Title (88 chars < 120): Punctuated evolution and transitional hybrid network in an ancestral cell cycle of fungi

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Abstract: (134 words < 150)

Although cell cycle control is an ancient, conserved, and essential process, some core animal and fungal cell cycle regulators share no more sequence identity than non-homologous proteins. Here, we show that evolution along the fungal lineage was punctuated by the early acquisition and entrainment of the SBF transcription factor through horizontal gene transfer. Cell cycle evolution in the fungal ancestor then proceeded through a hybrid network containing both SBF and its ancestral animal counterpart E2F, which is still maintained in many basal fungi. We hypothesize that a virally-derived SBF may have initially hijacked cell cycle control by activating transcription via the *cis*-regulatory elements targeted by the ancestral cell cycle regulator E2F, much like extant viral oncogenes. Consistent with this hypothesis, we show that SBF can regulate promoters with E2F binding sites in budding yeast.

Impact statement (30 words): Cell cycle network evolution in a fungal ancestor was punctuated by the arrival of a viral DNA-binding protein that was incorporated into the G1/S regulatory network controlling cell cycle entry.

Keywords: cell cycle / evolution / fungi / chytrid / virus / horizontal gene transfer

INTRODUCTION

The networks regulating cell division in yeasts and animals are highly similar in both physiological function and network structure (Figure 1) (Cross et al., 2011; Doonan and Kitsios, 2009). For example, the cell cycle controls proliferation in response to a variety of internal and external signals during the G1 phase, between cell division and DNA replication. These input signals, including cell growth, are integrated into a gradual increase in cyclin dependent kinase (Cdk) activity, which triggers a feedback loop at the basis of the all-or-none irreversible decision to proliferate (Bertoli et al., 2013).

Many of the molecular mechanisms underlying G1 regulation are highly conserved. In animal cells, Cyclin D, in complex with either Cdk4 or Cdk6, initiates cell cycle entry by phosphorylating the retinoblastoma protein, pRb. This begins the inactivation of pRb and the concomitant activation of the E2F transcription factors that induce transcription of downstream cyclins E and A, which complete the inhibition of pRb thereby forming a positive feedback loop (Bertoli et al., 2013). Similarly, in budding yeast, the G1 cyclin Cln3-Cdk1 complex initiates the transition by phosphorylating and partially inactivating Whi5, an inhibitor of the SBF transcription factor (Costanzo et al., 2004; de Bruin et al., 2004; Nasmyth and Dirick, 1991; Ogas et al., 1991; Sidorova and Breeden, 1993). This allows for SBF-dependent transcription of the downstream G1 cyclins *CLN1* and *CLN2*, which also inactivate Whi5 to complete a positive feedback loop (Skotheim et al., 2008). Thus, both the biochemical function of G1 regulators and their specific targets are highly conserved (Figure 1).

Many of the individual proteins performing identical roles are unlikely to be true orthologs, *i.e.*, it cannot be inferred from sequence identity that the proteins evolved from a common ancestral gene. In yeast, a single cyclin-dependent kinase, Cdk1, binds distinct cyclin partners to perform all the functions of three non-orthologous animal Cdks (Cdk2, 4 and 6) during cell cycle entry (Liu and Kipreos, 2000). Furthermore, no member of the transcription factor complex SBF-Whi5 exhibits amino acid sequence identity or structural similarity to any member of the E2F-pRb complex (Cross et al., 2011; Hasan et al., 2013; Taylor et al., 1997). Finally, Cdk inhibitors such as Sic1 and p27 play analogous roles in yeast and mammals despite a

total lack of sequence identity (Cross et al., 2011). Taken together, these examples imply significant evolution of cell cycle regulatory proteins in fungi and/or animals while the network topology remains largely intact. Although identification of network topology is restricted to a few model organisms and is not as broad as sequence analysis, the similar network topology in budding yeast and animals suggests that this feature is more conserved than the constituent regulatory proteins (Cross et al., 2011; Doonan and Kitsios, 2009).

The shared presence of E2F-pRb within plants (Archaeaplastida) and animal (Metazoa) lineages would suggest that this regulatory complex, rather than the fungal SBF-Whi5 complex, was present in the last eukaryotic common ancestor (Cao et al., 2010; Doonan and Kitsios, 2009; Fang et al., 2006; Harashima et al., 2013). The divergence of G1 regulator sequences is surprising because fungi and animals are more closely related to one another than either is to plants. This fungal-metazoan difference raises the question as to where the fungal components came from. Fungal components could either be rapidly evolved ancestral regulators or have a distinct evolutionary history, which would suggest convergent evolution of regulatory networks.

To address this question, we examine conserved and divergent features of eukaryotic cell cycle regulation. In contrast to previous work that considered a protein family of cell cycle regulators in isolation (Cao et al., 2010; 2014; Eme et al., 2011; Gunbin et al., 2011; Ma et al., 2013; Wang et al., 2004), we studied the evolutionary history of an entire regulatory network across hundreds of species. We examined a greater number of genomes covering most of eukaryotic diversity, including Excavata, Haptophyta, Cryptophyta, SAR (Stramenopiles, Alveolata, Rhizaria), Archaeplastida (plants), Amoebozoa, Apusozoa and the Opisthokonta (animals and fungi). This survey allowed us to estimate the cell cycle repertoire of the last eukaryotic common ancestor (LECA), a prerequisite to clarifying the evolutionary transitions of the cell cycle components of both animals and fungi.

Our results indicate that LECA likely had complex cell cycle regulation involving at least one Cdk, multiple cyclin families, activating and inhibitory E2F transcription factors, and pRb-family pocket proteins. Identifying the LECA repertoire helps establish that the emergence of SBF-Whi5 is abrupt and distinguishes fungi from all

105 other eukaryotes. We show that basal fungi can have both ancestral E2F-pRb and
106 fungal SBF-Whi5 components. Thus, fungal evolution appears to have proceeded
107 through a hybrid network before abruptly losing the ancestral components in the
108 lineage leading to Dikarya. This supports the hypothesis that network structure,
109 rather than the individual components, has been conserved through the transition to
110 fungi and argues against the case of convergent evolution.
111
112 Our data confirm that SBF shows homology to KilA-N, a poorly characterized domain
113 present in prokaryotic and eukaryotic DNA viruses. Thus, SBF is not derived from
114 E2F (its functional analog) and likely emerged through horizontal gene transfer in the
115 fungal ancestor. We show that SBF can regulate promoters with E2F binding sites in
116 budding yeast. We then then use high-throughput *in vitro* binding assay data to
117 elucidate the shared nucleotide preferences of E2F and SBF for DNA binding. These
118 data suggest that a viral SBF may have initially hijacked cell cycle control, activating
119 transcription via the *cis*-regulatory elements targeted by the ancestral cell cycle
120 regulator E2F, much like extant viral oncogenes.
121
122 **RESULTS**
123
124 **Reconstruction of complex cell cycle control in the ancestral eukaryote**
125 Recent work shows that the last eukaryotic common ancestor (LECA) already had a
126 complex repertoire of protein families (Dacks and Field, 2007; Eichinger et al., 2005;
127 Merchant et al., 2007). Indeed, all sequenced eukaryotic lineages have lost entire
128 gene families that were present in LECA (Fritz-Laylin et al., 2010). In contrast to the
129 growing consensus that LECA had an extensive repertoire of proteins, the prevailing
130 view of the cell cycle in LECA is that it was based on a simple oscillator constructed
131 with relatively few components (Coudreuse and Nurse, 2010; Nasmyth, 1995).
132 According to the 'simple' LECA cell cycle model, an ancestral oscillation in Cyclin B-
133 Cdk1 activity drove periodic DNA replication and DNA segregation, while other
134 aspects of cell cycle regulation, such as G1 control, may have subsequently evolved
135 in specific lineages. The model was motivated by the fact that Cdk activity of a single
136 Cyclin B is sufficient to drive embryonic cell cycles in frogs (Murray and Kirschner,
137 1989) and fission yeast (Stern and Nurse, 1996), and that many yeast G1 regulators
138 have no eukaryotic orthologs (Figure 1).
139

To determine the complexity of LECA cell cycle regulation, we examined hundreds of diverse eukaryotic genomes (Fungi, Amoebozoa, Excavata, SAR, Haptophyta, Cryptophyta). We first built sensitive profile Hidden Markov Models (Eddy, 2011) for each of the gene families of cell cycle regulators from model organisms *Arabidopsis thaliana, Homo sapiens, Schizosaccharomyces pombe,* and *Saccharomyces cerevisiae.* These HHMs were then used to query the sequenced eukaryotic genomes for homologs of both fungal and animal cell cycle regulators (Figures 2-3; see Methods). Phylogenetic analyses were performed on the detected homologs for accurate sub-family assignment of the regulators and inference of their evolutionary history (see Methods). If LECA regulation were simple, we would expect little conservation beyond the Cyclin B-Cdk1 mitotic regulatory module. However, if LECA regulation were more complex, we would expect to see broad conservation of a wider variety of regulators.

While we did not find either of the fungal regulators (SBF and Whi5) outside of Fungi, we did find proteins that regulate the animal cell cycle in Archaeplastida, Amoebozoa, SAR, Haptophyta, Cryptophyta, Excavata or Metazoa,. For example, the cyclin sub-families (A, B, D, and E) known to regulate the cell cycle in metazoans (for cyclin phylogeny see Figure 3-figure supplement 1) are found across the major branches of eukaryotes. We also found examples of all three sub-families of E2F transcription factors (E2F1-6, DP, E2F7/8) and the pRb family of pocket proteins (for E2F/DP and pRb phylogeny see Figure 3-figure supplement 2 and Figure 3-figure supplement 3). Nearly all species contain the APC specificity subunits Cdc20 and Cdh1/Fzr1, which regulate exit from mitosis and maintain low Cdk activity in G1 (for Cdc20-family APC phylogeny see Figure 3-figure supplement 4). Taken together, these data indicate that LECA cell cycle regulation was based on multiple cyclin families, as well as regulation by the APC complex and members of the pRb and E2F families. More broadly, our phylogenetic analyses tend to place the fungal regulators as sister groups to the metazoan regulators, as would be expected from the known eukaryotic species tree. These phylogenies are in agreement with the hypothesis that many fungal and metazoan regulators were vertically inherited from an opisthokont ancestor rather than loss of these regulators in fungi followed by secondary acquisition through horizontal gene transfer.

Members of the Cdk1-3 family (i.e. CdkA in plants) are also broadly conserved across eukaryotes, suggesting they were the primary LECA cell cycle Cdks (for CDK phylogeny see Figure 3-figure supplement 5). Other cell cycle Cdk families in animal (Cdk4/6) and plant (CdkB) are thought to be specific to those lineages. However, we found CdkB in Stramenopiles, which may have arrived via horizontal transfer during an ancient secondary endosymbiosis with algae as previously suggested (Cavalier-Smith, 1999). We excluded from our analysis other families of cyclin-Cdks, *e.g.,* Cdk7-9, which regulate transcription and RNA processing, and Cdk5 (yeast Pho85), which regulate cell polarity, nutrient regulation, and contribute to cell cycle regulation in yeast (Cao et al., 2014; Guo and Stiller, 2004; Ma et al., 2013; Moffat and Andrews, 2004). While interesting and important, an extensive examination of these cyclin-Cdk families is beyond the scope of this work.

A hybrid E2F-pRb-SBF-Whi5 network on the path of fungal evolution

To identify the possible origins of fungal SBF and Whi5, we searched for sequence homologs across eukaryotic genomes (see Methods). We were unable to find any eukaryotic homologs of SBF or Whi5 outside of fungi, with a few exceptions related to DNA viruses that we discuss later. SBF is an important member of a larger family of winged helix-turn-helix transcription factors in fungi that includes Xbp1, Bqt4, and the APSES family (Acm1, Phd1, Sok2, Egf1, StuA). The emergence of the new fungal components SBF, which includes the large APSES family, and Whi5 is abrupt and occurs near the split of basal fungi from metazoans (Figures 4-5; for SBF only, SBF+APSES, and Whi5 phylogeny see Figure 5-figure supplement 1, Figure 5-figure supplement 2, and Figure 5-figure supplement 3). The precise location remains unclear because we have only 1 Nucleard genome (*Fonticula alba*) and because Microsporidia are fast-evolving fungal parasites with reduced genomes (Cuomo et al., 2012). Interestingly, the new regulators (SBF and Whi5) and ancestral regulators (E2F and Rb) co-exist broadly across basal fungi and the lineages formerly known as "zygomycetes". Zoosporic, basal fungi such as Chytrids (*e.g.*, *Spizellomyces punctatus*) can have both fungal and animal cell cycle regulators, which likely represents the ancestral fungal hybrid network. SBF-Whi5 in budding yeast plays a similar role to E2F-pRb in animals, which suggests that these pathways were functionally redundant in an ancestral hybrid network. This redundancy would lead to the evolutionary instability of the hybrid network and could explain why different

209 constellations of components are present in the extant zygomycetes and basal fungi.
210 For example, the zygomycetes have lost pRb while retaining E2F, which was then
211 abruptly lost in the transition to Dikarya. However, with the possible exception of
212 Microsporidia, all fungi have retained SBF and never completely reverted back to the
213 original ancestral state; see Figure 5.
214
215 **The SBF and E2F family of transcription factors are unlikely to be orthologs.**
216
217 A simple scenario to explain the emergence of a hybrid network would be gene
218 duplication of the E2F pathway followed by rapid sequence evolution to create a
219 partially redundant SBF pathway. To this end, we scrutinized the highly conserved
220 E2F and SBF DNA-binding domains to detect any sequence and structural homology.
221 We used the Pfam HMMER model of the E2F/DP DNA-binding domain and SBF DNA-
222 binding domain (KilA-N.hmm), which is homologous to the KilA-N domain (Iyer et
223 al., 2002). We used KilA-N.hmm from Pfam for remote homology detection of
224 SBF+APSES because it was trained on a diverse set of KilA DNA-binding domains
225 across bacterial DNA viruses, eukaryotic DNA viruses, and fungal SBF+APSES
226 proteins. Thus, it should be a more sensitive HMM model to detect remote KilA-N
227 homologues in other eukaryotic genomes. Our controls (*H. sapiens* genome, Fig. 6A
228 and *S. cerevisiae* genome, Fig. 6B) demonstrate that E2F_TDP.hmm is specific to
229 E2F/DP and that KilA-N.hmm is specific to SBF+APSES. We show that genomes with
230 hybrid network (*S. punctatus*, Fig. 6C, and other basal fungi with both transcription
231 factors, Fig. 6D) have both E2F/DP and SBF+APSES. E2F_TDP.hmm never hits an
232 SBF+APSES transcription factor and KilA-N.hmm never hits an E2F transcription
233 factor (i.e. there are no scores on the diagonal of the panels in Figure 6). Thus, there
234 is no misclassification by the Pfam HMM models. These data suggest that SBF and
235 other KilA-N domains have no more sequence identity than non-homologous proteins
236 to E2F/DP. We find that non-fungal genomes only hit E2F/DP (Fig. 6E) with the
237 notable exception of *Trichomonas vaginalis*, the only non-fungal genome with E2F/DP
238 and KilA-N homologs (Fig. 6F). We will discuss the case of *T. vaginalis* in the next
239 section.
240
241 To test the possibility that SBF was a gene duplication of E2F/DP and evolution was
242 so rapid that sequence identity was lost, but structural and functional homology to
243 E2F/DP was maintained, we looked for possible evidence of structural homology. The

244 DNA-binding domains of SBF/MBF (Taylor et al., 2000; Xu et al., 1997) and E2F/DP
245 (Zheng et al., 1999) are structurally classified as members of the winged-helix-turn-
246 helix (wHTH) family, which is found in both prokaryotes and eukaryotes (Aravind and
247 Koonin, 1999; Aravind et al., 2005; Gajiwala and Burley, 2000). Although the DNA-
248 binding domains of E2F/DP and SBF/MBF are both classified as wHTH proteins, they
249 show important differences in overall structure and mode of protein-DNA complex
250 formation that lead us to conclude that it is highly unlikely that they are orthologs.
251
252 Many wHTH transcription factors, including the E2F/DP family, have a 'recognition
253 helix' that interacts with the major or minor grooves of the DNA. The E2F/DP family
254 has an RRXYD DNA-recognition motif in its helix that is invariant within the E2F/DP
255 family and is responsible for interacting with the conserved, core GCGC motif (Zheng
256 et al., 1999) (see Figure 7A: red structure). The RRXYD recognition motif is strikingly
257 conserved in E2F/DP across all eukaryotes, including the E2F/DP proteins uncovered
258 in basal fungi (Figure 7B, left). The first solved SBF/MBF crystal structure, Mbp1 from
259 *S. cerevisiae* in the absence of DNA, suggested Mbp1 recognizes its MCB (Mlu I cell
260 cycle box, ACGCGT) binding site via a recognition helix (Taylor et al., 1997; Xu et
261 al., 1997). However, a recent crystal structure of PCG2, an SBF/MBF homolog in the
262 rice blast fungus *Magnaporthe oryzae*, in complex with its MCB binding site does not
263 support this proposed mode of DNA binding (Liu et al., 2015). In striking contrast to
264 many wHTH structures, in which the recognition helix is the mediator of DNA binding
265 specificity, the wing of PCG2 binds to the minor groove to recognize the MCB binding
266 site. The two glutamines in the wing (Q82, Q89) are the key elements that recognize
267 the core MCB binding motif CGCG (Figure 7A, blue structure). Family-specific
268 conservation in the DNA-binding domain is observed for all members of the SBF and
269 APSES family, including basal fungal sequences (Figure 7B, right). In summary, the
270 incongruences in sequence, structure, and mode of DNA-interaction between E2F/DP
271 and SBF/MBF families strongly suggest that SBF is not derived from E2F.

