Size-Dependent Expression of the Fission Yeast Cdc13 Cyclin is Conferred by Translational Regulation

Samirul Bashir[1], Xi-Ming Sun[2,3], Yixuan Zhao[1,4], Nuria G. Martínez-Illescas[1,5], Isabella Gallego-López[1,6], Lauren Guerrero Negrón1, Daniel Keifenheim[1,7], Tatiana Karadimitriou[1,8], Thi Tran[1,], Mary Pickering[1,9], Samuel Marguerat[2,3] and Nicholas Rhind[1,10]

1) Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, 364 Plantation Street, Worcester, MA 01605, USA
2) MRC London Institute of Medical Sciences (LMS), Du Cane Road, London W12 0NN, UK
3) Institute of Clinical Sciences (ICS), Faculty of Medicine, Imperial College London, Du Cane Road, London W12 0NN, UK
4) Present address: The Rockefeller University, 1230 York Avenue, New York, NY 10065, USA
5) Present address: Department of Biochemistry and Molecular Biology, School of Biology, Complutense University, Madrid, Spain
Breast and Gynecologic Cancer Group, Research Institute i+12; Madrid, Spain
6) Present address: VIB-KU Leuven Center for Brain & Disease Research, 3000 Leuven, Belgium
7) Present address: Department of Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN 55455, USA
8) Present address: USC Keck School of Medicine, 1975 Zonal Avenue, Los Angeles, CA 90033, USA
9) RNA Therapeutics Institute, University of Massachusetts Medical School, 368 Plantation Street, Worcester, MA 01605, USA
10) Corresponding author: nick.rhind@umassmed.edu

Abstract

Two fission yeast mitotic activators, Cdc13 and Cdc25, have been shown to increase in concentration in correlation with cell size, and have been proposed to thereby regulate cell size at division. Here, we show that the expression of both Cdc13 and Cdc25 are, in fact, size dependent, as apposed to simply size-correlated due to time-dependent expression. However, we also find that their size dependence is regulated by different mechanisms. Cdc25 was known to be regulated transcriptionally. Here, we show that Cdc13 is regulated translationally. Its transcript is not expression is a size-dependent manner, rather a size-dependent concentration of protein is expressed from a size-independent concentration of mRNA. Moreover, the degradation rate of Cdc13 is not size dependent, implicating size-dependent translation in its regulation. We identify a 20-amino-acid motif, which includes the APC D-box degron, as necessary and sufficient for size-dependent expression, which allowed us to construct a size-independent allele of *cdc13*. Using this allele, in combination with a size-independent allele of *cdc25*, expressed from a size-independent promoter, we show that size-dependent expression of neither Cdc13 nor Cdc25 is required for size control, nor are the redundantly required for size control.

Introduction

How a population of cells maintains a stable cell size is a fundamental question of cell biology for which no general answer has been discovered (Amodeo and Skotheim, 2016; Rhind, 2021). One potential solution to the problem of cell-size control is the size-dependent expression of cell-cycle regulators (Fantes et al., 1975). Such proteins fall into two classes: Diluted Inhibitors and Accumulating Activators. Diluted inhibitors have been proposed to regulate size at the G1/S transition in budding yeast (Schmoller et al., 2015), algae (Liu et al., 2022), plants (D'Ario et al., 2021), and mammals (Zatulovskiy et al., 2020). In each case, an inhibitor of the G1/S transition—Whi5, TNY1, KRP4 and Rb, respectively—is proposed to be expressed at fixed number of molecules early in the cell cycle so that its concentration is dependent on the size of the cell, with small cells having a higher concentration of the inhibitor than large cells. The inhibitor has a concentration threshold above which it prevents the G1/S transition, so smaller cells must grow more in G1 to reach that threshold, but all cells will enter S at about the same size.

The other class of size-dependent regulators are accumulating activators, which are proposed to increase in concentration in proportion to cell size, such that they are only expressed at a sufficient concentration to drive cell cycle transitions when cells reach a critical size threshold. The existence of accumulating activators as regulators of size control was inferred from cell biology experiments in the 1950s, '60s and '70s (Prescott, 1956; Thormar, 1959; Herring, 1974; Fantes et al., 1975; Rhind, 2018). In the fission yeast, *Schizosaccharomyces pombe*, two mitotic activators—Cdc13, the B-type cycle required for mitotic cyclin-dependent kinase (CDK) activity, and Cdc25, the tyrosine phosphatase required to activate CDK at the G2/M transition—have been shown to increase in concentration in G2 in correlation with cell size and have thus been proposed to be accumulating activators that regulate the G2/M transition (Moreno et al., 1990; Creanor and Mitchison, 1996; Keifenheim et al., 2017; Patterson et al., 2019; Curran et al., 2022; Miller et al., 2022; Miller et al., 2022).

Although size control by size-dependent expression of cell cycle regulators is an attractive paradigm, such a system has not been shown to be required for cellular size control in any organism. Moreover, size-dependent expression is unusual—most proteins are expressed at a constant concentration as cells grown (Schmoller and Skotheim, 2015)—and in only one case, KRP4 in *Arabidopsis*, has a mechanism for size-dependent concentration been proposed (D'Ario et al., 2021). Therefore, many important questions remain regarding if and how size-dependent protein expression regulates cell size.

We have addressed such questions in fission yeast, with particular focus on the major B-type cycle, Cdc13. We have investigated three major questions: Is Cdc13 expressed in a size-dependent manner? How is Cdc13's size-dependent expression regulated? and Is Cdc13's size-dependent expression required of size control?

The first question is important in the case of proposed accumulating activators because, in growing cells, size is correlated with time. So, a protein that increases in concentration in correlation with size could do so directly, or it could increase as a function of time after induction of expression, and only be indirectly correlated with size. There are two ways to distinguish between size- and time-dependent increase in protein expression. The first way is to compare the timescale for the increase in concentration with the half life of the accumulating protein. For a protein to accumulate over time, it must be out of steady state, that is to say, its rate of synthesis (which is concentration independent) must be higher than its rate of degradation (which is concentration dependent) (REF). At some point, the concentration of the protein will raise to a level at which synthesis is balanced by degradation; at that point, steady state will be reached and the

increase in concentration will stop. The time it takes to achieve steady state is approximately the half life of the protein (or its message, if the message is significantly more stable than the protein) (Mehra et al., 2003; Belle et al., 2006). Therefore, in the case in which protein expression is turned on and the protein concentration increases from a low level to a high level over time, a protein can only accumulate in a time-dependent manner for about as long as its half life, or the half life of its message. Proteins that increase in concentration for longer than that cannot be doing so in a time-dependent manner, unless their rate of synthesis also increases in a time-dependent manner.

The second way to distinguish between size- and time-dependent expression of a protein is to uncouple the direct correlation between size and time in asynchronous cultures. In unperturbed cultures, there is a tight correlation between a cell's size and the time it has grown since division. This correlation can be uncoupled by manipulating the size at division, so that cells in different cultures begin the cell cycle at different lengths. If one culture of cells divides at twice the size of another, a protein expressed at in a size-dependent manner would be expected to be expressed at twice the concentration at the beginning of the subsequent cell cycle, whereas a protein expressed in a time-dependent manner would be expected to start at the same concentration in both cultures.

The second question we addressed is important because little is know about how proteins can be expressed in a size-dependent manner. Cdc25 size dependence is regulated at the transcriptional level (Keifenheim et al., 2017), although how its promoter is regulated by size is unknown. Cdc13 accumulates during S, G2 and metaphase (Creanor and Mitchison, 1996; Patterson et al., 2019; Miller et al., 2022) and then is degraded during anaphase and G1 by the anaphase-promoting complex (APC)-dependent proteolysis (Blanco et al., 2000). The APC recognizes Cdc13 via a short D-box motif in its N-terminus. However, how Cdc13 expression is regulated during S and G2 is unknown.

The third question is important because in no case is the mechanism of cell size control understood. Cell-size control is an essential cellular function. Cells unable to coordinate growth and division are inviable, either because they divide too often and come inviably small or because they do not divide often enough and become inviably large. Therefore, abrogating cell size control should lead to inviability. Loss of Rb leads to a loss of size dependence of the G1/S transition in mammalian cells (Zatulovskiy et al., 2020) and loss of Whi5 is presumed to have the same affect in budding yeast, but in neither case is cellular size control lost, presumably because of redundant size control mechanisms regulating other cell cycle transitions. A good candidate for such redundant regulation is size-dependent regulation of the G2/M transition. Therefore, it will be interesting to understand how size affects this transition in general and how Cdc13 is involved in its regulation in fission yeast in particular.

Results

Cdc13 is Expressed in a Size-Dependent Manner

To investigate the level of Cdc13 expression in individual cells and to test for a correlation with cell size, we tagged the 3' end of the endogenous *cdc13* gene with sequence encoding the green fluorescent protein NeonGreen (NG). Cdc13-NG localizes to the nucleus, as expected (Booher et al., 1989), and cells expressing it show only a slight increase in length (12.8±2.9 μm for *cdc13-NG* cells (yFS1036) v. 11.8±2.5 μm for wild-type cells (yFS109)), demonstrating that the tagged protein is close to fully functional. We used widefield fluorescence microscopy quantitate Cdc13-NG signal in cells and correlate it with cell length (Figure 1A).

