A genome-wide algal mutant library and functional screen identifies genes required for eukaryotic photosynthesis

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Photosynthetic organisms provide food and energy for nearly all life on Earth, yet half of their protein-coding genes remain uncharacterized 4–6. Characterization of these genes could be greatly accelerated by new genetic resources for unicellular organisms. Here we generated a genome-wide, indexed library of mapped insertion mutants for the unicellular alga *Chlamydomonas reinhardtii*. The 62,389 mutants in the library, covering 83% of nuclear protein-coding genes, are available to the community. Each mutant contains unique DNA barcodes, allowing the collection to be screened as a pool. We performed a genome-wide survey of genes required for photosynthesis, which identified 303 candidate genes. Characterization of one of these genes, the conserved predicted phosphatase-encoding gene CPL3, showed that it is important for accumulation of multiple photosynthetic protein complexes. Notably, 21 of the 43 higher-confidence genes are novel, opening new opportunities for advances in understanding of this biogeochemically fundamental process. This library will accelerate the characterization of thousands of genes in algae, plants, and animals.

The green alga *Chlamydomonas* has long been used for genetic studies of eukaryotic photosynthesis because of its rare ability to grow in the absence of photosynthetic function 1. In addition, it has made extensive contributions to basic understanding of light signaling, stress acclimation, and metabolism of carbohydrates, lipids, and pigments (Fig. 1a) 4–6. Moreover, *Chlamydomonas* has retained many genes from the plant–animal common ancestor, which has contributed to understanding of fundamental aspects of the structure and function of cilia and basal bodies 2,3. Like *Saccharomyces cerevisiae*, *Chlamydomonas* can grow as a haploid, facilitating genetic studies. However, until now, the value of *Chlamydomonas* had been limited by the lack of mutants for most of its nuclear genes.

In the present study, we sought to generate a genome-wide collection of *Chlamydomonas* mutants with known gene disruptions to provide mutants in genes of interest for the scientific community and then to leverage this collection to identify genes with roles in photosynthesis. To reach the necessary scale, we chose to random insertion mutagenesis and built on advances in insertion mapping and mutant propagation from our pilot study 9. To enable mapping of insertion sites and screening of pools of mutants on a much larger scale, we developed new tools leveraging unique DNA barcodes in each transforming cassette.

We generated mutants by transforming haploid cells with DNA cassettes that randomly insert into the genome and inactivate the genes into which they insert. We maintained the mutants as indexed colony arrays on agar medium containing acetate as a carbon and energy source to allow recovery of mutants with defects in photosynthesis. Each DNA cassette contained two unique barcodes, one on each side of the cassette (Supplementary Fig. 1a–d). For each mutant, the barcode and genomic flanking sequence on each side of the cassette were initially unknown (Supplementary Fig. 1e). We determined the sequence of the barcodes in each mutant colony by combinatorial pooling and deep sequencing (Supplementary Figs. 1f and 2). We then mapped each insertion by pooling all mutants and amplifying all flanking sequences together with their corresponding barcodes, followed by deep sequencing (Supplementary Fig. 1g). The combination of these datasets identified the insertion site(s) in each mutant. This procedure yielded 62,389 mutants on 245 plates, with a total of 74,923 insertions that were largely randomly distributed over the chromosomes (Fig. 1b,c, Supplementary Figs. 3 and 4, and Supplementary Table 5).

This library provides mutants for ~83% of all nuclear genes (Fig. 2a–d). Approximately 69% of genes are represented by an insertion in a 5′ UTR, an exon, or an intron—the regions in which disruption is most likely to cause an altered phenotype. Many gene sets of interest to the research community are well represented, including genes encoding proteins phylogenetically associated with the plant lineage (GreenCut2) 2, proteins that localize to the chloroplast (Fig. 2b), and proteins associated with the structure and function of flagella or basal bodies 3,4 (Fig. 2b). Mutants in this collection are available through the CLIP website (see URLs). Over 1,800 mutants have already been distributed to over 200 laboratories worldwide in the first 18 months of prepublication distribution (Fig. 2c). These mutants are facilitating genetic investigation of a broad range of processes, ranging from photosynthesis and metabolism to cilia structure and function (Fig. 2f).
To identify genes required for photosynthesis, we screened our library for mutants deficient in photosynthetic growth. Rather than phenotyping each strain individually, we pooled the entire library into one culture and leveraged the unique barcodes present in each strain to track the abundance of individual strains after growth under different conditions. This feature enables genome-wide screening with speed and depth unprecedented in photosynthetic eukaryotes. We grew the pool of mutants photosynthetically in the light in minimal Tris-phosphate (TP) medium with carbon dioxide (CO₂) as the sole source of carbon and heterotrophically in the dark in Tris-acetate-phosphate (TAP) medium, where acetate provides fixed carbon and energy (Fig. 3a). To quantify mutant growth under each condition, we amplified and performed deep sequencing of the barcodes from the final cell populations. We then compared the ability of each mutant to grow under the photosynthetic and heterotrophic conditions by comparing the read counts for each barcode in the two conditions (Supplementary Table 10 and Supplementary Note). Mutant phenotypes were highly reproducible (Fig. 3b and
In total, we identified 3,109 mutants deficient in photosynthetic growth (Fig. 3c and Supplementary Note). To identify genes with roles in photosynthesis, we developed a statistical analysis framework that leverages the presence of multiple alleles for many genes. This framework allows us to overcome several sources of false positives that have been difficult to account for with previous methods, including cases where the phenotype is not caused by the mapped disruption. For each gene, we counted the number of mutant alleles with and without a phenotype and evaluated the likelihood of obtaining these numbers by chance given the total number of mutants in the library that exhibited the phenotype (Supplementary Table 11 and Supplementary Note).

