

## Birth of a photosynthetic chassis: a MoClo toolkit enabling synthetic biology in the microalga *Chlamydomonas reinhardtii*

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3 1 **Birth of a photosynthetic chassis: a MoClo toolkit enabling synthetic biology in the**  
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5 2 **microalga *Chlamydomonas reinhardtii***

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3 27 **Abstract**  
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5 28 Microalgae are regarded as promising organisms to develop innovative concepts based on  
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7 29 their photosynthetic capacity that offers more sustainable production than heterotrophic  
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10 30 hosts. However, to realize their potential as green cell factories, a major challenge is to make  
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12 31 microalgae easier to engineer. A promising approach for rapid and predictable genetic  
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14 32 manipulation is to use standardized synthetic biology tools and workflows. To this end we  
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16 33 have developed a Modular Cloning toolkit for the green microalga *Chlamydomonas*  
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18 34 *reinhardtii*. It is based on Golden Gate cloning with standard syntax, and comprises 119  
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21 35 openly distributed genetic parts, most of which have been functionally validated in several  
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23 36 strains. It contains promoters, UTRs, terminators, tags, reporters, antibiotic resistance genes,  
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25 37 and introns cloned in various positions to allow maximum modularity. The toolkit enables  
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28 38 rapid building of engineered cells for both fundamental research and algal biotechnology.  
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30 39 This work will make *Chlamydomonas* the next chassis for sustainable synthetic biology.  
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35 41 **Keywords:** Algal biotechnology, *Chlamydomonas reinhardtii*, modular cloning, synthetic  
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3 45 There is an urgent need to decarbonize the world economy due to depletion of fossil fuel  
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5 46 reserves coupled with accumulation of greenhouse gases produced by their combustion.  
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7 47 One alternative to the use of fossil fuels is to use photosynthetic microorganisms, such as  
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9 48 microalgae, as green cell factories to produce fuels and chemicals from atmospheric CO<sub>2</sub> in a  
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11 49 sustainable process driven by sunlight<sup>1, 2</sup>. The fixed carbon can be redirected towards  
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13 50 compounds that can be used in the fuel, food, cosmetic and pharmaceutical industries, such  
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15 51 as proteins, alcohols, alkanes, lipids, sugars, pigments or terpenes<sup>3-5</sup>. By contrast with land  
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17 52 plant-based photoproduction, microalgae do not compete with agriculture and can be grown  
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19 53 at high yields even at large scale<sup>4, 6</sup>, including on waste streams, thus minimizing inputs<sup>3</sup>. The  
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21 54 green microalga *Chlamydomonas reinhardtii* (referred to hereafter as “Chlamydomonas”)  
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23 55 has been extensively engineered for basic research and industrial biotechnology<sup>4, 6-8</sup>. Its  
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25 56 nuclear and organellar genomes are sequenced and annotated, molecular biology  
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27 57 techniques and culture conditions are highly developed, and its physiology and metabolism  
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29 58 are well understood<sup>9-13</sup>. Moreover, the metabolic plasticity and cellular compartments of  
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31 59 *Chlamydomonas* offer great potential for advanced metabolic engineering strategies<sup>14, 15</sup>.  
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33 60 *Chlamydomonas* has already been engineered for production of the biodiesel precursor  
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35 61 bisabolene<sup>8</sup>, the terpene patchoulol<sup>7</sup>, and recombinant proteins as well as enzymes such as  
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37 62 an HIV antigen<sup>16</sup> and xylanase<sup>17</sup>. Despite these proofs of concept however, engineering of  
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39 63 *Chlamydomonas* is still slow due to a lack of standardized resources and tools<sup>11</sup>.  
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41 64 Development of the field of algal synthetic biology offers the means to enable design and  
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43 65 construction of microalgal cells with defined and predictable properties<sup>18</sup>. Besides  
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45 66 biotechnological applications, the transition from empirical to synthetic approaches also  
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47 67 provides the opportunity to answer fundamental biological questions using new concepts  
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49 68 and approaches based on understanding by construction rather than deconstruction.  
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3 69 Synthetic biology approaches, predicated on the Design-Build-Test-Learn cycle<sup>19</sup>, make  
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5 70 organisms easier to engineer through the use of standardized parts and their assembly to  
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7 71 simplify the building of designed DNA molecules<sup>19</sup>. Among available standards<sup>20</sup>, the Golden  
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9 72 Gate Modular Cloning (MoClo) technology, based on Type IIS restriction enzymes, offers  
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11 73 extensive standardization and allows the assembly of complex multigenic DNA from basic  
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13 74 gene parts (*e.g.* promoters, CDS, terminators) in just two steps<sup>21, 22</sup>. The method accelerates  
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15 75 and multiplies the possibilities to permute multiple genetic elements, and makes facile the  
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17 76 building of multigene constructs for full metabolic pathways<sup>23</sup>. MoClo is efficient and  
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19 77 versatile, but relies on intensive upfront generation of a standardized library of basic  
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21 78 building blocks, the gene parts, that have been domesticated to remove Type IIS sites, and  
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23 79 codon optimized for the host as appropriate. MoClo toolkits have already been developed  
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25 80 for a few model organisms<sup>24-29</sup> although not yet for microalgae.

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28 81 Here, we report the generation of a MoClo toolkit composed of more than 100 gene parts  
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30 82 codon-optimized for the *Chlamydomonas* nuclear genome. These genetic parts were  
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32 83 designed to provide maximum modularity to end-users, and to facilitate the development of  
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34 84 engineered strains for fundamental and green biotechnological applications, through  
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36 85 iterative design and testing. We provide functional validation and characterization of many  
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38 86 gene parts in several *Chlamydomonas* strains. This kit is available to the community, to allow  
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40 87 *Chlamydomonas* to become the next chassis for sustainable synthetic biology approaches.  
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## 47 **RESULTS**

### 48 **Standard and content of the *Chlamydomonas* MoClo kit**

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50 89 Standardization is the key to efficient building. The *Chlamydomonas* MoClo kit adopts the  
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52 90 syntax proposed by the plant synthetic biology community including the OpenPlant  
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3 92 Consortium<sup>30</sup> (Fig. 1). This syntax is defined for level 0 plasmids containing standard gene  
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5 93 parts (promoters, coding sequences, untranslated regions, etc.) and assigns strict fusion sites  
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7 94 for 10 cloning positions. In a single step, standardized parts can be assembled into modules  
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9 95 (Transcriptional Unit, TU, level 1) and modules into devices (multigenic construct, level M or  
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11 96 2) according to the original MoClo syntax<sup>22</sup> (Supplementary Fig. 1). Our *Chlamydomonas*  
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13 97 MoClo toolkit is composed of a set of 119 parts representing 67 unique genetic elements  
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15 98 available at different positions within the standard, thereby providing maximum modularity  
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17 99 to designers (Fig. 1, Fig. 2). The kit recapitulates most of the standard genetic elements  
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19 100 previously developed for *Chlamydomonas* which we “domesticated” by removing *Bpil* and  
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21 101 *Bsal* restriction sites (the two enzymes used by the MoClo strategy<sup>22</sup>, Supplemental Figure 1)  
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23 102 from their sequences by DNA synthesis or PCR-based mutagenesis. The available gene parts  
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25 103 encompass 7 promoters coupled or not to their original 5’UTR, the corresponding 5’UTR and  
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27 104 the *CrTH14* riboswitch, 8 immunological or purification tags in positions leading to N- or C-  
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29 105 terminal translational fusions, 9 signal and targeting peptides, 12 reporters, 5 antibiotic  
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31 106 resistance genes, the foot and mouth virus (FMDV) 2A peptide which allows expression of  
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33 107 two or more proteins from a single transcriptional unit<sup>17, 31, 32</sup>, 2 micro RNA (miRNA)  
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35 108 backbones and associated controls, and six 3’UTR-terminators (Fig. 1b, Fig. 2 and  
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37 109 Supplementary Table 1). All sequences and plasmids are available through the public  
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39 110 Addgene repository (<http://www.addgene.org/>).  
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#### 111 **Constitutive promoters and reporter genes**

112 Five antibiotic resistance genes are used as selectable markers for *Chlamydomonas* but also  
113 can function as reporter genes<sup>33, 34</sup>. We assembled three modules that allow control of the  
114 expression of the *aadA* gene, conferring spectinomycin resistance, by three constitutive

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3 115 promoters:  $P_{PSAD}$  and  $P_{\beta TUB2}$  with or without the first intron of  $\beta TUB2$  (pCM1-1 to 3,  
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5 116 Supplementary Table 3). The transformation efficiency of the three modules in UVM4<sup>35</sup> cells  
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7 117 was estimated by counting spectinomycin resistant colonies and showed resistance  
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9 118 frequencies within the same range (Fig. 3a). The presence of the first  $\beta TUB2$  intron  
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11 119 significantly increased the transformation efficiency as previously observed with the  
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13 120 presence of  $RBCS2$  introns in the *ble* marker<sup>33, 36, 37</sup>. Alternative reporters are bioluminescent  
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15 121 proteins, which allow more sensitive and quantitative analysis of gene expression. The kit  
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17 122 contains *Gaussia princeps* luciferase, the brightest luciferase established in  
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19 123 *Chlamydomonas*<sup>38</sup>, as well as the redesigned NanoLuciferase (NanoLuc) which provides a  
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21 124 stable and strong luminescence signal<sup>39</sup>. *Chlamydomonas* NanoLuc was specifically  
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23 125 developed for our MoClo kit through recoding to match the codon bias of *Chlamydomonas*,  
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25 126 and cloned at 6 different positions within the standard. This new part was first tested with  
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27 127 the most widely used promoter/terminator combination ( $P_{AR}$  promoter /  $T_{RBCS2}$  terminator)  
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29 128 for strong constitutive expression in *Chlamydomonas*. The corresponding module (pCM1-04)  
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31 129 was assembled with another module conferring paromomycin resistance (Supplementary  
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33 130 Fig. 2) into a device (pCMM-1) that was introduced into the genome of the D66 strain (CC-  
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35 131 4425, Fig. 3b). Among paromomycin resistant colonies,  $34.8\% \pm 8.3$  (N=48, mean  $\pm$  SEM)  
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37 132 were luminescent. The signal was variable between clones due to genomic position effects<sup>40</sup>,  
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39 133 <sup>41</sup> but was linear from 50 to  $5 \times 10^5$  cells (Fig. 3b and Supplementary Fig. 2). By contrast, non-  
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41 134 expressing transformants (resistant to paromomycin only) or the D66 recipient strain  
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43 135 displayed only a faint signal, 3 orders of magnitude lower, and saturating swiftly (Fig. 3b,  
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45 136 inset). The modularity of the MoClo strategy allows rapid assessment of combinations of  
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47 137 multiple parts. For example, we assembled 4 modules where NanoLuc expression is  
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49 138 controlled by all possible combinations of the two most common constitutive promoters ( $P_{AR}$   
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3 139 and  $P_{PSAD}$ ) and terminators ( $T_{RBCS2}$  and  $T_{PSAD}$ ) (Fig. 3b, pCM1-4 to 7, Supplementary Table 3).  
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5 140 Each module was assembled with the paromomycin resistance module (pCMM-1 to 4,  
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7 141 Supplementary Table 4) and introduced into the *Chlamydomonas* genome. Bioluminescence  
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9 142 levels were averaged over hundreds of transformants to account for the genome position  
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11 143 effect<sup>40, 41</sup>. The strengths of the two promoters were found to be comparable, whilst  $T_{PSAD}$   
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13 144 appeared to confer robust expression from both promoters, 10-fold higher than  $T_{RBCS2}$  (Fig.  
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15 145 3c). In a distinct context (strain, reporter sequence, culture conditions, etc.), the same  
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17 146 genetic element may perform differently<sup>31, 35</sup>. Such context sensitivity can be overcome by  
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19 147 taking advantage of the modularity of the *Chlamydomonas* MoClo kit, which allows for the  
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21 148 rapid characterization of all possible parts combinations. These results also confirmed the  
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23 149 performance of the *Chlamydomonas* NanoLuc reporter and its employability for detailed  
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25 150 understanding and characterization of genetic circuits especially if coupled with automated  
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27 151 cell-sorting microfluidic devices<sup>42</sup>.  
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### 33 152 **Control of gene expression**

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35 153 To build genetic circuits, the fine-tuning of gene expression is a prerequisite. Multiple parts  
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37 154 enabling controlled gene expression have therefore been implemented. The activity of the  
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39 155  $P_{NIT1}$  promoter can be controlled by switching the nitrogen source since it is strongly  
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41 156 repressed by ammonium and highly induced on nitrate<sup>34, 43, 44</sup>. A module where  $P_{NIT1}$  controls  
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43 157 expression of the *ble-GFP* gene (pCM1-8) conferred strong zeocin resistance in the CC-1690  
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45 158 strain but only when ammonium was replaced by nitrate as nitrogen source. By contrast, the  
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47 159  $P_{PSAD}$  promoter (pCM1-9) conferred strong antibiotic resistance on both nitrogen sources  
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49 160 (Fig. 4a and Supplementary Fig. 3a-c). The vitamin B<sub>12</sub>-repressible promoter  $P_{METE}$ <sup>45</sup> allowed  
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51 161 conditional functional complementation of the photosynthetic mutant *nac2-26* (CC-4421),  
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3 162 which lacks photosystem II due to the absence of the TPR-like protein NAC2 required for  
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5 163 stability of the *psbD* mRNA encoding the D2 reaction center protein<sup>46</sup>. *nac2-26* mutant cells  
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7 164 engineered with a module harboring the *NAC2* coding sequence under the control of the  
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9 165  $P_{METE}$  promoter (pCM1-10) could grow photoautotrophically in the absence of vitamin B<sub>12</sub>,  
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11 166 but growth was compromised by increasing its concentration by amounts as low as 5 ng/L  
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14 167 (Fig. 4b).

