HHS Public Access

Author manuscript

Curr Opin Plant Biol. Author manuscript; available in PMC 2019 December 01.

Published in final edited form as:

Curr Opin Plant Biol. 2018 December ; 46: 96–103. doi:10.1016/j.pbi.2018.08.003.

Sizing up the cell cycle: systems and quantitative approaches in Chlamydomonas

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Abstract

The unicellular green alga Chlamydomonas provides a simplified model for defining core cell cycle functions conserved in the green lineage and for understanding multiple fission, a common cell cycle variation found in many algae. Systems-level approaches including a recent groundbreaking screen for conditional lethal cell cycle mutants and genome-wide transcriptome analyses are revealing the complex relationships among cell cycle regulators and helping define roles for CDKA/CDK1 and CDKB, the latter of which is unique to the green lineage and plays a central role in mitotic regulation. Genetic screens and quantitative single-cell analyses have provided insight into cell-size control during multiple fission including the identification of a candidate 'sizer' protein. Quantitative single-cell tracking and modeling are promising approaches for gaining additional insight into regulation of cellular and subcellular scaling during the Chlamydomonas cell cycle.

Introduction

The cell cycle is a fundamental and ancient process that ensures faithful cellular replication by coordinating cell growth, genome duplication and cell division. A large amount of what we know about eukaryotic cell cycle control and its underlying mechanistic paradigms have come from studies done in fungi and animal cells conducted over the past several decades [1]. As the genomic era accelerates, increasing attention is being paid to cell cycle regulatory machinery in other eukaryotic groups that must be investigated in order to understand the shared ancestry of eukaryotic cell cycle regulation and how it has evolved and diverged in different taxa [2]. The eukaryotic microalga *Chlamydomonas reinhardtii* has been dubbed 'green yeast', but its advantages as a model for investigating cell cycle control have only recently begun to be fully exploited. One of these advantages is its amenability to systems-level and quantitative approaches that can be used to dissect essential processes such as cell division and to model cell division behavior. I focus here on recent advances and emerging opportunities for using such approaches in Chlamydomonas that include a large-scale systems-level screen for essential cell cycle genes, transcriptomic studies, and analysis of size control and scaling at the single-cell level.

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Papers of particular interest, published within the period of review, have been highlighted as of special interest

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Conserved and non-canonical features of the Chlamydomonas cell cycle

Chlamydomonas reinhardtii (Chlamydomonas) is a unicellular, haploid green alga belonging to the Chlorophytes, that to get her with their sister group the Streptophytes (Charophyte algae and land plants) comprise the Viridiplantae or 'green lineage'. While the green lineage is highly diversified, Chlamydomonas has retained what are likely ancestral features including an apical pair of flagella, a single large chloroplast, and a haploid-dominant life cycle [3,4]. Unlike angiosperms, such as the model plant *Arabidopsis thaliana*, which often have multiple paralogous duplications of cell cycle genes, most core cell cycle genes of Chlamydomonas are present in single copy, greatly simplifying genetic analyses of cell cycle proteins [5]. The Chlamydomonas mitotic cell cycle is a variant of the canonical cell cycle called multiple fission (also called palintomy) where cell growth and cell division are partially uncoupled. Multiple fission is found in many species of green algae as well as other single-celled eukaryotes [6], and has some similarity to elements of non-conventional cell cycles of plants and animals [5,7]. It begins with a prolonged G1 phase lasting around twelve hours during which newborn daughters may grow many-fold in mass (typically between two-fold and ten-fold) before Sphase and mitosis/cytokinesis commence. At the end of G1 phase mother cells move quickly through n rounds of alternating S phase and mitosis/cytokinesis (S/M) to produce 2^n daughter cells (Figure 1a). A control point in mid-G1 phase called Commitment gates cell cycle progression and is defined operationally as the point when cells are no longer dependent on light or external nutrients to complete at least one division. Passing Commitment requires that cells reach a minimal size (~2X average daughter size), but even after passing Commitment cells remain in G1 phase for several more hours and can grow substantially in size before initiating S/M [5].