272 **Viral origin and evolution of the fungal SBF and APSES family**

273 Since SBF is unlikely to be orthologous to the E2F family of transcription factors, we
274 considered the straightforward alternative. Previous work has shown that the DNA-
275 binding domain of the APSES and SBF proteins is homologous to a viral KilA-N
276 domain (Iyer et al., 2002). KilA-N is a member of a core set of "viral hallmark genes"
277 found across diverse DNA viruses that infect eubacteria, archaea, and eukaryotes
278 (Koonin et al., 2006). Outside the fungal SBF/APSES sub-family, little is known about

the KilA-N domain structure, its DNA-binding recognition sequence, and function (Brick et al., 1998). The wide distribution of DNA viruses and KilA-N across the three domains of life suggests that the fungal ancestor likely acquired SBF via horizontal gene transfer rather than the other way around.

To broaden the scope of analysis beyond the eukaryotic genomes that we studied, we carefully surveyed all KilA-N domains detected by the Pfam database. The majority of known KilA-N domains (weighted by species, not the number of sequences) are found in prokaryotes (85%) with a smaller fraction (10%) found in eukaryotes and a smaller fraction found in DNA viruses (5%). The KilA-N domains in prokaryotes appear to be either integrated by or derived from prokaryotic DNA viruses (i.e. bacteriophage), and thus, we will treat them as such. Within the eukaryotes, all known KilA-N domains are found in fungal genomes with three notable exceptions.

The first exception is *Trichomonas vaginalis*, a parasitic excavate with 1000+ KilA-N domains (Figure 6F). The *T. vaginalis* KilA-N domains have top blast hits to prokaryotic and eukaryotic DNA viruses, e.g. Mimivirus, a large double-stranded DNA virus of the Nucleo-Cytoplasmic Large DNA Viruses (Yutin et al., 2009). Mimiviruses are giant viruses known to infect simple eukaryotic hosts, such as *Acanthamoeba* and possibly other eukaryotes (Abrahão et al., 2014; Raoult and Forterre, 2008). The second and third exceptions are found in two insects, *Acyrthosiphon pisum* ('pea aphid') and *Rhodius prolixus* ('triatomid bug'). The one KilA-N domain in *A. pisum* genome has a top blast hit to eukaryotic DNA viruses (e.g. Invertebrate Iridescent Virus 6). The three KilA-N domains in *R. prolixus* have top blast hits to prokaryotic DNA viruses (e.g. Enterobacteria phage P1). The diverse and sparse distribution of KilA-N domains throughout the eukaryotic genomes is consistent with their horizontal gene transfer into hosts from eukaryotic DNA viruses and/or via engulfed bacteria that were infected with prokaryotic DNA viruses. In fact, the horizontal transfer of genes between Mimivirus and their eukaryotic host, or the prokaryotic parasites within the host, has been shown to be a more frequent event that previously thought (Moreira and Brochier-Armanet, 2008)

To gain further insight into the possible evolutionary origins of the SBF subfamily via horizontal gene transfer, we aligned diverse KilA-N sequences from the Uniprot and

314 PFAM database to the KilA-N domain of our most basal fungal SBF+APSES sequences
315 (Zoosporic fungi ("chytrids") and "Zygomycetes") and built a phylogenetic tree
316 (Figure 8). There are three major phylogenetic lineages of KilA-N domains: those
317 found in eukaryotic viruses, prokaryotic viruses, and the fungal SBF+APSES family.
318 Our results show that the fungal SBF family is monophyletic and is strongly
319 supported by multiple phylogenetic support metrics. This suggests a single HGT
320 event as the most likely scenario that established the SBF+APSES family in a fungal
321 ancestor. However, our current phylogeny is unable to distinguish whether the SBF
322 family arrived in a fungal ancestor through a eukaryotic virus or a phage-infected
323 bacterium. Structural and functional characterization of existing viral KilA-N domains
324 could help distinguish between these two hypotheses.

325 **SBF ancestor could regulate E2F-target genes**

326

327 Of all the members of the SBF+APSES family, the most likely candidate to be a
328 "founding" TF is SBF, as it is the only member present in all fungi (Figure 5). In
329 budding yeast and other fungi, SBF functions in G1/S cell cycle regulation and binds
330 a consensus site CGCGAA (Gordân et al., 2011), which overlaps with the consensus
331 site GCGSSAAA for the E2F family (Rabinovich et al., 2008); see Figure 9A. The
332 APSES regulators, Xbp1, and MBF in budding yeast bind TGCA, TCGA, ACGCGT
333 motifs, respectively. A viral origin of the SBF+APSES family—with the founding
334 member involved in cell cycle control—suggests the hypothesis that perhaps the
335 founder TF functioned like a DNA tumor virus protein and hijacked cell cycle control
336 to promote proliferation.

337

338 For the viral TF (SBF) to hijack cell cycle control in the fungal ancestor, it must have
339 been able to both bind E2F regulatory regions and then activate the expression of
340 genes under E2F in a cell cycle-regulated fashion. The overlap between the
341 conserved E2F and SBF consensus sites suggests that ancestral SBF could bind E2F
342 regulatory regions (Figure 9A). However, a single base pair substitution in the SBF
343 motif can reduce gene expression by up to ~95% (Andrews and Moore, 1992) and
344 flanking regions outside the core are often important for binding affinity and gene
345 expression (Nutiu et al., 2011). To our knowledge, no one has directly measured the
346 extent to which animal E2F and yeast SBF bind similar sites either *in vivo* or *in vitro*.
347 To first test whether yeast SBF can bind a canonical E2F binding site, we inserted
348 consensus E2F binding sites in the budding yeast genome. The hijacking hypothesis

349 would be supported *in vivo* if E2F binding sites could generate SBF-dependent cell
350 cycle regulated gene expression. We used the well-studied *CLN2* promoter, which
351 has three binding sites for SBF (SCB, Swi6-dependent cell cycle box) in a
352 nucleosome-depleted region (Figure 9B-9C). Removal of these SCB sites is known to
353 eliminate cell cycle-dependent gene expression (Bai et al., 2010). In our
354 experiment, the change in the SBF site was much more substantial than a single
355 base pair mutation. We replaced the complete SBF sites (TCACGAAA) of *CLN2* (Koch
356 et al., 1996) with a known E2F binding site (GCGCGAAA) from the promoters of the
357 histone gene cluster in mammals (Rabinovich et al., 2008). We observed significant
358 oscillations in GFP expression, which were coordinated with the cell cycle.
359 Importantly, the amplitude of these oscillations was dependent on the budding yeast
360 SBF (Swi4), but not MBF (Mbp1), and disappeared when the 3 binding sites were
361 removed (Figure 9C-9D). This experiment demonstrates that budding yeast SBF can
362 bind E2F-like sites, despite the fact that Dikarya lost ancestral E2F hundreds of
363 millions of years ago.
364
365 There are, of course, other possible E2F DNA binding sites that we could have used
366 in our experiment; we picked this one because it is a well-characterized E2F binding
367 site. To further explore the overlap in sequence specificity, we analyzed data from
368 high-throughput protein-binding microarray (PBM) assays (Afek et al., 2014; Badis
369 et al., 2008) of human E2F (E2F1) and budding yeast SBF (Swi4). PBM assays
370 measure, in a single experiment, the binding of recombinant proteins to tens of
371 thousands of synthetic DNA sequences, guaranteed to cover all possible 10-bp DNA
372 sequences in a maximally compact representation (each 10-mer occurs once and
373 only once). We used these PBM data to generate DNA motifs for E2F and SBF
374 (Berger et al., 2006), and to compute, for each possible 8-bp DNA sequence, an
375 enrichment score (or E-score) that reflects the specificity of the protein for that 8-
376 mer. E-scores vary between -0.5 and +0.5, with larger values corresponding to
377 higher affinity binding sites (Berger et al., 2006). As shown in Figure 10A, E2F1 and
378 Swi4 can bind a set of common motifs. For example, the E2F binding site variant that
379 we tested in budding yeast (GCGCGAAA, highlighted in red), is one of the sites
380 commonly bound *in vitro* by E2F and SBF.
381
382 Most notably, the *in vitro* PBM data show that there are specific motifs that can be
383 bound only by E2F or only by SBF. To identify the key nucleotide differences

384 between E2F-only and SBF-only binding, we created motifs of E2F-only and SBF-only
385 sites. The consensus E2F-only (NNSGCGSN) and SBF-only (NNCRCGNN) motifs
386 indicate that differential specificity between E2F1 and SBF is mediated by the
387 nucleotides in the 3rd and 4th positions (underlined) before the invariant CG at the
388 5th and 6th positions. E2F has a strict preference for G in the 4th position, where as
389 SBF has a strict preference for C in the 3rd position (Figure 10A).
390
391 We then scanned the promoters of known E2F target genes from the human genome
392 (*CCNE1*, *E2F1*, *EZH2*) with our empirically-defined DNA binding sites from PBM
393 assays (Afek et al., 2014; Badis et al., 2008) to predict putative E2F-only, SBF-only,
394 and common sites (Figure 10B). As expected, there are many predicted E2F-only
395 and common (E2F & SBF) sites that could be bound by E2F in these known target
396 genes. However, we could also find many potential SBF-only binding sites in these
397 same promoters. We then extended our analysis to 290 known E2F target genes in
398 the human genome to test the generality of SBF cross-binding to E2F sites (Figure
399 10C). Most E2F target promoters could be bound by SBF. Taken together, this set
400 of experiments lends support to the hijacking hypothesis, where an ancestral SBF
401 may have taken control of several E2F-regulated genes.
402
403 **DISCUSSION**
404
405 Cell division is an essential process that has been occurring in an uninterrupted chain
406 for billions of years. Thus, one expects strong conservation in the regulatory network
407 controlling the eukaryotic cell division cycle. Consistent with this idea, cell cycle
408 network structure is highly similar in budding yeast and animal cells. However, many
409 components performing similar functions, such as the SBF and E2F transcription
410 factors, lack sequence identity, suggesting a significant degree of evolution or
411 independent origin. To identify axes of conservation and evolution in eukaryotic cell
412 cycle regulation, we examined a large number of genome sequences in
413 Archaeplastida, Amoebozoa, SAR, Haptophyta, Cryptophyta, Excavata, Metazoa and
414 Fungi. Across eukaryotes, we found a large number of proteins homologous to
415 metazoan rather than fungal G1/S regulators. Our analysis indicates that the last
416 eukaryotic common ancestor likely had complex cell cycle regulation based on Cdk1,
417 Cyclins D, E, A and B, E2F, pRb and APC family proteins.
418

419 In contrast, SBF was not present in the last common eukaryotic ancestor, and
420 abruptly emerged, with its regulator Whi5, in fungi likely due to the co-option of a
421 viral KilA-N protein at the base of the fungal lineage. The origin of Whi5 is unclear
422 because we found no homologs outside of fungi. Whi5 is a mostly unstructured
423 protein, which, like pRb, recruits transcriptional inhibitor proteins to specific sites on
424 DNA via transcription factor binding (Huang et al., 2009; Wang et al., 2009). The
425 relatively simple structure of Whi5 suggests that it may have been subsequently co-
426 opted as a phosphopeptide to entrain SBF activity to cell cycle regulated changes in
427 Cdk activity (Figure 11).
428
429 The replacement of E2F-Rb with SBF-Whi5 at the core of the cell cycle along the
430 fungal lineage raises the question as to how such a drastic change to fundamental
431 regulatory network could evolve. One answer can be found in the evolution of
432 transcription factors. When the function of an essential transcription factor does
433 change, it often leaves behind a core part of its regulon for another factor (Brown et
434 al., 2009; Gasch et al., 2004; Lavoie et al., 2009). This process of handing off
435 transcription factor function has been observed to proceed through an intermediate
436 state, present in some extant genomes, in which both factors perform the function
437 (Tanay et al., 2005). The logic of proceeding through an intermediate state has been
438 well-documented for the regulation of genes expressed only in yeast of mating type
439 **a** (asgs) (Tsong et al., 2006). In the ancestral yeast and many extant species, asgs
440 expression is activated by a protein only present in **a** cells, while in other yeasts,
441 expression is repressed by a protein only present in α cells and **a**/α diploids. The
442 replacement of the ancestral positive regulation by negative regulation occurred via
443 yeast that contained both systems illustrating how an essential function can evolve
444 through a hybrid state (Baker et al., 2012).
445
446 Clearly, something similar happened during cell cycle evolution. It appears that the
447 replacement of the E2F-pRb transcription regulatory complex with the SBF-Whi5
448 complex proceeded via a hybrid intermediate that preserved its function. In the
449 hybrid intermediate, E2F-Rb and SBF-Whi5 may have evolved to be parallel
450 pathways whose functions overlapped to such an extent that the previously essential
451 E2F-Rb pathway could be lost in the transition to Dikarya. Interestingly, many basal
452 fungi (e.g. Chytrids) have preserved rather than lost this hybrid intermediate, which
453 suggests that each pathway may have specialized functions. Chytrids exhibit both

454 animal (e.g. centrioles, flagella, amoeboid movement) and fungal features (e.g. cell
455 walls, hyphal morphology) whose synthesis needs to be coordinated with cell
456 division. The preservation of the hybrid network in chytrids could then be explained if
457 animal-like and fungal features are regulated by the E2F-Rb and SBF-Whi5 pathways
458 respectively (Figure 11). Once Fungi lost many of the ancestral animal-like features
459 (flagellated cells, amoeboid movement) during the emergence of the "zygomycetes"
460 or Dikarya, the ancestral E2F-pRb components could have evolved new functions or
461 have been lost.
462
463 The origin of the hybrid network at the base of Fungi is abrupt and may have been
464 initiated by the arrival of SBF via virus. Many tumor viruses activate cell
465 proliferation. For example, the DNA tumor viruses Adenovirus and SV40 highjack cell
466 proliferation in part by activating the expression of E2F-dependent genes by binding
467 pRb to disrupt inhibition of E2F (DeCaprio, 2009). While the specific mechanisms
468 may differ, when SBF entered the fungal ancestor cell it might have activated the
469 transcription of E2F target genes. Rather than inhibiting the inhibitor of E2F, SBF
470 may have directly competed for E2F binding sites with transcriptionally inactive E2F-
471 Rb complexes (Figure 11). Consistent with this model, we have shown here that SBF
472 can directly regulate gene expression in budding yeast via a consensus E2F binding
473 site. Thus, the cooption of a viral protein generated a hybrid network to ultimately
474 facilitate dramatic evolution of the core cell cycle network in fungi.

