Transcriptional Regulatory Circuitries in the Human Pathogen *Candida albicans* Involving Sense–Antisense Interactions

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ABSTRACT *Candida albicans*, a major human fungal pathogen, usually contains a diploid genome, but controls adaptation to a toxic alternative carbon source L-sorbose, by the reversible loss of one chromosome 5 (Ch5). We have previously identified multiple unique regions on Ch5 that repress the growth on sorbose. In one of the regions, the *CSU51* gene determining the repressive property of the region was identified. We report here the identification of the *CSU53* gene from a different region on Ch5. Most importantly, we find that *CSU51* and *CSU53* are associated with novel regulatory elements, *ASUs*, which are embedded within *CSUs* in an antisense configuration. *ASUs* act opposite to *CSUs* by enhancing the growth on sorbose. In respect to the *CSU* transcripts, the *ASU* long antisense transcripts are in lesser amounts, are completely overlapped, and are inversely related. *ASUs* interact with *CSUs* in natural *CSU/ASU cis* configurations, as well as when extra copies of *ASUs* are placed in *trans* to the *CSU/ASU* configurations. We suggest that *ASU* long embedded antisense transcripts modulate *CSU* sense transcripts.

GENOME-WIDE surveys of eukaryotic transcriptomes have led to the identification of abundant noncoding transcription in plants, mammals (Lee *et al.* 2009), and fungi (David *et al.* 2006; Samanta *et al.* 2006; Dutrow *et al.* 2008; Nagalakshmi *et al.* 2008; Sellam *et al.* 2010). The noncoding transcriptome of higher eukaryotes includes a class of various small RNAs that were extensively studied and that were shown to directly modulate gene expression (reviewed by Lee *et al.* 2009; Olejniczak *et al.* 2010; Taft *et al.* 2010). It was not initially appreciated that another class of long noncoding RNAs appears in 10- to 12-fold larger amounts than the coding transcripts (Nagano and Fraser 2011). Initially neglected, the long noncoding RNAs are now given much attention, being recognized as important regulators that are implicated in a large range of various functions, including epigenetic control, enhancing or mediating long-range chromatin interactions, as well as serving as scaffolds of chromatin-modifying complexes (Hongay *et al.* 2006; Camblong *et al.*

2007; Houseley *et al.* 2008; Guttman *et al.* 2009; Khalil *et al.* 2009; Mahmoudi *et al.* 2009; reviewed by Morris 2009; Nagano and Fraser 2011).

Characterization of transcription in *Candida albicans*, which is considered to be the most common fungal opportunistic pathogen of humans, is in its infancy. RNA interference (RNAi) in this organism has been recently discovered (Drinnenberg *et al.* 2009). First reports on the transcriptome have been published, revealing an extensive antisense transcriptome (Bruno *et al.* 2010; Sellam *et al.* 2010; Tuch *et al.* 2010).

C. albicans is a single-cell organism with a diploid genome organized into eight pairs of chromosomes. Survival in various adverse environments is an important property of this pathogen. Integral parts of *C. albicans* adaptation and survival strategies are alterations of large portions of the genome, including monosomy or trisomy of entire chromosomes. Similar alterations of the same chromosomes occur in the same environments, allowing survival (Rustchenko 2007, 2008). A well-known example, which is the topic of this article, is survival on the toxic sugar L-sorbose, when it is available as a sole source of carbon. The loss and gain of chromosome 5 (Ch5) up- and downregulates, respectively, the *SOU1* (sorbose utilization) gene (orf19.2896) on Ch4 and confers growth, *Sou*⁺, and no growth, *Sou*[−], on sorbose (Rustchenko and Sherman 2002; Rustchenko 2007, 2008).

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Our early studies already indicated the complexity of regulation by copy number of Ch5. This chromosome carries multiple unique regions for negative control of growth on sorbose; the final number of regions is yet to be established (Kabir *et al.* 2005). A total of five regions A–C, 135, and 139 (Figure 1) have been rigorously confirmed by an analysis of Ch5 deletions. The regions were proposed to each encompass at least one unique negative controlling element *CSU* (control of sorbose utilization). The first *CSU*, *CSU51* (orf19.1105.2), has been identified in region A (Kabir *et al.* 2005). Another *CSU*, *CSU53* (orf19.3931) from region 135, is presented in this work.

