photosynthetic carbon fixation. The upper cyanobacterial  
135 oxygenic-photosynthesis layer is typically followed by a reddish layer dominated by oxygen-tolerant  
136 anoxygenic photosynthesizers (Alpha- and Gammaproteobacteria) and often an underlying green layer of  
137 photosynthetic Chloroflexi and/or Chlorobi. Organic matter fixed in the upper mat layers is progressively  
138 degraded in deeper, anoxic layers, by extremely diverse microbial communities<sup>42,50,51</sup>. Two broad zones  
139 can be distinguished in vertical anoxic profiles where, respectively, sulfate reduction and methanogenesis  
140 dominate<sup>46</sup> (**Fig. 1a**). Here, like in anoxic sediments, the degradation of organic matter involves  
141 syntrophy<sup>52</sup>, mostly implicating interspecies hydrogen (or, directly, electron<sup>53</sup>) transfer. In anoxic  
142 environments, pairs of electron donors and acceptors display low redox potential differences such that  
143 many energy-generating metabolic reactions can only proceed in the presence of syntrophic sinks<sup>54</sup>.  
144 Methanogenic archaea and sulfate-reducing bacteria (SRB) belonging to the Deltaproteobacteria are  
145 frequently engaged in syntrophy. Deltaproteobacteria are metabolically diverse and can use or produce  
146 hydrogen or, directly, electrons<sup>55</sup>, being frequently involved in interspecies hydrogen or electron  
147 transfer<sup>53</sup>. Many of them oxidize organic compounds with sulfate, but they can also be autotrophic<sup>56</sup>  
148 (including in syntrophy<sup>57</sup>), use other electron donors and acceptors (including metals, such as arsenic<sup>58</sup>),  
149 ferment or switch between metabolisms depending on the environmental conditions<sup>59</sup>.  
150 Deltaproteobacteria establish widespread syntrophies with archaea; with methanogens when acting as  
151 hydrogen producers, with methanotrophic archaea when acting as hydrogen-consuming sulfate-  
152 reducers<sup>60</sup>. Mutualistic interactions between methanogens and deltaproteobacteria can be rapidly  
153 selected, leading to specialized syntrophy<sup>61,62</sup>. Deltaproteobacterial SRB also establish symbioses with  
154 sulfide-oxidizing or other bacteria and eukaryotes<sup>2,59,63</sup>. In addition to methanogens and SRB, a wide  
155 variety of uncultured lineages occurs in anoxic sediment and microbial mat layers, where archaea thrive.  
156 Many of these archaea seem to be involved in cycling organics, particularly alkanes, being likely engaged  
157 in syntrophies with hydrogen-scavengers<sup>49,52,64</sup>. Indeed, the first cultured Asgard archaeon can grow by  
158 degrading amino acids in syntrophy with either a sulfate-reducing deltaproteobacterium and/or a  
159 methanogen<sup>18</sup>.

160

## 161 Eukaryogenetic syntrophies

162 Considering this historical and ecological context, we favor the idea that eukaryogenesis occurred in  
163 microbial mats (or similarly stratified shallow sediments) with marked redox gradients (**Fig. 1a**),  
164 potentially mildly warm. Oxygenic photosynthesis (and in close proximity, aerobic respiration) might have  
165 first evolved in warm environments. Indeed, most deep-branching cyanobacteria are thermophilic<sup>65,66</sup>.  
166 Although universal molecular mechanisms to cope with reactive oxygen species exist<sup>67,68</sup> and might have  
167 been co-opted from antioxidant-prone compounds very early<sup>69,70</sup>, oxygen toxicity would have been

168 advantageously relieved in thermophilic mats by its rapid release into the atmosphere (oxygen is poorly  
169 soluble at high temperature). The original (HM) Syntrophy hypothesis postulated, on solid microbial  
170 ecology grounds, the evolution of eukaryotes from well-known widespread symbioses between  
171 fermenting (hydrogen-producing), ancestrally SRB, deltaproteobacteria and methanogenic archaea. SRB  
172 and methanogens, which compete for hydrogen, can readily evolve stable syntrophy in co-culture<sup>61,62</sup>. We  
173 additionally favored a myxobacterial-like deltaproteobacterium due to the similarities shared by these  
174 complex social bacteria and eukaryotes<sup>19,26</sup>. This symbiosis would have established at the sulfate-methane  
175 transition zone but evolved upwards in the redox gradient, where an additional symbiosis formed with a  
176 versatile methanotrophic alphaproteobacterium that scavenged the methane released by the primary  
177 consortium. We cannot completely reject such tripartite metabolic symbiosis at the origin of eukaryotes  
178 since methanogenesis, originally thought exclusive of Euryarchaeota, occurs across archaeal phyla and  
179 might have been ancestral to the archaeal domain<sup>71-74</sup>. However, although methanogenesis might be  
180 eventually discovered in Asgard archaea (some Asgard archaea do have methyl-coenzyme M reductases  
181 probably involved in the reverse, anaerobic alkane oxidation, reaction<sup>41</sup>), current genomic comparisons  
182 seem to exclude it from their ancestral metabolic capacities<sup>16</sup>.

183 In this context, we now favor a similar eukaryogenetic process but based on alternative, albeit  
184 equally ecologically relevant, metabolic symbioses (**Fig. 1**). In our HS-Syntrophy hypothesis, we propose  
185 that eukaryotes evolved from the syntrophic interaction of a sulfate-reducing (hydrogen/electron-  
186 requiring) deltaproteobacterium, possibly sharing some complex traits with myxobacteria, and a  
187 hydrogen-producing Asgard-like archaeon. This deltaproteobacterium may have been metabolically  
188 versatile or mixotrophic, but in symbiosis with the archaeon, it respired sulfate. This initial facultative  
189 symbiosis was stabilized by the incorporation of the archaeon as endosymbiont (**Fig. 1b-c**). This  
190 consortium likely established first in deeper anoxic layers and subsequently migrated upwards in the  
191 redox gradient, where it established a second (initially facultative) symbiosis with a sulfide-oxidizing  
192 alphaproteobacterium that acted as both, sulfide sink and sulfate donor for the Asgard-  
193 deltaproteobacterium consortium (**Fig. 1b-c**). Alternatively, the two facultative symbioses might have co-  
194 existed although, in this case, we favor a later obligatory endosymbiosis of the alphaproteobacterial  
195 ancestor<sup>19</sup>. This would be in line with genomic evidence suggesting a late mitochondrial symbiosis<sup>75</sup>.  
196 Given the dominance of H<sub>2</sub>S-dependent anoxygenic photosynthesizing bacteria in microbial mats and  
197 their interaction with SRB for sulfur cycling in upper layers<sup>76,77</sup>, the versatile, facultatively aerobic  
198 mitochondrial ancestor was likely also photosynthetic (or mixotrophic). Interestingly, the possibility that  
199 mitochondrial cristae evolved from intracytoplasmic membranes typical of photosynthetic  
200 Alphaproteobacteria has been highlighted<sup>78</sup>. This tripartite symbiotic consortium became definitely  
201 stabilized when the alphaproteobacterium became an endosymbiont within the deltaproteobacterium  
202 (**Fig. 1d**). In our view, the first eukaryotic common ancestor (FECA) is neither an archaeon nor a  
203 bacterium, but the first obligatory symbiogenetic consortium. Strictly speaking, this would correspond to  
204 the integrated symbiosis of the three partners that contributed to the final making of the eukaryotic cell  
205 and genome. But the FECA stage could also be decoupled in time in two subsequent stages corresponding  
206 to the integration of the Asgard archaeon within the deltaproteobacterium (FECA 1) and the acquisition  
207 of the mitochondrial endosymbiont (FECA 2).

