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Rearrangements of the transcriptional regulatory networks of metabolic pathways in fungi

Hugo Lavoie^{1,2}, Hervé Hogues¹ and Malcolm Whiteway^{1,2}

Growing evidence suggests that transcriptional regulatory networks in many organisms are highly flexible. Here, we discuss the evolution of transcriptional regulatory networks governing the metabolic machinery of sequenced ascomycetes. In particular, recent work has shown that transcriptional rewiring is common in regulons controlling processes such as production of ribosome components and metabolism of carbohydrates and lipids. We note that dramatic rearrangements of the transcriptional regulatory components of metabolic functions have occurred among ascomycetes species.

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Introduction

Much of the global coordination of cellular processes occurs at the point of transcriptional regulation of functionally related genes. This global control strategy is used by bacteria, which generally organize common functions into operons to put them under coordinated control [1], as well as by eukaryotes that typically make use of *trans*-acting transcription factors to coordinate regulons of functionally related genes not arranged in physically linked units.

Changing gene expression levels is now recognized as a major mechanism to generate biological diversity, and recent years have seen an explosion in the number of large-scale studies highlighting transcript abundance variations between closely related species [2[•],3,4]. However, the strategies employed by the cell to implement these changes are poorly understood and need to be studied by both experimental and bioinformatical approaches. The

fungi provide an excellent opportunity to investigate gene expression circuitries chosen by organisms living in a variety of niches: the genome sequences of a growing number of fungal species have been determined, starting with the ascomycetes models *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and the human fungal pathogen *Candida albicans*, and many fungi can be manipulated with advanced molecular tools [5–7]. Furthermore, sequencing efforts dedicated to species of the *Saccharomyces*, *Candida* and *Aspergillus* geni allows genome-wide comparisons and phylogenetic analyses of transcriptional circuits [8,9[•],10]. The techniques of transcriptional profiling [11] and of chromatin immunoprecipitation coupled with microarrays (ChIP-CHIP) [7,12] have provided a detailed picture of a variety of transcriptional circuits within fungal model organisms, and in these species, since sequence information can be coupled to experimental validations, we are in the position of directly comparing the regulatory circuits of central cellular processes.

The combination of experimental and bioinformatical strategies already allowed researchers to contrast regulatory circuits among species. Early evidence for changes in gene regulation among fungi came from transcriptional profiling studies comparing *S. cerevisiae*, *S. pombe* and *C. albicans* [13–15]. Furthermore, predictions of *cis*-regulatory sequences, and meta-analyses of gene co-expression revealed that transcription networks have a great deal of flexibility [4,16–18]. A well characterized example of comparative genomics applied to gene expression evolution is mating type identity that is controlled by distinct circuits with identical logic in *S. cerevisiae* and *C. albicans* [19[•]]. Also, ChIP-CHIP analysis of filamentous growth regulators in related *Saccharomyces* species [20^{••}], of the Mcm1 factor in three fungal species [21[•]] and of ribosomal regulators in *C. albicans* [22[•]] showed that transcriptional networks are extremely plastic.

The reorganization of transcriptional networks between species has been termed rewiring [23] and it is becoming evident that many regulons essential for cellular viability have been rewired in the fungal lineage. In this review we will place a primary focus on genome-scale analyses of transcriptional circuits regulating basal metabolic processes between the budding yeast and the fungal pathogen *C. albicans*. We will specifically emphasize the circuits controlling lipid metabolism, carbohydrate utilization, amino-acid starvation response and expression of the ribosomal regulon. These comparisons will allow us to investigate the way cells with different lifestyles

diversify the control of conserved processes by exploiting the flexibility of transcriptional networks.