We increased the range of cell length over which we measured Cdc13 concentration by overexpressing a temperature-sensitive allele of Wee1, the kinase that inhibits CDK during G2, to regulate cell size. At the permissive temperature of 25˚C, the high level of Wee1 prevents dephosphorylation of Cdc2 and arrests cell in G2 (Russell and Nurse, 1987). By increasing the temperature between 27˚C and 33˚C, we can titrate down the specific activity of Wee1 and achieve levels of Wee1 activity that result in asynchronous cultures with cell lengths that range from about 4 μm to 8 μm at 33˚C to from about 15 μm to 30 μm at 27˚C (Figure 1D). These cultures are otherwise healthy and have doubling times similar to wild-type cells (Russell and Nurse, 1987). By combining data from this range of sizes, we observed that Cdc13 concentration in cells correlates linearly cell size over at least a 3.5-fold range (Figure 1A). In contrast, Cdc2 maintains a constant concentration over a similar size range (Figure 1A), as has be reported for almost every over protein in fission yeast (Marguerat et al., 2012).

As discussed in the introduction, the correlation between Cdc13 concentration and cell size could be directly due to size-dependent expression of Cdc13, or indirectly due to Cdc13 accumulating over time during the cell cycle. To determine if Cdc13 and its transcript are stable enough to accumulate in a time-dependent manner over the 3 hours fission yeast cell cycle, we measured their half lives. We did so by blocking their synthesis, with cycloheximide or thiolutin, respectively, and assaying their levels over time to

infer their decay rates (Figures 1B, C). The half life of Cdc13 is about 30 minutes, consistent with previous reports (Christiano et al., 2014; Esposito et al., 2022), and it does not change much with cell size (Figure 1C). The difference between cells averaging 10.7±0.3 μm in length and those averaging 21.2±1.3 μm is only 28% (Figure 1C). The half life of the *cdc13* transcript is less than 10 minutes (Figure 1B), consistent with previous reports (Eser et al., 2016). Therefore, Cdc13 is unable to accumulate in a time-dependent manner for more than about 30 minutes. Therefore, the correlation we see between Cdc13 concentration and size over a 180 minute cell cycle is unlikely be due to time-dependent accumulation.

The second way to distinguish between size- and time-dependent expression of Cdc13 is to uncouple the direct correlation between size and time in asynchronous cultures. We did so using the approach described above of overexpressing a temperature-sensitive allele of Wee1. Using four temperatures (27˚C, 29˚C, 31˚C and 33˚C), we can produce four cultures that begin the cell cycle at a range of lengths (about 4 μm, 6 μm, 8 μm and 15 μm, respectively), but that all grow with the similar doubling times. By measuring Cdc13 concentration in these cultures, we find that Cdc13 correlates with the size of a cell, not the amount of time it has been growing since the beginning of the cell cycle. (Figure 1D). For instance, cells that are 14 μm long have about the same concentration of Cdc13 regardless of whether they are at the beginning of their cell cycle (as is the case for the culture grown at 27˚C), the middle of their cell cycle (as is the case for the culture grown at 29˚C), or at the end of their cell cycle (as is the case for the culture grown at 31˚C). These results, along with the short half life of Cdc13 and its transcript, lead us to conclude that Cdc13 is expressed in a size-dependent manner.

Cdc13 Expression is Regulated Translationally

To determine how Cdc13 size-dependent expression is achieved, we examined the size dependence of its transcript. We measured steady-state transcript levels in individual cells using single-molecule RNA fluorescence in situ hybridization (smFISH) (Keifenheim et al., 2017; Sun et al., 2020). As is the general case for transcripts in fission yeast and other organisms (Zhurinsky et al., 2010; Marguerat et al., 2012; Padovan-Merhar et al., 2015), the *cdc13* transcript maintains a constant concentration with respect to cell size (Figure 2). This is the case both in unperturbed, wild-type, asynchronous cells (Figure 2A) and in cells that vary over 5-fold in length obtained by manipulation of Wee1 levels in five separate asynchronous cultures (Figure 2B). In this case, we used a strain expressing wild-type Wee1 under the control of the estradiol-responsive ZEV promoter (Ohira et al., 2017) to grow asynchronous cultures with cells from 4.1 to 28.8 μm in length. These results demonstrate that the size dependence of Cdc13 expression is achieved post-transcriptionally. Combined with the observation that Cdc13 half life is not strongly size dependent (Figure 1C), these results suggest that Cdc13 size-dependent expression is achieve by the regulation of protein synthesis, presumably the regulation of translational initiation, but possibly translational elongation.

Size-Dependence is Encoded in an N-Terminal Motif of the Cdc13 Protein

We next undertook a structure-function analysis of the *cdc13* gene to identify the region required for its size-dependent expression. Initially, we tested whether size dependence we encoded in the untranscribed, untranslated or translated parts of the gene. To do so in a way that allowed us to test essential parts of *cdc13*, we built a exogenous, NeonGreen-tagged copy of *cdc13* and integrated it in a strain containing the *wee1-ts* overexpression construct. Cdc13-NG expressed from its own promoted, with its own 5'- and 3'-UTRs, shown size dependence similar to expression from its endogenous locus (Figure 3A). Replacement of the *cdc13* promoter, 5'- and/or 3'-UTR with regulatory sequences from the *cdc2* or *adh1* genes does not affect size dependence of Cdc13 expression (Figure 3A). Together, these results demonstrate that size dependence is encoded in the *cdc13* ORF.

To further localize the sequence required for size-dependent expression within the *cdc13* ORF, we performed a deletion analysis, this time using the *ZEV:wee1* system, which allows us to control cell size with estradiol (Ohira et al., 2017). To be able to quantitatively distinguish size-dependent from size-independent expression, we developed a metric that represents the correlation between size and relative protein concentration. The metric, which is effectively the slope of a linear fit to the data on a relative size to relative concentration plot, is 1 if size is perfectly correlated with size and is 0 if it is uncorrelated with size. In practice, we find a clear distinction between size-dependent expression, with a score of greater than 0.5, and size-independent expression, with a score less than 0.15 (Figure 3C).

We initially split the *cdc13* ORF in the two parts that encode its N-terminal regulatory domain and its C-terminal cyclin domain (REF), and found that the N-terminal region is necessary for size-dependent expression (Figure 3B). Further deletions narrowed the critical region down to a 20-codon region that encodes the D-box degron, which is responsible for the APC-dependent degradation of Cdc13 during late mitosis and G1.

Finding that a small region encoding the N-terminus of Cdc13 is required for its size-dependent expression could indicate that size-dependence is conferred by the amino acids in that part of the protein or by the RNA that encodes them. To distinguish between these two possibilities, we recoded the first 122 codons of *cdc13*, replacing every ambiguous nucleotide to change the RNA sequence as much as possible without changing the encoded protein sequence. Such recoding does not affect the size-dependent expression of Cdc13, suggesting that size-dependence is encoded in Cdc13s amino acids, not the nucleotides of its transcript.

Constructs that retain the degron region retain size-dependent expression. However, it is more difficult to determine if constructs that lack this region are expressed in a size-dependent manner because they are not degraded during mitosis. Therefore, the amount of Cdc13 in cells reflects Cdc13 inherited from the parental cell, in addition to newly synthesized Cdc13, obscuring the kinetics of Cdc13 synthesis. This complication is manifest in the fact that a construct expressing only the N-terminal 50 amino acids of Cdc13 shows no size dependence (correlation metric = 0.09) but has an overall signal that is more that fifteen-fold higher than the full-length construct (Figure 3B). This level of Cdc13 overexpression would plausible be lethal, due to interference with mitotic exit (REF)(Murray, 1989), and may explain why we have been unable to create strains carrying constructs that would express the C-terminal cyclin domain without the N-terminal regulatory domain.

Nonetheless, we were able to create a strain that expresses a Cdc13 allele lacking just amino acids 51-70 (Cdc13Δ51-70), which contain the D-box degron. This construct is expressed at lower levels—only about twice the full-length level—and is not toxic. From these results, we surmise that the rest of the N-terminal regulatory region confers instability to Cdc13, relative to that of its C-terminal cyclin domain. Cdc13Δ51-70 appears to be expressed in a size-independent manner, suggesting that those 20 amino acids are necessary and sufficient for size-dependent expression. To confirm that the slightly increased stability of Cdc13Δ51-70 is not obscuring size-dependent expression, we fused a ubiquitin degron to the N-terminus of the protein. The N-terminal ubiquitin is expected to be removed by constitutive ubiquitin hydrolases, exposing an N-terminal tyrosine that destabilizes the protein (Houser et al., 2012). This ubiquitin degron decreases the average fluorescence intensity of cells carrying Cdc13Δ51-70 about tenfold, but does not affect its size-independence. Therefore, we conclude that the 20 amino acids that include the D-box degron are necessary and sufficient for size dependent expression.

Size-Dependent Expression of Cdc13 and Cdc25 is not Necessary for G2 Size Control in Fission Yeast

Based on the correlation between concentration and cell size for both Cdc13 (Creanor and Mitchison, 1996; Patterson et al., 2019; Curran et al., 2022) and Cdc25 (Moreno et al., 1990; Creanor and Mitchison, 1996; Keifenheim et al., 2017; Curran et al., 2022; Miller et al., 2022), these two positive regulators of the G2/M transition have been proposed to be involved in fission yeast G2 size control. We tested whether Cdc25 is in fact expressed in a size-dependent manner, as we had done for Cdc13, and we find it is (Figure 4A), consistent with the short half life of the Cdc25 protein and transcript (Keifenheim et al., 2017) and with a recent report (Miller et al., 2022). We therefore tested if either Cdc13, Cdc25, or the two redundantly are required for G2 size control in fission yeast by expressing either or both proteins in size-independent manners.