Systems biology and genomics defines a core cell cycle regulatory framework

One very fruitful approach taken recently has involved systematic identification of essential cell cycle regulatory genes and pathways using saturating screens for temperature-sensitive (ts) lethal mutations that cause cell cycle arrest at high temperature [8••,9••]. This approach is extremely useful because it ensures that most essential cell cycle pathways will have one or more ts mutations that can be used for conditional inactivation. Innovations in use of robotics and semi-automated screening sped up the process of identifying and classifying a large collection of ts lethal mutants; and a microscopic screening method was employed to distinguish cell cycle arrest phenotypes from non-specific growth arrest [9••,10]. A subsequent hurdle that had to be overcome for each cell cycle ts allele was identification of the causative mutation amidst a heavily mutagenized strain background containing hundreds of 'passenger' mutations [11]. Even though high- throughput genome re-sequencing costs are relatively low, the scale required for this approach would be prohibitively expensive without the development of more efficient methods that involve a combination of pooled screening and association mapping [9••].

Cell cycle arrest mutations identified in the ts screen were classified into two categories termed *gex* and *div*, with *gex* mutants unable to exit G1 phase (but relatively unimpaired in

cell growth) and *div* mutants arresting after entering and completing some portion of S and M. Reassuringly, many of the *div* mutations were in genes whose homologs or orthologs have conserved functions in fungi, metazoans or plants — including cyclin dependent kinases (CDKs), mitotic cyclins, DNA replication factors, and the anaphase promoting complex (APC) [8••,9••]. Equally exciting was the identification of genes that had not previously been associated with cell cycle regulation such as the conserved green lineage phosphatase BSL1 [8••] whose Arabidopsis homologs were first identified in a screen for genes related to brassinosteroid signaling [12]. The *gex* genes are less thematically coherent than *div* genes, and may be involved in coupling growth, biomass or biosynthetic processes to cell cycle progression.

An important set of results emerging from these studies was a clarification of the roles for two key cell cycle regulators, CDKA and CDKB, and their associated cyclins, CYCA and CYCB, respectively (Figure 1b). In earlier studies with Arabidopsis, CDK1/CDKA appeared to be essential as it is in metazoans and fungi [13]; however, more detailed studies revealed that Arabidopsis *cdka* null plants were viable but severely compromised for growth and development, with compensatory CDK activity being supplied by CDKB [14]. The single *CDKA* gene in Chlamydomonas (*CDKA1*) was identified in an initial ts screen with a *gex*-like phenotype, but cell cycle arrest for this allele required a second mutation without which the *cdka* single mutant showed a non-lethal delayed cell cycle entry phenotype [8••]. Subsequently, *cdka* null alleles with phenotypes similar to the original segregated ts allele were generated in a suppressor screen for delayed lethality of a topoisomerase mutant, *div19*, that rapidly loses viability upon entry into S/M phase [15••]. The non-essentiality of CDKA in Chlamydomonas suggests that CDK1/CDKA is indeed dispensable across the entire green lineage, and this has implications when considering how cell cycle control operates in eukaryotic groups outside of opisthokonts (fungi and animals) which cannot be assumed to provide a universal ‘template’ for understanding eukaryotic cell cycles [2,5]. *cdkb* ts strains arrested after a single round of DNA replication at the non-permissive temperature [8••], and it appears that CDKB is the central driver of mitotic cell cycle progression in plants and algae, with CDKA/CDK1 playing an important but somewhat less critical role as a ‘kick-starter’ for transitioning into S phase, after which CDKB-CYCB largely take over [15••]. Using a combination of double mutants, cytological markers, transcriptome analyses and biochemical characterization, a plausible model for control of the multiple fission cell cycle was developed [15••,16•] and somewhat extended here based on additional inferences and some speculation (Figure 1b and c).

Transcriptome studies elucidate diurnal and cell cycle dynamics

The application of omics-level analyses (e.g. transcriptomics, proteomics, metabolomics etc.) has been very useful in dissecting plant cell cycle regulation [17,18], and in Chlamydomonas omics tools are also starting to be exploited. A special advantage of Chlamydomonas is that large quantities of highly synchronous cells can be generated for omics studies, and may make it easier to bridge the gap between population-level omics data and data from single-cells. Initial studies which examined a handful of cell cycle genes [19] have now been expanded to full transcriptome studies on synchronous cultures at modest temporal resolution [20], and subsequently to much higher temporal resolution [21]. In the