208 In our model, up to the FECA stage, the eukaryogenetic syntrophies were based on the same  
209 metabolic exchange that occurred in the corresponding facultative symbioses (hydrogen between the

210 archaeon and the SRB; sulfide/sulfate between the SRB and SOB). However, the incorporation of the  
211 mitochondrial ancestor as obligatory endosymbiont implied a radical change in the metabolism of the  
212 whole consortium, constraining the outcome of the eukaryogenetic process. Because the mitochondrial  
213 ancestor was also aerobic and could get a much higher energy yield by directly oxidizing organics, the  
214 consortium started to rely solely on aerobic respiration (Fig. 1e). This resulted in the loss of the less-  
215 efficient anaerobic archaeal metabolism and bacterial sulfate-reduction. At the same time, the proto-  
216 eukaryote migrated to the fully oxic layers of the mats, spreading on oxic surfaces and, upon the  
217 development of motility mechanisms, colonizing the planktonic realm. Cellular changes that included the  
218 development of an extensive endomembrane system (see below) led to the LECA stage (Fig. 1f).

219 The HS-Syntrophy model implies three prokaryotic partners that became integral part of the future  
220 eukaryotic cell. However, other pre-eukaryogenetic symbioses might have occurred at the facultative  
221 syntrophy stage, eventually leaving historical traces in the form of transferred genes to the  
222 eukaryogenetic symbiotic partners. One traditional criticism to symbiogenetic models proposing the  
223 endosymbiosis of one prokaryote within another prokaryote is the absence of phagocytosis in  
224 prokaryotes<sup>2,4,6,79,80</sup>. Mainstream models now accept a symbiogenetic origin of eukaryotes but only under  
225 the premise that an endomembrane system, a developed cytoskeleton and phagocytosis evolved in the  
226 archaeal ancestor prior to the engulfment of the mitochondrial ancestor<sup>6,81</sup>. However, prokaryotes  
227 harboring endosymbiotic prokaryotes are known and might be more frequent than currently thought. In  
228 addition to the well-known cases of gammaproteobacterial symbionts within betaproteobacterial  
229 endosymbionts in mealybugs<sup>82</sup> and rickettsiales in tick mitochondria<sup>83</sup>, old electron microscopy studies<sup>84-  
230 86</sup> and more recent observations<sup>87</sup> suggest the potential occurrence of prokaryotic endosymbionts in  
231 bacteria. Interestingly, a recent report of prey engulfment by planctomycetes<sup>88</sup> suggests that bona fide  
232 bacterial phagocytosis exists, albeit based on different molecular grounds than eukaryotic phagocytosis<sup>88</sup>.  
233 In the case of archaea, although Nanoarchaeota can be associated to the inter-membrane space in the  
234 archaeon *Ignicoccus hospitalis*<sup>89</sup>, true endosymbionts remain to be observed. Consequently, regardless  
235 the mechanism, these collective observations suggest that prokaryotic endosymbioses, at least within  
236 bacteria, are feasible.

237

## 238 Membranes and endomembranes

239 The eukaryotic plasma membrane and endomembrane system, including the endoplasmic reticulum (ER),  
240 the nuclear membrane, the Golgi apparatus and other vesicular components (vacuoles, lysosomes, etc.)  
241 are interconnected (are either continuous or can fuse and merge). They share a similar composition, with  
242 typical bacterial-like phospholipids<sup>2,90</sup>. The phospholipid bilayer in eukaryotes is particularly flexible and  
243 can undergo deformation, bending, fusion and fission. This is achieved thanks to a highly developed  
244 cytoskeleton<sup>91</sup>, coating components (e.g. clathrin/AP1-5, COPI, TSET, COPII, retromer, ESCRT complexes),  
245 ARF/ARF-like GTPases and their regulators, and fusion machinery (involving SNARE complex, multisubunit  
246 tethering complexes, Rab GTPases and regulatory factors)<sup>92-94</sup>. Both, the cytoskeleton and membrane  
247 remodeling and fusion complexes were already present in LECA, which was capable of phagocytosis,  
248 secretion and trafficking, and have a chimeric origin<sup>20,93,95-97</sup>. In addition to innovations<sup>21,95</sup>, some  
249 cytoskeletal and membrane-remodeling proteins are archaeal-like (e.g. actin, profilin, ESCRT proteins<sup>6,98</sup>,  
250 perhaps some GTPases<sup>99</sup>, although some of these might be bacterial<sup>100,101</sup>) but a significant number of



251 endomembrane system-related proteins could also be of bacterial, though not alphaproteobacterial,  
252 origin<sup>75</sup>. The biosynthesis of sterols is notably of bacterial origin<sup>102</sup>.

253 Most eukaryogenetic models propose a two-partner symbiosis in which the archaeal host  
254 incorporated the alphaproteobacterial ancestor of mitochondria. This implies a shift of the host  
255 membrane from the more rigid archaeal, glycerol-1-phosphate (G1P)-based ether-linked isoprenoid  
256 phospholipids to the more flexible and permeable bacterial glycerol-3-phosphate (G3P)-based, usually  
257 ester-linked, fatty acid phospholipids<sup>2,90</sup> (**Box 2**). However, such a transition with, in particular, a G1P-to-  
258 G3P-based phospholipid shift, has never been observed in nature (some thermophilic bacteria use ether-  
259 links, long-term-known exceptions<sup>90</sup>). Recently, an engineered *Escherichia coli* strain was forced to  
260 express archaeal phospholipids, making up to 30% of the total membrane phospholipids<sup>103</sup>. The  
261 engineered heterochiral-membrane strain was viable and, interestingly, the expressed archaeal lipids  
262 recruited G1P, suggesting that stereospecificity is somehow linked to the phospholipid composition, in a  
263 peculiar form of membrane heredity. However, if more than 30% archaeal lipids incorporated to the  
264 membrane, severe growth impairment was observed and the shape of cells became aberrant; they  
265 produced numerous vesicles and underwent asymmetric cell division<sup>103</sup>. One could therefore ask how the  
266 expression of archaeal phospholipids affects *E. coli* fitness and whether such engineered strain would be  
267 able to survive competition with normal bacteria in natural environments. Archaeal and bacterial  
268 phospholipids impose very different local physicochemical conditions that constrain integral membrane  
269 proteins<sup>104</sup>. As a consequence, a membrane lipid composition shift implies an extensive adaptation of the  
270 whole membrane-associated proteome<sup>105</sup>. In this context, neither the stability of heterochiral  
271 liposomes<sup>106</sup> nor the (partial) expression of archaeal phospholipids in engineered *E. coli*<sup>103</sup> can be taken as  
272 evidence for an archaeal-to-bacterial membrane transition. While the bacterial nature of eukaryotic  
273 phospholipids represents a serious difficulty for models invoking an archaeal host, it is naturally explained  
274 by the bacterial nature of the host in the Syntrophy hypothesis (**Box 2, Fig. 1**).

275 In the HS-Syntrophy model, the endomembrane system results from the invagination of the  
276 deltaproteobacterium inner membrane and the internalization of the periplasm. The outer bacterial  
277 plasma membrane would be retained as the eukaryotic plasma membrane. Many bacteria harbor  
278 endomembrane compartments linked to specialized biochemical functions<sup>107</sup>. These include the well-  
279 known cyanobacterial thylakoids, but also compartments in magnetotactic bacteria<sup>108</sup>, anammox  
280 bacteria<sup>109</sup> and Poribacteria<sup>110</sup>. Some Planctomycetes develop a thoroughly studied nuclear-like  
281 compartment<sup>111</sup> and a similar structure has been recently described in the candidate phylum  
282 Atribacteria<sup>112</sup>. This implies that the internalization of membranes is relatively common across bacterial  
283 phyla. Although Deltaproteobacteria with endomembranes have not been described, their diversity is far  
284 from fully explored and they have membrane-remodeling potential. For instance, developed  
285 cytoskeletons (a prerequisite for extensive membrane remodeling) exist in the predatory *Bdellovibrio*<sup>113</sup>  
286 but also in myxobacteria, which are able to generate protruding membrane tubes that interconnect  
287 cells<sup>114</sup>. In the HS-Syntrophy model, similarly to the former HM-Syntrophy<sup>19</sup>, the initial driving force for  
288 endomembrane evolution is the establishment of a secretory system that connected the endosymbiotic  
289 archaeon with the periplasm (**Fig. 1c-d**). As in contemporary heterotrophic deltaproteobacteria, the  
290 periplasm was the digestive space of the host deltaproteobacterium in which complex organics uptaken  
291 from the environment were hydrolyzed to simpler organics. Some of these simpler organics (e.g. amino  
292 acids, short hydrocarbons) were used by the archaeon for its organoheterotrophic metabolism, which