The fact that *cdc25* is regulated transcriptionally(Keifenheim et al., 2017) makes it straightforward to change its expression to size-independent by replacing its promoter at its endogenous locus with the size-independent *nmt41* promoter (Figure 4B). Such strains are healthy, grow at a normal rate and maintain robust size homeostasis (Figure 4C,D). Therefore, size-dependent expression of Cdc25 does not appear to be required for G2 size control.

Our structure-function analysis of Cdc13 identified deletions that are expressed at size-independent concentrations (Figure 3). In particular, it seemed plausible that Cdc13-Δ51-70, which is expressed in a size-independent manner and lacks only 20 amino acids from its N-terminal regulatory domain, would be a functional allele. To test this possibility, we deleted the endogenous *cdc13* in strains carrying both *cdc13-Δ51-70* and *ubi-cdc13-Δ51-70*. In both cases, we obtained strains that are healthy, grow at a normal rates and maintain robust size homeostasis (Figure 4C,D). Therefore, size-dependent expression of Cdc13 does not appear to be required for G2 size control.

To test whether size-dependent expression of Cdc13 and Cdc25 might be redundantly required of G2 size control, we built a *nmt41:cdc25 ubi-cdc13-Δ51-70* double mutant. The strain is healthy, grows at a normal rate and maintains robust size homeostasis (Figure 4C,D). We built a similar strain with *cdc13-Δ51-70* and got similar results. The single and double mutant strains are longer than wild type (Figure 4C) indicating that the size-independent alleles of *cdc13* and *cdc25* have reduced activity. However, that reduction could be due simply to their levels of expression. Consistent with that explanation, when

nmt41:cdc25 cells (yFS887) are grown in thiamine-free medium, which induces the *nmt41* promoter, the average length at septation is reduced from 25.7±2.3 μm to 13.9±2.5. The single and double mutations also have a higher coefficient of variation (CV) in length at septation than wild type (15-25% v. 9%, Figure 4C), which might suggest weaker size homeostasis. However, the majority of mutant cells actually divide in a narrow range (Figure 4D). The observed increase in CV is driven largely outliers, not a general loss of size homeostasis. In any case, fission yeast can still clearly initiate the G2/M transition in the absence of size-dependent expression of Cdc13 and Cdc25.

Discussion

We have characterized the size dependence of the fission yeast B-type cycle, Cdc13, and investigated its role in size-dependent regulation of the G2/M transition. We find that Cdc13 is expressed in a size-dependent manner and that it's size dependence seems to be conferred by size-dependent translation. However, we find that size-dependent expression of Cdc13 is not required, either on its own or redundantly with size-dependent expression of Cdc25, for size control of the G2/M transition.

We conclude that Cdc13 is expressed in a size-dependent manner, and specifically that the correlation between its concentration and cell size is not an indirect result of time-dependent expression, from two sets of results. The first is the short half life of Cdc13 and its transcript (Figure 1B,C). When expression of a protein is turned on, the concentration of that protein increases over time until it reaches its stable steady-state concentration. The reason that it takes time for a protein to reach its steady-state concentration, and the reason that its steady-state is stable once reached, is that, initially, its rate of synthesis (which is concentration independent) is higher than its rate of degradation (which is concentration dependent). As its concentration increases, it reaches its steady-state level at which synthesis and degradation are balance. The increase in concentration up to that point is time dependent and should occur at the same rate regardless of when during the cell cycle, or at what cell size, expression is initiated. And, critically, the time it take to reach steady state is determined by the half lives of the protein and its transcript. Proteins with short half lives come to steady state quickly; a long half life is required to increase in a time-dependent manner for an extended period of time. The short half life of Cdc13 and its transcript (Figure 1B,C) therefore lead us to conclude that its size-dependent expression over the several hours of the fission yeast cell cycle cannot be time dependent, in the standard understanding of item dependence.

There is a more complicated scenario that could reconcile the short half lives of Cdc13 with time-dependent expression. That scenario is one in which the rate of Cdc13 synthesis is not constant, but instead increases with time. In such a scenario, Cdc13 expression would not come to steady state (or would do so more slowly, depending on rate of change of Cdc13 synthesis) and so would continue increasing beyond the timescale of its half life. One straightforward mechanism for such scenario would be a positive feedback loop in which CDK activity increases the rate of Cdc13 synthesis.

To rule out this more complicated scenario, we took a second experimental approach, which also supports our conclusion that Cdc13 is expressed in a directly size-dependent manner. By varying the size at division in asynchronous cultures thought the manipulation of the activity of the Wee1 kinase, we obtained culture that begin the cell cycle at various sizes. If Cdc13 is expressed in a time-dependent manner, it should be expressed at the same level in all cells at the beginning of the cell cycle, independent of cell size. If, on the other hand, it is expressed in a size-dependent manner, it should exhibit similar concentrations at similar sizes, independent of how long the cells have been in the cell cycle. We observe the latter result, with cells that are 14 μm long have about the same concentration of Cdc13 regardless of whether they are at the beginning, the middle, or the end of their cell cycle (Figure 1D). A recent report describes a similar experiment using deletion of *cdr2*, which encodes a negative regulator of Wee1, to affect cell size, but reaches the opposite conclusion (Miller et al., 2022). We obtained the *cdr2Δ cdc13-NG* strain from Miller et al., repeated their experiment and got ambiguous results. We find that Cdc13 concentrations are slightly lower in similarly sized *cdr2Δ* cells, when compared to *cdr2+* cells, but not as much lower as time-dependent expression predicts. The ambiguity is caused by the fact that the difference in size between the *cdr2+* and *cdr2Δ* cells is only about 35%, making it difficult to distinguish time- from size-dependence. The difference in the size of the cultures in our experiments is 350% (Figure 1D), making the distinction obvious. Nonetheless, Miller et al. present other data in support of their conclusion that Cdc13 is expressed in a time-dependent manner, which we have yet to directly address.

The Cdc13 transcript is not expressed in a size-dependent manner (Figure 2A), therefore Cdc13's size dependence must be regulated at the level of protein synthesis or degradation. Since the half life of Cdc13 does not vary greatly with cell size (Figure 1C), we conclude that the size-dependent step in Cdc13 expression must be protein synthesis, probably translational initiation, but perhaps translational elongation.

To investigate how Cdc13 translation is regulated in a size-dependent manner, we narrowed down the sequence necessary and sufficient for size-dependent expression to a 20-codon motif in the region encoding its N-terminal regulatory domain (Figure 3B,C). To determine if the RNA sequence of this motif, or the protein sequence that it encodes, is responsible for size-dependent expression, we recoded the first 122 codons of Cdc13, replacing every ambiguous nucleotide with one that would not change the encoded protein sequence. With this recoding, we hoped to disrupt any regulatory motifs or secondary structures contained in the RNA sequence without affecting the encoded protein. The fact that the recoded sequence is expressed in a size-dependent manner leads us to conclude that size dependence is conferred by the protein sequence, although it is possible that the recoding failed to disrupt a critical RNA regulatory sequence.

The 20-amino-acid motif that we identified as being necessary and sufficient for size-dependence contains the D-box motif recognized by the APC for degradation of Cdc13 during post-anaphase mitosis and G1. It is not expected that the APC would regulate Cdc13 stability during G2, because it is not though to be active then. However, we have yet to determine if the APC degron, *per se*, is required for size dependence, and, even if it is, it is possible that it could be recognized be different regulator during G2. It is also surprising that Cdc13Δ51-70 is viable. Removal of the APC degron from cyclin B in budding yeast and *Xenopus* extracts causes anaphase arrest with high CDK levels (REF)(Murray, 1989). The fact that we are unable to create strains carrying just the C-terminal cyclin domain of Cdc13 suggests that overexpression of a stable Cdc13 is also lethal. The viability of strains expressing Cdc13Δ51-70 may be due to a secondary APC degron that has been identified in the very N-terminus of Cdc13 (REF) or to the ability of Wee1 to inhibit CDK post anaphase (REF); either of both of the mechanisms may allow sufficient reduction in CDK activity to allow cell to enter G1.

More surprising is that fact that Cdc13 seems to be regulated translationally via an internal protein motif. If Cdc13 concentration is regulated by size-dependent translational initiation, it is unclear how an internal peptide sequence could be required. Even if its regulation affects elongation, the involvement of protein motif, particularly one implicated in protein degradation, is unexpected. More experiments will be required to investigate these apparently inconsistent conclusions.

Regardless of the mechanism of Cdc13's translational regulation, how any such mechanism could be size-dependent is hard to conceive. To the extent that proteins and RNA generally maintain a constant concentration as cells grow (Zhurinsky et al., 2010; Marguerat et al., 2012; Schmoller and Skotheim, 2015), there should no difference in translation (or any other biochemistry) between small and large cells. The one thing that does universally change with cell size is the concentration of DNA, with the ratio of protein to DNA increasing as cells grow. This change has been proposed as the basis for a number of different mechanisms by which transcription could be made to be size dependent (Wang et al., 2009; Schmoller and Skotheim, 2015; Schmoller et al., 2015; Dorsey et al., 2018). Therefore, we speculate that it is unlikely that Cdc13's translation is directly size-dependent. Instead, we offer the hypothesis that Cdc13's translation is regulated by a size-dependent translation factor that is itself regulated by size-dependent transcription.