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16 168 Regulation of gene expression can also be controlled by vitamin B<sub>1</sub> (thiamine) at the level of  
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18 169 the transcript through riboswitches<sup>47, 48</sup>. Binding of thiamine pyrophosphate to the *THI4*  
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20 170 riboswitch (RS) results in alternative splicing and retention of an 81 bp upstream open  
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22 171 reading frame, ultimately interfering with translation<sup>47, 48</sup>. The RS also responds when cells  
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24 172 are grown in the presence of the thiamine biosynthetic intermediate 4-methyl-5-(2-  
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26 173 hydroxyethyl) thiazole (HET), but not with 4-amino-5-hydroxymethyl-2-methylpyrimidine  
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28 174 (HMP)<sup>47</sup>. A module combining  $P_{AR}$  and *THI4* (RS) to drive expression of the *ble-GFP* gene  
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30 175 (pCM1-11) conferred conditional zeocin sensitivity in the UVM4 strain<sup>35</sup>. Resistance was  
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32 176 compromised by thiamine or HET but not HMP (Fig. 4c and Supplementary Fig. 3d), thereby  
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34 177 demonstrating the efficient repression of the transgene through the *THI4* riboswitch.

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36 178 Finally, to allow targeted repression of gene expression, a microRNA precursor sequence  
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38 179 derived from the pre-miR1157 and used for the generation of artificial miRNAs (amiRNA)<sup>49</sup>  
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40 180 was re-designed for compatibility with the Golden Gate cloning method. To demonstrate its  
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42 181 effectiveness in driving gene repression, a specific amiRNA sequence directed against the  
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44 182 *MAA7* gene, whose repression provides resistance to 5'-fluoroindole (5'-FI)<sup>50</sup>, was inserted  
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46 183 into the microRNA precursor. A control random sequence ("scrambled") amiRNA was  
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48 184 inserted into the same backbone. These parts were placed under the control of  $P_{PSAD}$  and  
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50 185  $T_{PSAD}$  (pCM1-12 and 13) and assembled with a paromomycin resistance module (pCM1-27).  
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3 186 The same amiRNA sequences were introduced into the previously established pChlamiRNA3  
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5 187 vector<sup>49</sup> as controls. After transformation of the CC-1690 strain, 36% of paromomycin-  
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7 188 resistant cells displayed resistance to 5'-FI with the device targeting *MAA7* (pCMM-5) but  
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9 189 not with the scrambled amiRNA (pCMM-6) (Fig. 4d and Supplementary Fig. 3f). A modified 5'  
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11 190 rapid amplification of cDNA ends (5'-RACE) assay revealed that the *MAA7* transcript was  
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13 191 most frequently cleaved at a site corresponding to positions 10 and 11 of the amiRNA, as  
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15 192 expected for a specific action of the miRNA (Fig. 4d). The properties of controllable parts can  
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17 193 also be combined as shown for *P<sub>NIT1</sub>* control of amiRNA-dependent gene repression<sup>34</sup>. An  
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19 194 amiRNA strategy recently proved useful for concerted metabolic engineering of a biodiesel  
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21 195 precursor in *Chlamydomonas*<sup>8</sup>. The versatility of the MoClo kit opens new possibilities for  
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23 196 sophisticated metabolic engineering strategies, *e.g.* the specific downregulation of up to six  
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25 197 target genes with one level M assembly.

### 31 198 **Multiple fusion tags for detection and purification of gene products.**

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33 199 Protein fusion tags are indispensable tools used to improve protein expression yields, enable  
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35 200 protein purification, and accelerate the characterization of protein structure and function<sup>51</sup>.  
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37 201 Our MoClo kit includes multiple epitope and affinity tags known to be functional in  
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39 202 *Chlamydomonas*. The modularity of the MoClo assembly allows rapid assessment of the best  
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41 203 tagging strategy through a rapid design/build/test/learn cycle. We took advantage of the  
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43 204 well characterized *rap2* mutant ( $\Delta$ *FKBP12*), which is insensitive to rapamycin<sup>52</sup>, to test the  
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45 205 functionality of five tags (Fig. 5a,b). We designed and built 5 devices allowing strong  
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47 206 constitutive expression of N- or C-terminal tagged FKBP12 coupled to a paromomycin  
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49 207 module (pCMM-7 to 11, Fig. 5c-h and Supplementary Table 4). The engineered strains were  
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51 208 selected on paromomycin and the functionality of the fusion protein was tested by assessing  
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3 209 sensitivity to rapamycin. Protein extracts were probed by immunoblotting using FKBP12-  
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5 210 specific and tag-specific antibodies (Fig. 5d-h). All tags allowed detection (Fig. 5d-h) or  
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7 211 purification (Fig. 5i) of FKBP12 even though some were not functional for restoring  
8  
9 212 rapamycin sensitivity. The test revealed that pCMM-9 outperforms other devices since it  
10  
11 213 provides a WT-like phenotype and expression level coupled to a strong and specific Myc  
12  
13 214 signal with no significant processing of the protein. These results demonstrate the  
14  
15 215 importance of the modularity provided by the Chlamydomonas MoClo toolkit for designing  
16  
17 216 optimal fusion proteins.

### 217 **Visualization and targeting of proteins in living cells**

218 Fluorescent protein tags allow the temporal and spatial monitoring of dynamic expression  
219 patterns at cellular and subcellular scales<sup>53</sup>. Natural and synthetic metabolic pathways can  
220 be optimized through spatial organization since cell compartments offer many advantages,  
221 such as isolation of metabolic reactions and generation of concentration gradients<sup>14</sup>. In a  
222 eukaryotic chassis like Chlamydomonas, organelles such as microbodies, mitochondria and  
223 chloroplasts can be engineered to implement or improve metabolic pathways<sup>15</sup>. The  
224 Chlamydomonas MoClo kit includes 11 targeting and signal peptides that allow the targeting  
225 of fusion proteins to mitochondria, chloroplast, nucleus, secretory pathway, ER and  
226 peroxisome-like microbodies. The functionality of the targeting and signal peptides and of  
227 the five fluorescent proteins (mVenus - yellow, mCherry - red, mRuby2 - red, Clover - green,  
228 mCerulean3 - cyan) included in the toolkit was tested. Eight modules (pCM1-19 to 26,  
229 Supplementary Table 3) combining diverse fluorescent proteins and targeting sequences  
230 were assembled into devices with an antibiotic resistance module (pCMM-12 to 19). All  
231 devices were found to behave as expected and provided the expected fluorescent signal in

1  
2  
3 232 the targeted compartment (Fig. 6). The fluorescent and targeting parts of the  
4  
5 233 Chlamydomonas MoClo toolkit, most of which have been validated here, enable engineering  
6  
7 234 in the third dimension<sup>14</sup> *i.e.* isolation and organization in multiple cellular compartments,  
8  
9 235 and offer new tools for biological design/build/test cycles.

## 12 13 236 **Discussion**

14  
15 237 The Chlamydomonas MoClo toolkit presented here provides more than 100 domesticated  
16  
17 238 gene parts to allow advanced synthetic biology in microalgae. Numerous parts of multiple  
18  
19 239 types have been characterized and validated in different genetic backgrounds<sup>10</sup> and culture  
20  
21 240 conditions, and can be readily used for biological design without further development. With  
22  
23 241 the efficiency and modularity of the MoClo strategy, molecular cloning is no longer a limiting  
24  
25 242 step for engineering Chlamydomonas cells. Indeed, from design to building, a complex  
26  
27 243 device of up to six different genes/modules can be obtained within a week using the  
28  
29 244 standardized parts provided in our kit. The modularity will also enable combinatorial  
30  
31 245 assembly by shuffling part libraries<sup>54</sup> and determine *a posteriori* which combination is the  
32  
33 246 most relevant. The development of gene-editing technologies in Chlamydomonas, including  
34  
35 247 Zinc-finger nucleases<sup>55, 56</sup> and several CRISPR-Cas9 approaches<sup>55, 57-59</sup>, together with the  
36  
37 248 development of high-throughput microfluidics<sup>42</sup> are beginning to gather pace. Coupling  
38  
39 249 these resources to our standardized MoClo toolkit will facilitate the use of Chlamydomonas  
40  
41 250 as the photosynthetic chassis for innovative synthetic biology approaches aimed at  
42  
43 251 fundamental and biotechnological applications. We expect that the creativity of designers,  
44  
45 252 released from the time constraints associated with classical cloning strategies, will allow  
46  
47 253 rapid expansion of the standard gene parts, modules and devices through open distribution,  
48  
49 254 notably using the Addgene repository. We invite the community to openly share their parts  
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3 255 through Addgene and/or our consortium (contact M. Schroda). The development of the  
4  
5 256 *Chlamydomonas* MoClo toolkit constitutes a complete step-change in the fields of microalgal  
6  
7 257 biology and biotechnology. The parts developed for the MoClo toolkit may also be employed  
8  
9 258 in other microalgal species since the orthogonality of several *Chlamydomonas*  
10  
11 259 transcriptional units has been demonstrated in multiple hosts, including the industrially  
12  
13 260 relevant species *Chlorella ellipsoidea*, *Nannochloropsis sp.* and *Dunaliella salina*<sup>60</sup>. Synthetic  
14  
15 261 approaches will allow engineering of microalgae in a predictable and efficient manner and  
16  
17 262 thereby offer great potential to couple environmental protection, energy transition and  
18  
19 263 bioeconomic growth<sup>4</sup>.  
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264

## 265 **Methods**

266 All chemicals were obtained from Sigma-Aldrich, unless otherwise specified.

267

### 268 ***Escherichia coli* and *Chlamydomonas reinhardtii* strains, transformation and growth** 269 **conditions.**

270 Bacterial growth was performed at 37°C in LB broth supplemented with agar (20% m/V),  
271 spectinomycin (50 µg/mL), ampicillin or carbenicillin (50 or 100 µg/mL, respectively) and X-  
272 gal (40 µg/mL) when required. Chemically competent *E. coli* DH10β (New England Biolabs)  
273 were used for transformation (by heat shock following the manufacturer's instructions) and  
274 maintenance of plasmids. All plasmids of the kit were maintained and amplified in TOP10  
275 *E. coli* strain prior to submission to Addgene.

