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Extra View

Evolution of Cell Cycle Control

Same Molecular Machines, Different Regulation

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ABSTRACT

Decades of research has together with the availability of whole genomes made it clear that many of the core components involved in the cell cycle are conserved across eukaryotes, both functionally and structurally. These proteins are organized in complexes and modules that are activated or deactivated at specific stages during the cell cycle through a wide variety of mechanisms including transcriptional regulation, phosphorylation, subcellular translocation and targeted degradation. In a series of integrative analyses of different genome-scale data sets, we have studied how these different layers of regulation together control the activity of cell cycle complexes and how this regulation has evolved. The results show surprisingly poor conservation of both the transcriptional and the post-translation regulation of individual genes and proteins; however, the changes in one layer of regulation are often mirrored by changes in other layers, implying that independent layers of control coevolve. By taking a bird's eye view of the cell cycle, we demonstrate how the modular organization of cellular systems possesses a built-in flexibility, which allows evolution to find many different solutions for assembling the same molecular machines just in time for action.

INTRODUCTION

Since the discovery of the cyclins it has been clear that regulated transcription is used to restrict the synthesis of key components to specific phases of the cell cycle. Such cell cycle-regulated genes thus display a cyclic or periodic pattern of expression when monitored over consecutive cell cycles. Although many cases of periodic expression were documented over the years, the extent of this regulation first became clear in 1998 when DNA microarrays were used to probe the entire transcriptome of synchronously growing yeast cells.[1,2] These studies indicated that the cell uses a just-in-time manufacturing principle similar to the car industry: genes whose protein products are needed for a specific process or phase of the cell cycle tend to be expressed right before the cell enters that phase. These observations were later supported by insight, mainly from yeast, showing that a network of transcription factors act to induce or inhibit groups of genes involved in a specific process or stage of the cell cycle.[3-8] The transcription factors themselves form a regulatory circuit with feed-forward activation and feedback inhibition. Later DNA microarray studies with human cell cultures,[9,10] *Arabidopsis thaliana* cell suspensions[11-13] and fission yeast cells[14-16] have shown that periodic transcription is a universal feature of the eukaryotic cell cycle. Taken together with the high degree of structural and functional conservation of cell cycle proteins, one might expect to find that the periodically expressed genes from each organism are orthologs of each other. Much to our surprise, we found the exact opposite when investigating this question.[17]

In order to compare cell cycle-regulated expression across organisms, we first reanalyzed the available microarray data from four organisms and estimated that there are about 600 periodically expressed genes in budding yeast,[18] 500 in fission yeast,[19] 600 in human[17] and 400 in the plant *A. thaliana*.[17] We then identified groups of orthologous genes for the four organisms based on genome-wide comparisons of the protein sequences. Three hundred eighty-one of these orthologous groups had at least one member from each organism and one or more members that were periodically expressed. Comparing the gene expression of proteins within these groups of highly conserved proteins, we found that the majority of the proteins were only periodic in one organism. This means that although transcriptional regulation per se is widely used in all organisms to control the synthesis of key proteins during the cell cycle, each organism regulates a different set of proteins. This conclusion