Cdc13's size-dependent expression makes it a prime candidate for a central cell-size regulator as an accumulating activator of the G2/M transition in fission yeast (Figure 1A,D and Patterson et al., 2019; Creanor and Mitchison, 1996; Curran et al., 2022). Our analysis, which identified Cdc13Δ51-70 as functional allele that is expressed is a size-independent manner, allowed us to test that hypothesis directly. The fact that cells expressing Cdc13Δ51-70 as their only copy of Cdc13 are viable, healthy and maintain size homeostasis demonstrates that size-dependent expression of Cdc13 is not required for cell-size control (Figure 4C,D). Cdc25, the other mitotic activator that accumulates in a size-dependent manner (Figure 4A and Keifenheim et al., 2017; Creanor and Mitchison, 1996; Moreno et al., 1990; Curran et al., 2022; Miller et al., 2022), is likewise a prime candidate for a central cell-size regulator as an accumulating activator of the G2/M transition in fission yeast. We similarly tested Cdc25's role, using a promoter-swap allele that expresses Cdc25 in a size-independent manner (Figure 4B), and find similarly that such cells are viable, healthy and maintain size homeostasis, demonstrating that size-dependent expression of Cdc25 is not required for cell-size control (Figure 4C,D). To test the hypothesis that size-dependent of either Cdc13 or Cdc25 could redundantly regulate size, we built the *cdc13Δ51-70 nmt41:cdc25* double mutant. That strain is also viable, healthy and maintains size homeostasis, demonstrating that size-dependent expression of Cdc13 and Cdc25 are not redundantly required for cell-size control (Figure 4C,D).

The fact that Cdc13 and Cdc25 are not required for G2/M size control in fission yeast does not mean that they are not involved in the process. Cdr2 has been proposed to regulate G2/M size control in fission yeast, via its regulation of Wee1 (Pan et al., 2014; Facchetti et al., 2019; Miller et al., 2022), although it too has been shown to be dispensable for such regulation (Facchetti et al., 2019; Miller et al., 2022). It has been proposed that all three—Cdc13, Cdc25 and Cdr2—could be redundantly required for G2/M size control in fission yeast (Miller et al., 2022). Alternatively, it is possible that many positive and negative regulators of

the G2/M transition collaborate to regulate size control (Chen et al., 2020). Either was, detailed understanding of the mechanisms of size-dependent expression of cell-cycle regulators will be crucial for unpicking the strategies that cell use to main stable cell size.

Methods

Strain and plasmid construction

Strains and plasmids were created using standard approaches (Forsburg and Rhind, 2006). Cultures were grown in YES at 30˚C, unless otherwise noted. Strains, plasmids and primers used in this study are listed in Tables 1, 2 and 3. Plasmids were created using Gibson Assembly (NEB) as detailed in Table 4.

To create a recoded version for *cdc13* in pFS524, the following sequence, designed to replace every ambiguous nucleotide without changing the encoded amino acids, in which the replaced nucleotides are lowercase and codons 51-70 are italic, was purchased from Genewiz (Azenta Life Sciences).

ATGACcACtCGcaGaTTgACcCGtCAaCAtCTgTTaGCgAAcACtTTaGGtAAtAAcGAtGAgAA
cCAcCCcTCtAAtCAcATcGCtaGaGCgAAgAGtTCcTTaCAtTCcTCgGAgAAcTCcTTgGTg
AAcGGtAAaAAAGCtACcGTa*TCcTCtACtAAtGTcCCcAAaAAaCGcCAcGCaTTaGAcGAcG*
*TcTCtAAcTTcCAtAAt*AAgGAgGGcGTgCCcTTgGCgAGcAAgAAtACgAAcGTtAGgCAtAC
cACaGCgTCcGTgAGcACtCGcaGaGCgCTgGAaGAgAAaTCaATcATtCCcGCgACtGAcGAc
GAgCCaGCcTCtAAaAAaCGcCGtCAgCCcTCcGTcTTcAAcTCg

cdc13 was deleted by PCR-mediated cassette deletion using a NatMX6 cassette amplified from pFS272 plasmid with SB40 and SB41 primers and transformed into yFS1120. *ZEV:cdc25* was built by PCR-mediated N-terminal tagging using a KanMX ZEV promoter cassette amplified from pFS478 with primers SB124 and SB155 and transformed into yFS1105. *nmt41:cdc25-NG* was built by PCR-mediated C-terminal tagging using a NeonGreen HygMX cassette amplified from pFS508 with primers SB36 and SB37 and transformed into yFS887 strain.

Fluorescent Microscopy

Cells were grown in YES medium to mid log phase. To obtain different sized cells, cells were either grown at different temperatures or in presence of different levels of beta-estradiol, as indicated in the figure legends. Cells were fixed in 100% methanol at -80°C for at least 16 hours. Cells were rehydrated in 1X PBS and imaged by widefield fluorescence microscopy with a DeltaVision-enabled Olympus IX71 inverted microscope with a 60x/1.42 oil-immersion objective. Images acquired were analyzed using ImageJ (Schneider et al., 2012) and pomBseen (Ohira and Rhind, 2022).

Transcript Half Life

For calculation of transcript half-life, log phase cultures of wild-type cells (yFS105) were treated with 15 mg/ml thiolutin to inhibit polymerase II (Mendell et al., 2000) and 10 OD samples were taken at 0, 5, 10 and 30 min. Samples were pelleted and frozen in liquid nitrogen. Total RNA was isolated from pellets using the Direct-zol kit (Zymo Research). First strand synthesis was performed using random hexamers and SuperScript III first strand synthesis kit (Invitrogen). qPCR was performed using SYBR Fast qPCR kit (Kappa). Transcripts were normalized to the 0' time point and *srp7* as an internal control for a stable transcript (Mendell et al., 2000). Primers—DK368 and DK369 for *cdc13*, DK374 and DK375 for *upf2*, and DK372 and DK373 for *srp7*—are listed in Table 2. Half life data was fit with exponential decay curves using Igor Pro (WaveMetrics).

Protein Half Life

To measure the half-life of Cdc13 in cells of different sizes, a strain expressing Cdc13-NG and an overexpressed temperature-sensitive allele of Wee1 (yFS1036) was grown to log phase at 28ºC, 30ºC, and 32ºC. 100ug/ml cycloheximide was added to each culture and samples were taken at 0, 15, 30 and 60 min. Samples were fixed with methanol prior to imaging as described above. Half life data was fit with exponential decay curves using Igor Pro (WaveMetrics).

Single Molecule RNA Fluorescence In Situ Hybridization (smFISH)

smFISH samples were prepared according to a modification of published protocols (Trcek et al., 2012; Heinrich et al., 2013). Briefly, cells were fixed in 4% formaldehyde and the cell wall was partially digested using Zymolyase. Cells were permeabilized in 70% EtOH, pre-blocked in BSA and salmon sperm DNA, and incubated over-night with custom Stellaris oligonucleotides sets (Biosearch Technologies) designed against cdc25 (CAL Fluor Red 610) and rpb1 (Quasar 670) mRNAs (Table S2). Cells were mounted in ProLong Gold antifade reagent with DAPI (Life Technologies) and imaged on a Leica TCS Sp8 confocal microscope, using a 63x/1.40 oil-immersion objective. Optical z sections were acquired (z-step size 0.3 microns) for each scan to cover the depth of the cells. Cell boundaries were outlined manually and single mRNA molecules were identified and counted using the FISH-quant MATLAB package (Mueller et al., 2013). Cell area, length and width were quantified using custom-made ImageJ macros. The FISH-quant detection technical error was estimated at 6%–7% by quantifying rpb1 mRNAs simultaneously with two sets of probes labeled with different dyes.

Acknowledgements

We are grateful to Christiana Baer and the Sanderson Center for Optical Experimentation (SCOpE) for access to, and technical support for, the DeltaVision microscope. This work was supported by NIGMS R01 GM134300 to NR.

Figure 1. Cdc13 is Expressed in a Size-Dependent Manner
A) Cdc13 Concentration Correlates with Size. Asynchronous cells expressing Cdc13-NG from its endogenous locus (yFS1036) were analyzed by widefield fluorescence microscopy. Asynchronous cultures of cells spanning various size ranges were obtained by growth between 27˚C and 33˚C to regulate the activity of Wee1-50ts overexpressed from the *adh1* promoter. Data from such cultures was combined and data from binucleate cells, which are in late mitosis, G1 and early S phase, and in which Cdc13 is degraded by APC-dependent proteolysis, was removed. Cdc2-GFP cells (yFS1133) were similarly analyzed. The small symbols represent individual cell; the large symbols represent an average of the data in 50-cell bins.
B) The *cdc13* Transcript has a Short Half Life. Wild-type cells (yFS105) were treated with 15 mg/mL thiolutin and sampled as indicated for RNA quantitation by qRT-PCR. The mean and SEM, relative to time 0 and to *srp7*, a control stable RNA(Mendell et al., 2000), are shown. n = 3. Data was fit with exponential decay curves using Igor Pro (WaveMetrics). The half life estimates are likely underestimates both because the lag time required for thiolutin to inhibit transcription is not accounted for in the fits and because the analysis assumes that *spr7* is perfectly stable, which it may not be.
C) Cdc13 has a Short Half Life. Cdc13-NG cells (yFS1036) were analyzed as in A. Asynchronous cultures of various lengths were treated with 100 mg/mL cycloheximide and sampled at the indicated times. The mean and SEM of the background-subtracted nuclear signal is shown. n = 3. Data was fit with exponential decay curves using Igor Pro (WaveMetrics).
D) Cdc13 is Expressed in a Size-Dependent Manner. Cdc13-NG cells (yFS1036) were analyzed as in A. Individual cultures grown at different temperatures are plotted in different colors. Light and dark colors represent biologically independent repeats.