293 yielded hydrogen used in turn by the SRB host. By being an endosymbiont, the archaeon maximized its  
294 uptake surface for small organics from the bacterial cytoplasm. In turn, the deltaproteobacterial host  
295 maintained an optimal uptake surface for complex organics from the environment while having a ready  
296 internal source of hydrogen (or electrons) for sulfate reduction. As the symbiosis evolved, many genes  
297 were transferred from the deltaproteobacterium to the archaeon, which progressively centralized genes  
298 and gene expression for the whole consortium. This included, notably, many hydrolytic enzymes required  
299 for the periplasmic degradation of complex organics. These enzymes were transported from the archaeal  
300 compartment towards the original bacterial periplasm via an incipient endomembrane system that,  
301 eventually, fully surrounded the archaeon and constituted the future nuclear membrane (see below). This  
302 implied the evolution of a transport system only through the archaeal membrane since, on the bacterial  
303 side, transporters for the export of newly synthesized hydrolytic enzymes to the periplasm and the  
304 environment already existed. Bacterial transporters might have initially been inserted also in the archaeal  
305 membranes (following gene transfer to the archaeal genome) but later replaced by channels  
306 communicating with bacteria-derived pore-like structures and allowing the export of increasingly bigger  
307 and varied substrates (see below). The transfer of hydrolytic enzymes to the digestive periplasmic space  
308 via the endomembrane system was essential to prevent the hydrolysis of cytoplasmic components (**Fig.**  
309 **1d**). At the same time, in this way, the digestive space largely increased. Hence, the initial digestive and  
310 trafficking-related endomembrane system was the precursor of the nuclear membrane and the ER but  
311 also of the different eukaryotic vesicles related to digestive processes (lysosomes, peroxisomes, digestive  
312 vacuoles). Upon the endosymbiosis of the mitochondrial ancestor and the loss of the archaeal and SRB  
313 metabolism, the organics were directly oxidized via aerobic respiration by the alphaproteobacterium and  
314 the endomembrane system was retained for the trafficking of proteins synthesized in the proto-nucleus  
315 (**Fig. 1e**) and, with time, in association with the ER itself (**Fig. 1f**). At the same time, the ancient periplasm  
316 was completely internalized and the former digestive periphery transferred to independent vesicular  
317 compartments (**Fig. 1f**). The secretory Golgi apparatus as well as other endocytotic and exocytotic  
318 systems developed in parallel. The association of archaeal membrane-bending systems (e.g. ESCRT) with  
319 the host bacterial membranes facilitated the process of endomembrane formation.

320

## 321 **The origin of the nucleus**

322 Most eukaryogenetic models fail to advance convincing selective forces to explain why the nucleus  
323 evolved<sup>2</sup>. We propose, like in the HM-Syntrophy<sup>19</sup>, a two-step process entailing two sequential selective  
324 forces. First, a proto-nucleus evolved as a different metabolic compartment. This chimeric compartment  
325 was composed of the endosymbiotic archaeon and the surrounding proto-nuclear membrane of  
326 deltaproteobacterial origin (**Fig. 1c-d**). The first selective force for the evolution of the nuclear membrane  
327 was the need to export bacterial enzymes already synthesized by the archaeon (after their genes were  
328 transferred to the archaeal genome) to the periplasmic space. Its first role was therefore secretory  
329 (export towards the trafficking endomembrane system). Other proteins of archaeal origin also started to  
330 be exported, contributing to the evolution of several chimeric eukaryotic systems. Once the archaeal  
331 genome started to host essential genes from its symbiotic partners, and these genes were lost from the  
332 donor genomes, the archaeon started to centralize protein synthesis for the whole consortium. This  
333 entailed the development of a transport mechanism from the archaeal cytoplasm to the bacterial

334 endomembrane system, which was at the origin of the nuclear pore. This implied the formation of  
335 coordinated apertures through the archaeal membrane and the proto-nuclear membrane, although these  
336 apertures might have formed only on the bacterial membrane (future nuclear pores) with archaeal  
337 membrane transporters facilitating export prior to archaeal membrane loss. The potential to establish  
338 communicating pores exists in both, archaea and deltaproteobacteria. Archaea are able to establish  
339 intercellular cytoplasmic bridges and fuse<sup>115</sup>. Myxobacterial deltaproteobacteria are also able to fuse their  
340 membranes<sup>116,117</sup> and develop contact-dependent abilities, including coordinated gliding via junctional  
341 pore complexes<sup>118-121</sup>. Progressively, ribosomes concentrated around these incipient communicating  
342 pores, eventually migrating to the host's cytoplasm along the endomembrane system (future ER), where  
343 protein synthesis started to take place. This led to a progressive decoupling of translation, which became  
344 associated to the ER, and transcription, which took place in the archaeal cytoplasm (future nucleoplasm).

345 As the consortium evolved between FECA and LECA, after the mitochondrial ancestor was fixed in the  
346 consortium and the archaeal and SRB metabolisms were lost in favor of the more efficient mitochondrial  
347 respiration, the archaeal membrane became useless and was completely lost (membrane loss is not  
348 infrequent in the framework of endosymbiosis<sup>122</sup>). However, during this evolutionary process, extensive  
349 genome evolution took place<sup>19</sup>. This involved (endo)symbiotic gene transfer (EGT) to the archaeal  
350 genome, likely accompanied by other HGT, which was largely facilitated by active processes fostering  
351 genome evolution, such as gene and genome fragment duplication and reshuffling. As transcription and  
352 translation decoupled, introns invaded the future eukaryotic genome. They likely derived from the  
353 original self-splicing introns of the alphaproteobacterial endosymbiont<sup>123</sup>, possibly complemented by  
354 other mechanisms<sup>124,125</sup>. Once introns invaded the genome, the proto-nuclear membrane was selectively  
355 retained (exapted) to maintain the transcription-translation uncoupling. Therefore, preventing the  
356 deleterious massive synthesis of aberrant proteins was the second selective force acting during nuclear  
357 evolution<sup>19</sup>. Intron invasion has been proposed as exclusive selective force for the origin of the nucleus<sup>126</sup>.  
358 However, in our view, transcription-translation uncoupling, and therefore a nuclear membrane, must pre-  
359 exist in order for introns to spread and not the opposite<sup>2</sup>. Not only the insertion of one or a few introns in  
360 essential genes would be immediately deleterious, but the evolution of a continuous nuclear membrane  
361 requires intermediate steps during which transcription and translation are still coupled that intron  
362 invasion as selective force cannot explain.

363 The initial chimeric proto-nuclear pore evolved into the modern nuclear pore as traffic check-point  
364 and hub of gene regulation<sup>127</sup>. We view the nucleolus and the ribosomal particle assembly process as  
365 remnants of the archaeal origin of the nuclear compartment<sup>19</sup>. The assembly of eukaryotic ribosomes is a  
366 complex and energy-costly process that takes place in the nucleolus. Ribosomal proteins are synthesized  
367 in the cytoplasm and transported to the nucleus. After assembly with rRNA in the nucleolus, ribosomal  
368 particles are transported back to the cytoplasm, where they associate to the ER for function<sup>128</sup>. The set of  
369 proteins involved (processome) is essentially of archaeal origin<sup>129</sup>.