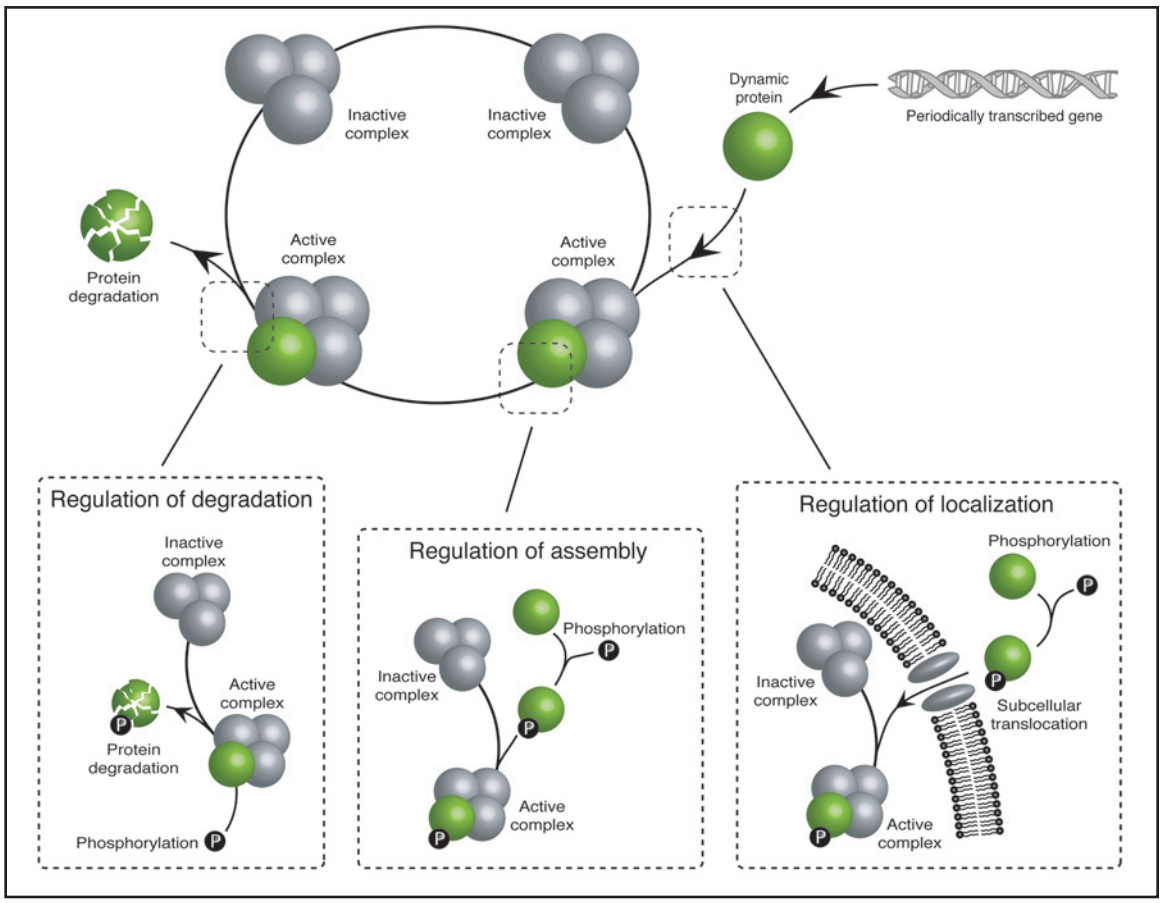

Figure 1. Just-in-time assembly of protein complexes. A single cell cycle-regulated subunit (shown in green) is sufficient to govern the activation of an entire complex, as proper assembly cannot take place until this final subunit is expressed. Phosphorylation often serves as an extra layer of regulation of the same subunits and may be required for assembly of the complex, for subcellular translocation into the proper compartment, and/or for targeted degradation and thus disassembly of the protein complex.

has been hinted at by other, less comprehensive studies.[14,15,20-22] It was nonetheless difficult to reconcile with the massive body of evidence demonstrating that the core molecular machinery of the cell cycle is both functionally and structurally highly conserved.

JUST-IN-TIME ASSEMBLY

In order to find a model, which could explain these apparently conflicting observations, we turned to a principle for regulation of cell cycle protein complexes in budding yeast that we uncovered recently.[23] By constructing a cell cycle interaction network from protein-protein interaction data and information on gene expression during the cell cycle, we found that protein complexes were generally formed as a combination of static (constitutively expressed) and dynamic (periodically expressed) proteins. The latter showed a clear tendency to be expressed right before the complex is known to become active and thus, by their change in abundance, control the assembly of the functional complex. This mechanism, which we termed "just-in-time assembly," is illustrated in Figure 1. One could argue that periodic transcription is not sufficient evidence to conclude that the protein abundance changes similarly, as is implicitly assumed in our just-in-time assembly model. When studying protein complexes of known function across all four organisms, however, we observed a clear tendency for transcription of genes encoding dynamic subunits to peak just before the complex is known to function. This is evident in the figures of references 17 and 23, as well as in the many examples covered in the accompanying supplementary information, which we encourage the reader to study (www.cbs.dtu.dk/cellcycle). This link between the timing of transcription and complex activity strongly suggests that translational control plays only a minor role and supports a model in which protein synthesis is temporally controlled at the transcriptional level. This model is supported by a recent genome-wide study of transcription and translation in fission yeast by Bähler and coworkers[24] as well as by the many known examples where the temporal pattern of gene expression correlates closely with the changes in abundance of the protein (for example, cyclins and histones). Similarly, the lack of periodic fluctuation in the expression level of other complex subunits suggests that these proteins reside inside the cell at a constant level, either as individual components or as subcomplexes; hence the name static proteins.