Figure 2. *cdc13* is not Transcribed in a Size-Dependent Manner
A) The *cdc13* Transcript Maintains a Constant Concentration in Asynchronous Wild-Type Cells. Wild-type cells (yFS105) were analyzed by smFISH for transcript numbers of *cdc13* and *rpb1*, a standard smFISH control. The small symbols represent individual cell; the large symbols represent the average of the data in 20-cell bins.
B) The *cdc13* Transcript Maintains a Constant Concentration Over a Wide Range of Cell Sizes. Asynchronous cultures of *ZEV:wee1* cells (yFS970) spanning various size ranges were obtained by growth 0 to 100 mM beta-estradiol to regulate the expression of Wee1. *cdc13* and *rpb1* transcript numbers in individual cells was measured by smFISH. Data from all of the cultures was combined. The small symbols represent individual cell; the large symbols represent the average of the data in 20-cell bins.

Figure 3. Cdc13 Size-Dependent Regulation is Encoded in a 20 Amino Acid N-terminal Motif
A) Cdc13 Size-Dependent Regulation is Encoded in Its ORF. The size dependence of Cdc13 expressed from various depicted constructs was assessed as in Figure 1A.
B) Deletion Analysis of the Cdc13 ORF. The size dependence of Cdc13 expressed from various ORF deletions was assessed as in Figure 1A, except that cell size was manipulated by growing *ZEV:wee1* cells in 0 to 100 mM beta-estradiol.
C) Data from the Deletion Analysis of the Cdc13 ORF. The data from B is plotted as the average values of 50-cell bins. Error bars represent the SEM of fluorescent signal and size. The legend states the strain name and the correlation metric for the data.

Figure 4. Cdc13 and Cdc25 are Not Redundantly Required for Size Control
A) Cdc25 is Expressed in a Size-Dependent Manner. The size dependence of Cdc25 was assessed as in Figure 1A, except that cell size was manipulated by growing *ZEV:wee1* cells (yFS078) in 0 to 30 mM beta-estradiol.
B) *nm141:cdc25* Expresses Cdc25 in a Size-Independent Manner. The size dependence of Cdc25 expressed from the *nmt41* promoter (yFS1132) was assessed as in Figure 1A.
C) Size-Dependent Expression of Neither Cdc13 nor Cdc25 is Required for Viability. Wild-type (yFS110), *nmt41:cdc25* (yFS887), *cdc13-ubi-Δ51-70* (yFS1123) and *nmt41:cdc25 cdc13-ubi-Δ51-70* (yFS1123) cells were grown to mid log and photographed.
D) Size-Dependent Expression of Neither Cdc13 nor Cdc25 is Required for Size Homeostasis. The distribution of lengths at septation for the cultures shown in C. n = 50 for each strain.

Table 1: Strains used

Strain	Genotype
yFS105	h- leu1-32 ura4-D18
yFS110	h- leu1-32 ura4-D18 ade6-216 his7-366
yFS887	h- leu1-32 ura4-D18 nmt41:cdc25 (KanMX)
yFS970	h- leu1-32::pFS461 (adh1:ZEV leu1) ura4-D18 ZEV:wee1 (KanMX)
yFS1036	h+ leu1-32 ura4-D18 cdc13-NG (HygMx) wee1::pWAU-50 (adh1:wee1-50 ura4) nhp6-mcherry (Ura4)
yFS1078	h- leu1-32::pFS461 his7:pFS462 (adh1:ZEV his7) ura4-D18 ade6-216 cdc25-NG (HphMX) ZEV:wee1 (KanMX) nph6-mCherry (ura4)
yFS1104	h+ leu1-32::pFS514 (ade4:cdc13ΔC-NG leu1) ura4-D18 wee1::pWAU-50 (adh1:wee1-50 ura4)
yFS1106	h- leu1-32::pFS513 (cdc2:cdc13-NG leu1) ura4-D18 wee1::pWAU-50 (adh1:wee1-50 ura4)
yFS1109	h+ leu1-32::pFS515 (cdc2:1-122 cdc13-NG leu1) ura4-D18 his7::pFS462 (adh1:ZEV his7) ZEV:wee1 (KanMX)
yFS1110	h- leu1-32::pFS516 (cdc2:1-50 cdc13-NG leu1) ura4-D18 his7::pFS462 (adh1:ZEV his7) ZEV:wee1 (KanMX)
yFS1111	h- leu1-32::pFS517 (cdc2:51-122 cdc13-NG leu1) ura4-D18 his7::pFS462 (adh1:ZEV his7) ZEV:wee1 (KanMX)
yFS1112	h- leu1-32::pFS518 (cdc2:Δ71-122 cdc13-NG leu1) ura4-D18 his7::pFS462 (adh1:ZEV his7) ZEV:wee1 (KanMX)
yFS1113	h- leu1-32::pFS519 (cdc2:Δ1-50 cdc13-NG leu1) ura4-D18 his7::pFS462 (adh1:ZEV his7) ZEV:wee1 (KanMX)
yFS1114	h- leu1-32::pFS521(cdc2:ubiquitin+Δ51-70 cdc13-NG leu1) ura4-D18 his7::pFS462 (adh1:ZEV his7) ZEV:wee1 (KanMX)
yFS1115	h- leu1-32::pFS522 (cdc2:Δ51-70 cdc13-NG:adh1 3'UTR leu1) ura4-D18 his7::pFS462 (adh1:ZEV his7) ZEV:wee1 (KanMX)
yFS1116	h- leu1-32::pFS523 (cdc2:Δ2-50/Δ71-122 cdc13-NG leu1) ura4-D18 his7::pFS462 (adh1:ZEV his7) ZEV:wee1 (KanMX)
yFS1117	h- leu1-32::pFS524 (cdc2:1-122 recoded cdc13-NG leu1) ura4-D18 his7::pFS462 (adh1:ZEV his7) ZEV:wee1 (KanMX)
yFS1118	h- leu1-32::pFS526 (cdc2:Ubiquitin+D-boxNG leu1) ura4-D18 his7::pFS462 (adh1:ZEV his7) ZEV:wee1 (KanMX)
yFS1119	h- leu1-32::pFS527(cdc2:Ubiquitin+mutant D-box-NG leu1) ura4-D18 his7::pFS462 (adh1:ZEV his7) ZEV:wee1 (KanMX)
yFS1121	h+ leu1-32::pFS522 (cdc2:Δ51-70 cdc13-NG:adh1 3'UTR leu1) ura4-D18 cdc13Δ::NatMX
yFS1122	h+ leu1-32::pFS522 (cdc2:Δ51-70 cdc13-NG:adh1 3'UTR leu1) ura4-D18 cdc13Δ::NAT nmt41:cdc25 (KanMX)
yFS1123	h- leu1-32::pFS521 (cdc2:ubiquitin+Δ51-70 cdc13-NG leu1) ura4-D18 cdc13del::NatMX
yFS1124	h+ leu1-32 ura4-D18 his7-366 nmt41:cdc25 (KanMX)
yFS1125	h- leu1-32::pFS521 (cdc2:ubiquitin+Δ51-70 cdc13-NG leu1) ura4-D18 cdc13Δ::NAT nmt41:cdc25 (KAN)
yFS1126	h+ leu1-32 ura4-D18 (adh1:ZEV his7) ZEV:cdc25 (KanMX)
yFS1127	h- leu1-32::pFS522 (cdc2:Δ51-70 cdc13-NG:adh1 3'UTR leu1) ura4-D18 cdc13Δ::NAT
yFS1128	h- leu1-32::pFS521 (cdc2:Ubiquitin+Δ51-70 cdc13-NG leu1) ura4-D18 his7::pFS462 (adh1:ZEV his7) cdc13Δ::NAT ZEV:cdc25::KanMX
yFS1129	h+ leu1-32::pFS522 (cdc2:Δ51-70 cdc13-NG:adh1 3'UTR leu1) ura4-D18 his7::pFS462 (adh1:ZEV his7) cdc13Δ::NatMX ZEV:cdc25 (KanMX)
yFS1132	h- leu1-32 ura4-D18 nmt41:cdc25-NG (KanMX, HphMX) wee1::pWAU-50 (adh1:wee1-50 ura4)
yFS1133	h- leu1-32::pFS461 (adh1:ZEV leu1) ura4-D18 cdc2-GFP (KanMX) ZEV:wee1 (KanMX)
yFS1134	h- leu1-32::pFS530 (cdc2:cdc13-NG:adh1 3'UTR leu1) ura4-D18 his7::pFS462 (adh1:ZEV his7) ZEV:wee1 (KanMX)