We report here a new genetic element *ASU* (activation of sorbose utilization), adding an additional layer of control from Ch5. *ASUs* are embedded in *CSU51* and *CSU53* in the opposite orientation and are associated with a distinct, albeit weak, overexpression phenotype of the enhanced growth on sorbose, thus counteracting the repressive phenotype by *CSUs*. Antisense *ASU* transcripts are long, can be capped and polyadenylated, and seem to act as noncoding transcripts. We present evidence that, as expected, complementary sense *CSU* and antisense *ASU* transcripts interact. The final number of the *CSU/ASU* configurations on Ch5 has yet to be determined.

Materials and Methods

The co-overexpressing system

We used an important tool, a low copy number replicative plasmid pCA88 overexpressing the metabolic gene *SOU1*, thus causing the *Sou*[−] recipient strain to utilize sorbose, *Sou*[−] → *Sou*⁺ (Wang *et al.* 2004). This plasmid was previously used for preparing a Ch5 DNA library and subsequently cloning a negative regulatory gene *CSU51*, as well as unique regions carrying other putative *CSUs* on Ch5, as based on the reversal of sorbose utilization, *Sou*⁺ → *Sou*[−] (Kabir *et al.* 2005). In this work, similarly, we used pCA88 to co-overexpress *SOU1* and different Ch5 sequences, as presented in the nine diagrams in Figure 2. Care was taken to assure that inserted genes had up to 1.5 kb of the upstream regions, as *C. albicans* is known to have long promoters (Gaur *et al.* 2004; Srikantha *et al.* 2006; Vines *et al.* 2006). In addition, genes contained ~100 bp of the downstream region.

Strains, media, plasmids, and primers

We used the *C. albicans* *Sou*[−] sequencing strain SC5314 and its relatively genetically stable *Sou*[−] *Ura*[−] derivative CAF4-2 (Ahmad *et al.* 2008). Also, we used the well-characterized prototrophic *Sou*[−] strain 3153A (Rustchenko-Bulgac and Howard 1993). The strains carrying in their genome either a control empty vector pAK156 or a vector with one *ASU53*, pEA249, or two *ASU53s*, pEA254, were prepared by individually integrating the plasmids into the *LEU2* locus on Ch7.

Yeast extract/peptone/dextrose (YPD) and synthetic dextrose (SD) media were previously described (Sherman

Figure 1 Schematic presentation of two *CSU/ASU* sense/antisense configurations in the context of Ch5. *CSU51/ASU51* configuration from region A or *CSU53/ASU53* configuration from region 135 is located within a 209-kb portion of Ch5, which is critical for growth on sorbose. This portion also carries other regions B, C, and 139, as indicated (Kabir *et al.* 2005). The size of Ch5 is indicated and the centromere (C) and telomeres (T) of Ch5 are shown. Also indicated are the ORF sizes of *CSUs* and *ASUs*.

2002). A total of 1 M sorbitol was added in SD medium, when growing transformants. Synthetic sorbose or sorbitol media were the same as SD medium, but contained 2% of either L-sorbose or sorbitol, as a sole carbon source (Rustchenko *et al.* 1994). To prepare solid medium, 2% (wt/vol) agar or agarose was added. Uridine (50 µg/ml) was added when needed. The proper growth and handling of cells preventing chromosomal instability was previously reported (Rustchenko-Bulgac 1991; Perepnikhatka *et al.* 1999; Wang *et al.* 2004; Ahmad *et al.* 2008).

All plasmids or primers that were used in this study are described in Supporting Information, File S1 and are also presented in Table S1 and Table S2, respectively. All replicative plasmids are derivatives of pCA88 (see The co-overexpressing system in Results), which is pRC2312 carrying *SOU1* (Wang *et al.* 2004).

