The Eukaryotic CO$_2$-Concentrating Organelle is Liquid-Like and Exhibits Dynamic Reorganization

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Summary

Approximately 30-40% of global CO$_2$ fixation occurs inside a poorly understood organelle called the pyrenoid, which is found in the chloroplasts of most eukaryotic algae. The pyrenoid matrix is densely packed with the CO$_2$-fixing enzyme Rubisco, and is thought to be a crystalline or amorphous solid. Here, we show that the pyrenoid matrix of the unicellular alga *Chlamydomonas reinhardtii* is not crystalline, but behaves as a liquid that dissolves and condenses during cell division. Furthermore, we show that the pyrenoid is inherited both by fission and *de novo* assembly. A model inspired by this system predicts the existence of a "magic number" effect that influences phase separation in liquid-like organelles. This view of the pyrenoid matrix as a phase-separated compartment provides a new paradigm for understanding its structure, biogenesis, and regulation. More broadly, our findings expand our understanding of the principles that govern the architecture and inheritance of liquid-like organelles.

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Introduction

Although pyrenoids mediate approximately one third of global carbon fixation (Mackinder *et al.*, 2016), the molecular structure and biogenesis of these biogeochemically fundamental organelles remain largely unknown. Pyrenoids are non-membrane-bound, proteinaceous structures that contain a matrix packed with the CO_2-fixing enzyme Rubisco. In many species, the matrix is traversed by membrane tubules that are continuous with the photosynthetic thylakoid membranes (Gibbs, 1962; Ohad *et al.*, 1967; Griffiths, 1970; Engel *et al.*, 2015). Pyrenoids are a central feature of the algal CO_2 concentrating mechanism, which supplies Rubisco with a high concentration of its substrate CO_2, enabling more efficient carbon capture than that of most land plants (Badger *et al.*, 1998; Meyer *et al.*, 2012; Wang *et al.*, 2015). Pyrenoids are found within the chloroplasts of a diverse array of photosynthetic eukaryotes, including nearly all freshwater and marine algae, as well as a group of non-vascular plants (Brown *et al.*, 1967; Griffiths, 1980; Wang *et al.*, 2015). Although pyrenoids were among the first organelles to be scientifically documented (Vaucher, 1803), they have remained largely uncharacterized at a molecular level due to the limited availability of genetic tools for algae.

Based on results in the model unicellular green alga *Chlamydomonas reinhardtii* (*Chlamydomonas* hereafter), we recently proposed that a protein called Essential Pyrenoid Component 1 (EPYC1; also known as LCI5) links Rubisco holoenzymes together to form the pyrenoid matrix (Mackinder *et al.*, 2016). EPYC1 localizes to the pyrenoid matrix, is of similar abundance to Rubisco, and is required for Rubisco's localization in the pyrenoid matrix, supporting a possible structural role. EPYC1 binds Rubisco, and the EPYC1 protein sequence consists of four nearly identical ~60 amino acid repeats, suggesting a model in which each of

61   EPYC1's repeats contains a Rubisco binding site, allowing EPYC1 to link multiple Rubisco

62   holoenzymes together. The molecular details of the EPYC1-Rubisco binding interaction, and the

63   packing organization of EPYC1 and Rubisco in the matrix, remain unknown.

64   Electron micrographs of several species suggest that the densely-packed pyrenoid matrix

65   is crystalline (Holdsworth, 1968; Kowallik, 1969; Bertagnolli and Nadakavukaren, 1970), while

66   micrographs of other species suggest the matrix is amorphous (Griffiths, 1970; Meyer *et al.*,

67   2012). Our recent cryo-electron tomography (cryo-ET) study of *Chlamydomonas* revealed that

68   the average packing of Rubisco molecules within the pyrenoid matrix resembles a hexagonal

69   close packed lattice (Engel *et al.*, 2015). We have proposed that EPYC1 could link Rubisco

70   holoenzymes together into this arrangement (Mackinder *et al.*, 2016). However, if Rubisco is

71   immobilized within a rigid lattice, it is not clear how Rubisco Activase, which regenerates

72   Rubisco's active sites (Pollock *et al.*, 2003; Portis, 2003) but is significantly less abundant than

73   Rubisco in pyrenoids (Mckay *et al.*,1991; Wienkoop *et al.*, 2010; Mackinder *et al.*, 2016), could

74   reach enough Rubisco molecules to maintain efficient carbon fixation.

75   Moreover, pyrenoid biogenesis has not been definitively observed in living cells. Until

76   recently, the only tool available for visualization of the pyrenoid matrix was electron

77   microscopy, which precludes direct observation of dynamics. Classic electron microscopy

78   studies on fixed cells have suggested that pyrenoids may arise *de novo* (Bisalputra and Weier,

79   1964; Brown *et al.*, 1967; Retallack and Butler, 1970) in some species and by fission (Buffaloe,

80   1958; Brown *et al.*, 1967; Goodenough, 1970) in others.

81   Here, we describe our study of pyrenoid inheritance in living cells, and how our findings

82   inspired us to investigate the *Chlamydomonas* pyrenoid as a liquid. Examination of pyrenoid

83   ultrastructure with improved *in situ* cryo-ET revealed that the pyrenoid matrix is not crystalline,

84   but exhibits liquid-like local order. Further live-cell fluorescence experiments showed that the

85   matrix mixes internally and disperses into the surrounding stroma during cell division,

86   demonstrating that the matrix exhibits liquid-like properties. Finally, using a simple model

87   inspired by the pyrenoid, we predict that phase transitions in some multicomponent, liquid-like

88   biological systems are governed by changes in the ratio of available multivalent binding sites on

89   the constituent macromolecules. This work provides insights into both algal CO_2 concentrating

90   mechanisms and liquid-like biological systems.

91

92   **Results**

93

94   **The Pyrenoid Exhibits Both Fission and *de novo* Assembly**

95   To enable the first observations of pyrenoid matrix dynamics in living cells, we expressed

96   pyrenoid matrix proteins tagged with the fluorescent protein Venus and imaged them in 3D with

97   fluorescence time-lapse microscopy during photoautotrophic growth. We tracked inheritance of

98   the pyrenoid matrix by monitoring Venus-tagged Rubisco small subunit 1 (RbcS1) or Venus-

99   tagged EPYC1, and recorded chlorophyll autofluorescence to follow cellular orientation and

100   chloroplast division. The fusion proteins localized to the pyrenoid (Figure 1A,D), and did not

101   measurably perturb pyrenoid function (Figure S1A-B). During our observation, cells often

102   divided twice in rapid succession, which is typical for *Chlamydomonas* (Cross and Umen, 2015).

103   We observed that roughly two thirds of daughter cells inherited their pyrenoid by

104   elongation and then fission of the mother cell's pyrenoid (Figure 1; Figure 2E-F; Movie S1;

105   Figure S2). The duration of pyrenoid fission, defined as the time between visible elongation of

106   the mother pyrenoid and separation of the daughter pyrenoids, was ~7 minutes, much faster than

107 chloroplast division (~30-80 minutes, Figure 1F). All pyrenoid fissions occurred during the final

108 minutes of chloroplast division (Figure 1G-H).

109   In all 88 cell divisions in which pyrenoid fission was observed, the completion of

110 pyrenoid division was quickly followed by a gap in the chlorophyll signal between the daughter

111 pyrenoids, generated by the chloroplast cleavage furrow (Figure 1E,G-H). Conversely, in the 44

112 cell divisions in which the furrow did not bisect the pyrenoid, pyrenoid fission did not occur.

113 These findings support the hypothesis that pyrenoid fission is driven by the constrictive force of

114 the chloroplast division furrow (Goodenough, 1970; Hirakawa and Ishida, 2015).

115   In cases where pyrenoid fission did not occur, one of the daughter cells typically

116 inherited the mother cell's whole pyrenoid (~16-21% of daughter cells; Figure 2A-F; Movie S2),

117 leaving the other daughter without a pyrenoid. Some of the cells that failed to inherit a pyrenoid

118 remained without a visible pyrenoid for the duration of observation (~8-9% of all daughter cells;

119 Figure 2A,C,E-F; Movie S2). In other cases, one or more fluorescent puncta appeared *de novo*

120 and grew or coalesced into an apparent pyrenoid (~6-7% of all daughter cells; Figure 2B,D,E-I;

121 Movie S3). The proportions of each inheritance pattern in RbcS1-Venus and EPYC1-Venus were

122 not significantly different (Figure 2E-F), indicating that these patterns are not artifacts related to

123 a particular construct.

124   Several characteristics of pyrenoid behavior during division are reminiscent of liquids.

125 Towards the end of fission, a "bridge" of matrix material connecting the two lobes of a dividing

126 pyrenoid is briefly visible (Figure 3). After the bridge ruptures, daughter pyrenoids quickly

127 revert to spherical shapes, similar to the behavior of liquid droplets (Stone, 1994; Yanashima *et*

128 *al.*, 2012). Furthermore, during apparent *de novo* pyrenoid formation, we typically observed that

129 smaller puncta shrank while larger ones grew until the cell contained a single pyrenoid (Figure

130   2B,D,G-I) – much like Ostwald ripening in systems containing multiple liquid droplets (Hyman

131   *et al.*, 2014), indicating that components likely exchange between the puncta. Both fission and *de*

132   *novo* formation has been observed in established liquid-like organelles such as *C. elegans* P

133   granules (Brangwynne *et al.*, 2009; Brangwynne, 2011; Saha *et al.*, 2016) and *Xenopus* oocyte

134   nucleoli (Brangwynne *et al.*, 2011; Weber and Brangwynne, 2015). The similarity of the

135   pyrenoid's behavior to that of such phase-separated biological liquid droplets suggested the

136   possibility that pyrenoids may also be liquid, rather than static solids or crystals, as has been

137   proposed (Holdsworth 1968; Kowallik 1969; Bertagnolli & Nadakavukaren 1970). We therefore

138   decided to investigate the fine-scale structure and potential liquid-like dynamics of the pyrenoid

139   matrix.

140

141   **The Matrix is not Crystalline, and has Liquid-Like Organization**

142   The different reports of crystalline and amorphous pyrenoid matrices emerged from

143   classical electron micrographs (Holdsworth, 1968; Kowallik, 1969; Bertagnolli and

144   Nadakavukaren, 1970; Griffiths, 1970, 1980). However, these micrographs have limited

145   resolution, and the native arrangement of Rubisco may be compromised by artifacts from the

146   sample preparation procedure, which involves chemical fixation, dehydration, plastic

147   embedding, and heavy metal staining (Crang and Klomparens, 1988).

148   To avoid such artifacts, we analyzed the positions of individual Rubisco holoenzymes

149   within the *Chlamydomonas* pyrenoid matrix by *in situ* cryo-ET (Figure 4A, Movie S4), which

150   provides 3D views of the native cellular environment at molecular resolution (Asano *et al.*, 2016;

151   Schaffer *et al.*, 2017). In order to achieve the complete freezing vitrification required for high-

152   quality cryo-ET, we examined *mat3-4* cells (Umen and Goodenough, 2001), which are smaller

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153   due to premature cell division but have normal pyrenoid morphology and a functional CO_2

154   concentrating mechanism. The high fidelity of direct electron detector imaging enabled us to

155   localize ~97.5% of the Rubisco holoenzymes within five pyrenoid tomograms, totaling 192,100

156   particles (Figure 4B-C; Figures S3, S4A-C).

157   As a quantitative metric for the packing of Rubisco within the matrix, we calculated the

158   local concentration of holoenzymes in an expanding shell around every Rubisco. Our analysis

159   sampled only the pyrenoid matrix, avoiding potential effects from the traversing membrane

160   tubules and pyrenoid borders. Quasiperiodic near-neighbor peaks of Rubisco density eroded and

161   then vanished as distance increased from the reference particle (Figure 4D), suggesting that the

162   pyrenoid lacks the long-range order characteristic of a crystal.

163   We previously reported that the average local neighborhood in the pyrenoid matrix

164   resembles a lattice of hexagonal close packing (HCP) with ~15 nm between particle centers

165   (Engel *et al.*, 2015). However, due to limitations in imaging, this conclusion was not reached by

166   determining the positions of individual Rubisco holoenzymes, but rather by averaging together

167   larger volumes that contained neighborhoods of multiple Rubisco particles. The improved

168   quality of our new tomograms enabled us to pinpoint the positions of each Rubisco, revealing

169   that the "HCP lattice" is actually an average of a much more heterogeneous environment. In

170   HCP, the 12 nearest neighbors to any given particle are equidistant. However, when we plotted

171   the center-to-center distances between holoenzymes and their 12 nearest neighbors, we observed

172   a specific peak corresponding to 4.4 ± 1.8 neighbors that were 13.9 ± 1.5 nm away, with most of

173   the remaining neighbors at a range of longer distances, for an average distance of 15.9 ± 2.9 nm

174   (Figure 4E). The diameter of Rubisco is ~10-13 nm depending on orientation (Taylor *et al.*,

175   2001); thus, there is ~1-4 nm between a Rubisco and its nearest neighbors in the specific peak.