In a complementary study on the evolution of the transcriptional regulation that controls core-histone expression during S-phase, Landsman and coworkers found that the cis-regulatory motifs and transcription factors responsible for the regulation are not conserved across eukaryotes.[25] This is intriguing, because the core histone genes belong to some of the very few orthologous groups for which both transcriptional regulation per se and the timing of expression is conserved.[17] Whereas our results show how different solutions have evolved to regulate the same conserved proteins complexes and modules, the work of Landsman and coworkers thus demonstrates

that many different solutions exist for generating the same transcriptional dynamics. Another recent analysis of high-throughput data from yeast and fly also suggests poor conservation of the transcription factors and cis-regulatory elements responsible for regulation of conserved protein complexes.[26]

Just as the changes in the abundance of the dynamic proteins are assumed to control the assembly of the active complexes, it also logically follows from the just-in-time assembly model that the complexes disassemble at some later stage. However, microarray expression data cannot be used to determine when this process takes place as they only describe the timing of synthesis. With the recent advances in proteomics, data on the abundance of individual proteins during the cell cycle may not be far into the future. In fact, the first such studies have already emerged.[27]

As a biological design principle, just-in-time assembly (Fig. 1) holds a built-in flexibility in the sense that regulating any subset of the complex members could in principle control the assembly of a complex, as long as these dynamic subunits are expressed just in time. To investigate this hypothesis, we carefully annotated a set of evolutionarily conserved protein complexes related to DNA replication, deoxynucleotide biosynthesis, sister chromatid cohesion and targeted protein degradation, all of which were well characterized with respect to their function and protein composition.[17] Visualizing the temporal dynamics of the orthologous proteins in these complexes revealed that "just-in-time assembly" is an evolutionarily conserved mechanism for complex regulation: Within each complex, only some of the subunits are subject to transcriptional regulation, and these are generally expressed just in time for the known activity of the complex. When comparing across organisms, however, we found that the identity of the transcriptionally activated subunits changes dramatically (see Fig. 2), which explains the poor conservation of periodic regulation at the level of individual genes.

The DNA replication machinery is a prime example of how regulation can be conserved at the level of a whole process while allowing for widespread variation in the regulation of individual components. The composition, function and order of assembly of the different subcomplexes involved in DNA replication is almost completely conserved within eukaryotes, and the dynamic subunits of the protein complexes involved in DNA replication are expressed right before entry into the S phase in all organisms. However, the identity of the regulated subunits varies; either within a single complex or between complexes that perform similar or complementary functions. Figure 2A shows an example of the latter situation. Both Cdc45 and Dbf4-dependent kinase (DDK) are involved in regulating the recruitment of the final parts of the replisome after the prereplication complex has assembled on the origins. In human, *A. thaliana* and budding yeast cells, Cdc45 expression peaks just prior to DNA replication (Fig. 2A), whereas it is static in fission yeast. Instead, both subunits of the fission yeast DDK complex are regulated with expression right before S phase (which in *S. pombe* corresponds to M/G_1 phase). Both solutions ensure temporal control of the overall process, but act via regulation of different subunits and complexes. For a complete picture of how regulation of the DNA replication machinery has evolved, we refer the reader to Figure 2 of reference 17.

COEVOLUTION OF MULTIPLE LAYERS OF REGULATION

Transcriptional regulation is only one mechanism by which the cell cycle is regulated. Post-translational modifications and targeted protein degradation also play a major role, but although many examples are known, it has been unclear how these different layers of regulation work together as a system.

Phosphorylation, in particular by the cyclin-dependent kinases (CDKs), is well established as a major regulator of protein function, and many substrates of the CDKs have been identified already. By mapping a set of CDK substrates onto the yeast cell cycle interaction network, we found that regulation by phosphorylation specifically targets the dynamic proteins.[23] This may not seem surprising, since CDK phosphorylation should be expected to target cell cycle proteins, many of which are periodically expressed. The surprising conclusion was that the static subunits of the cell cycle complexes showed no overrepresentation of CDK substrates compared to the entire yeast genome.