276 *C. reinhardtii* strains<sup>35, 46, 52, 61, 62</sup>, culture and transformation conditions are recapitulated in  
277 Supplementary Table 5. They were grown in Tris-Acetate-Phosphate (TAP) medium<sup>63</sup>  
278 supplemented with agar (1.6 % m/V), spectinomycin (100 µg/mL), paromomycin (15 µg/mL),

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3 279 zeocin (ThermoFisher Scientific, 10 to 15  $\mu\text{g}/\text{mL}$ ), 5-fluoroindole (20  $\mu\text{M}$ ) or rapamycin (LC  
4  
5 280 Laboratories, 1  $\mu\text{M}$ ) when required. For *NIT1* promoter characterization (Figure 4a), a  
6  
7 281 modified TAP medium lacking nitrogen source (TAP-N) was used instead, and was  
8  
9 282 supplemented with 4 mM  $\text{KNO}_3$  (nitrate) or 7.5 mM  $\text{NH}_4\text{Cl}$  (ammonium). For NAC2  
10  
11 283 autotrophy test (Figure 4b), cells were grown in minimal media (HSM) for selection of  
12  
13 284 complemented strains. The responsiveness to B12 was assessed on plate and then in liquid.  
14  
15 285 Cells were grown for 15 days in HSM until  $1\text{-}5 \times 10^7$  cells/mL concentration prior to  
16  
17 286 inoculation in a 96-well plate at a concentration of  $10^5$  cells/mL in 200  $\mu\text{L}$  of HSM. For  
18  
19 287 response assays (Figure 4c) thiamine (Melford Laboratories Ltd.), 4-methyl-5-(2-  
20  
21 288 hydroxyethyl) thiazole (HET) and 4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP,  
22  
23 289 Fluorochem UK) were added to TAP media at a final concentration of 10  $\mu\text{M}$ .  
24  
25  
26  
27  
28 290 For transformation by electroporation (see Supplementary Table 5), a TAP culture of  $1\text{-}5 \times$   
29  
30 291  $10^6$  cells/mL was concentrated 100 times in TAP complemented with 60 mM sucrose or the  
31  
32 292 MAX Efficiency Transformation reagent for Algae (ThermoFisher scientific) and 25-250  $\mu\text{L}$   
33  
34 293 were incubated with 80-300 ng of DNA for 10-30 min on ice in a 0.4 cm gapped cuvette  
35  
36 294 (BioRad) prior to electroporation (BioRad Gene Pulser Xcell). The cells were then left to  
37  
38 295 recover in TAP complemented with 40-60 mM sucrose for 16 h under appropriate light and  
39  
40 296 shaking conditions (typically  $50 \mu\text{mol photon m}^{-2} \text{s}^{-1}$  at 100 rpm) prior to plating on TAP-agar  
41  
42 297 plates with adapted antibiotics. Transformation by glass-beads method followed previously  
43  
44 298 published protocols<sup>7, 64</sup>. Briefly, after growth in TAP until  $5 \times 10^6$  cells/mL, cells were  
45  
46 299 concentrated 30 times and  $5 \times 10^7$  cells were mixed with DNA using glass beads. After 2-fold  
47  
48 300 dilution with TAP,  $2.5 \times 10^7$  cells were spread onto TAP agar plates containing  $100 \mu\text{g ml}^{-1}$   
49  
50 301 spectinomycin. Plates were incubated for 16 h in the dark prior to light exposition ( $30 \mu\text{mol}$   
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52 302  $\text{photon.m}^{-2}.\text{s}^{-1}$ ). When colony counting was performed (Figure 3a), it was 8 days after the  
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3 303 beginning of light. In both cases, the transformation protocol leads to insertion of a linear  
4  
5 304 DNA in a random location within the nuclear genome.

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9 306 **Design.** All *in silico* sequence designs and analysis were performed with Serial Cloner,  
10  
11 307 Benchling, SnapGene, ApE or Genome Compiler. For exogenous parts, reverse translation  
12  
13 308 was performed with Serial Cloner using *C. reinhardtii* nuclear genome codon frequency  
14  
15 309 (<http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=3055>).

16  
17  
18 310 amiRNAs can be generated using DNA parts pCM0-068 and pCM0-069. Both are derived  
19  
20 311 from the endogenous pre-miR1157<sup>65</sup>, but differ in the way in which the amiRNA specific  
21  
22 312 sequence is introduced. pCM0-069/pCMM-20 is analogous to pChlamyRNA3<sup>65</sup>, and a dsDNA  
23  
24 313 fragment containing the amiRNA/loop/amiRNA\* sequence is introduced into a *SpeI* site  
25  
26 314 inside the miRNA precursor sequence. pCM0-068 presents two divergently oriented *BpiI*  
27  
28 315 sites, allowing the cloning of the dsDNA fragment by Golden Gate. In this last case, the  
29  
30 316 dsDNA fragment is formed by the annealing of two oligos with the following sequence: 1)

31  
32 317 sense oligo (5' AGTA-(MIRNA\*SEQ)-  
33  
34 318 TCTCGCTGATCGGCACCATGGGGGTGGTGGTGATCAGCGCTA-(MIRNA SEQ)-T 3'), 2) anti-sense

35  
36 319 oligo (5'CAGT-A-(rev com MIRNA SEQ)-  
37  
38 320 TAGCGCTGATCACCACCACCCCATGGTGCCGATCAGCGAGA-(rev com MIRNA\*SEQ) 3'). There

39  
40  
41 321 are online tools that help with the design of the amiRNA sequence  
42  
43 322 (<http://wmd3.weigelworld.org/cgi-bin/webapp.cgi>).

44  
45 323

46  
47 324 **Parts repository.** All sequences listed in Supplementary Table 2 were deposited in Addgene.  
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49 325 Physical distribution of the DNA is performed through Addgene. We invite the community to



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2  
3 326 share their future parts through Addgene and/or with our consortium (contact M. Schroda)  
4  
5 327 which will make them available to the community.  
6

7 328

8  
9 329 **Parts cloning.** All PCR reactions were performed using the Phusion DNA polymerase, KOD  
10  
11 330 Xtreme Hot Start DNA polymerase (Merck) or Q5 DNA polymerase purchased from New  
12  
13 331 England Biolabs (NEB) following the manufacturer's instructions adapted to GC-rich DNA,  
14  
15 332 typically duration of hybridization and polymerization was doubled and/or GC enhancer  
16  
17 333 solution was used. Molecular biology kits were purchased from Macherey-Nagel, peqLab,  
18  
19 334 NEB or QIAGEN (gel extraction and miniprep kits). Primers were produced by Eurofins  
20  
21 335 Genomics or Sigma-Aldrich while synthesized parts were obtained from Genecust, DC  
22  
23 336 Biosciences, IDTDNA or Sigma-Aldrich.  
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29  
30 338 **MoClo Assembly Conditions.** All Restriction/ligation reactions were performed using *BbsI* or  
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32 339 *BbsI*-HF (*BpiI* is an isoschizomer) or *BsaI*-HF (NEB or ThermoFisher) together with T4 ligase  
33  
34 340 (NEB) in a medium containing the NEB CutSmart buffer and 1 mM ATP (with stock of 10 mM  
35  
36 341 solubilized in 0.1 M Tris-HCl, pH 7.9). Typical ratio between destination plasmid (100 fmol)  
37  
38 342 and entry plasmid/parts was 1:2. To facilitate handling of the kit for end-users, we provide  
39  
40 343 detailed protocols and reaction mix calculators for each type of assembly: level 0 for parts  
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42 344 (Supplementary Table 6), level 1 for modules (Supplementary Table 7) and level M for  
43  
44 345 devices (Supplementary Table 8).  
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50 347 **Quality Control of generated DNAs.** All plasmids were controlled by differential restriction.  
51  
52 348 In addition, all level 0 plasmids were sequenced with specific primers. Sequencing was  
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3 349 performed by Eurofins Genomics, Source BioSciences UK, Seqlab, Macrogen, Microsynth,  
4  
5 350 GATC Biotech or Core Facility (CeBiTec, Bielefeld University).  
6

7 351  
8

9 352 **NanoLuc activity determination.** Reagents were purchased from Promega (ref. N1110) and  
10  
11 353 activity was determined as previously described<sup>39</sup>. For screening, *C. reinhardtii* colonies were  
12  
13  
14 354 transferred into a 96-well plate containing 100  $\mu$ L of TAP in each well. After gentle  
15  
16 355 resuspension, 50  $\mu$ L was transferred into a solid white 96-well plate to which 50  $\mu$ L of Nano-  
17  
18 356 Glo substrate diluted in the provided buffer (2% V/V) was added and gently mixed by  
19  
20 357 pipetting. Luminescence was measured at 460 nm with a CLARIOstar plate reader (BMG  
21  
22  
23 358 Labtech). For promoter/terminator combination assessment experiment (Figure 3c), all *C.*  
24  
25 359 *reinhardtii* colonies from a transformation event were pooled and resuspended in TE buffer  
26  
27 360 (50 mM Tris-HCl pH 7.9, 1 mM EDTA) complemented with anti-protease (1 tablet per 50 mL,  
28  
29 361 Sigma-Aldrich: S8830). The cells were lysed by vortexing 10 seconds twice in the presence of  
30  
31 362 glass beads (about 1:5 ratio beads/cells V/V) prior to two centrifugations (20000 g for 10 min  
32  
33 363 at 4°C) to clarify the supernatant. The protein concentration was then determined using  
34  
35 364 Bradford reagent with a Bovine Serum Albumin standard curve and the concentration was  
36  
37 365 standardized to 0.5 g/L. The activity was determined in a 96-well plate in a final volume of  
38  
39 366 50  $\mu$ L (1:1 with nano-Glo resuspended in provided lysis buffer) per well. NanoLuc activity was  
40  
41 367 determined on 6 different increasing protein quantities (0.1 to 2.5  $\mu$ g) for each assay,  
42  
43 368 allowing to assess linearity of the signal.  
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49 370 **Absorbance measurement of cultures growing in microtiter plates**

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51 371 Growth in microtiter plates was determined by measuring the optical density of each well at  
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53 372 730 nm. Microtiter plates containing 180-200  $\mu$ L culture were incubated under constant light  
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3 373 (125  $\mu\text{mol photon}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) at 25°C and 40 rpm orbital shaking. For density determination,  
4  
5 374 cultures were resuspended by pipetting and 100  $\mu\text{L}$  of cell suspension was transferred to a  
6  
7 375 new microtiter plate containing 50  $\mu\text{L}$  TAP 0.03% Tween-20. Optical density of each well was  
8  
9 376 determined at 730 nm in a CLARIOstar plate reader (BMG Labtech). Plates were shaken for  
10  
11 377 6-10 sec at 600 rpm before measurement.  
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14 378

### 16 379 **RNA extraction and miRNA-mediated cleavage mapping**

18 380 RNA isolation was carried out as previously described<sup>49</sup> (a detailed protocol can be found at  
19 381 <http://www.plantsci.cam.ac.uk/research/davidbaulcombe/methods/downloads/smallrna.pdf>  
20  
21 382 [f/view](#)), with the following modifications: Cells were centrifuged and resuspended in 0.25 mL  
22  
23 383 of water and mixed with 0.25 mL Lysis buffer (50 mM Tris-HCl pH 8.0, 200 mM NaCl, 50 mM  
24  
25 384 EDTA, 2% SDS, 1 mg/mL proteinase K). Lysis buffer was incubated at 50°C for 5 min prior  
26  
27 385 mixing with cells. Cell suspension was then incubated at 25°C for 20 min. Finally, 2 mL of  
28  
29 386 PureZol (Biorad) was added and samples were snap-frozen. RNA quality was assessed in gel  
30  
31 387 and quantified in Nanodrop (ThermoFisher scientific).  
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36  
37 388 miRNA cleavage site determination was performed as previously described<sup>66</sup>. Briefly, 10  $\mu\text{g}$   
38  
39 389 of total RNA was ligated with an RNA oligo (5' CGACUGGAGCACGAGGACACUGACAUGGAC  
40  
41 390 UGAAGGAGUAGAAA 3') using T4 RNA ligase for 1 h at 37°C. RNA was extracted with  
42  
43 391 phenol:chloroform and precipitated with ethanol and sodium acetate. The precipitated RNA  
44  
45 392 was retrotranscribed into cDNA by SuperScript IV reverse transcriptase (ThermoFisher  
46  
47 393 scientific), using random hexamers and following manufacturer's recommendations. Two  $\mu\text{L}$   
48  
49 394 of the cDNA was used as template of a PCR using primers FJN456 (5'-  
50  
51 395 CGACTGGAGCACGAGGACACTGA) and FJN495 (5'- TGGGGTAGGGGTGGGGGCCAG). Two  $\mu\text{L}$  of  
52  
53 396 this PCR was used as template of a second PCR with primers FJN457 (5'-  
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3 397 GGACTGACATGGACTGAAGGAGTA) and FJN496 (5'- TGACCCAGTCGCGGATGGCCT). PCR  
4  
5 398 was resolved in a 2% agarose gel and the specific band was isolated from the gel and cloned  
6  
7 399 into pGEM-T easy (Promega) for sequencing.  
8

9  
10 400

11  
12 401 **Immuno-blotting.** Chlamydomonas cells expressing FKB12 fusion proteins from liquid  
13  
14 402 cultures were collected by centrifugation 4000 g for 5 min at room temperature (RT),  
15  
16 403 washed in 50 mM Tris-HCl pH 7.5, and resuspended in a minimal volume of the same  
17  
18 404 solution. Cells were lysed by two cycles of slow freezing to -80°C followed by thawing at RT.  
19  
20  
21 405 The soluble cell extract was separated from the insoluble fraction by centrifugation (15000 g  
22  
23 406 for 20 min at 4°C). Total protein extracts (15 µg) were then subjected to 15% SDS-PAGE.

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25  
26 407 mCherry-expressing cells were harvested at 3500 rpm for 2 min (4 °C) and resuspended in  
27  
28 408 60 µL of DTT-carbonate buffer (0.1 M DTT, 0.1 M Na<sub>2</sub>CO<sub>3</sub>). After freezing at -20 °C and  
29  
30 409 thawing, 55 µL of SDS-Sucrose buffer were added (5 % SDS, 30 % sucrose). Samples were  
31  
32 410 then boiled for 45 s at 95 °C, followed by 2 min incubation on ice and 13000 g centrifugation  
33  
34 411 for 2 min at RT. Protein extracts corresponding to 2 µg of Chlorophyll were then separated  
35  
36 412 using 12% SDS-PAGE.