475 **MATERIALS AND METHODS**

476 **Identification of potential protein family homologs.**
477 We used Profile-Hidden Markov Models (profile-HMMs) to detect homologs for each of
478 the families studied, using the HMMER 3 package (Eddy, 2011). Profile-HMMs are
479 sensitive tools for remote homology detection. Starting with a set of diverse yet
480 reliable protein homologs is fundamental for detecting remote protein homology and
481 avoiding "model poisoning" (Johnson et al., 2010). To this end, we used reliable
482 training-set homologs from the cell cycle model organisms *Arabidopsis thaliana,*
483 *Homo sapiens, Schizosaccharomyces pombe,* and *Saccharomyces cerevisiae*, to build
484 the profile-HMMs used to detect homologs. Our profile-HMM search used a stringent
485 e-value threshold of 1e-10 to detect putative homologs in the "best" filtered protein
486 sets (where available) of our 100+ eukaryotic genomes (see Supplementary File 1A
487 for genome details). All putative homologs recovered through a profile-HMM search

488 were further validated (or rejected) using an iterative search algorithm (Jackhmmer)
489 against the annotated SwissProt database using the HMMER web server (Finn et al.,
490 2011).
491
492 Our profile-HMM for E2F/DP family only detects E2F or DP, where as our profile-HMM
493 for SBF/MBF family only detects SBF/MBF (or APSES). The same protein was never
494 identified by both profile-HMMs because the sequence profiles and the structure are
495 non-homologous. In the case of basal fungi, which have both E2F/DP and SBF/MBF,
496 all proteins classified as an E2F/DP had clear homology to E2F or DP (see alignment
497 in Figure 7B) and all proteins that we classified as SBF/MBF had clear homology to
498 SBF/MBF (see alignment in Figure 7B).

499 **Phylogenetic-based classification of protein homologs in sub-families.**

500 A phylogenetic analysis and classification was built in four stages. In the first stage,
501 we used MAFFT-L-INS-i (-maxiterate 1000) to align the sequences of eukaryotic
502 protein family members (Katoh and Standley, 2013). We then used probabilistic
503 alignment masking using ZORRO (Wu et al., 2012) to create different datasets with
504 varying score thresholds. Next, we used ProtTest 3 to determine the empirical
505 amino-acid evolutionary model that best fit each of our protein datasets using
506 several criteria: Akaike Information Criterion, corrected Akaike Information Criterion,
507 Bayesian Information Criterion and Decision Theory (Darriba et al., 2011). Last, for
508 each dataset and its best-fitting model, we ran different phylogenetic programs that
509 use maximum-likelihood methods with different algorithmic approximations (RAxML
510 and PhyML) and Bayesian inference methods (PhyloBayes-MPI) to reconstruct the
511 phylogenetic relationships between proteins.
512
513
514 For RAxML analyses, the best likelihood tree was obtained from five independent
515 maximum likelihood runs started from randomized parsimony trees using the
516 empirical evolutionary model provided by ProtTest. We assessed branch support via
517 rapid bootstrapping (RBS) with 100 pseudo-replicates. PhyML 3.0 phylogenetic trees
518 were obtained from five independent randomized starting neighbor-joining trees
519 (RAND) using the best topology from both NNI and SPR moves. Non-parametric
520 Shimodaira-Hasegawa-like approximate likelihood ratio tests (SH-aLRTs) and
521 parametric *à la Bayes* aLRTs (aBayes) were calculated to determine branch support
522 from two independent PhyML 3.0 runs. For Bayesian inference we used PhyloBayes

523 (rather than the more frequently used MrBayes) because it allows for site-specific
524 amino-acid substitution frequencies, which better models the level of heterogeneity
525 seen in real protein data (Lartillot and Philippe, 2004; Lartillot et al., 2009). We
526 performed Phylobayes analyses by running three independent chains under CAT and
527 the exchange rate provided by ProtTest 3 (e.g. CAT-LG), four discrete gamma
528 categories, and with sampling every 10 cycles. Proper mixing was initially confirmed
529 with Tracer v1.6 (2014). The first 1000 samples were discarded as burn-in, and
530 convergence was assessed using bipartition frequencies and summary statistics
531 provided by bpcomp and tracecomp from Phylobayes. These were visually inspected
532 with an R version of AWTY (https://github.com/danlwarren/RWTY) (Nylander et al.,
533 2008). The best phylogenies are shown in Figure 3-figure supplement 1-5 and Figure
534 5-figure supplement 1-3, and were used to tentatively classify sequences into sub-
535 families and create Figures 2-5, 8.
536
537 We note that the confidence of each node in the phylogenetic trees was assessed
538 using multiple, but complementary support metrics: (1) posterior probability for the
539 Bayesian inference, (2) rapid bootstrap support (Stamatakis, 2006; Stamatakis et
540 al., 2008) for RAxML, and (3) non-parametric Shimodaira-Hasegawa-like
541 approximate likelihood ratio tests (SH-aLRTs) and parametric *à la Bayes* aLRTs
542 (aBayes) for PhyML. These different support metrics complement each other in their
543 advantages and drawbacks. SH-aLRT is conservative enough to avoid high false
544 positive rates but performs better compared to bootstrapping (Guindon et al., 2010;
545 Simmons and Norton, 2014). aBayes is powerful compared to non-parametric tests,
546 but has a tendency to increase false-positive rates under serious model violations,
547 something that can be balanced with SH-aLRTs (Anisimova and Gascuel, 2006;
548 Anisimova et al., 2011).
549

550 **Strain construction:**

551
552 Our *CLN2pr-GFP-CLN2PEST* constructs were all derived from pLB02-0mer (described
553 in (Bai et al., 2010) and obtained from Lucy Bai). To create pLB02-CLN2, a synthetic
554 DNA fragment (IDT, Coralville, IA) encompassing a region of the *CLN2* promoter
555 from 1,130 bp to 481 bp upstream of the *CLN2* ORF was digested with BamHI and
556 SphI and ligated into pLB02-0mer digested with the same enzymes. To create
557 pLB02-E2F, which contains E2F binding sites, the same procedure was applied to a

558 version of the promoter fragment in which the SCBs at 606bp, 581bp, and 538bp
559 upstream of the ORF were replaced with the E2F binding site consensus sequence
560 GCGCGAAA (Thalmeier et al., 1989). All these plasmids were linearized at the BbsI
561 restriction site in the *CLN2* promoter and transformed. Both *swi4Δ* and *mbp1Δ*
562 strains containing pLB02-0mer, pLB02-Cln2, pLB02-E2F fluorescent expression
563 reporters were produced by mating lab stocks using standard methods. JE103 was a
564 kind gift from Dr. Jennifer Ewald. Plasmids and strains are listed in Supplementary
565 File 1B and 1C, respectively.

566 **Imaging and analysis:**

567

568 Imaging proceeded essentially as described in Bean *et al*., 2006. Briefly, early log-
569 phase cells were pre-grown in SCD and gently sonicated and spotted onto a SCD
570 agarose pad (at 1.5%), which was inverted onto a coverslip. This was incubated on a
571 heated stage on a Zeiss Observer Z.1 while automated imaging occurred (3 minute
572 intervals, 100-300 ms fluorescence exposures). Single-cell time-lapse fluorescence
573 intensity measurements were obtained using software described in (Doncic and
574 Skotheim, 2013; Doncic et al., 2011), and oscillation amplitudes were obtained
575 manually from the resulting traces. The single-cell fluorescence intensity traces used
576 mean cellular intensity with the median intensity of the entire field of view
577 subtracted, to control for any fluctuations in fluorescent background. The resulting
578 measurements were analyzed in R.

579

580 **Bioinformatic analysis of protein binding microarrays and human promoters:**

581

582 Universal PBM data for Swi4 was downloaded from the cis-BP database (Weirauch et
583 al., 2014). We used the PBM 8-mer E-scores reported in cis-BP for the data set
584 M0093_1.02:Badis08:SWI4_4482.1_ArrayB. Universal PBM data for E2F1 (Afek et
585 al., 2014) is available in the GEO database (accession number GSE61854, probes
586 UnivV9_*). E2F1 8-mer E-scores were computed using the Universal Protein Binding
587 Microarray (PBM) Analysis Suite (Berger and Bulyk, 2009). An E-score cutoff of 0.37
588 was used to call SBF and E2F binding sites. This cutoff corresponds to a false positive
589 discovery rate of 0.001 (Badis et al., 2009). To generate DNA motifs for E2F-only,
590 SBF-only, and common sites, we used the Priority software (Gordân et al., 2010)
591 with a uniform prior to align the 8-mers with E-score > 0.37 for E2F only, SBF only,
592 or both E2F and SBF, respectively (Figure 10A). Promoter sequences (1000bp

upstream of transcription start) for known E2F targets (Eser et al., 2011) were retrieved from *Homo sapiens* genome as provided by UCSC (hg38) (BSgenome.Hsapiens.UCSC.hg38) and the annotation package org.Hs.eg.db from Bioconductor (v3.2) (Gentleman et al., 2004; Huber et al., 2015). Only regions for which at least two consecutive sliding windows of 8-mers (1 nucleotide step; 7 overlapping nucleotides) were high scoring (E-score ≥ 0.37) were called as potential SBF or E2F binding regions. Overlapping or "common" binding regions between E2F and SBF were defined as regions that, regardless of their difference in length (nested or partially overlapping), overlapped in at least one full 8-mer (Figure 10B-C).

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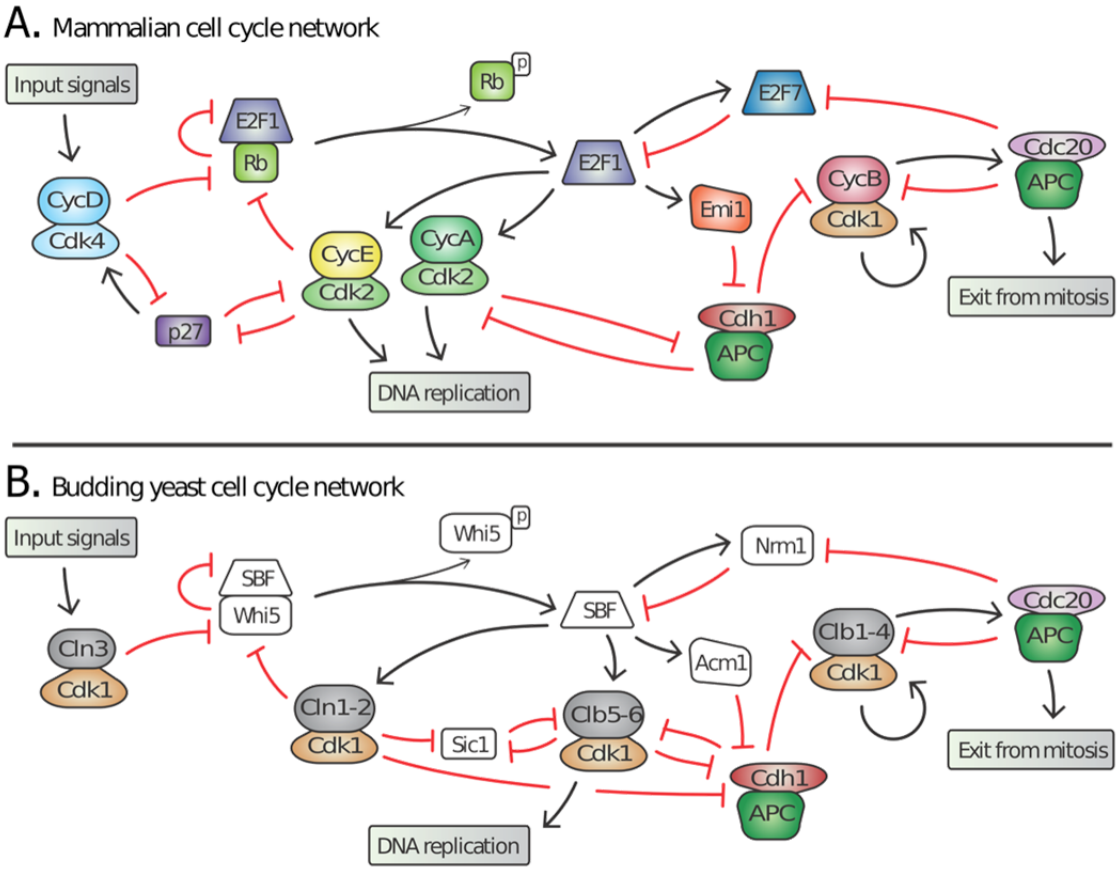


Figure 1. Topology of G1/S regulatory network in mammals and budding yeast is conserved, yet many regulators exhibit no detectable sequence homology. Schematic diagram illustrating the extensive similarities between animal (A) and budding yeast (B) G1/S cell cycle control networks. Similar coloring denotes members of a similar family or sub-family. Fungal components colored white denote proteins with no identifiable animal orthologs.