In our recent comparative analysis of human, *A. thaliana*, fission and budding yeast, we expanded the analysis and found that CDK phosphorylation does indeed show a clear preference in all organisms for the dynamic proteins, rather than cell cycle proteins per se. The same proteins are thus controlled by two independent mechanisms, namely transcriptional regulation and phosphorylation. Combined with our demonstration of the poor conservation of periodic transcription among orthologs, these results imply that transcriptional regulation and CDK phosphorylation must have changed in a coordinated fashion during evolution. When comparing the CDK phosphorylation of dynamic proteins with static orthologs to static proteins with dynamic orthologs in all organisms, we indeed found that loss or gain of periodic transcription of a gene correlates with loss or gain of CDK phosphorylation of the corresponding protein.[17] This is, to the best of our knowledge, the first documented case of coevolution of two independent layers of regulation in a biological system.

Figure 2B shows an example of coevolution, where the gain of phosphorylation of a subunit correlates with gain of transcriptional regulation. The ribonucleotide-diphosphate reductase (RNR) complex consists of two large and two small subunits and is responsible for making building blocks for DNA replication by converting ribonucleotides to their deoxy form. Our analysis shows that the transcriptional regulation of the RNR complex is primarily mediated through the large subunits, which are periodically expressed in all the four organisms, whereas human RRM2 is the only small subunit of RNR to be periodically expressed (Fig. 2B). Like the large subunit, RRM1, it is maximally expressed during S phase, which is consistent with what has been observed in mouse.[28] Notably, RRM2 is also the only RNR small subunit to be phosphorylated by cyclin-dependent kinase 1 (Cdk1). A multiple alignment reveals that the phosphorylation site does not exist in the orthologous proteins from any of the three other organisms, and none of the large-scale screens in budding yeast suggest Rnr2 or Rnr4 to be Cdc28 substrates.[29,30] The small subunit of RNR is thus a clear example of the coevolution of transcriptional and post-translational regulation, which our systematic analysis showed to have taken place at the global scale.[17]

We also investigated the possibility that cell cycle-regulated proteolysis of the dynamic proteins too has coevolved with other levels of regulation. Very little experimental data is available on which proteins are targeted for degradation during the cell cycle, and even less data exists on the timing of such targeted degradation. In order to