37  
38  
39 413 For immunoblot analyses, proteins were then transferred to nitrocellulose membranes (Bio-  
40  
41 414 Rad, 162-0115 or Amersham Protran). After blocking with 3 to 5% low-fat Milk in PBS for 1 h  
42  
43  
44 415 at RT, membranes were incubated with primary antibody in 5% low-fat Milk in PBS for 16 h  
45  
46 416 at 4°C. After 4 washes in PBS - 0.1% tween-20 (TPBS), the membranes were incubated with  
47  
48 417 secondary antibody in 5% low-fat Milk in PBS for 1 h at RT, and subsequently washed 4 time  
49  
50  
51 418 in TPBS prior to chemi-luminescence revelation using ECL. Primary antibodies used were  
52  
53 419 anti-FKBP12<sup>52</sup> (1/5000 dilution; secondary was anti-rabbit 1/10000), anti-FLAG (Sigma-  
54  
55 420 Aldrich F1804, 1/5000 dilution; secondary was anti-mouse 1/5000), anti-STREP (IBA, Catalog

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2  
3 421 N. 2-1509-001, 1/5000 dilution; conjugated to HRP), anti-cMYC (Sigma-Aldrich M4439,  
4  
5 422 1/2500 dilution; secondary was anti-mouse 1/5000), anti-HA (Sigma-Aldrich H9658, 1/5000  
6  
7 423 dilution; secondary was anti-mouse 1/2500) and anti-PRPL1<sup>67</sup>. For mCherry serum, rabbits  
8  
9 424 were immunized against purified full-length mCherry protein containing an N-terminal His<sub>6</sub>-  
10  
11  
12 425 tag.

13  
14 426  
15  
16 427 **Microscopy.** For mCherry experiments (Figure 6b-e), images were taken at 100x  
17  
18 428 magnification with a BX53F microscope (Olympus). Fluorescence images for the detection of  
19  
20 429 mCherry were taken using a TRITC filter. For other fluorescent proteins (Figure 6f-h),  
21  
22 430 microscopy was performed as previously described<sup>7,68</sup>.

23  
24 431  
25  
26 432 **Accession numbers.** All parts accession numbers and the corresponding references are listed  
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28 433 in Supplementary Table 2.

29  
30 434

## 31 435 **ASSOCIATED CONTENT**

### 32 436 **Supporting Information.**

33  
34 437 Supplementary Figure 1 - MoClo assembly workflow reflecting the abstraction hierarchy

35 438 Supplementary Figure 2 - Variability of Nanoluc expression in pCMM-1 transformants.

36 439 Supplementary Figure 3 - Control of gene expression, complementary data.

37 440 Supplementary Table 1 - list of all unique parts of the Chlamy MoClo kit

38 441 Supplementary Table 2 - list of all parts of the Chlamy MoClo kit: level 0 plasmids

39 442 Supplementary Table 3 - list of all modules used for the Chlamy MoClo kit validation: level 1  
40 443 plasmids

41 444 Supplementary Table 4 - list of all modules used for the Chlamy MoClo kit validation: level M  
42 445 plasmids

43 446 Supplementary Table 5 - list of Chlamydomonas reinhardtii strains and associated  
44 447 transformations

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2  
3 448 Supplementary Table 6 - level 0 ligation file: protocol and reaction mix calculator to clone  
4 449 parts.

5  
6 450 Supplementary Table 7 - level 1 ligation file: protocol and reaction mix calculator to  
7 451 assemble modules.

8  
9 452 Supplementary Table 8 - level M ligation file: protocol and reaction mix calculator to assemble  
10 453 devices.

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13  
14 455 **Abbreviation.**

15  
16 456 MoClo: Modular Cloning, TU: Transcriptional Unit, RBCS2: Ribulose Bisphosphate

17  
18 457 Carboxylase oxygenase Small subunit 2, HSP70: Heat Shock Protein 70, AR: HSP70A/RBCS2,

19  
20 458 TUB2: Tubulin 2, PSAD: Photosystem I reaction center subunit II, HET: 4-methyl-5-(2-

21  
22 459 hydroxyethyl) thiazole, HMP: 4-amino-5-hydroxymethyl-2-methylpyrimidine, amiRNA:

23  
24 460 artificial micro RNA, TAP: Tris Acetate Phosphate

25  
26 461

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28 462 **Author Information.**

29  
30 463 K. Vavitsas current address is: Australian Institute for Bioengineering and Nanotechnology

31  
32 464 (AIBN), The University of Queensland, Australia

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34 465

35  
36 466 **Author Contribution.**

37  
38 467 SDL, AGS, MS, PEJ, OK, JLC and GP created the consortium that led this study.

39  
40 468 PC, FJN, FW, PM, DCB, GP, JLC, OK, PEJ, MS, AGS and SDL designed the study and wrote the

41  
42 469 manuscript.

43  
44 470 PC, FJN, FW, PM, KB, KJL, MEPP, PA, AGR, SSG, JN, BS, JT, RT, LW, KV, TB, KS, MC, FdC, AD,

45  
46 471 MdM, JH, WH, CHM designed parts, modules and devices, performed the experiments,

47  
48 472 and/or analyzed data.

49  
50 473

1  
2  
3 474 **Competing Financial Interests statement.**

4  
5 475 The authors declare no competing financial interest.

6  
7 476

8  
9  
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16  
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18  
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20  
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22  
23 484 (Project no. 13363) (to PEJ, KB, KV), by the Technology Platforms at the Center for

24  
25 485 Biotechnology (CeBiTec) Bielefeld University (to KL, TB, and OK), by UK Biotechnology and

26  
27 486 Biological Sciences Research Council (BBSRC) (to PM and AGR) and by ERA-SynBio project

28  
29 487 Sun2Chem (to PA and GP).

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31 488

32  
33 489 **REFERENCES.**

34  
35 490 (1) Zargar, A., Bailey, C. B., Haushalter, R. W., Eiben, C. B., Katz, L., and Keasling, J. D. (2017)  
36 491 Leveraging microbial biosynthetic pathways for the generation of 'drop-in' biofuels, *Curr*  
37 492 *Opin Biotechnol* 45, 156-163.

38  
39 493 (2) Georgianna, D. R., and Mayfield, S. P. (2012) Exploiting diversity and synthetic biology for the  
40 494 production of algal biofuels, *Nature* 488, 329-335.

41  
42 495 (3) Wijffels, R. H., Kruse, O., and Hellingwerf, K. J. (2013) Potential of industrial biotechnology with  
43 496 cyanobacteria and eukaryotic microalgae, *Curr Opin Biotechnol* 24, 405-413.

44  
45 497 (4) Gimpel, J. A., Henríquez, V., and Mayfield, S. P. (2015) In Metabolic Engineering of Eukaryotic  
46 498 Microalgae: Potential and Challenges Come with Great Diversity, *Front Microbiol* 6.

47  
48 499 (5) Scranton, M. A., Ostrand, J. T., Fields, F. J., and Mayfield, S. P. (2015) Chlamydomonas as a model  
49 500 for biofuels and bio-products production, *Plant J* 82, 523-531.

50  
51 501 (6) Gangl, D., Zedler, J. A., Rajakumar, P. D., Martinez, E. M., Riseley, A., Wlodarczyk, A., Purton, S.,  
52 502 Sakuragi, Y., Howe, C. J., Jensen, P. E., and Robinson, C. (2015) Biotechnological exploitation  
53 503 of microalgae, *J Exp Bot* 66, 6975-6990.

54  
55 504 (7) Lauersen, K. J., Baier, T., Wichmann, J., Wordenweber, R., Mussgnug, J. H., Hubner, W., Huser, T.,  
56 505 and Kruse, O. (2016) Efficient phototrophic production of a high-value sesquiterpenoid from  
57 506 the eukaryotic microalga *Chlamydomonas reinhardtii*, *Metab Eng* 38, 331-343.

- 1  
2  
3 507 (8) Wichmann, J., Baier, T., Wentnagel, E., Lauersen, K. J., and Kruse, O. (2017) Tailored carbon  
4 508 partitioning for phototrophic production of (E)-alpha-bisabolene from the green microalga  
5 509 *Chlamydomonas reinhardtii*, *Metab Eng*.
- 6 510 (9) Merchant, S. S., Prochnik, S. E., Vallon, O., Harris, E. H., Karpowicz, S. J., Witman, G. B., Terry, A.,  
7 511 Salamov, A., Fritz-Laylin, L. K., Marechal-Drouard, L., Marshall, W. F., Qu, L. H., Nelson, D. R.,  
8 512 Sanderfoot, A. A., Spalding, M. H., Kapitonov, V. V., Ren, Q., Ferris, P., Lindquist, E., Shapiro,  
9 513 H., Lucas, S. M., Grimwood, J., Schmutz, J., Cardol, P., Cerutti, H., Chanfreau, G., Chen, C. L.,  
10 514 Cognat, V., Croft, M. T., Dent, R., Dutcher, S., Fernandez, E., Fukuzawa, H., Gonzalez-Ballester,  
11 515 D., Gonzalez-Halphen, D., Hallmann, A., Hanikenne, M., Hippler, M., Inwood, W., Jabbari, K.,  
12 516 Kalanon, M., Kuras, R., Lefebvre, P. A., Lemaire, S. D., Lobanov, A. V., Lohr, M., Manuell, A.,  
13 517 Meier, I., Mets, L., Mittag, M., Mittelmeier, T., Moroney, J. V., Moseley, J., Napoli, C.,  
14 518 Nedelcu, A. M., Niyogi, K., Novoselov, S. V., Paulsen, I. T., Pazour, G., Purton, S., Ral, J. P.,  
15 519 Riano-Pachon, D. M., Riekhof, W., Rymarquis, L., Schroda, M., Stern, D., Umen, J., Willows, R.,  
16 520 Wilson, N., Zimmer, S. L., Allmer, J., Balk, J., Bisova, K., Chen, C. J., Elias, M., Gendler, K.,  
17 521 Hauser, C., Lamb, M. R., Ledford, H., Long, J. C., Minagawa, J., Page, M. D., Pan, J.,  
18 522 Pootakham, W., Roje, S., Rose, A., Stahlberg, E., Terauchi, A. M., Yang, P., Ball, S., Bowler, C.,  
19 523 Dieckmann, C. L., Gladyshev, V. N., Green, P., Jorgensen, R., Mayfield, S., Mueller-Roeber, B.,  
20 524 Rajamani, S., Sayre, R. T., Brokstein, P., Dubchak, I., Goodstein, D., Hornick, L., Huang, Y. W.,  
21 525 Jhaveri, J., Luo, Y., Martinez, D., Ngau, W. C., Otilar, B., Poliakov, A., Porter, A., Szajkowski, L.,  
22 526 Werner, G., Zhou, K., Grigoriev, I. V., Rokhsar, D. S., and Grossman, A. R. (2007) The  
23 527 *Chlamydomonas* genome reveals the evolution of key animal and plant functions, *Science*  
24 528 *318*, 245-250.
- 25 529 (10) Gallaher, S. D., Fitz-Gibbon, S. T., Glaesener, A. G., Pellegrini, M., and Merchant, S. S. (2015)  
26 530 *Chlamydomonas* Genome Resource for Laboratory Strains Reveals a Mosaic of Sequence  
27 531 Variation, Identifies True Strain Histories, and Enables Strain-Specific Studies, *Plant Cell* *27*,  
28 532 2335-2352.
- 29 533 (11) Scaife, M. A., Nguyen, G. T., Rico, J., Lambert, D., Helliwell, K. E., and Smith, A. G. (2015)  
30 534 Establishing *Chlamydomonas reinhardtii* as an industrial biotechnology host, *Plant J* *82*, 532-  
31 535 546.
- 32 536 (12) Li, X., Zhang, R., Patena, W., Gang, S. S., Blum, S. R., Ivanova, N., Yue, R., Robertson, J. M.,  
33 537 Lefebvre, P. A., Fitz-Gibbon, S. T., Grossman, A. R., and Jonikas, M. C. (2016) An Indexed,  
34 538 Mapped Mutant Library Enables Reverse Genetics Studies of Biological Processes in  
35 539 *Chlamydomonas reinhardtii*, *Plant Cell* *28*, 367-387.
- 36 540 (13) Jinkerson, R. E., and Jonikas, M. C. (2015) Molecular techniques to interrogate and edit the  
37 541 *Chlamydomonas* nuclear genome, *Plant J* *82*, 393-412.
- 38 542 (14) Agapakis, C. M., Boyle, P. M., and Silver, P. A. (2012) Natural strategies for the spatial  
39 543 optimization of metabolism in synthetic biology, *Nat Chem Biol* *8*, 527-535.
- 40 544 (15) Chen, A. H., and Silver, P. A. (2012) Designing biological compartmentalization, *Trends Cell Biol*  
41 545 *22*, 662-670.
- 42 546 (16) Barahimipour, R., Neupert, J., and Bock, R. (2016) Efficient expression of nuclear transgenes in  
43 547 the green alga *Chlamydomonas*: synthesis of an HIV antigen and development of a new  
44 548 selectable marker, *Plant Mol Biol* *90*, 403-418.
- 45 549 (17) Rasala, B. A., Lee, P. A., Shen, Z., Briggs, S. P., Mendez, M., and Mayfield, S. P. (2012) Robust  
46 550 expression and secretion of Xylanase1 in *Chlamydomonas reinhardtii* by fusion to a selection  
47 551 gene and processing with the FMDV 2A peptide, *PLoS One* *7*, e43349.
- 48 552 (18) Scaife, M. A., and Smith, A. G. (2016) Towards developing algal synthetic biology, *Biochem Soc*  
49 553 *Trans* *44*, 716-722.
- 50 554 (19) Endy, D. (2011) Building a new biology, *C R Chim* *14*, 424-428.
- 51 555 (20) Casini, A., Storch, M., Baldwin, G. S., and Ellis, T. (2015) Bricks and blueprints: methods and  
52 556 standards for DNA assembly, *Nat Rev Mol Cell Biol* *16*, 568-576.
- 53 557 (21) Engler, C., Kandzia, R., and Marillonnet, S. (2008) A one pot, one step, precision cloning method  
54 558 with high throughput capability, *PLoS One* *3*, e3647.