370

## 371 **The make-up of a composite genome**

372 During eukaryogenesis, various mechanisms shaped the evolving eukaryotic genome. These included  
373 HGT, EGT, gene duplication, loss and new gene creation, accompanied by the invasion of introns and  
374 mobile selfish elements. The directionality of gene transfer to the archaeal genome and its establishment  
375 as future nuclear genome might have been simply dictated by chance; as the consequence of an essential

376 gene transfer from one genome to the other genome followed by loss in the donor<sup>19</sup>. The retention of the  
377 archaeal endosymbiont genome as future nuclear genome is often criticized on the ground that in extant  
378 cases of endosymbioses, endosymbionts tend to reduce their genomes in favor of the host's. However,  
379 known extant endosymbioses occur within eukaryotes, which are already composite cells harboring  
380 mitochondria and eventually chloroplasts for which many essential genes already reside in the nuclear  
381 genome. Therefore, the eukaryotic nuclear genome is essential and must centralize genes coming from  
382 any new incoming endosymbiont. The situation was radically different at the origin of eukaryotes when  
383 organelle reliance on the nuclear genome was not yet established and symbiotic partners were mutually  
384 dependent. In the Syntrophy hypothesis, the archaeal genome became the future nuclear genome.  
385 Bacterial components were thus included in an archaeal genomic background, leading to the long-term  
386 recognized mixed heritage of eukaryotic genomes, with 'informational' genes (related to DNA replication,  
387 transcription and translation) being archaeal-like, and 'operational' genes (involved in energy and carbon  
388 metabolism) bacterial-like<sup>130</sup>. While true in general terms, a closer look to the bacterial-like genes in  
389 eukaryotes poses some questions.

390 Most symbiogenetic models invoke only two partners, an archaeal host and the alphaproteobacterial  
391 ancestor of mitochondria (**Box 1**). Consequently, two predictions follow: i) host (archaeal-like) genes must  
392 dominate over the endosymbiont (alphaproteobacterial-like) genes and ii) most bacterial-like eukaryotic  
393 genes must be of alphaproteobacterial origin. However, neither of them holds. Bacterial-like genes are  
394 more abundant than archaeal-like genes in eukaryotic genomes<sup>131</sup> and genes with alphaproteobacterial  
395 ancestry only represent a minority of bacterial-like genes in modern eukaryotes<sup>75,132</sup> and LECA<sup>133</sup>. To  
396 explain this 'silent' non-alphaproteobacterial bacterial majority in eukaryotic genomes, the progressive  
397 erosion of ancient phylogenetic signal making it difficult to pinpoint the precise origin of those genes and  
398 massive HGT from diverse bacterial donors to the archaeal and/or the alphaproteobacterial symbiotic  
399 partners have been invoked<sup>131,134</sup>. High bacteria-to-archaea HGT levels have been observed in several  
400 phyla<sup>135</sup>, including the Asgard archaea<sup>9,10</sup>. However, the patterns observed in eukaryotic genomes could  
401 be only explained if genes transferred to the archaeal and/or alphaproteobacterial ancestors of  
402 eukaryotes had been subsequently lost in all their sister lineages, which is unlikely<sup>133</sup>. In addition,  
403 eukaryotic alphaproteobacterial-like genes have significantly shorter branches than other bacterial-like  
404 genes in phylogenetic trees including prokaryotic homologues<sup>75</sup>. This suggests a late mitochondrial arrival  
405 in a host with an already chimeric genome<sup>75</sup>. Moreover, if alphaproteobacterial-like genes mostly relate  
406 to mitochondrial functions, bacterial genes of non-alphaproteobacterial ancestry seem to be involved in  
407 other essential eukaryotic traits such as the endomembrane system, reinforcing the idea that they  
408 evolved prior to the mitochondrion<sup>75</sup>. Non-alphaproteobacterial genes appear to derive from various  
409 bacterial phyla (with Deltaproteobacteria and Actinobacteria among the most frequent donors),  
410 suggesting successive ancient waves of HGT from these phyla and/or the implication of several bacterial  
411 symbionts during eukaryogenesis (**Box 2e**). Symbiogenetic models involving more than two partners are  
412 often dismissed applying a simplistic parsimony argument. However, parsimony is not evolutionary  
413 evidence and, in most complex ecosystems, multiple symbioses are widespread<sup>3</sup>. The first cultured  
414 Asgard archaeon can indeed grow in symbiosis with one sulfate-reducing deltaproteobacterium and one  
415 methanogenic archaeon<sup>18</sup>. If we transpose a similar symbiosis at the onset of eukaryogenesis, a significant  
416 number of deltaproteobacterial-like genes in eukaryotes might be explained by HGT during the long-term  
417 coexistence with a symbiont that later disappeared without integrating the consortium<sup>18</sup>. Additional

418 bacterial ecto- or endo-symbionts might have also transferred genes, leading to the mosaic origin of  
419 eukaryotic bacterial-like genes as proposed in the 'pre-mitochondrial symbiosis' model<sup>133</sup> and on the line  
420 of the 'shopping-bag model' proposed for the evolution of plastid genomes<sup>136</sup>.

421 However, the presence of many non-alphaproteobacterial bacterial-like genes in eukaryotes is  
422 compatible with the HS-syntrophy model. Accordingly, deltaproteobacterial genes would have been  
423 acquired by EGT. Interestingly, deltaproteobacterial-like genes seem to be the most abundant non-  
424 alphaproteobacterial category, and also older<sup>75</sup>. Inferring the precise phylogenetic origin of genes of  
425 different ages in eukaryotic genomes is far from trivial due to mutational saturation and the erosion of  
426 phylogenetic signal in increasingly older genes<sup>137</sup>. Furthermore, each potential additional symbiont could  
427 contribute a number of genes acquired by HGT from different donors in such a way that the apparent  
428 number of eukaryogenetic symbiotic partners would appear inflated. Nonetheless, strong phylogenetic  
429 signal supports the deltaproteobacterial origin of many eukaryotic genes involved in diverse functions and  
430 structures. In addition to early identified deltaproteobacterial-like eukaryotic genes<sup>26</sup>, the list also  
431 includes antimicrobial defensins<sup>138</sup>, Ser/Thr/Tyr protein kinases<sup>139</sup>, PPP protein phosphatases<sup>140</sup>, high  
432 mobility group A proteins<sup>141</sup>, isoprenoid biosynthesis enzymes<sup>142</sup>, cyclitol synthases<sup>143</sup> and some  
433 kinetochore proteins<sup>97</sup>. Mitochondria also recruited some deltaproteobacterial proteins, potentially  
434 reflecting an alpha-delta-proteobacterial symbiosis, such as thiolases<sup>144</sup>, fatty acid beta-oxidation  
435 enzymes<sup>145</sup>, and possibly, some proteins involved in anaerobic metabolism. Stemming from the versatility  
436 of many alphaproteobacteria, we view the mitochondrial ancestor as a facultative aerobe able to carry  
437 out anaerobic respiration with various electron acceptors but also substrate-level phosphorylation<sup>27</sup>.  
438 Several genes involved in these reactions seem ancestral in eukaryotes, branching close to  
439 deltaproteobacteria and other anaerobic bacteria<sup>146</sup>. Although they are usually interpreted as  
440 independent HGT acquisitions from various donors<sup>146</sup>, these observations can alternatively support a  
441 deltaproteobacterial anaerobic respiration toolkit in ancestral eukaryotic mitochondria, subsequently lost  
442 to different degrees in aerobic lineages.

443

## 444 Future prospects

445 Any model on eukaryogenesis must account for the evolution of key eukaryotic traits (e.g. genome  
446 complexity, nature and origin of eukaryotic membranes/endomembranes and the nucleus), in a way that  
447 i) is mechanistically plausible and ii) explains the observed patterns and the causes (selective forces) for  
448 the evolution of those traits in iii) a realistic ecological context. In this framework, our HS-Syntrophy  
449 model takes into account constraints imposed by the discovery of Asgard archaea and their ancestral  
450 metabolic potential to put forward one of the most comprehensive eukaryogenetic models. This model  
451 presents some difficulties, notably in the centralization of the genome and protein synthesis by the  
452 archaeon with subsequent export to the deltaproteobacterial host. However, it is ecologically relevant,  
453 fits well with the observed chimerism of eukaryotic genomes and has the advantage, over archaeal host-  
454 based models (**Box 1**), of readily explaining the bacterial-like nature of eukaryotic membranes<sup>2,90</sup>. The HS-  
455 Syntrophy hypothesis makes several predictions that differentiate it from other hypotheses (**Table 1**).  
456 Some of these are shared with the HM-Syntrophy model and include the presence of EGT-derived  
457 deltaproteobacterial genes in eukaryotes that should be mostly involved in membrane, cell-signaling and  
458 cytoplasmic functions. Others, such as the involvement of a potentially photosynthetic S-oxidizing

459 alphaproteobacterium, specifically characterize the HS-Syntrophy. These predictions suggest that such  
460 specific Alpha- and Deltaproteobacterial lineages, phylogenetically closer to eukaryotes than other  
461 bacterial lineages, might exist. The nature of the alphaproteobacterial ancestor of mitochondria is indeed  
462 still cryptic<sup>31</sup>. If such lineages were discovered through environmental studies in microbial mats or  
463 sediments, the HS-Syntrophy would gain support. These Syntrophy models are realistically based on well-  
464 known metabolic interactions in microbial mats or sediments, but metabolic variants involving a similar  
465 tripartite symbiosis and eukaryogenetic process might be also envisaged within a more general Syntrophy  
466 model; its distinctive features being the nuclear origin from an archaeal endosymbiont in a bacterial  
467 cytoplasm and the independent acquisition of the mitochondrial ancestor.