176   To directly compare the observed Rubisco packing to that of a crystal, we performed the

177   same local density analysis on simulated data in which the exact volume of the pyrenoid matrix

178   from the tomograms was replaced with one of two crystalline matrix models: one based on HCP

179   with 13.9 nm between particle centers, and the other on the structure observed in crystallized

180   Rubisco (Taylor *et al.*, 2001) (Figure 4F; Figure S4E-F). Compared to the experimental data,

181   both crystalline simulated data sets produced much taller short-range peaks along with

182   pronounced long-range peaks. Furthermore, the Rubisco concentration observed in the pyrenoid

183   matrix (377,856 ± 37,823 Rubiscos/μm^3; 628 ± 63 μM) was 28% lower than in the HCP

184   simulated data (526,039 ± 709 Rubiscos/μm^3; 874 ± 1 μM) (Figure S3). Therefore, we conclude

185   that the arrangement of Rubisco in the pyrenoid is neither HCP nor that observed in crystallized

186   Rubisco.

187   To explore whether the pyrenoid matrix has liquid-like organization, we next compared

188   the observed Rubisco packing to the distribution of molecules within known fluids. The local

189   density of Rubisco within the pyrenoid matrix fit well to the radial distribution function of a

190   simple model for liquid molecular interactions, the Lennard-Jones fluid (Johnson *et al.*, 1993)

191   (Figure 4G), determined both analytically (Morsali *et al.*, 2005) and by molecular dynamics

192   simulations (Plimpton, 1995). Additionally, similar radial distribution functions have been

193   measured for colloidal liquids (Gu *et al.*, 2010; Thorneywork *et al.*, 2014). Therefore, we

194   conclude that the radial distribution of Rubisco within the matrix resembles that of a liquid.

195   We sought to determine whether effects at the macromolecular scale could produce the

196   liquid-like organization that we observed in the pyrenoid matrix. We first examined whether

197   random packing of Rubisco particles could yield the local density seen in our experimental data.

198   Using Monte Carlo simulations, we randomly placed the same number of Rubisco particles as

199   the experimental data throughout identical pyrenoid matrix volumes (Figure 4H, Figure S4G).

200   The local density of this simulated data fit our experimental data poorly, showing that the

201   organization of pyrenoid matrix is not random.

202   We next randomly mapped Rubisco particles into the matrix volumes while imposing

203   specific distance constraints between either linked pairs of particles or interconnected linked

204   networks (Figure 4H, Figure S4H-J). As we increased the number of constraints on particle

205   spacing from the single constraint between paired particles to the numerous constraints between

206   particles in a network, the local density of Rubisco much more closely resembled the observed

207   distribution in the pyrenoid matrix. This result suggests that holoenzymes could be linked in a

208   network with a favored Rubisco-to-Rubisco distance. EPYC1 may be the molecular structure

209   that enforces this spacing.

210

211   **The Pyrenoid Mixes Internally**

212   The Rubisco packing distribution we observed is consistent with either a liquid or an

213   amorphous solid state. In order to discriminate between these states, a dynamic assay is

214   necessary. The most direct test of liquid-like behavior is whether the constituent components

215   exhibit internal mixing (Brangwynne *et al.*, 2009; Hyman *et al.*, 2014), which is traditionally

216   assayed *in vivo* by fluorescence recovery after photobleaching (FRAP) (Reits and Neefjes, 2001;

217   van Royen *et al.*, 2009; Ishikawa-Ankerhold *et al.*, 2012; Hyman *et al.*, 2014).

218   Strikingly, our FRAP experiments revealed that the pyrenoid matrix mixes internally

219   (Figure 5). After bleaching approximately half the volume of the matrix (Figure S5A-C), we

220   observed signal re-homogenization on a timescale of ~20 seconds (Figure 5A,C,G,I-L; Movie

221   S5), similar to that of established liquid-like compartments (Brangwynne *et al.*, 2009; Li *et al.*,

222   2012; Hubstenberger *et al.*, 2013; Kroschwald *et al.*, 2015; Nott *et al.*, 2015; Patel *et al.*, 2015;

223   Saha *et al.*, 2016). During recovery, the signal from the unbleached region decreased as the

224   signal in the bleached region increased, and a "wave" of fluorescence could be seen moving from

225   the unbleached to the bleached region (Figure 5C,E), suggesting that recovery is primarily due to

226   internal rearrangements, rather than import of stromal RbcS1-Venus. In contrast, pyrenoids in

227   chemically crosslinked cells showed no recovery (Figure 5B,D,F,G; Movie S5).

228   Further FRAP experiments demonstrated that the other major components of the

229   pyrenoid matrix, EPYC1 (Mackinder *et al.*, 2016) and Rubisco Activase (Mckay *et al.*, 1991;

230   Mackinder *et al.*, 2016) (RCA1), are also mobile (Figure 5G-L, Figure S5D-F). Differences in

231   the initial recovery rates of the three matrix components (Figure 5H) suggest that they may have

232   distinct mobilities within the matrix, apparently inversely proportional to their molecular weights

233   (Rubisco holoenzymes are ~540 kDa; RCA1 hexamers are ~270 kDa; EPYC1 is ~35 kDa). The

234   different mobilities suggest that although these matrix components can bind to each other, the

235   major mobile unit is not a complex between EPYC1 and Rubisco; thus, EPYC1-Rubisco binding

236   interactions (Mackinder *et al.*, 2016) are transient. Alongside our observations of liquid-like

237   fission (Figures 1,3) and local order (Figure 4), the internal mixing of matrix components

238   demonstrates that the *Chlamydomonas* pyrenoid matrix behaves like a liquid.

239

240   **The Pyrenoid Disperses During Cell Division**

241   A remarkable property of liquid-like protein compartments is their ability to transition

242   between an aggregated liquid phase and a dispersed soluble phase (Brangwynne *et al.*, 2009; Li

243   *et al.*, 2012; Weber and Brangwynne, 2012). Surprisingly, our experiments revealed that the

244   *Chlamydomonas* pyrenoid matrix also appears to undergo such a phase transition during

245   division. A portion of the RbcS1-Venus and EPYC1-Venus signals rapidly dispersed from the

246   pyrenoid matrix into the stroma for ~20 minutes near the end of chloroplast and pyrenoid

247   division (Figures 6, S6). During this time, the Venus signal in the chloroplast stroma rose

248   dramatically, while the signal in the pyrenoid dropped by ~35-50%. The Venus intensity in the

249   pyrenoid subsequently recovered, coincident with a reduction in the stromal signal (Figure 6B-

250   C). The increased stromal signal during chloroplast division is unlikely to be due to new protein

251   synthesis because these transitions occurred on the timescale of 1-5 minutes, and were not

252   associated with an increase in the total fluorescence signal (Figure 6B, Figure S6). Pyrenoid

253   fission nearly always occurred during the time of increased stromal signal (Figure 6B,D; Figure

254   S6), raising the intriguing possibility that the partial dispersal may be associated with a decrease

255   in matrix surface tension or viscosity, which could facilitate the progression of the cleavage

256   furrow through the pyrenoid.

257   We observed pyrenoid component dispersal regardless of the mode of pyrenoid

258   inheritance (Figure S6). During dispersal, puncta of matrix material often appeared transiently

259   throughout the stroma (Figures 1D, S1C-D; Movie S3). These *de novo* matrix protein puncta

260   may correspond to the "dense regions" that were observed in electron micrographs of dividing

261   *Chlamydomonas* cells (Goodenough, 1970). In some daughter cells that failed to inherit part or

262   all of the maternal pyrenoid during chloroplast division, we observed that these puncta grew into

263   apparent new pyrenoids (Figure 2B,D,G-I). Our observations suggest a model in which the

264   dispersal of the building blocks of the matrix serves as a redundant mechanism to fission,

265   facilitating equal partitioning of the pyrenoid matrix to daughter chloroplasts. The inheritance of

266   dispersed pyrenoid components enables rapid *de novo* pyrenoid assembly in daughter

267   chloroplasts that fail to inherit a pyrenoid by other means.

268

A "Magic Number" Effect in Multicomponent Phase-Separation

The liquid-like nature of the pyrenoid fits well with the principle that biological liquid

compartments are formed by weak multivalent binding between constituent proteins (Li *et al.*,

2012). The two major constituents of the pyrenoid, Rubisco and EPYC1, likely have multiple

binding sites for each other. The octameric symmetry of the Rubisco holoenzyme makes it

plausible that Rubisco has eight binding sites for EPYC1. Additionally, the four repeats of the

EPYC1 protein suggest that EPYC1 has four binding sites for Rubisco (Mackinder *et al.*, 2016).

We have previously proposed that dynamic regulation of the availability of EPYC1's binding

sites could cause a change in the aggregation state of the pyrenoid (Mackinder *et al.*, 2016).

In the model liquid-like system involving binding between the SRC homology 3 (SH3)

domain and a proline-rich motif ligand (PRM), it has been established that decreasing the

number of binding sites on flexible linker proteins promotes a phase transition from an

aggregated to a dispersed state (Li *et al.*, 2012). Surprisingly, modeling of the EPYC1-Rubisco

interaction predicts the existence of an additional effect that governs phase transitions in

multicomponent liquid-like biological systems.

Because the binding mechanism between Rubisco and EPYC1 is uncharacterized, we

developed a simplified computational model to investigate how interactions between these two

components may drive pyrenoid aggregation and dissolution. In our model, "Rubisco

holoenzymes" and "EPYC1 molecules" occupy a 2D square grid. Each Rubisco holoenzyme is

modeled as a 2x4 rectangle of 8 EPYC1 binding sites, and each EPYC1 is modeled as a flexible

chain of 4 Rubisco binding sites. Each binding site on Rubisco can bind to a single EPYC1 site,

and *vice versa.* Binding occurs when a Rubisco site and an EPYC1 site occupy the same grid

291   position. While our model is simplified and abstract, it allows the observation of certain

292   fundamental behaviors.

293   Our simulation produced the expected increased aggregation when we increased the

294   number of binding sites on EPYC1 from 4 to 5 (Figure 7B-C). However, contrary to the

295   established paradigm for liquid-like systems, we also observed increased aggregation when we

296   decreased the number of binding sites on EPYC1 from 4 to 3 (Figure 7A-B).

297   Further investigation into this previously unappreciated behavior revealed a "magic

298   number" effect in the simulated EPYC1-Rubisco system. Magic numbers occur in various

299   contexts in chemistry and physics (Sakurai *et al.*, 1999; Steppenbeck *et al.*, 2013), in which a

300   certain number of particles form an unusually stable state, such as filled electronic shells in

301   atoms. However, to our knowledge, magic numbers have not previously been observed in a

302   biological system. In our model, the magic number effect arises because all 8 binding sites of one

303   Rubisco can be exactly saturated by two EPYC1s with 4 binding sites each to form a stable

304   trimer, resulting in minimal aggregation. When the magic number stoichiometry is broken either

305   with more or fewer binding sites on EPYC1, larger aggregates form.

306   To ensure that the magic number effect is not an artifact of the lattice geometry or of two

307   dimensions, we also simulated the system using a more computationally intensive but more

308   realistic three-dimensional, off-lattice representation. We modeled each Rubisco as a sphere with

309   4 small spherical binding sites on each end, and each EPYC1 molecule as a polymer with 3, 4, or

310   5 binding sites (Figure 7G-I, Movie S6). When the EPYC1 and Rubisco binding sites

311   overlapped, specific bonds were formed. Similar to the lattice simulation results, we observed

312   more aggregation when we increased the number of binding sites on EPYC1 from 4 to 5 (Figure

313   7H, I) and when we decreased it from 4 to 3 (Figure 7G, H). Additionally, small trimer

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314   complexes containing one Rubisco and two EPYC1s were commonly observed when the number

315   of binding sites on EPYC1 was 4, but these complexes were rarely seen when the number of

316   binding sites on EPYC1 was 3 or 5. Thus, the magic number effect persists in three-dimensions

317   and off-lattice. Furthermore, the effect is robust for a wide range of protein concentrations and

318   model parameters (Figure 7D-F, Figure S7, Table S1, Movie S6).

319   To explore the generality of this effect, we used the 2D lattice model to consider an even

320   wider range of the number of Rubisco binding sites on EPYC1 (Figure 7J-L), and found

321   additional magic numbers at 2 and 8 binding sites per EPYC1. When the number of binding sites

322   on EPYC1 is a magic number, small heterocomplexes are favored; consequently, higher protein

323   concentrations are required in order to form large aggregates (Figure 7J-L). These results are

324   robust to constraints such as restricting EPYC1 to only bind one end of each Rubisco (Figure

325   S7). Thus, in addition to the previously established general trend that increasing the number of

326   binding sites in a flexible linker protein promotes phase separation (Li *et al.*, 2012), there are

327   strong magic number effects that impact the phase diagram.

328

329   **Discussion**

330
331   **Phase Separation Helps Explain Known Pyrenoid Physiology**

332   Phase separation provides a new framework for understanding the structure, biogenesis,

333   and inheritance of the pyrenoid. Our observations suggest that the re-localization of Rubisco

334   from the pyrenoid to the stroma in response to high CO_2 (Borkhsenious *et al.*, 1998) or darkness

335   (Mitchell *et al.*, 2014) is a phase transition of the pyrenoid matrix. The use of phase transitions

336   could allow rapid reorganization of Rubisco to enhance CO_2 fixation in fluctuating

337   environmental conditions. Furthermore, the fluidity of the pyrenoid matrix resolves the paradox

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338   of how Rubisco Activase chaperones can efficiently access the more abundant Rubisco active

339   sites throughout the matrix.

340

341   **EPYC1-Rubisco Interactions May Drive Pyrenoid Phase Separation**

342   The pyrenoid's liquid-like nature may be mediated by binding between Rubisco and

343   EPYC1's sequence repeats. Each EPYC1 repeat displays low complexity, with 73% of the repeat

344   regions consisting of just alanine, serine, proline, and arginine (Mackinder *et al.*, 2016). Low

345   complexity domains and repeat regions have been widely implicated in mediating the liquid-like

346   properties and phase separation of nonmembrane-bound organelles (Li *et al.*, 2012; Fromm *et*

347   *al.*, 2014; Bergeron-Sandoval *et al.*, 2016; Mitrea and Kriwacki, 2016). The exact nature of the

348   binding between Rubisco and EPYC1 is unknown, but it is likely that surface-exposed α-helices

349   on the Rubisco small subunit play a role in the binding interaction because a specific sequence in

350   these helices is required for Rubisco aggregation in *Chlamydomonas* (Meyer *et al.*, 2012). Weak

351   multivalent interactions have been implicated in mediating biological phase separation in other

352   liquid-like organelles (Li *et al.*, 2012; Hyman *et al.*, 2014). Thus, if EPYC1-Rubisco interactions

353   are weak, then these two binding partners could explain both the structure and the fluidity of the

354   matrix.