latter study, almost every annotated cell cycle gene showed a strong periodic expression peak coincident with cells entering and transiting through S and M phases, with further temporal sub-clustering by shared function (e.g. replication genes peaked slightly before core mitotic regulators, and flagella genes peaked later). However, on their own these transcriptome studies (as well as other -omics-based diurnal studies[22,23]) could not formally rule out diurnal or circadian control. A third transcriptome study made use of comparisons between arrested and non-arrested cultures of *cdka1* and *cdkb1* mutants, thereby allowing a more definitive assignment of genes to cell-cycle-controlled regulons [16•]. Moreover, the genes controlled by CDKA and CDKB fell into two classes — those dependent on CDKA activity (early genes) and a set that were dependent on both CDKA and CDKB (late genes), with most of the key cell cycle genes in the early category, and the flagella genes — which are transcribed post-mitotically — in the late category (Figure 1c).

The targets of CDK-dependent gene expression control in Chlamydomonas are likely to be transcription factors (TFs), though identification of such TFs has remained elusive, and post-transcriptional mechanisms may also play a role in setting mRNA levels. Retinoblastoma related (RBR) protein complexes are important cell cycle regulators in animals and plants [24] where they associate with DNA binding proteins from the E2F-DP family and with additional co-repressors and activators to control cell cycle gene transcription [25–27]. Mutants in genes for Chlamydomonas RBR pathway have cell cycle defects that are consistent with a repressor role for RBR (encoded by the *MAT3* gene) and activator roles for E2F-DP; but the mutants had no obvious defects in cell cycle transcription of candidate target genes [28] or in genome-wide transcriptome comparisons (JM Zones *et al.*, unpublished data). Although additional testing remains to be done, the data in Chlamydomonas suggest a potential role for the RBR pathway via non-transcriptional mechanisms such as altering chromatin structure to directly impact cell cycle progression, for example through direct influence on S phase initiation [29]. While this idea may seem to put the proposed role of Chlamydomonas RBR at odds with its canonical role as a TF in plants and metazoans, the connections between RBR and chromatin structure are well established [30,31]. It is the relative contributions of RBR to non-transcriptional versus transcriptional mechanisms in driving the cell cycle which are not entirely clear. For example Arabidopsis *cdka* null mutants showed severe cell cycle defects and reductions of RBR target gene expression; but the near-complete rescue of these cell cycle defects in *cdka rbr* double mutants was accompanied by generally small or modest increases in expression of RBR target genes, a finding which could reflect contributions of the RBR pathway to cell cycle control that extend beyond transcription [14].

In Arabidopsis a subfamily of triplet Myb domain TFs (Myb3Rs) governs the mitotic transcription program and also interacts with RBR in higher order complexes [26,27]. The Chlamydomonas genome encodes at least one and possibly two Myb3R proteins [16•,32], but their possible role(s) in cell cycle transcription have not been investigated. Flagella resorption and regrowth occur coordinately with the cell cycle (Figure 1a) [5], and a Chlamydomonas TF controlling flagella gene expression, XAP5, was identified recently and shown to undergo changes in phosphorylation that appear to be coincident with cell cycle progression [33], thus making XAP5 a good candidate as a direct or indirect target of CDKs.

Several additional TFs whose transcripts showed diurnal or cell cycle controlled expression have been identified as candidate cell cycle regulators [21], but not investigated further.

Single cell quantitative tracking to model stochastic behavior and cell size control

Single-cell tracking has revolutionized our ability to model and understand cell cycle behaviors in yeasts, animals and plants [34–38,39•,40•,41], but is still under-utilized in Chlamydomonas, partly due to technical challenges of immobilizing and visualizing cells as they go through highly dynamic changes during a single cell cycle. However, various methods have been developed for visualizing single-cell behaviors in Chlamydomonas [15••, 42,43•,44]. A recent study using a novel micro-droplet system where individual droplets could be inoculated with single cells or a few cells revealed inter-clonal heterogeneity in growth rates, maximum biomass density or cell number, implying intrinsic stochastic differences in cell physiology [43•]. Another study on starch content of synchronous cultures revealed an unexpectedly broad range of starch densities in individual cells during G1 phase that greatly surpassed the heterogeneity observed for cell sizes (Figure 2a) [45]. Further studies on the origins and connections between metabolic and cell cycle heterogeneity may shed light on these aspects of cell behavior that are poorly understood.