RNA preparation and Northern blot analysis

C. albicans cells were grown for independent colonies at 37° on plates with sorbitol medium (see above) and opened with glass beads. Total RNA was isolated according to Russo *et al.* (1991) or with RNeasy Midi kit (Qiagen) and additionally treated with RNase free DNase to remove all traces of genomic DNA. mRNA was isolated from total RNA using Oligotex mRNA kit (Qiagen), as recommended by the manufacturer. Either 15 µg of total RNA or 3–4 µg of mRNA was denatured and size fractionated on 1% formaldehyde gel and then transferred to positively charged nylon membrane from Ambion according to Russo *et al.* (1991). For loading controls, blots were hybridized with double stranded (ds) DNA probes that were prepared from the *PGK1* gene (orf19.3651) or 18S rRNA and labeled with ³²P (Russo *et al.* 1991). ³²P-labeled strand-specific single-stranded RNAs (riboprobes) were generated by *in vitro* transcription with MAXIScript T7 kit (Ambion) according to the manufacturer's instructions. Riboprobes were hybridized with blots, according to Clements

Figure 2 Diagrams representing various co-overexpression plasmids that were derived from the backbone plasmid pCA88. Shown are the following: (1) an original low copy number replicative plasmid pRC2312; (2) its derivative pCA88 overexpressing the metabolic *SOU1* gene; (3–9) various plasmids co-overexpressing *SOU1* with a sequence of interest. The sequences are indicated by open, shaded, and solid blocks. These correspond to the Ch5 region, to the portion of region encompassing the *CSU* gene, which always contains an embedded *ASU* gene (*CSU* + *ASU*), and to the *ASU* gene, as indicated. Mutations are marked with an X. The phenotype of the recipient *Sou*[−] cells of the strain CAF4-2 conferred by each type of plasmid on L-sorbose medium, is indicated by *Sou*[−], *Sou*⁺, *Sou*⁺⁺, and *Sou*^{+/-}. The *Sou*[−] or *Sou*⁺ phenotypes due to the original pRC2312 or pCA88 (*SOU1*), respectively, are considered as the general negative or positive controls for growth. Note that in the *CSU/ASU* configuration, the stronger repression phenotype of *CSU*, *Sou*[−], dominates the weaker phenotype of *ASU*, *Sou*⁺⁺, in the *CSU/ASU* configuration. Also note that the intact *CSU* always contains the embedded *ASU*. However *ASU* can be separated by destroying *CSU*, while the intact *CSU* cannot be separated from *ASU*.

et al. (1988). Briefly, blots were incubated in QuikHyb Hybridization solution from Stratagene overnight at 68° and washed with low- and high-stringency solutions at room temperature and at 68°, respectively. Images were processed using PhosphorImager Storm-820 (Molecular Dynamics). Individual bands were quantified using ImageQuant 5 software (Molecular Dynamics). Transcript sizes were estimated with RNA Millenium size markers–formamide (Ambion). General approaches for performing Northern analyses were adopted from Ding *et al.* (2007). For example, each experiment was repeated several times with different batches of RNA that were prepared from independently grown cultures.

RT-PCR and semiquantitative analysis

To synthesize strand-specific cDNA, we set the RT reaction as a duplex with gene-specific primers for a gene of interest

and a control gene. The following genes were used as controls: *CDC6* (orf19.5242), *EMP24* (orf19.6293), *TPK2* (orf19.2277), and *PGK1* (orf19.3651). Synthesis was conducted with the MonsterScript Reverse Transcriptase (Epicentre Biotechnologies), as recommended by the manufacturer. The PCR amplification was also set as a duplex and was conducted for different number of cycles with Phusion Hot Start High-Fidelity DNA Polymerase (New England BioLabs), as recommended by the manufacturer (also see Cerazin-Leroy *et al.* 1998; Kuai *et al.* 2004). Prior to that, pilot duplex PCR amplifications were undertaken to optimize reaction conditions for efficiency of amplification and the lack of nonspecific bands.

Semiquantitative (sq)RT-PCR analysis was performed according to Kuai *et al.* (2004) and Cerazin-Leroy *et al.* (1998). Briefly, images of ethidium bromide-stained gels loaded with amplification products from different number of cycles were prepared with the AlphaImager IS2000 Digital Imaging system (Alpha Innotech). ImageQuant 5 software (Molecular Dynamics) was used to measure the brightness of the bands (Ahmad *et al.* 2008). At least three consecutive amplicons in exponential phase, as estimated by the determination coefficient value 0.97 or more were used to normalize the experimental values against the values of the control genes and then to calculate the ratios test/control genes from the averaged values.

