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***Chlamydomonas* cell cycle mutant *crcdc5* over-accumulates starch and oil**

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Abstract

The use of algal biomass for biofuel production requires improvements in both biomass productivity and its energy density. Green microalgae store starch and oil as two major forms of carbon reserves. Current strategies to increase the amount of carbon reserves often compromise algal growth. To better understand the cellular mechanisms connecting cell division to carbon storage, we examined starch and oil accumulation in two *Chlamydomonas* mutants deficient in a gene encoding a homolog of the *Arabidopsis* Cell Division Cycle 5 (CDC5), a MYB DNA binding protein known to be involved in cell cycle in higher plants. The two *crcdc5* mutants (*crcdc5-1* and *crcdc5-2*) were found to accumulate significantly higher amount of starch and oil than their corresponding parental lines. Flow cytometry analysis on synchronized cultures cultivated in a diurnal light/dark cycle revealed an abnormal division of the two mutants, characterized by a prolonged S/M phase, therefore demonstrating its implication in cell cycle in *Chlamydomonas*. Taken together, these results suggest that the energy saved by a slowdown in cell division is used for the synthesis of reserve compounds. This work highlights the importance in understanding the interplay between cell cycle and starch/oil homeostasis, which should have a critical impact on improving lipid/starch productivity.

Keywords

Algae; Cell division; Day/night cycle; Mutants; Starch; Oil

Abbreviations

CDC5, cell division cycle 5; CLIP, Chlamydomonas Library Project; CHT7, compromised hydrolysis of triacylglycerols 7; HP-TLC, high performance-thin layer chromatography; MM, minimum media, PBS, phosphate-buffer-saline, RESDA-PCR, restriction enzyme site-directed amplification-polymerase chain reaction; RT-PCR, reverse transcription-PCR; RT, room temperature; TAG, triacylglycerol; TAP, tris-acetate-phosphate.

1. Introduction

Cleaner energy alternatives are highly needed considering the increasing energy demand and the harmful environmental impact of fossil fuels. Microalgae have the potential to satisfy global oil demands due to their high biomass productivity and their capacity to synthesize naturally energy-rich compounds [1,2]. Triacylglycerols (TAG, oil) and starch are the two major forms of carbon and energy storage in green microalgae, and they can be converted to biodiesel and bioethanol, respectively. However, in optimal growth conditions, oil and starch represent only a small percentage of algal biomass. Massive oil and starch accumulation mostly occurs under stress conditions, with nitrogen (N) starvation being the most effective and therefore one of the most studied triggers [3–6]. Unfortunately, N deprivation undermines biomass productivity by inducing cellular quiescence [7]. This tradeoff between the yield of energy reserves and biomass productivity compels a two-step cultivation process where logarithmic growth provides enough algal biomass and a stress phase diverts energy uptake into TAG and starch accumulation [8]. Such process is energetically demanding which renders this process very expensive [9]. Economically viable production of biofuel relies therefore largely on strain engineering, which in turn requires a comprehensive knowledge on the interplay between reserve formation and cell growth [10].

For unicellular organisms, biomass accumulation is directly linked to the cell division and formation of reserves. Cell division is known to be one of the most energy-consuming events during the life of a cell [11]. Understanding the cell cycle and its regulations could have an impact on biomass productivity and oil and starch yield [12]. The molecular mechanisms regulating cell cycle in microalgae have been well studied [13–17], and this resulted in the identification of >50 genes required for cell division [15]. Despite the recent studies describing the influence of photoperiod on algal growth and biomass production and composition [18–21], little is known about the connection between cell cycle and formation of energy reserves.

Chlamydomonas reinhardtii has emerged as an established model to study cell cycle [11,15,17] and also a model to study lipid and starch metabolism [5,22–26]. To dissect the relationships between cell division on the formation of energy-rich compounds, here we took advantage of mutants already available in our laboratory

and studied the cell growth and formation of carbon reserves in two insertional mutants of *C. reinhardtii* shown to be impaired in cell cycle progression. We provide evidence that a genetic lesion affecting the cell division led to the redirection of cellular energy and assimilated carbon to formation of starch and oil in *Chlamydomonas*.

2. Materials and methods

2.1. Strains and culture conditions

C. reinhardtii cell-wall-less strain dw15 (*cw15*, *nit1-305*, *mt+*) is the parental line to generate the library of insertional mutants compromised in oil homeostasis [27]. Using restriction enzyme site-directed amplification PCR (RESDA-PCR) [28], one mutant *Mb1F11* was identified as containing an insertion in the locus Cre03.g197350 encoding a putative protein CDC5. Mutant *crcdc5-2* (LMJ.RY0402.255682_1) and its background strain CC-4533 were obtained from the Chlamydomonas Library Project (CLIP: <https://www.chlamylibrary.org>) of indexed insertional mutants [29] and streaked to isolate single cell clones.

Strains were maintained in agar plates containing Tris-Acetate-Phosphate (TAP) media [30] under constant light at 25°C. For mutants, TAP was supplemented with 10 µg mL⁻¹ paromomycin. Cell cultures were grown in glass flasks in incubators (Multitron, Infors HT) shaking at 120 rpm, 25°C, under continuous light (100 µmol photons m⁻² s⁻¹), unless otherwise specified. Light was supplied by fluorescent tubes (Fluora Osram). Cells were cultivated either in mixotrophic conditions, TAP media, or in photoautotrophic conditions, Minimum Media (MM; similar to the TAP media but without acetate where Tris was replaced by MOPS) with additional supply of 2% CO₂ in the air. Cell concentration was followed with a Beckman Coulter Multisizer 4 (MultisizerTM3 Coulter Counter, Beckman Coulter). To calculate cell doubling time, formulas (1) and (2) were used on the exponential phase of cell growth:

$$N(t) = N(0)e^{\mu t} \quad (1)$$

where $N(t)$ = the number of cells at time t , $N(0)$ = the number of cells at time 0, μ = growth rate, and t = time in hours.

$$\text{doubling time} = \frac{\ln(2)}{\mu} \quad (2)$$

For N starvation, log-phase cultures were washed with fresh media without N (NH₄Cl replaced by the same amount of NaCl) at 450 *g* for 3 min. N was resupplied to N-starved cells (48 h) and the cultures were set in the dark for 24 h to evaluate oil and starch catabolism.

2.2. Quantification of gene expression in *crcdc5-1* and *crcdc5-2* mutants

The null expression of the gene CrCDC5 was verified by semi-quantitative reverse transcription-PCR (RT-PCR). Mid-log phase cultures (concentration around 5×10⁶ cells mL⁻¹) were pelleted and snap-frozen in liquid nitrogen. RNA extraction was performed as detailed in [31]. Briefly, pellets were resuspended in 0.5 mL RNA reagent containing phenol (Invitrogen 12322-012) and were shaken with 2.8 mm beads for lysis using a Precellys Homogenizer (Bertin technologies). After a 2-min centrifugation (12000 *g* at 4°C), supernatant was laid over 100 µL NaCl (5 M stock), mixed up and down, and 300 µL chloroform were added. Tubes were vortexed, spun down for 10 min, 4°C at 12000 *g*, and upper phase was transferred to a new tube, where RNA was precipitated using 1 vol of isopropanol and kept for 10 min at room temperature (RT) before centrifuging again. RNA pellet was washed with 500 µL 75% ethanol, then dried and resuspended in water. DNA was degraded by TURBO DNase (Ambion) for 30 min at 37°C and further inactivated with RNA CleanUp (protocol NucleoSpin® RNA Clean-up) (Macherey Nagel). Yield was checked by Nanodrop and 1 µL of total RNA was retrotranscribed with the cDNA synthesis kit SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen).