In a canonical binary fission cell cycle, size homeostasis is thought to be maintained by triggering division when cells pass a minimum size threshold (sizer model or titration model)and/or after they accrue a fixed amount of biomass(adder model) [46,47]. Single-cell tracking has been employed recently in studying size homeostasis in plant meristems where there is evidence of active size control, though the contributions of sizer or adder mechanisms are not clear [39•,40•]. Cell size homeostasis in Chlamydomonas has been studied in different ways, including measurements of cells from bulk synchronous cultures ,genetic perturbation, and by limited single cell tracking in wild-type cultures [5]. Although the accuracy of size control varies across species, size ranges can be maintained in a relatively narrow range. For example in fission yeast nearly all division occurs at mother cell lengths of 14 μm with a coefficient of variation of 6% [48]. Mitotic size control in a multiple fission cell cycle must cope with a very large range of mother cell sizes (>ten fold), but can only decrease cell sizes by multiples of 1/2; consequently, the largest and smallest daughters in a population must span a size range that is at least two-fold, but is typically even broader, which means that mothers of a given size do not always divide the same number of times (Figure 2a and b). Despite stochastic variation in population-level division behavior, behavior of daughter cells within one mother cell is highly correlated: after each round of division the two resulting sister cells will either both continue dividing or both exit S/M. This observation suggests that division number may be pre-programmed in a mother cell prior to initiation of S/M so that all sister cells undergo the same number of divisions (Figure 2b). This idea is also consistent with a model for size control based on titration of a ‘sizer’ molecule that is synthesized in mother cells prior to starting S/M (see below). One recent study modeled division behavior and accurately reproduced empirical results, but the model was only fit to a single mother–daughter dataset [49]. A combination of additional empirical observations and probabilistic/stochastic modeling may help generate a more

general framework to describe size control during multiple fission and spur further mechanistic studies that help tie stochastic or variable cell behavior to quantitative measures of cell cycle regulators [47,50].

Some progress in quantitatively connecting molecular events to cell size in Chlamydomonas has been made using size control mutants. Recently, a viable null mutant, *cdkg1–1*, was found to undergo insufficient numbers of mitotic divisions leading to large daughters, while forced mis-expression of *CDKG1* caused the opposite phenotype of small daughters. *CDKG1* encodes a sub-class of cyclin dependent kinase that is sister to the CDKA and CDKB families, and has orthologs in related green algae [51••], but no clear orthologs elsewhere. Genetic epistasis experiments placed *CDKG1* function upstream of the retinoblastoma pathway in mitotic size control, a finding that fits with its in vitro biochemical activity as a D-cyclin stimulated kinase that can phosphorylate RBR, presumably to antagonize RBR function as a cell cycle repressor. How CDKG1 and the RBR pathway interface with the core cell cycle machinery and possibly with GEX-protein functions remains unclear (Figure 2c).

In bulk cultures CDKG1 was synthesized just prior to S/M and disappeared completely upon exit back into G0 or G1 phase [51••]. Moreover, its relative concentration scaled with mother cell size. Quantitative single-cell imaging on individual mother cells of different sizes and stages of division revealed that CDKG1 is predominantly nuclear-localized and its concentration relative to nuclear DNA progressively decreased as cells completed each round of division during (Figure 2d). The quantitative and qualitative features of CDKG1 dynamics during multiple fission make it an appealing candidate for a ‘sizer protein’ whose abundance relative to nuclear DNA could control the activity of the RBR complex, thereby influencing the decision to undergo another round of S/M or exit into G0/G1 phase [51••]. Although the bases for titration models of size control differ among organisms, they are an emerging theme in cell size homeostasis [52]. Live-cell quantitative imaging of CDKG1 and other cell cycle regulators to investigate dynamics during multiple fission is a promising direction for understanding how stochastic behaviors of regulatory proteins are translated into binary decisions such as initiation of cell division versus mitotic exit [50].