Mapping of 5'- or 3'-untranslated region with rapid amplification of cDNA ends

Total RNA was used to analyze 5'- and 3'-untranslated regions (UTRs) by rapid amplification of cDNA ends (RACE) with the gene-specific primers and with the FirstChoice RLM RACE kit (Ambion), according to the manufacturer's specifications. The RACE products were electrophoretically separated on agarose gel, purified, and subsequently ligated into pJET1.2/Blunt vector (Fermentas Life Sciences) for further transformation into *Escherichia coli* 5-alpha competent cells supplied by New England BioLabs. Plasmids from individual transformants were analyzed for the presence of inserts with expected size and sequenced using BigDye Terminator v3.1 from Applied Biosystems.

Assay for the *Sou* phenotype

Spot dilution assay was performed on solid sorbose medium (see above), as described by Wellington and Rustchenko (2005).

Handling transformants carrying replicative plasmids for the growth assay and for RNA isolation

Several transformants grown as colonies on solid SD medium supplied with sorbitol were combined and streaked as patches on solid SD medium. After incubation, some cells were taken for sorbose growth assays, whereas the other cells were suspended in distilled water, plated on solid SD medium for independent colonies, incubated, colonies grown, harvested, and total RNA isolated (see above).

Miscellaneous

Amplicons or plasmid inserts were routinely sequenced in the core facility at the University of Rochester using BigDye Terminator v3.1 Cycle Sequencing kit on ABI 3730 PRISM Genetic Analyzer. Transformation of *C. albicans* cells was conducted according to Kabir and Rustchenko (2005). Site-directed mutagenesis was performed with a QuickChange Site-Directed Mutagenesis kit (Stratagene), according to the manufacturer's recommendations.

Results

Identification of the *CSU53* gene in region 135 of Ch5

In this work, we continue characterizing five well-established regions on Ch5 that are involved in the repression of the growth on sorbose (see the Introduction and Figure 1). One of the approaches used here is the replicative test-plasmid pCA88 carrying metabolic *SOU1*. As previously reported (see also *The co-overexpressing system in Materials and Methods*), this plasmid confers growth on sorbose medium to the Sou^- recipient cells, $Sou^- \rightarrow Sou^+$ (Figure 2, diagrams 1 and 2). However, the introduction of the 4.3-kb portion of Ch5 that is designated region 135 (Figure 1) into pCA88 results in the plasmid pCA135 (Figure 2, diagram 3) that shifts the Sou^+ phenotype of the recipient cells due to *SOU1* back to no growth, $Sou^+ \rightarrow Sou^-$ (Kabir *et al.* 2005). Compare the repressive Sou^- phenotype due to region 135 with the control Sou^+ phenotype due to *SOU1*, as shown with the spot assay in Figure 3A (see *Materials and Methods* for the assay and Figure S1 for the control growth on glucose medium). Analysis of the sequence of region 135 indicated a single large ORF of 912 bp previously annotated, as the *SFC1* (orf19.3931) gene. We demonstrated that this ORF is implicated in the repression of growth on sorbose, as presented below, and thus designated this gene as *CSU53*. In figure 4A showing the diagram of region 135, the *CSU53* ORF is presented with a shaded box.

To evaluate whether *CSU53* is relevant to the Sou phenotype, we interfered with the *CSU53* translation product by creating three independent frameshift mutations in region 135 within the *CSU53* ORF at positions +66, +112, or +233, as indicated by stars in Figure 4A (see [supporting information File S1](#) for details). The mutations were verified by sequencing and each mutated region 135 was individually subcloned into pCA88 resulting in plasmids pEA227, pEA158, and pEA201, respectively. Note that in Figure 4A the plasmid names are given to the corresponding inserts, a nomenclature that will be kept throughout the text. CAF4-2 cells were individually transformed with the above plasmids and tested for the growth on sorbose with the spot assay. For the handling of *C. albicans* transformants, see *Materials and Methods*. Each mutation consistently abolished the repressive Sou^- phenotype of region 135 and restored the control Sou^+ phenotype due to *SOU1*, as presented in Figure 3B (compare pEA227, pEA158, and pEA201 with pCA135

and pCA88; also see Figure S2A for the control growths on glucose medium). These data strongly implicated the *CSU53* (*SFC1*) gene with the repression of growth on sorbose and indicated that the putative *Csu53p* is important for the Sou phenotype.