Fatty-acid catabolism and phospholipid biosynthesis

The breakdown and synthesis of lipids is important in the metabolism, growth, development, and pathogenicity of many fungi. Catabolism of long-chain fatty-acids occurs via the beta-oxidation pathway in specialized organelles called peroxisomes [24] and, in the presence of an abundant source of fatty-acids, *S. cerevisiae* turns on a regulon leading to the expression of the beta-oxidation pathway and to the biogenesis of peroxisomes [25]. The regulatory proteins involved in this process comprise the transcription factors (TFs) Adr1, Cat8, Oaf1 and Pip2 [26]. In *Aspergillus nidulans*, the TFs FarA and FarB were recently identified as central regulators of lipid utilization genes and were shown to bind the CCGAGG sequence: a farA mutant has a reduced peroxisomal proliferation and perturbs the oleate gene expression response [27]. Interestingly, both the CCGAGG cis-regulatory element and the FarA protein are conserved in most ascomycetes, with the exception of *Ashbya gossypii*, *Kluyveromyces lactis*, *C. glabrata*, *S. cerevisiae* and other *Saccharomyces* species [27] (Figure 1a). The ortholog of FarA in *C. albicans*, Ctf1, is required for growth on oleate and different lipidic carbon sources while the TFs Cat8 and Adr1 are dispensable and Oaf1 and Pip2 are missing in *C. albicans* and related species [28,60]. The FarA/Ctf1 gene regulatory switch thus appears to have been lost in the budding yeast lineage and replaced by a distinct set of TFs.

In *S. cerevisiae*, inositol and choline (IC) production is induced by a transcriptional complex composed of the Ino2, Ino4 and Opi1 TFs. The Ino2/4 basic Helix–Loop–Helix (bHLH) heterodimer binds the IC response element (ICRE: CATGTG) upstream of structural genes of the phosphatidyl-inositol and phosphatidyl-choline synthesis pathways [29,30]. Opi1 is a repressor that monitors imbalances in the phosphatidic acid pool, and when inositol is limiting it directly interacts with phosphatidic acid and is tethered in the endoplasmic reticulum. This derepresses the Ino2/4 activatory complex that can then turn on the transcription of biosynthetic enzyme genes [30,31]. The Ino4 and Ino2 proteins as well as the structural genes of the inositol/choline (IC) regulon are highly conserved between *S. cerevisiae* and *C. albicans*. But in addition to the IC regulon, it was noticed that in *C. albicans* several genes involved in fatty-acid beta-oxidation and others encoding peroxisomal proteins contain an ICRE in their intergenic regions [32]. Analysis of the ICRE element across the fungal phylogeny shows that indeed, it is enriched in the peroxisomal and lipid utilization regulons in many species but the association with genes involved in fatty-acid breakdown is mostly absent in the *S. cerevisiae* branch (Figure 1a). The Ino2/4 hetero-

dimer was suspected to regulate ribosomal protein (RP) genes through an element resembling the ICRE but this hypothesis was invalidated in a recent study that identified Cbf1 as the TF-binding RP CACGTG elements [22^{*}]. It is thus likely that the Ino2/4 complex has conserved its function in the regulation of phosphatidyl-inositol and phosphatidyl-choline synthesis pathways, but in addition it appears to regulate targets involved in beta-oxidation and peroxisomal biogenesis in many fungal species.

Carbohydrate metabolism

The metabolism of sugars provides a central source of energy and essential metabolites for most organisms. *S. cerevisiae* has a highly specialized strategy of sugar metabolism that is of great interest to humans, as yeast cells efficiently ferment glucose even in the presence of oxygen, sacrificing short-term energy production for rapid exploitation of the carbon source and favoring the production of ethanol as a byproduct [33]. The transcriptional circuit controlling hexose metabolism has been extensively investigated in the brewer's yeast and the key activators of the glycolytic pathway that directs the fermentation of hexose sugars are Gcr1 and Gcr2, which associate with the CT box motif upstream of the glycolytic genes along with the Rap1 general transcription factor [34,35]. As well, *S. cerevisiae* and its close relatives exhibit the Crabtree effect that results from repressors such as Mig1 and Rgt1 inhibiting the non-fermentative use of glucose [26,36].