Table 2: Plasmids used

Plasmid	Description	Yeast Marker
pFS272	NatMX cassette	
pFS435	Ubiquitin degron sequence	
pFS461	ZEV transcription faction driven by adh1 promoter	leu1
pFS462	ZEV transcription faction driven by adh1 promoter	his7
pFS478	KanMX ZEV promoter cassette	
pFS508	NeonGreen-HPH cassette and adh1 3'UTR	
pFS513	Cdc13 driven by cdc2 promoter and 5'UTR	leu1
pFS514	Cdc13 1-205 driven by ade4 promoter and 5'UTR	leu1
pFS515	Cdc13 1-122 driven by cdc2 promoter and 5'UTR	leu1
pFS516	Cdc13 1-50 driven by cdc2 promoter and 5'UTR	leu1
pFS517	Cdc13 N51-122 driven by cdc2 promoter and 5'UTR	leu1
pFS518	Cdc13 Δ71-122 driven by cdc2 promoter and 5'UTR	leu1
pFS519	Cdc13 Δ2-50 driven by cdc2 promoter and 5'UTR	leu1
pFS520	Cdc13 Δ51-70 driven by cdc2 promoter and 5'UTR	leu1
pFS521	Ubiquitin-Δ51-70 driven by cdc2 promoter and 5'UTR	leu1
pFS522	Cdc13 Δ51-70 driven by cdc2 promoter and 5'UTR and adh1 3'UTR	leu1
pFS523	Cdc13 Δ2-50/Δ71-122 driven by cdc2 promoter and 5'UTR	leu1
pFS524	Cdc13 1-122_recodeddriven by cdc2 promoter and 5'UTR	leu1
pFS525	Ubiquitin+ NeonGreen driven by cdc2 promoter and 5'UTR	leu1
pFS526	Ubiquitin+ D-box-NeonGreen driven by cdc2 promoter and 5'UTR	leu1
pFS530	Cdc13 driven by cdc2 promoter and 5'UTR and adh1 3'UTR	leu1

Table 3: Primers Used

Primer	Sequence 5' to 3'
SB20	TTTTAATTCAATGGTGAGCAAGGGCGAG
SB21	TGCTCACCATTGAATTAAAAACAGAAGGTTGGCG
SB36	TTGGCCAAAGTGTGTTAGCTTCCCCAGACGTTAATGATTCTCCTACTGCCATG CATTCCCTCTCTACACTTAGAAGATTTCGGATCCCCGGGTTAATTAA
SB37	AGAAAAAACTTAGGTTTAGAAAGTTGAATATATAAGAGTATACTTCAGGCTA GGTAAAGTATTGAGTCAGCCTAAAATCAGAATTCGAGCTCGTTTAAAC
SB40	TCTGTTACCTGAACCAATTAATATTTTAATTTCCCTTCCTCTCTTTCAATCCCC GACAGAATTCGTTTCTTCTTTTCCTCCGGATCCCCGGGTTAATTAA
SB41	CACATATAAAGAGCGCTTGAACAAGTTGGAATATTCACAATTGAAAGAGGTT GAGATAGTGATATGCACAATACACTAAAGAATTCGAGCTCGTTTAAAC
SB55	AGCCACTGTGAAAGAAGGTGTTCCATTAGC
SB56	CACCTTCTTTCACAGTGGCTTTCTTGCC
SB59	AGCCACTGTGATGGTGAGCAAGGGCGAG
SB60	TGCTCACCATCACAGTGGCTTTCTTGCC
SB61	GTTTGCAATGTCTTCCACCAACGTTCCTAAG
SB62	TGGTGGAAGACATTGCAAACCACTAAAAGAAG
SB64	GAGTGGTCATTGCAAACCACTAAAAGAAGCAAC
SB73	CAATACGAGTATGGTGAGCAAGGGCGAG
SB74	AAATCTGCATTGCAAACCACTAAAAGAAGCAAC
SB75	GTGGTTTGCAATGCAGATTTTCGTCAAGACTTTG
SB76	TGCTCACCATACTCGTATTGGGCGCCAG
SB89	TTTTCACAACTCAGTCCCCTCGTTACCTC
SB90	AGGGGACTGAGTTGTGAAAATTGGAAACATCATCC
SB97	ATGACTACCCGTCGTTTAAC
SB98	TGCAAACCACTAAAAGAAG
SB99	GCTTCTTTTAGTGGTTTGCAATGCAGATTTTCGTCAAGACTTTG
SB100	GTTAAACGACGGGTAGTCATACTCGTATTGGGCGCCAG
SB109	GTGGTTTGCAATGACCACTCGCAGATTG
SB121	CTTCAACTCGTCAGTCCCCTCGTTACCTC
SB122	AGGGGACTGACGAGTTGAAGACGGAGGG
SB124	AATTCACGGGCAGCAGGACGCAATACATTCCGTTTGCCAGATAGAGTGTTGG TAAAGGAAAGTGAAGAAAGCGGAGAATCCATTATAGTTTTTTCTCCTTGAC
SB132	ATATAGATCTGAGCTCCAGCTTTTGTTC
SB133	GTGGCGCGCCTTACTTGTACAGCTCGTC
SB155	AACATATTTTATCTCGCTGCTCGTGTTACATATCAGGAGTGCGGGTATGAGAT TATGGGGATTTAATGGCTGTCAAGCAGGAATTCGAGCTCGTTTAAAC
SB157	TTTTCACAACATGGTGAGCAAGGGCGAG
SB158	TGGTGGAAGAACTCGTATTGGGCGCCAG
SB159	CAATACGAGTTCTTCCACCAACGTTCCTAAG
SB160	TGCTCACCATGTTGTGAAAATTGGAAACATCATC
DK368	ACCACGAGCTGTCCTTAACC
DK369	TGCTTAACCGACCAGGTTCC
DK372	GTGCATGTTCGGTGGTCTCG
DK373	AAGACCCGGTAGTGATGTGC
DK374	ATCCGCCAAAGCGTGGTATC
DK375	AAGCGCACTAAGCAGACGAG

Table 4: Plasmid Construction Details

Plasmid	Sequence Source	Sequence used	Primers
pFS515	pFS513	Δ123-482	SB20, SB21
pFS516	pFS515	Δ51-122	SB59, SB60
pFS517	pFS515	Δ2-50	SB61, SB62
pFS518	pFS513	Δ71-122	SB89, SB90
pFS519	pFS513	Δ2-50	SB61, SB62
pFS520	pFS513	Δ51-70	SB55, SB56
pFS521	pFS520	Δ51-70	SB97, SB98
	pFS435	Ubiquitin degron	SB99, SB100
pFS522	pFS520	Δ51-70	SB109, SB122
	pFS508	adh1 3'UTR	SB134, SB135
pFS523	pFS519	Δ71-122	SB89, SB90
pFS524	pFS528	recoded 1-122	SB132, SB133
	pFS513	Δ2-122	SB64, SB121
pFS525	pFS513	NeonGreen	SB73, SB74
	pFS435	Ubiquitin degron	SB75, SB76
pFS526	pFS525	Ubiquitin degron - NeonGreen	SB157, SB158
	pFS513	51-70	SB159, SB160

References

Amodeo, A. A., and Skotheim, J. M. (2016). Cell-Size Control. Cold Spring Harb Perspect Biol *8*, a019083.

Belle, A., Tanay, A., Bitincka, L., Shamir, R., and O'Shea, E. K. (2006). Quantification of protein half-lives in the budding yeast proteome. Proc Natl Acad Sci U S A *103*, 13004-13009.

Blanco, M. A., Sanchez-Diaz, A., de, P. J. M., and Moreno, S. (2000). APC(ste9/srw1) promotes degradation of mitotic cyclins in G(1) and is inhibited by cdc2 phosphorylation. EMBO J *19*, 3945-3955.

Booher, R. N., Alfa, C. E., Hyams, J. S., and Beach, D. H. (1989). The fission yeast cdc2/cdc13/suc1 protein kinase: Regulation of catalytic activity and nuclear localization. Cell *58*, 485-497.

Chen, Y., Zhao, G., Zahumensky, J., Honey, S., and Futcher, B. (2020). Differential Scaling of Gene Expression with Cell Size May Explain Size Control in Budding Yeast. Mol Cell *78*, 359-370.e6.

Christiano, R., Nagaraj, N., Fröhlich, F., and Walther, T. C. (2014). Global proteome turnover analyses of the Yeasts S. cerevisiae and S. pombe. Cell Rep *9*, 1959-1965.

Creanor, J., and Mitchison, J. M. (1996). The kinetics of the B cyclin p56cdc13 and the phosphatase p80cdc25 during the cell cycle of the fission yeast *Schizosaccharomyces pombe*. J Cell Sci *109*, 1647-1653.

Curran, S., Dey, G., Rees, P., and Nurse, P. (2022). A quantitative and spatial analysis of cell cycle regulators during the fission yeast cycle. Proc Natl Acad Sci U S A *119*, e2206172119.

D'Ario, M., Tavares, R., Schiessl, K. et al. (2021). Cell size controlled in plants using DNA content as an internal scale. Science *372*, 1176-1181.