468 Beyond the Syntrophy hypothesis, progress in several areas is needed to answer open questions and  
469 differentiate major model types (**Table 1**). In archaeal host-based symbiotic models<sup>16,18,147,148</sup>, three major  
470 issues need attention. First, the archaeal-to-bacterial membrane transition remains a major drawback.  
471 Cases of membrane transitions implying complete phospholipid-type replacement and concomitant  
472 membrane proteome adaptation are not observed in nature. If bacteria and, more particularly archaea,  
473 can be engineered to i) accomplish the full replacement of membrane phospholipids for the opposite  
474 type and ii) be competitive in real environmental conditions (permissive fitness cost), the historical  
475 feasibility of such transition would be supported. So far, the evidence is lacking. In some models, the  
476 emission of cell protrusions and a progressive engulfment of an alphaproteobacterium is preferred over  
477 immediate phagocytosis<sup>18,147</sup>. That long process would favor phospholipid exchange and replacement of  
478 the rigid archaeal phospholipids for more flexible bacterial ones<sup>147</sup>. However, it is unclear how such a  
479 “slow phagocytosis” process would occur across prokaryotic generations, which are needed for evolution  
480 to take place (phospholipid replacement and proteome adaptation) prior to true engulfment. Second,  
481 two-partner models need to propose convincing detailed evolutionary mechanisms and selective forces  
482 for the origin of the eukaryotic nucleus, which are so far lacking. Finally, eukaryogenetic models need to  
483 explain the ‘silent bacterial majority’ in eukaryotic genomes. In two-partner models, HGT from different  
484 bacteria to any of the partners might be the most logical explanation, but this does not necessarily  
485 explain why groups of functionally-related genes seem to come from a few bacterial groups<sup>75,133</sup>, even if  
486 some HGT and phylogenetic reconstruction noise are likely involved in these observations. In-depth  
487 phylogenomic analyses including a broad taxon sampling and, if identified, the closest bacterial and  
488 archaeal relatives of eukaryotes will be needed. Collectively, the information gathered from  
489 environmental, experimental cell biology and phylogenomic studies should help to discriminate existing  
490 models, refine them or create new ones. More than ever, solving the eukaryogenesis riddle seems at  
491 hand.

492  
493

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801

## 802 **Author contributions**

803 P.L.-G. and D.M. conceived and discussed the ideas presented in the manuscript. P.L.-G. wrote the  
804 manuscript with critical input from D.M.

805

## 806 **Competing interests**

807 The author declares no competing financial interests.

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## 812 Figure Legend

813 **Figure 1 | Environmental context, metabolic interactions and (endo)membrane evolution during**  
814 **eukaryogenesis according to the HS-Syntrophy hypothesis. a**, eukaryogenesis took place in phototrophic  
815 microbial mats where steep redox gradients occur. Syntrophic interactions based on interspecies  
816 hydrogen and/or sulfur transfer are widespread depending on local physicochemistry; they notably  
817 involve methanogens, Asgard archaea, sulfate-reducing bacteria (SRB) and sulfide-oxidizing bacteria  
818 (SOB). **b-f**, different eukaryogenesis steps. **b**, initial facultative symbiosis stage involving a hydrogen-  
819 producing Asgard archaeon, a sulfate-reducing deltaproteobacterium and a sulfide-oxidizing  
820 alphaproteobacterium possibly able to carry out oxygenic photosynthesis. **c**, first integration of the  
821 Asgard archaeon as an endosymbiont (future nucleus). **d**, second integration step involving the  
822 endosymbiosis of the alphaproteobacterium. Stages c and d might have been coetaneous (first eukaryotic  
823 common ancestor – FECA – stage) or, more likely, decoupled in time (FECA 1 and 2). **e**, advanced  
824 integration stage involving important changes in metabolism (the consortium relies on aerobic  
825 respiration, all other previous metabolic interactions between partners being lost) and endomembrane  
826 evolution. **f**, last eukaryotic common ancestor stage (LECA). The position of H<sub>2</sub> or any other substrate by  
827 an arrow (over or under) implies transfer in the sense of the arrow; when it is on two arrows of opposed  
828 directionality, transfer may occur either way. HGT, horizontal gene transfer; EGT, endosymbiotic gene  
829 transfer; aa, amino acids; ER, endoplasmic reticulum; conc., concentration; Ox., oxygenic; Anox.,  
830 anoxygenic; SRB, sulfate-reducing bacteria; SOB, sulfide-oxidizing bacteria; FECA, first eukaryotic common  
831 ancestor; LECA, last eukaryotic common ancestor; hν, photon-derived energy.

832

833 **Box 1 | Symbiogenetic models for the origin of eukaryotes based on metabolic exchange.**

834 A variety of models propose that the eukaryotic cell evolved from a metabolic symbiosis (or syntrophy)  
835 established between archaeal and bacterial cells in anoxic or microoxic environments. Most of them  
836 involve only two partners, one archaeon and the alphaproteobacterial ancestor of mitochondria.  
837 However, some models invoke the participation of one additional bacterium, either transiently, as  
838 facilitator of the eukaryogenetic symbiosis<sup>18</sup>, or as an integral part of it<sup>19,26</sup>. One of the oldest proposals  
839 based on explicit syntrophy was that of D. Searcy, who stated that eukaryotes derived from a sulfur-  
840 mediated symbiosis between a wall-less, sulfur-respiring *Thermoplasma*-like archaeon and photo- or  
841 chemoautotrophic H<sub>2</sub>S-utilizing bacterium<sup>149,150</sup>. The original Hydrogen hypothesis postulated a hydrogen-  
842 mediated symbiosis between a hydrogenoclastic methanogenic archaeon and a hydrogen-producing  
843 alphaproteobacterium, hydrogen being used to reduce the CO<sub>2</sub> also released by the bacterium for  
844 methanogenesis<sup>25</sup>. In a more recent version of the Hydrogen hypothesis, the initial methanogenic host  
845 was abandoned in favor of an autotrophic, non-methanogenic, archaeon that would use the Wood-  
846 Ljungdahl pathway to fix carbon using the hydrogen released by the mitochondrial ancestor<sup>148</sup>. Based on  
847 the inferred ancestral metabolism of Asgard archaea, which likely were organoheterotrophs with flexible  
848 potential for hydrogen consumption and production, Spang and co-workers put forward the Reverse Flow  
849 model. In this model, the eukaryogenetic syntrophy was based on hydrogen transfer (or electrons, i.e.  
850 reducing equivalents, which might be also mediated by formate or acetate) from the anaerobic  
851 heterotrophic archaeon to the alphaproteobacterium<sup>16</sup>. The recent Entangle-Engulf-Enslave – E<sup>3</sup> model<sup>18</sup>  
852 favors a dual symbiosis of an Asgard archaeon that degraded amino acids to short-chain fatty acids and  
853 hydrogen with a sulfate-reducing bacterium (SRB) and an aerobic organotrophic alphaproteobacterium in  
854 microoxic environments that scavenged toxic O<sub>2</sub>. As the consortium progresses towards increasingly oxic  
855 zones, the interaction with the alphaproteobacterium becomes stronger until it is engulfed. The SRB  
856 symbiosis is transient and eventually lost<sup>18</sup>. Finally, the original version of the Syntrophy hypothesis (HM-  
857 Syntrophy) postulated a tripartite integrative symbiosis. First, a syntrophy based on interspecies H<sub>2</sub>-  
858 transfer was established between a fermentative, ancestrally sulfate-reducing, myxobacterium  
859 (Deltaproteobacteria) and a methanogenic archaeon using the fermentation-derived hydrogen for  
860 methanogenesis. Subsequently, a metabolically versatile alphaproteobacterium able to carry out  
861 facultative aerobic respiration but also to oxidize methane (methanotroph) incorporated stably into the  
862 consortium<sup>19,26</sup>. In the revised variant of the Syntrophy model (HS-Syntrophy model), we hypothesize a  
863 symbiosis between a hydrogen-releasing Asgard archaeon able to degrade small organics and a complex,  
864 myxobacterial-like, deltaproteobacterial host scavenging hydrogen (reducing equivalents) for sulfate  
865 reduction. The alphaproteobacterial ancestor of mitochondria was a sulfide-oxidizing facultative aerobe,  
866 recycling sulfur in the consortium. Possibly, it was also a mixotrophic organism able to carry out  
867 anoxygenic photosynthesis using H<sub>2</sub>S as electron donor.

