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Intercalation of a new tier of transcription regulation into an ancient circuit

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Abstract

Changes in gene regulatory networks are a major source of evolutionary novelty^{1–3}. Here we describe a specific type of network rewiring event, one that intercalates a new level of transcriptional control into an ancient circuit. We deduce that, over evolutionary time, the direct ancestral connections between a regulator and its target genes were broken and replaced by indirect connections, preserving the overall logic of the ancestral circuit but producing a new behaviour. The example was uncovered through a series of experiments in three ascomycete yeasts: the bakers' yeast *Saccharomyces cerevisiae*, the dairy yeast *Kluyveromyces lactis* and the human pathogen *Candida albicans*. All three species have three cell types: two mating-competent cell forms (*a* and α) and the product of their mating (*a/α*), which is mating-incompetent. In the ancestral mating circuit, two homeodomain proteins, *Mata1* and *Mata2*, form a heterodimer that directly represses four genes that are expressed only in *a* and α cells and are required for mating^{4–6}. In a relatively recent ancestor of *K. lactis*, a reorganization occurred. The *Mata1*–*Mata2* heterodimer represses the same four genes (known as the core haploid-specific genes) but now does so indirectly through an intermediate regulatory protein, *Rme1*. The overall logic of the ancestral circuit is preserved (haploid-specific genes ON in *a* and α cells and OFF in *a/α* cells), but a new phenotype was produced by the rewiring: unlike *S. cerevisiae* and *C. albicans*, *K. lactis* integrates nutritional signals, by means of *Rme1*, into the decision of whether or not to mate.

In *S. cerevisiae*, *K. lactis* and *C. albicans*, three cell types (*a*, α and *a/α*) are specified by transcriptional regulators (sequence-specific DNA-binding proteins) encoded at the mating type locus. An important part of this cell-type-specific circuit is the regulation of the haploid-specific genes (hsgs), a group of genes that are expressed in *a* and α cells but not in *a/α* cells⁷. The full sets of hsgs were previously identified in *S. cerevisiae*⁴ and *C. albicans* (ref. ⁵, and B.B.T., Q. M. Mitrovich, F. M. De La Vega, C. K. Monighetti and A.D.J., unpublished observations) but not in the related species *K. lactis*. To examine the evolution of this portion of the mating circuit, we identified the genes in the *K. lactis* hsg regulon and compared them to those in *S. cerevisiae* and *C. albicans*. By profiling the expression patterns of wild-type *a*, α and *a/α* *K. lactis* cells genome-wide, we identified 12 genes that

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are clear hsgs under the conditions tested (Fig. 1a), two of which—*RME1* (referred to previously as *MTS1*) and *STE4*—were previously identified as hsgs in *K. lactis*⁸. Comparison of all the hsgs in the three species revealed a substantial level of turnover in the regulon; in other words, an hsg in one species is not necessarily an hsg in the other two (Fig. 1b). However, an ancestral core of four hsgs (*GPA1*, *STE4*, *STE18* and *FAR1*) share a common expression pattern in all three species. The first three genes encode the heterotrimeric G protein that, in the presence of mating pheromone, activates a downstream mitogen-activated protein kinase (MAPK) cascade required for mating^{9–11}. Far1 lies further downstream in this pathway and mediates two responses needed as a prelude to mating, cell cycle arrest¹² and the formation of mating projections^{13,14}.

In *S. cerevisiae* and *C. albicans* *a/a* cells, all four genes of the hsg core regulon are directly repressed by the transcription regulator *a1*–*a2* (refs ^{4, 6}), a heterodimer encoded by one gene at the *MATa* locus and one gene at the *MATα* locus. To determine whether this was also true in *K. lactis*, genome-wide chromatin immunoprecipitations (ChIP-chip) of *a1* and *a2* were performed in *K. lactis* *a/a* cells. In total, the upstream regions of 14 genes were observed to be occupied by both *a1* and *a2*, including the *RME1* gene (Fig. 1c), which is also *a1*–*a2* regulated in *S. cerevisiae*. *a1* and *a2* ChIP peaks were not observed at the promoters of any of the four core hsgs in *K. lactis*, indicating that, unlike in *S. cerevisiae* and *C. albicans*, these genes are not directly regulated by *a1*–*a2*.