Dorsey, S., Tollis, S., Cheng, J. et al. (2018). G1/S Transcription Factor Copy Number Is a Growth-Dependent Determinant of Cell Cycle Commitment in Yeast. Cell Syst *6*, 539-554.e11.

Eser, P., Wachutka, L., Maier, K. C. et al. (2016). Determinants of RNA metabolism in the Schizosaccharomyces pombe genome. Mol Syst Biol *12*, 857.

Esposito, E., Weidemann, D. E., Rogers, J. M. et al. (2022). Mitotic checkpoint gene expression is tuned by codon usage bias. EMBO J *41*, e107896.

Facchetti, G., Knapp, B., Flor-Parra, I., Chang, F., and Howard, M. (2019). Reprogramming Cdr2-Dependent Geometry-Based Cell Size Control in Fission Yeast. Curr Biol *29*, 350-358.e4.

Fantes, P. A., Grant, W. D., Pritchard, R. H., Sudbery, P. E., and Wheals, A. E. (1975). The regulation of cell size and the control of mitosis. J Theor Biol *50*, 213-244.

Forsburg, S. L., and Rhind, N. (2006). Basic methods for fission yeast. Yeast *23*, 173-183.

Heinrich, S., Geissen, E. M., Kamenz, J. et al. (2013). Determinants of robustness in spindle assembly checkpoint signalling. Nat Cell Biol *15*, 1328-1339.

Herring, A. J. A Study of the Induced Delay in the Division of Yeast *Schizosccharomyces pombe*. University of Edinburgh; 1974. p. Dissertation.

Houser, J. R., Ford, E., Chatterjea, S. M. et al. (2012). An improved short-lived fluorescent protein transcriptional reporter for Saccharomyces cerevisiae. Yeast *29*, 519-530.

Keifenheim, D., Sun, X. M., D'Souza, E. et al. (2017). Size-Dependent Expression of the Mitotic Activator Cdc25 Suggests a Mechanism of Size Control in Fission Yeast. Curr Biol *27*, 1491-1497.e4.

Liu, D., Lopez-Paz, C., Li, Y., Zhuang, X., and Umen, J. G. (2022). Subscaling of a cytosolic RNA binding protein governs cell size homeostasis in the multiple fission alga Chlamydomonas. bioRxiv 2022.11.02.514835.

Marguerat, S., Schmidt, A., Codlin, S. et al. (2012). Quantitative analysis of fission yeast transcriptomes and proteomes in proliferating and quiescent cells. Cell *151*, 671-683.

Mehra, A., Lee, K. H., and Hatzimanikatis, V. (2003). Insights into the relation between mRNA and protein expression patterns: I. Theoretical considerations. Biotechnol Bioeng *84*, 822-833.

Mendell, J. T., Medghalchi, S. M., Lake, R. G., Noensie, E. N., and Dietz, H. C. (2000). Novel Upf2p orthologues suggest a functional link between translation initiation and nonsense surveillance complexes. Mol Cell Biol *20*, 8944-8957.

Miller, K. E., Vargas-Garcia, C., Singh, A., and Moseley, J. B. (2022). The fission yeast cell size control system integrates pathways measuring cell surface area, volume, and time. bioRxiv 2022.11.17.516946.

Moreno, S., Nurse, P., and Russell, P. (1990). Regulation of mitosis by cyclic accumulation of p80cdc25 mitotic inducer in fission yeast. Nature *344*, 549-552.

Mueller, F., Senecal, A., Tantale, K. et al. (2013). FISH-quant: automatic counting of transcripts in 3D FISH images. Nat Methods *10*, 277-278.

Murray, A. W. (1989). Cyclin synthesis and degradation and the embryonic cell cycle. J Cell Sci Suppl *12*, 65-76.

Ohira, M. J., Hendrickson, D. G., McIsaac, R. S., and Rhind, N. (2017). An estradiol-inducible promoter enables fast, graduated control of gene expression in fission yeast. Yeast *34*, 323-334.
Ohira, M. J., and Rhind, N. (2022). pomBseen: An Automated Pipeline for Analysis of Fission Yeast Images. bioRxiv 2022.12.27.521403.
Padovan-Merhar, O., Nair, G. P., Biaesch, A. G. et al. (2015). Single mammalian cells compensate for differences in cellular volume and DNA copy number through independent global transcriptional mechanisms. Mol Cell *58*, 339-352.
Pan, K. Z., Saunders, T. E., Flor-Parra, I., Howard, M., and Chang, F. (2014). Cortical regulation of cell size by a sizer cdr2p. Elife *3*, e02040.
Patterson, J. O., Rees, P., and Nurse, P. (2019). Noisy Cell-Size-Correlated Expression of Cyclin B Drives Probabilistic Cell-Size Homeostasis in Fission Yeast. Curr Biol *29*, 1379-1386.e4.
Prescott, D. M. (1956). Relation between cell growth and cell division. III. Changes in nuclear volume and growth rate and prevention of cell division in Amoeba proteus resulting from cytoplasmic amputations. Exp Cell Res *11*, 94-98.
Rhind, N. (2018). Cell Size Control via an Unstable Accumulating Activator and the Phenomenon of Excess Mitotic Delay. Bioessays *40*, 1700184.
Rhind, N. (2021). Cell-size control. Curr Biol *31*, R1414-R1420.
Russell, P., and Nurse, P. (1987). Negative regulation of mitosis by wee1+, a gene encoding a protein kinase homolog. Cell *49*, 559-567.
Schmoller, K. M., and Skotheim, J. M. (2015). The Biosynthetic Basis of Cell Size Control. Trends Cell Biol *25*, 793-802.
Schmoller, K. M., Turner, J. J., Koivomagi, M., and Skotheim, J. M. (2015). Dilution of the cell cycle inhibitor Whi5 controls budding-yeast cell size. Nature *526*, 268-272.
Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. Nat Methods *9*, 671-675.
Sun, X. M., Bowman, A., Priestman, M. et al. (2020). Size-Dependent Increase in RNA Polymerase II Initiation Rates Mediates Gene Expression Scaling with Cell Size. Curr Biol *30*, 1217-1230.e7.
Thormar, H. (1959). Delayed division in tetrahymena pyriformis induced by temperature changes. C R Trav Lab Carlsberg *31*, 207-225.
Trcek, T., Chao, J. A., Larson, D. R. et al. (2012). Single-mRNA counting using fluorescent in situ hybridization in budding yeast. Nat Protoc *7*, 408-419.
Wang, H., Carey, L. B., Cai, Y., Wijnen, H., and Futcher, B. (2009). Recruitment of Cln3 cyclin to promoters controls cell cycle entry via histone deacetylase and other targets. PLoS Biol *7*, e1000189.
Zatulovskiy, E., Zhang, S., Berenson, D. F., Topacio, B. R., and Skotheim, J. M. (2020). Cell growth dilutes the cell cycle inhibitor Rb to trigger cell division. Science *369*, 466-471.
Zhurinsky, J., Leonhard, K., Watt, S. et al. (2010). A Coordinated Global Control over Cellular Transcription. Curr Biol *20*, 2010-2015.