A fragment of the CrCDC5 coding sequence (762 bp, locus Cre03.g197350) was amplified by KOD Xtreme Hot start polymerase (Novagen) (primers M-b1-F11_F: CGCTCAACCTGGACTCGCTGCCG and M-b1-F11_R: CTAGTGCCGCCGCCAGCTCGTC; cycling conditions: 94°C for 2 min, then 30 cycles at 98°C for 10 s, 66°C for 30 s, and 68°C for 90 s). The G protein beta subunit-like polypeptide (CBLP) gene (279 bp, Cre06.g278222) was used as a housekeeping gene [28] (primers CBLP_F: GAGTCCAACTACGGCTACGCC and CBLP _R: CTCGCCAATGGTGTACTTGAC; cycling conditions: 94°C for 2 min, then 25 cycles at 98°C for 10 s, 60°C for 30 s, and 68°C for 30 s).

2.3. Lipid extraction and analyses

Freshly grown cells were harvested via centrifugation in a glass tube (8 mL with Teflon-lined cap) at 3220 *g* for 5 min at 4°C. Lipids were either extracted immediately or kept frozen at -80°C for later extractions. Total lipids were extracted with a modified Bligh and Dyer method [32] and TAG amount was quantified by High Performance-Thin Layer Chromatography (HP-TLC) as previously described [5].

2.4. Starch quantification

Cells of *Chlamydomonas* were collected in 2-mL screw top Eppendorf tubes and centrifuged for 10 min at 13000 *g*. Pellets were resuspended in 1 mL of methanol and kept at -80°C for at least 1 h. Cells were then centrifuged (5 min at 13000 *g*) and methanol phase was removed. Pellets were dried under a fume hood, resuspended in 400 µL of water, and samples were autoclaved for 20 min at 121°C. Based on [33] protocol to convert starch into sugar monomers, 200 µL of amyloglucosidase (1 U mL⁻¹, Roche) were added to samples and incubated for 90 min at 60°C. After spinning down, soluble glucose was measured using a YSI2700 select sugar analyzer (YSI Life Sciences) with commercial glucose as calibration standard.

2.5. Cell cycle analysis using Flow cytometry

Diurnal synchronization was achieved by growing cultures photoautotrophically on a 12h-light/12h-dark regime for at least 3 days [34] maintaining exponential growth by multiple back-dilution in flasks. DNA content per cell was monitored throughout a whole diurnal cycle by staining with SYBR Green I (ThermoFisher) and collecting fluorescent signal of each cell particle by Flow cytometry to analyze the cell cycle [35]. Cells were counted with the Beckman Coulter Multisizer 4 to harvest the same number of cells. After harvesting, samples were fixated with 2.5% (v/v) glutaraldehyde and stored at 4°C. Cells were spun down (3000 *g*, 30 s), washed in Phosphate-Buffer-Saline (PBS) and incubated in PBS containing 0.1 mg mL⁻¹ RNase A at 37°C for 2 h [15]. Then treated cells were stained in dark for 30 min with SYBR Green I diluted in PBS (1:3000). In triplicate, green fluorescence (laser excitation: 488 nm; emission filter band pass: 525/30 nm) of at least 10000 cells was read with the flow cytometer Guava easyCyte 5HT (Merck Millipore). Population histograms were plotted in function of logarithmic values of green fluorescence signal and normalized by count percentage. For CC-4533 strains, a 70% ethanol solution step (RT for 2 h) was added prior to RNase treatment to enhance cell permeability.

3. Results

3.1. Isolation of a *Chlamydomonas* mutant over-accumulating starch and oil

To identify novel factors implicated in oil homeostasis, we carried out a forward genetic screen in the model microalga *C. reinhardtii* to isolate strains impaired in oil content in one or all of the following three conditions [27]: during optimal growth (TAP), under N starvation (TAP-N) or upon N resupply in the dark (MM24h) following a period of N starvation. Among all mutants isolated, the *Mb1F11* was one of the few mutants that showed an increased oil content under all three cultivation conditions. The mutant was found to accumulate three times more TAG during optimal growth, accumulated 1.3 times more oil 24 h after N starvation, and showed a reduced capacity to degrade its oil reserves, i.e. 30% remaining in the mutant cells 24h after N resupply as compared to 20% remaining in the WT (**Figure 1**).

The perturbation in lipid homeostasis under all three cultivation conditions suggests likely a defect in general cellular metabolism rather than specific processes related to lipid synthesis or degradation. We therefore quantified also starch amount under these conditions. Interestingly, changes in cellular starch amount paralleled those changes in oil content, i.e. the mutant *Mb1F11* over-accumulated starch during optimal growth and under N starvation (1.3 - 1.4 times more), and were also defected in starch degradation, from 16% remaining in the WT compared to 32% remaining in the mutant 24 h after N resupply (**Figure 2**).

3.2. The mutation is identified to be in the locus Cre03.g197350 encoding a putative cell division cycle 5 (CDC5) protein

The mutant library was generated via insertional mutagenesis where the paromomycin resistance gene *AphVIII* was randomly integrated into the nuclear genome [27]. To identify the genetic lesion in the mutant *Mb1F11*, RESDA-PCR was used [36]. The *AphVIII* cassette was found to be located in the 11th intron of the locus Cre03.g197350 (**Fig. 3A**). This gene encodes a 91.7-kDa protein containing two domains (a MYB-like DNA binding domain and a pre-mRNA-splicing factor domain). This protein is annotated as a CDC5 in the v5.5 of *C. reinhardtii* genome [37]. The zero expression of the *CrCDC5* gene in the mutant *Mb1F11* was confirmed by RT-

PCR using gene specific primers designed on the Cre03.g197350 coding sequence (**Figure 3B**). The mutant *Mb1F11* was therefore named as *crcdc5-1* from here on.

3.3. An independent insertional event in the same locus resulted in a similar phenotype as *crcdc5-1*

In the meantime, an indexed insertional mutant library of *C. reinhardtii* released to the public [29]. A strain, LMJ.RY0402.255682_1, was identified as potentially harboring an insertion in the 13th exon of the same locus as in *crcdc5-1* (Cre03.197350) (**Figure 3A**). Using gene specific primers, RT-PCR analysis showed zero expression of *CrCDC5* in the *crcdc5-2* mutant (**Figure 3B**). To further explore the interplay between cell cycle and reserve formation, we focused on conditions when active cell division occurs. The *crcdc5-2* mutant was found to over-accumulate both starch and oil compared to the WT CC-4533 during optimal growth (**Figure 4**), thus firmly establishing that the disruption in the expression of the putative *CrCDC5* gene in *C. reinhardtii* is responsible for a higher storage of carbon reserves (oil and starch).

3.4. The *crcdc5* mutants divide slower

The mutant *cdc5-1* was found to grow slower than WT (**Figure 5A**). To investigate the growth behavior, cells were grown under various conditions, and their doubling times were compared. Under all conditions tested, the two *crcdc5* mutants divided significantly slower, as indicated by the time (h) that is needed for cell doubling (**Figure 5B**). We observed that the time that is required to achieve one cell division is generally longer (from 10% - 60% longer) in the two *crcdc5* mutants compared to their respective parental lines under various growth conditions (photoautotrophic, heterotrophic and mixotrophic, constant light versus day/night cycle).

3.5. The S/M phase of the cell cycle is prolonged in the two mutants compared to their respective parental lines

To investigate impairment in cell division, we analyzed WT and mutant cell cycle by monitoring DNA replication. For this purpose, DNA was stained with SYBR Green I and fluorescence signal of each cell particle was collected with the aid of a Flow cytometer. In most microalgae, mitotic cycles are amenable to diurnally induced synchronization. Thus, we took advantage of day/night cycles to obtain a uniform cell population in order to detect population abnormalities as in other previous cell cycle

studies [15,34,38]. As in these studies, to obtain highly synchronized cell cultures, we avoided external organic carbon sources influencing cell size, which could potentially affect the time at which cells pass the commitment point [39], a control point in mid-G1 when cells could complete at least one division without further energy input. Since commitment gates cell cycle progression and interferes in cell synchronization, for this experiment we have cultivated cells photoautotrophically using MM instead of TAP.