Another well-studied carbohydrate-use circuit of *S. cerevisiae* is that of the Leloir pathway genes involved in the metabolism of galactose. When fed with glucose, yeast cells repress the galactose utilization genes through the action of the transcriptional repressor Mig1 [37,38] but in the presence of galactose and once available glucose is exhausted, genes containing a galactose upstream activating sequence (UAS; CGG(N₁₁)CCG) are activated by Gal4; this is probably the most studied and applied gene regulatory switch of *S. cerevisiae* [38]. The allosteric mechanism of activation of the Gal pathway relies on the co-factor Gal80, which represses Gal4 activity until Gal3 bound to galactose sequesters it in the cytoplasm. Gal3 is an enzymatically inactive glucose sensor paralogous to the galactokinase Gal1, and Gal80 is a sensor of the NADP/NADPH balance [38–40].

Recent analysis of the transcriptional circuits controlling carbohydrate metabolism in *C. albicans* showed that the *S. cerevisiae* regulation has been significantly rewired relative to that of the fungal pathogen. The *C. albicans* Gal4 regulatory protein, which has only conserved its DNA-binding domain relative to its budding yeast's ortholog, is not involved in regulation of the Leloir pathway genes, but is connected to the glycolytic pathway genes [41^{*},61] (Figure 1b). On the other hand, the

Figure 1

Evolution of major metabolic regulatory circuits in the fungal lineage. The conservation of a regulatory motif in the ascomycetes lineage is depicted as a colored bar. The enrichment of TF-binding sites in each metabolic function in each species was tested with a hypergeometric distribution and a simplified representation of the data was created. **(a–d)** Heatmap display of various transcription factor-binding motifs in seven functional categories. Orthologous gene sets involved in phospholipid biosynthesis (Lipid), beta-oxidation and peroxisome (Perox), galactolysis (Galacto), glycolysis (Glyco), tRNA-aminoacyl-transfer (tRNA), amino-acid biosynthesis (AA) and nucleolar genes (Nucleolus) from 15 completely sequenced fungal genomes were tested for enrichment of the Ctf1, Ino4, Gal4, GalX, Gcn4 and PAC-Pbf1/2 motifs. Color saturation follows $\log_{10}$ enrichment p -values given by the hypergeometric distribution starting with faint color ($p = 10^{-2}$) to saturation ($p = 10E^{-6}$). **(e)** Transcription factors involved in the rewiring of the different biological functions reviewed here.

Figure 2

Evolution of transcription factor connections with metabolic sensors and intracellular cellular signaling. **(a)** Reorganization of the galactose-sensing co-regulators and their co-evolution with the Gal4 DNA-binding transcription factor. **(b)** The translational control of the *GCN4* mRNA is highly stringent in *S. cerevisiae* and depends on the eIF2 α kinase Gcn2. This post-transcriptional mechanism of Gcn4 regulation seems to have been lost in *C. albicans*. **(c)** The ribosomal transcriptional regulatory complex is connected to TOR and PKA signaling through the Fhl1-Ifh1 heterodimer in both *S. cerevisiae* and *C. albicans* even though the central DNA-binding regulators have been exchanged between species.

Leloir pathway genes in *C. albicans* have another candidate regulatory motif (TGTAACGTT) associated to a yet elusive TF (GalX) and specifically conserved in the *C. albicans* branch [41[•]] (Figure 1b); reporter gene assays suggested that this motif could be a target of the Cph1 transcription factor but ChIP-CHIP analysis did not provide any support for this hypothesis [20]. In addition to the Gal4 protein linked to the glycolytic circuit, recent evidence suggests that the *C. albicans* ortholog of the bHLH transcription factor Tye7 is a key regulator of the transcriptional control of carbohydrate metabolism in the pathogen. ChIP-CHIP analysis shows that both Tye7 and Gal4 bind upstream the glycolytic pathway genes, and loss of these two factors prevents *C. albicans* growth under anaerobic conditions and when the cells are forced to grow fermentatively due to decoupling of oxidative phosphorylation [61]. Because Tye7 is a minor element in the regulation of glycolytic genes in *S. cerevisiae* and Gal4 is limited to the regulation of the Leloir pathway genes required for galactose catabolism, the yeast and *C. albicans* regulatory circuits have significantly diverged. Concomitant with the importance of Gal4 and Tye7 in *C. albicans* glycolytic regulation is the absence of the Gcr1 and Gcr2 regulators in most branches of the ascomycetes phylogeny except in *S. cerevisiae* and relatives [28] (Figure 1b).