355

356   **A Magic Number Effect Could Facilitate Phase Transitions**

357   We speculate that a magic number effect could help explain the rapid phase transitions of

358   the pyrenoid matrix that we observed experimentally (Figure 6). The rapid partial dissolution and

359   reorganization of the pyrenoid suggests regulation at the level of EPYC1-Rubisco interactions.

360   The EPYC1 phosphorylation state changes under conditions that require a pyrenoid (Turkina *et*

361   *al.*, 2006; Wang *et al.*, 2014), suggesting that phosphorylation could be a mechanism to alter the

362   availability of Rubisco binding sites on EPYC1. In our simulations, the magic number effect

363   produces a dramatic phase change in response to the addition or removal of a single binding site,

364   from aggregation with 3 binding sites on EPYC1 to dissolution with 4 binding sites and back to

365   aggregation with 5 binding sites (Figure 7). Thus, as a result of the magic number effect, a

366   change in the affinity of only a single binding site on each EPYC1 may trigger a phase change.

367   Both lattice and off-lattice models indicate that clustering can be strongly suppressed if

368   the number of binding sites on one species (*e.g.*, Rubisco) is an integral multiple of the number

369   of binding sites on the other (*e.g.*, EPYC1), as this favors the assembly of small oligomers in

370   which all binding sites are saturated. In general, we expect this magic number effect to pertain to

371   multicomponent, multivalent binding systems where bonds are one-to-one and saturable.

372   Moreover, the effect requires these specific bonds to have an energy of several k_BT, strong

373   enough for most small oligomers to be fully bonded without defects (Figure 5 D-F). Since such

374   binding energies are quite common, magic number effects can be predicted broadly for

375   interacting protein pairs such as SH3-PRM (Li *et al.*, 2012) and SIM-SUMO (Banani *et al.*,

376   2016) as well as for RNA-protein droplets (Lin *et al.*, 2015). Additional insights could be gained

377   in the future by investigating the influence of polymer flexibility, stoichiometry, and mixed

378   valency populations on the magic number effect described in this work.

379

380   **Interactions Between the Pyrenoid Matrix and Other Chloroplast Structures Remain to be**

381   **Explored**

382   In *Chlamydomonas*, the pyrenoid matrix is located at the base of the chloroplast, is

383   traversed by tubules that are continuous with the thylakoid membranes, and is surrounded by a

16

384   starch sheath (Griffiths, 1970, 1980; Meyer and Griffiths, 2013; Engel *et al.,* 2015). It is not yet

385   known what controls the location of the pyrenoid, but we hypothesize that the matrix's liquid-

386   like nature may promote its localization around cellular components that have affinity for the

387   matrix, including possibly the pyrenoid tubules. Future investigations into the division and

388   possible *de novo* formation of the starch sheath and thylakoid tubules may provide insights into

389   the principles that underlie the interface between liquid-like organelles and other cellular

390   structures.

391

392   **Other Species May Also Have Liquid-Like Pyrenoids**

393   Our results suggest that the pyrenoid "regression" (reduction in size and disappearance)

394   observed in some algae during chloroplast division in zoosporogenesis (Brown and Arnott, 1970)

395   may be a phase transition from an aggregated to a soluble phase. Previous reports suggested that

396   different species of algae undergo either pyrenoid fission or pyrenoid regression during cell

397   division (Brown et al., 1967; Griffiths, 1970, 1980). However, our observations demonstrate that

398   both phenomena can occur simultaneously in the same cell.

399   The observation of pyrenoid fission and regression in other species of algae (Brown,

400   1964; Brown and Arnott, 1970; Griffiths, 1970, 1980), combined with the amorphous appearance

401   of the matrix of many species (Evans, 1966; Griffiths, 1970, 1980; Meyer *et al.*, 2012), suggests

402   that the liquid-like nature may be a general property of all pyrenoids. However, our data does not

403   exclude the possibility that the pyrenoid matrix of some species is more permanently crosslinked

404   or crystalline. As new genetic tools are developed, it will be particularly interesting to perform

405   experiments similar to those presented here on the diatom *Achnanthes brevipes*, where some of

406   the most striking crystalline matrix packing was observed (Holdsworth, 1968). If solid pyrenoids

407   do exist, it will be interesting to understand what benefits the solid phase can provide that

408   outweigh the advantages of a liquid for inheritance and protein mobility.

409   Pyrenoids are not only broadly distributed in the Chloroplastida, but are also found in

410   five out of the seven supergroups of the eukaryotic tree of life [Excavates, Stramenopiles,

411   Alveolates, Rhizaria and Archaeplastids (Burki, 2014)], and it is thought that pyrenoids have

412   evolved multiple times (Villarreal and Renner, 2012; Meyer and Griffiths, 2013). Intriguingly,

413   across sequenced algae, the presence of a pyrenoid is correlated with the presence of a protein

414   with EPYC1-like physiochemical properties, such as a low-complexity repeat sequence, a high

415   pI, and no transmembrane domains (Mackinder *et al.*, 2016). If the only requirement for

416   aggregating diffuse Rubisco into a liquid-like pyrenoid matrix is the evolution of a linker protein

417   with multiple weak binding sites for Rubisco, pyrenoids could be relatively simple to evolve.

418

419   **Pyrenoid Behavior is Distinct from that of Carboxysomes**

420   Cyanobacteria have a functionally similar structure to the pyrenoid called the

421   carboxysome. Like the pyrenoid, carboxysomes contain aggregated Rubisco and are not bound

422   by a membrane. Unlike the *Chlamydomonas* pyrenoid, carboxysomes are not traversed by

423   membrane tubules or surrounded by a starch sheath, but are instead surrounded by an icosahedral

424   protein shell. There are two classes of carboxysomes, α and β, which are thought to have arisen

425   through convergent evolution (Rae *et al.,* 2013).

426   Rubisco in β-carboxysomes is thought to be linked together by CcmM, a protein

427   containing multiple Rubisco small subunit-like domains, each of which is thought to be

428   incorporated into a different Rubisco holoenzyme (Long *et al.*, 2010). Such incorporation would

429   be expected to preclude internal mixing. Indeed, in contrast to our results in pyrenoids,

430   fluorescently-tagged Rubisco in β-carboxysomes does not recover after photobleaching (Chen *et*

431   *al.*, 2013).

432   Less is known about the arrangement of Rubisco in α-carboxysomes. The proposed α-

433   carboxysome Rubisco linker protein CsoS2 (Cai *et al.*, 2015) is a disordered repeat protein,

434   raising the possibility that the α-carboxysome may have liquid-like characteristics. Whereas we

435   have observed pyrenoid biogenesis by both fission and *de novo* assembly, biogenesis of new α-

436   and β-carboxysomes has only been observed to occur *de novo* (Iancu *et al.*, 2010; Cameron *et al.*,

437   2013; Chen *et al.*, 2013; Kerfeld and Melnicki, 2016). It will be interesting to see whether α-

438   carboxysomes do exhibit liquid-like behavior, or whether the underlying structural and

439   biogenesis principles are a general distinguishing feature between carboxysomes and pyrenoids.

440

441   **The Pyrenoid Provides Insights into Liquid-Like Organelle Biology**

442   To our knowledge, the pyrenoid is now the first example of a liquid-like organelle

443   specific to photosynthetic organisms. Like other previously-characterized liquid-like organelles,

444   new pyrenoids can be generated by either *de novo* aggregation or fission into two daughters

445   (Brangwynne, 2011; Brangwynne *et al.*, 2011; Weber and Brangwynne, 2015; Saha *et al.*, 2016).

446   The first characterized liquid-like organelle, the *C. elegans* P granule, leverages the properties of

447   phase transitions to promote its own asymmetric inheritance by preferentially condensing at one

448   end of the cell before cell division (Brangwynne *et al.*, 2009; Saha *et al.*, 2016). In contrast, the

449   pyrenoid appears to leverage the liquid-like properties of partial dissolution and fission to

450   promote symmetric inheritance, allowing both daughter cells to inherit a pyrenoid.

451

452

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Main Figure Titles and Legends

Figure 1. The Pyrenoid is Inherited Primarily by Fission.

(**A** and **B**) Confocal Z-sum images of pyrenoid divisions by fission, with chlorophyll

autofluorescence shown in magenta, and RbcS1-Venus in green; t=0 is the first observation of a

gap in chlorophyll between the daughter pyrenoids in the first division shown. Dashed curves

represent approximate chloroplast outlines in the mother (white) and daughter (pink) cells.

(**C**) A cartoon of the approximate locations of the pyrenoid (green), chloroplast (magenta), and

cell membrane (black outline).

(**D**) Example of pyrenoid fission in EPYC1-Venus, annotated as in (B).

(**E**) Example of the progressing chloroplast cleavage furrow (arrows) appearing to separate

daughter pyrenoids. Images are 2D snapshots of 3D Z-stack reconstructions.

(**F**) Average and standard deviation of the durations of chloroplast (magenta) and pyrenoid

(green) fissions in RbcS1-Venus (left; $n = 28$ 1st and 2nd divisions) and EPYC1-Venus (right; $n =$

22 1st and 25 2nd divisions).

(**G** and **H**) Duration and relative timing of chloroplast (magenta) and pyrenoid (green) division

for the pyrenoid fissions plotted in (F). Each bar represents a different division.

See also Figures S1-2, and Movie S1.

750 **Figure 2. Pyrenoids Can Also be Inherited by Other Means.**

751 (**A** – **D**) Examples of other types of pyrenoid inheritance patterns observed in RbcS1-Venus (A,

752 B) and EPYC1-Venus (C, D) cells. (A, C) One daughter (blue) inherits an entire pyrenoid from

753 the mother cell (white) and another daughter (orange) inherits neither a pyrenoid nor puncta. (B,

754 D) One daughter (blue) inherits the entire pyrenoid, and puncta appear in the other daughter

755 (yellow) and coalesce into a new pyrenoid.

756 (**E** – **F**) Proportion of observed RbcS1-Venus (E) and EPYC1-Venus (F) daughter cells that

757 exhibited each observed inheritance pattern; the distribution of inheritance patterns in EPYC1-

758 Venus cells was not significantly different from that of RbcS1-Venus cells (Chi-square test, p =

759 0.8).

760 (**G** – **I**) Stills from time course image captures in which pyrenoids were observed to grow or

761 coalesce from puncta that appeared in the chloroplast stroma during division. (G – H) RbcS1-

762 Venus; (I) EPYC1-Venus. Images are 2D projections of the sum of pixel values in each channel

763 in a Z-stack through the whole cell at each time point. The chloroplast of the dividing cell of

764 interest in each series is outlined in white. Arrows point to growing or coalescing pyrenoids. t=0

765 is defined as the first minute at which the daughter chloroplasts are observed to be distinct in 3D.

766 See also Figures S1-2, and Movies S2-3.

767

768   **Figure 3. A "Bridge" of Matrix Material Connects Nascent Daughter Pyrenoids During**

769   **Fission.**

770   (**A** – **E**) Examples of pyrenoid fissions in five RbcS1-Venus cells. Magenta is chlorophyll

771   autofluorescence; green is RbcS1-Venus. Images are 2D projections of the sum of pixel values in

772   each channel from a Z-stack through the whole cell.

773   (**F**) Example of pyrenoid fission in an EPYC1-Venus cell. Magenta is chlorophyll

774   autofluorescence; green is EPYC1-Venus. Images are 2D projections of the sum of pixel values

775   in each channel from a Z-stack through the whole cell.

776   See also Movie S1.

777

778 **Figure 4. The Pyrenoid Matrix is not Crystalline but Exhibits Short-Range Liquid-Like**

779 **Order.**

780 (**A**) Slice through a tomographic volume of the native *Chlamydomonas* pyrenoid.

781 (**B**) Segmentation of the tomogram shown in (A) with localized positions of 46,567 Rubisco

782 holoenzymes (magenta) mapped into the volume. Green and yellow: pyrenoid tubule

783 membranes.

784 (**C**) *In situ* subtomogram average of Rubisco (16.5 Å resolution; Figure S4A) generated from

785 30,000 particles extracted from the tomogram shown in (A).

786 (**D**) The local density of neighbor Rubisco particles as a function of the distance from the

787 reference particle. Each line represents a separate tomogram, showing the sum of the local

788 densities around every Rubisco. The distances to peaks of high local Rubisco concentration are

789 indicated.

790 (**E**) Histogram of distances from reference particles to their nearest neighbors (NN), summed

791 from all five tomograms. Red dashed line: Gaussian distribution fit to the 13.9 nm NN peak.

792 Light blue bars: distance to the 12 NN within 1 standard deviation (<1 SD) of the 13.9 nm peak,

793 dark blue bars: distance to the 12 NN beyond 1 standard deviation (>1 SD) from the 13.9 nm

794 peak, grey bars: distance to further (13+ NN) neighbors. Mean distance to the 12 NN = 15.9 nm.

795 Inset: distribution of the number of neighbors per reference particle (mean = 4.4 neighbors) that

796 are <1 SD from the 13.9 nm peak.