We identified 303 candidate photosynthesis genes on the basis of our statistical analysis. These genes are enriched for membership in a diurnally regulated photosynthesis-related transcriptional cluster \( (P < 1 \times 10^{-11}) \), are enriched for upregulation upon dark-to-light transitions \( (P < 0.003) \), and encode proteins enriched for predicted chloroplast localization \( (P < 1 \times 10^{-7}) \). As expected\(^{15}\), the candidate
Fig. 3 | A high-throughput screen using the library identifies many genes with known roles in photosynthesis and many novel components. a, Unique barcodes allow screening of mutants in a pool. Mutants deficient in photosynthesis can be identified because their barcodes will be less abundant after photosynthetic growth than they are after heterotrophic growth. b, Biological replicates were highly reproducible, with a Spearman’s correlation coefficient of 0.982. Each dot represents one barcode. See also Supplementary Fig. 5. c, The phenotype of each insertion was determined by comparing its read counts under the photosynthetic and heterotrophic conditions. Insertions that fell below the phenotype cutoff were considered to result in a defect in photosynthetic growth. d, Biological replicates were highly reproducible, with a Spearman’s correlation coefficient of 0.982. Each dot represents one barcode. See also Supplementary Fig. 5. c, The phenotype of each insertion was determined by comparing its read counts under the photosynthetic and heterotrophic conditions. Insertions that fell below the phenotype cutoff were considered to result in a defect in photosynthetic growth. e, Exon and intron insertions are most likely to show strong phenotypes, whereas 3' UTR insertions rarely do. The plot is based on all insertions for the 43 higher-confidence genes. e, The photosynthetic/heterotrophic ratios for all alleles are shown for higher-confidence photosynthesis-screen hit genes and control genes. Each column is a gene; each horizontal bar is an allele. f, The 303 candidate genes were categorized on the basis of statistical confidence in this screen and whether they had a previously known function in photosynthesis (Supplementary Note). g, Known higher-confidence genes, novel higher-confidence genes, and lower-confidence genes are all enriched in predicted chloroplast-targeted proteins (P < 0.011). h, A schematic summary illustrating the numbers of candidate genes in each category (as in f) and the specific functions of the genes with a known role in processes related to photosynthesis.
genes also encode a disproportionate number of GreenCut2 proteins (P < 1 × 10^{-10}), which are conserved among photosynthetic organisms but absent from non-photosynthetic organisms: 32 GreenCut2 proteins are encoded by the 303 candidate genes (11%), as compared to ~3% of genes in the entire genome.

Photosynthesis occurs in two stages: the light reactions and carbon fixation. The light reactions convert solar energy into chemical energy and require the coordinated action of photosystem II (PSII), cytochrome b/f, photosystem I (PSI), ATP synthase complexes, and a plastocyanin or cytochrome c₆ metalloprotein, as well as small-molecule cofactors⁶. PSII and PSI are each assisted by peripheral light-harvesting complexes (LHCs) known as LHCl and LHCl, respectively. Carbon fixation is performed by enzymes in the Calvin–Benson–Bassham (CBB) cycle, including the CO₂-fixing enzyme Rubisco. In addition, most eukaryotic algae have a mechanism to concentrate CO₂ around Rubisco to enhance its activity⁷.

Sixty-five of the genes we identified encode proteins that were previously shown to have a role in photosynthesis or chloroplast function in *Chlamydomonas* or vascular plants (Fig. 3). These include 3 PSI–LHClI subunits (PSBP1, PSBP2, and PSB27) and 7 PSI–LHClII biosynthesis factors (CGL54, CPLD10, HCF136, LPA1, MBBI, TBC2, and Cre02.g105650), 2 cytochrome b/f complex subunits (PETC and PTEM) and 6 cytochrome b₆ biosynthesis factors (CCB2, CCS5, CPLD43, CPLD49, MCD1, and MGC1), 5 PSI–LHClI subunits (LHClA, LHClA1, PSAD, PSAE, and PSAL) and 9 PSI–LHClII biosynthesis factors (CGL71, CPLD46, OPR120, RAAl, RAa2, RAa3, RAT2, Cre01.g045902, and Cre09.g389615), a protein required for ATP synthase function (PET3), plastocyanin (PCY1) and 2 plastocyanin biosynthesis factors (CPT2 and PCC1), 12 proteins involved in the metabolism of photosynthesis cofactors or signaling molecules (CHLD, CTH1, CYP745A1, DVR1, HMOX1, HPD2, MTF1, PLAP6, UORD3, Cre08.g358538, Cre13.g501850, and Cre16.g659050), 3 CBB cycle enzymes (FBP1, PRK1, and SEB1), 2 Rubisco biosynthesis factors (MR11 and RMT2), and 3 proteins involved in the algal carbon-concentrating mechanism (CAG3, CAS1, and LGIB), as well as proteins that have a role in photosrespiration (GSF1), CO₂ regulation of photosynthesis (Cre02.g146851), chloroplast morphogenesis (Cre14.g616600), chloroplast protein import (SDR17), and chloroplast DNA, RNA, and protein metabolism (DEG9, MSF11, MSRA1, TSM2, and Cre01.g010864) (Fig. 3b and Supplementary Table 12). We caution that not all genes previously demonstrated to be required for photosynthetic growth were detectable by this approach, especially the ones with paralogous copies in the genome, such as RBCS1 and RBCS2, which encode the small subunit of Rubisco. Nonetheless, the large number of known factors recovered in our screen is a testament to the power of this approach.

In addition to recovering these 65 genes with known roles in photosynthesis, our analysis identified 238 candidate genes with no previous involvement in photosynthesis, our analysis identified 238 candidate genes with no previous involvement in photosynthesis, our analysis identified 238 candidate genes with no previous involvement in photosynthesis, our analysis identified 238 candidate genes with no previous involvement in photosynthesis, our analysis identified 238 candidate genes with no previous involvement in photosynthesis, our analysis identified 238 candidate genes with no previous involvement in photosynthesis, our analysis identified 238 candidate genes with no previous involvement in photosynthesis, our analysis identified 238 candidate genes with no previous involvement in photosynthesis, our analysis identified 238 candidate genes with no previous involvement in photosynthesis, our analysis identified 238 candidate genes with no previous involvement in photosynthesis. These 238 genes represent a rich set of targets to better understand photosynthesis. Because our screen likely yielded some false positives, we divided all genes into ‘higher-confidence’ (P < 0.0011; false-discovery rate (FDR) < 0.27) and ‘lower-confidence’ genes on the basis of the number of alleles that supported each gene’s involvement in photosynthesis (Fig. 3d–f, Tables 1 and 2, and Supplementary Note). The 21 higher-confidence genes with no previously reported role in photosynthesis are enriched in chloroplast localization (9/21, P < 0.011; Fig. 3g) and transcriptional upregulation during dark-to-light transition (5/21, P < 0.005), similarly to the known photosynthesis genes. Thus, these 21 higher-confidence genes are particularly high-priority targets for the field to pursue.