Cells were pre-cultured for at least three days under a 12h-light followed by a 12h-dark regime to synchronize cell division. In synchronized cells, *C. reinhardtii* divides at the beginning of the night. Then cell cultures were sampled at multiple time-points throughout the diurnal cycle to determine cell cycle progression. We first confirmed that under this condition both mutants over-accumulated starch and oil (**Supplemental Figure S1**), similar to the observation under mixotrophic growth (**Figures 1, 2 and 4**). We then monitored cell size and number with a cell counter (**Figure 6A**). A single narrow peak at the beginning of the day phase indicates that every cell had divided during the night phase (G0 to G1 transition), afterward during the day cells would grow and pull the peak towards greater cell diameters (G1 phase). And at the end of the day, cells would start replicating their genomes (S phase) and undertaking mitosis and cell division(s) (M phase) consecutively to go back to their initial minimum size (G0 phase) and remain quiescent until the end of the night.

During the diurnal cycle, population peaks attain a minimum of DNA content per cell by the end of the dark phase, where *dw15* cells have already divided and cannot give birth to daughter cells: they contain a single DNA copy (1C) (**Figure 6B**). By the end of the light phase, cells replicate their genomes and as a result, DNA peaks shift to 2C intensity. Next, cells undertake a series of divisions (around 3 per cell usually). We observed that *crcdc5-1* population lingered at the 2C state, suggesting that *crcdc5-1* struggles to carry on division after the S phase (**Figure 6B**). We observed that WT populations had already finished the S/M stage 2 h entering the night phase whereas *crcdc5-1* displayed a second fluorescence peak corresponding to the double of DNA content, indicating the presence of an additional cell subpopulation containing two nuclei (**Figure 6B**).

Strain dw15 does not display more than 2C signal, and this is probably because it is defective in cell wall and cells are readily separated after cytokinesis without hatching. In contrast, CC-4533 cells, having also an impaired cell wall but probably less severe compared to the dw15, need to hatch to release the daughter cells and can therefore reach higher DNA content per cell particle (**Supplemental Figure S2**). We found that CC-4533 attained lower synchronization rates than dw15 under the same diurnal cycles. However, should G2/M phase be lagged, the overall frequency of >1C peaks throughout the cycle is expected to increase. Indeed, *crcdc5-2* population profiles are enriched in >1-nucleus cells, which confirms division S/M phase is slowed down.

4. Discussion

C. reinhardtii is unicellular, and its biomass accumulation is directly linked to the cell division and formation of reserves (starch and oil). Conditions favoring reserve accumulation often caused a compromise in cell division and growth, for example, nutrient deprivation. However, the metabolic consequence of perturbation in cell cycle on reserve metabolism has not been studied by itself. Understanding the underlying relationship between cell cycle and formation of carbon reserves should have significant impact on genetic engineering studies to improve cellular lipid/starch amount, and to the biotechnological utilization of microalgae.

Here we show that a protein involved in the cell division cycle (CDC5) affects reserve metabolism. Using Flow cytometry analysis, we demonstrated that the *Arabidopsis* AtCDC5 homolog in *C. reinhardtii* is indeed a functional protein and plays a role in the transition from S to M phase in the cell cycle. Furthermore, through the study of the *crcdc5* mutants, we provided biochemical, genetic and cytological evidence that perturbation in cell cycle can have multiple consequences in reserve turnover, resulting in cells over-accumulating oil and starch during optimal growth, and are perturbed in the reutilization of carbon reserves upon N resupply after a period of N starvation. These results and their implications on algal biotechnology are discussed below.

4.1. CrCDC5 is actively involved in cell cycle progression in *C. reinhardtii*

Although cell cycle processes have been relatively well studied in *C. reinhardtii* [11,14,17,34,40], this study provides a first experimental evidence for the implication of CrCDC5 in cell cycle in *C. reinhardtii*. CrCDC5 shared a 51% identity with

Arabidopsis thaliana CDC5 (AtCDC5; At1g09770) and 37% with the *Schizosaccharomyces pombe* CDC5 (SpCDC5), both known to act as a positive regulator of cell cycle in the G2/M progression [41–43]. Additionally, SpCDC5 regulates mitotic entry, chromosome segregation, mitotic exit, and cytokinesis [44,45]. AtCDC5 is mainly expressed in tissues with high cell division activity and during embryo formation. The *Arabidopsis* knockout mutant of *atcdc5* is embryo lethal, and in RNAi silencing lines, endoreduplication was increased and shoot apical meristem functions greatly affected [46]. This suggests G2/M phase could be affected [43]. However, unlike fission yeast or plants, *C. reinhardtii* divides by multiple fission concatenating S/M phases without a distinct G2 phase [11,14]. The consequence of a putative CrCDC5 knock-out is thus difficult to predict. Here we show that two independent *crcdc5* mutants are impaired in cell cycle progression, presumably during or immediately before M phase, therefore establishing the role of CrCDC5 in cell cycle in *C. reinhardtii*. Consistent with its function in the S/M phase in a cell cycle, CrCDC5 showed a peak expression at the end of the day during a 24 h day/night cycle, with protein levels increasing towards the night period (**Supplemental Figure S3**) [17,34].

4.2. Carbon storage and cell cycle in unicellular algae

Photosynthetic cells have to adjust their metabolism to ensure cell survival and optimize energy production and use in an ever-changing environment. Starch and oil are two major sinks serving as a buffering role to store energy as reduced carbon when produced in excess or to reuse it when the cellular energy status is low. During optimal growth, starch and oil are usually present in small amounts, and their cellular content have been found to follow a diurnal cell cycle [20,28,33,47]. The alterations in starch amount are further found not to be related to photosynthesis carbon fixation, but regulated by cell cycle [33]. Indeed, starchless mutants of *C. reinhardtii* divide less and grow slower under constant light, and cannot grow at all under diurnal light, indicating that starch provides an essential part of energy that is required for normal cell division [48–50]. This serves as additional evidence that starch and oil provide energy and carbon that are vital for cell growth and reproduction. Our study here provides genetic evidence that, when cell cycle is perturbed and cells spend less their energy for cell division, intracellular starch and lipid amounts increase as a result.

Compared to normal growth, carbon allocation has been subjected to intensive investigations under N starvation. N starvation has been identified as the most effective inducer in triggering cells to accumulate substantial amount of starch and oil [5,6,51]. This seems not surprising since in active growing cells, N metabolism represents a major sink for C utilization, and when N is absent, C is diverted to starch and lipids. N starvation-induced starch or oil accumulation is less attractive in terms of biotechnology, because N starvation also induces quiescence, a dormant state where cell growth is arrested. The interplay between cell quiescence and lipid metabolism has lately been demonstrated through the identification of the Compromised Hydrolysis of Triacylglycerols (CHT7) protein, a repressor that affects the exit of the quiescent state in *C. reinhardtii* upon N resupply. The *cht7* mutant was isolated because it showed a delay in TAG degradation following N resupply [7]. Analyses of starch dynamics in the *cht7* mutant would be interesting and could provide additional information on the interplay between cell quiescence and carbon reserve metabolism.