To produce other evidence of the repressive function of *CSU53*, we interfered with its transcription. Portions of region 135 encompassing the *CSU53* ORF with various sizes of the sequence in front of the start codon, were PCR amplified, individually co-overexpressed with *SOU1*, and tested for growth on sorbose, as above. We found that diminishing the sequence upstream to the ORF led to diminution of the repressive property. For example, an entire upstream sequence of ~2 kb, pEA144, rendered the same repressive phenotype, as the entire region 135 (Figures 3A, bottom, and 4A; also Figure 2, diagram 4). Diminishing the upstream sequence to 1.4 kb, pEA105, or to 0.5 kb, pEA143 (Figure 4A) decreased the repression, as indicated by multiple Sou^+ colonies, Sou^{-*} , which appeared after prolonged incubation (Figure 3A, top or bottom, respectively). On the other hand, various portions of region 135 lacking the *CSU53* ORF, as, for example, pEA145, pEA146, pEA141, pEA140, pEA130, pEA132, or pEA133 (Figure 4A) consistently displayed the control Sou^+ phenotype (see Figure 3A, bottom, for representative pEA145 and pEA140), thus demonstrating no relevance to the repressive property. These results provided clear evidence for a single *CSU53* in region 135.

Identification of new elements, ASUs, which are associated with CSUs, and which act opposite to CSUs by enhancing the growth on sorbose

By co-overexpressing *SOU1* and different portions of region 135, as described above, we found a phenotype that was different from the repressive Sou^- or the control Sou^+ . This phenotype was an increased growth on sorbose medium, Sou^{++} , which occurred when the region upstream to *CSU53* was removed, thus resulting in pEA104, pEA156, and pEA155 (Figure 4A), as exemplified with the growth assay of pEA104 in Figure 3A (top).

We addressed the question of whether the Sou^{++} phenotype depends on the insert orientation toward *SOU1* by preparing plasmid pEA234, which carried a 2158-bp insert from plasmid pEA104 in opposite orientation (see [File S1](#) and [Table S1](#) for the pEA234 construction). As exemplified in Figure 3C, plasmids with different orientation of the insert, pEA104 and pEA234, rendered the same increase of growth, which was independent of insert orientation, in multiple experiments. See [Figure S3A](#) for the control growth on glucose medium.

We next addressed the question of whether the Sou^{++} phenotype depends on the large insert, acting, for example, as a stabilizing factor. We prepared and analyzed plasmid pEA261 carrying an insert of the same size, 2158 bp, as the above pEA104, encompassing the sequence upstream to *CSU53* with an adjacent 169 bp extending to outside the

Figure 4 Schematic presentation of the analyses of *CSU/ASU* configuration in region 135 or region A of Ch5 (Figure 1). The entire region or a portion of the region was individually co-overexpressed with *SOU1* on a plasmid (Figure 2) and assayed on sorbose medium, as exemplified in Figure 3. The co-overexpression phenotypes of portions fell into three categories: repression of growth *Sou*⁻, enhanced growth *Sou*⁺⁺, and control growth *Sou*⁺, as indicated on the right (see Figure 3A legend for more explanations). (A) *CSU53/ASU53* configuration in the context of region 135, which is also designated pCA135 by the name of the corresponding plasmid. Also shown are the sense *CSU53* and antisense *ASU53* transcripts, as determined by RACE in the strains 3153A and CAF4-2 (see Mapping UTRs of sense and antisense transcripts). Also shown are representative portions of region 135 that were co-overexpressed with *SOU1* to identify *CSU53* and *ASU53*. Mutations within an entire region 135 in *CSU53* ORF or within a portion of the region in *ASU53* ORF are indicated by stars. (B) *CSU51/ASU51* configuration in the context of region A, which is also designated pAK65 by the name of the corresponding plasmid. Also shown are the sense *CSU51* and antisense *ASU51* transcripts that were determined as indicated in A. Also shown are representative portions of region A that were co-overexpressed with *SOU1* to identify *ASU51*.

region 135 (Figure 4A). Unlike pEA104, *Sou*⁺⁺, new pEA261 conferred the control *Sou*⁺ phenotype.