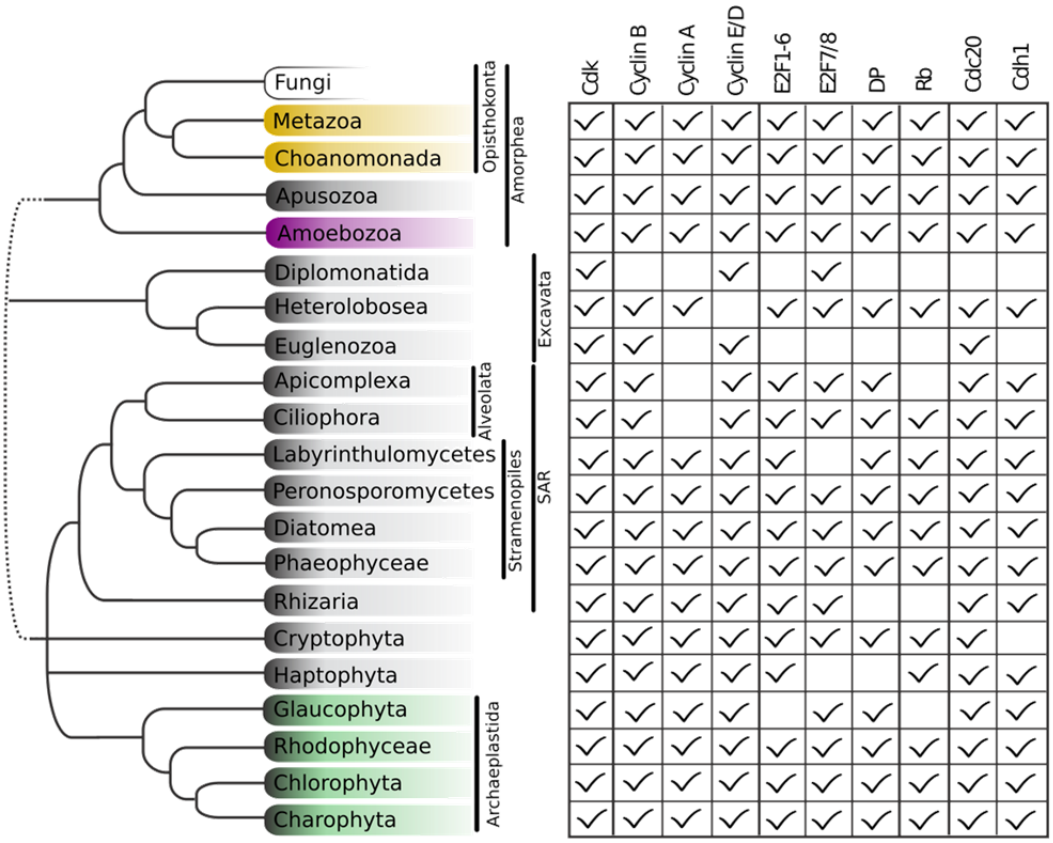


Figure 2. Animal and plant G1/S regulatory network was present in the last eukaryotic common ancestor. Distribution of cell cycle regulators across the eukaryotic species tree (Adl et al., 2012). Animals (Metazoa) and yeasts (Fungi) are sister groups (Opisthokonta), and are distantly related to plants (Charophyta), which are members of the Archaeplastida. Check marks indicate the presence of at least one member of a protein family in at least one sequenced species from the corresponding group. See Figure 3 for a complete list of components in all species analyzed.

			(CycB3)		(CycO/F/J/G/I)						
	(H. sapiens)	**Cdk1-4,6**	**CycB**	**CycA**	**CycE/D**	**E2F1-6**	**E2F7-8**	**DP**	**Rb**	**Cdc20**	**Fzr1**
Metazoa	*H. sapiens (human)*	5	3	2	13	6	2	3	3	2	1
	G. gallus (chicken)	4	2	2	13	6	2	2	3	2	1
	D. rerio (zebrafish)	5	3	2	12	5	2	3	3	1	2
	B. floridae (lancelet)	5	1	1	6	2	2	1	2	1	2
	C. intestinalis (sea squirt)	5	1	2	5		1	1	1	1	1
	S. purpuratus (urchin)	3	2	1	6	2		1	3	2	1
	L. gigantea (sea snail)	3	2	1	7	2	1	1	2	2	1
	D. melanogaster (fly)	3	2	1	3	2		1	2	1	2
	C. elegans (nematode)	3	4	1	2	2	1	1	1	1	1
	N. vectensis (anemone)	3	3	1	5	5	3	1	1	2	1
	A. queenslandica (sponge)	2	1	1	5	2	1	1	3	2	1
	T. adhaerens (placozoa)	3	2	1	3	2	1	1	2	1	1
Choanomonada	*Sphaeroforma arctica*		1		3	2		2	1	1	1
	Capsaspora owczarzaki		1	1	4	1	1	1	1	1	1
	Monosiga brevicollis	1	1	1	2	1	1	1	1	1	1
	Salpingoeca rosetta	2	1	1	2	2	1	1	2	1	1
Apusozoa	*Thecamonas trahens*	1	1	1	3	1	1	1	1	1	1
Amoebozoa	*Dictyostelium discoideum*	1	1	1	1	1		1	1	1	1
	Dictyostelium purpureum	1	1	1	1	1		1	1	1	1
	Dictyostelium fasciculatum	1	1	1	1	1	1	1	1	1	1
	Polysphondylium pallidum	1	1	1		1	2		1	1	1
	Entamoeba histolytica	6	2		3						2
	Entamoeba nuttalli	6	1		1						2
Diplomonatida	*Giardia intestinalis*	2			2		1				
Heterolobosea	*Naegleria gruberi*	3	3	3		1	1	1	1	3	1
Euglenozoa	*Trypanosoma brucei*	3	1		2					3	
	Leishmania major	3	2		1					2	
	Leishmania donovani	2	2		1					2	
Apicomplexa	*Symbiodinium minutum*	7	2							1	1
	Plasmodium falciparum	2									1
	Cryptosporidium muris	3			2	1	1	1			1
	Toxoplasma gondii	2									1
Ciliophora	*Tetrahymena thermophila*	7	5		11	2	2	3	2	1	8
Labyrinthulomycetes	*Aurantiochytrium limacinum*	3	1	1	3			1	1	1	1
	Schizochytrium aggregatum	3	1	1	2					1	1
	Aplanochytrium kerguelense	3	1	1	2	1		1	1	2	1
Peronosporomycetes	*Phytophthora infestans*	5	1	1	3	1	1	1	1	1	1
	Pythium ultimatum	1	1	1	4	1	1	1	1	1	1
	Hyaloperonospora parasitica	3		2	4			2	1	1	
	Saprolegnia parasitica	3	1	2	4	1	1	2	1	4	1
Diatomea	*Fragilariopsis cylindrus*	2	4	1	10	1	1	1		2	1
	Phaeodactylum tricornutum	2	1	1	10	2	1	1	1	2	1
	Thalassiosira pseudonana	2	3	1	26	1	1	1	1	2	2
Eustigmatales	*Nannochloropsis gaditana*	3	2	1	2			1	1	1	
Phaeophyceae	*Ectocarpus siliculosus*	3	2	1	2	1	1	1	1	2	1
Pelagophyceae	*Aureococcus anophaefferens*	2	2		2	1		1		2	1
Rhizaria	*Bigelowiella natans*	1	2	1	3	1	2			2	1
Cryptophyta	*Guillardia theta*	4	3	1	3	1	1	2	1	3	
Haptophyta	*Emiliania huxleyi*	2	2	3	1	2			1	6	2
Glaucophyta	*Cyanophora paradoxa*	1	1	1	1		1	1		1	2
Rhodophyceae	*Porphyridium cruentum*	3	1	1	1	1	1	1		1	1
	Cyanidioschyzon merolae	2		1	2	1	1	2	1	1	1
Chlorophyta	*Ostreococcus tauri*	2	1	1	2	1	1	1	1	1	1
	Ostreococcus lucimarinus	2	1	1	2	1	1	1	1	1	1
	Micromonas pusilla	2	1	1	2	1	1	1	1	1	1
	Coccomyxa subellipsoidea	4	1	1	1	1	1	1	1	1	1
	Volvox carteri	3	1	1	4	1		1	1	1	1
	Chlamydomonas reinhardtii	3	1	1	4	1		1	1	1	1
Charophyta	*Physcomitrella patens*	9	2	8	2	3	2	2	3	6	4
	Selaginella moellendorffii	3	1	3	3	2	1	1	2	3	2
	Brachypodium distachyon	5	5	6	14	3	2	2	2	5	2
	Oryza sativa	5	6	6	12	4	2	2	2	3	2
	Arabidopsis thaliana	5	11	10	11	3	3	2	1	6	3
	(A. thaliana)	**CdkA/B**	**CycB**	**CycA**	**CycD**	**E2FA-C**	**DEL**	**DP**	**RBR**	**Cdc20**	**Fzr1**
					(CycSDS)						

Figure 3. Comparative genomic data of G1/S regulators across eukaryotes. We developed profile-HMMs to detect cell division cycle regulators in eukaryotic genomes. For each cell cycle regulatory family (*e.g.*, cyclins), we used molecular phylogeny to classify eukaryotic sequences into sub-families (*e.g.*, Cyclins B, Cyclin A, Cyclins E/D). See Methods for details and Figure 3-supplement 1 (Cyclin), Figure 3-supplement 2 (E2F/DP), Figure 3-supplement 3 (pRb), Figure 3-supplement 4 (Cdc20-family), and Figure 3-supplement 5 (CDK) for final phylogenies. Each entry lists the number of sub-family members (column) for each eukaryotic genome (row). Grey rows list the sub-family gene names in *H. sapiens* and *A. thaliana*. Additional cyclin sub-family members are listed in parentheses.

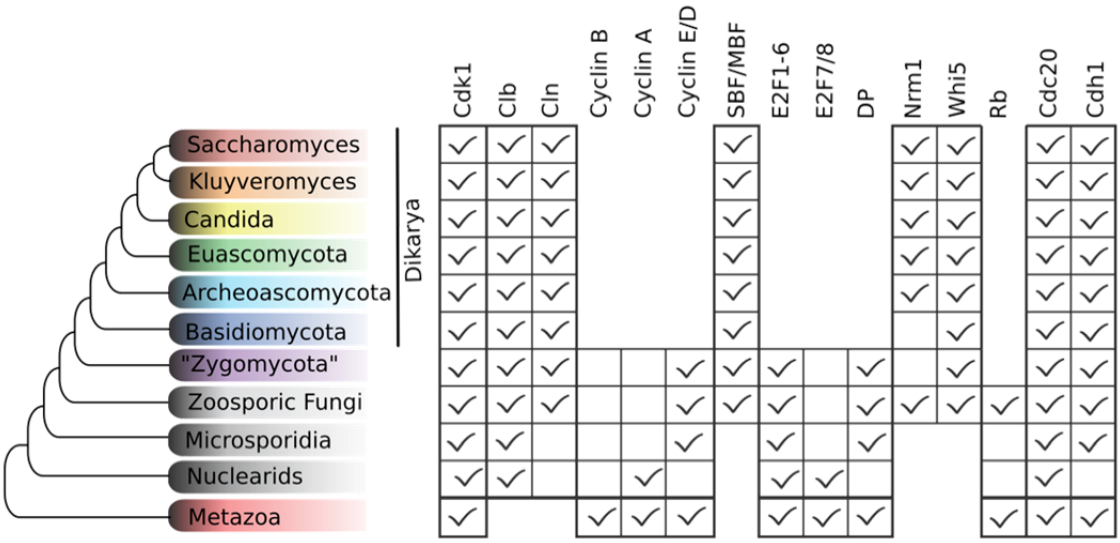


Figure 4. Fungal ancestor evolved novel G1/S regulators, which eventually replaced ancestral cyclins, transcription factors, and inhibitors in Dikarya. Basal fungi and "Zygomycota" contain hybrid networks comprised of both ancestral and fungal specific cell cycle regulators. Check marks indicate the presence of at least one member of a protein family in at least one sequenced species from the group. Cells are omitted (rather than left unchecked) when a family is completely absent from a clade. See Figure 5 for a complete list of components in all fungal species analyzed.

(S. cerevisiae)	CLB	CLN				SBF/MBF	APSES	Xbp1					Nrm1	Whi5	
Saccharomyces cerevisiae	6	3				3	2	1					1	2	
Saccharomyces mikatae	6	3				3	2	1					1	2	
Saccharomyces bayanus	6	3				3	2	1					1	2	
Candida glabrata	6	3				3	2	1					1	1	
Zygosaccharomyces rouxii	3	3				3	1	1					1	1	
Kluyveromyces waltii	3	4				3	1	1					1	1	
Kluyveromyces thermotolerans	3	3				3	1	1					1	1	
Saccharomyces kluyveri	3	3				3	1	1					1	1	
Ashbya gossypii	3	2				3	1	1					1	1	
Kluyveromyces lactis	3	3				3	1	1					1	1	
Wickerhamomyces anomalus	3	3				3	2	1					1	1	
Candida parapsilosis	2	3				3	2	1					1		
Candida albicans	2	3				3	2	1					1		
Candida tropicalis	2	3				3	1	1					1		
Candida guilliermondii	2	3				3	2	1					1	1	
Debaryomyces hansenii	2	3				3	2	1					1		
Candida lusitaniae	2	4				3	2	1					1		
Yarrowia lipolytica	2	2				2	2	1					1	1	
Neurospora crassa	2	1				2	1	1	1				1	2	
Podospora anserina	2	1				2	1	1	1				1	2	
Magnaporthe grisea	2	1				2	1	1	1				1	1	
Fusarium graminearum	2	1				2	1	1	1				1	2	
Cladonia grayi	2	1				2	1	1	1				1	2	
Aspergillus nidulans	2	1				2	1	1	1					1	
Cocciodioides immitis	2	1				2	1	1	1					2	
Saitoella complicata	2	1				2	1	1	1				1	2	
Pneumocystis jirovecii	2	1				2	1		1						
Taphrina deformans	2	1				2	1	1	1					1	
Schizosaccharomyces pombe	4	1				3			1				1	1	
Schizosaccharomcyes octosporus	4					3			1				1	1	
Schizosaccharomyces japonicus	4	1				3			1				1	1	
(S. pombe)	Cdc13	Puc1				Cdc10/Res	APSES		Bqt4					Whi5	
Coprinopsis cinerea	3	1				2		1	1					1	
Laccaria bicolor	3	1				2		1	1					2	
Schizophyllum commune	2	1				2		1	1						
Phanerochaete chrysosporium	2	1				2			1					2	
Cryptococcus neoformans	2	1				2			1					1	
Ustilago maydis	2	1				2	1	1	1					2	
Puccinia graminis	2	1				2	1	1	1					1	
Mortierella alpina	3	1			1	4	1	1	2	1		2		3	
Mortierella verticillata	3	1			1	3	1	1	2	1		1		1	
Rhizophagus irregularis	1					2	1	1		1		1			
Umbelopsis ramanniana	1				1	3	3	1		1				2	
Lichtheimia hyalospora	1				1	4	9	1		2		3		5	
Mucor circineolloides	1					4	6	2		1		2		5	
Phycomyces blakesleeanus	1					4	6	1		2		2		4	
Rhizopus oryzae	1					6	10	1		2		1		4	
Coemansia reversa	4				1	2	1								
Conidiobolus coronatus	1				2	1	2	1							
Allomyces macrogynus	1				4	6	3								
Catenaria anguillulae	1				1	2	1								
Gonapodya prolifera	3	1			8	2									1
Piromyces sp E2	3	1				1	2	1							
Batrachochytrium dendrobatidis	2	1			2	3							1	1	1
Spizellomyces punctatus	1	1			3	3				1		1		2	1
Rozella allomycis	1				2	1				1		1			1
Encephalitozoon cuniculi	1				1					1		1			
Nosema ceranae	1				1					1		1			
Edhazardia aedis	1				1					1		1			
Vittaforma corneae	1				1					1		1			
Nematocida parisii	1				1					1		1			
Fonticula alba	2				2						1	1			
Thecamonas trahens			1	1	3					1	1	1			1
Capsaspora owczarzaki			1	1	4					1	1	1			1
H. sapiens (human)			3	2	13					6	2	3			3
(H. sapiens)			CycB	CycA	CycE/D					E2F1-6	E2F7-8	DP			pRB

Figure 5. Comparative genomic data of G1/S regulators across fungi. We developed profile-HMMs to detect fungal-specific cell division cycle regulators in eukaryotic genomes. For each cell cycle regulatory family (*e.g.*, SBF+APSES), we used molecular phylogeny to classify eukaryotic sequences into sub-families (*e.g.*, SBF/MBF, APSES, Xbp1, Bqt4). Corresponding table shows the number of regulators of each class for all species analyzed. See Methods for details and Figure 5-supplement 1 (SBF only), Figure 5-supplement 2 (SBF+APSES), and Figure 5-supplement 3 (Whi5) for final phylogenies. Grey rows list the sub-family gene names in *S. cerevisiae*, *S. pombe*, and *H. sapiens*