Functional annotations for 15 of the 21 higher-confidence genes suggest that these genes could have roles in regulation of photosynthesis, photosynthetic metabolism, and biosynthesis of the photosynthetic machinery. Seven of the genes likely have roles in regulation of photosynthesis: GEF1 encodes a voltage-gated channel, Cre01.g080550 and Cre02.g111150 encode putative protein kinases, CPL3 encodes a predicted protein phosphatase, the protein encoded by TRX21 contains a thioredoxin domain, Cre12.g542569 encodes a putative glutamate receptor, and the protein encoded by Cre13.g586750 contains a predicted nuclear importin domain. Six of the genes are likely involved in photosynthetic metabolism: the *Arabidopsis thaliana* homolog of Cre10.g448950 modulates sucrose and starch accumulation⁹, the protein encoded by Cre11.g467712 contains a starch-binding domain, Cre02.g073900 encodes a putative carotenoid oxygenase, VTE₅ encodes a putative phosphatase, the protein encoded by Cre10.g429650 encodes a putative alpha/beta hydrolase, and the protein encoded by Cre50.g761497 contains a magnesium transporter domain. Finally, two of the genes are likely to have roles in the biogenesis and function of photosynthetic machinery: the protein encoded by *EIF2* has a translation initiation factor domain and *CD12* encodes a protein with a chloroplast DnaJ domain. Future characterization of these genes by the community is likely to yield fundamental insights into photosynthesis.

As an illustration of the value of the genes identified in this screen, we sought to explore the specific function of one of the higher-confidence candidate genes, CPL3 (conserved in plant lineage 3; Cre03.g185200, also known as *MPA6*), which encodes a putative protein phosphatase (Fig. 4a and Supplementary Fig. 6). Many proteins in the photosynthetic apparatus are phosphorylated, but the role and regulation of these phosphorylation events are poorly understood⁹. An insertion junction that mapped to the 3’ UTR of CPL3 was previously found in a collection of acetate-requiring mutants, although it was not determined whether this mutation caused the phenotype⁴. In our screen, three mutants with insertion junctions in CPL3 exons or introns exhibited a deficiency in photosynthetic growth (Fig. 3c and Supplementary Table 13). We chose to examine one allele (LMI_RY0402.153647, referred to hereafter as cpl3; Fig. 4a and Supplementary Fig. 6a) for phenotypic confirmation, genetic complementation, and further studies.

Consistent with the pooled growth data, the cpl3 mutant showed a severe defect in photosynthetic growth on agar, which was rescued under heterotrophic conditions (Fig. 4b). We confirmed that the CPL3 gene was disrupted in the cpl3 mutant and found that complementation with a wild-type copy of the CPL3 gene rescued the phenotype, demonstrating that the mutation in CPL3 was the cause of the growth defect of the mutant (Supplementary Note and Supplementary Fig. 6a–d).

We then examined photosynthetic performance, morphology of the chloroplast, and composition of photosynthetic pigments and proteins in cpl3. The photosynthetic electron transport rate was decreased under all light intensities, suggesting a defect in the photosynthetic machinery (Fig. 4c). The chloroplast morphology of cpl3 appeared similar to that of the wild type on the basis of chlorophyll fluorescence microscopy (Supplementary Fig. 7a). However, we observed a lower chlorophyll a/chlorophyll b ratio in cpl3 than in the wild type (Supplementary Fig. 7b), which suggests a defect in the accumulation or composition of the protein–pigment complexes involved in the light reactions⁹. By using whole-cell proteomics, we found that cpl3 was deficient in accumulation of all detectable subunits of the chloroplast ATP synthase (ATPC, ATPD, ATPG, AtpaA, AtPB, AtpE, and AtpF), some subunits of PSI (D1, D2, CP43, CP47, PbsE, and PbsH), and some subunits of PSI (PsaaA and PsabB) (FDR < 0.31 for each subunit; Fig. 4d, f and Supplementary Table 14). We confirmed these findings with western blots for CP43, PsaaA, and ATPC (Fig. 4e and Supplementary Fig. 7c). Our results indicate that CPL3 is required for normal accumulation of thylakoid protein complexes (PSI, PSI, and ATP synthase) involved in the light reactions of photosynthesis.

Our finding that 21 of the 43 higher-confidence photosynthesis genes identified were uncharacterized suggests that nearly half of
### Table 1 | Higher-confidence genes from the photosynthesis screen with a previously known role in photosynthesis

| Category                                      | Gene                        | Definition or description in Phytozome | PredAlgo Alleles in two replicates | Arabidopsis homolog | Reference and corresponding organism(s) |
|-----------------------------------------------|-----------------------------|----------------------------------------|-----------------------------------|---------------------|----------------------------------------|
| Calvin-Benson-Bassham cycle                   | Cre03.g185550 (SEBP1, SBP1) | Sedoheptulose-1,7-bisphosphatase       | C 3 0 0.021                       | AT3G55800.1 (SBP42) | Arabidopsis                            |
|                                              | Cre12.g524500 (RMT2)        | Rubisco small subunit N-methyltransferase | O 3 0 0.018 | AT3G07670.1 | Pium                                   |
|                                              | Cre06.g298300 (MRL1, PPR2)  | Pentatricopeptide-repeat protein, stabilizes rcr mRNA | C 1 1 1.000 | AT4G34830.1 (MRL1) | Chlamydomonas and Arabidopsis          |
| Carbon-concentrating mechanism                | Cre12.g497300 (CA31, TEF2)  | Rhodanese-like calcium-sensing receptor | C 2 0 0.260 | AT5G23060.1 (CaS) | Chlamydomonas                          |
|                                              | Cre10.g452800 (LCIB)        | Low-CO2-inducible protein              | C 2 0 0.260 | AT5G09820.2 | Arabidopsis                            |
|                                              | Cre14.g616600               | –                                      | M 4 3 0.021 | AT1G03160.1 (FZI) | Arabidopsis                            |
| Chloroplast and thylakoid morphogenesis      | Cre13.g581850               | –                                      | M 5 5 0.010 | AT4G31390.1 | Arabidopsis                            |
|                                              | Cre10.g423500 (HMOX1, HMO1) | Heme oxygenase                        | C 3 0 0.021 | AT1G69720.1 (HO3) | Chlamydomonas                          |
|                                              | Cre03.g188700 (PLAP6, PLP6) | Plastid lipid-associated protein, fibrillin | C 3 1 0.070 | AT5G09820.2 | Arabidopsis                            |
|                                              | Cre16.g659050               | –                                      | C 4 6 0.098 | AT1G68890.1 | Chlamydomonas                          |
| PSI protein synthesis and assembly           | Cre12.g524300 (CGL71)       | Predicted protein                      | C 2 0 0.260 | AT1G22700.1 | Synechocystis, Arabidopsis, Chlamydomonas | Arabidopsis |
|                                              | Cre01.g045902               | –                                      | C 2 0 0.239 | AT3G24430.1 (HCF101) | Arabidopsis |
| PSI RNA splicing and stabilization           | Cre09.g389615               | –                                      | M 5 0 0.0002 | AT3G17040.1 (HCF107) | Chlamydomonas, Arabidopsis |
|                                              | Cre01.g027150 (CPLD46, HEL5) | DEAD/DEAH-box helicase                | M 5 1 0.0004 | AT1G70070.1 (EMB25, ISE2) | Arabidopsis |
|                                              | Cre09.g394150 (RAA1)        | –                                      | M 5 1 0.0004 | AT1G70070.1 (EMB25, ISE2) | Chlamydomonas |
|                                              | Cre12.g531050 (RAA2)        | psaA mRNA maturation factor 3          | C 3 0 0.021 | – | Chlamydomonas |
|                                              | Cre10.g440000 (OPR120)      | –                                      | C 2 0 0.260 | – | Chlamydomonas |
| PSII protein synthesis and assembly          | Cre13.g578650 (CPLD10, NUOA5) | Similar to complex I intermediate-associated | C 3 3 0.208 | AT1G16720.1 (HCF173) | Arabidopsis |
|                                              | Cre02.g073850 (CGL54)       | Predicted protein                      | C 2 0 0.260 | AT1G05385.1 (LPA19, Psb27-HT) | Arabidopsis |
|                                              | Cre02.g105650               | –                                      | C 2 0 0.260 | AT5G51545.1 (LPA2) | Arabidopsis |
|                                              | Cre06.g273700 (HCF136)      | –                                      | C 2 0 0.260 | AT5G23120.1 (HCF136) | Arabidopsis, Synechocystis |
|                                              | Cre10.g430150 (LP1A1, REP27) | –                                      | C 2 0 0.260 | AT1G02910.1 (LPA1) | Arabidopsis |