797 (**F**) The normalized local density of neighbor particles (local density divided by the global

798 density), showing the mean value ± 99% CI of the experimental data (black) compared to

799 crystalline simulated data generated within the same tomogram volumes (Figure S4E,F): crystal

800 structure packing (Taylor *et al.*, 2001) (red), 13.9 nm-spaced HCP (blue).

36

801   (**G**) The mean value ± 99% CI of the experimental data's normalized local density (black) fit

802   with the radial distribution function of a Lennard-Jones fluid generated by an analytical model

803   (Morsali *et al.*, 2005) (red) and by molecular dynamics simulations (Plimpton, 1995) (blue).

804   (**H**) The normalized local density of neighbor particles, showing the mean value ± 99% CI of the

805   experimental data (black) compared to random simulated data generated within the same

806   tomogram volumes (Figure S4G-J): single particles (red), pairs linked by 13.9 ± 1.5 nm

807   (yellow), and linked networks (blue).

808   See also Figures S3-4, and Movie S4.

809

810 **Figure 5. Pyrenoid Matrix Components Mix Internally.**

811 (**A** and **B**) FRAP in live (A) and fixed (B) RbcS1-Venus pyrenoids. Cartoons depict the

812 approximate bleached region (dark gray). Different intensity display scales are used in the pre-

813 and post-bleach image sets.

814 (**C – D**) Kymographs of the pyrenoids shown in parts (A-B), respectively. From left to right: the

815 pyrenoid cartoons from (A-B), showing the region used to create the kymographs (dashed

816 rectangle); the pre-bleach section of the kymographs; and the post-bleach kymographs.

817 (**E – F**) Fluorescence recovery occurs from within the pyrenoid in live pyrenoids (E), but does

818 not occur in fixed pyrenoids (F). The *x*-axis is μm along the dashed regions in (C- D).

819 (**G**) Average fluorescence recovery profiles ± SEM for pyrenoids in live RbcS1-Venus (blue),

820 RCA1-Venus (red), or EPYC1-Venus (yellow) cells, and in fixed RbcS1-Venus cells (gray).

821 (**H**) Average recovery rates ± SEM over the first 12 seconds in (G). ** $p < 0.005$; * $p < 0.05$

822 (one-way ANOVA & post-hoc Bonferroni means comparison;).

823 (**I – L**) Examples of half-pyrenoid FRAP in live RCA-Venus (I, K) and EPYC1-Venus (J, L)

824 cells, with images from the recovery time-courses (I, J) and corresponding kymographs (K, L) as

825 shown in (A – D).

826 See also Figure S5 and Movie S5.

827   **Figure 6. The Pyrenoid Matrix Disperses and Re-Aggregates During Division.**

828   (**A**) Heat maps of the RbcS1-Venus signal during the divisions in Figure 1A-C. Times are

829   defined as in Figure 1**.**

830   (**B**) Raw signal from (A) plotted over time by regions of interest, representing the sum through

831   the whole Z-stack in each masked region over time. Times of pyrenoid divisions are highlighted

832   in gray.

833   (**C**) The average signal in the pyrenoid during division is significantly lower than that 15 minutes

834   later (Wilcoxon Matched-Pairs Signed-Ranks Test; $* p \leq 1.50^{-6}$; $n = 31$), shown $\pm$ SEM.

835   (**D**) Timeline of an average cell division with pyrenoid fission. Chloroplast division (magenta),

836   pyrenoid dissolution (cyan), and pyrenoid fission (green) are displayed relative to the moment

837   the chloroplast division furrow passes between the daughter pyrenoids (t=0). Cartoons depicting

838   the aggregation state of the pyrenoid matrix are shown above each stage, with the chloroplast

839   outlined in black, aggregated matrix components shown as filled black circles, and partially

840   dispersed matrix components as speckles.

841   See also Figure S6, and Movies S1-S3.

842 **Figure 7. Simulations of an EPYC1-Rubisco System Reveal an Effect of Binding Site**

843 **Stoichiometry on the Aggregation State.**

844 (**A – C**)**,** Snapshots of simulations with 3 (A), 4 (B), and 5 (C) Rubisco binding sites on EPYC1.

845 "Rubiscos" (blue rectangles) and "EPYC1s" (red polymers) bind when they occupy the same

846 sites in a 2D grid. Snapshots are from simulations with 10 $k_B T$ specific bonds and 0.1 $k_B T$

847 nearest-neighbor non-specific bonds.

848 (**D – F**) Heat maps of the fraction of Rubiscos that are in clusters of >10 Rubiscos connected by

849 EPYC1s with 3 (D), 4 (E), or 5 (F) Rubisco binding sites. The fraction of grid sites occupied by

850 Rubiscos *(x-axis)* is varied from ~10% to ~70%, with an equal fraction of grid sites occupied by

851 EPYC1s. The specific bond energy *(y-axis)* is varied from 0 to 10 $k_B T$, while the nonspecific

852 bond energy is fixed at 0.1 $k_B T$. Red dots indicate the parameters used for the snapshots in (A –

853 C).

854 (**G – I**)**,** Snapshots of off-lattice 3D simulations with 3 (G), 4 (H), and 5 (I) Rubisco binding sites

855 on EPYC1 for Rubisco. "Rubiscos" (blue spheres with 4 binding sites on each end) and

856 "EPYC1s" (red polymers) bind when their binding sites overlap. Inset in (I): zoom-in with one

857 Rubisco and one EPYC1 with 5 binding sites; 4 of the 5 binding sites of the EPYC1 are in

858 specific bonds with the Rubisco. Snapshots are from simulations with 10 $k_B T$ specific bonds and

859 a Lennard-Jones nonspecific interaction with $\epsilon = 0.1\ k_B T$. The molecules occupy ~2% of the

860 total space in these simulations, with equal total numbers of EPYC1 and Rubisco binding sites.

861 (**J**) Fraction of Rubiscos in clusters of >10 Rubiscos for EPYC1s with different numbers of

862 binding sites, in the 2D model. The specific bond energy is 10 $k_B T$ and the nonspecific bond

863 energy is 0.1 $k_B T$.

864   (**K**) The concentration of Rubisco at which clustering begins for systems with different numbers

865   of EPYC1 binding sites in the 2D model. The onset is determined from the curves in (J) (see

866   Figure S7).

867   (**L**) Heat map of the distribution of cluster sizes for different numbers of EPYC1 binding sites in

868   the 2D model. Each column depicts the normalized cluster-size distribution at [Rubisco] = 0.15,

869   with 10 k_BT specific bond energy and 0.1 k_BT nonspecific bond energy.

870   (**M**) Schematic of a possible mechanism by which magic numbers could regulate the formation

871   and dissolution of pyrenoids: EPYC1s with <4 binding sites favor Rubisco clustering in the

872   pyrenoid (left), while EPYC1s with <4 binding sites form stable 2:1 complexes of

873   EPYC1:Rubisco that dissolve into the chloroplast stroma (right).

874   See also Figure S7, Table S1, and Movie S6.

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Supplemental Figure Titles and Legends

879

880 **Figure S1. Fluorescently-Tagged Lines Grow Normally, and Specks Can Appear**

881 **Transiently Even During Fission; Related to Figures 1-3 and 5-6.**

882 (**A** – **B**) Spot tests on cells grown in photoautotrophic conditions that require a functional

883 pyrenoid (A) versus heterotrophic conditions that do not require a functional pyrenoid (B) show

884 that tagging pyrenoid matrix proteins (RbcS1, EPYC1, RCA1) with the fluorescent protein

885 Venus results in wild-type-like growth. In comparison, the *epyc1* mutant, which has perturbed

886 pyrenoid structure (Mackinder *et al.*, 2016), displays a growth defect under conditions that

887 require a functional pyrenoid. TP air cells were imaged ~5 days after spotting; TAP dark cells

888 were imaged ~12 days after spotting.

889 (**C** – **D**) Additional example of pyrenoid fission in an EPYC1-Venus cell, with transient puncta

890 appearing in the stroma. Images are summed through Z, with chlorophyll autofluorescence

891 shown in magenta, and RbcS1-Venus in green; t=0 is the first observation of a gap in chlorophyll

892 between the daughter pyrenoids in the first division shown. Dashed curves represent approximate

893 chloroplast outlines in the mother (white) and daughter (pink) cells.

894 **Figure S2. The Mode and Lineage of Pyrenoid Inheritance were Tracked for RbcS1-Venus**

895 **and EPYC1-Venus Daughter Cells; Related to Figures 1-3 and 6.**

896 (**A – B**) Pyrenoid inheritance was observed in daughter cells resulting from the first division

897 (middle columns) or second division (right columns) of 17 original RbcS1-Venus cells, resulting

898 in 86 daughters (A) and 41 original EPYC1-Venus cells, resulting in 178 daughters (B). Colors

899 correspond to the pattern of pyrenoid inheritance in that cell. As in Figures 1 and 2, pyrenoid

900 fissions are displayed in magenta, and inheritance of an entire previously existing pyrenoid is

901 blue. Cases in which no pyrenoid was inherited and puncta appeared are shown in yellow; cases

902 in which neither a pyrenoid nor puncta were inherited are shown in orange. In some lineages, the

903 mother cell contained two pyrenoids at the start of observation, allowing both daughters to be

904 labeled in blue. Some observed cells only divided once. The observed proportions of these

905 inheritance patterns are displayed in Figure 2. Images from selected divisions in this figure are

906 presented elsewhere as follows: Fig 1A-C, Figure 6A-B, and Movie S1 are RbcS1-Venus

907 original cell 7 divisions 1 and 2; Figure 1D is EPYC1-Venus original cell 9 division 1; Figure 1E

908 is RbcS1-Venus cell 9 division 1; Figure 2A and Movie S2 are RbcS1-Venus cell 14 division 2;

909 Figure 2B is RbcS1-Venus cell 9 division 2; Figure 2C is EPYC1-Venus cell 19 division 2;

910 Figure 2D is EPYC1-Venus cell 40 division 1; Figure 2G-I are RbcS1-Venus cell 15 division 2,

911 RbcS1-Venus cell 10 division 2, and EPYC1-Venus cell 8 division 2, respectively; Figure 3A is

912 RbcS1-Venus cell 12 division 1, B is RbcS1-Venus cell 6 division 1, C is RbcS1-Venus cell 5

913 division 1, D is RbcS1-Venus cell 13 division 1, E is RbcS1-Venus cell 3 division 1, and F is

914 EPYC1-Venus cell 19 division 1; Figure S1C-D are EPYC1-Venus cell 29 division 1; Figure

915 S6A is RbcS1-Venus cell 5 divisions 1 and 2; Movie S3 and Figure S6B are RbcS1-Venus cell 9

916 divisions 1 and 2; Figure S6C is RbcS1-Venus cell 14 divisions 1 and 2, D is RbcS1-Venus cell

917   13 divisions 1 and 2, E is RbcS1-Venus cell 12 divisions 1 and 2, and H is EPYC1-Venus cell 5

918   divisions 1 and 2.

919 **Figure S3. *In situ* Localization of Rubisco Holoenzymes Within Tomograms; Related to**

920 **Figure 4.**

921 (**A**) Tomogram volumes were masked to separate the pyrenoid matrix (yellow), which was

922 template matched for Rubisco particles, from the rest of the volume (blue), which was excluded

923 from the search. 2D projection images of the 3D masks are shown. The volumes contained

924 within the matrix masks are underlined in yellow.

925 (**B**) Following template matching, particles were exhaustively extracted and plotted in

926 histograms by their cross-correlation coefficient (CCC) to the Rubisco template. Clear true-

927 positive peaks with higher CCC values than the false-positive noise were apparent for all

928 tomograms. Each true-positive peak was fit with a Gaussian distribution (red dashed line), and a

929 two standard deviation (2s) cutoff was used to separate particles to be processed further (yellow,

930 ~97.5% of true positives) from those to be discarded (blue). The numbers of particles that were

931 kept are underlined in yellow.

932 (**C**) Following 3D subvolume alignment, the particles were subjected to hierarchical

933 classification to remove contaminating false positives. Between 6.5% and 11.8% of particles

934 were removed per tomogram (blue averages), leaving only clean true-positives (yellow

935 averages). The averages were all filtered to 30 Å resolution for clear comparison. The numbers

936 of particles remaining after classification are underlined in yellow.

937 (**D**) Calculation of the concentration of Rubisco particles within each masked pyrenoid matrix

938 region, adjusted for the ~97.5% sampling of true positives in (B). For comparison to lower-

939 resolution light microscopy measurements, concentrations that do not omit the volume of the

940 pyrenoid tubules were also calculated. Concentrations for the HCP simulated data generated

941 within the same masked matrix volumes (Figure S4E) are also listed.

942 **Figure S4. Assessment of the Rubisco *in situ* Subtomogram Average and Generation of the**

943 **Crystalline and Randomly Packed Simulated Pyrenoid Data; Related to Figure 4.**

944 (**A**) Fourier shell correlation (FSC) resolution estimates for the subtomogram average shown in

945 Figure 4C. Resolution was calculated both by cross-resolution of the full dataset to the crystal

946 structure (Taylor *et al.*, 2001) (blue line, 15.5 Å, 0.3 cutoff) and by randomly splitting the dataset

947 and comparing the consistency of the two half-sets (red line, 16.5 Å, 0.143 cutoff).

948 (**B**) The Rubisco crystal structure (Taylor *et al.*, 2001) fitted into the subtomogram average.

949 (**C**) A difference map between the crystal structure and the subtomogram average. There is only

950 a very small significant difference (green volume: 2.5s variance) near the Rubisco small

951 subunits. Half of the subtomogram average has been cut away to more clearly show the position

952 of the difference density relative to the crystal structure.