868

869 ----- insert Fig. Box 1 here -----

870

871

872 **Box 2 | Symbiogenetic models for the origin of eukaryotes according to the timing and mode of evolution of**  
873 **key eukaryotic traits.**

874 Regardless the metabolic basis of the symbiosis established between the Asgard archaeon and its  
875 bacterial partner(s) during eukaryogenesis, models differ in the proposed mechanisms that resulted in the  
876 physical integration of two or more cells in one (future eukaryotic cell) and the evolution of typical  
877 eukaryotic traits as well as in the relative timing of the involved events (**a-e**). The Syntrophy hypothesis is  
878 the only model where a membrane transition is not needed, since the host (future cytoplasm) is a  
879 deltaproteobacterium naturally endowed with bacterial phospholipids (**a**). The future nucleus has an early  
880 origin in this model; it would derive from a distinct metabolic compartment (endosymbiotic archaeon)  
881 that is progressively confined by a host-derived secretory membrane (Med., medium). Mitochondria  
882 appear relatively late. In the Hydrogen hypothesis<sup>25</sup>, the endosymbiosis of the alphaproteobacterial  
883 ancestor of mitochondria within an archaeon by means independent of classical, eukaryotic-like  
884 phagocytosis (Ek-phagocytosis\* in the figure) is the starting event triggering eukaryogenesis (**b**). The  
885 nucleus and the associated membrane system would form de novo from lipid vesicles produced by the  
886 alphaproteobacterium<sup>126,151</sup>. The archaeal membrane phospholipids would have been fully replaced by  
887 the bacterial phospholipids by the fusion of those bacterial vesicles with the outer plasma membrane and  
888 the progressive displacement of archaeal lipids<sup>151</sup>. In the currently most widely accepted type of  
889 eukaryogenetic models, which include the 'phagocytosing archaeon'<sup>81</sup> hypothesis (**c**) and the 'reverse  
890 flow' model (**d**), the development of a complex cytoskeleton and endomembrane system predate the  
891 acquisition of the mitochondrial ancestor by classical phagocytosis<sup>6,9,16</sup>. Although these two models show  
892 similarities, in the 'phagocytosing archaeon' model the nucleus would appear before the mitochondrial  
893 acquisition, in contrast with the 'reverse flow' model . At any rate, in these models (**c, d**) the  
894 mitochondrial endosymbiosis would be the consequence of an already well engaged eukaryogenetic  
895 process. From this perspective, these models represent the transposition of past scenarios based on the  
896 existence of a proto-eukaryotic lineage different from archaea and bacteria endowed with all typical  
897 eukaryotic traits but mitochondria<sup>4,5,79,152</sup> to a nucleus-lacking proto-eukaryotic Asgard archaeon that is  
898 already seen as the first eukaryotic common ancestor (FECA)<sup>6,153</sup>. These two models and the hydrogen  
899 hypothesis clearly differ in the timing of the mitochondrial acquisition and the endomembrane system  
900 but, in the three cases, the archaeal membrane phospholipids are replaced relatively late by bacterial-  
901 type phospholipids (**b-d**). In another set of models, including the inside-out<sup>147</sup> and the E<sup>3</sup> hypotheses, the  
902 mitochondrial ancestor is acquired relatively late by a slow process of engulfment. This involves archaeal  
903 membrane extrusions that progressively surround, entangle and end up by enslaving the future  
904 mitochondria (**e**). At least in the inside-out model, the archaeal-to-bacterial membrane phospholipid  
905 transition would occur relatively early, facilitated by close cell-cell contact and symbiotic gene transfer.  
906 According to this model, only bacterial membranes, much more flexible than the archaeal ones, would be  
907 able to form an endomembrane system and carry out phagocytosis<sup>147</sup>. Finally, in the Serial Endosymbiosis  
908 model, several sequential symbioses intervene<sup>75,133</sup>. Although the details remain undetermined, the  
909 mitochondrion would be acquired late (**f**).

910  
911  
912  
913 ----- insert Fig. Box 2 here -----

914



915 **Table 1** | Key open questions and possible ways of progress to discriminate or refine current  
 916 symbiogenetic models of eukaryogenesis