- 1  
2  
3 559 (22) Weber, E., Engler, C., Gruetzner, R., Werner, S., and Marillonnet, S. (2011) A modular cloning  
4 560 system for standardized assembly of multigene constructs, *PLoS One* 6, e16765.
- 5 561 (23) Smanski, M. J., Bhatia, S., Zhao, D., Park, Y., L. B. A. W., Giannoukos, G., Ciulla, D., Busby, M.,  
6 562 Calderon, J., Nicol, R., Gordon, D. B., Densmore, D., and Voigt, C. A. (2014) Functional  
7 563 optimization of gene clusters by combinatorial design and assembly, *Nat Biotechnol* 32,  
8 564 1241-1249.
- 9 565 (24) Celinska, E., Ledesma-Amaro, R., Larroude, M., Rossignol, T., Pauthenier, C., and Nicaud, J. M.  
10 566 (2017) Golden Gate Assembly system dedicated to complex pathway manipulation in  
11 567 *Yarrowia lipolytica*, *Microb Biotechnol* 10, 450-455.
- 12 568 (25) Engler, C., Youles, M., Gruetzner, R., Ehnert, T. M., Werner, S., Jones, J. D., Patron, N. J., and  
13 569 Marillonnet, S. (2014) A golden gate modular cloning toolbox for plants, *ACS Synth Biol* 3,  
14 570 839-843.
- 15 571 (26) Iverson, S. V., Haddock, T. L., Beal, J., and Densmore, D. M. (2016) CIDAR MoClo: Improved  
16 572 MoClo Assembly Standard and New E. coli Part Library Enable Rapid Combinatorial Design for  
17 573 Synthetic and Traditional Biology, *ACS Synth Biol* 5, 99-103.
- 18 574 (27) Lee, M. E., DeLoache, W. C., Cervantes, B., and Dueber, J. E. (2015) A Highly Characterized Yeast  
19 575 Toolkit for Modular, Multipart Assembly, *ACS Synth Biol* 4, 975-986.
- 20 576 (28) Martella, A., Matjusaitis, M., Auxillos, J., Pollard, S. M., and Cai, Y. (2017) EMMA: An Extensible  
21 577 Mammalian Modular Assembly Toolkit for the Rapid Design and Production of Diverse  
22 578 Expression Vectors, *ACS Synth Biol*.
- 23 579 (29) Moore, S. J., Lai, H. E., Kelwick, R. J., Chee, S. M., Bell, D. J., Polizzi, K. M., and Freemont, P. S.  
24 580 (2016) EcoFlex: A Multifunctional MoClo Kit for E. coli Synthetic Biology, *ACS Synth Biol* 5,  
25 581 1059-1069.
- 26 582 (30) Patron, N. J., Orzaez, D., Marillonnet, S., Warzecha, H., Matthewman, C., Youles, M., Raitskin, O.,  
27 583 Leveau, A., Farre, G., Rogers, C., Smith, A., Hibberd, J., Webb, A. A., Locke, J., Schornack, S.,  
28 584 Ajioka, J., Baulcombe, D. C., Zipfel, C., Kamoun, S., Jones, J. D., Kuhn, H., Robatzek, S., Van  
29 585 Esse, H. P., Sanders, D., Oldroyd, G., Martin, C., Field, R., O'Connor, S., Fox, S., Wulff, B.,  
30 586 Miller, B., Breakspear, A., Radhakrishnan, G., Delaux, P. M., Loque, D., Granell, A., Tissier, A.,  
31 587 Shih, P., Brutnell, T. P., Quick, W. P., Rischer, H., Fraser, P. D., Aharoni, A., Raines, C., South, P.  
32 588 F., Ane, J. M., Hamberger, B. R., Langdale, J., Stougaard, J., Bouwmeester, H., Udvardi, M.,  
33 589 Murray, J. A., Ntoukakis, V., Schafer, P., Denby, K., Edwards, K. J., Osbourn, A., and Haseloff, J.  
34 590 (2015) Standards for plant synthetic biology: a common syntax for exchange of DNA parts,  
35 591 *New Phytol* 208, 13-19.
- 36 592 (31) Lopez-Paz, C., Liu, D., Geng, S., and Umen, J. G. (2017) Identification of Chlamydomonas  
37 593 reinhardtii endogenous genic flanking sequences for improved transgene expression, *Plant J*  
38 594 92, 1232-1244.
- 39 595 (32) Plucinak, T. M., Horken, K. M., Jiang, W., Fostvedt, J., Nguyen, S. T., and Weeks, D. P. (2015)  
40 596 Improved and versatile viral 2A platforms for dependable and inducible high-level expression  
41 597 of dicistronic nuclear genes in Chlamydomonas reinhardtii, *Plant J* 82, 717-729.
- 42 598 (33) Lumberras, V., Stevens, D. R., and Purton, S. (1998) Efficient foreign gene expression in  
43 599 Chlamydomonas reinhardtii mediated by an endogenous intron, *Plant J* 14, 441-447.
- 44 600 (34) Strenkert, D., Schmollinger, S., and Schroda, M. (2013) Heat shock factor 1 counteracts  
45 601 epigenetic silencing of nuclear transgenes in Chlamydomonas reinhardtii, *Nucleic Acids Res*  
46 602 41, 5273-5289.
- 47 603 (35) Neupert, J., Karcher, D., and Bock, R. (2009) Generation of Chlamydomonas strains that  
48 604 efficiently express nuclear transgenes, *Plant J* 57, 1140-1150.
- 49 605 (36) Schroda, M., Beck, C. F., and Vallon, O. (2002) Sequence elements within an HSP70 promoter  
50 606 counteract transcriptional transgene silencing in Chlamydomonas, *Plant J* 31, 445-455.
- 51 607 (37) Eichler-Stahlberg, A., Weisheit, W., Ruecker, O., and Heitzer, M. (2009) Strategies to facilitate  
52 608 transgene expression in Chlamydomonas reinhardtii, *Planta* 229, 873-883.

- 1  
2  
3 609 (38) Shao, N., and Bock, R. (2008) A codon-optimized luciferase from *Gussia principis* facilitates the  
4 610 in vivo monitoring of gene expression in the model alga *Chlamydomonas reinhardtii*, *Curr*  
5 611 *Genet* 53, 381-388.
- 6 612 (39) Hall, M. P., Unch, J., Binkowski, B. F., Valley, M. P., Butler, B. L., Wood, M. G., Otto, P.,  
7 613 Zimmerman, K., Vidugiris, G., Machleidt, T., Robers, M. B., Benink, H. A., Eggers, C. T., Slater,  
8 614 M. R., Meisenheimer, P. L., Klaubert, D. H., Fan, F., Encell, L. P., and Wood, K. V. (2012)  
9 615 Engineered luciferase reporter from a deep sea shrimp utilizing a novel imidazopyrazinone  
10 616 substrate, *ACS Chem Biol* 7, 1848-1857.
- 11 617 (40) Lodha, M., Schulz-Raffelt, M., and Schroda, M. (2008) A new assay for promoter analysis in  
12 618 *Chlamydomonas* reveals roles for heat shock elements and the TATA box in HSP70A  
13 619 promoter-mediated activation of transgene expression, *Eukaryot Cell* 7, 172-176.
- 14 620 (41) Barahimipour, R., Strenkert, D., Neupert, J., Schroda, M., Merchant, S. S., and Bock, R. (2015)  
15 621 Dissecting the contributions of GC content and codon usage to gene expression in the model  
16 622 alga *Chlamydomonas reinhardtii*, *Plant J* 84, 704-717.
- 17 623 (42) Best, R. J., Lyczakowski, J. J., Abalde-Cela, S., Yu, Z., Abell, C., and Smith, A. G. (2016) Label-Free  
18 624 Analysis and Sorting of Microalgae and Cyanobacteria in Microdroplets by Intrinsic  
19 625 Chlorophyll Fluorescence for the Identification of Fast Growing Strains, *Anal Chem* 88, 10445-  
20 626 10451.
- 21 627 (43) Ohresser, M., Matagne, R. F., and Loppes, R. (1997) Expression of the arylsulphatase reporter  
22 628 gene under the control of the nit1 promoter in *Chlamydomonas reinhardtii*, *Curr Genet* 31,  
23 629 264-271.
- 24 630 (44) Schmollinger, S., Strenkert, D., and Schroda, M. (2010) An inducible artificial microRNA system  
25 631 for *Chlamydomonas reinhardtii* confirms a key role for heat shock factor 1 in regulating  
26 632 thermotolerance, *Curr Genet* 56, 383-389.
- 27 633 (45) Helliwell, K. E., Scaife, M. A., Sasso, S., Araujo, A. P., Purton, S., and Smith, A. G. (2014)  
28 634 Unraveling vitamin B12-responsive gene regulation in algae, *Plant Physiol* 165, 388-397.
- 29 635 (46) Boudreau, E., Nickelsen, J., Lemaire, S. D., Ossenbuhl, F., and Rochaix, J. D. (2000) The Nac2 gene  
30 636 of *Chlamydomonas* encodes a chloroplast TPR-like protein involved in psbD mRNA stability,  
31 637 *EMBO J* 19, 3366-3376.
- 32 638 (47) Croft, M. T., Moulin, M., Webb, M. E., and Smith, A. G. (2007) Thiamine biosynthesis in algae is  
33 639 regulated by riboswitches, *Proc Natl Acad Sci U S A* 104, 20770-20775.
- 34 640 (48) Moulin, M., Nguyen, G. T., Scaife, M. A., Smith, A. G., and Fitzpatrick, T. B. (2013) Analysis of  
35 641 *Chlamydomonas* thiamin metabolism in vivo reveals riboswitch plasticity, *Proc Natl Acad Sci*  
36 642 *U S A* 110, 14622-14627.
- 37 643 (49) Molnar, A., Schwach, F., Studholme, D. J., Thuenemann, E. C., and Baulcombe, D. C. (2007)  
38 644 miRNAs control gene expression in the single-cell alga *Chlamydomonas reinhardtii*, *Nature*  
39 645 447, 1126-1129.
- 40 646 (50) Palombella, A. L., and Dutcher, S. K. (1998) Identification of the gene encoding the tryptophan  
41 647 synthase beta-subunit from *Chlamydomonas reinhardtii*, *Plant Physiol* 117, 455-464.
- 42 648 (51) Young, C. L., Britton, Z. T., and Robinson, A. S. (2012) Recombinant protein expression and  
43 649 purification: a comprehensive review of affinity tags and microbial applications, *Biotechnol J*  
44 650 7, 620-634.
- 45 651 (52) Crespo, J. L., Diaz-Troya, S., and Florencio, F. J. (2005) Inhibition of target of rapamycin signaling  
46 652 by rapamycin in the unicellular green alga *Chlamydomonas reinhardtii*, *Plant Physiol* 139,  
47 653 1736-1749.
- 48 654 (53) Crivat, G., and Taraska, J. W. (2012) Imaging proteins inside cells with fluorescent tags, *Trends*  
49 655 *Biotechnol* 30, 8-16.
- 50 656 (54) Engler, C., Gruetzner, R., Kandzia, R., and Marillonnet, S. (2009) Golden gate shuffling: a one-pot  
51 657 DNA shuffling method based on type IIs restriction enzymes, *PLoS One* 4, e5553.
- 52 658 (55) Greiner, A., Kelterborn, S., Evers, H., Kreimer, G., Sizova, I., and Hegemann, P. (2017) Targeting of  
53 659 Photoreceptor Genes in *Chlamydomonas reinhardtii* via Zinc-finger Nucleases and  
54 660 CRISPR/Cas9, *Plant Cell*.