To confirm the absence of direct *a1*–*a2* regulation at *K. lactis* hsgs, we identified the *a1*–*a2* recognition motif in *K. lactis* from the ChIP data, using a *de novo* motif-finding program¹⁵. The highest-scoring motif was similar to the *a1*–*a2* motifs previously identified in *S. cerevisiae*¹⁶ and *C. albicans*¹⁷ (Fig. 1d). Indeed, the *S. cerevisiae* motif is efficiently recognized by the *C. albicans* *a1*–*a2* protein¹⁷, confirming that key features of this sequence have remained largely unchanged in the three species. We searched the regions 2 kilobases upstream of each *K. lactis* core hsg for the *K. lactis* *a1*–*a2* motif but did not find significant matches, confirming the absence of direct *a1*–*a2* regulation of these genes. (Whereas the *a1*–*a2* site upstream of *RME1* had a log₁₀-odds score of 4.98, the best matches at the core hsgs ranged from –0.70 to 0.93.) These results indicate that although the ancestral core hsg expression pattern is conserved in *K. lactis*, the mechanism of the regulation has changed.

To understand how the *K. lactis* hsgs are cell-type regulated we searched the upstream regions of the 12 genes identified as hsgs by expression array (Fig. 1a) for *cis*-regulatory motifs¹⁵. The second-highest ranking motif (the top-ranking motif was a repeat sequence) was found in 11 out of 12 of the promoters (Fig. 2a) and was similar to the *S. cerevisiae* Rme1 motif (*K. lactis* consensus, GAACCNMAA; *S. cerevisiae* consensus, GAACCTCAA^{18,19}). This motif is also similar to, although longer than, the *K. lactis* Rme1 motif derived previously²⁰. The Rme1 motif is absent from *S. cerevisiae* and *C. albicans* hsg promoters.

In *S. cerevisiae*, Rme1 was initially identified as a repressor of meiosis and sporulation^{21,22}, and was later shown to act as a transcriptional activator of other genes¹⁹. In *K. lactis*, Rme1 was shown to regulate mating-type interconversion²⁰ (the switching of *a* and *α* cells to the opposite cell-type by means of DNA rearrangement). We speculated that Rme1 was co-opted in the *K. lactis* lineage to positively regulate the core hsgs.

To test this hypothesis, we knocked out the *RME1* gene in *K. lactis* *a* cells and examined the gene expression profile by microarray. We found that 20 genes were downregulated in the knockout strain (Fig. 2b), including all four of the core hsgs ($P = 2 \times 10^{-10}$, hypergeometric distribution). We also observed a set of genes that was upregulated in the absence of Rme1 (Supplementary Fig. 1), including a significant number of genes orthologous to *S. cerevisiae*

sporulation genes (Gene Ontology (GO): 0043934, $n = 14$, $P = 10^{-14}$, hypergeometric distribution) indicating that the function of Rme1 in regulating meiosis is shared with *S. cerevisiae*. Thus, whereas some of the targets of Rme1 have remained the same as in the common ancestor of *S. cerevisiae* and *K. lactis*, Rme1 has gained new targets in the *K. lactis* lineage, including the core hsgs.

To test whether Rme1 directly regulates the core hsgs in *K. lactis*, we performed a genome-wide ChIP of Rme1 in **a** cells. ChIP peaks were observed at the promoters of the four core hsgs (Fig. 2c) and were centred over the Rme1 motifs (Fig. 2c). Thus, in the *K. lactis* lineage, Rme1 was gained as a direct activator of the core hsgs by the acquisition of Rme1 *cis*-regulatory sequences at all four genes. We note that Rme1 is not the only regulator of the *K. lactis* hsgs; for example, *STE18* is repressed by Sir2 (ref. ²³).