Cellular architecture and scaling during the Chlamydomonas cell cycle

Besides cell size, there are other scaling relationships in Chlamydomonas that vary during the cell cycle and offer opportunities for quantitative analysis and modeling. For example, Chlamydomonas flagella-length seems to have a set point, and after severing, flagella rapidly regrow and reach their original size within 30–60 min [53]. A less well-understood observation is the kinetically slow process of continuous flagella lengthening as cells enlarge, with small, early G1-phase cells having shorter flagella than large, late G1 phase cells [54]. The relationship between nuclear volume (N), cell size (C) and the cell cycle has been studied in yeasts and plants where the N:C ratio remains constant [40•,55–57], but this relationship had not been extensively explored in cells that divide by multiple fission. During G1 phase in Chlamydomonas the nuclear volume expands in proportion to cell size while haploid nuclear DNA content remains constant. During S/M phase the large nucleus is subdivided with each round of cell division to maintain a constant N:C across the cell cycle

[51••]. A corollary of this scaling relationship is that nuclear DNA concentration can vary by many-fold during G1 phase, and the impact of this change on processes such as chromatin organization, nuclear transport and transcription is not known. Land plants have multiple plastids that appear to divide and segregate somewhat autonomously during the cell cycle [58]; whereas Chlamydomonas and many other algae have a single large chloroplast which grows and divides in tight coordination with the cell cycle [5,58]. Predicted chloroplast division genes are under coordinate cell cycle control [16•,21], but details about how the chloroplast and other organelles scale during G1 phase and segregate to daughters during the cell cycle are not known.

Future directions

Systems level, quantitative and modeling approaches to the Chlamydomonas cell cycle are still in early stages, but have already influenced our understanding of the conserved and unique features of multiple fission. Current models for the Chlamydomonas cell cycle are mostly qualitative descriptions. However, cell cycle mutants that affect different pathways and newly developed methods for quantitatively tracking individual cell cycle proteins in real time are just starting to be combined. New resources and methods such as an indexed insertional mutants and genome editing are also now available and offer additional opportunities to dissect and model cell cycle pathways [59–63]. The use of these new resources and genomics tools promise in sights into how deterministic and stochastic processes combine to control cell cycle behavior.

I highlight below some long-standing and unresolved questions about control of the Chlamydomonas cell cycle. Many of these also have relevance or remain unanswered for land plants and other taxa, and their continued investigation in Chlamydomonas using systems-level and single-cell approaches holds great promise.

- What occurs at the Commitment point, and what mechanisms generate a timer-like behavior in cells during the delay between passing Commitment and entering S/M phase?
- How do circadian and diurnal cues integrate with cell cycle machinery?
- How does CDKA activation occur during the transition from G1phase to S/M?
- How does the RBR pathway interface with chromatin and the cell cycle machinery to gate size-dependent cell cycle progression?
- How do *GEX* genes control cell cycle entry?
- What triggers exit from S/M phase and reentry into G1 or G0?
- What are the sources of stochastic variability in cell size control?

Acknowledgements

I thank Dr. Yi-Hsiang Chou, Dr. Elizabeth Haswell, Dianyi Liu and Sarah Rommelfanger for helpful feedback and editorial suggestions on this review.

This work was supported by the National Science Foundation [MCB 1515220] and National Institutes of Health [R01GM126557].