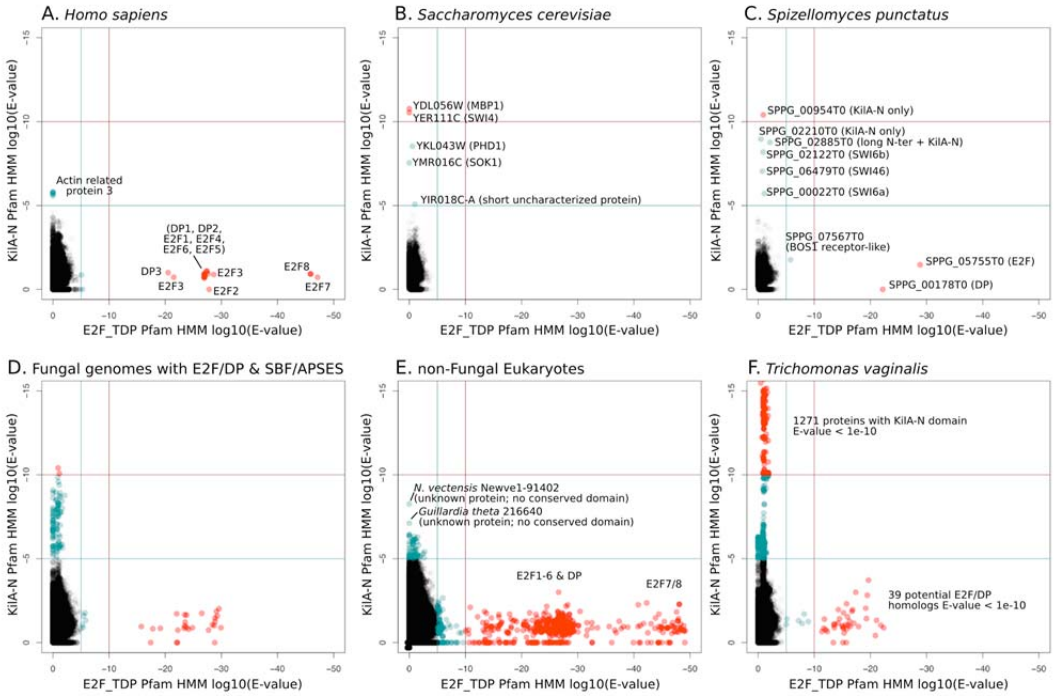


Figure 6. E2F/DP and KilA-N HMMs detect different sequences. We used the Pfam HMMER model of the E2F/DP DNA-binding domain (E2F_TDP.hmm) and the SBF+APSES DNA-binding domain (KilA-N.hmm). We used KilA-N.hmm for remote homology detection of SBF-like proteins because pSMRC.hmm (our HMM model trained on fungal SBFs to detect SBF; see Figure 5 - supplement 1) includes two well-conserved fungal ankyrin repeats in addition to the KilA-N DNA binding domain. Every protein in the query genome (listed at top) was scored using hmmsearch with E2F/DP HMM (x-axis) and KilA-N HMM (y-axis). All scores below 1E-5 (i.e., marginally significant) are blue and those below 1E-10 (i.e. highly significant) are red. All hits with E-values between 1e-5 and 1e-10 were further validated (or rejected) using an iterative search algorithm (Jackhmmer) against the annotated SwissProt database using the HMMER web server (Finn et al., 2011). We then inspected these sequences manually for key conserved KilA-N residues. (A) *Homo sapiens* only has E2F/DP, (B) *Saccharomyces cerevisiae* only has KilA-N (i.e. SBF, MBF, APSES). (C) *Spizellomyces punctatus* and (D) other basal fungi have both E2F/DP and KilA-N. (E) All the non-fungal eukaryote genomes that we surveyed only have E2F/DP. (F) *Trichomonas vaginalis* is one of the few eukaryotes outside of fungi that has both E2F/DP and KilA-N. The E2F/DP HMM and KilA-N HMM always have orthogonal hits (i.e. no protein in our dataset significantly hits both HMMs).

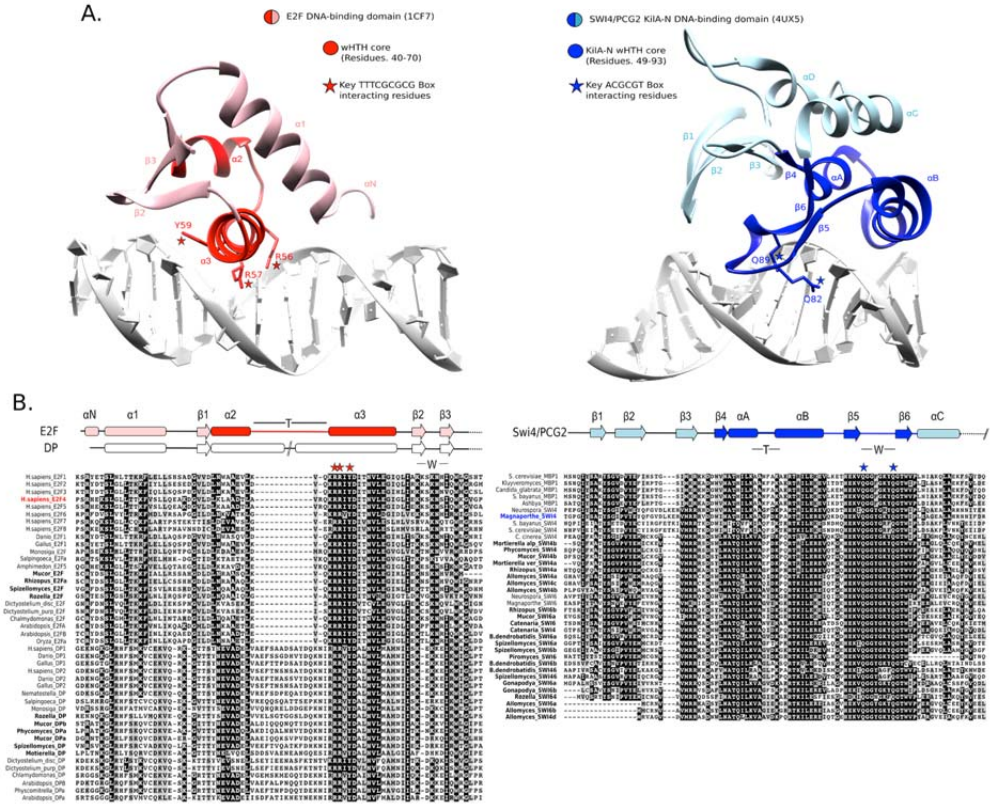


Figure 7. E2F and SBF show incongruences in sequence, structure, and mode of DNA binding. (A) Although both proteins share a winged helix-turn-helix (wHTH) domain, the E2F/DP and SBF/MBF superfamilies do not exhibit significant sequence identity or structural similarity to suggest a common recent evolutionary origin according to CATH or SCOP databases. Furthermore, each wHTH has a different mechanism of interaction with DNA: the arginine and tyrosine side-chains of recognition helix-3 of E2F (E2F4 from *Homo sapiens* (Zheng et al., 1999)) interact with specific CG nucleotides, where as the glutamine side-chains of the "wing" of SBF/MBF (PCG2 from *Magnaporthe oryzae* (Liu et al., 2014)) interact with specific CG nucleotides. (B) Sequence alignment of the DNA binding domain of representative eukaryotic E2F/DP (left) and fungal SBF/MBF (right). The corresponding secondary structure is above the sequence alignment. Evolutionary conserved residues of sequence aligned DNA binding domains are highlighted in black. Bold sequence names correspond to E2F/DP and SBF/MBF sequences from basal fungi. Colored sequence names correspond to sequences of the structures shown in panel A. PDB IDs for the structures used are shown in parentheses. W = wing; T= turn.

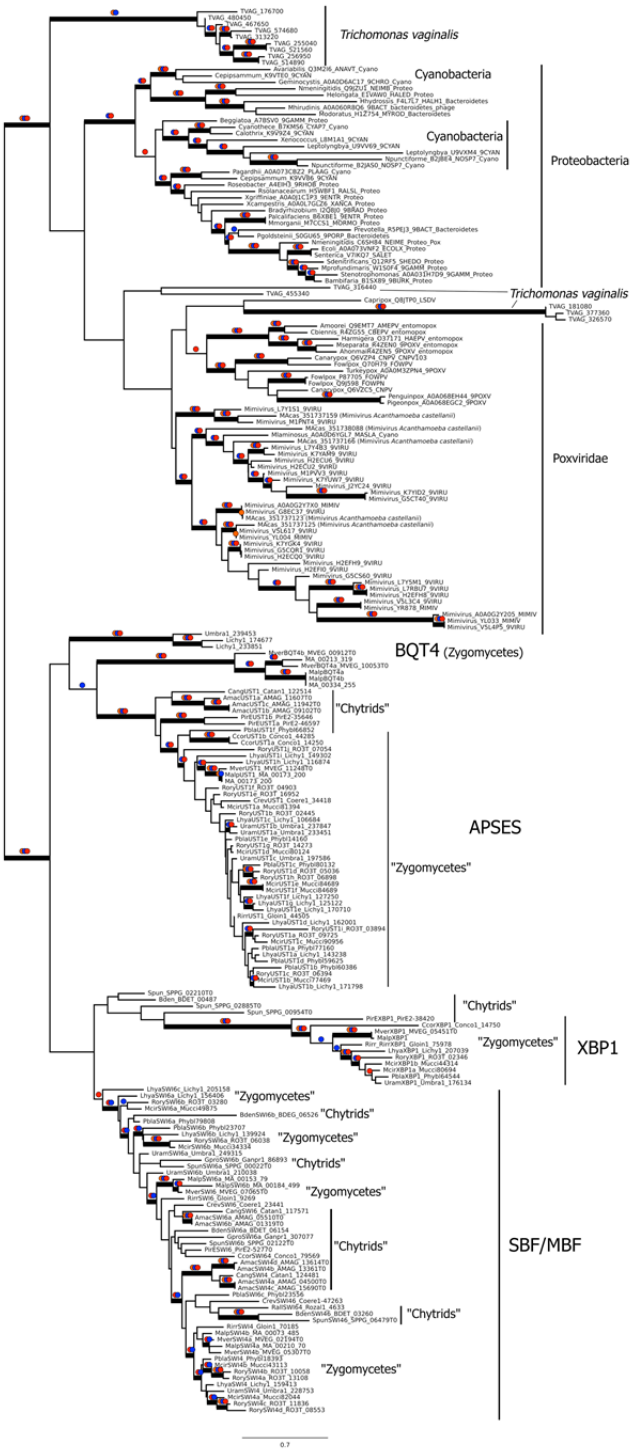


Figure 8. Viral origin of yeast cell cycle transcription factor SBF. Maximum likelihood unrooted phylogenetic tree depicting relationships of fungal SBF-family proteins, KilA-N domains in prokaryotic and eukaryotic DNA viruses. The original dataset was manually pruned to remove long-branches and problematic lineages. Our reduced KilA-N dataset has a total of 219 sequences, 130 positions. Confidence at nodes was assessed with multiple support metrics using different phylogenetic programs under LG model of evolution. Colored dots in branches indicate corresponding branch supports (red dots: PhyML aBayes ≥ 0.9; blue dots: PhyML

961 SH-aLRT ≥ 0.80; orange: RAxML RBS ≥ 70%). Thick branches indicate significant
962 support by at least two metrics, one parametric and one non-parametric; Scale bar
963 in substitutions per site; (See Methods).
964

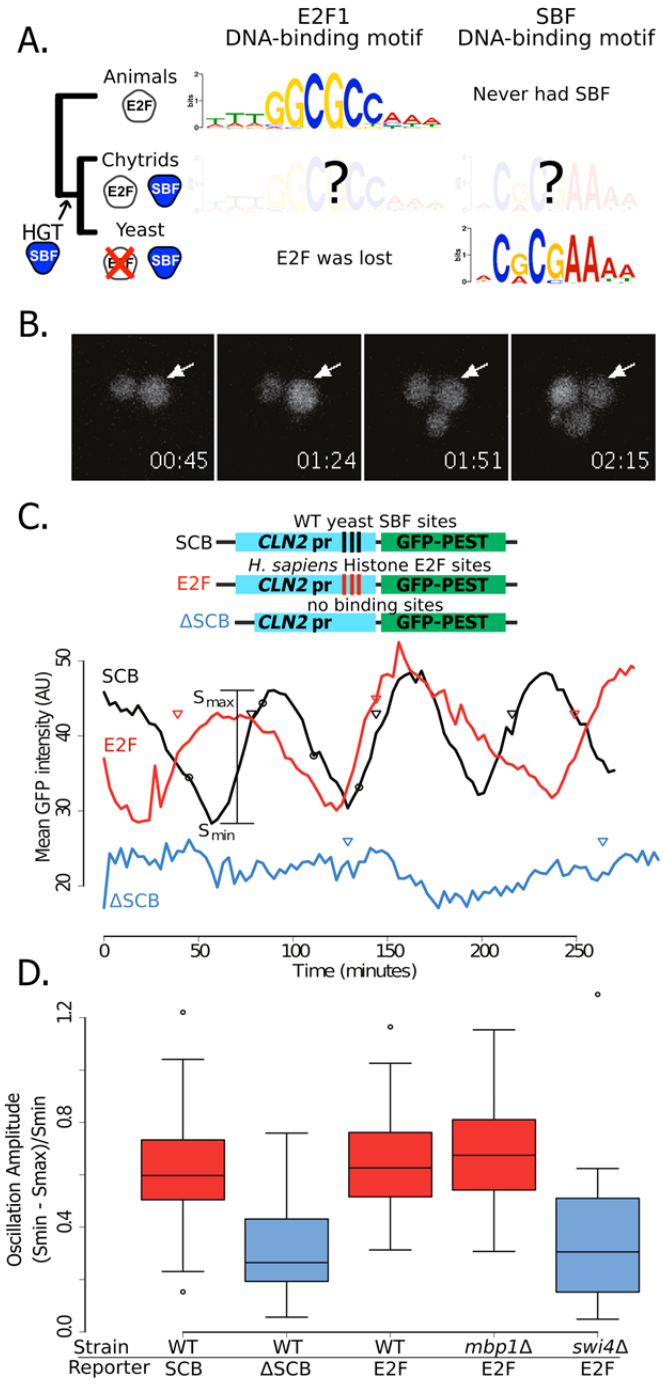
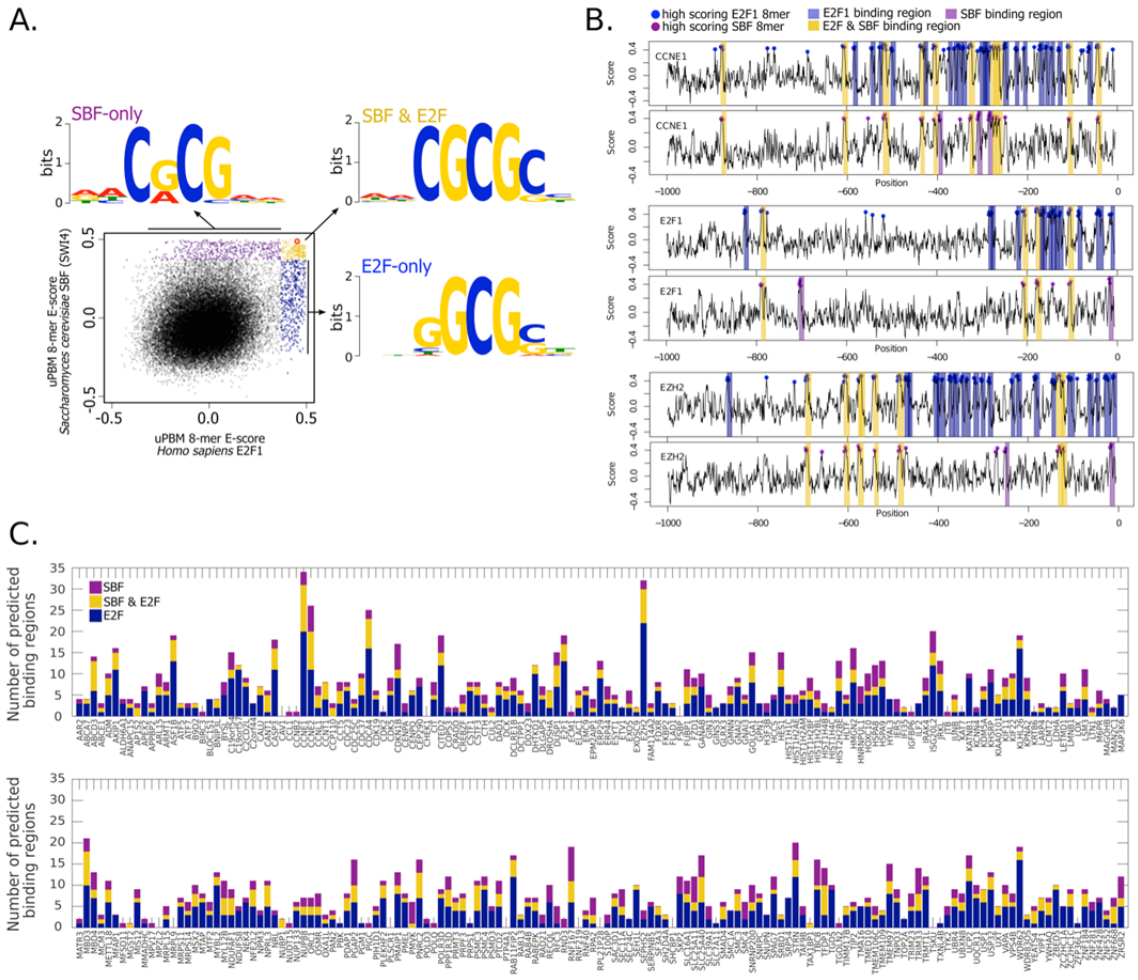