We next tested the biological role of Rme1 in mating in *K. lactis*, *S. cerevisiae* and *C. albicans* by comparing wild-type and *RME1* knockout **a** cells. In response to α pheromone, **a** cells form mating projections (polarized growth towards the source of pheromone). When *S. cerevisiae* and *C. albicans* wild-type and *Arme1* **a** cells were exposed to α mating pheromone, both strains formed mating projections normally (Fig. 2d). In contrast, whereas *K. lactis* wild-type **a** cells produced mating projections in response to pheromone, *Arme1* **a** cells did not, indicating that this biological response was dependent on Rme1 (Fig. 2d). As a second test of the role of Rme1, we examined mating directly using a quantitative mating assay. No difference was observed between the mating efficiencies of wild-type **a** cells and those of *Arme1* **a** cells for *S. cerevisiae* and *C. albicans* (Fig. 2e). In contrast, the *K. lactis* *Arme1* **a** cell mating efficiency was decreased, relative to the wild type, by a factor of at least 10^6 (Fig. 2e). Thus, the ability to mate is critically dependent on Rme1—but only in *K. lactis*.

Unlike *S. cerevisiae* and *C. albicans*, *K. lactis* requires a starvation signal to mate²⁴ and to respond to pheromone²⁵. Although several different types of starvation signal can prime *K. lactis* to respond to pheromone^{24,25}, we found that phosphate starvation is particularly potent, and it was used in subsequent experiments. Our expression profiling experiments (Fig. 1a) revealed that *K. lactis* requires starvation to express most of its mating genes. *RME1* was also highly induced (24-fold) by phosphate starvation (Fig. 1a). We note that *S. cerevisiae* *RME1* transcript levels also increase tenfold under starvation conditions¹⁸, suggesting that regulation of *RME1* by starvation may be ancestral to *S. cerevisiae* and *K. lactis*.

We next investigated in greater detail how the starvation signal is incorporated in the *K. lactis* mating regulatory circuit. The simplest model consistent with the data presented so far is that starvation upregulates *RME1*, which in turn activates transcription of the hsgs. A prediction of this model is that ectopic expression of *RME1* in *K. lactis* should override the requirement for starvation in expressing the hsgs. We created an **a** strain overexpressing *RME1* to levels that were within tenfold of the level in starved wild-type cells (using the *KI* LAC4 promoter) and found that overexpression of *RME1* (in rich YEP-galactose medium) was sufficient to induce expression of the heterotrimeric G protein subunits (Fig. 3a). Overexpression of *RME1* is also sufficient to allow *K. lactis* to form mating projections in response to pheromone in rich medium (Fig. 3b). These results strongly support the model by showing that upregulation of *RME1* is sufficient to cause biologically relevant upregulation of the heterotrimeric G proteins.

Thus, the rewiring of the *K. lactis* hsg circuit (summarized in Fig. 4) resulted in a new network configuration and a novel phenotype, relative to the ancestor. Our results suggest a possible evolutionary path for this rewiring. In the ancestor of all three yeasts, the hsgs were

directly repressed by $\alpha 1$ – $\alpha 2$. Either in an ancestor to *S. cerevisiae* and *K. lactis* or independently in each lineage, *RME1* was brought under nutritional regulation. Finally, in the *K. lactis* lineage alone, two steps occurred: the hsgs lost the *cis*-regulatory sequences for $\alpha 1$ – $\alpha 2$ and gained the *cis*-regulatory sequences for Rme1. As described in Supplementary Fig. 2, it is possible to determine more precisely when the rewiring of the core hsgs occurred. We can infer that direct $\alpha 1$ – $\alpha 2$ regulation of the core hsgs was probably lost several times in the ascomycete lineage, and that the *K. lactis* form of regulation of the hsgs probably arose after *K. lactis* and the closely related species *L. kluyveri* branched from their common ancestor.