|   | Test - Means of obtaining answers   | Models favored or disfavored   |
|---|---|--|
| <b>Predictions of the HS-syntrophy hypothesis</b>   |   |  |
| Existence of a versatile SRB deltaproteobacterial lineage, possibly sharing complex traits in common with myxobacteria, closer to eukaryotes  | Explore microbial ecosystems relevant for eukaryogenesis (sediments, microbial mats) in search for novel deltaproteobacterial lineages followed by phylogenomic analyses  | The detection of deltaproteobacterial lineages sharing a common and stronger phylogenetic signal with eukaryotes as compared to other bacteria would be consistent with the HS-Syntrophy model   |
| Existence of an alphaproteobacterial lineage of versatile S-oxidizers, perhaps photosynthetic, closer to mitochondria   | Explore microbial ecosystems relevant for eukaryogenesis in search for novel alphaproteobacterial lineages followed by phylogenomic analyses  | The detection of sulfide-oxidizing, potentially photosynthetic, alphaproteobacterial lineages sharing a common phylogenetic signal with eukaryotes to the exclusion of other bacteria would be consistent with the HS-Syntrophy model  |
| A large fraction of bacterial genes in eukaryotes predates the mitochondrial endosymbiosis and derives from Deltaproteobacteria   | Improve phylogenetic analyses of bacterial genes across eukaryotes, particularly those present in LECA  | The presence, function and relative amount of deltaproteobacterial-like genes in eukaryotes as compared to other bacterial-like genes might support the involvement of a deltaproteobacterial symbiont in eukaryogenesis   |
| Deltaproteobacterial genes mostly relate to membrane, cell signaling and cytoplasm functions  | Improve phylogenetic analyses and functional annotation of deltaproteobacterial-like genes in eukaryotes  | The involvement of deltaproteobacterial genes in membrane, cell signaling and cytoplasmic functions would be supportive of the HS-Syntrophy model  |
| Bacterial genes widespread in eukaryotes (present in LECA) largely derive from EGT, not HGT   | Improve phylogenetic analyses of bacterial genes present in LECA and look for potential homologues in Asgard archaea and in the closest alphaproteobacterial ancestors of mitochondria                            | If those bacterial-like genes in eukaryotes are missing in Asgard archaea or in Alphaproteobacteria, or the potential homologues are more distantly related than genes from other prokaryotic lineages, models invoking bacterial symbioses prior to the mitochondrial symbiosis would be favored  |
| <b>More general questions / problems</b>  |   |  |
| <b>Prokaryotic endosymbiosis and origin of nucleus</b>  |   |  |
| Do endosymbiotic prokaryotes exist in free-living prokaryotes? Do endosymbiotic archaea exist within bacteria?  | Look for potential prokaryotic endosymbionts in anoxic/redox-transition ecosystems such as sediments or microbial mats.   | If prokaryotic endosymbionts occur within prokaryotes, models proposing early prokaryotic endosymbionts would be as favored as models for which eukaryotic-like phagocytosis is a prerequisite. Finding archaeal endosymbionts within bacteria would relieve constraints for models proposing the endosymbiosis of one archaeon within a bacterium during eukaryogenesis   |
| <b>Mitochondria: original metabolism and timing</b>   |   |  |
| What was the metabolism of the alphaproteobacterial mitochondrial ancestor like? Did it have genes from other bacteria?   | Explore microbial ecosystems in search for novel alphaproteobacterial lineages closely related to the mitochondrial lineage   | If the closest alphaproteobacteria to the mitochondrion are identified, they might provide clues about the metabolic properties of the mitochondrial ancestor, potentially favoring specific eukaryogenesis models. If non-alphaproteobacterial genes in eukaryotes can be mapped back to these alphaproteobacteria, the origin of those genes would be more easily explained by HGT to the mitochondrial ancestor from other bacteria |
| Will the inclusion of more bacterial and archaeal genomes potentially more closely related to eukaryotes lead to the discovery of genes displaying similarly long branches in phylogenetic trees as compared to alphaproteobacterial genes? | Enrich the taxonomic sampling of Asgard archaea and bacteria having close homologues in eukaryotic genomes  | The discovery of an Asgard and/or bacterial lineage closer to eukaryotes and displaying branches of equivalent length to that of alphaproteobacterial-like genes in eukaryotes in phylogenetic trees might imply a simultaneous or temporally close symbiotic interaction of archaea and/or other bacteria during eukaryogenesis   |
| <b>Origin and nature of eukaryotic membranes</b>  |   |  |
| Can bacteria expressing archaeal phospholipids be stably maintained?  | Carry out experiments progressively expressing more archaeal phospholipids in bacteria until the complete replacement of bacterial phospholipids (eventually knocking out bacterial phospholipid synthesis genes) | If bacteria bearing membranes where bacterial phospholipids have been fully replaced by archaeal phospholipids can be experimentally produced, a bacterial-to-archaeal membrane transition would have been historically feasible   |
| If so, what is the fitness cost?  | Study fitness of bacteria with archaeal phospholipids in their membrane in long-term experiments with and without competition with wild/other strains and as a function of environmental conditions               | If fitness decreases and if bacteria cannot compete under any tested environmental conditions with wild strains and/or other bacteria, a membrane transition would have been historically unlikely   |
| Does the whole proteome evolve?   | Study how the proteome change in experimental evolution as a function of archaeal phospholipid content in bacterial membranes   | If important changes in the proteome are observed, this imposes constraints for models invoking a membrane-transition  |
| Can we answer in the same way to the three previous questions for archaea expressing bacterial phospholipids?   | Engineer archaeal cells with bacterial phospholipids and carry out similar experiments as described above for bacteria expressing archaeal phospholipids  | If if archaea bearing membranes with only bacterial phospholipids can be produced, an archaeal-to-bacterial type membrane transition could have been historically feasible. If not, the fitness is too high for archaea to compete with wild-type archaea and/or in natural environments and the proteome is significantly affected, an archaeal-to-bacterial type membrane transition would have been historically unlikely           |

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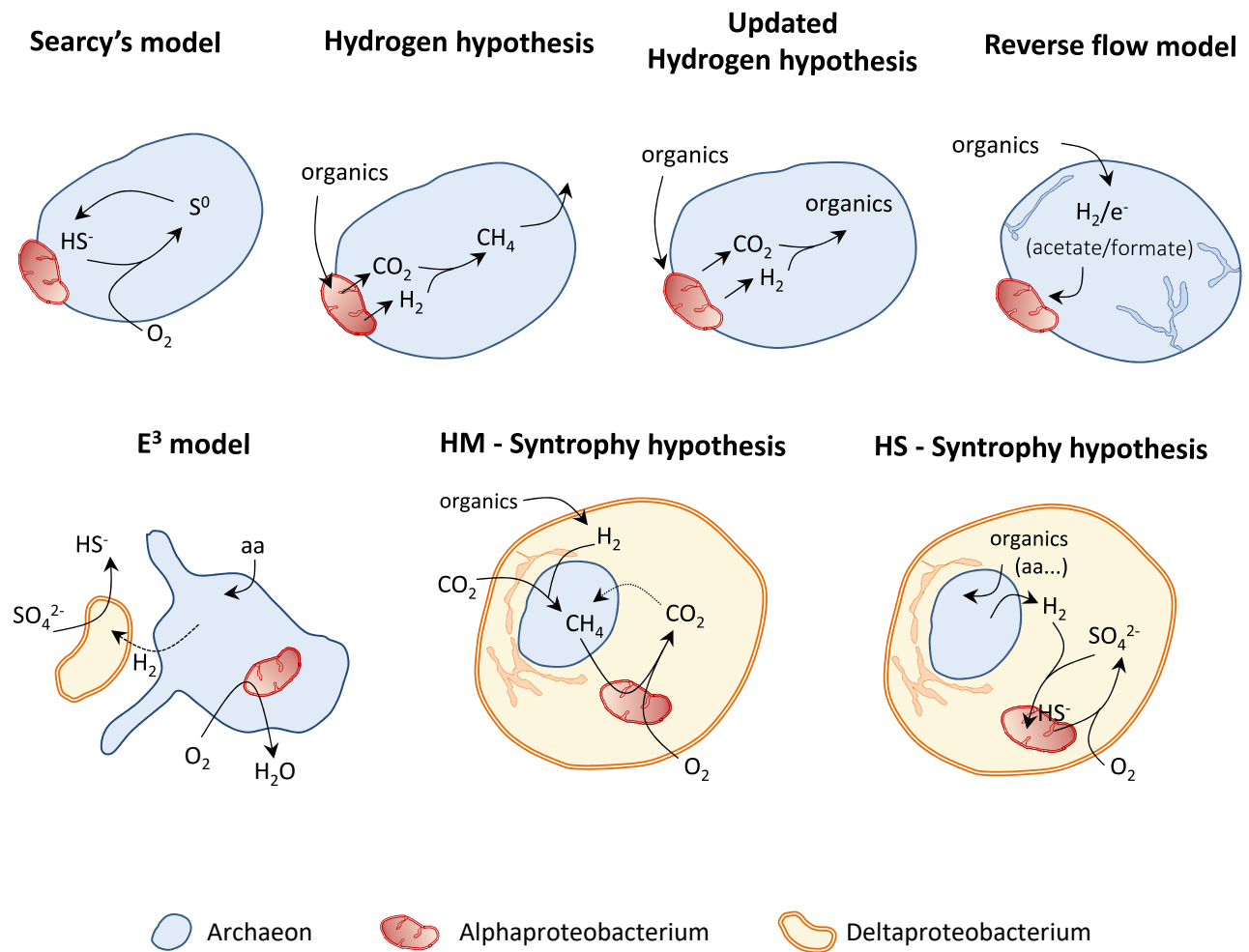


Figure Box 1. Lopez-Garcia & Moreira (R2)