953 (**D – I**) Experimental Rubisco positions (D), HCP with 13.9 nm spacing between Rubisco centers

954 (E), Rubisco packing within a Rubisco crystal (F), randomly positioned single Rubisco particles

955 (G), randomly positioned linked pairs of Rubisco with 13.9 ± 1.5 nm between pair centers (H),

956 and a randomly positioned network of linked Rubisco (I), each mapped into the same tomogram

957 volume (corresponding slices are shown for each volume). In (G – I), the rotation symbols with

958 "?°" indicate that the particles have random orientations in addition to their random positions.

959 (**J**) Schematic for the generation of the random linked network in (I), illustrated from left to

960 right. A seed Rubisco template is randomly placed into the matrix volume, and a zone for the

961 potential placement of neighbors (yellow) is defined in a shell occupying the volume that is 5.5-7

962 nm from the seed particle surface. A second particle is randomly placed within the neighbor zone

963 (center position of particle indicated by "X"), and the zone for potential neighbor placement is

964 then updated to occupy a shell of 5.5-7 nm around both particles. The rounds of random neighbor

965   placement and zone updating are iteratively repeated to grow the random linked network. After a

966   maximum of 8 particles have been added to a network, a new seed is randomly placed elsewhere

967   in the matrix volume and another network is formed.

968 **Figure S5. The Bleached Region During FRAP Experiments Extends Vertically Through**

969 **the Pyrenoid Volume, and RCA1-Venus and EPYC1-Venus are also Mobile Within the**

970 **Pyrenoid; Related to Figure 5.**

971 (**A – B**) Serial sections through a fixed RbcS1-Venus pyrenoid before (A) and after (B) a bleach

972 event. Sections are 0.23 μm apart.

973 (**C**) Average total intensity and volume of the Venus signal after bleaching, as a percent of the

974 initial value, in 3D reconstructions of 10 fixed RbcS1-Venus pyrenoids before and after a bleach

975 event. Error bars are standard deviation.

976 (**D**) Average FRAP recovery profiles ± SEM for 28 pyrenoids in live *epyc1* mutants

977 complemented with EPYC1-Venus. The *y*-axis is intra-pyrenoid homogeneity.

978 (**E – F**) Examples of FRAP in an *epyc1* EPYC1-Venus pyrenoid. Cartoons depict the

979 approximate bleached region (dark gray). Image stills from the recovery time-course (E) and

980 corresponding kymograph (F) as shown in Figure 5.

981

982 **Figure S6. Pyrenoid Matrix Components Undergo Relocalization out of and into the**

983 **Pyrenoid During Cell Division; Related to Figures 1, 2, and 6.**

984 (**A** – **E**) Examples of the changing localization of the RbcS1-Venus signal during divisions in

985 five lineages. As in Figure 6A-B, the plots represent the sum of the fluorescence through the

986 whole Z-stack in each masked region over time. The time window during which the pyrenoid is

987 undergoing fission is highlighted in gray; note that the pyrenoids in the second division of (E) do

988 not undergo fission, and thus lack a gray highlight. $t=0$ is the minute in which a chlorophyll gap

989 first appears between daughter pyrenoids in the first division. Underneath each plot, snapshots of

990 the RbcS1-Venus signal at several different points are displayed in two ways: on the top, the

991 RbcS1-Venus signal is false-colored based on intensity, with the approximate chloroplast

992 boundaries outlined in white, and signal from outside the chloroplast masked out. Beneath that,

993 an overexposed version of each image is displayed, with the daughter cells colored to match the

994 mode of pyrenoid inheritance in that cell. The second division in (C) is also shown in Figure 2A;

995 the second division in (E) is also shown in Figure 2B.

996 (**F**) Duration and relative timing of the RbcS1-Venus chloroplast (magenta) and pyrenoid (green)

997 division for the pyrenoid fissions, as in Figure 1G, with the duration and relative timing of

998 pyrenoid dissolution superimposed in blue. Left: 1st divisions; right: 2nd divisions. $t=0$ is the

999 first observation of a gap in chlorophyll between the daughter pyrenoids. We observed only one

1000 instance in which pyrenoid dissolution and fission appeared to be temporally distinct; in this

1001 case, the mother cell contained two pyrenoids (which is rare, but does occur), and one daughter

1002 cell inherited both an entire pyrenoid and a pyrenoid by fission.

1003   (**G**) Average and standard deviation of the durations of chloroplast (magenta) and pyrenoid

1004   (green) fissions, as well as pyrenoid dissolution (blue) for the RbcS1-Venus divisions shown in

1005   (F) ($n = 28$).

1006   (**H**) Example of the changing localization of Venus signal during a series of divisions in EPYC1-

1007   Venus cells, displayed as in (A – E).

1008   **Figure S7. Moves in Monte Carlo Simulations, Determination of Onset of Clustering, and**

1009   **Snapshots of Simulations with Binding of Each EPYC1 Restricted to one end of a Rubisco;**

1010   **Related to Figure 7.**

1011   (**A – I**) Schematics of Rubisco and EPYC1 moves in Monte Carlo simulations.

1012   (**J**) Determination of clustering onsets in Monte Carlo simulations. Data points are from the

1013   simulation data in Figure 7J. Each curve for a fixed number of EPYC1 interacting sites is fitted

1014   with a 4th order polynomial, and the highest zero-crossing is taken as the onset of clustering in

1015   Figure 7H.

1016   (**K**) Fraction of Rubiscos in clusters of >10 Rubiscos for EPYC1s with 3, 4, or 5 binding sites in

1017   the off-lattice 3D simulation. The specific bond energy is 10 k_BT and the Lennard-Jones

1018   nonspecific interaction energy is $\epsilon = 0.1\ k_BT$.

1019   (**L – N**) Snapshots of an EPYC1-Rubisco system with 8 binding sites on Rubisco holoenzymes

1020   for EPYC1 with four binding sites, with the constraint that a given EPYC1 cannot bind to both

1021   ends of one Rubisco. Percentage of occupied grid sites is 20% in each layer.

STAR★ METHODS

CONTACT FOR REAGENT AND RESOURCE SHARING

Please contact the Lead Contact, Martin C. Jonikas (mjonikas@princeton.edu), with any

requests regarding reagents used in this study.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

RbcS1-, RCA1-, epyc1 EPYC1-, and EPYC1-Venus *Chlamydomonas reinhardtii* strain

generation and culture conditions.

All strains expressing fluorescent proteins were generated in Mackinder, *et al.* (2016), as

follows: the DNA encoding the protein of interest (RbcS1, RCA1, or EPYC1) was amplified

from *Chlamydomonas reinhardtii* gDNA and cloned behind the PsaD promoter with a Venus-

3xFLAG on the C-terminus, in a construct containing the *aphVIII* gene for paromomycin

resistance. Vector sequences have been deposited at GenBank under accession numbers

KY550376 (pLM005-RBCS1-Venus), KX077944 (pLM005-EPYC1-Venus), and KY550375

(pLM005-RCA1-Venus). Linearized constructs were transformed into wild-type (cMJ030; also

known as CC-4533 cw15; mating type minus) or *epyc1* (also in the cMJ030 background)

(Mackinder *et al.*, 2016) *Chlamydomonas reinhardtii* by electroporation, which results in random

integration into the nuclear genome (Zhang *et al.*, 2014; Mackinder *et al.*, 2016). Strains

expressing the construct were selected by growth on agar plates containing Tris-Acetate-

Phosphate (TAP) plus paromomycin, and subsequent fluorescence screening for Venus on a

1045   Typhoon Trio fluorescence scanner (GE Healthcare) with excitation at 532 nm and emission at

1046   555/20 nm. Strains containing RbcS1- and EPYC1-Venus were authenticated by Western Blots

1047   in Mackinder *et al.* (2016). The above strains have been deposited at the Chlamydomonas

1048   Resource Center under accession numbers CC-5357 (RbcS1-Venus), CC-5359 (EPYC1-Venus),

1049   CC-5358 (RCA1-Venus), CC-5360 (*epyc1*), and CC-5361 (*epyc1* EPYC1-Venus).

1050   For microscopy, photoheterotrophic pregrowth cultures were inoculated from ~two-

1051   week-old TAP plates into 50 mL liquid TAP media in 250 mL glass Erlenmeyer flasks, and

1052   grown under continuous white light (~40 μmol photons m^{-2} s^{-1}) while shaking at 125 rpm until

1053   cells reached log phase growth (2-5 x 10^6 cells/mL; 3-4 days after inoculation). Photoautotrophic

1054   cultures were inoculated by centrifuging the pregrowth cultures at 1000 g for 5 minutes at room

1055   temperature and then resuspending the pellet in 50 mL liquid minimal Tris-Phosphate (TP)

1056   media (Kropat *et al.*, 2012). Photoautotrophic cultures were grown in 50 mL liquid TP in glass

1057   tubes bubbled with air (~0.04% CO_2) under continuous light (150 μmol photons m^{-2} s^{-1} red and

1058   blue LumiGrow LumiBar LED lights). After several rounds of dilution and growth in TP, cells

1059   were imaged when they reached a density of ~1-2x10^6 cells/mL.

1060

1061   **Vitrification, cryo-FIB, cryo-ET, and tomogram reconstruction**

1062   *Chlamydomonas mat3-4* cells (Umen and Goodenough, 2001), acquired from the

1063   Chlamydomonas Resource Center (Univ. of Minnesota, CC-3994, mt+), were used because they

1064   vitrify better than wild-type cells due to their smaller size (~5 μm) but have normal pyrenoid

1065   morphology and a functional carbon concentrating mechanism, shown by growth in low-CO_2

1066   conditions (Umen and Goodenough, 2001), which inhibit carbon concentrating-deficient strains

1067   such as the *epyc1* mutant (Mackinder *et al.*, 2016). Cells were grown in TAP media with

1068   constant light (~90 μmol photons m^{-2} s^{-1}) and aeration with normal atmosphere until mid-log

1069   phase.

1070

1071   **METHOD DETAILS**

1072
1073   **Division microscopy & analysis**

1074   *Image acquisition*

1075   Strains were grown and prepared for microscopy as described above. Cells were imaged

1076   at room temperature every minute for 8-16 hours on a Leica TCS SP8 laser scanning confocal

1077   microscope in resonant scanning mode with LASX software and a 63X NA 1.4 oil objective,

1078   while illuminated with a 626 nm red LED circle affixed to the condenser (LED Angel Eye

1079   Headlight Accent Light Kit; SuperBrightLeds.com, AE80-RGB12). Venus fluorescence was

1080   imaged by exciting with a white light laser at 488 nm and collecting emission from 499-551 nm

1081   on a HyD SMD hybrid detector (Leica) with lifetime gate filter (1-6 ns) to reduce background

1082   due to chlorophyll autofluorescence. Chlorophyll autofluorescence was imaged simultaneously

1083   with the same 488 nm excitation, and emission was collected from 680-750 nm on a PMT

1084   (Leica). Images were collected with 8-line averaging, 0.3 μm steps through the entire cell

1085   volume, and adaptive focus control. Displayed division analysis data represent images collected

1086   over at least two independent biological replicates for each strain. Sample sizes were chosen

1087   based on similar published studies (Conduit *et al.*, 2010; Chen *et al.*, 2013; Weber and

1088   Brangwynne, 2015; Eichel *et al.*, 2016; Feric *et al.*, 2016).

1089   *Image analysis*

1090   4D TIFFs were exported to FIJI as virtual stacks and dividing cells were manually

1091   marked in MaxZ projections over time. The pyrenoid inheritance pattern and timing of division

1092   was manually noted for each dividing cell in 3D in Imaris (Bitplane). If a cell exhibited more

1093   than one mode of inheritance (for instance, both pyrenoid fission and puncta), then fission took

1094   precedence for categorization. The duration of chloroplast division and pyrenoid fission (as

1095   defined in the text) were measured for all observed RbcS1-Venus pyrenoid fission events ($n = 14$

1096   first divisions and 14 second divisions) and randomly-selected EPYC1-Venus fission events ($n =$

1097   22 first divisions and 25 second divisions). The extent of pyrenoid dissolution and increase in

1098   stromal Venus signal was measured in FIJI as follows: For each dividing cell, a SumZ projection

1099   was created through the whole cell. Then, any saturated pixels were masked out, and intensity-

1100   based masks were created from SumZ projections of the chlorophyll and Venus channels to mask

1101   out any signal in the Venus channel from outside the chloroplast or outside the pyrenoid,

1102   respectively. Finally, for each masked region of each cell, the total intensity (sum of the

1103   unmasked pixels in the entire image; RawIntDen) was measured at each time point and exported

1104   to Excel. The Wilcoxon Matched-Pairs Signed-Ranks Test was used to quantify the difference in

1105   the average pyrenoid signal between maximal dimming and 15 minutes beforehand in 31

1106   divisions (with the error bars denoting SEM) as described below.

1107

1108   **Vitrification, cryo-FIB, cryo-ET, and tomogram reconstruction**

1109   Using a Vitrobot Mark 4 (FEI), cells were blotted onto carbon-coated EM grids

1110   (Quantifoil Micro Tools; 4 μL cell culture per grid) and plunge-frozen into a liquid

1111   ethane/propane mixture. Cryo-FIB milling was performed on a dual-beam Quanta3D FIB/SEM

1112   microscope (FEI) by scanning the cells with Gallium ions, as previously described (Schaffer *et*

1113   *al.*, 2015, 2017). Thinned samples were transferred to a Titan Krios 300 kV transmission electron

1114   microscope (FEI) equipped with a 968 Quantum post-column energy filter (Gatan), and imaged

1115 with a K2 Summit direct detector (Gatan) operated in movie mode at 12-17 frames per second.