Given the reutilization and swapping of DNA-binding TFs involved in glycolysis and galactolysis (Figure 1b), the allosteric mechanism responsible for galactose sensing must also have evolved between *S. cerevisiae* and *C. albicans* [39^{••},42^{••}]. These findings raise the question of how the *C. albicans* galactose-sensing transcriptional regulatory switch is controlled since orthologs of Gal80 and Gal3 are absent in *C. albicans*, and a recent study has shown that galactose sensing in *C. albicans* relies on the glucose sensor Hgt4 [42^{••}] (Figure 2a). How then did the former glycolytic regulator Gal4 gain its connection to galactose-sensing proteins following the glycolysis to galactolysis rewiring event? It has been shown that in the *S. cerevisiae* lineage, following the whole-genome duplication, the galactokinase (Gal1) paralogue Gal3 was acquired and specialized in the transcriptional regulation of Gal genes, losing its enzymatic function [39^{••}] (Figure 2a). Altogether, these lines of evidence suggest that the galactose-sensing protein Gal3 co-evolved with the TF Gal4 and its co-repressor Gal80 to specifically control galactose metabolism in the budding yeast lineage while Gal4 in *C. albicans* and many ascomycetes regulates glycolysis.

Amino-acid biosynthesis

Starvation for amino acids stimulates the expression of genes of the amino-acid (AA) biosynthetic pathways by

the general control non-derepressible (GCN) response (reviewed in [43]). This response depends on the bZIP transcription factor Gcn4. In conditions of AA availability the *GCN4* mRNA is transcribed, but its translation is repressed by four small upstream ORFs (uORFs) in its 5'-UTR, and the Gcn4 protein is rapidly degraded. *S. cerevisiae* Gcn4 expression is mainly regulated at the translational level and when AAs become limiting, general translation is repressed but the *GCN4* mRNA is selectively translated in a eIF2 α kinase (Gcn2)-dependent manner. Once translated, Gcn4 binds its element (TGASTCA) upstream of AA biosynthetic genes and activates their transcription.

This *S. cerevisiae* AA starvation response governed by Gcn4 appears globally conserved throughout the ascomycetes phylogeny [44[•],45]. However, transcriptional profiling of the amino-acid starvation response in *Neurospora crassa* suggested that in these cells AA biosynthesis genes and tRNA-aminoacyl-transferases are highly co-regulated [44[•]] and a meta-analysis of expression co-regulation between *S. cerevisiae* and *C. albicans* identified distinctions in the mode of tRNA-aminoacyl-transferases co-regulation between the two species [4]. Consistently, phylogenetic analyses of the Gcn4 element (TGASTCA) enrichment showed that it is conserved in the amino-acid biosynthesis regulon in most ascomycetes and that it is found upstream of genes encoding tRNA-aminoacyl-transferases in many species but excluded from the *S. cerevisiae* branch (Figure 1c) [16,44] (Figure 1).

The regulation of the Gcn4 transcriptional switch in response to AA limitation appears to also have changed in the ascomycete phylogeny since the translational regulation of the *GCN4* mRNA does not occur in *C. albicans* [45] (Figure 2b). In fact, the *C. albicans* ortholog of the eIF2 α kinase Gcn2 has limited involvement in the AA starvation response since its inactivation only partially attenuates growth under starvation conditions [46] (Figure 2b). This suggests that Gcn4 is a conserved amino-acid biosynthesis regulatory protein in ascomycetes, that it coordinates the AA and tRNA-aminoacyl-transferase regulons in a subset of ascomycetes species including *C. albicans* and *N. crassa* and that direct post-transcriptional regulation of *GCN4* mRNA translation by the Gcn2 kinase is lost or less stringent in some fungal species like *C. albicans*.