*Predicton of protein localization by PredAlgo; C, chloroplast; M, mitochondrion; S, secretory pathway; O, other. The number of exon, intron, or 5′ UTR mutant alleles for the gene that satisfied our minimum read count requirement but not the requirement for at least tenfold depletion in the TP-light condition. The FDR for the gene in comparison to all alleles for all genes (Supplementary Note). Arabidopsis homolog, obtained from the 'best_arabidopsis_TAIR10_Hit_name' field in Phytozome. AT3G17040.1 is required for functional PSII in Arabidopsis, whereas Cre09.g389615 was shown to be involved in PSI accumulation in Chlamydomonas.*
the genes required for photosynthesis remain to be characterized. This finding is notable considering that genetic studies on photosynthesis extend back to the 1950s22. Our validation of the role of CPL3 in photosynthesis illustrates the value of the uncharacterized genes identified in this study as a rich set of candidates for the community to pursue.

More broadly, it is our hope that the mutant resource presented here will serve as a powerful complement to newly developed

| Gene | Definition or description in Phytozome | PredAlgo | Alleles in two replicates | Arabidopsis homolog |
|------|---------------------------------------|----------|--------------------------|---------------------|
| Cre01.g008550 | Serine/threonine kinase related | O | 2 0 0.260 | AT1G73450.1 |
| Cre01.g014000 | – | C | 3 0 0.021 | – |
| Cre01.g037800 (TRX21) | ATP-binding protein; thioredoxin domain | O | 3 3 0.260 | AT2G18990.1 (TXND9) |
| Cre02.g073900 | All-trans-10'-apo-β-carotenal 13,14-cleaving dioxygenase | C | 3 1 0.070 | AT4G32810.1 (ATCCD8, CCD8, MAX4) |
| Cre02.g111550 | Serine/threonine kinase related | SP | 10 8 <10−6 | AT4G24480.1 |
| Cre03.g185200 (CPL3, MPA6) | Metallophosphoesterase/metallo-dependent phosphatase | C | 3 4 0.260 | AT1G07010.1 |
| Cre06.g259100 | Domain of unknown function (DUF1995) | C | 1 4 1.000 | – |
| Cre06.g281800 | Domain of unknown function (DUF1995) | C | 3 0 0.021 | – |
| Cre07.g316050 (CDJ2) | Chloroplast DnaJ-like protein | M | 2 0 0.260 | AT5G59610.1 |
| Cre07.g341850 (EIF2, INF3) | Translation initiation factor IF-2, chloroplastic | C | 2 0 0.260 | AT1G17220.1 (FUG1) |
| Cre08.g358350 (TDA1, OPR34) | Fast leucine-rich domain containing* | C | 3 2 0.152 | – |
| Cre09.g396250 (VTES) | Phosphatidate cytidylyltransferase | SP | 2 0 0.260 | AT5G04490.1 (VTES) |
| Cre10.g429650 | Alpha/beta hydrolase family (Abhydrolase_5) | O | 2 0 0.260 | – |
| Cre10.g448950 | Nocturnin | C | 1 1 1.000 | AT3G58560.1 |
| Cre11.g467712 | Structural maintenance of chromosomes smc family member; starch-binding domain | M | 7 7 0.0003 | AT5G05180.1 |
| Cre12.g542569 | Ionotropic glutamate receptor | O | 0 2 1.000 | AT1G05200.1 (ATGLR3.4, GLR3.4, GLUR3) |
| Cre13.g566400 (OPR55) | Fast leucine-rich domain containing* | M | 4 2 0.018 | – |
| Cre13.g574000 (GEF1, CLV1) | Voltage-gated chloride channel | O | 1 11 1.000 | AT5G62640.1 (ATCLC-D, CLC-D) |
| Cre13.g586750 | Transportin 3 and importin | O | 3 4 0.260 | AT5G62600.1 |
| Cre16.g658950 | – | C | 2 2 0.909 | – |
| Cre50.g761497 | Magnesium transporter mrs2 homolog, mitochondrial | M | 2 0 0.260 | AT5G22830.1 (ATMRG10, GGN10, MGT10, MR52-11) |

*The annotation of ‘fast leucine-rich domain containing’ cannot be confirmed by BLASTP analysis at NCBI57.
gene-editing techniques\textsuperscript{21–28} and that, together, these tools will help the research community generate fundamental insights in a wide range of fields, from organelle biogenesis and function to organism–environment interactions.

URLs. CLiP website for mutant distribution, \url{https://www.chlamyli-brary.org/}; Jonikas Lab GitHub repositories of scripts, \url{https://github.com/Jonikas-Lab/Li-Patena-2019/}.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at \url{https://doi.org/10.1038/s41588-019-0370-6}.

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Fig. 4 | CPL3 is required for photosynthetic growth and accumulation of photosynthetic protein complexes in the thylakoid membranes.