Figure 1

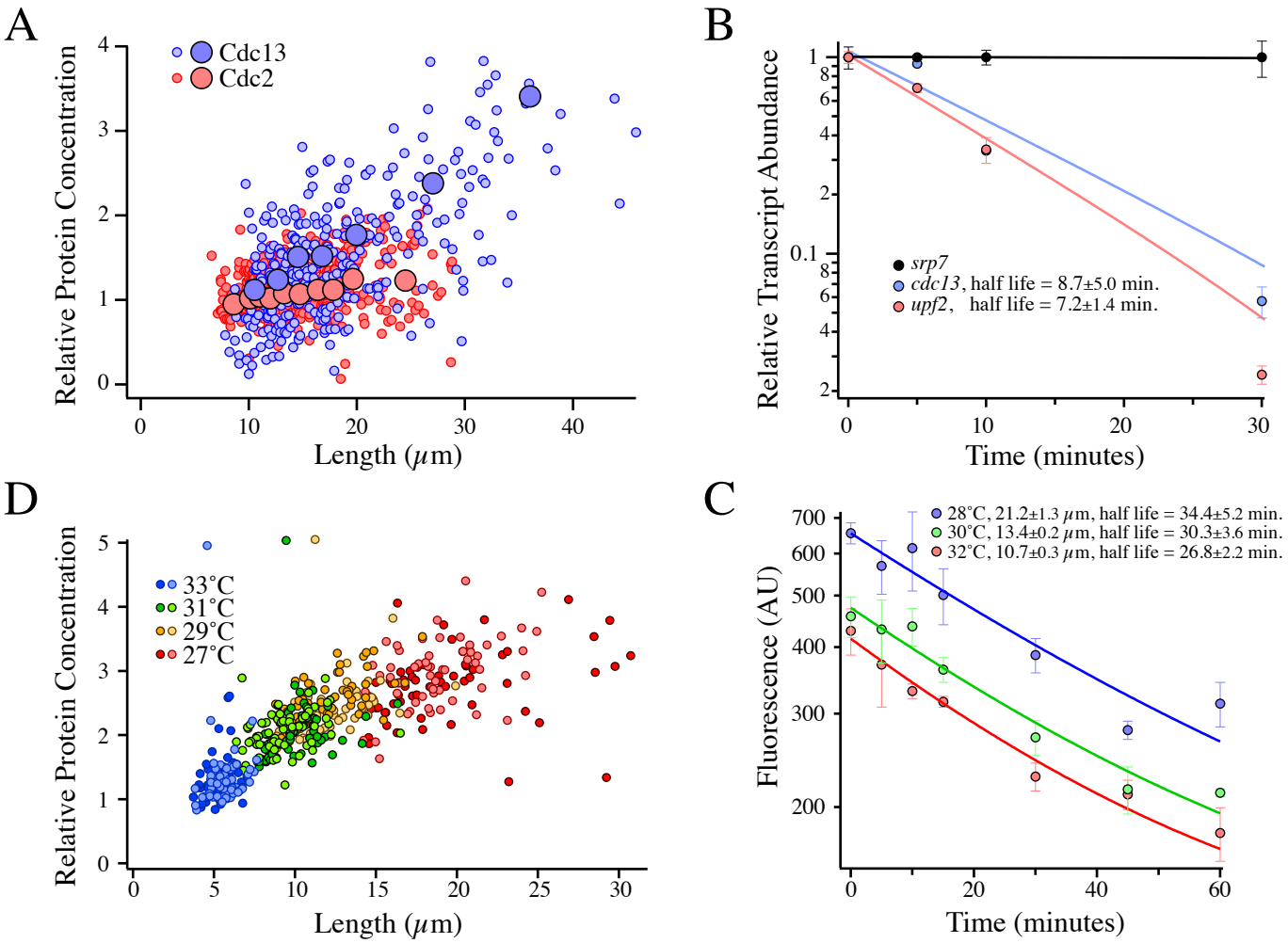

Figure 2

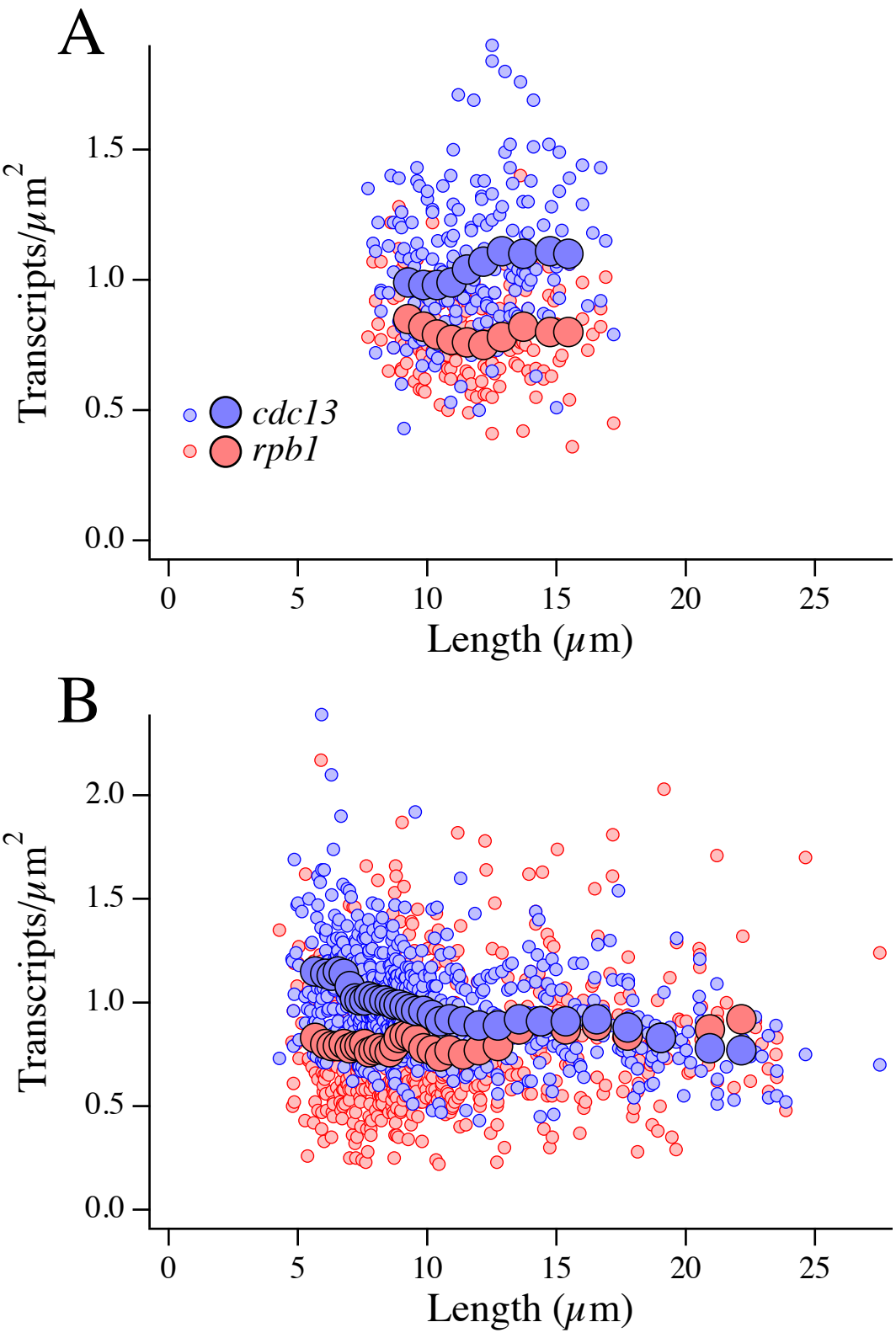

Figure 3

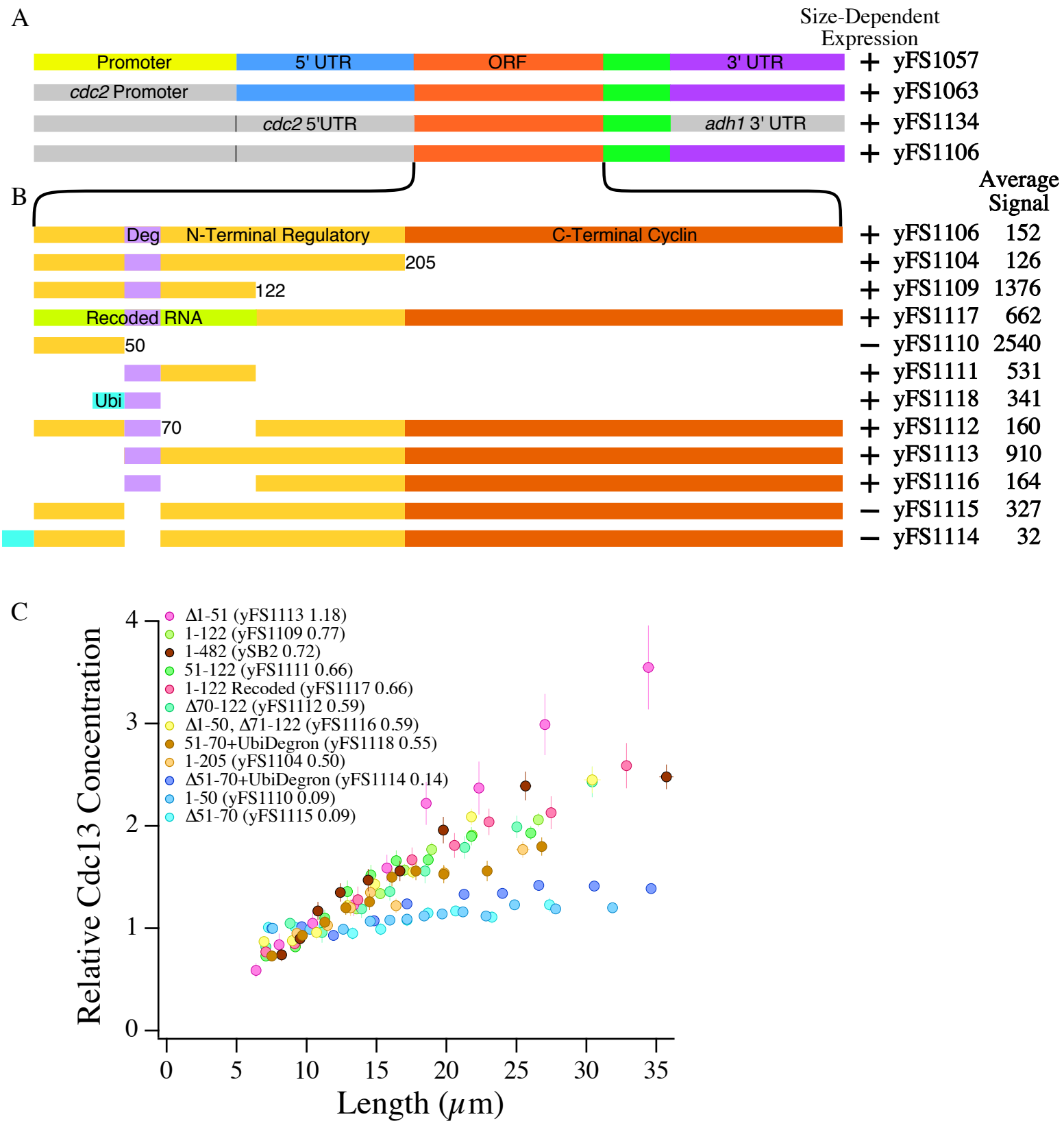

Figure 4