Ribosomal proteins and ribosome biogenesis

A further central metabolic process that has been significantly rewired between *S. cerevisiae* and *C. albicans* is that directing the production of ribosomal proteins. While the utilization of metabolites such as sugars and lipids could be easily seen to be subject to differential regulatory pressures based on the metabolic niche of the organism, the expression of ribosomal protein genes is a highly conserved and universal process, and thus it is remarkable

that the regulatory circuits controlling this process have been fundamentally rewired between *S. cerevisiae* and *C. albicans*. In *S. cerevisiae* the regulatory circuit controlling the expression of ribosomal proteins consists of the generalist myb-domain containing transcription factor Rap1p together with the high-mobility group protein Hmo1p and a condition-sensitive switch complex made up of Ifh1 and Fhl1 [47–51]. In *C. albicans*, the Ifh1/Fhl1 switch appears to be conserved, but the key DNA-binding element of the circuit is the myb-domain protein Tbf1 ([22[•]] Lavoie *et al.*, submitted) (Figure 2c). As well, while the Ifh1/Fhl1 complex has a DNA-binding target (the IFHL box) in *S. cerevisiae*, there is no evidence for this motif in *C. albicans*, suggesting that all the DNA targeting function of the circuit is supplied by the Tbf1 *cis*-regulatory motifs.

It is intriguing that all these regulatory players are found in both species; but their functions have in many cases been rearranged. Hmo1, which has a strong association with ribosomal protein gene promoters in *S. cerevisiae* is not linked to any particular class of promoters in *C. albicans*; Tbf1, which is highly associated with promoters of ribosomal protein genes in *C. albicans*, is bound to subtelomeric sites and an important number of targets of unrelated functions in *S. cerevisiae* (Lavoie *et al.*, submitted). Cbf1 maintains a connection with the promoters of sulfur metabolic genes in both species, but is connected to ribosomal protein gene expression in *C. albicans* and centromere binding and chromosome segregation specifically in *S. cerevisiae*. Thus the overall impression is that while the cells are reluctant to discard a DNA-binding protein, they can switch the TF function from that of a specialist coordinating the regulation of a unique cellular process, to that of a generalist where the binding sites are not linked to any obvious class of protein-coding genes. The presence of Mcm1 binding sites upstream of most RP genes in close proximity with Rap1 elements uniquely in *Kluyveromyces lactis* suggests that many such recruitments of TFs have occurred within the fungal RP regulon [21].

A recent study of the nucleolar regulon involved in the processing and maturation of rRNA species and in the assembly of ribosomal particles has shown that the regulatory elements responsible for the transcription of nucleolar genes have been constantly reorganized over the long evolutionary road separating ascomycetes and metazoans [52^{••}]. These authors suggested that hemiascomycetes evolved a unique regulatory signature made of the polymerase $\underline{A}$ and $\underline{C}$ element (PAC) and RRPE elements, compared to metazoans that possess a distinct set of *cis*-regulatory elements. The TFs binding the PAC were recently identified in two independent studies [53[•],54[•]] and the phylogenetic conservation of these two PAC-binding TFs Pbf1 and Pbf2 follows the conservation of the PAC element exclusively in the hemiascomycetes

Figure 3

Reutilization of regulatory switches in *C. albicans* and *S. cerevisiae*. Possible scenarios of evolutionary tinkering with transcriptional regulatory switches in ascomycetes connecting related biological processes situated within the lipid (green), hexose (cyan), amino acid (red) and protein synthesis (orange) 'regulatory fields'.

lineage [28,52^{••}] (Figure 1d). Thus, remarkably, the ribosomal transcriptional regulatory machinery dictating the pace of ribosomal protein and nucleolar components expression might be one of the most plastic components of the fungal regulatory network.