4.3. From cell cycle to biotechnology

In addition to N starvation, a common feature underlying most other stress treatments is the arrest in cell division and growth. We provide evidence that complete arrest in cell division is not an absolute requirement for oil and starch accumulation. Findings made in this study show the importance of understanding cell cycle on reserve metabolism. Our findings will be of use for industrial cultivation, where it is preferable to use strains that simultaneously store oil and starch while continuing cell growth. In addition to the *coa1* mutant isolated previously from our laboratory [52] and the *cht7* mutant [7], this serves as another evidence that through mutation in a single gene, strains over-accumulating both starch and oil without the need for N starvation can be achieved. These set a precedent for new engineering strategies to the current two-step microalgal oil production paradigm in which biomass productivity must be sacrificed for an oil accumulation phase [53,54]. The discovery of the CrCDC5 protein provides a starting point to explore other factors regulating the nexus of circadian rhythm, cell division and carbon partitioning.

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6. Author contributions

I.T.R., F.B., G.P. and Y.L.B. conceived the work; I.T.R. performed all experiments except the ones listed below; F.K. isolated the *crdc5-1* mutant; B.L. performed lipidomics analyses; I.T.R., Y.L.B. wrote the paper with comments from F.B. and G.P. All authors have approved the final article.

Figure legend:

Figure 1. The mutant Mb1F11 over-accumulated oil during optimal growth, N starvation and also showed delay in oil reutilization upon N resupply during the recovery phase.

A. Oil content.

B. A zoom-in for oil content quantification during optimal growth.

C. % TAG remaining 24 h after N resupply.

Data are means of three biological replicates with two technical repeats each, with standard deviations shown. Student's t-test: * <0.05, ** <0.01, *** <0.001. Mutant Mb1F11 was named as *crdc5-1* as later identified.

Figure 2. Starch accumulation and remobilization is also affected in the mutant Mb1F11.

A. Starch content.

B. Percentage of starch remaining 24 h after N resupply.

Data are means of three biological replicates with two technical repeats each, with standard deviations shown. Student's t-test: * <0.05, ** <0.01, *** <0.001. Mutant Mb1F11 was named as *crdc5-1* as later identified.

Figure 3. Genetic validations.

A. The insertion site of the *AphVIII* cassette in the *crdc5-1* mutant (background: dw15) and in *crdc5-2 mutant* (CC-4533).

B. RT-PCR confirms zero expression of *CrCDC5* in both mutants.

Abbreviations: CBLP: Chlamydomonas beta subunit-like polypeptide.

Figure 4. The *crcdc5-2* mutant over-accumulated oil (A) and starch (B) during optimal growth.

Data are means of three biological replicates with three technical replicates each. Bars indicate one standard deviation.

Student's t-test: * <0.05, ** <0.01, *** <0.001.

Figure 5. *crcdc5* mutants divide slower

A. Cell growth as a function of increase in cellular number under mixotrophic growth. Data are means of two biological replicates, with standard deviations shown.

B. Cell doubling times in different growth conditions. Time (h) for cultures to double in cell number and relative increase in mutant compared to their respective parental lines.

Figure 6. Cell cycle analysis showed a prolonged S/M phase in *crcdc5-1*.

A. Synchronization was assessed by monitoring cell population diameter in a Coulter counter.

B. DNA content was analyzed throughout one day/night cycle with a flow cytometer for the dw15 WT and *crcdc5-1* mutant.

Axis 1C represents the fluorescence intensity of one DNA copy and subsequent C series are multiples of this, which were set in function of WT population after cell division. Remaining presence of a subpopulation with two nuclei in the *crcdc5-1* reveals cell division phase had not concluded after 2 h, suggesting a delay in the G2/M phase.

Supplemental Figures:

Supplemental Figure S1. The *crcdc5* mutants over-accumulate oil (A) and starch (B) during photoautotrophic growth.

Data are means of three biological replicates with three technical replicates each. Bars indicate one standard deviation. Student's t-test: * <0.05, ** <0.01, *** <0.001.

Supplemental Figure S2. Cell cycle analysis using Flow cytometer.

DNA content was analyzed throughout one day/night cycle with a flow cytometer for the dw15 WT and *crcdc5-1* mutant and for the CC-4533 WT and *crcdc5-2* mutant. X-

Axis 1C represents the fluorescence intensity of 1 cell with 1 nucleus. Unlike dw15, CC-4533 strains need to hatch after S/M to be counted as different particles, do not manage to adjust its division to the beginning of the dark period and have a softer synchronization instead. Thus division occurs also during the day.

The two mutants accumulate more DNA during the entire cell cycle, suggesting a delay in the G2/M phase of cell division.

Supplemental Figure S3. Abundance of transcript and protein levels of CrCDC5 during a 12h:12h day/night cycle.

A. RPKM values from two biological replicates from Zones et al., 2015 [34], and FPKM values from Strenkert et al., 2019 [17].

RPKM: reads per kilobase per million mapped reads; FPKM: kilobase of transcript per million mapped reads.

B. Data shows normalized peptide abundance of CrCDC5 protein from two biological replicates. Bars represent standard deviation.

7. References

- [1] R.H. Wijffels, M.J. Barbosa, An Outlook on Microalgal Biofuels, *Science*. 329 (2010) 796–799. doi:10.1126/science.1189003.
- [2] A. Kirrolia, N.R. Bishnoi, R. Singh, Microalgae as a boon for sustainable energy production and its future research & development aspects, *Renew. Sustain. Energy Rev.* 20 (2013) 642–656. doi:10.1016/j.rser.2012.12.003.
- [3] R. Miller, G. Wu, R.R. Deshpande, A. Vieler, K. Gärtner, X. Li, E.R. Moellering, S. Zäuner, A.J. Cornish, B. Liu, B. Bullard, B.B. Sears, M.-H. Kuo, E.L. Hegg, Y. Shachar-Hill, S.-H. Shiu, C. Benning, Changes in Transcript Abundance in *Chlamydomonas reinhardtii* following Nitrogen Deprivation Predict Diversion of Metabolism, *Plant Physiol.* 154 (2010) 1737–1752. doi:10.1104/pp.110.165159.
- [4] E.R. Moellering, C. Benning, RNA Interference Silencing of a Major Lipid Droplet Protein Affects Lipid Droplet Size in *Chlamydomonas reinhardtii*, *Eukaryot. Cell.* 9 (2010) 97–106. doi:10.1128/EC.00203-09.
- [5] M. Siaut, S. Cuiné, C. Cagnon, B. Fessler, M. Nguyen, P. Carrier, A. Beyly, F.

Beisson, C. Triantaphylidès, Y. Li-Beisson, G. Peltier, Oil accumulation in the model green alga *Chlamydomonas reinhardtii*: characterization, variability between common laboratory strains and relationship with starch reserves, *BMC Biotechnol.* 11 (2011) 7. doi:10.1186/1472-6750-11-7.

[6] S. Schmollinger, T. Mühlhaus, N.R. Boyle, I.K. Blaby, D. Casero, T. Mettler, J.L. Moseley, J. Kropat, F. Sommer, D. Strenkert, D. Hemme, M. Pellegrini, A.R. Grossman, M. Stitt, M. Schroda, S.S. Merchant, Nitrogen-Sparing Mechanisms in *Chlamydomonas* Affect the Transcriptome, the Proteome, and Photosynthetic Metabolism[W], *Plant Cell.* 26 (2014) 1410–1435. doi:10.1105/tpc.113.122523.

[7] C.-H. Tsai, J. Warakanont, T. Takeuchi, B.B. Sears, E.R. Moellering, C. Benning, The protein Compromised Hydrolysis of Triacylglycerols 7 (CHT7) acts as a repressor of cellular quiescence in *Chlamydomonas*, *Proc. Natl. Acad. Sci.* 111 (2014) 15833–15838. doi:10.1073/pnas.1414567111.

[8] L.D. Zhu, Z.H. Li, E. Hiltunen, Strategies for Lipid Production Improvement in Microalgae as a Biodiesel Feedstock, *BioMed Res. Int.* 2016 (2016) e8792548. doi:10.1155/2016/8792548.