\textbf{a}. The \textit{cpl3} mutant contains cassettes inserted in the first exon of CPL3. The locations of conserved protein phosphatase motifs are indicated (Supplementary Fig. 6e). \textbf{b}. \textit{cpl3} is deficient in growth under photosynthetic conditions and can be rescued upon complementation with the wild-type CPL3 gene (\textit{comp1–comp3} represent three independent complemented lines). WT, wild type. \textbf{c}. \textit{cpl3} has a lower relative photosynthetic electron transport rate than the wild-type strain and \textit{comp1}. Error bars, s.d. (\(n = 3\) for WT and \textit{comp1}; \(n = 7\) for \textit{cpl3}). \textbf{d}. Whole-cell proteomics (Supplementary Table 14) indicates that \textit{cpl3} is deficient in accumulation of PSII, PSI, and the chloroplast ATP synthase. Each dot represents one \textit{Chlamydomonas} protein; PSII, PSI, and ATP synthase subunits are highlighted as black and red symbols. \textbf{e}. Western blots showing that CPL3 is required for normal accumulation of the PSII subunit CP43, the PSI subunit PsaA, and the chloroplast ATP synthase subunit ATPC. \(\alpha\)-tubulin was used as a loading control. To facilitate estimation of protein abundance in the \textit{cpl3} and \textit{comp1} samples, 50\%, 25\%, and 12.5\% dilutions of the wild-type sample were loaded. See also Supplementary Fig. 7c. \textbf{f}. A heat map showing the protein abundance of subunits in the light reaction protein complexes and enzymes in the CBB cycle in \textit{cpl3} relative to the wild type based on proteomics data. Depicted subunits that were not detected by proteomics are filled in gray (N.D.). Nuclear- and chloroplast-encoded proteins are labeled in black and red, respectively. A stack of horizontal ovals indicates different isoforms for the same enzyme, such as FBA1, FBA2, and FBA3.
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Author contributions

X.L. developed the method for generating barcoded cassettes. R.Y. and S.R. optimized the multiplex PCR amplification protocol. R.Y. and X.L. generated the library. J.V.-B., A.G., and R.Y. maintained, consolidated, and cryopreserved the library. X.L. developed the barcode sequencing method. W.P. developed the barcode sequencing method. X.L. developed the method for generating barcoded cassettes. R.Y. and S.R. optimized the multiplex PCR amplification protocol. R.Y. and X.L. generated the library. J.V.-B., A.G., and R.Y. maintained, consolidated, and cryopreserved the library. X.L. developed the barcode sequencing method. W.P. developed the barcode sequencing method. X.L. and S.R. performed physiological characterizations of Arabidopsis thaliana and Arabidopsis thaliana psbA mRNA in Arabidopsis thaliana. Plant Cell 20, 1182–1199 (2016).

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Additional information

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Methods

Generation of the indexed and barcoded mutant library. A three-step pipeline was developed for generation of an indexed, barcoded library of insertional mutants in Chlamydomonas (Fig. 1 and Supplementary Fig. 1).

To generate mutants, CC-4533 cells (wild type in the text and figures) were transformed with DNA cassettes that randomly insert into the genome, confer paromomycin resistance for selection, and inactivate the genes into which they insert. Each cassette contained two unique 22-nucleotide barcodes, one at each end of the cassette (Supplementary Fig. 1a–d and Supplementary Note). Transformants were arrayed on agar plates, and each insertion in a transformant would contain two barcodes. The barcode sequences as well as the insertion site were initially unknown (Supplementary Fig. 1e).

To determine the sequences of the barcodes in each colony, we generated combinatorial pools of the individual mutants; DNA was then extracted from each pool, and barcodes were amplified and deep-sequenced. The combinatorial pooling patterns were inferred so that each colony was included in a different combination of pools, allowing us to determine the barcode sequences associated with individual colonies on the basis of which pools the sequences were found in (Supplementary Figs. 1f and 2a–e, and Supplementary Note). This procedure was similar in concept to the approach we used in our pilot study, but it consumed considerably less time because we used a simple PCR amplifying only the barcodes instead of a multistep flanking sequence extraction protocol (ChlMmeSeq®) on each combinatorial pool.

To determine the insertion site associated with each barcode, the library was pooled into a single sample or divided into six separate samples. Barcodes and their flanking genomic regions were amplified using PCR amplifications (Supplementary Figs. 1g and 2f–j, and Supplementary Note). The flanking sequences associated with each barcode were obtained by paired-end deep sequencing of the final product.

The final product is an indexed library in which each colony has known flanking sequences that identify the genomic insertion site and barcode sequences that facilitate pooled screens in which individual mutants can be tracked by deep sequencing (Fig. 1b).

Insertion verification PCR. PCRs were performed in two steps to verify the insertion site (Supplementary Table 6): (i) genome locus amplification and (ii) genome–cassette junction amplification. In the first step, genomic primers that were 1 kb away from the flanking genomic sequence reported by LEAP-Seq were used to amplify the genomic locus around the flanking sequence. If the wild type produced the expected PCR band but the mutant did not or yielded a much larger product, this indicated that the genomic locus reported by LEAP-Seq might be disrupted by the insertional cassette and we proceeded to the second step. In this step, a primer binding to the cassette (oM913 for the 5′ side and oM944 for the 3′ side; Supplementary Table 6) and a second primer binding to flanking Chlamydomonas genomic DNA (one of the genomic primers from the first step) were used to amplify the genome–cassette junction. If the mutant produced a PCR band of the expected size that was confirmed by sequencing but the wild type did not, we categorized the insertion as ‘confirmed’. For some mutants, genomic primers surrounding the site of insertion did not yield any PCR products in the wild type or mutant even after several trials, possibly owing to incorrect reference genome sequence or local PCR amplification difficulties. These cases were grouped as ‘failed PCR’ and were not further analyzed.

72 mutants (24 insertions each for confidence levels 1 and 2, confidence level 3, and confidence level 4) were chosen randomly from the library and tested. The genomic DNA template was prepared from a single colony of each mutant by using the DNeasy Plant Mini kit (69106, Qiagen). PCRs were performed using the Taq PCR Core kit (201225, Qiagen) as described previously. PCR products of the expected size were verified by Sanger sequencing.