Figure 9. Yeast cell cycle transcription factor SBF can regulate cell cycle-dependent transcription via E2F binding sites in vivo (A) Phylogenetic tree of animals, chytrids, yeast labelled with E2F, SBF or both transcription factors (TF) if present in their genomes. The known DNA-binding motifs of animal E2F (E2F1) and yeast SBF (Swi4) were taken from the JASPAR database, where as Chytrid E2F and SBF motifs are unknown. (B) Fluorescence images of cells expressing a destabilized GFP from the SBF-regulated *CLN2* promoter. (C) Oscillation of a transcriptional reporter in budding yeast. Characteristic time series of GFP expression from a *CLN2* promoter (SCB), a *CLN2* promoter where the SBF binding sites were deleted (ΔSCB), or a *CLN2* promoter where the SBF binding sites were replaced with E2F binding sites

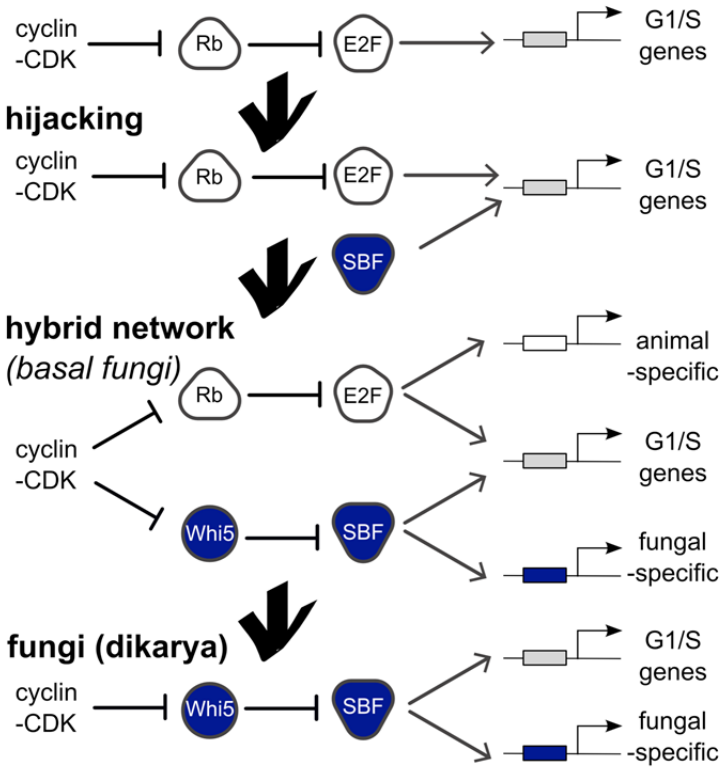
976 from the human gene cluster promoters (E2F). Oscillation amplitudes were quantified
977 by scaling the mean fluorescence intensity difference from peak to trough divided by
978 the trough intensity $(S_{max} - S_{min})/S_{min}$. Circles denote time points corresponding to
979 (b). Triangles denote budding events. (D) Distribution of oscillation amplitudes for
980 different genotypes and GFP reporters. *swi4Δ* and *mbp1Δ* strains have deletions of
981 the SBF and MBF DNA-binding domain subunits respectively. t-test comparisons
982 within and across red and blue categories yield p-values > 0.3 or < 0.01
983 respectively. Boxes contain 25th, median and 75th percentiles, while whiskers extend
984 to 1.5 times this interquartile range.



985

986 **Figure 10: High-throughput DNA binding data for yeast SBF and human E2F**
987 **shows that SBF could bind many E2F-regulated promoters in the human**
988 **genome** (A) Plot of *in vitro* protein binding microarray 8-mer E-scores for *Homo*
989 *sapiens* E2F1 (Afek et al., 2014) versus *S. cerevisiae* SBF protein Swi4 (Badis et al.,
990 2008). All 8-mer motifs colored (E-score > 0.37) are considered significant targets
991 with a false positive discovery rate of 0.001 (Badis et al., 2009). Yellow are common
992 8-mer motifs bound by both E2F1 and SBF, blue are E2F-only motifs, and purple are
993 SBF-only motifs. The E2F motif from histone cluster promoters used in Figure 9 is
994 circled in red. (B) E2F1 (top) and SBF (bottom) PBM motifs were used to scan the
995 proximal (1000bp) promoters of E2F-regulated promoters (CCNE1, E2F1, and EZH2).
996 Promoter regions with a significant hit (8-mer E-score > 0.37) to a E2F or SBF motif
997 have blue or purple dot, respectively. Predicted TF-binding regions were defined as
998 at least 2 consecutive overlapping 8-mers (7 nucleotide overlap) and shaded as blue
999 (E2F binding region) or purple (SBF-binding region). Common or E2F & SBF binding
1000 regions were colored yellow and defined as regions that overlap in at least one full 8-
1001 mer (C) Summary of E2F-only regions (blue), SBF-only regions (purple), and E2F
1002 and SBF co-regulated regions (yellow) for a set of 290 E2F-regulated promoters
1003 (Eser et al., 2011).

1004



1005
1006
1007 **Figure 11. Punctuated evolution of a conserved regulatory network.**
1008 Evolution can replace components in an essential pathway by proceeding through a
1009 hybrid intermediate. Once established, the hybrid network can evolve dramatically
1010 and lose previously essential regulators, while sometimes retaining the original
1011 network topology. We hypothesize that SBF may have hijacked the cell cycle of a
1012 fungal ancestor by binding cis-regulatory DNA sites of E2F and activating expression
1013 of G1/S genes, thus promoting cell cycle entry. Cell cycle hijacking in a fungal
1014 ancestor was followed by evolution of Whi5 to inhibit SBF and Whi5 was
1015 subsequently entrained to upstream cell cycle control through phospho-regulation by
1016 old or new cyclin-CDKs to create a hybrid network with parallel pathways. The hybrid
1017 network likely provided redundant control of the G1/S regulatory network, which
1018 could explain the eventual loss of E2F and its replacement by the SBF pathway in
1019 more derived fungi (Dikarya). Interestingly, Chytrids have hybrid networks and are
1020 transitional species because they exhibit animal-like features of the opisthokont
1021 ancestor (centrioles, flagella) and fungal-like features (cell wall, polarized growth).
1022 We hypothesize that E2F and SBF also bind and regulate a subset of animal-specific
1023 and fungal-specific G1/S genes, which could help explain the preservation of the
1024 hybrid network in Chytrids. Ancestral SBF expanded to create an entire family of
1025 transcription factors (APSES) that regulate fungal-specific traits such as sporulation,
1026 differentiation, morphogenesis, and virulence.
1027

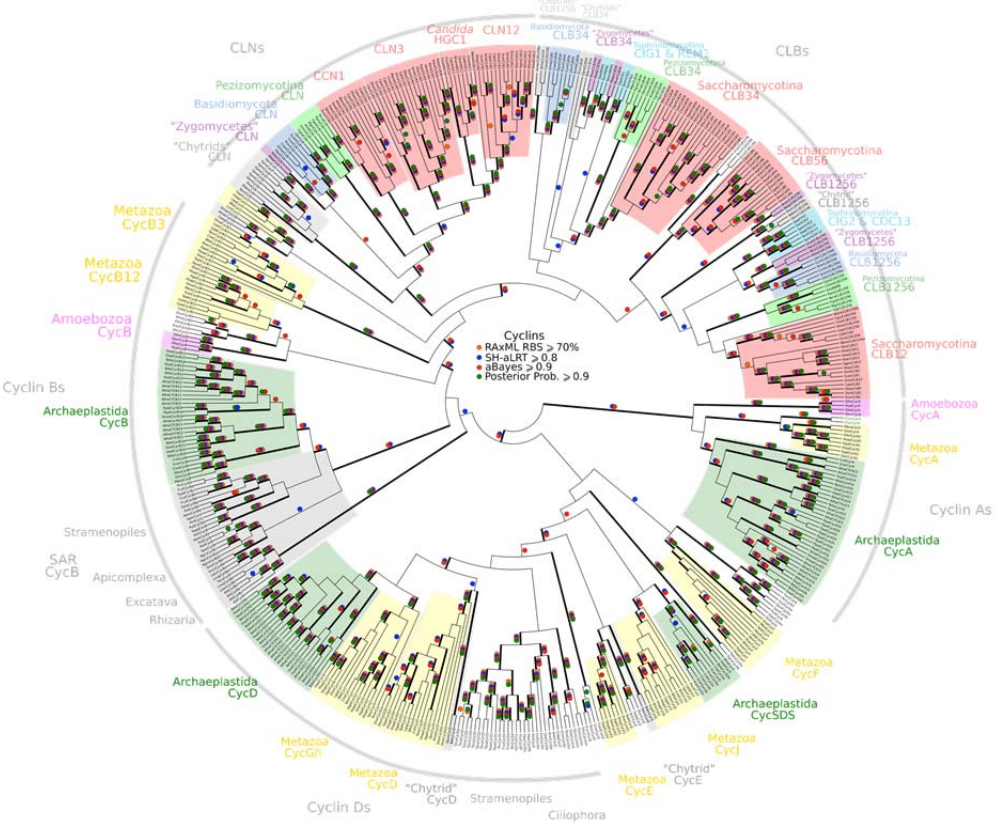


Figure 3 - supplement 1: Reduced phylogeny of eukaryotic cell cycle cyclins. The cell division cycle (CDC) cyclin family consists of several sub-families with a well-characterized cyclin box: (1) CycA (*H. sapiens, A. thaliana*), (2) CycB and CLB (*H. sapiens, A. thaliana, S. cerevisiae, S. pombe*), (3) CycD (*H. sapiens, A. thaliana*), (4) CycE (*H. sapiens*), and (5) CLN (*S. cerevisiae, S. pombe*). We combined CycA, CycB, CycD, CycE, CLB, and CLN sequences from *H. sapiens* (10 cyclins)*, A. thaliana* (13 cyclins)*, S. cerevisiae* (9 cyclins)*,* and *S. pombe* (5 cyclins) to create a eukaryotic CDC cyclin profile-HMM (pCYC.hmm) following the procedure outlined in the Methods. Our HMM profile was sensitive enough to discover known, but uncharacterized cyclin sub-families (CycO, CycF, CycG, CycI, CycJ, CycSDS) as *bona fide* CDC cyclins. A domain threshold of E-20 was used to identify potential CDC cyclin homologs. We first made a phylogeny of all cyclins to classify them. This dataset was then manually pruned to remove long-branches and problematic lineages. Our reduced CDC cyclin dataset has a total of 499 sequences. Columns with the top 10% Zorro score (496 positions) were used in our alignment. Confidence at nodes was assessed with multiple support metrics using different phylogenetic programs under LG model of evolution (aBayes and SH-aLRT metrics with PhyML, RBS with RAxML, Bayesian Posterior Probability with Phylobayes (23,163 sampled trees, meandiff= 0.01, maxdiff= 0.5)). Colored dots in branches indicate corresponding branch supports. Thick branches indicate significant support by at least two metrics, one parametric and one non-parametric; branch support thresholds are shown in the center of the figure; see Methods.