- 1  
2  
3 661 (56) Sizova, I., Greiner, A., Awasthi, M., Kateriya, S., and Hegemann, P. (2013) Nuclear gene targeting  
4 662 in *Chlamydomonas* using engineered zinc-finger nucleases, *Plant J* 73, 873-882.
- 5 663 (57) Shin, S. E., Lim, J. M., Koh, H. G., Kim, E. K., Kang, N. K., Jeon, S., Kwon, S., Shin, W. S., Lee, B.,  
6 664 Hwangbo, K., Kim, J., Ye, S. H., Yun, J. Y., Seo, H., Oh, H. M., Kim, K. J., Kim, J. S., Jeong, W. J.,  
7 665 Chang, Y. K., and Jeong, B. R. (2016) CRISPR/Cas9-induced knockout and knock-in mutations  
8 666 in *Chlamydomonas reinhardtii*, *Sci Rep* 6, 27810.
- 9 667 (58) Baek, K., Kim, D. H., Jeong, J., Sim, S. J., Melis, A., Kim, J. S., Jin, E., and Bae, S. (2016) DNA-free  
10 668 two-gene knockout in *Chlamydomonas reinhardtii* via CRISPR-Cas9 ribonucleoproteins, *Sci*  
11 669 *Rep* 6, 30620.
- 12 670 (59) Ferenczi, A., Pyott, D. E., Xipnitou, A., and Molnar, A. (2017) Efficient targeted DNA editing and  
13 671 replacement in *Chlamydomonas reinhardtii* using Cpf1 ribonucleoproteins and single-  
14 672 stranded DNA, *Proc Natl Acad Sci U S A* 114, 13567-13572.
- 15 673 (60) Doron, L., Segal, N., and Shapira, M. (2016) Transgene Expression in Microalgae-From Tools to  
16 674 Applications, *Front Plant Sci* 7, 505.
- 17 675 (61) Sager, R. (1955) Inheritance in the Green Alga *Chlamydomonas Reinhardi*, *Genetics* 40, 476-489.
- 18 676 (62) Kuchka, M. R., Goldschmidt-Clermont, M., van Dillewijn, J., and Rochaix, J. D. (1989) Mutation at  
19 677 the *Chlamydomonas* nuclear NAC2 locus specifically affects stability of the chloroplast psbD  
20 678 transcript encoding polypeptide D2 of PS II, *Cell* 58, 869-876.
- 21 679 (63) Gorman, D. S., and Levine, R. P. (1965) Cytochrome f and plastocyanin: their sequence in the  
22 680 photosynthetic electron transport chain of *Chlamydomonas reinhardi*, *Proc Natl Acad Sci U S*  
23 681 *A* 54, 1665-1669.
- 24 682 (64) Kindle, K. L. (1990) High-frequency nuclear transformation of *Chlamydomonas reinhardtii*, *Proc*  
25 683 *Natl Acad Sci U S A* 87, 1228-1232.
- 26 684 (65) Molnar, A., Bassett, A., Thuenemann, E., Schwach, F., Karkare, S., Ossowski, S., Weigel, D., and  
27 685 Baulcombe, D. (2009) Highly specific gene silencing by artificial microRNAs in the unicellular  
28 686 alga *Chlamydomonas reinhardtii*, *Plant J* 58, 165-174.
- 29 687 (66) Llave, C., Xie, Z., Kasschau, K. D., and Carrington, J. C. (2002) Cleavage of Scarecrow-like mRNA  
30 688 targets directed by a class of Arabidopsis miRNA, *Science* 297, 2053-2056.
- 31 689 (67) Ries, F., Carius, Y., Rohr, M., Gries, K., Keller, S., Lancaster, C. R. D., and Willmund, F. (2017)  
32 690 Structural and molecular comparison of bacterial and eukaryotic trigger factors, *Sci Rep* 7,  
33 691 10680.
- 34 692 (68) Lauersen, K. J., Kruse, O., and Mussnug, J. H. (2015) Targeted expression of nuclear transgenes  
35 693 in *Chlamydomonas reinhardtii* with a versatile, modular vector toolkit, *Appl Microbiol*  
36 694 *Biotechnol* 99, 3491-3503.
- 37 695 (69) Roehner, N., Beal, J., Clancy, K., Bartley, B., Misirli, G., Grunberg, R., Oberortner, E., Pocock, M.,  
38 696 Bissell, M., Madsen, C., Nguyen, T., Zhang, M., Zhang, Z., Zundel, Z., Densmore, D., Gennari, J.,  
39 697 H., Wipat, A., Sauro, H. M., and Myers, C. J. (2016) Sharing Structure and Function in  
40 698 Biological Design with SBOL 2.0, *ACS Synth Biol* 5, 498-506.

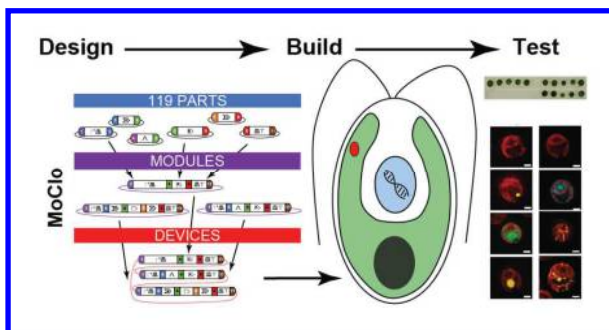
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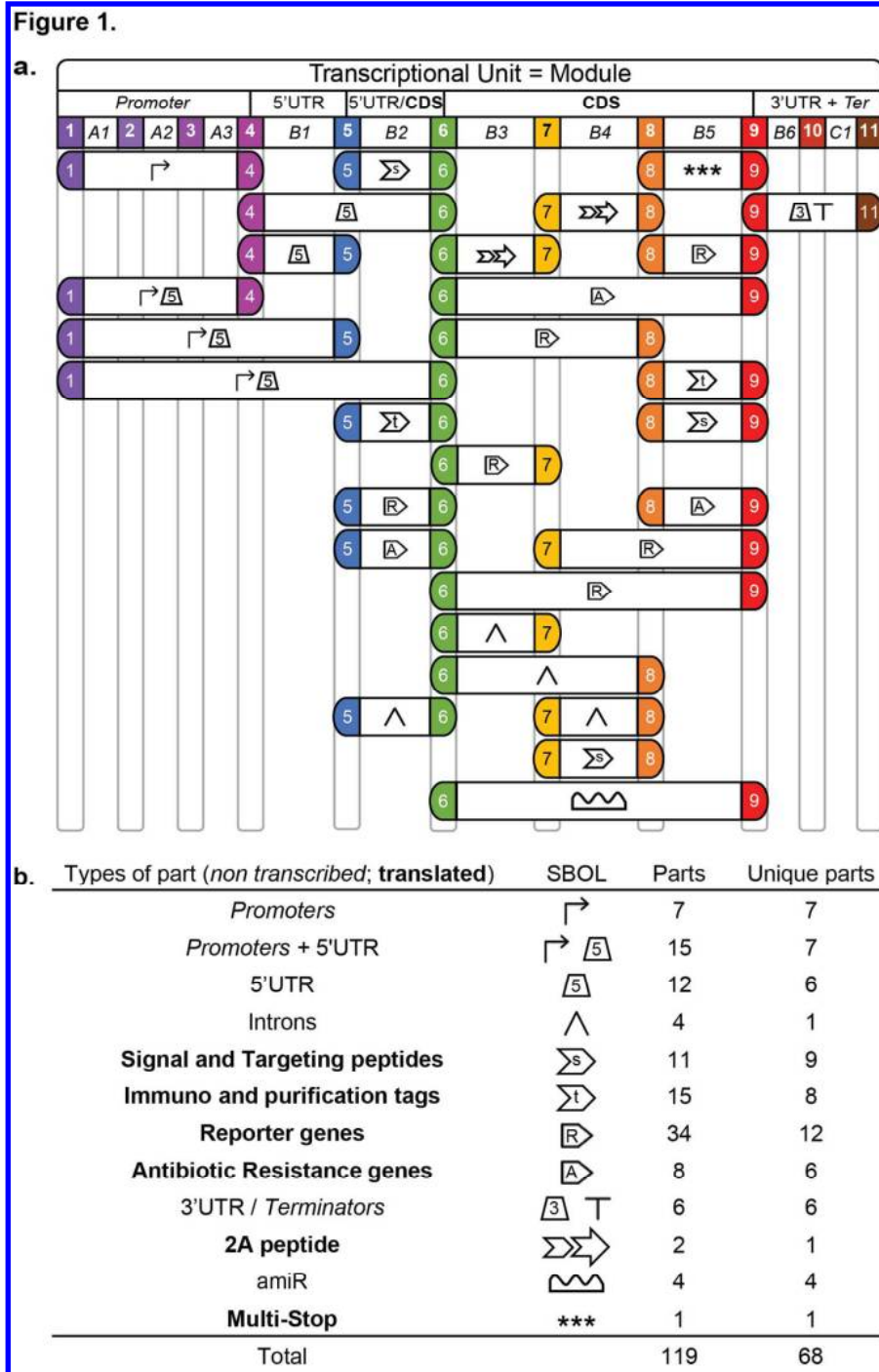
702 **Graphical Abstract**

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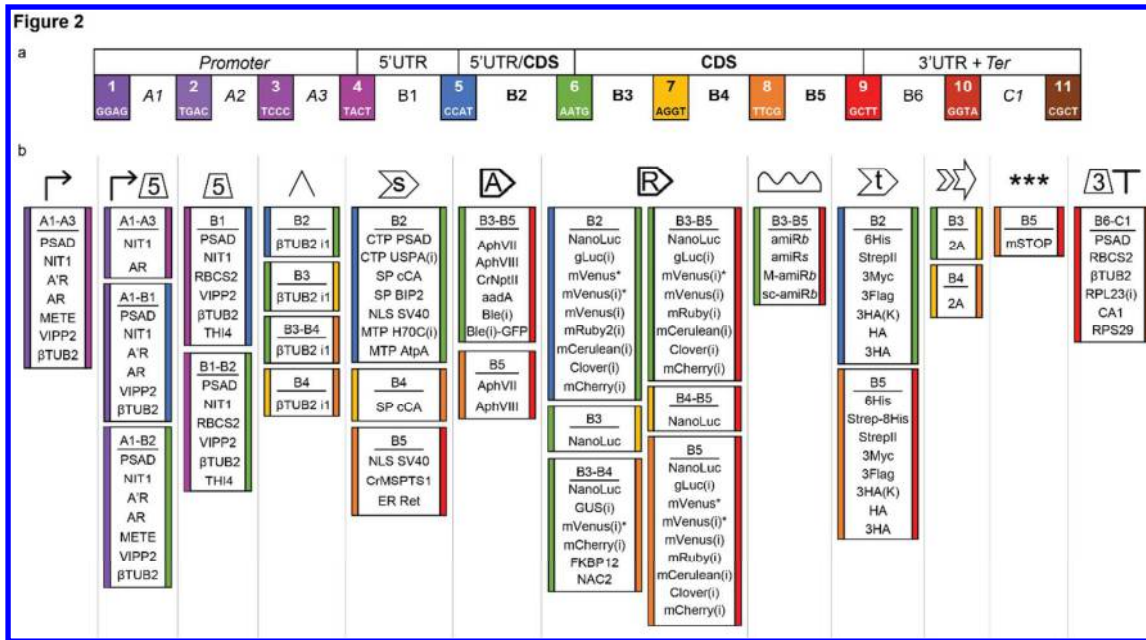
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707 **Figure 1.** Overview of the Chlamydomonas MoClo toolkit.

708 (a) Type and position of parts used following the Plant MoClo Syntax<sup>30</sup>. Symbols correspond to the SBOL2.0  
 709 visual<sup>69</sup> representation described in **b**. Each of the 11 fusion sites defining a part position is represented with a  
 710 color and a number. Positions presented are representative of the whole set of each part type. Parts in  
 711 italicized letters are non-transcribed, parts in regular letters are transcribed and parts in bold letters are  
 712 transcribed and translated.

713 (b) Table summarizing unique and total gene parts available. The SBOL2.0 symbols are indicated for each type.  
 714 When the SBOL2.0 standard was not existing for a part type, the symbol proposed before<sup>28</sup> was used, or  
 715 defined here.