Southern blotting. Southern blotting was performed as previously described in detail. Genomic DNA was digested with StuI enzyme (R0187L, New England Biolabs) and separated on a 0.7% Triis-borate-EDTA (TBE) agarose gel. The DNA in the gel was depurinated in 0.25 M HCl, denatured in a bath of 0.5 M NaOH and 1 M NaCl followed by neutralization in a bath of 1.5 M Triis-HCl (pH 7.4) and 1.5 M NaCl, and finally transferred onto a Zeta-probe membrane (16201059, Bio-Rad) overnight, by using the alkaline transfer protocol given in the manual accompanying the membrane. On the next day, the membrane was gently washed with saline–sodium citrate (SSC) buffer (2× SSC: 0.3 M NaCl and 0.03 M sodium citrate), dried by paper towel, and UV cross-linked with a Stratalinker 1800 (Stratagene). For probe generation, the AphVIII gene on CIR1 was amplified by using primers oM58J88 and oM58J89 (Supplementary Table 1). The PCR product was purified and labeled according to the protocol of the Amer sham Gene Images AlkPhos Direct Labeling and Detection System (RPN3690, GE Healthcare). The membrane was hybridized at 60°C overnight with 10 ng/ml probe in hybridization buffer. On the next day, the membrane was washed with primary and secondary wash buffers and signal was visualized with CL-XPosure film (34093, Thermo Fisher).

Analyses of insertion distribution and identification of hotspots and coldspots. A mappability metric was defined to quantify the fraction of all possible flanking sequences from any genomic region that could be uniquely mapped to that region. Calculation of mappability, hotspot and coldspot analysis, and simulations of random insertions were performed as described previously, except that a 30-bp flanking sequence length instead of a mixture of 20-bp and 21-bp lengths was used (because we were now using 30-bp flanking sequence data derived from LEAP-Seq rather than 20-bp and 21-bp ChlMmeSeq sequences) and the v5.5 Chlamydomonas genome was used instead of the v5.3 genome. This analysis was done on the original full set of mapped insertions, to avoid introducing bias from the choice of mutants for the consolidated set. The hotspot and coldspot analysis was performed on confidence level 1 insertions only, to avoid introducing bias caused by junk fragments and their imperfect correction. The full list of statistically significant hotspots and coldspots is provided in Supplementary Table 7.

Identification of under-represented gene ontology terms. For each Gene Ontology (GO) category, we calculated the total number of insertions in all genes annotated with the GO term and the total mappable length of all such genes, and we compared these values to the total number of insertions in and total mappable length of the set of flagellar proteome genes. Comparison was performed by using Fisher’s exact test with correction for multiple comparisons to obtain the FDR. This analysis was done on the original full set of mapped insertions, to avoid introducing bias from the choice of mutants for the consolidated set. We decided to use the flagellar proteome as the comparison set because flagellar genes are unlikely to be essential; we did not use intergenic insertions or the entire genome because we knew that the overall insertion density differed between genes and intergenic regions. The statistically significant results are listed in Supplementary Table 8.

Prediction of essential genes. To predict essential genes in Chlamydomonas, we sought to generate a list of genes that had fewer insertions than would be expected randomly. Among those, those with no insertions were considered candidate essential genes.

For each gene, we calculated the total number of insertions in the gene and the total mappable length of the gene, and we compared these values to the total number of insertions in and total mappable length of the set of flagellar proteome genes, as was done for each GO category. The resulting list of genes with significantly fewer insertions than expected is discussed in the Supplementary Note and provided in Supplementary Table 9; the list includes 203 genes with no insertions and 558 genes with at least one insertion. However, only genes 5 kb or longer yielded an FDR of 0.05 or less when we had no insertion; our overall density of insertions was not high enough to detect smaller essential genes.

Pooled screens. Library plates that were replicated once every 4 weeks onto fresh medium were switched to a 2-week replication interval to support uniform colony growth before pooling. Cells were pooled from 5-d-old library plates. First, for each set of eight agar plates, cells were scraped using the blunt side of a razor blade (55411-050, VWR) and resuspended in 40 ml of liquid TAP medium in a 50-ml conical tube. Second, cell clumps were broken up by pipetting, by using a P200 pipette tip attached to a 10-ml serological pipette. In addition, cells were pipetted through a 100-μm cell strainer (431752, Corning). Third, subpools were combined into a master pool representing the full library.

The master pool was washed and resuspended in TP. Multiple aliquots of 2 × 106 cells were pelleted by centrifugation (1,000g, 5 min, room temperature), and the supernatant was removed by decanting. Some aliquots were used for inoculation of pooled cultures, whereas other aliquots were frozen at −80°C as initial pool samples for later barcode extraction to enable analysis of reproducibility between technical replicates. For pooled growth, 20 liters of TAP or TP in a transparent Carboy container (2251-0050, Nalgene) was inoculated with the initial pool to a final concentration of 2 × 106 cells ml−1. Cultures were grown at 22°C, mixed by using a conventional magnetic stirrer, and aerated with air filtered by using a 1-μm bacterial air venting filter (4308, Pall Laboratory).

The TAP culture was grown in the dark. For the two replicate TP cultures, the light intensity measured at the surface of the growth container was initially 1000 μmol m−2 s−1 photons and was then increased to 500 μmol m−2 s−1 photons after the cells reached ~2 × 106 cells ml−1. When the culture reached a final cell density of 2 × 107 cells ml−1 after several doublings, 2 × 108 cells were pelleted by centrifugation (1,000g, 5 min, room temperature) for DNA extraction and barcode sequencing.

Molecular characterization of the cpl3 mutant. Mutant genotyping PCRs were performed as previously described. To complement the cpl3 mutant, the wild-type CPL3 gene was PCR amplified and cloned into the pRAM118 vector containing the aph7 gene, which confers resistance to hygromycin B. In this construct, expression of CPL3 is under the control of the PSAD promoter. The construct was linearized before being transformed into the cpl3 mutant. Transformants were allowed to grow on colony plates in the presence of selective and nonselective acetate (Supplementary Fig. 6c,d). Three representative lines that showed rescued photosynthetic growth were used in further phenotype analyses (Fig. 4).