Although we do not know whether acquisition of the *K. lactis* form of regulation was adaptive, this type of regulation makes logical sense given that the primary mode of growth of *K. lactis* is as a haploid²⁶. The formation of spores is a strategy employed by many yeasts to survive harsh environments. For starvation to give rise to spores, *K. lactis* would first have to mate (to form the sporulation-competent α/α cell type), thus rationalizing the link between starvation and mating. In contrast, *S. cerevisiae* in the wild is typically at least diploid²⁷ and forms spores directly in response to starvation. Thus, the coupling of mating and starvation makes conceptual sense for *K. lactis* in comparison with *S. cerevisiae*.

We have described a case in which a new tier of regulation has been intercalated into an ancient transcription circuit consisting of a regulator (a homeodomain heterodimer) and a set of target genes. This change involved breaking the original connections between the regulator and its target genes and replacing them with a more complex type of hierarchy (Fig. 4). Intercalation may be a common way in which regulatory circuits evolve. This type of ‘intercalary evolution’ was first proposed²⁸ to account for a common origin of eyes. In a wide variety of species, the transcription regulator Pax6 lies at the top of the eye development hierarchy, and rhodopsins occupy the bottom. According to the proposal, different types of eye arose from evolutionary intercalation of a variety of regulatory and structural genes within this simple, deeply conserved, regulatory relationship. The change we describe here is less complex and provides a concrete example of evolutionary intercalation, one that is responsible for an important feature of modern mating behaviour in *K. lactis*. It has been known for decades that *K. lactis* (unlike its relatives *S. cerevisiae* and *C. albicans*) requires starvation to mate²⁴, and we have shown that this behaviour is due to the new configuration of the *K. lactis* mating circuit.

METHODS SUMMARY

Gene expression array

RNA was isolated by hot phenol extraction and reverse transcribed, and the resulting complementary DNAs were coupled to Cy5. A pooled mixture of the cDNAs was coupled to Cy3 and used as a reference. Labelled cDNAs were hybridized to Agilent arrays for visualization.

ChIP-chip

ChIP experiments were performed as described previously²⁹, with minor modifications.

Pheromone response assays

Exponential-phase cultures were exposed to 13-mer α -mating pheromone, and the formation of mating projections was monitored by microscopy.

Quantitative mating assay

a and **α** cultures were grown independently to exponential phase and then mixed together with **α** cells in fivefold excess under mating conditions. The mating products were selected for on medium that either **a** and **a/α** cells or only **a/α** cells could grow on, and efficiencies were calculated as $\text{efficiency} = (\mathbf{a}/\alpha \text{ colonies})/(\mathbf{a} \text{ and } \mathbf{a}/\alpha \text{ colonies})$.

RT–quantitative PCR

Cultures were grown to exponential phase in YEP-galactose medium, and RNA was isolated by extraction with hot phenol. RNA was reverse transcribed and the cDNAs were quantified by quantitative PCR.

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Figure 1. The core hsgs are not directly regulated by $a1-a2$ in *K. lactis*

a, The expression profiles of the set of 12 hsgs identified in *K. lactis*. Note that phosphate starvation induces expression of the hsgs and is required to identify these genes. For example, when starved for phosphate, the heterotrimeric G protein genes are expressed in a and α cells at levels about fivefold higher than in a/α cells. **b**, A comparison of hsgs defined by transcriptional profiling in *S. cerevisiae* (Sc)⁴, *K. lactis* (Kl) (panel **a**) and *C. albicans* (Ca) (ref. ⁵, and B.B.T., Q. M. Mitrovich, F. M. De La Vega, C. K. Monighetti and A.D.J., unpublished observations) shows a conserved subset of hsgs (*GPA1*, *STE4*, *STE18*, *FAR1*), which we refer to as the core hsgs (bold in **a** and **b**). **c**, ChIP enrichment profiles from experiments using haemagglutinin (HA)-tagged *MATa1* a/α cells (magenta), HA-tagged *MATa2* a/α cells (blue) and, as a control, untagged a/α cells (green). The ChIP enrichment was determined by hybridization to a tiling microarray. The location of the $a1-a2$ motif in the *RME1* promoter is indicated by the orange star. The genes (tan boxes) are all transcribed in the reverse direction. Data were visualized with MochiView³⁰. **d**, The *K. lactis* $a1-a2$ motif determined from the ChIP-chip data. For comparison, the *S. cerevisiae* and *C. albicans* motifs (derived from published ChIP data^{4,6}) are also shown.