1116 Using SerialEM software (Mastronarde, 2005), single-axis tilt-series were recorded from -60° to

1117 +60° (in two halves separated at 0°) at 2° increments, with an object pixel size of 3.42 Å, a target

1118 defocus of -4 or -5 mm, and a cumulative electron dose of ~100 electrons/$Å^2$. Image frames were

1119 aligned using in-house developed software to correct for beam-induced motion. Using IMOD

1120 software (Kremer *et al.*, 1996), tilt-series were aligned by patch-tracking and reconstructed by

1121 weighted back projection. The biggest gain in image quality compared to the previous study

1122 (Engel *et al.*, 2015) was the result of using a direct electron detector instead of a CCD camera.

1123

1124 **Localization of Rubisco within tomograms and subtomogram averaging**

1125   *Tomogram masking and template matching*

1126   Tomograms were binned to a pixel size of 13.68 Å and subjected to template matching

1127 using the PyTom software (Hrabe *et al.*, 2012). The template was generated from the X-ray

1128 crystallography structure of *Chlamydomonas* Rubisco (Taylor *et al.*, 2001) (PDB entry: 1GK8)

1129 by lowpass filtering the electron density map to a resolution of 33 Å, a value determined by the

1130 approximate first zero of the contrast transfer function in the tomograms (Förster *et al.*, 2010).

1131 Tomogram masks were manually segmented in Amira software (FEI), restricting the search area

1132 to only the pyrenoid matrix (Figure S3A). For each tomogram, the template matching cross-

1133 correlation function was filtered by the tomogram mask, and the remaining cross-correlation

1134 peaks were exhaustively extracted with a mask radius of 9.5 nm. Histograms of cross-correlation

1135 scores from the extracted peaks revealed Gaussian distributions of true-positive particles at the

1136 high values. A cutoff was set to two standard deviations towards the low-valued tail of each

1137 Gaussian, and all particles with scores below this cutoff were discarded (Figure S3B).

1138　　　　　*Subtomogram averaging and classification*

1139　　　　　Subvolumes corresponding to the extracted peaks were binned to a pixel size of 6.84 Å

1140　　and aligned in PyTom using a real-space expectation maximization method that implements

1141　　gold-standard alignment (Hrabe *et al.*, 2012). This alignment procedure did not impose D_4

1142　　symmetry. The number of iterations was set to 10, the initial angular increment to 3°, and the

1143　　angular shells to 3. Next, subtomogram classification was performed using constrained principal

1144　　component analysis (CPCA) (Förster *et al.*, 2008). PyTom was used to calculate similarity

1145　　matrices and for hierarchical clustering, while CPCA and k-means clustering were performed in

1146　　Matlab (MathWorks). Subtomograms were lowpass filtered to 38 Å, 5 eigenvectors were used,

1147　　and the number of classes was set between 99 and 165, depending on the number of particles in

1148　　each tomogram. Classes were then merged by hierarchical clustering, using constrained cross-

1149　　correlation as distance measure, yielding a "positive" and "negative" class for each tomogram

1150　　(Figure S3C).

1151　　　　　*High-resolution subtomogram averaging*

1152　　　　　Unbinned subtomogram volumes (3.42 Å pixel size) from "tomogram 1" corresponding

1153　　to the 30,000 highest template matching cross-correlation scores in the "positive" class were

1154　　used to generate the average shown in Figure 4C. The subvolumes were corrected for the

1155　　contrast transfer function by phase-flipping in IMOD, then aligned with imposed D_4 symmetry

1156　　using gold-standard alignment in RELION (Bharat and Scheres, 2016). This procedure was

1157　　restricted to a local angular search, using initial angle and offset sampling rates of 3.7° and 0.34

1158　　nm, respectively, while the maximal offset value was set to 1.7 nm. The initial reference was a

1159　　subtomogram average filtered to 4 nm resolution. In the final average, the B-factor variable was

1160   set to 4 Å^2 for each 1 electron/Å^2, and the cumulative electron dose of the subtomograms was

1161   restricted by only using the central 60° of the tilt-series (-30° to +30°).

1162

1163   **Local density analysis and generation of simulated data**

1164   *Measurement of radial local Rubisco density*

1165   Pyrenoid matrix organization was quantified by Radial Local Density (RLD) estimation,

1166   defined as:

$$\lambda(r) = \frac{\sum_{i=0}^{n} C\left(S_h(\vec{x}_{p_i}, r)\right)}{V\left(S_h(\vec{x}_{p_i}, r)\right)}$$

1167   The coordinates of the reference particle are represented by the vector $\vec{x}_{p_i}$. Function C counts the

1168   number of particles contained within the S_h subvolume, which is a radial shell subset of the S_P

1169   total masked matrix volume ($S_h \subset S_P$). Function V computes the volume of S_h. Since all

1170   particles in S_P are used, then $\{\vec{x}_{p_0}, \dots, \vec{x}_{p_n}\} \in S_P$. The definition of the local subvolume S_h

1171   associated to a particle $\vec{x}_{p_i}$ and a radius r is:

$$S_h(\vec{x}_{p_i}, r) = \left\{\vec{x} \in S_P | \{d(\vec{x}, \vec{x}_{p_i}) \geq (r - \Delta r/2)\} \wedge \{d(\vec{x}, \vec{x}_{p_i}) \leq (r + \Delta r/2)\}\right\}$$

1172   where d is the Euclidean distance function. In an unbounded space, S_h would correspond with a

1173   spherical shell centered at $\vec{x}_{p_i}$, with radius r (distance to reference particle p_i) and a shell

1174   thickness of $\Delta r > 0$. However, because the pyrenoid matrix volume S_P is actually a finite

1175   irregularly bounded space, $V(S_h)$ cannot be accurately estimated by analytical formulas. Thus,

1176   we used a numerical estimator analogous to Wiegand and Moloney (2004), but adapted for 3D

1177   volumes instead of 2D areas. This approach restricts S_h to the masked pyrenoid matrix volume,

1178   eliminating edge effects that would have arisen from the inclusion of the membrane tubules and

1179   areas outside of the pyrenoid. For all RLD measurements in Figure 4 (experimental and

1180   simulated data in panels D, F and H, experimental data in panel G), we set $\Delta r = 1.4$ nm as a

1181   compromise between precision and graph smoothness.

1182   As the global pyrenoid matrix Rubisco density, ρ, is slightly different for each pyrenoid

1183   (Figure 4D, Figure S3D), we normalized each RLD by the global density so that RLD shape

1184   could be directly compared between multiple tomograms: $\lambda' = \lambda/\rho$.

1185   *Generation of simulated tomogram volumes*

1186   For direct comparison to the experimental data, all of the simulated tomograms were

1187   created by placing Rubisco particles into the same pyrenoid matrix volumes as those in the real

1188   tomograms (Figures S3A, S4D-I), as defined by the manual masking step described above.

1189   *Crystal*

1190   This data was generated by propagating the unit cell of crystalized *Chlamydomonas*

1191   Rubisco (Taylor *et al.*, 2001) throughout the matrix volumes. Despite their "noisy" appearance,

1192   the crystalline profiles have less variance than the experimental data – their 99% confidence

1193   intervals are almost too small to see in Fig. 4F. Two factors cause the simulated data to look

1194   "noisy": 1) Unlike the heterogeneous organization of the experimental data, the crystalline

1195   packing is exact, leading to dramatic peaks. 2) RLD is sampled with a spherical shell (of 1.4 nm

1196   width), whereas the crystalline organization does not propagate in a spherical pattern. This

1197   mismatch between the spherical RLD measurement and the crystalline packing causes the

1198   "noisy" jagged appearance of the plots.

1199   *Hexagonal close packing*

1200   Within the matrix volumes, an HCP lattice was generated with 13.9 nm between the

1201   centers of all nearest neighbors, a distance based on the preferred nearest neighbor spacing

1202   measured in Figure 4E.

59

1203 *Random singles*

1204 A binary Rubisco template was generated by thresholding our Rubisco subtomogram

1205 average (Figure 4C) to allow the minimum near neighbor distance found in our experimental

1206 data (Figure 4E). Using Monte Carlo simulations, these templates were sequentially mapped into

1207 the matrix volumes, using random positions and orientations but forbidding overlap between

1208 templates. The procedure was stopped upon reaching the same number of Rubisco particles as

1209 the experimental data.

1210 *Random linked pairs*

1211 Pairs of two Rubisco templates, each with random orientation, were placed together with

1212 an inter-center distance defined by the experimental data's distribution (mean = 13.9 nm,

1213 standard deviation = 1.5 nm; Figure 4E). Pairs were sequentially mapped into the matrix

1214 volumes, using random positions and orientations, while avoiding overlap with previously

1215 inserted particles. These steps were repeated until the same number of particles as the

1216 experimental data was reached.

1217 *Random linked network*

1218 First, a seed Rubisco template was randomly placed into the matrix volume, while

1219 avoiding overlap with already inserted particles. Second, a zone for the potential placement of

1220 neighbors was defined as a shell around the seed template in a range of 5.5-7 nm from the seed

1221 particle surface (Figure S4J). Third, a neighbor Rubisco particle was randomly placed with its

1222 center inside the zone. Fourth, the zone for potential neighbor placement was updated to

1223 encompass a range of 5.5-7 nm around both particles. The rounds of random neighbor placement

1224 and zone updating were sequentially repeated until a maximum of 8 networked particles were

1225 placed. After 8 particles were placed or the network failed to place a particle due to lack of

1226   space, a new seed was randomly created within the matrix volume and the procedure was

1227   repeated. When two networks encountered each other, they merged their neighbor placement

1228   zones. The procedure was stopped when the same number of Rubisco particles as the

1229   experimental data was reached.

1230   *Fitting Lennard-Jones fluid models to the experimental data*

1231   RLD is a robust estimator of the Radial Distribution Function (RDF) for proteins in a

1232   finite, bounded, and irregularly shaped space like the pyrenoid matrix. Thus, analytical models

1233   for RDF can also be applied to RLD results. We fit our experimental data with the Lennard-

1234   Jones fluid analytical model proposed in Morsali *et al.* (2005). This RDF model is constrained by

1235   65 constants that Morsali *et al.* (2005) calculated from 353 molecular dynamics simulations of

1236   argon atoms interacting via the Lennard-Jones potential under a range of state variables. We set

1237   the Lennard-Jones length parameter (σ = 13.9 nm) to scale the x-axis to reduced r^*, and

1238   optimized the variables for reduced temperature ($T^* = 17.296$) and reduced density ($\rho^* = 1.143$)

1239   by minimizing the mean squared error between the model and our data with the Python SciPy

1240   library's global optimization algorithm described in Wales and Doye (1997). With these values

1241   for σ, T^* and ρ^*, the Morsali RDF model fit our data well (root mean squared error: 0.0935,

1242   mean squared error: 0.0087, maximum squared error: 0.5308, standard deviation of squared

1243   error: 0.05819). Our optimized value for T^* fell outside the range of molecular dynamics data

1244   that Morsali *et al.* (2005) used to develop their model; therefore, we also determined the

1245   Lennard-Jones RDF for these conditions using a molecular dynamics simulation (Plimpton,

1246   1995).

1247   The simulation was performed using the LAMMPS Molecular Dynamics Simulator

1248   (Plimpton, 1995). The simulation space was a 10x10x10 box with hard-wall boundary conditions

1249   and $N = 1000$ particles. We employed a Lennard-Jones interaction between particles, defined by:

1250 $$V_{LJ} = \epsilon \left[\left(\frac{\sigma}{r} \right)^{12} - \left(\frac{\sigma}{r} \right)^{6} \right].$$

1251   We set the interaction strength at $\epsilon = 1$, and the Lennard-Jones length scale σ was chosen to

1252   reproduce the reduced density via $\rho^* = N\sigma^3/V$, where V is the system volume. The reduced

1253   density ($\rho^* = 1.143$) and reduced temperature ($T^* = 17.296$) were set to the values determined by

1254   the analytical fit of the experimental RLD to the Lennard-Jones fluid RDF formula from Morsali

1255   *et al.* (2005) as described above. To avoid boundary artifacts, the RDF was computed from the

1256   particle-to-particle distances around a set of reference particles occupying a small 2x2x2 box at

1257   the center of the full simulation volume. To compare the simulated RDF with the experimental

1258   RLD curve, we set the vertical scale by matching the far distance amplitude, and then set the

1259   horizontal scale by a least-squares fit to the experimental data.

1260   There is an important geometrical difference between the Lennard-Jones RDF models

1261   and our experimental measurements of the pyrenoid's RLD. Lennard-Jones models consider

1262   spherical particles [such as argon (Morsali *et al.*, 2005)] that always have one minimum-energy

1263   distance between particle centers (r_m). However, Rubisco particles are not spherical, and have a

1264   minimum diameter of ~10 nm and a maximum diameter of ~13 nm. Thus, unlike spherical

1265   particles, a single mimimum-energy distance between the surfaces of neighboring Rubiscos (as

1266   shown in our linked-network model, Figure S4J) yields a range of minimum-energy distances

1267   between particle centers (a distribution of r_m values) instead of a single, discrete distance. This

1268   may explain why our experimental data had a broader first peak than that observed in Lennard-

1269   Jones models.

1270   The Lennard-Jones potential describes the attractive and repulsive forces between small

1271   molecules (our models used argon), the balance of which results in a single preferred distance

1272   between neighbors. Rubisco particles exist on a much larger size scale than argon atoms, and

1273   thus are subject to different molecular forces such as protein interactions. However, we reasoned

1274   that this simple Lennard-Jones fluid might nonetheless serve as an informative analogy for liquid

1275   organization. Interestingly, the Lennard-Jones distribution appears to be robust to changes in

1276   scale, matching well not only to ~12 nm pyrenoid Rubisco and 120 nm colloidal particles (Gu *et*

1277   *al.*, 2010), but also to 4 μm colloidal particles (Thorneywork *et al.*, 2014). Due to differences in

1278   the underlying molecular forces, the analogy between the pyrenoid and a Lennard-Jones fluid is

1279   limited to the interpretation that the pyrenoid matrix may be liquid; the analogy is not intended

1280   for the application of other Lennard-Jones descriptors to properties of the pyrenoid.