Analyses of growth, chlorophyll, and photosynthetic electrophoretic construct. For all physiological and biochemical characterizations of cpl3 described below,
we grew cells heterotrophically in the dark to minimize secondary phenotypes due to defects in photosynthesis. For spot assays, cells were grown in TAP medium in the dark to log phase (−10^6 cells ml^-1). Cells were washed in TAP and spotted onto solid TAP or TP medium. The TAP plates were incubated in the dark for 12 d before being imaged. The TP plates were incubated under 300 µm chloroacetamide, 100 mM Tris (pH 8.5), 1× MS-Safe protease inhibitor, and 1× phosphatase inhibitor cocktail II) and grinding in liquid nitrogen, followed by sonication. Protein lysates were then digested with trypsin (Promega) into peptides. Three biological replicates were processed for each strain. Samples were labeled with tandem mass tags (TMTs), multiplexed, and then fractionated before tandem mass spectrometry (MS/MS) analyses. Briefly, each sample was labeled by using TMT labeling reagent (Thermo Fisher) according to the manufacturer’s instructions. Samples were then mixed in equimolar amounts and desalted with C18 stage tips. The dried peptide mixture was separated with strong cation exchange (SCX) stage tips into four fractions. Each of the four fractions was diluted with 1% trifluoroacetic acid (TFA) and separated into three fractions with SDB-RPS stage tips. This procedure initially resulted in a total of 12 fractions. Fractions 1–3 (derived from the first SCX fraction) were pooled together, yielding ten final fractions. Each final fraction was diluted and injected into an Easy-nLC 1200 UPLC system (Thermo Fisher). Samples were loaded onto a nano capillary column packed with 1.9-µm C18-AQ (Dr. Maisch) mated to a metal emitter in line with a Fusion Lumos (Thermo Fisher). Samples were eluted using a split gradient of 10–20% solution B (80% acetonitrile and 0.1% formic acid) in 32 min and 20–40% solution B in 92 min, followed by column washing with 100% solution B for 10 min. The mass spectrometer was operated in data-dependent mode with the MS1 scan at 60,000 resolution (mass range of 380–1,500 m/z), an automatic gain control (AGC) target of 4×10^6, and a maximum injection time of 50 ms. Peptides above the threshold of 5×10^4 with charges of 2−7 were selected for fragmentation with dynamic exclusion after one run for 60 s with tolerance of 10 p.p.m. MS1 isolation windows of 1.6 m/z, MS2 isolation windows of 2.2 m/z, and higher-energy-collisional dissociation (HCD) normalized collision energy (NCE) of 55% were selected. MS3 fragments were detected in the Orbitrap at 50,000 resolution in the mass range of 120–500 m/z with AGC at 5×10^5 and a maximum injection time of 86 ms. The total duty cycle was set to 5.0 s. Raw files were searched with MaxQuant while using default settings for MS3 reporter. TMT 10-plex data. Files were searched against sequences of nucleus-, mitochondrial-, and chloroplast-encoded Chlamydomonas proteins supplemented with common contaminants [1,2,4]. Raw files were also analyzed within Proteome Discoverer (Thermo Fisher) by using the Byonic search node (Protein Metrics). Data from MaxQuant and Proteome Discoverer were combined in Scaffold Q+ (Proteome Software). Scaffold was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 80% probability by the scaffold local FDR algorithm. Protein identifications were accepted if they could be established at greater than 96.0% probability and contained at least two identified peptides. Scaffold Q+ non-normalized data were exported in the format of the log2 values for the reporter ion intensities, which reflect the relative abundance of the same protein among different samples multiplexed. Each sample was then normalized to a median of 0 (by subtracting the original median from the raw values, as the values are log, transformed). For each gene and for each pair of samples, the normalized log, intensity values from the three replicates for one sample were compared against those for the other sample using a standard t test. The resulting P values were adjusted for multiple testing 2, yielding an FDR for each gene in each pair of samples. We note that our calculation of FDR does not take into account the spectral count for each protein (provided in Supplementary Table 14), which is related to the absolute abundance of the protein and impacts the accuracy of proteomic measurements. Specifically, proteins with a low spectral count are likely of low abundance in cells and often exhibit large variation in the intensity value between biological replicates. **Western blotting.** Cells grown in TAP in the dark were pelleted by centrifugation, resuspended in extraction buffer containing 5 mM HEPES-KOH (pH 7.5), 100 mM dithiothreitol, 100 mM NaCl, 2% SDS, and 12% sucrose, and lysed by boiling for 1 min. Extracted proteins were separated by SDS–PAGE (12% precast polyacrylamide gels, Bio-Rad) and α-tubulin was used as a loading and normalization control. Polyacrylamide gels were transferred onto PVDF membranes with a semidy blotting apparatus (Bio-Rad) at 15 V for 30 min. For western blot analyses, membranes were blocked for 1 h at room temperature in Tris-buffered saline with 0.1% Tween (TBST) containing 5% powdered milk followed by incubation for 1 h at room temperature with primary antibodies in TBST containing 3% powdered milk. Primary antibodies were diluted according to the manufacturer’s recommendations. All antibodies were from Agrisera; the catalog numbers for the antibodies against CP43, PsA, ATCP, and α-tubulin were AS11-1787, AS06-172-100, AS08-312, and AS10-680, respectively. Proteins were detected by enhanced chemiluminescence (K-12045-D20, Advansta) and imaged on a medical film processor (Konica) as previously described. Additional methods. Additional method details are provided in the Supplementary Note. **Statistical analyses.** The statistical methods and tests used are indicated throughout the manuscript. Fisher’s exact test with Benjamini–Hochberg correction for multiple comparisons was used to identify under-represented GO terms, essential genes, and hit genes in the photosynthesis screen and for the analysis of candidate gene enrichment. The binomial test with Benjamini–Hochberg correction for multiple comparisons was used for the hotspot and coldspot analysis. A chi-square test of independence was used for insertion distributions. A t test with Benjamini–Hochberg correction for multiple comparisons was used for analysis of the proteomics data. Please see the corresponding Methods or Supplementary Note section for details on each analysis. **References** 58. Zhang, R. et al. High-throughput genotyping of green algal mutants reveals random distribution of mutagenic insertion sites and endonucleolytic cleavage of transforming DNA. *Plant Cell* 26, 1398–1409 (2014). 59. Rubin, B. E. et al. The essential gene set of a photosynthetic organism. *Proc. Natl. Acad. Sci. USA* 112, E6634–E6643 (2015). 60. Wernette, K. M. et al. Rapid quantification of mutant fitness in diverse bacteria by sequencing randomly bar-coded transposons. *MBio* 6, e00306–e00315 (2015). 61. Benjamini, Y. & Hochberg, Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Statist. Soc. B* 57, 289–300 (1995). 62. 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Data collection  No software was used in data collection in this study.

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Sample size  We empirically determined the sample sizes based on published research.

Data exclusions  We generated a library containing ~210,000 mutants and cherry-picked 62,389 mutants for long-term maintenance. We used these 62,389 mutants for analyses of library coverage.

Replication  We performed replicates and used orthogonal approaches where appropriate.

Randomization  We randomly picked mutants for validation of insertion site mapping.

Blinding  Blinding and randomization were not used for this study.

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|-----|-----------------------|
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| ☑   | Antibodies |
| ☑   | Eukaryotic cell lines |
| ☑   | Palaeontology |
| ☑   | Animals and other organisms |
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Methods

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| ☑   | Flow cytometry |
| ☑   | MRI-based neuroimaging |

Unique biological materials

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Obtaining unique materials  The mutants described in this manuscript are available from the Chlamydomonas Resource Center: https://www.chlamylibrary.org/

Antibodies

Antibodies used  Antibodies used in Fig. 4 are commercially available from Agrisera. The catalog numbers are provides in Methods.