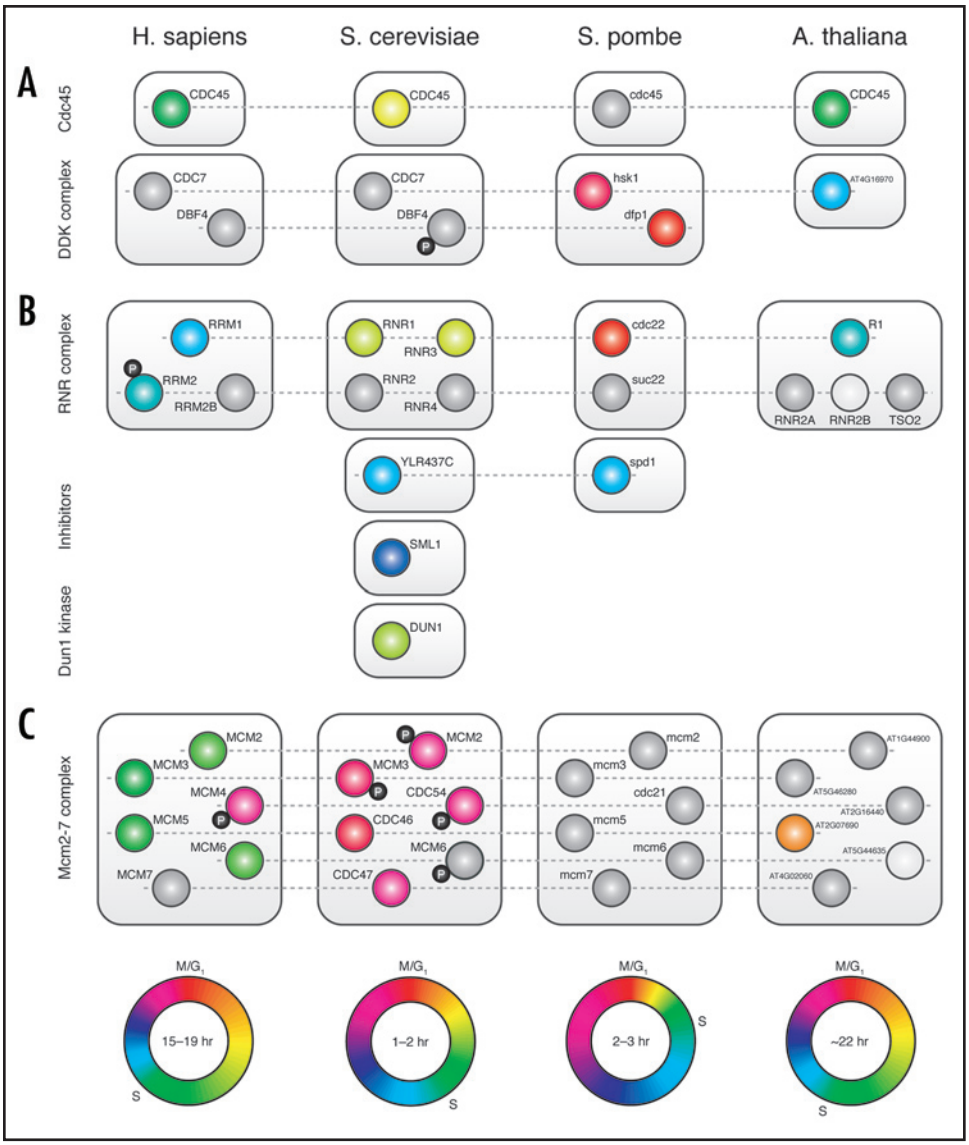

Figure 2. Regulation is conserved at the level of protein complexes rather than the single proteins. The subunits (circles) of a small set of cell-cycle complexes (boxes) are shown for four organisms (columns). Proteins that are located on the same horizontal line are sequence orthologs and are believed to perform the same function. Dynamic subunits are colored according to their time of peak expression, static subunits are shown in gray, and subunits for which no expression data was available are displayed in white. (A) The Cdc45 and Dbf4-dependent kinase (DDK) are together involved in recruiting the replisome; whereas most eukaryotes regulate the expression of Cdc45, fission yeast instead regulates the expression of DKK. (B) The RNR complex makes the building blocks for DNA replication. In contrast to the orthologous subunits in other organisms, human RRM2 is both transcriptionally regulated and phosphorylated by CDK, and it thus exemplifies the coevolution of transcriptional and post-translational cell-cycle regulation. Also, the yeast-specific RNR inhibitors are expressed out of phase with the dynamic RNR subunits, whereas the Dun1 kinase that controls the degradation of one of these inhibitors is expressed in phase. (C) The MCM is involved in DNA replication and associates with DNA-bound origin-of-replication complexes (ORC). Whereas most human MCM subunits are expressed in S-phase, MCM4 is expressed in M phase when the MCM complex is known to assemble. As Mcm4 is also the only known CDK substrate in the human MCM complex, this supports a mechanism where Mcm4 plays a rate-limiting role in controlling its assembly and disassembly.

perform a global analysis of degradation, we instead relied on computational predictions of PEST regions (regions in the protein sequence rich in the amino acids P, E, S, T and D), KEN boxes and D boxes as a proxy for cell cycle-regulated proteolysis. Whereas these predictions are too unreliable to prove that all three layers of regulation have coevolved, it did show a statistical overrepresentation of degradation targets among the periodic proteins in all four organisms.[17] Since then, we have been able to obtain a list of 304 experimentally verified ubiquitination targets in *S. cerevisiae*.[31] Although ubiquitination does not directly imply targeted degradation during the cell cycle, we find that ubiquitinated proteins are significantly overrepresented among the dynamic *S. cerevisiae* proteins ($p < 0.003$). In addition, this data set supports coevolution of transcriptional regulation and targeted protein degradation when compared to *A. thaliana* ($p < 0.018$) and *S. pombe* ($p < 0.083$), but not when compared to human. Although these data do not provide decisive proof for coevolution of transcriptional regulation and targeted protein degradation, they certainly support and extend the results obtained from sequence-based predictions.

POSSIBLE ROLES OF PHOSPHORYLATION

The vast majority of data available on phosphorylation of cell cycle proteins is inherently static; it tells us that a particular protein is phosphorylated, and sometimes also about the particular residue(s) and the responsible kinase. But for the majority of the known sites we do not know when in the cell cycle a particular phosphorylation event occurs or what the functional consequence of it is. Some of the latest approaches within quantitative phosphoproteomics seem very promising towards obtaining large-scale data sets on changes in protein phosphorylation during the cell cycle.[32-34] The just-in-time assembly mechanism does, however, in itself hint at a number of distinct roles for phosphorylation (see panels in Fig. 1).