It did not seem that the *Sou*⁺⁺ phenotype was an artifact due to a certain sequence, as various shorter portions encompassing the region downstream to *CSU53* alone or with a portion of the *CSU53* ORF, such as pEA145, pEA146, pEA141, pEA140, and pEA130 (Figure 4A) showed the control *Sou*⁺ phenotype (see Figure 3A, bottom, for the representative pEA145 and pEA140).

Importantly, all three portions conferring the *Sou*⁺⁺ phenotype, pEA155, pEA156, and pEA104 contained a relatively small ORF of 120 bp embedded in the *CSU53* ORF of 912 bp

in opposite orientation, 461 bp downstream from the start codon (Figure 4A, solid box). Apparently, there is a critical 20-bp region, which causes the difference between pEA155 (*Sou*⁺⁺) and pEA142 (*Sou*⁺). This critical region lies in front of a putative transcription start site of the embedded ORF. We thus designated the ORF embedded in *CSU53* of region 135, as *ASU53* (activation of sorbose utilization).

Similar to *CSU53* from region 135, we previously reported that *CSU51* from region A, Figure 1, also has a smaller ORF of 105 bp embedded in opposite orientation, 32 bp downstream from the start codon (Kabir *et al.* 2005) (Figure 4B). Also similarly, the authors introduced frameshift mutations in

region A to target either *CSU51* or the embedded ORF and demonstrated that the embedded ORF lacked the repressive property. Importantly, when the repressive property of *CSU51* was abolished, the phenotype shifted to *Sou*⁺, i.e., no *Sou*⁺⁺ growth occurred. In this work, we changed the approach and analyzed portions of region A, instead of introducing mutations in the intact region. We found that the portions pEA219 of 792 bp, pEA221 of 802 bp, and pEA209 of 404 bp encompassing *CSU51* with the embedded ORF, but lacking the region upstream to the *CSU51* ORF, rendered, as expected, the *Sou*⁺⁺ phenotype (Figure 4B and Figure S3B). Increased growth was independent of the insert orientation, as shown with plasmid pEA236 carrying the same insert as in pEA209, but in opposite orientation (Figure S3B). We thus designated the ORF embedded in *CSU51* of region A as *ASU51*, by analogy with *ASU53* of region 135.

We confirmed the *Sou*⁺⁺ phenotype of *ASU51* by trimming the region upstream to its ORF to 20 bp, a portion pEA240 (Figure 4B) that shifted the phenotype *Sou*⁺⁺ → *Sou*⁺. We also extended the region downstream to the *ASU51* ORF from a portion of pEA209 (*Sou*⁺⁺) into the region upstream to the *CSU51* ORF, a portion of pEA183, which shifted the phenotype *Sou*⁺⁺ → *Sou*⁺ (the phenotypes are indicated on the schematics in Figure 4B). The 44-bp sequence downstream to the *ASU51* ORF on pEA209 or 75 bp on pEA183 included 11 bp or 44 bp, respectively, from the region upstream to *CSU51*. Sequence analysis of the critical 33-bp difference between pEA209 (*Sou*⁺⁺) and pEA183 (*Sou*⁺) revealed a putative TATA box between position −12 to −15 in the 5'-UTR of *CSU51*, suggesting that this TATA box was sufficient for some transcriptional activity of *CSU51* that prevailed over the weaker *Sou*⁺⁺ phenotype.

Importantly, the *Sou*⁺⁺ phenotype consistently occurred in multiple independent experiments, as well as in the series of experiments that were conducted at 37°, 30°, and 22°, as established with pEA104 or pEA234 carrying *ASU53* or with pEA104 or pEA234 carrying *ASU51*. This phenotype could be clearly observed within approximately the first 3–5 days of incubation, until the control cells caught up with the growth, thus obscuring a convenient comparison. At 37°, the phenotype due to *ASU51* was not as pronounced, as those at two other temperatures. Also, overall, the phenotype due to *ASU51* was not as pronounced as the phenotype due to *ASU53* (Figure S3).