1281

1282   **Spot tests**

1283   Pre-growth cultures were inoculated in TAP as described above. After 4 days, 50 mL of

1284   each culture were washed of acetate twice by centrifuging for 5 minutes at 2000 g and

1285   resuspending the pellet in 20 mL TP. After washing, cells were counted three times (Countess II

1286   Automated Cell Counter; ThermoFisher Scientific) and diluted serially to contain 10^4, 10^3, 10^2,

1287   or 10 cells per 15 μL. 15 uL of each strain in each concentration were spotted in replicate onto

1288   TAP and TP plates and left to dry in the dark for one hour. TAP plates were then wrapped in

1289   Parafilm and aluminum foil and kept at room temperature in the dark for 12 days before imaging;

1290   TP plates were incubated in custom containers with filtered air flow (~0.04% CO_2) with 40 hours

1291   of acclimation at 50 μmol photons m^{-2} s^{-1} red and blue LED light, and then 3 days in ~100 μmol

1292   photons m^{-2} s^{-1} before imaging. Representative samples out of 4 total replicates (2 biological

1293   replicates, each with two technical replicates) per condition are shown.

1294

1295   **Fluorescence Recovery After Photobleaching microscopy & analysis**

1296   *Slide preparation*

1297   For live cell imaging, 300 μL of photoautotrophic culture at ~2x10^6 cell/mL were plated

1298   onto poly-L-lysine-coated 8 well μ-slides (ibidi, 80824) and allowed to settle for ~5 min. The

1299   supernatant was removed, and the remaining cells were coated with 300 μL of TP in 1.5%

1300   UltraPure Low Melting Point Agarose (Invitrogen) at ~40°C and allowed to cool for ~20

1301   minutes. For fixed cell imaging, cells were fixed in 10% glutaraldehyde as follows: ~70 x 10^6

1302   cells were pelleted by centrifugation for 10 minutes at 1000 g at room temperature, resuspended

1303   in 6 mL of 10 mM HEPES-KOH (pH 7) at 4°C, and transferred to a cold 20 mL glass beaker. 1.5

1304   mL of 10% glutaraldehyde solution (300 uL of 50% glutaraldehyde in 1.2 mL of 10 mM

1305   HEPES-KOH, pH 7) was added in successive 100 uL drops every five seconds while swirling

1306   vigorously. The mixture was incubated on ice and agitated every 10 minutes for one hour, after

1307   which the fixed cells were centrifuged (1000 g, 5 minutes, 4°C), resuspended in 6 mL cold 10

1308   mM HEPES-KOH (pH 7), centrifuged again, and resuspended in 1 mL of 10 mM HEPES-KOH

1309   (pH 7). 300 μL were plated for microscopy, as described above.

1310   *FRAP image acquisition*

1311   Pyrenoids were imaged at mid-plane at room temperature on a spinning disk confocal

1312   microscope (Leica DMI6000B custom-adapted with a Yokogawa CSU-X1 spinning disk head; a

1313   Photometrics Evolve 512 camera; and Intelligent Imaging Innovations SlideBook software,

1314   Vector FRAP, LaserStack, and mSAC spherical aberration systems), with a 100X oil objective

1315 (HCX Pl APO, 1.4-0.7 NA; Leica). Venus fluorescence was imaged by excitation at 514 nm and

1316 emission with a YFP 540/15 filter (Semrock) and 445/514/561 nm Yokogawa dichroic

1317 beamsplitter (Semrock) under the following conditions: 100 ms exposure every 3 seconds for

1318 100 images, with <2 mW laser power (measured at the fiber), and a camera gain of 3 and EM

1319 gain of 300, with Adaptive Focus Control active before every acquisition. FRAP experiments

1320 were conducted one by one on individual pyrenoids centered in the field of view and far from

1321 previously bleached pyrenoids on the slide. Vector was used to direct the 514 nm laser at full

1322 power for photobleaching (~18 mW, measured at the Vector fiber), which took place between

1323 the third and fourth image captures. on a sub-resolution region of interest positioned at the edge

1324 of each selected pyrenoid; this bleached ~⅓-½ of the cross-section of the pyrenoid. For the

1325 comparative recoveries of RbcS1-, RCA1-, and EPYC1-Venus graphed in Figure 5, the

1326 photobleaching event consisted of one repetition of a 4 ms exposure on a 2x2 pixel region of

1327 interest. Due to alterations in the light path of the microscope, subsequent acquisition and bleach

1328 conditions were altered to achieve the same cross-sectional proportion of bleaching: For the

1329 images of live and fixed RbcS1-Venus FRAP pyrenoids shown in Figure 5, the photobleaching

1330 event consisted of 2 repetitions of a 10 ms exposure directed to a 4x4 pixel region of interest; for

1331 *epyc1* EPYC1-Venus FRAP, 1 repetition of a 10 ms exposure on a 2x2 pixel region of interest

1332 was used. Displayed results are from independent experiments conducted at least three (live

1333 RbcS1-, RCA1-, and EPYC1-Venus), two (fixed RbcS1-Venus), or one (*epyc1* EPYC1-Venus)

1334 times. Sample sizes were chosen based on similar published studies (Conduit *et al.*, 2015;

1335 Klammt *et al.*, 2015; Eichel *et al.*, 2016).

1336   *FRAP quantitative image analysis*

1337   FRAP images were analyzed in FIJI software (Schindelin *et al.*, 2012). The StackReg

1338   translation registration plug-in (Thévenaz and Ruttimann, 1998) was used to align image sets that

1339   shifted in XY during imaging. For each bleached pyrenoid that was analyzed, a kymograph was

1340   generated using the "Reslice" function on a 3-pixel-wide, 3.5 μm-long rectangle that spanned the

1341   bleached and unbleached regions (shown in Figure 5), avoiding any saturated pixels. To plot the

1342   fluorescence recoveries, the signal along 3-pixel-wide rectangles over the bleached and

1343   unbleached regions of each kymograph were measured, respectively; these signals were exported

1344   to Excel and compared to calculate fluorescence recovery, shown as "intra-pyrenoid

1345   homogeneity" over time for each bleached pyrenoid, as follows: for each time point, the signal

1346   from the bleached region of the kymograph was divided by that from the unbleached region.

1347   The signal ratio at the fourth time point ($t = 0$; immediately post-bleach) was defined as y

1348 $= 0$ for each pyrenoid by subtraction. Each recovery plot was then normalized to the average of

1349   the ratios of the three pre-bleach time points, which was defined as $y = 1$. This normalized

1350   recovery was averaged over the stated number of pyrenoids and displayed with the standard error

1351   of the mean for each strain.

1352   Thus, it is important to note that our FRAP curves represent homogeneity over the

1353   bleached and unbleached regions, correcting for signal loss due to repeated measurements. In our

1354   plots, therefore, a return to $y = 1$ is not a return to the initial signal intensity, but a return to the

1355   initial signal homogeneity. This is different from how FRAP recovery is often plotted, and

1356   results in seemingly "higher" recoveries than, for example, those in Shin *et al.* (2016). However,

1357   our FRAP images and kymographs show that the final signal intensities are less than the initial

1358   intensities, but that the signal disparity between the bleached and unbleached regions relaxes,

1359   and our FRAP kymographs resemble those of other liquid-like organelles (Kroschwald *et al.*,

1360   2015; Patel *et al.*, 2015).

1361   Additionally, plotting "homogeneity" allows us to control for potential variations in the

1362   proportion of the pyrenoid that was bleached in each event. Because the fluorescence recovery in

1363   the pyrenoid cannot be said to draw from an infinite pool, variation in the ratio of the bleached to

1364   unbleached pools could produce artifacts in the observed final recovery.

1365   To calculate the fraction of the total pyrenoid volume that was bleached, Z-stacks of

1366   glutaraldehyde-fixed RbcS1-Venus pyrenoids were captured before and after a bleaching event;

1367   in live cells, fluorescence recovered too quickly to section through the entire pyrenoid. 23 planes

1368   were imaged with a 0.23-µm step size (spanning 5.06 µm) before and after bleaching, and pre-

1369   and post-bleach 3D volumes were reconstructed for 10 pyrenoids in Imaris (Bitplane), using the

1370   same intensity thresholds for pre- and post-bleach volumes. The total fluorescence intensity and

1371   volume of each reconstructed pre- and post-bleach pyrenoid was exported and analyzed in Excel.

1372

1373   **"Magic number" modeling**

1374   *Model parameters*

1375   Simulations were performed using a square grid system of 50x50 sites with periodic

1376   boundary condition. In the model, each Rubisco is represented as a 4 by 2 rectangle, and each

1377   EPYC1 occupies several connected (nearest neighbor) grid sites. Rubisco and EPYC1 are self-

1378   avoiding. However, each grid site can be simultaneously occupied by EPYC1 and Rubisco, and

1379   if so, the two are considered to form a specific bond. This scheme allows for stoichiometric

1380   bonding between EPYC1s and Rubiscos without the artifacts due to crowding that would occur if

1381   the two were prevented from occupying the same sites. The simulations also include a weak non-

1382 specific attractive interaction between all occupied nearest-neighbor sites, EPYC1-EPYC1,

1383 EPYC1-Rubisco, and Rubisco-Rubisco.

1384   We performed Markov-Chain Monte Carlo simulations using the Metropolis algorithm

1385 (Metropolis *et al.*, 1953). Briefly, in each simulation step we randomly propose a move of the

1386 EPYC1-Rubisco configuration. The move is always accepted if it reduces system energy, and

1387 accepted with probability $e^{-(E_f-E_i)/k_BT}$, where E_f and E_i are the final and initial energies, if the

1388 move increases system energy. Three categories of moves are proposed: single-EPYC1 moves,

1389 single-Rubisco moves and EPYC1-Rubisco joint moves (Figure S7). Single-EPYC1 moves are

1390 standard lattice-polymer local moves: the end-point move, the corner move, and the reptation

1391 move. Single-Rubisco moves consist of one-step translations in the four cardinal directions and a

1392 90-degree rotation around the Rubisco's center. In the regime of strong specific bonds, EPYC1s

1393 and Rubiscos are typically held together by multiple specific bonds, which leads to dynamical

1394 freezing. To enable the system to better explore configuration space, we include EPYC1-Rubisco

1395 joint moves such that connected clusters of EPYC1s and Rubiscos move together, without

1396 breaking any specific bonds. The joint moves consist of translating a connected cluster of

1397 EPYC1s and Rubiscos together or rotating the whole cluster by 90-degrees around any point. To

1398 obtain thermalized ensembles, we follow a simulated two-step procedure: we keep k_BT constant

1399 and gradually increase bond strength. We first increase the non-specific bond from 0 to 0.1 k_BT

1400 in 0.005 k_BT increments, keeping the specific bond energy at 0 k_BT. Then the specific bond

1401 energy is increased from 0 to 11 k_BT in 0.04 k_BT increments, while the non-specific bond energy

1402 is kept at 0.1 k_BT. Each step of annealing is simulated with 50,000-150,000 Monte-Carlo steps

1403 and results are averaged over 20-100 of the resulting thermalized snapshots.

1404   *Analysis of clustering in the 2D simulations*

1405   To assess the extent of clustering of Rubiscos, we consider a cluster to be a group of

1406   Rubiscos that are connected by EPYC1s via specific bonds. To quantify the fraction of Rubiscos

1407   in large clusters, we employ a cluster-size cutoff of 10+ Rubiscos (Figure 7D-F). This avoids

1408   ambiguities due to smaller clusters that form independent units with all specific bonds satisfied

1409   (*e.g.*, 3 Rubiscos with 8 EPYC1s each of 3 binding sites). To determine the onset of clustering,

1410   we fit the fraction of Rubiscos in large clusters with a degree 4 spline (Figure S7); for the case of

1411   EPYC1s with 8 binding sites, because of the delayed onset of clustering we only use the data for

1412   concentrations > 40% for the fit.

1413   *Three-dimensional off-lattice model*

1414   Molecular dynamics simulations were performed using the LAMMPS Molecular

1415   Dynamics Simulator (Plimton *et al.*, 1995). We simulate a cubic box of 120 nm in each

1416   dimension, with periodic boundary conditions. In the simulation, particles representing Rubisco

1417   and EPYC1 interact both non-specifically and via specific EPYC1-Rubisco bonds. Particles

1418   denoted by A and B interact non-specifically with each other through the Lennard-Jones

1419   potential with a cutoff

$$V(r) = 4\epsilon_{AB}\left[\left(\frac{\sigma_{AB}}{r}\right)^{12} - \left(\frac{\sigma_{AB}}{r}\right)^{6}\right] - 4\epsilon\left[\left(\frac{\sigma_{AB}}{r_c}\right)^{12} - \left(\frac{\sigma_{AB}}{r_c}\right)^{6}\right], r < r_c$$

1420   where $\sigma_{AB} = \sigma_A + \sigma_B$ is the sum of the effective radii of the two particles, ϵ_{AB} is the interaction

1421   strength between the two particles, and r_c is the cutoff of the interaction range.