[9] F. Delrue, Y. Li-Beisson, P.-A. Setier, C. Sahut, A. Roubaud, A.-K. Froment, G. Peltier, Comparison of various microalgae liquid biofuel production pathways based on energetic, economic and environmental criteria, *Bioresour. Technol.* 136 (2013) 205–212. doi:10.1016/j.biortech.2013.02.091.

[10] S.S. Merchant, J. Kropat, B. Liu, J. Shaw, J. Warakanont, TAG, You're it! *Chlamydomonas* as a reference organism for understanding algal triacylglycerol accumulation, *Curr. Opin. Biotechnol.* 23 (2012) 352–363. doi:10.1016/j.copbio.2011.12.001.

[11] F.R. Cross, J.G. Umen, The *Chlamydomonas* cell cycle, *Plant J.* 82 (2015) 370–392. doi:10.1111/tpj.12795.

[12] M. Vitova, K. Bisova, S. Kawano, V. Zachleder, Accumulation of energy reserves in algae: From cell cycles to biotechnological applications, *Biotechnol. Adv.* 33 (2015) 1204–1218. doi:10.1016/j.biotechadv.2015.04.012.

[13] K. Bisova, Genome-Wide Annotation and Expression Profiling of Cell Cycle

Regulatory Genes in *Chlamydomonas reinhardtii*, *PLANT Physiol.* 137 (2005) 475–491. doi:10.1104/pp.104.054155.

[14] K. Bišová, V. Zachleder, Cell-cycle regulation in green algae dividing by multiple fission, *J. Exp. Bot.* (2014) ert466. doi:10.1093/jxb/ert466.

[15] F. Tulin, F.R. Cross, A Microbial Avenue to Cell Cycle Control in the Plant Superkingdom, *Plant Cell.* 26 (2014) 4019–4038. doi:10.1105/tpc.114.129312.

[16] Y. Li, D. Liu, C. López-Paz, B.J. Olson, J.G. Umen, A new class of cyclin dependent kinase in *Chlamydomonas* is required for coupling cell size to cell division, *ELife.* 5 (2016) e10767. doi:10.7554/eLife.10767.

[17] D. Strenkert, S. Schmollinger, S.D. Gallaher, P.A. Salomé, S.O. Purvine, C.D. Nicora, T. Mettler-Altmann, E. Soubeyrand, A.P.M. Weber, M.S. Lipton, G.J. Basset, S.S. Merchant, Multiomics resolution of molecular events during a day in the life of *Chlamydomonas*, *Proc. Natl. Acad. Sci.* 116 (2019) 2374–2383. doi:10.1073/pnas.1815238116.

[18] I. Krzemińska, B. Pawlik-Skowrońska, M. Trzcińska, J. Tys, Influence of photoperiods on the growth rate and biomass productivity of green microalgae, *Bioprocess Biosyst. Eng.* 37 (2014) 735–741. doi:10.1007/s00449-013-1044-x.

[19] L. de Winter, I.T.D. Cabanelas, D.E. Martens, R.H. Wijffels, M.J. Barbosa, The influence of day/night cycles on biomass yield and composition of *Neochloris oleoabundans*, *Biotechnol. Biofuels.* 10 (2017) 104. doi:10.1186/s13068-017-0762-8.

[20] R.T. Smith, D.J. Gilmour, The influence of exogenous organic carbon assimilation and photoperiod on the carbon and lipid metabolism of *Chlamydomonas reinhardtii*, *Algal Res.* 31 (2018) 122–137. doi:10.1016/j.algal.2018.01.020.

[21] Y. Kato, Y. Fujihara, C.J. Vavricka, J.-S. Chang, T. Hasunuma, A. Kondo, Light/dark cycling causes delayed lipid accumulation and increased photoperiod-based biomass yield by altering metabolic flux in oleaginous *Chlamydomonas* sp., *Biotechnol. Biofuels.* 12 (2019) 39. doi:10.1186/s13068-019-1380-4.

[22] S.G. Ball, The Intricate Pathway of Starch Biosynthesis and Degradation in the Monocellular Alga *Chlamydomonas reinhardtii*, *Aust. J. Chem.* 55 (2002) 49–59.

doi:10.1071/ch02052.

[23] B. Liu, C. Benning, Lipid metabolism in microalgae distinguishes itself, *Curr. Opin. Biotechnol.* 24 (2013) 300–309. doi:10.1016/j.copbio.2012.08.008.

[24] Y. Li-Beisson, F. Beisson, W. Riekhof, Metabolism of acyl-lipids in *Chlamydomonas reinhardtii*, *Plant J.* 82 (2015) 504–522. doi:10.1111/tpj.12787.

[25] M.A. Scranton, J.T. Ostrand, F.J. Fields, S.P. Mayfield, *Chlamydomonas* as a model for biofuels and bio-products production, *Plant J.* 82 (2015) 523–531.

doi:10.1111/tpj.12780.

[26] F. Kong, Y. Yamaoka, T. Ohama, Y. Lee, Y. Li-Beisson, Molecular Genetic Tools and Emerging Synthetic Biology Strategies to Increase Cellular Oil Content in *Chlamydomonas reinhardtii*, *Plant Cell Physiol.* 60 (2019) 1184–1196.

doi:10.1093/pcp/pcz022.

[27] C. Cagnon, B. Mirabella, H.M. Nguyen, A. Beyly-Adriano, S. Bouvet, S. Cuiné, F. Beisson, G. Peltier, Y. Li-Beisson, Development of a forward genetic screen to isolate oil mutants in the green microalga *Chlamydomonas reinhardtii*, *Biotechnol. Biofuels.* 6 (2013) 178. doi:10.1186/1754-6834-6-178.

[28] F. Kong, Y. Liang, B. Légeret, A. Beyly-Adriano, S. Blangy, R.P. Haslam, J.A. Napier, F. Beisson, G. Peltier, Y. Li-Beisson, *Chlamydomonas* carries out fatty acid β -oxidation in ancestral peroxisomes using a bona fide acyl-CoA oxidase, *Plant J.* 90 (2017) 358–371. doi:10.1111/tpj.13498.

[29] X. Li, R. Zhang, W. Patena, S.S. Gang, S.R. Blum, N. Ivanova, R. Yue, J.M. Robertson, P. Lefebvre, S.T. Fitz-Gibbon, A.R. Grossman, M.C. Jonikas, An indexed, mapped mutant library enables reverse genetics studies of biological processes in *Chlamydomonas reinhardtii*, *Plant Cell.* (2016) TPC2015-00465-LSB.

doi:10.1105/tpc.15.00465.

[30] E.H. Harris, *Chlamydomonas* as a Model Organism, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 52 (2001) 363–406. doi:10.1146/annurev.arplant.52.1.363.

[31] H.M. Nguyen, S. Cuiné, A. Beyly-Adriano, B. Légeret, E. Billon, P. Auroy, F. Beisson, G. Peltier, Y. Li-Beisson, The Green Microalga *Chlamydomonas reinhardtii*

Has a Single ω -3 Fatty Acid Desaturase That Localizes to the Chloroplast and Impacts Both Plastidic and Extraplastidic Membrane Lipids, *Plant Physiol.* 163 (2013) 914–928. doi:10.1104/pp.113.223941.

[32] E.G. Bligh, W.J. Dyer, A Rapid Method of Total Lipid Extraction and Purification, *Can. J. Biochem. Physiol.* 37 (1959) 911–917. doi:10.1139/o59-099.

[33] U. Klein, Intracellular Carbon Partitioning in *Chlamydomonas reinhardtii*, *Plant Physiol.* 85 (1987) 892–897. doi:10.1104/pp.85.4.892.