First of all, cell cycle-related phosphorylation of dynamic proteins must logically occur after the time of peak expression of the gene that encodes the protein. It may serve as an additional control of assembly, if only the phosphorylated form of the protein can join the complex. As a variant of this theme, phosphorylation may not be required for assembly but instead for activation of the complex. In both of these scenarios, the timing of phosphorylation and transcription would have to coincide, as most complexes become active shortly

after the expression of their dynamic subunits. This design would confer robustness to the temporal control of complex activation, as two independent layers of regulation act in concert to ensure that a complex can only be assembled at the correct time in the cell cycle.

Phosphorylation may also play a role in regulating the subcellular localization of proteins and thereby potentially the assembly and disassembly of complexes. As illustrated in Figure 1, phosphorylation may be required for translocation of one or more subunits to the compartment where the active complex is assembled. Similarly, phosphorylation may play a role in inactivation of the complex by causing the phosphorylated form of a subunit to be sequestered in a different compartment. Comprehensive screens of protein subcellular localization are currently only available for *S. cerevisiae*, and although many proteins have several documented locations, these data do not provide any temporal information. It therefore remains unclear to which extent phosphorylation (or other post-translational modifications) regulates subcellular localization and thereby complex assembly/disassembly.

Finally, phosphorylation is known to play a role in targeted degradation of proteins during the cell cycle (Fig. 1). For instance, several examples are known where CDK phosphorylation of residues located in PEST regions makes the protein a substrate for the SCF ubiquitin ligases, which tag proteins for destruction by the proteasome. Under such conditions, phosphorylation triggers protein degradation and thereby ensures that the active complex cannot reassemble until new unmodified proteins are synthesized in the next cycle. Due to the limited coverage of proteome-wide data sets on targeted degradation and phosphorylation, however, it remains unclear how often degradation is dependent on, or triggered by, phosphorylation of the protein.

The regulation of the RNR complex (Fig. 2B) includes several examples of tightly regulated degradation of key components. To prevent large amounts of dNTP from accumulating once DNA replication has been completed, the RNR complex (Fig. 2B) must be deactivated shortly thereafter. In budding and fission yeast this happens primarily through the nonhomologous RNR inhibitors Sml1[35-37] and Spd1,[38,39] which are both expressed druing early G_2 phase as should be expected given their function (Fig. 2B). In order to allow reactivation of RNR function in the next cycle, budding yeast Sml1 is phosphorylated by the Dun1 kinase and is thereby targeted for degradation[40] during G_1 phase. Notably, our analysis shows that DUN1 is expressed at almost exactly the same time point in the cell cycle as the large subunit of RNR, thus ensuring that the RNR inhibitor Sml1 is inactivated exactly at the time when RNR activity is again desired. In mammals, the deactivation of RNR function is accomplished through allosteric inhibition by dATP.[41] As shown in our analysis, however, the human RRM2 is, unlike in the other organisms, both transcriptionally regulated and phosphorylated by Cdk1. Rrm2 has furthermore been identified as an APC/C target in mice,[28] which suggest that several of the mechanisms exemplified in Figure 1 may be relevant in the regulation of the mammalian RNR complex.

It is important to note, that these distinct roles for phosphorylation are not mutually exclusive. Any combination of them may be in play for a given protein and the precise mechanisms vary between proteins.

NEW INSIGHT INTO THE DETAILS OF REGULATION

Although the main focus of our work has been on understanding global aspects of cell cycle regulation, our data-integration approaches and visualizations have also proven very useful for discovering new correlations and generating new hypotheses about the regulation of individual proteins and complexes. For all the complexes treated in reference 17, we have thoroughly compared our results to the literature with regard to what was known about their function and regulation; this work is documented in the extensive supplementary material that accompanies the paper. Here we will bring a few examples of the value of integrating different (published) data sets.

As mentioned above, the two yeasts deactivate the RNR complex via two nonhomologous inhibitors, Sml1 and Spd1, respectively. Interestingly, we have found that spd1 has a putative ortholog in budding yeast, YLR437C, which according to our analysis is expressed at the same time point (early G_2 phase) in the cell cycle as both spd1 itself and the other budding yeast inhibitor, Sml1 (Fig. 2B). We thus propose that the uncharacterized protein Ylr437c could be an unrecognized functional homolog of spd1, which together with Sml1 ensures that RNR activity is suppressed after DNA replication has been completed.