Conclusions

Transcriptional regulatory networks have been subjected to numerous evolutionary changes ranging from total rewiring and replacement to subtler couplings of biological functions by reutilization of conserved transcriptional regulatory switches. It is thus becoming apparent that the level of plasticity of these networks during evolution is very high. In addition to these spectacular rearrangements of metabolic regulators, other studies provide support for massive rearrangements of the proteasome regulatory network and subtler rewirings of cell cycle regulatory circuits [16,55]. Surprisingly, large-scale modifications in transcriptional regulatory network connections occur in the control of pathways and complexes essential for cellular growth like the ribosomal and glycolytic regulons, while other apparently less constrained pathways are conserved [55]. This paradoxical observation is potentially

the result of constant and strong selective pressure on these central constituents of the cell metabolic machinery and on their exploration of various regulatory possibilities. These changes in transcription factor-regulon interactions involved massive reorganization of *cis*-regulatory elements as well as exquisite modifications in the assembly of regulatory complexes. In fact, the harnessing of DNA-binding molecules to new promoters or chromosome structural elements have been coupled with the formation of distinct combinatorial assemblies of transcription factors in *C. albicans* versus *S. cerevisiae* at the glycolytic (Gal4-Tye7 versus Rap1-Gcr1/2), galactolytic (GalX versus Gal4-Gal80-Gal3) and ribosomal (Tbf1-Cbf1-Fhl1-Ifh1 versus Rap1-Hmo1-Fhl1-Ifh1) regulons and at centromeres (? versus Cbf1-CBF3) ([21], Lavoie *et al.*, submitted) (Figure 2). As well, the integration of rewired transcription factors within cellular signaling networks and metabolites sensing pathways remains to be studied.

It is also interesting that DNA-binding proteins can be switched from having a function in the regulation of gene expression to being connected to structural elements of chromosomes such as centromeres and telomeres as seen

in the case of Cbf1, Rap1 and Tbf1. While these transitions in function are often associated with changes in the exact binding specificity of the regulatory proteins, the major driving force behind these regulatory rearrangements is the reassociation of DNA elements with a coherent group of central metabolic machines or chromosome structural elements.

The way cells reutilize transcription factors may seem chaotic at first sight but in an evolutionary perspective, it makes sense that an adapted nutrient-sensitive switch controlled by a given environmental condition would be reused in all situations where condition-dependent transcriptional control is advantageous. For example, the evolution of the regulons of Gcn4, Ino4, Gal4 and of the rapid growth element (RGE) (connecting the MRP and RP regulons in *C. albicans* but not *S. cerevisiae*) [18] suggests that conserved regulatory switches are used to mediate evolvable connections between related biological processes (Figure 3). Gal4 has swapped between two hexose utilization regulons (galactolysis and glycolysis), Ino4 connects two lipid-related processes (the Inositol/choline regulon and the peroxisomal regulon), Gcn4 connects amino-acid-related regulons (AA biosynthesis and tRNA aminoacylation) and the RGE controls the cytosolic and the mitochondrial ribosomes (Figure 3). Therefore, many transcriptional regulators appear to rewire within functionally related 'fields' of the cell's metabolism (Figure 2). These observations are well in line with the idea of François Jacob that evolution uses the tools at its disposal to promote adaptations rather than engineering new components from scratch [56,57].

Altogether, the comparison of the transcriptional regulatory networks of ascomycetes suggests that both *cis*- and *trans*-regulatory elements involved in the control of metabolic pathways have dramatically changed between species. The regulators of the most central components of a eukaryotic cell's metabolism like the ribosome and the glycolytic pathway have been reorganized and this process of transcriptional rewiring of metabolic pathways probably generates a great diversity of biological responses and evolutionary novelties. Fungi occupy a great diversity of ecological niches and have therefore developed a variety of physiologies. Of particular interest for human health is the metabolic regulation in fungal pathogens like *C. albicans* or *A. nidulans* that are adapted to live as commensals or to colonize and grow efficiently within their host tissues during infection [58,59]. Similarly, species closely related to *S. cerevisiae* have distinct physiologies and a picture that arises is that very often, this branch of ascomycetes stands as one with unique adaptations in terms of the regulatory circuitry of metabolic pathways. It is thus very likely that adaptations to establish the host-pathogen interaction as well as other important physiological parameters found in ascomycetes have involved the reorganization of regulatory circuits controlling metabolic machineries. But a complete

picture of the span, nature and pace of transcriptional rearrangements among species and a comprehension of these modifications in the context of organism's adaptation to their environments will require that experimental strategies be applied on a genome scale in model systems representing the different branches of the ascomycetes phylogeny.

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