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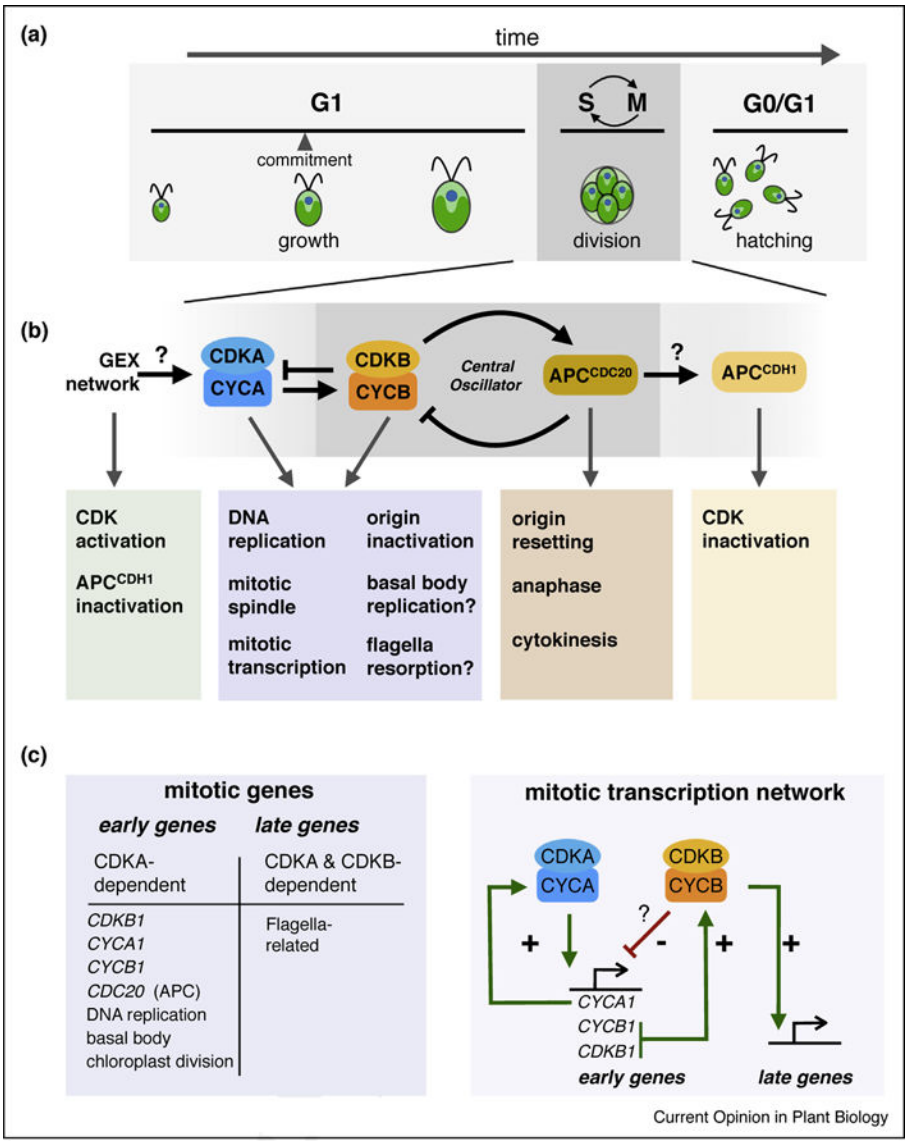

Figure 1.

Regulation of the multiple fission cell cycle. **(a)** Schematic of key stages in multiple fission with cell growth (G1) followed by alternating rounds of S phase and mitosis/cytokinesis (S/M) to produce 2^n daughters (four daughters pictured here), that hatch upon mitotic exit and reenter G0 or G1. Commitment is described in the main text. **(b)** Upper panels show a possible framework for the multiple fission cell cycle with major regulatory activities CDKA-CYCA, CDKB-CYCB, the CDC20-activated anaphase promoting complex (APC^{CDC20}), an E3 ubiquitin ligase which is unstable due to CDC20 itself being a APC substrate, and APC^{CDH1} whose activator subunit, CDH1, is not a APC substrate [1]. Arrows indicate positive regulation, and repression bars indicate inhibition. Question marks indicate inferred relationships. Dark gray arrows pointing downward show processes that are thought to be promoted by each of the major regulators in the upper panel. **(c)** Cell-cycle controlled genes or groups of genes, their relative expression timing, and their CDKA or CDKB dependencies are listed in the left panel. The inferred regulatory structure of cell cycle

transcription controlled by CDKs is diagrammed, with green arrows and plus signs showing positive regulation, and with red repression bars minus signs showing negative regulation. CDKA-CYCA forms a positive feedback loop via CYCA transcription and a feed forward loop that helps activate CDKB-CYCB. CDKB-CYCB activity negatively influences early cell cycle gene transcription, but this may be through indirect mechanisms.