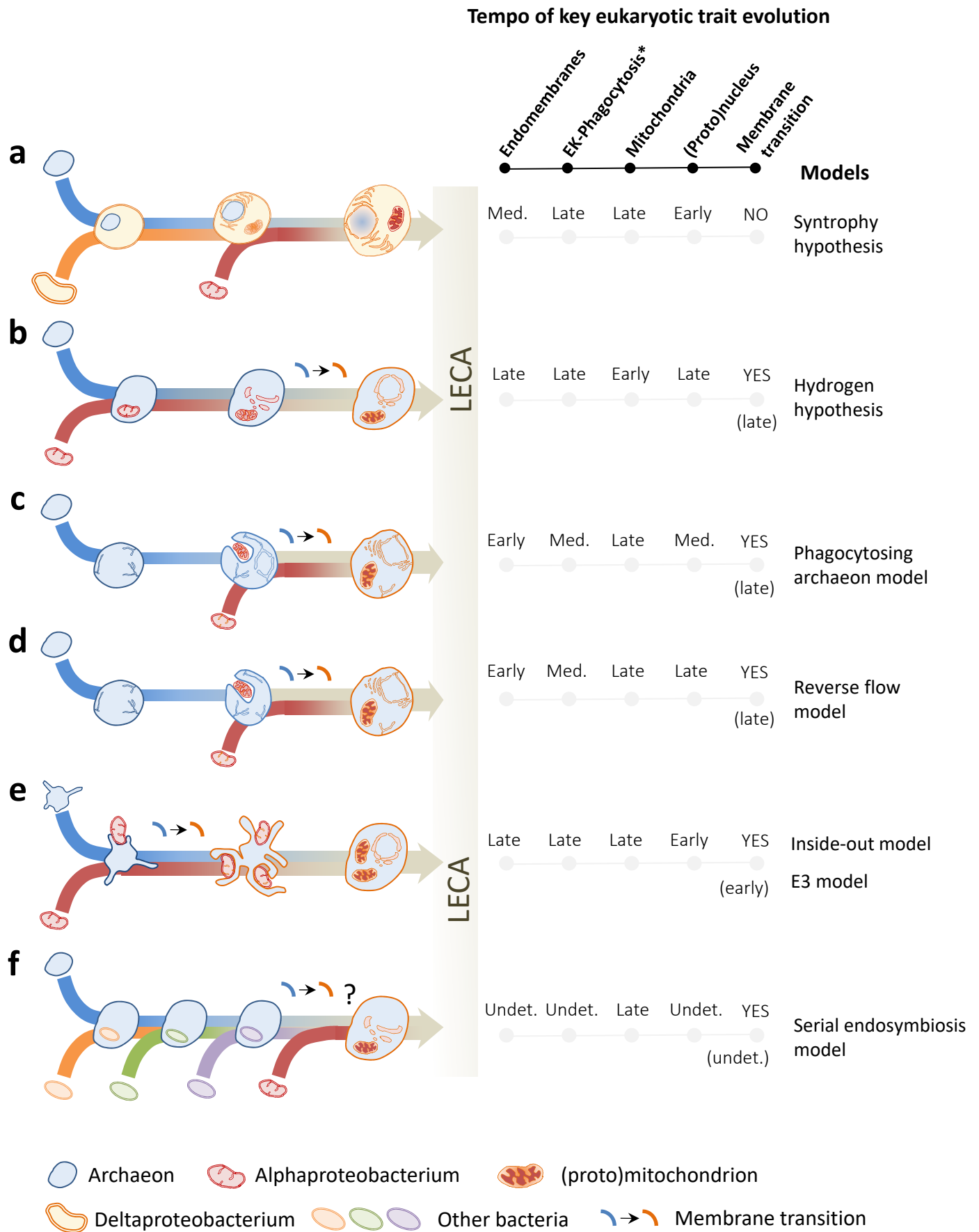


Figure Box 2. Lopez-Garcia & Moreira (R2)

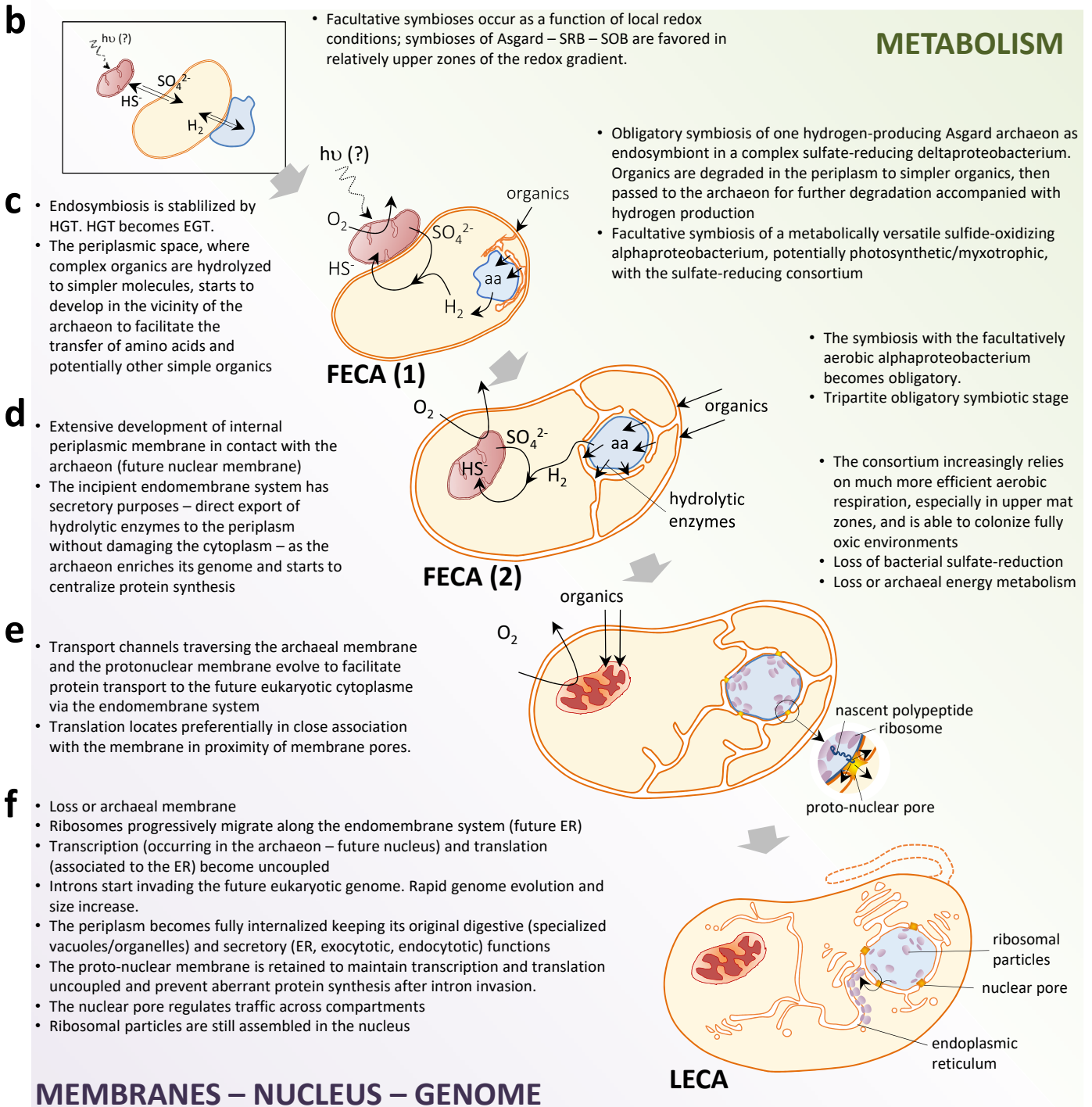
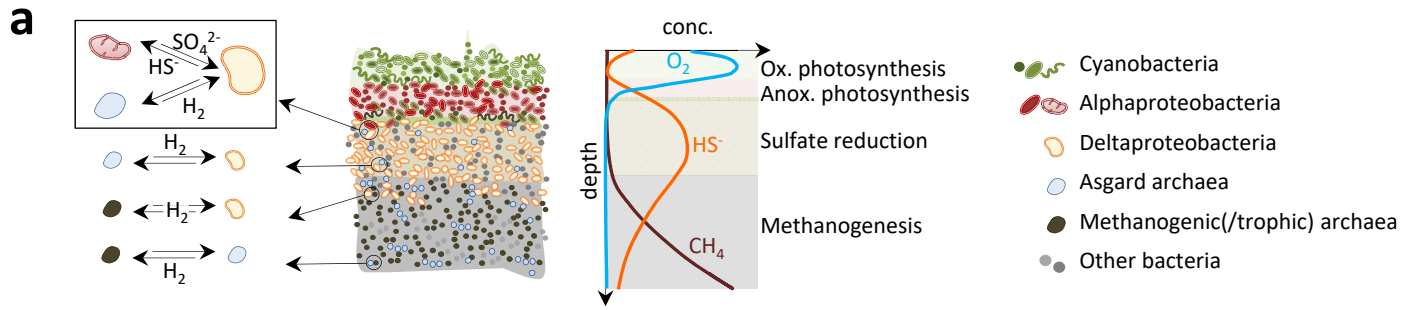


Figure 1. Lopez-Garcia & Moreira