[34] J.M. Zones, I.K. Blaby, S.S. Merchant, J.G. Umen, High-Resolution Profiling of a Synchronized Diurnal Transcriptome from *Chlamydomonas reinhardtii* Reveals Continuous Cell and Metabolic Differentiation, *Plant Cell.* (2015) tpc.15.00498. doi:10.1105/tpc.15.00498.

[35] D. Marie, F. Partensky, S. Jacquet, D. Vaultot, Enumeration and Cell Cycle Analysis of Natural Populations of Marine Picoplankton by Flow Cytometry Using the Nucleic Acid Stain SYBR Green I, *Appl. Environ. Microbiol.* 63 (1997) 186–193.

[36] D. González-Ballester, A. de Montaigne, A. Galván, E. Fernández, Restriction enzyme site-directed amplification PCR: A tool to identify regions flanking a marker DNA, *Anal. Biochem.* 340 (2005) 330–335. doi:10.1016/j.ab.2005.01.031.

[37] S.S. Merchant, S.E. Prochnik, O. Vallon, E.H. Harris, S.J. Karpowicz, G.B. Witman, A. Terry, A. Salamov, L.K. Fritz-Laylin, L. Maréchal-Drouard, W.F. Marshall, L.-H. Qu, D.R. Nelson, A.A. Sanderfoot, M.H. Spalding, V.V. Kapitonov, Q. Ren, P. Ferris, E. Lindquist, H. Shapiro, S.M. Lucas, J. Grimwood, J. Schmutz, P. Cardol, H. Cerutti, G. Chanfreau, C.-L. Chen, V. Cognat, M.T. Croft, R. Dent, S. Dutcher, E. Fernández, P. Ferris, H. Fukuzawa, D. González-Ballester, D. González-Halphen, A. Hallmann, M. Hanikenne, M. Hippler, W. Inwood, K. Jabbari, M. Kalanon, R. Kuras, P.A. Lefebvre, S.D. Lemaire, A.V. Lobanov, M. Lohr, A. Manuell, I. Meier, L. Mets, M. Mittag, T. Mittelmeier, J.V. Moroney, J. Moseley, C. Napoli, A.M. Nedelcu, K. Niyogi, S.V. Novoselov, I.T. Paulsen, G. Pazour, S. Purton, J.-P. Ral, D.M. Riaño-Pachón, W. Riekhof, L. Rymarquis, M. Schroda, D. Stern, J. Umen, R. Willows, N. Wilson, S.L. Zimmer, J. Allmer, J. Balk, K. Bisova, C.-J. Chen, M. Elias, K. Gendler, C. Hauser, M.R. Lamb, H. Ledford, J.C. Long, J. Minagawa, M.D. Page, J. Pan, W. Pootakham, S. Roje, A. Rose, E. Stahlberg, A.M. Terauchi, P. Yang, S. Ball, C. Bowler, C.L.

Dieckmann, V.N. Gladyshev, P. Green, R. Jorgensen, S. Mayfield, B. Mueller-Roeber, S. Rajamani, R.T. Sayre, P. Brokstein, I. Dubchak, D. Goodstein, L. Hornick, Y.W. Huang, J. Jhaveri, Y. Luo, D. Martínez, W.C.A. Ngau, B. Otilar, A. Poliakov, A. Porter, L. Szajkowski, G. Werner, K. Zhou, I.V. Grigoriev, D.S. Rokhsar, A.R. Grossman, The *Chlamydomonas* Genome Reveals the Evolution of Key Animal and Plant Functions, *Science*. 318 (2007) 245–250. doi:10.1126/science.1143609.

[38] S. Lemaire, M. Hours, C. Gerard-Hirne, A. Trouabal, O. Roche, J.-P. Jacquot, Analysis of light/dark synchronization of cell-wall-less *Chlamydomonas reinhardtii* (Chlorophyta) cells by flow cytometry, *Eur. J. Phycol.* 34 (1999) 279–286. doi:10.1080/09670269910001736332.

[39] J.G. Umen, Sizing up the cell cycle: systems and quantitative approaches in *Chlamydomonas*, *Curr. Opin. Plant Biol.* 46 (2018) 96–103. doi:10.1016/j.pbi.2018.08.003.

[40] Y. Li, D. Liu, C. López-Paz, B.J. Olson, J.G. Umen, A new class of cyclin dependent kinase in *Chlamydomonas* is required for coupling cell size to cell division, *ELife*. 5 (2016) e10767. doi:10.7554/eLife.10767.

[41] R. Ohi, A. Feoktistova, S. McCann, V. Valentine, A.T. Look, J.S. Lipsick, K.L. Gould, Myb-Related *Schizosaccharomyces pombe* cdc5p Is Structurally and Functionally Conserved in Eukaryotes, *Mol. Cell. Biol.* 18 (1998) 4097–4108.

[42] Z. Lin, K. Yin, D. Zhu, Z. Chen, H. Gu, L.-J. Qu, AtCDC5 regulates the G2 to M transition of the cell cycle and is critical for the function of Arabidopsis shoot apical meristem, *Cell Res.* 17 (2007) 815–828. doi:10.1038/cr.2007.71.

[43] S. Zhang, M. Xie, G. Ren, B. Yu, CDC5, a DNA binding protein, positively regulates posttranscriptional processing and/or transcription of primary microRNA transcripts, *Proc. Natl. Acad. Sci.* 110 (2013) 17588–17593. doi:10.1073/pnas.1310644110.

[44] V.V. Botchkarev, J.E. Haber, Functions and regulation of the Polo-like kinase Cdc5 in the absence and presence of DNA damage, *Curr. Genet.* 64 (2018) 87–96. doi:10.1007/s00294-017-0727-2.

[45] P.K. Mishra, G. Olafsson, L. Boeckmann, T.J. Westlake, Z.M. Jowhar, L.E.

Dittman, R.E. Baker, D. D'Amours, P.H. Thorpe, M.A. Basrai, Cell cycle–dependent association of polo kinase Cdc5 with CENP-A contributes to faithful chromosome segregation in budding yeast, *Mol. Biol. Cell.* 30 (2019) 1020–1036. doi:10.1091/mbc.E18-09-0584.

[46] H. Lin, U.W. Goodenough, Gametogenesis in the *Chlamydomonas reinhardtii minus* Mating Type Is Controlled by Two Genes, *MID* and *MTD1*, *Genetics.* 176 (2007) 913–925. doi:10.1534/genetics.106.066167.

[47] E. Poliner, N. Panchy, L. Newton, G. Wu, A. Lapinsky, B. Bullard, A. Zienkiewicz, C. Benning, S.-H. Shiu, E.M. Farré, Transcriptional coordination of physiological responses in *Nannochloropsis oceanica* CCMP1779 under light/dark cycles, *Plant J.* 83 (2015) 1097–1113. doi:10.1111/tpj.12944.

[48] I.K. Blaby, A.G. Glaesener, T. Mettler, S.T. Fitz-Gibbon, S.D. Gallaher, B. Liu, N.R. Boyle, J. Kropat, M. Stitt, S. Johnson, C. Benning, M. Pellegrini, D. Casero, S.S. Merchant, Systems-Level Analysis of Nitrogen Starvation–Induced Modifications of Carbon Metabolism in a *Chlamydomonas reinhardtii* Starchless Mutant, *Plant Cell.* 25 (2013) 4305–4323. doi:10.1105/tpc.113.117580.

[49] M.P. Davey, I. Horst, G.-H. Duong, E.V. Tomsett, A.C.P. Litvinenko, C.J. Howe, A.G. Smith, Triacylglyceride Production and Autophagous Responses in *Chlamydomonas reinhardtii* Depend on Resource Allocation and Carbon Source, *Eukaryot. Cell.* 13 (2014) 392–400. doi:10.1128/EC.00178-13.