1422   Each Rubisco in the simulation is represented by a sphere of radius $R_R = 5$ nm, which is

1423   the radius of gyration of Rubisco (Keown, *et al.,* 2013). Since the specific Rubisco binding sites

1424   for EPYC1 are not yet known, we employ a simple model in which each Rubisco has 4 binding

1425   sites, forming a rigid square of edge length 3.4 nm, on each of its two ends. The radius of each

1426   binding site is 0.9 nm and the center of the binding site is 0.9 nm away from the Rubisco sphere

1427 (i.e., 5.9 nm from the center of the Rubisco sphere). Each EPYC1 is represented by 3, 4, or 5

1428 connected binding sites: the binding sites are spheres of radius $R_E = 0.9$ nm, which is the radius

1429 of a compact region of 18 amino acids representing the repeat region of EPYC1. We model the

1430 unstructured chain of 34 amino acids separating these repeats as harmonic springs with zero rest

1431 length and stiffness 0.24 $k_B T/\text{nm}^2$, reflecting the entropic elasticity of a worm-like polymer chain

1432 consisting of 34 units of size 0.35 nm (the approximate size of an amino acid) with a persistence

1433 length of 0.5 nm (a rough consensus for polypeptide chains [Hofmann *et al.*, 2012, Cheng *et al.*,

1434 2010]).

1435 We set the non-specific Lennard-Jones interactions between two EPYC1 binding sites,

1436 between two Rubisco cores, and between one EPYC1 binding site and one Rubisco core to be

1437 the Lennard-Jones potential with a cutoff with interaction energy $\epsilon = 0.1\ k_B T$ and σ the sum of

1438 the radii of the two interacting particles. We set $r_c = 1.4\sigma$ as a cutoff so that the system does not

1439 include long-range interactions.

1440 In addition to the non-specific interaction, we model the attractive specific interaction

1441 between Rubisco and EPYC1 binding sites as a soft potential

$$V(r) = \epsilon_b \left(1 + \cos\left(\frac{\pi r}{r_c}\right) \right), r < r_c \quad \#(1)$$

1442 with binding energy $\epsilon_b = -10\ k_B T$, r the distance between binding site centers, and a cutoff

1443 distance $r_c = 0.45$nm. The minimum of energy for this interaction corresponds to fully

1444 overlapping binding sites. The one-to-one character of EPYC1-Rubisco specific bonds is

1445 guaranteed by the Lennard-Jones repulsion between EPYC1 binding sites, which prevents them

1446 from binding to the same Rubisco binding site, and by an additional soft repulsion (with the

1447 same functional form as Equation (1) and $\epsilon = +50\ k_B T$) between two Rubisco binding sites,

1448 which prevents them from binding to the same EPYC1 binding site. There is no Lennard-Jones

1449   interaction between a Rubisco binding site and an EPYC1 binding site, in order to allow them to

1450   overlap and form a specific bond.

1451   We performed molecular dynamics simulations with Langevin dynamics in the NVT

1452   ensemble. For convenience, we attributed equal mobilities to all particles, chose a simulation unit

1453   length of 1 nm and a time step of 0.008 in LJ units. Each simulation consisted of a total of

1454   150,000,000 time steps. The first 100,000,000 steps were used to let the system reach thermal

1455   equilibrium and a snapshot was taken every 10,000 steps after that for clustering analysis.

1456   *Analysis of clustering in the 3D simulations*

1457   To identify specific bonds between EPYC1 and Rubisco binding sites, we first computed

1458   the distance distribution histogram between all EPYC1 and Rubisco binding sites, and found a

1459   clear gap around 0.5 nm. Below this gap, the EPYC1 and Rubisco binding sites form a specific

1460   bond; above this gap, the binding sites are farther away from each other than the binding site

1461   diameter, and thus do not form a specific bond. As in the 2D case, for each snapshot we

1462   tabulated the Rubiscos connected by EPYC1s through specific bonds and plotted the fraction of

1463   Rubiscos in clusters with 10+ Rubiscos (Figure S10K).

1464   *Robustness of the magic-number effect*

1465   To confirm that the magic-number effect is robust with respect to our choice of

1466   interaction parameters, we performed additional 3D simulations in which we set the EPYC1 and

1467   Rubisco binding-site radii to be the same, and equal to either 0.8 nm, 1 nm, or 1.25 nm (i.e.

1468   factor of two volume changes), with the distance between the center of the Rubisco core and the

1469   Rubisco binding site as 5.8 nm, 6 nm, or 6.25 nm, correspondingly. We also varied the non-

1470   specific Lennard-Jones interaction energy and the EPYC1 inter-binding-site spring stiffness by a

1471   factor of 2 (increase or decrease) for a fixed concentration of Rubisco (2% volume fraction) and

1472   an equal number of Rubisco and EPYC1 binding sites. In Table S1, we report the resulting

1473   percentage of Rubiscos in clusters with 10+ Rubiscos for EPYC1s with 3, 4, or 5 binding sites

1474   for each of these parameter sets.

1475

1476

1477   **QUANTIFICATION AND STATISTICAL ANALYSIS**

1478

1479   **Division image analysis**

1480   Chi-square statistic calculations on the relative proportions of each observed inheritance

1481   pattern in RbcS1- and EPYC1-Venus were performed using an online calculator

1482   (http://www.socscistatistics.com/tests/chisquare2/Default2.aspx).

1483   The Wilcoxon Matched-Pairs Signed-Ranks Test was used to quantify the difference in

1484   the pyrenoid signal during division using an online calculator

1485   (http://www.fon.hum.uva.nl/Service/Statistics/Signed_Rank_Test.html). This nonparametric test

1486   assumes data are paired, but that pairs are independent of each other. In this case, each pair was

1487   made up of the signal from the pyrenoind of a single mother cell at 1) the minimal value during

1488   dimming, and 2) the value 15 minutes after that point (including both daughters); thus, different

1489   pairs represent different mother cells. Sample size and error definitions can be found in the

1490   relevant figure legends.

1491
1492   **Tomogram analysis**

1493   Nine tomograms were screened for their reconstruction quality based on IMOD patch-

1494   tracking scores and the resolution of Rubisco subtomogram averages produced from each

72

1495 tomogram's extracted particles. The final dataset used for quantitative analysis was five

1496 tomograms, representing five cells from three separate liquid cultures.

1497   Following the localization of Rubisco holoenzymes within pyrenoid tomograms by

1498 volume masking, template matching, subtomogram averaging and classification (described

1499 above), Rubisco concentrations for each tomogram were calculated by dividing the number of

1500 particles in the "positive" class by the masked volume of the pyrenoid matrix. Error values

1501 displayed in the text are standard deviations, unless marked otherwise.

1502

1503 **FRAP image analysis**

1504   Images were screened for quality before quantitative analysis: any pyrenoids with

1505 indistinct bleach regions, many saturated pixels, or loss of focus during recovery were discarded.

1506 If a pyrenoid contained 1-2 saturated pixels, those pixels were not included in quantification.

1507 Average recovery rates for $n = 44$ (EPYC1-Venus), 48 (RCA1-Venus), 42 (live RbcS1-Venus), 8

1508 (fixed RbcS1-Venus), or 28 (*epyc1* EPYC1-Venus) pyrenoids are shown with error bars or

1509 shading representing standard error of the mean (SEM). FRAP data were collected over 1 (*epyc1*

1510 EPYC1-Venus) or 3 (RbcS1-, EPYC1-, and RCA1-Venus) biological replicates. For comparison

1511 of recovery rates between strains, a one-way ANOVA with post-hoc Bonferroni means

1512 comparison was performed using OriginPro software; the variances for these recovery rates were

1513 6.36×10^{-4} (RbcS1-Venus), 6.58×10^{-4} (RCA1-Venus), and 8.88×10^{-4} (EPYC1-Venus). A one-way

1514 ANOVA test assumes normal distributions, independent samples, and equal variances, all of

1515 which appeared to be met in our data

1516

1517 **DATA AND SOFTWARE AVAILABILITY**

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1518   The computer code used in modeling Rubisco-EPYC1 aggregation and fitting the

1519   Lennard-Jones RDF to experimental data will be made available through

1520   https://github.com/binarybin/RubiscoSimulation and https://github.com/anmartinezs/LJRDFfit/,

1521   respectively.

1522   The datasets generated during and/or analyzed during the current study are available from

1523   the corresponding authors upon reasonable request. Sequence information for the fluorescent

1524   constructs is available from GenBank under accession numbers KY550376 (pLM005-RBCS1-

1525   Venus), KX077944 (pLM005-EPYC1-Venus), and KY550375 (pLM005-RCA1-Venus). The *in*

1526   *situ* subtomogram average of *Chlamydomonas* Rubisco has been deposited in the EMDataBank

1527   with accession code EMD-3694

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1532 **Supplemental Item Titles and Legends**

1533

1534 **Movie S1. Example of Pyrenoid Inheritance by Fission; Related to Figures 1 and 6.**

1535 Movie of the division shown in Figure 1A-C and Figure 6A-B. Left: an overlay of the Venus

1536 (green) and chlorophyll autofluorescence (magenta) channels, with saturated pixels masked out.

1537 Right: heat map of the Venus channel alone, with the scale identical to that in Figure 6A. Images

1538 are 2D projections of the sum of pixel values in each channel in a Z-stack through the whole cell

1539 at each time point. Scale bar = 2 μm. Time stamps correspond to Figures 1A-B and 6A-B.

1540

1541 **Movie S2. Example of Whole Pyrenoid Inheritance and Inheritance of no Apparent**

1542 **Pyrenoid; Related to Figure 2.**

1543 Movie of the division shown in Figure 2A. Left: an overlay of the Venus (green) and chlorophyll

1544 autofluorescence (magenta) channels, with saturated pixels masked out. Right: heat map of the

1545 Venus channel alone, with the scale identical to that in Figure 6A. Images are 2D projections of

1546 the sum of pixel values in each channel in a Z-stack through the whole cell at each time point.

1547 Scale bar = 2 μm. Time stamps correspond to Figure 2A.

1548

1549 **Movie S3. Example of Whole Pyrenoid Inheritance and *de novo* Pyrenoid Formation;**

1550 **Related to Figure 2.**

1551 The second divisions in this Movie are highlighted in Figure 2B; first division (not shown in

1552 Figure 2B, but shown in Figure S6B) exhibits pyrenoid fission; Left: an overlay of the Venus

1553 (green) and chlorophyll autofluorescence (magenta) channels, with saturated pixels masked out.

1554 Right: heat map of the Venus channel alone, with the scale identical to that in Figure 6A. Images

1555   are 2D projections of the sum of pixel values in each channel in a Z-stack through the whole cell

1556   at each time point. Scale bar = 2 μm. Time stamps correspond to Figure 2B.

1557

1558   **Movie S4. Sub-Nanometer Localization of Rubisco Holoenzymes within the Native**

1559   ***Chlamydomonas* Pyrenoid Matrix by *in situ* Cryo-Electron Tomography; Related to Figure**

1560   **4.**

1561   Sequential sections through the tomogram displayed in Figure 4A, followed by a reveal of the

1562   segmented membranes shown in Figure 4B, corresponding to the pyrenoid tubules and

1563   minitubules (green and yellow, respectively). Sequential sections are then shown through a

1564   binary volume with white spheres indicating localized Rubisco positions, followed by a reveal of

1565   every Rubisco position within the tomogram (magenta). Finally, there is a short tour of

1566   segmented volume, showing the interconnected pyrenoid membranes embedded within the

1567   pyrenoid matrix. Scale bar = 200 nm.

1568

1569   **Movie S5. Fluorescence Recovery After Photobleaching in Live and Fixed Pyrenoids;**

1570   **Related to Figure 5.**

1571   Movies of the RbcS1-Venus FRAP experiments on the live (top) and fixed (bottom) pyrenoids

1572   shown in Figure 5A-F. Left: images scaled to pre-bleach values. Right: images scaled to post-

1573   bleach values. Color scaling is displayed in Figure 5. Each image is the RbcS1-Venus signal in a

1574   single mid-volume plane in the pyrenoid. Scale bar = 1 μm. Time stamps correspond to Figure 5.

1575

1576   **Movie S6. "Magic number" EPYC1-Rubisco Aggregation in Simulated 3D Systems with**

1577   **EPYC1s with 3, 4, or 5 Binding Sites; Related to Figure 7.**

1578   Top: Examples of off-lattice 3D-modeled aggregation over time in EPYC1-Rubisco systems,

1579   starting from random dispersed initial configurations, in which EPYC1 contains 3 (left), 4

1580   (middle), or 5 (right) binding sites for Rubisco. Bottom: Plots of the fraction of Rubiscos in a

1581   cluster of 10 or more through time in the above movies.

1582

1583   **Table S1: Robustness of the Magic-Number Effect in 3D Off-Lattice Simulations; Related**

1584   **to Figure 7.**

1585   The EPYC1 and Rubisco binding-site radii are set as 0.8 nm, 1 nm, and 1.25 nm, and the non-specific

1586   Lennard-Jones interaction energy and the EPYC1 inter-binding-site spring stiffness are varied by a factor

1587   of 2 (increase or decrease) for a fixed concentration of Rubisco (2% volume fraction) and an equal

1588   number of Rubisco and EPYC1 binding sites. Reported is the resulting fraction of Rubiscos in clusters

1589   with 10+ Rubiscos for EPYC1s with 3, 4, or 5 binding sites for each of these parameter sets. The magic-

1590   number effect, *i.e.* strong reduction of clustering for EPYC1s with 4 binding sites, is consistently

1591   observed, except at the stiffest value of spring constant (0.48 kBT/nm2), where strong clustering is

1592   always observed. At this high stiffness, adjacent EPYC1 binding sites cannot readily bind to the same

1593   Rubisco but can still bridge different Rubiscos, favoring cluster formation.

1594

1595