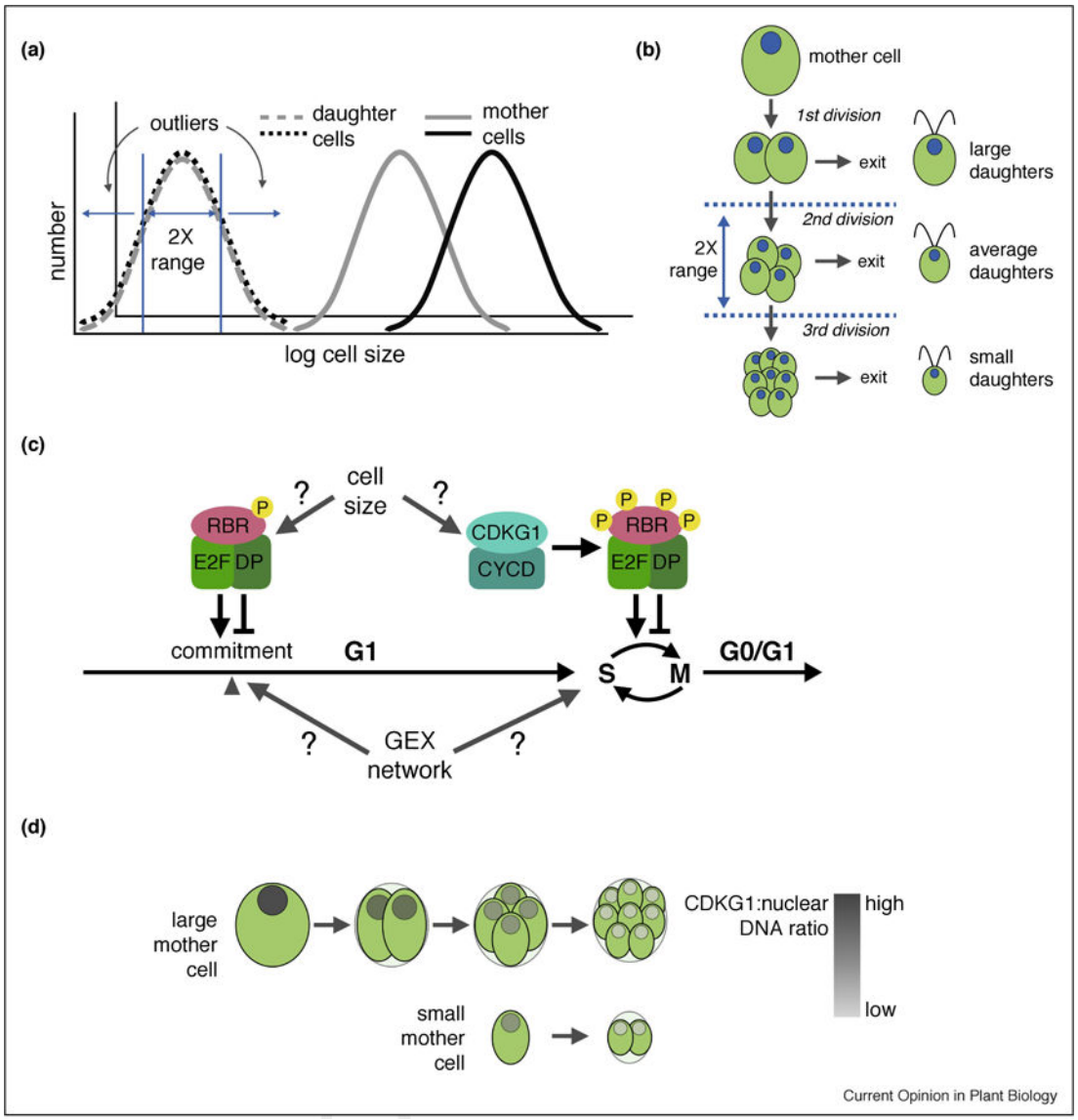

Figure 2.

Cell size control and the RBR complex. **(a)** Graph showing log-normal distributions of mother cells (solid lines) and their resulting daughters (dashed lines) from cultures with large (black) or small (gray) mother cells. Stochastic variation results in outliers that are larger or smaller than the idealized theoretical two-fold size range. **(b)** Example of stochastic behaviors of a mother cell that should divide two times to produce four daughters in the target size-range. Exiting S/M after only one division produces two large daughters, while exiting after three divisions produces eight small daughters. Note that within an individual mother cell there is little or no stochastic variation in division behavior between daughter pairs in the first or subsequent divisions, so daughter number is nearly always a power of two. **(c)** Schematic similar to that in Figure 1a showing the RBR complex and CDKG1 influencing size control at Commitment and during S/M. It is unknown how RBR in Chlamydomonas interfaces with the other cell cycle regulatory machinery depicted in Figure 1b. The GEX network influences entry into S/M phase, but may also operate by activating

the Commitment step. **(d)** Adapted from [50]. CDKG1 levels scale with mother cell size and are limiting for cell division number. Scale bar depicts ratios of nuclear localized CDKG1:DNA in mother cells and its decrease in daughters with each subsequent cell division. When this ratio falls below a threshold cells exit S/M and return to a G0 or G1 state.