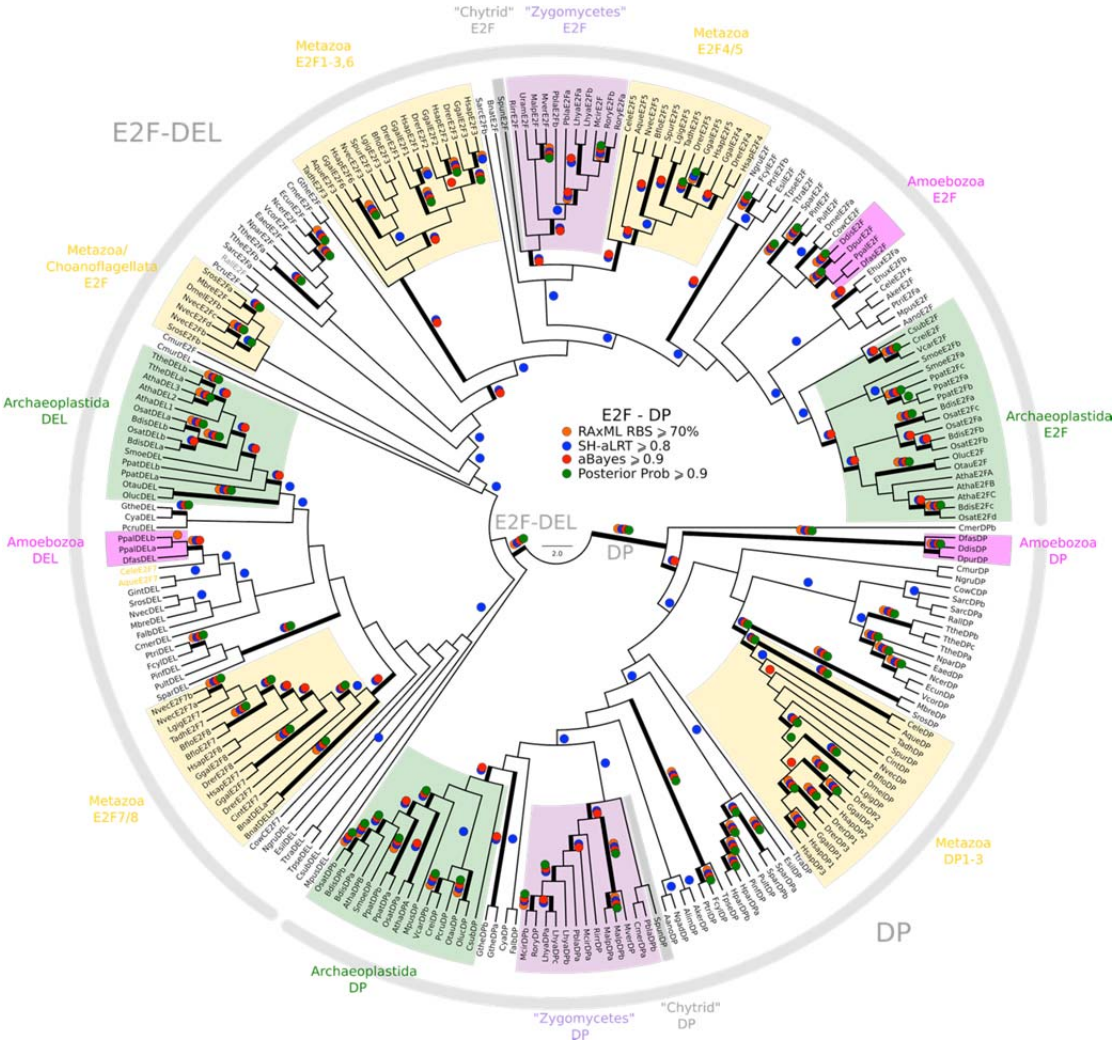


Figure 3 - supplement 2: Phylogeny of eukaryotic E2F/DP transcription factors. E2F-DP is a winged helix-turn-helix DNA-binding domain that is conserved across eukaryotes (van den Heuvel and Dyson, 2008). There are three sub-families within the E2F-DP family: (1) the E2F subfamily, (2) the E2F7-8/DEL subfamily, and (3) the DP subfamily. The E2F family consists of E2F1-6 (*H. sapiens*) and E2FA-C (*A. thaliana*). The E2F7-8/DEL family consists of E2F7-8 (*H. sapiens*) and DEL1-3 (*A. thaliana*). The DP family consists of DP1-3 (*H. sapiens*) and DPA-B (*A. thaliana*). The members of E2F form heterodimers with DP, whereas the DEL family has two DNA-binding domains and does not require DP to bind DNA. We used the E2F_TDP.hmm profile from PFAM to uncover members of the E2F/DP family across eukaryotes. A domain threshold of E-10 was used to identify potential E2F/DP homologs. Our E2F/DP dataset (pE2FDP.fasta) has 248 sequences. Columns with the top 8% Zorro score (284 positions) were used in our alignment. Confidence at nodes was assessed with multiple support metrics using different phylogenetic programs under LG model of evolution (aBayes and SH-aLRT metrics with PhyML, RBS with RAxML, Bayesian Posterior Probability with Phylobayes (53,009 sampled trees, meandiff=0.0064, maxdiff=0.18)). Colored dots in branches indicate corresponding branch supports. Thick branches indicate significant support by at least two metrics, one parametric and one non-parametric; branch support thresholds are shown in the center of the figure; see Methods.

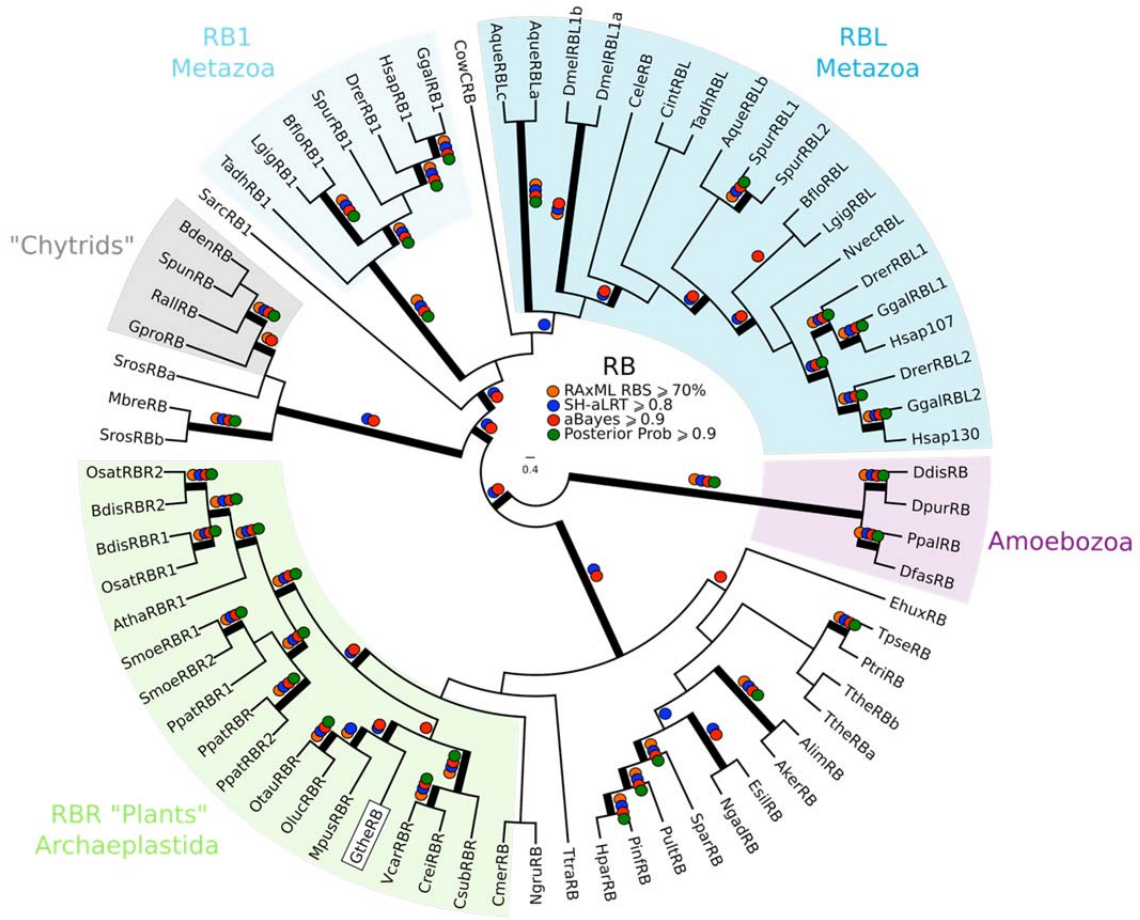


Figure 3 - supplement 3: Phylogeny of eukaryotic Rb inhibitors. *H. sapiens* has Rb1, RBL1 (p107), and RBL2 (p130), and *A. thaliana* has RBR1. The model fungi *S. cerevisiae* and *S. pombe* do not have any obvious retinoblastoma pocket proteins. We needed more eukaryotic sequences to create a robust HMM profile (pRb.hmm) for the pRb family. Based on the pRb sequences collected in (Hallmann, 2009), we built a profile-HMM using putative pRb homologs from *H. sapiens, G. gallus, C. intestinalis, D. melanogaster, C. elegans, N. vectensis, T. adhaerens* (metazoa)*; B. dendrobatidis* (fungi); *D. discoideum, D. purpureum, T. pseudonana, P. tricornutum, N. gruberi, E. huxleyi* (protists)*; C. merolae, O. tauri, O. lucimarinus, M. pusilla, V. carteri, C. reinhartdii, P. patens, S. moellendorfii, A. thaliana* (plants). A domain threshold of E-20 was used to identify pRB homologs. Our pRB dataset (pRb.fasta) has 72 sequences. Columns with the top 15% Zorro score (566 positions) were used in our alignment. Confidence at nodes was assessed with multiple support metrics using different phylogenetic programs under LG model of evolution (aBayes and SH-aLRT metrics with PhyML, RBS with RAxML, Bayesian Posterior Probability with Phylobayes (23,219 sampled trees, meandiff=0.0035, maxdiff=0.067)). Colored dots in branches indicate corresponding branch supports. Thick branches indicate significant support by at least two metrics, one parametric and one non-parametric; branch support thresholds are shown in the center of the figure; see Methods.

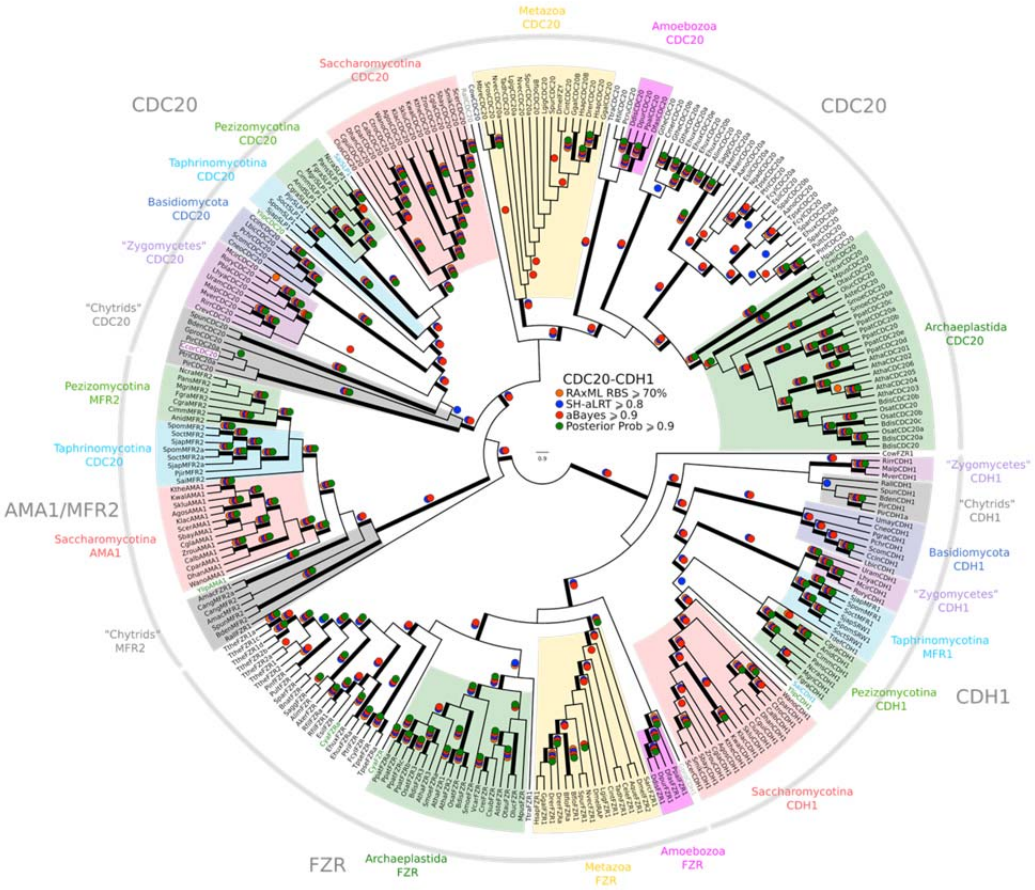


Figure 3 - supplement 4: Reduced phylogeny of eukaryotic Cdc20-family APC regulators. We combined CDC20 and CDH1/FZR1 sequences from *H. sapiens* (3 members), *A. thaliana* (9 members), and *S. cerevisiae* (3 members) to create a eukaryotic CDC20-family APC regulator profile-HMM (pCDC20.hmm) following the procedure outlined in the Methods. A domain threshold of E-50 was used to identify CDC20 homologs. Our pCDC20 dataset (pCDC20.fasta) has 350 sequences. This dataset was manually pruned to remove long-branches and problematic lineages. Our reduced CDC20 dataset has a total of 289 sequences. Columns with the top 20% Zorro score (608 positions) were used in our alignment. Confidence at nodes was assessed with multiple support metrics using different phylogenetic programs under LG model of evolution (aBayes and SH-aLRT metrics with PhyML, RBS with RAxML, Bayesian Posterior Probability with Phylobayes (13,638 sampled trees, meandiff=0.015, maxdiff=0.37)). Colored dots in branches indicate corresponding branch supports. Thick branches indicate significant support by at least two metrics, one parametric and one non-parametric; branch support thresholds are shown in the center of the figure; see Methods.

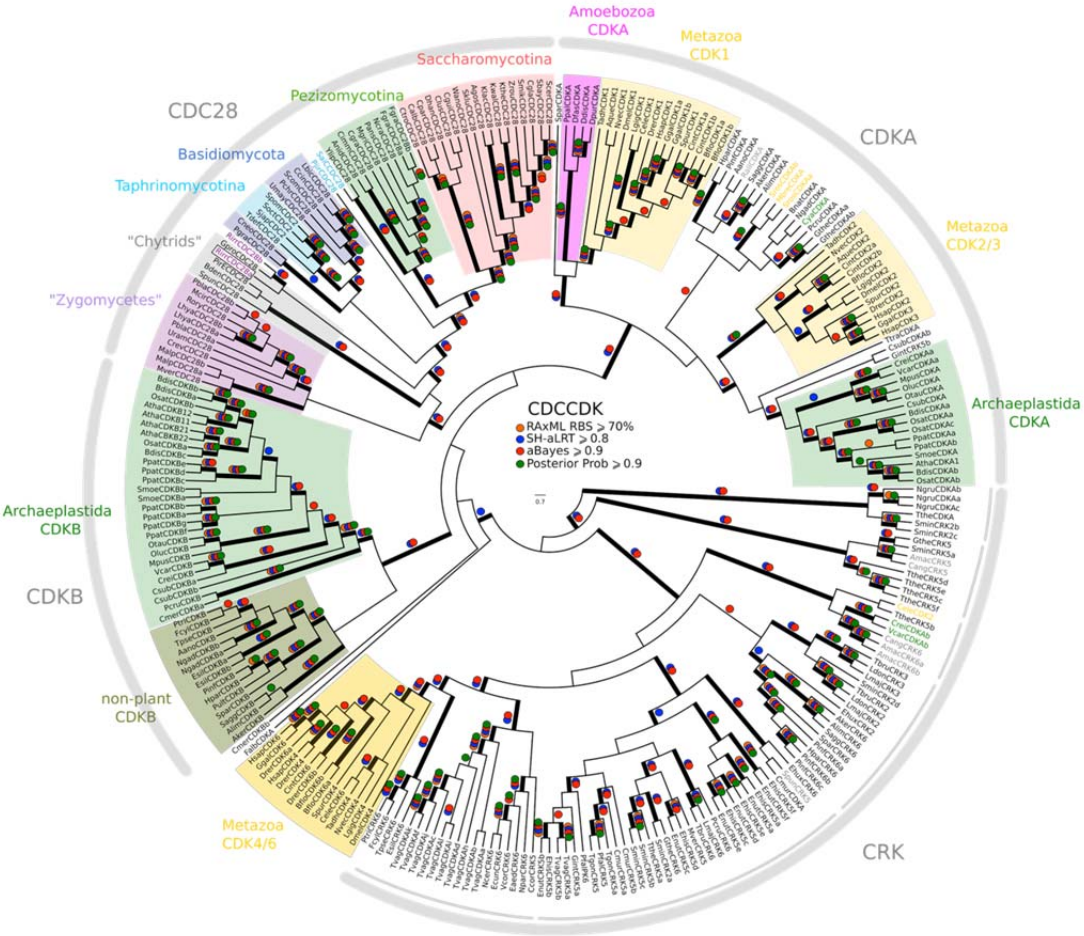


Figure 3 - supplement 5: Phylogeny of eukaryotic cyclin-dependent kinases. To create a profile-HMM (pCDCCDK.hmm) for eukaryotic cell cycle CDK, we combined Cdk1-3, Cdk4, Cdk6 sequences from *H. sapiens,* CdkA and CdkB from *A. thaliana,* Cdc28 from *S. cerevisiae,* and Cdc2 from *S. pombe*. A domain threshold of E-20 was used to identify CDK homologs. Our cell cycle CDK dataset (pCDCCDK.fasta) has 272 sequences. Columns with the top 15% Zorro score (473 positions) were used in our alignment. Confidence at nodes was assessed with multiple support metrics using different phylogenetic programs under LG model of evolution (aBayes and SH-aLRT metrics with PhyML, RBS with RAxML, Bayesian Posterior Probability with Phylobayes (28,193 sampled trees, meandiff=0.015, maxdiff=0.53)). Colored dots in branches indicate corresponding branch supports. Thick branches indicate significant support by at least two metrics, one parametric and one non-parametric; branch support thresholds are shown in the center of the figure; see Methods.