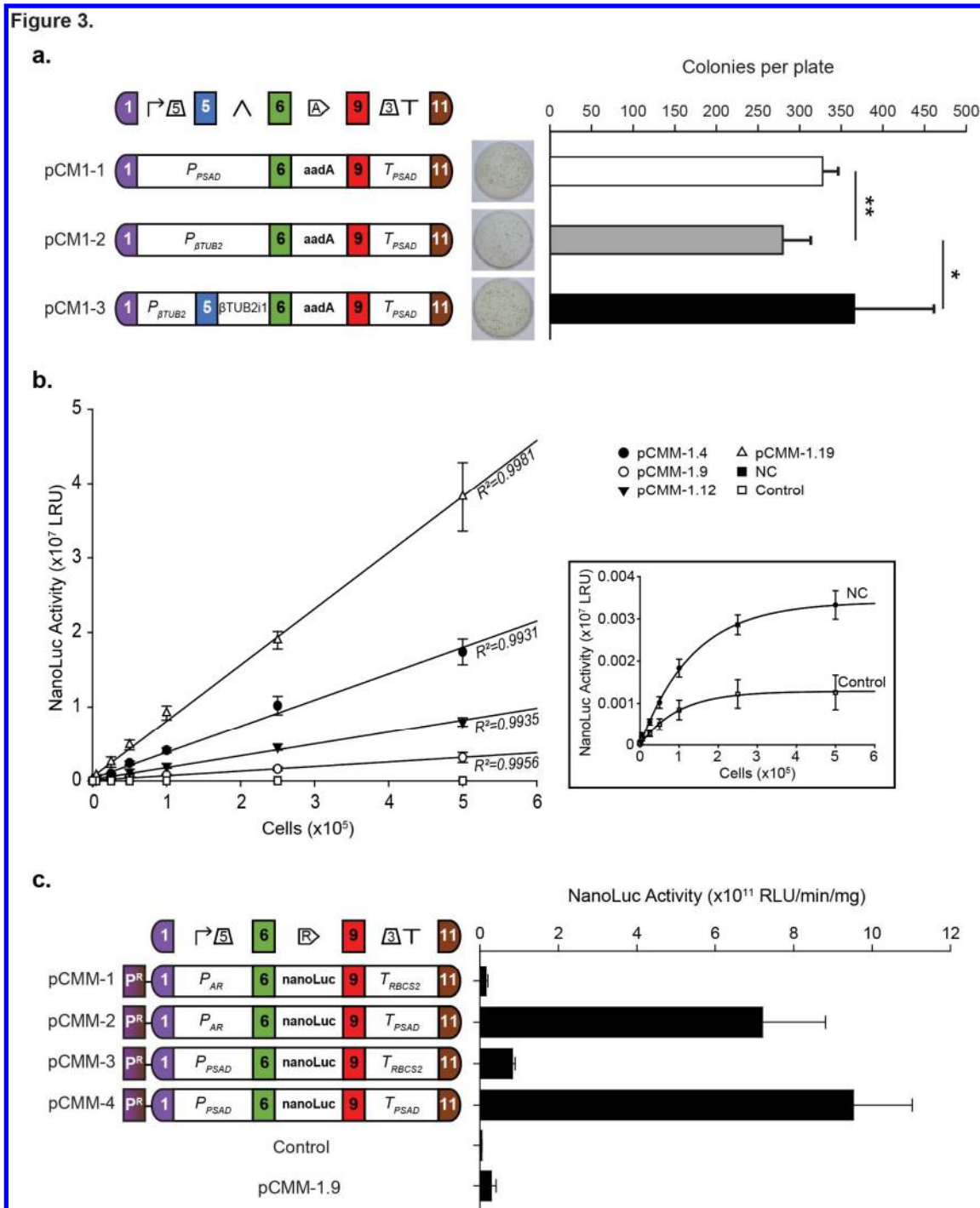
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717 **Figure 2. List of parts in function of their type and assembly position.**718 (a) Plant MoClo syntax<sup>30</sup> indicating the color code for fusion sites used in this figure.

719 (b) All parts in the Chlamydomonas MoClo kit are classified primarily by their function, indicated by  
 720 SBOL2.0 visual code<sup>69</sup> as in Fig. 1 (from left to right: promoters, promoter+5'UTR, 5'UTR, introns,  
 721 antibiotic resistance genes, reporter genes, artificial microRNA, immunological and purification tags,  
 722 2A peptide, and 3'UTR+terminators). Colored stripes on the left and right sides of each box represent  
 723 the fusion sites in 5' and 3' of the position, respectively, and follow the color code on top. AR and A'R  
 724 stand for HSP70A/RBCS2 and HSP70A467/RBCS2, respectively<sup>36</sup>. A star (\*) indicates that the part  
 725 contains extra restriction sites as in pOpt vectors<sup>68</sup> while the same part unmarked does not. An (i)  
 726 indicates the presence of an intron within the part (cf. Supplementary Table 2). For amiRNA (amiR)  
 727 backbones, *b* and *s* mean that *Bpi*I and *Spe*I site are within the backbone for amiR cloning,  
 728 respectively, while M and sc mean that the target amiR sequence for *MAA7* and the control  
 729 scrambled sequence were introduced into the miR1157 backbone, respectively (cf. Fig. 4). mSTOP  
 730 stands for multi-STOP.



731

732 **Figure 3.** Constitutive promoters and reporter genes.

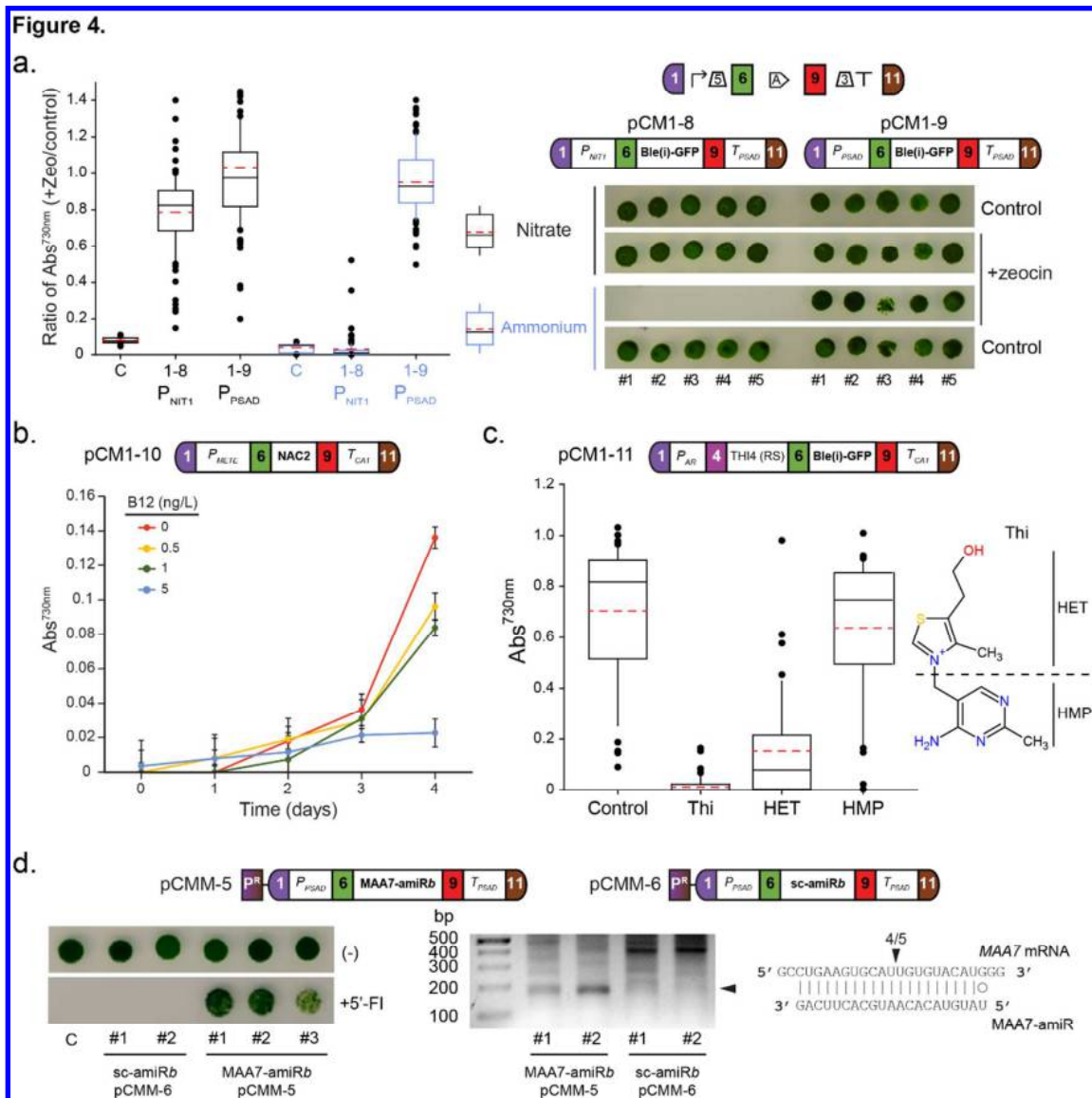
733 (a) Average number of spectinomycin resistant colonies after transformation of UVM4 cells (mean  $\pm$   
 734 SD, N=11) for the three modules (pCM1-3, where pCM stands for plasmid Chlamydomonas Moclo).  
 735 Representative transformation plates are shown.

736 (b) Linearity of NanoLuc activity as a function of cell number. NanoLuc activity for 4 independent  
 737 clones transformed with the pCMM-1 device (pCMM-1.X), one non-expressing clone (NC) and the  
 738 recipient strain (CC-4425 noted as control) are presented (N=3, mean  $\pm$  SEM). Linear regression and  
 739 correlation coefficient ( $R^2$ ) are shown. The NC and control are shown in the inset on a different scale.

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3 740 (c) Average NanoLuc activity of D66 (CC-4425) cells transformed with 4 devices (pCMM-1 to 4)  
4 741 harboring promoter/terminator combinations to drive NanoLuc expression coupled to a  
5 742 paromomycin resistance module (represented as P<sup>R</sup>, left, Supplementary Fig. 2). Luminescence levels  
6 743 are represented as mean ± SEM (average of a total of more than 400 clones from 3 biological  
7 744 replicates). The negative and positive controls are the recipient strain and the pCMM-1.9 strain  
8 745 (shown in **b**), respectively.  
9 746 **a,c** \*p<0.05; \*\*p< 0.01 assessed by Student's t-test, SBOL2.0<sup>69</sup> visual of module designs are shown  
10 747 above the devices.  
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749 **Figure 4.** Control of gene expression.