Validation  Antibodies used in Fig. 4 have been tested in photosynthetic organisms (references are on the Agrisera website).

Eukaryotic cell lines

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Cell line source(s)  We have been using the CC-4533 Chlamydomonas strain generated in our lab.

Authentication  When we generated the CC-4533 strain in 2012, we froze dozens of copies and now revive one stock each time. The identity of our line is validated by sequencing.

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## Animals and other organisms

### Policy information about studies involving animals

- **ARRIVE guidelines** recommended for reporting animal research.

#### Laboratory animals

- For laboratory animals, report species, strain, sex and age OR state that the study did not involve laboratory animals.

#### Wild animals

- Provide details on animals observed in or captured in the field; report species, sex and age where possible. Describe how animals were caught and transported and what happened to captive animals after the study (if killed, explain why and describe method; if released, say where and when) OR state that the study did not involve wild animals.

#### Field-collected samples

- For laboratory work with field-collected samples, describe all relevant parameters such as housing, maintenance, temperature, photoperiod and end-of-experiment protocol OR state that the study did not involve samples collected from the field.

## Human research participants

### Policy information about studies involving human research participants

#### Population characteristics

- Describe the covariate-relevant population characteristics of the human research participants (e.g. age, gender, genotypic information, past and current diagnosis and treatment categories). If you filled out the behavioural & social sciences study design questions and have nothing to add here, write "See above."

#### Recruitment

- Describe how participants were recruited. Outline any potential self-selection bias or other biases that may be present and how these are likely to impact results.

## ChIP-seq

### Data deposition

- Confirmed that both raw and final processed data have been deposited in a public database such as GEO.

- Confirmed that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

#### Data access links

- For "Initial submission" or "Revised version" documents, provide reviewer access links. For your "Final submission" document, provide a link to the deposited data.

#### Files in database submission

- Provide a list of all files available in the database submission.

#### Genome browser version

- Provide a link to an anonymized genome browser session for "initial submission" and "Revised version" documents only, to enable peer review. Write "no longer applicable" for "Final submission" documents.

## Methodology

### Replicates

- Describe the experimental replicates, specifying number, type and replicate agreement.

### Sequencing depth

- Describe the sequencing depth for each experiment, providing the total number of reads, uniquely mapped reads, length of reads and whether they were paired- or single-end.

### Antibodies

- Describe the antibodies used for the ChIP-seq experiments; as applicable, provide supplier name, catalog number, clone name, and lot number.

### Peak calling parameters

- Specify the command line program and parameters used for read mapping and peak calling, including the ChIP, control and index files used.

### Data quality

- Describe the methods used to ensure data quality in full detail, including how many peaks are at FDR 5% and above 5-fold enrichment.
Flow Cytometry

Plots

Confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation
Describe the sample preparation, detailing the biological source of the cells and any tissue processing steps used.

Instrument
Identify the instrument used for data collection, specifying make and model number.

Software
Describe the software used to collect and analyze the flow cytometry data. For custom code that has been deposited into a community repository, provide accession details.

Cell population abundance
Describe the abundance of the relevant cell populations within post-sort fractions, providing details on the purity of the samples and how it was determined.

Gating strategy
Describe the gating strategy used for all relevant experiments, specifying the preliminary FSC/SSC gates of the starting cell population, indicating where boundaries between “positive” and “negative” staining cell populations are defined.

☐ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.

Magnetic resonance imaging

Experimental design

Design type
Indicate task or resting state; event-related or block design.

Design specifications
Specify the number of blocks, trials or experimental units per session and/or subject, and specify the length of each trial or block (if trials are blocked) and interval between trials.

Behavioral performance measures
State number and/or type of variables recorded (e.g. correct button press, response time) and what statistics were used to establish that the subjects were performing the task as expected (e.g. mean, range, and/or standard deviation across subjects).

Acquisition

Imaging type(s)
Specify: functional, structural, diffusion, perfusion.

Field strength
Specify in Tesla

Sequence & imaging parameters
Specify the pulse sequence type (gradient echo, spin echo, etc.), imaging type (EPI, spiral, etc.), field of view, matrix size, slice thickness, orientation and TE/TR/flip angle.

Area of acquisition
State whether a whole brain scan was used OR define the area of acquisition, describing how the region was determined.

Diffusion MRI
☐ Used
☐ Not used

Preprocessing

Preprocessing software
Provide detail on software version and revision number and on specific parameters (model/functions, brain extraction, segmentation, smoothing kernel size, etc.).

Normalization
If data were normalized/standardized, describe the approach(es): specify linear or non-linear and define image types used for transformation OR indicate that data were not normalized and explain rationale for lack of normalization.

Normalization template
Describe the template used for normalization/transformation, specifying subject space or group standardized space (e.g. original Talairach, MNI305, ICBM152) OR indicate that the data were not normalized.

Noise and artifact removal
Describe your procedure(s) for artifact and structured noise removal, specifying motion parameters, tissue signals and physiological signals (heart rate, respiration).
### Volume censoring

Define your software and/or method and criteria for volume censoring, and state the extent of such censoring.

### Statistical modeling & inference

#### Model type and settings

Specify type (mass univariate, multivariate, RSA, predictive, etc.) and describe essential details of the model at the first and second levels (e.g. fixed, random or mixed effects; drift or auto-correlation).

#### Effect(s) tested

Define precise effect in terms of the task or stimulus conditions instead of psychological concepts and indicate whether ANOVA or factorial designs were used.

#### Specify type of analysis:

- [ ] Whole brain
- [ ] ROI-based
- [ ] Both

#### Statistic type for inference

Specify voxel-wise or cluster-wise and report all relevant parameters for cluster-wise methods.

#### Correction

Describe the type of correction and how it is obtained for multiple comparisons (e.g. FWE, FDR, permutation or Monte Carlo).

### Models & analysis

| n/a | Involved in the study |
|-----|------------------------|
|     | Functional and/or effective connectivity |
|     | Graph analysis |
|     | Multivariate modeling or predictive analysis |

#### Functional and/or effective connectivity

Report the measures of dependence used and the model details (e.g. Pearson correlation, partial correlation, mutual information).

#### Graph analysis

Report the dependent variable and connectivity measure, specifying weighted graph or binarized graph, subject- or group-level, and the global and/or node summaries used (e.g. clustering coefficient, efficiency, etc.).

#### Multivariate modeling and predictive analysis

Specify independent variables, features extraction and dimension reduction, model, training and evaluation metrics.