[50] A. Krishnan, G.K. Kumaraswamy, D.J. Vinyard, H. Gu, G. Ananyev, M.C. Posewitz, G.C. Dismukes, Metabolic and photosynthetic consequences of blocking starch biosynthesis in the green alga *Chlamydomonas reinhardtii* *sta6* mutant, *Plant J.* 81 (2015) 947–960. doi:10.1111/tpj.12783.

[51] S. Himanshu, R.S. Manish, J.R. Basuthkar, V.R.C. Kandala, Regulation of starch, lipids and amino acids upon nitrogen sensing in *Chlamydomonas reinhardtii*, *Algal Res.* 18 (2016) 33–44. doi:10.1016/j.algal.2016.05.028.

[52] H.D. Goold, H.M. Nguyen, F. Kong, A. Beyly-Adriano, B. Légeret, E. Billon, S. Cuiné, F. Beisson, G. Peltier, Y. Li-Beisson, Whole Genome Re-Sequencing Identifies a Quantitative Trait Locus Repressing Carbon Reserve Accumulation

during Optimal Growth in *Chlamydomonas reinhardtii*, *Sci. Rep.* 6 (2016).
doi:10.1038/srep25209.

[53] E.C. Goncalves, A.C. Wilkie, M. Kirst, B. Rathinasabapathi, Metabolic regulation of triacylglycerol accumulation in the green algae: identification of potential targets for engineering to improve oil yield, *Plant Biotechnol. J.* 14 (2016) 1649–1660.
doi:10.1111/pbi.12523.

[54] K. Zienkiewicz, Z.-Y. Du, W. Ma, K. Vollheyde, C. Benning, Stress-induced neutral lipid biosynthesis in microalgae — Molecular, cellular and physiological insights, *Biochim. Biophys. Acta BBA - Mol. Cell Biol. Lipids.* 1861 (2016) 1269–1281.
doi:10.1016/j.bbalip.2016.02.008.

Figure 1.

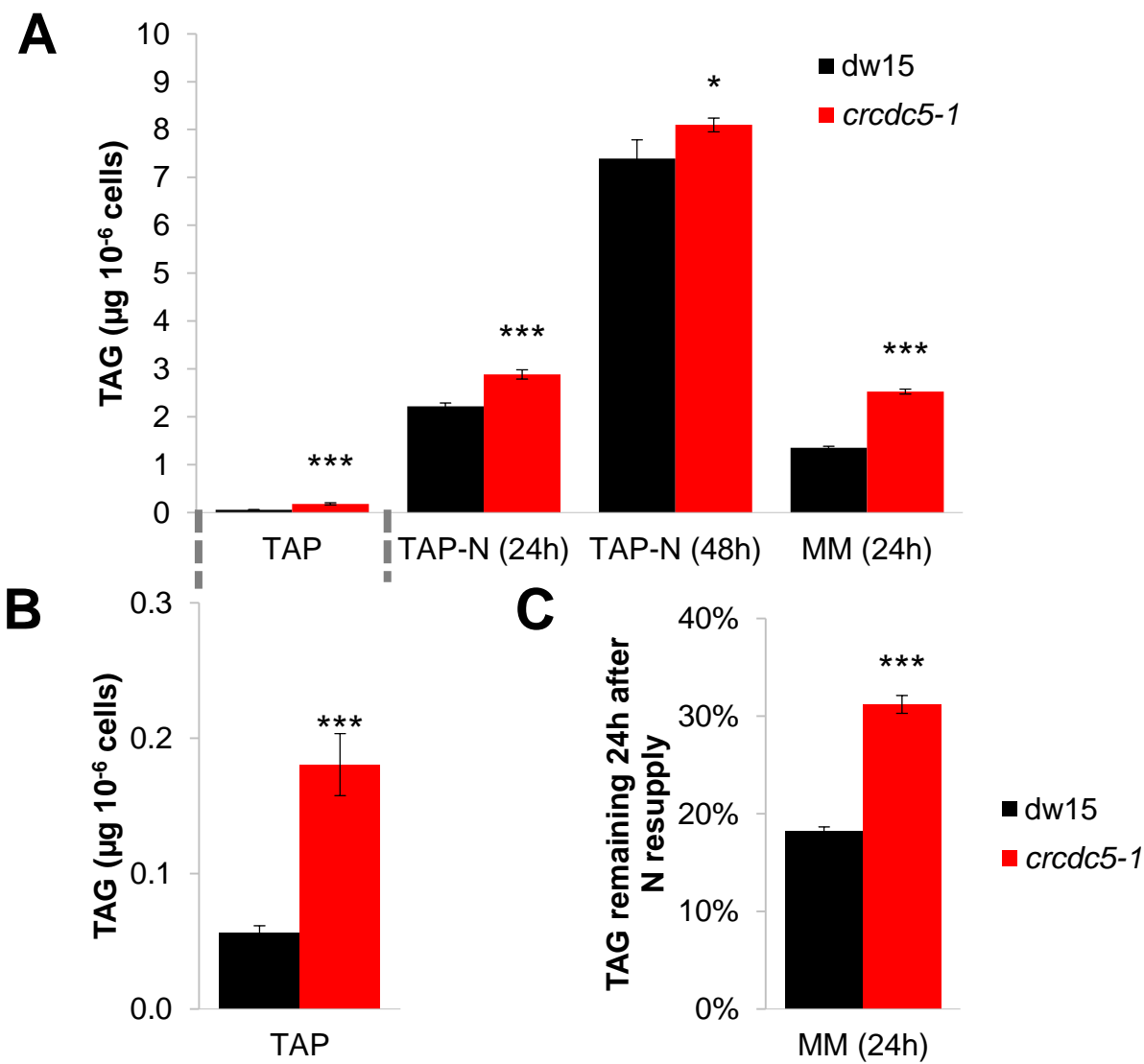


Figure 2.

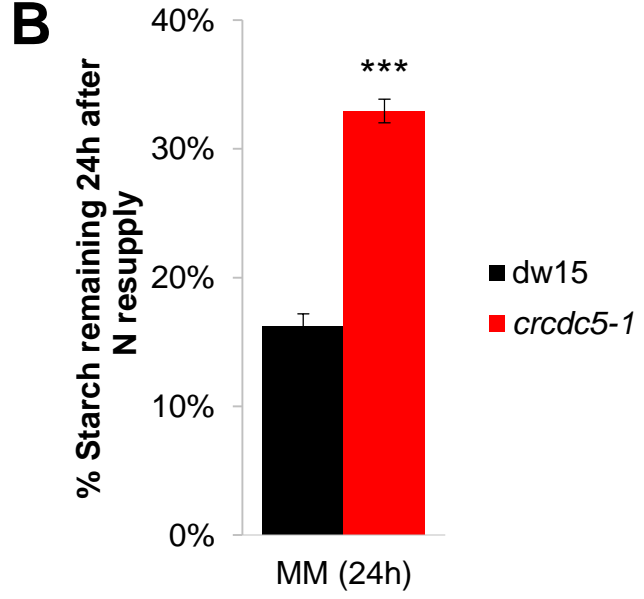
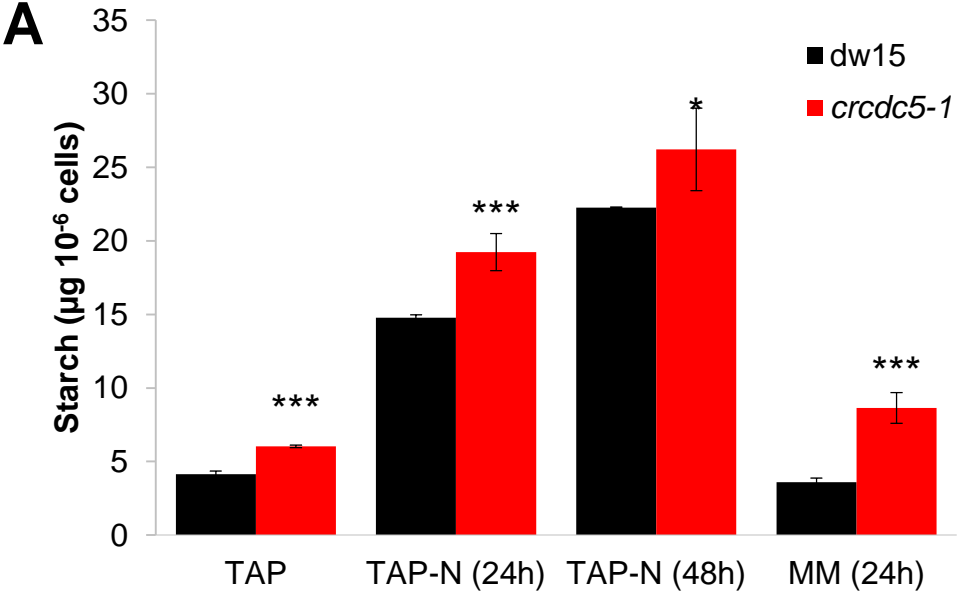