Figure 2. RME1 is a direct activator of hsg expression and is required for *K. lactis* mating
a, The *K. lactis* Rme1 motif found by a *de novo* search¹⁵ of the 12 *Kl* hsgs and the *S. cerevisiae* motif derived from two experimentally characterized binding sites^{18,19}. **b**, The set of 19 genes repressed twofold or greater relative to wild type when *RME1* is absent and the cells are phosphate-starved. In bold are the core hsgs. **c**, Rme1 is a direct regulator of the core hsgs. ChIP of Rme1 was performed in *K. lactis* c-Myc-tagged *RME1* **a** cells (blue and green lines, two biological replicates) and untagged, control **a** cells (orange line). The immunoprecipitated DNA was hybridized to a tiling microarray. The genes (tan boxes) above the line are transcribed in the forward direction and those below are transcribed in the reverse direction. The location of the *Kl* Rme1 motif is indicated by a purple star. **d**, Rme1 is required only in *K. lactis* to respond to mating pheromone. Wild-type or *RME1* knockout **a** cells were exposed to either α mating pheromone or a mock treatment of dimethylsulphoxide (DMSO). Mating projections form readily when both wild-type and *Δrme1* *S. cerevisiae* and *C. albicans* cells are exposed to mating pheromone. Only the *K. lactis* *Δrme1* **a** cells were unable to respond to the presence of α mating pheromone. **e**, Rme1 is required for mating in *K. lactis* but not in *S. cerevisiae* nor *C. albicans*. Quantitative mating assays were performed by mating wild-type or *Δrme1* **a** cells to wild-type α cells. In *S. cerevisiae* and *C. albicans* the percentage of **a** cells that was able to mate is similar for wild-type and *Δrme1*. *K. lactis* *Δrme1* **a** cells are mating incompetent; no mating products were isolated from the *Δrme1* **a** × wild-type α mating.

Figure 3. Overexpression of *RME1* is sufficient for hsg expression in the absence of nutrient starvation

a, In the overexpression strain (pLAC4-*RME1*), *RME1* transcription is induced by galactose-containing medium, a condition that does not cause expression of the heterotrimeric G proteins or pheromone response in wild-type (WT) cells. A strain using the empty pLAC4 vector was used as a control. The transcripts were measured relative to *ACT1* transcript levels by RT-quantitative PCR (means and s.d., $n = 3$). In the absence of a starvation signal the hsgs, but not *CKB1* (a non-hsg control), are upregulated when *RME1* is overexpressed.

b, *RME1* overexpression allows cells to respond to mating pheromone in the absence of a starvation signal. *K. lactis* **a** cells that contained only the endogenous *RME1* copy and an empty pLAC4 vector (WT), the endogenous copy of *RME1* and *RME1* driven by the pLAC4 promoter (WT + pLAC4-*RME1*) or only *RME1* driven by the pLAC4 promoter ($\Delta rme1$ 1pLAC4-*RME1*) were grown in YEP-galactose and exposed to α mating pheromone. Wild-type cells were unable to form mating projections in the absence of a starvation signal, but both strains overexpressing *RME1* (pLAC4-*RME1*) formed mating projections in the absence of a starvation signal.

Figure 4. A simplified model for the evolution of regulation of core hsgs in three yeasts

In all three species the core hsgs are repressed by $\mathbf{a1-\alpha2}$; thus, they are ON in $\mathbf{a}$ and α cells and OFF in $\mathbf{a/\alpha}$ cells. In *S. cerevisiae* and *C. albicans* the repression is direct ($\mathbf{a1-\alpha2}$ binds to the promoters of these genes), but in *K. lactis* it is indirect, through Rme1. The circuit rewiring in the *K. lactis* lineage has resulted in a new mating behaviour; this species is able to mate only when starved. We show that this behaviour is due to the intercalation of Rme1, which is upregulated by starvation in *K. lactis*.