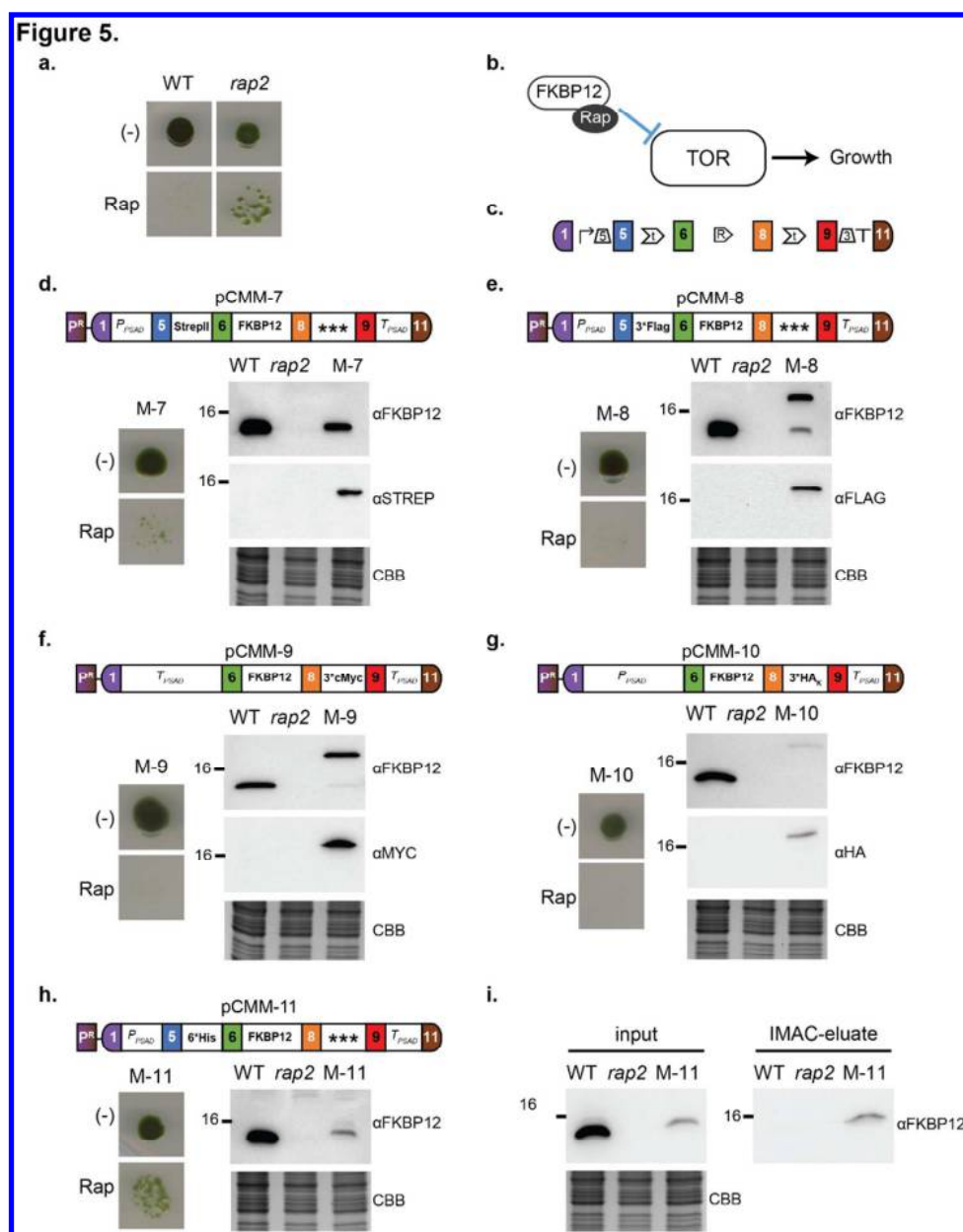
750 (a) Control of gene expression by the nitrogen source. Zeocin resistant colonies (conferred by *Ble(i)-GFP*)  
 751 selected after transformation of CC-1690 cells with each of the two represented modules (“1-8” for pCM1-8  
 752 and “1-9” for pCM1-9) were grown in TAP-nitrogen  $\pm$  zeocin (15  $\mu\text{g}/\text{mL}$ ) supplemented with either 7.5 mM  
 753 ( $\text{NH}_4$ )Cl (ammonium, blue) or 4 mM  $\text{KNO}_3$  (nitrate, black) and their growth was followed (Absorbance at 730  
 754 nm). The plot shows the ratio between the growth in the presence and absence of zeocin (C is the non-  
 755 transformed CC-1690 strain). The right panel shows cells grown in similar conditions but on solid media. Results  
 756 presented (N=16 for control CC-1690 and N=86 for each other conditions) correspond to one out of three  
 757 independent transformations (for the other two, see Supplementary Fig. 3).

758 (b) Control of gene expression by vitamin  $\text{B}_{12}$ . Conditional complementation of *nac2-26* cells with the pCM1-10  
 759 module expressing NAC2 under  $P_{\text{METE}}$  control. Complemented strains were selected for photoautotrophic  
 760 growth on solid minimal medium and the cells were grown in liquid minimal medium supplemented with the  
 761 indicated amount of vitamin  $\text{B}_{12}$ . Data are mean  $\pm$  SD (N=3).

762 (c) Control of gene expression by vitamin  $\text{B}_1$ . Average growth (absorbance at 730 nm after 7 days of growth,  
 763 N=40) of UVM4 cells transformed with the pCM1-11 module designed to express constitutively *Ble(i)-GFP*  
 764 transcripts containing the *THI4* riboswitch in the 5'UTR. After culture in TAP, the cells were transferred to  
 765 TAP+zeocin (10  $\mu\text{g}/\text{mL}$ ) supplemented with 10  $\mu\text{M}$  thiamine (Thi), 10  $\mu\text{M}$  4-methyl-5-(2-hydroxyethyl) thiazole

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3 766 (HET) or 10  $\mu$ M 4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP) or not (control). The chemical structure  
4 767 of Thi is represented on the right and the HET and HMP moieties are indicated (See also Supplementary Fig. 3).  
5 768 (d) Targeted gene knockdown with artificial miRNA. Paromomycin resistant cells selected after transformation  
6 769 of CC-1690 cells by each of the two represented devices (pCMM-5 and pCMM-6), carrying an amiRNA cloned  
7 770 with *Bpil* and directed against *MAA7* (*MAA7-amiRb*) or a random sequence ('scrambled': *sc-amiRb*), were  
8 771 grown in the absence (denoted (-)) or presence of 5'-fluoroindole (+5'-FI) (left panel). C indicates non-  
9 772 transformed cells. Clones resistant to 5'FI were analyzed by a modified 5'-RACE assay. A specific 173 bp PCR  
10 773 band (black arrowhead) was amplified only from the 5'-FI resistant transformants and not from ones expressing  
11 774 the amiRNA with scrambled sequence (middle panel and Supplementary Fig. 3). Sequencing revealed that the  
12 775 most frequent cleavage occurred at positions opposed to positions 10 and 11 of the amiRNA (right panel, black  
13 776 arrowhead). P<sup>R</sup> represents the paromomycin resistance module (pCM1-27, Supplementary Fig. 2a).  
14 777 a, c The box and whisker plots show the 10<sup>th</sup> (lower whisker), 25<sup>th</sup> (base of box), 75<sup>th</sup> (top of box) and 90<sup>th</sup> (top  
15 778 whisker) percentiles. The line within the box is the median, the dashed red line is the mean. Outliers are  
16 779 plotted as individual data points.  
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**Figure 5.** Design, build and test of five fusion tags

(a) Phenotype of recipient (WT) and  $\Delta$ FKBP12 (*rap2*) strains in the presence (Rap) or absence (-) of 1  $\mu$ M rapamycin.

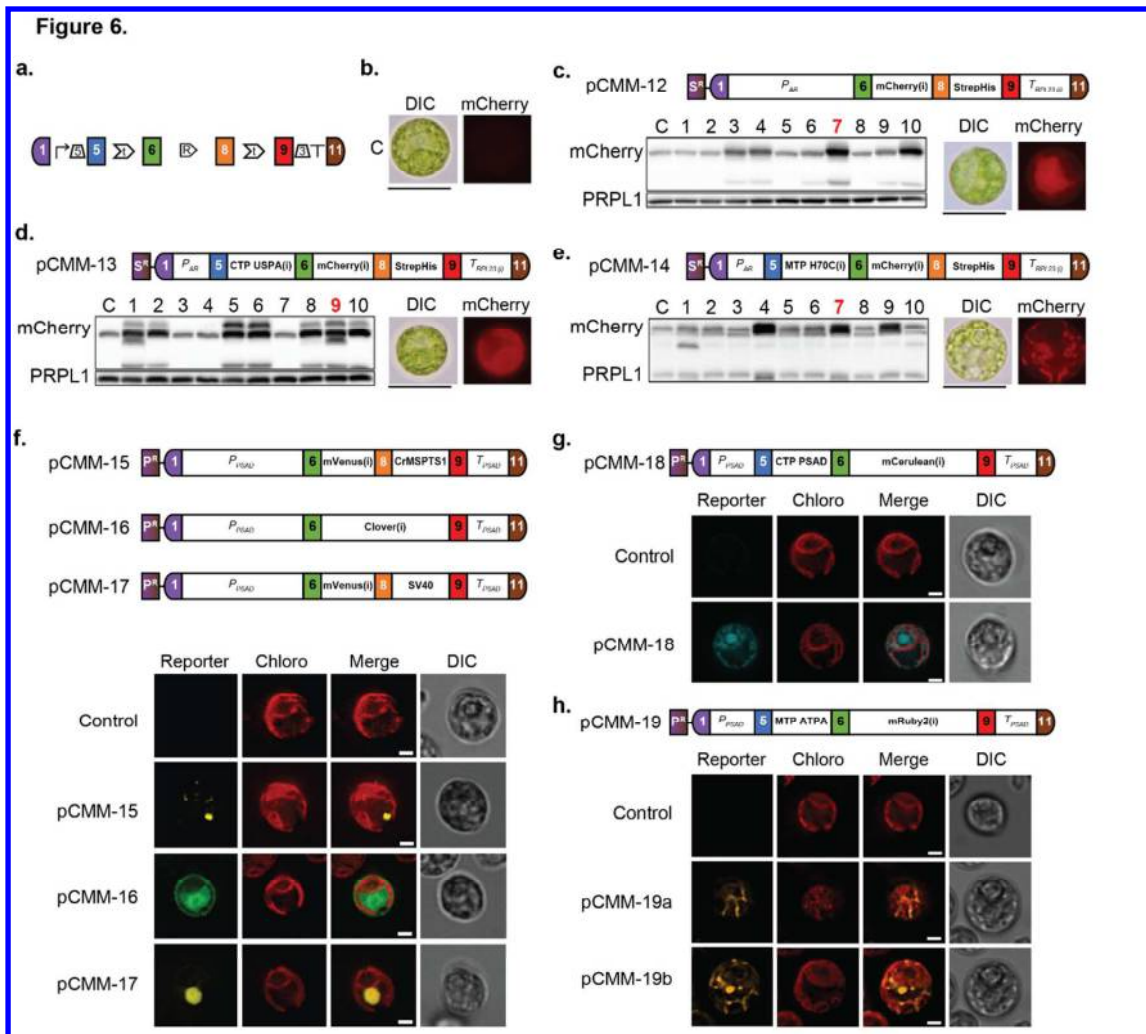
(b) Molecular mechanism underlying the *rap2* phenotype. Target Of Rapamycin (TOR) is inhibited by rapamycin only in the presence of FKBP12 (mutated in *rap2*). Upon formation of the tripartite TOR/FKBP12/rapamycin complex, TOR is inhibited and growth is arrested<sup>52</sup>.

(c) SBOL2.0 visual<sup>69</sup> of module designs for functional complementation of *rap2*.

(d-h) Phenotype in the presence (Rap) or absence (-) of 1  $\mu$ M rapamycin and detection of tagged proteins in soluble extracts by immunoblotting with antibodies against either FKBP12 ( $\alpha$ FKBP12) or the appropriate tag (indicated within each panel). Each device is indicated in the upper part of the panel. CBB: Coomassie Brilliant Blue staining of a duplicate gel loaded with the same samples and shown as loading control.

(i) Purification through ion-metal affinity chromatography (IMAC) of 6His-FKBP12 expressed from the same device as in h.

P<sup>R</sup> represents the paromomycin resistance module (pCM1-27, Supplementary Fig. 2a). *rap2* cells transformed with pCMM-X are indicated as M-X in each panel. Data are representative of 3 biological replicates.



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798 **Figure 6. Targeting reporter genes to different subcellular compartments.**799 (a) SBOL2.0<sup>69</sup> visual syntax for modules used.800 (b) Visible light ("DIC") and fluorescence signal ("mCherry") of the UVM4 recipient strain used as control ("C")  
801 for panels c-e.802 (c-e) mCherry targeting to the (c) cytosol with no transit peptide, (d) chloroplast with CTP USPA (Chloroplast  
803 Transit Peptide of Universal Stress Protein A) or (e) mitochondria with MTP H70C (Mitochondrial Transit  
804 Peptide of HSP70C) in UVM4 cells transformed with the indicated devices (pCMM-12 to 14). In each panel, an  
805 anti-mCherry immunoblot analysis of transformants is shown. Note that the anti-mCherry antibody cross-reacts  
806 with a protein of similar size present in control cells (C). An anti-PRPL1 immunoblot is shown as loading control.  
807 The transformant strain number indicated in red corresponds to the images (bars are 10  $\mu$ m) presented on the  
808 right.809 (f-h) Fluorescent marking of (f) microbodies with mVenus-CrMSPTS1 (Malate Synthase PTS1-like sequence),  
810 cytosol with Clover or the nucleus with mVenus-SV40 (Simian Virus 40 nuclear localization signal), (g) the  
811 chloroplast with CTP PSAD-mCerulean (Chloroplast Transit Peptide of PSAD), (h) mitochondria with MTP ATPA-  
812 mRuby2 (Mitochondrial Transit Peptide of ATPA) after transformation of UVM4 cells with the indicated devices  
813 (pCMM-15 to 19). Images of representative transformants are grouped with the corresponding control image  
814 (recipient strain) according to the filter used. pCMM-19a and pCMM-19b show two images taken on different  
815 z-axis on the same cell. "Chloro" refers to chlorophyll autofluorescence. The Scale bars represent 2  $\mu$ m.816 S<sup>R</sup> and P<sup>R</sup> represent respectively modules conferring resistance to spectinomycin (S<sup>R</sup>=pCM1-1, Fig. 3a and  
817 Supplementary Fig. 2a) and paromomycin (P<sup>R</sup>=pCM1-27, Supplementary Fig. 2a).