Figure 3.

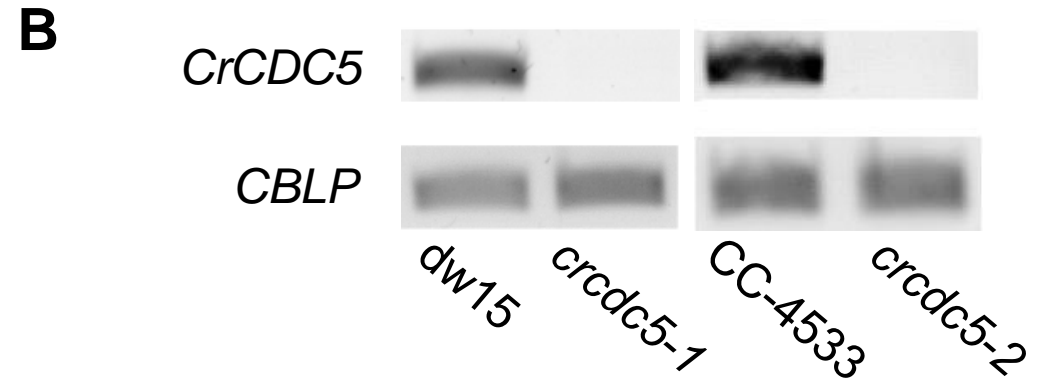
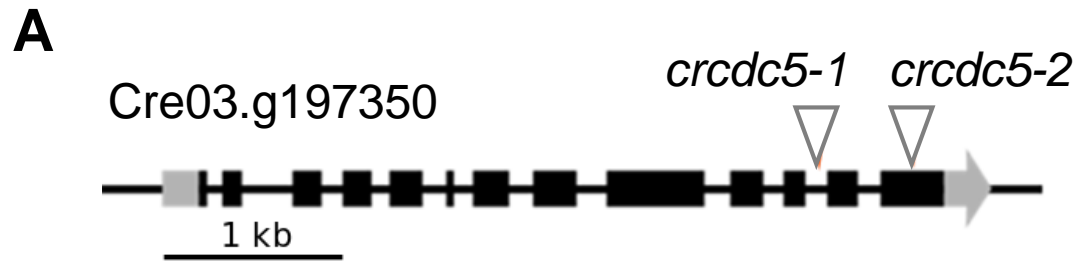


Figure 4.

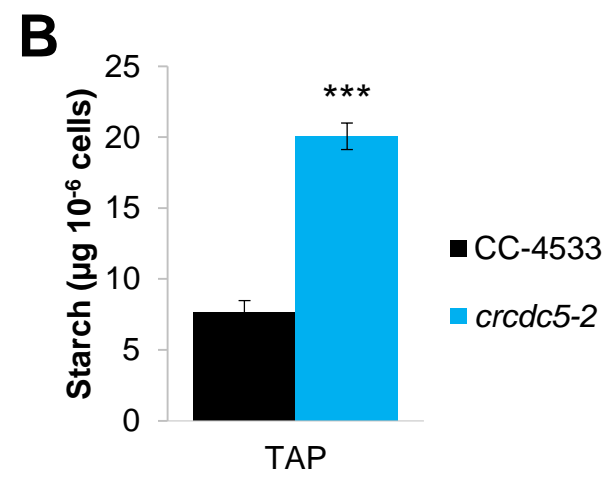
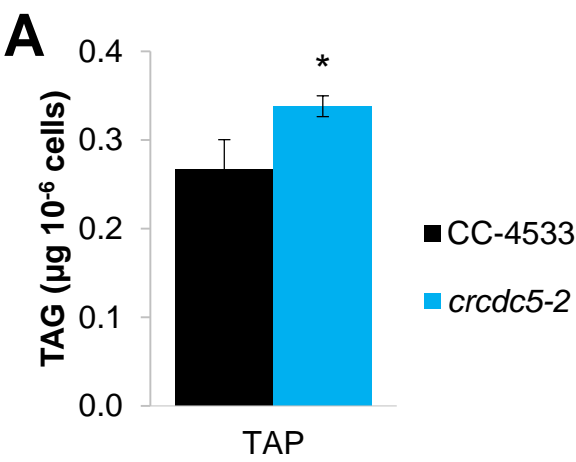
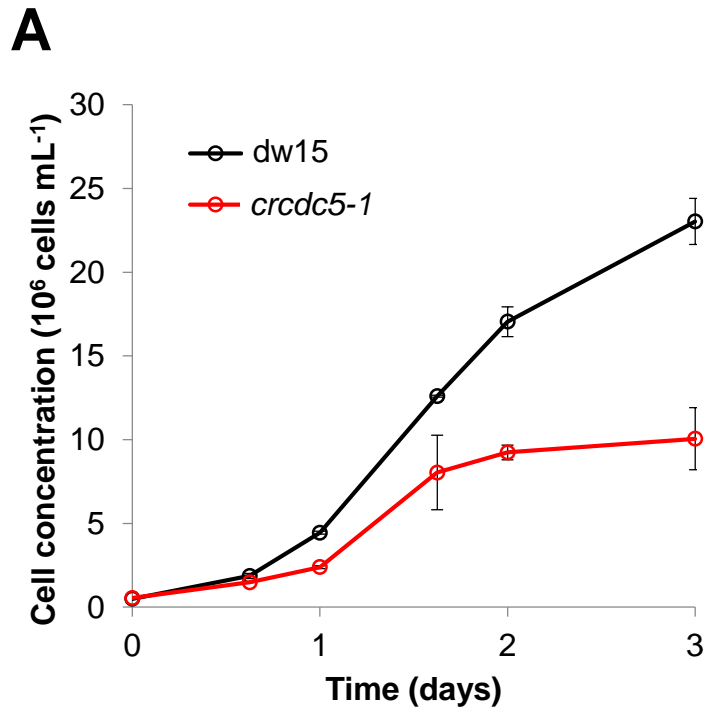


Figure 5.



B

Growth conditions	dw15	<i>crcdc5-1</i>	+/-	CC-4533	<i>crcdc5-2</i>	+/-
Photoautotrophy +2% CO ₂	15	23	+53%	11	14	+25%
Photoautotrophy + Air	19	24	+26%	24	33	+38%
Heterotrophy 	19	21	+10%	25	40	+60%
Mixotrophy 	9	11	+22%	10	11	+16%

Figure 6.