In eukaryotes, DNA replication is a tightly controlled process that involves a large number of protein complexes and regulators that assemble on the DNA in a sequential manner. Our work shows that although most of these components are conserved through evolution, their regulation differs between organisms (Fig. 2 in ref. 17). One example is the regulation of the hexameric MCM complex, which is conserved across all eukaryotes and associates with the origin of replication complexes (ORC) already bound to DNA. In budding yeast, five of the six MCM genes are periodically transcribed, peaking during M phase (Fig. 2C). At the time our papers were published, this was an enigma as the MCM proteins are all present throughout the budding yeast cell cycle; however, Braun and Breeden have recently shown that only the newly synthesized MCM proteins localize to the nucleus, whereas those from the previous cell cycle are unable to reenter the nucleus and are degraded through ubiquitin-mediated proteolysis.[42] This explains how transcriptional regulation can control MCM complex assembly at origins of replication despite the high constitutive protein levels.

In mammals, the MCM complexes are known to assemble during telophase (late M phase)[43,44] and their association with chromatin becomes progressively weaker as cells proceed through S phase.[45] The literature is, however, vague on the issue of transcriptional regulation of the complex. We find that although MCM7 is static, the majority of the other subunits are dynamic with expression in G_1/S phase (Fig. 2C), similar to the rest of the dynamic DNA replication proteins. MCM4 is also dynamic in humans, but its expression in late M phase is distinct from the rest of the complex (Fig. 2C) and from all the other dynamic components of the DNA replication machinery (Fig. 2 in Ref. 17). This suggest that Mcm4 plays a rate-limiting role in controlling the assembly of the human MCM complex as it is the only subunit expressed right at the time when the complex is known to assemble. Consistent with this, recent data have shown that Mcm4 is phosphorylated at multiple sites, most likely by Cdk2, and that the phosphorylation promotes the dissociation of Mcm4 from chromatin.[46-48] Whereas assembly of the MCM complex appear to be controlled transcriptionally through MCM4, phosphorylation of this subunit thus appears to be important for disassembly of the complex; possibly be triggering the degradation of Mcm4 (as illustrated in Fig. 1).

To the best of our knowledge the unique pattern of expression of the human MCM genes, and in particular of MCM4, has not been reported by others.

These examples illustrate how detailed hypotheses can be formulated by systematic integration of temporal data from large-scale experiments with information on orthology and complex composition.

CONCLUSIONS

By taking a bird's eye view on the regulation of the eukaryotic cell cycle, we have uncovered a new design principle, just-in-time assembly, which explains how the cell ensures that the right protein complexes are active at the right time in the cell cycle. We found that the assembly, and hence the activity, of a cell cycle complex is typically controlled by tightly regulating only a few of its subunits. This gives a high degree evolutionary flexibility, since different organisms may control the assembly of the same protein complexes via different subunits, which explains the observed lack of conservation of cell-cycle-regulated transcription at the single-gene level.

To understand the fundamental question of how the eukaryotic cell cycle as we know it today came about in the first place, comparison with data on the archaeal cell cycle, such as the recently published microarray study on *Sulfolobus acidocaldarius*,[49] and the bacterial cell cycle[50] will likely play a crucial role. The four eukaryotes included in our study are, however, already rather distantly related and thus provide little insight into how fast regulation evolves, only that it has evolved considerably within eukaryotes. To assess changes in transcriptional regulation over shorter evolutionary time-scales, gene expression data would be needed from more closely related organisms rather than more distantly related ones; in particular, it would be interesting to address the conservation of regulation between humans and another mammal, such as mouse or rat. As highlighted by Gavin Sherlock,[51] widespread transcriptional variation has indeed been observed also between closely related organisms.[52-54]

Our comparative analysis demonstrates that other types of regulation also change considerably and that they tend to coevolve with transcriptional regulation. To attempt to assess the timescale at which evolutionary changes in cell cycle regulation take place, we selected proteins, which are dynamic and phosphorylated by CDK in one of the four organisms, but static and not phosphorylated in any of the other three. For two such examples, the specific CDK site was known, allowing us to use sequence alignment for estimating when during evolution the CDK phosphorylation may have been gained. These analyses indicated that changes can occur on a time-scale of only 100-300 million years.[17] This has major implications for the use of model organisms to unravel complex regulatory networks in humans, since even other vertebrates such as zebrafish may be too distantly related for regulatory mechanisms to be transferable.

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