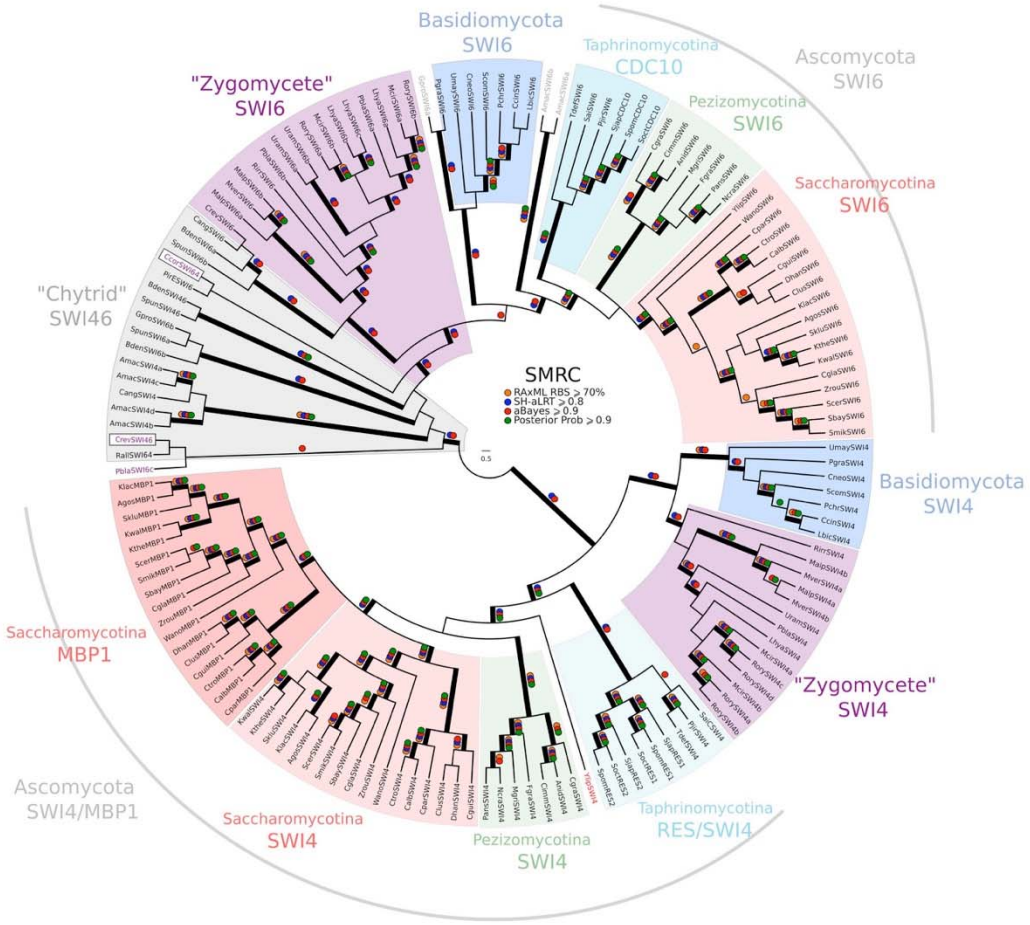


Figure 5 - supplement 1: Phylogeny of fungal SBF transcription factors. SBF and MBF are transcription factors that regulate G1/S transcription in budding and fission yeast. To detect SMRC (Swi4/6 Mbp1 Res1/2 Cdc10) across fungi, we built a sensitive profile-HMM (pSMRC.hmm) by combining well-characterized SMRC sequences from *S. cerevisiae, C. albicans, N. crassa, A. nidulans,* and *S. pombe*. A domain threshold of E-10 was used to identify SMRC homologs. Our SMRC dataset (pSMRC.fasta) has 147 sequences. Columns with the top 20% Zorro score (709 positions) were used in our alignment. Confidence at nodes was assessed with multiple support metrics using different phylogenetic programs under LG model of evolution (aBayes and SH-aLRT metrics with PhyML, RBS with RAxML, Bayesian Posterior Probability with Phylobayes (19,457 sampled trees, meandiff=0.0056, maxdiff=0.145)). Colored dots in branches indicate corresponding branch supports. Thick branches indicate significant support by at least two metrics, one parametric and one non-parametric; branch support thresholds are shown in the center of the figure; see Methods.

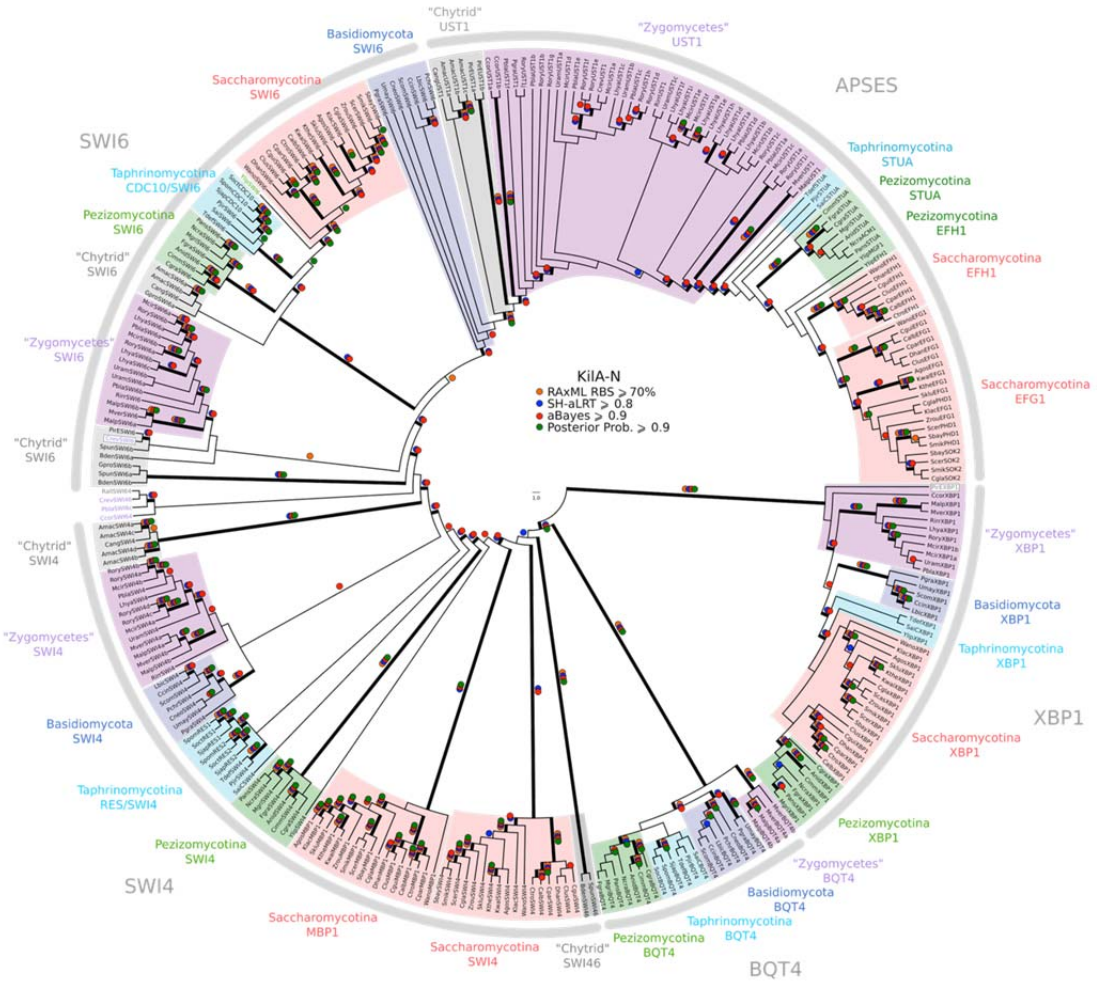


Figure 5 - supplement 2: Phylogeny of fungal SBF and APSES transcription factors. SBF and APSES transcription factors (Asm1, Phd1, Sok2, Efg1, StuA) share a common DNA-binding domain (KilA-N), which is derived from DNA viruses. During our search for SBF and APSES homologs, we consistently detected two additional fungal sub-families with homology to KilA-N: XBP1 (family name taken from *S. cerevisiae*) and BQT4 (family name taken from *S. pombe*). To detect APSES, XBP1, and BQT4 homologs, we built profile-HMMs (APSES.hmm, XBP1.hmm, and BQT4.hmm) by combining APSES, XBP1, and BQT4 homologs from *S. cerevisiae, C. albicans, N. crassa, A. nidulans,* and *S. pombe*. A domain threshold of E-10 was used to identify APSES, XBP1, and BQT4 homologs. Our final dataset (pKILA.fasta) contains all fungal KILA sub-families (SBF, APSES, XBP1, BQT4) and has a total of 301 sequences. Columns with the top 10% Zorro score (447 positions) were used in our alignment. Confidence at nodes was assessed with multiple support metrics using different phylogenetic programs under LG model of evolution (aBayes and SH-aLRT metrics with PhyML, RBS with RAxML, Bayesian Posterior Probability with Phylobayes (15,251 sampled trees, meandiff=0.012, maxdiff=0.25)). Colored dots in branches indicate corresponding branch supports. Thick branches indicate significant support by at least two metrics, one parametric and one non-parametric; branch support thresholds are shown in the center of the figure; see Methods.

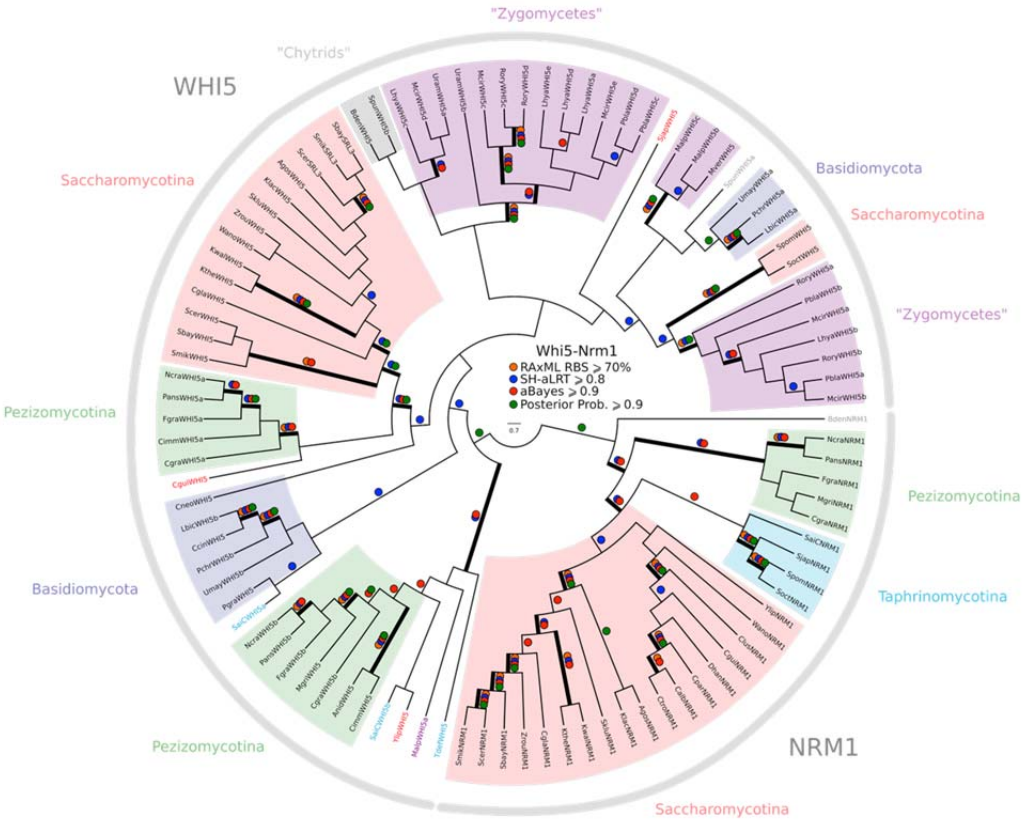


Figure 5 - supplement 3: Phylogeny of fungal Whi5 inhibitors. WHI5 and NRM1 are a yeast-specific protein family that has been identified and functionally characterized across *S. cerevisiae*, *C. albicans*, and *S. pombe.* Both WHI5 and NRM1 are fast evolving proteins. There is a small conserved region of 25 amino-acids (known as the GTB domain) that is responsible for interacting with Swi6/Cdc10 (Travesa et al., 2013). Unfortunately, the Whi5.hmm profile from PFAM is unable to detect an SRL3 paralogue in *S. cerevisiae* or the NRM1 orthologues in *A. gossypii* or *C. albicans.* We built a more sensitive profile-HMM (pWHI5.hmm) by combining WHI5/NRM1 sequences across ascomycetes (including SRL3 from Saccharomyces genomes and NRM1 from Candida genomes). A domain threshold of E-05 was used to identify WHI5 homologs. Our WHI5 dataset (pWHI5.fasta) has 98 sequences. Columns with the top 15% Zorro score (260 positions) were used in our alignment. Confidence at nodes was assessed with multiple support metrics using different phylogenetic programs under the LG model of evolution (aBayes and SH-aLRT metrics with PhyML, RBS with RAxML, Bayesian Posterior Probability with Phylobayes (77,696 sampled trees, meandiff=0.0068, maxdiff=0.11)). Colored dots in branches indicate corresponding branch supports. Thick branches indicate significant support by at least two metrics, one parametric and one non-parametric; branch support thresholds are shown in the center of the figure; see Methods.

1197 **Supplementary File 1A: List of eukaryotic genomes.** We downloaded and
1198 analyzed the following annotated genomes using the "best" filtered protein sets when
1199 available. We gratefully acknowledge the Broad Institute, the DOE Joint Genome
1200 Institute, Génolevures, PlantGDB, SaccharomycesGD, AshbyaGD, DictyBase, JCV
1201 Institute, Sanger Institute, TetrahymenaGD, PythiumGD, AmoebaDB,
1202 NannochloroposisGD, OrcAE, TriTryDB, GiardiaDB, TrichDB, CyanophoraDB, and
1203 CyanidioschizonDB for making their annotated genomes publicly available. We
1204 especially thank D. Armaleo, I. Grigoriev, T. Jeffries, J. Spatafora, S. Baker, J.
1205 Collier, and T. Mock for allowing us to use their unpublished data.
1206
1207 **Supplementary File 1B: Plasmids.**

1208 **Supplementary File 1C: Strains.** All yeast strains were derived from W303 and
1209 constructed using standard methods.
1210
1211 **Supplementary File 1D: Protein sequences of cell cycle regulators.** All protein
1212 sequences from different genome that were used to create molecular phylogenies
1213 can be downloaded as FASTA files.
1214