In conclusion, we have found that each studied locus, *CSU51* or *CSU53*, contains a *CSU* element and an *ASU* element, which is embedded in *CSU* in the opposite orientation. In the natural *CSU/ASU* configurations, the *Sou*[−] repressive phenotype of *CSUs* dominates the weaker *Sou*⁺⁺ phenotype of the *ASUs* (Figure 2, diagram 3 or 4, Figure 3A, Figure S3, and Figure 4). The *ASU* element can be revealed by interfering with the transcription, but not the translation of the *CSU* element. A simple explanation would be that mutations destroying the *Csu* proteins leave the corresponding sense RNAs nearly intact. However, the elimination of the upstream regions, hence promoters, interferes with the pro-

duction of the sense *CSU* transcripts, which subsequently affects translation products. Such phenotypic differences are expected if sense *CSU* and antisense *ASU* transcripts interact, for example, by forming dsRNA molecules that subsequently either inhibit translation or lead to degradation. Then, the lack of the *CSU* transcript from the *CSU/ASU* configuration from the plasmid would lead to the increased abundance of the *ASU* transcript from the plasmid and ultimately would lead to the increase of combined amount of plasmid and chromosomal *ASU* transcripts. This would increase the interaction with the chromosomal *CSU* transcripts and cause more depletion of *CSU* inhibitory transcripts, thus upregulating *SOU1*, and finally resulting in the better growth.

Visualization of sense *CSU* and antisense *ASU* transcripts with Northern blots

We determined *CSU* and *ASU* transcripts from chromosomal *CSU/ASU* configurations in the strains SC5314, 3153A, and CAF4-2 with Northern blots from three or four independent cultures of each strain (*Materials and Methods*), as exemplified by SC5314 and 3153A in Figure 5A. Highly abundant *CSU51* sense transcript was revealed with total RNA and dsDNA probe prepared from *CSU51*. However, the low-abundance complementary *ASU51* antisense transcript could be revealed only with mRNA and a riboprobe prepared from *ASU51* (Figure 5B). The low-abundance sense *CSU53* and antisense *ASU53* were also revealed with mRNA and riboprobes prepared from the corresponding genes (Figure 5B). Each probe repeatedly produced hybridization signal(s) of the pattern, which was identical in all three examined strains. Specifically, the *CSU51* probe revealed more abundant transcript of ~700 nt and less abundant transcript of ~1200 nt. The *ASU51* probe revealed a pool of transcripts that were distributed around a 1000-nt size. A *CSU53/ASU53* pair produced one sense and one antisense transcript of ~1300 nt and 1200 nt, respectively.

Relative amounts of antisense *ASU* and sense *CSU* transcripts

We estimated comparative amounts of sense *CSU* and antisense *ASU* transcripts from chromosomal *CSU/ASU* configurations in the strains SC5314, 3153A, and CAF4-2 using sqRT-PCR analysis with gene-specific primers (*Materials and Methods*). The transcript levels of *ASU51*, as compared to *CSU51*, were 1.5% in SC5314, 4% in CAF4-2, and 3.8 and 4% in 3153A in two independent experiments, thus ranging from 1.5 to 4%. Also, the transcript levels of *ASU53*, as compared to *CSU53*, were 16% in SC5314, 8% in CAF4-2, and 37 and 44% in 3153A in two independent experiments, thus ranging from 8 to 44%.

The RT-PCR data are in agreement with the Northern blot analyses that indicated the lower abundance of the antisense transcript for at least *CSU51/ASU51* configuration (see above and Figure 5A). Furthermore, the low amounts of the *ASU* transcripts are consistent with their phenotype being recessive in respect to the phenotype of *CSUs* (see

Figure 5 Northern blot analyses reveal the *CSU53/ASU53* and *CSU51/ASU51* sense/antisense transcripts, as exemplified with the strains 3153A and SC5314. (A) Hybridization signals obtained with total RNA and dsDNA probe for *CSU51* transcript, as well as mRNA and riboprobes for *CSU53*, *ASU51*, and *ASU53* transcripts, as indicated. For quantitative estimate of amounts of the corresponding *CSU* and *ASU* transcripts, see *Relative amounts of antisense ASU and sense CSU transcripts*. (B) Nucleotide sequence of *CSU53/ASU53* or *CSU51/ASU51* ORF configuration. Start codons of *CSU* sense elements, as well as sequences comprising *ASU* antisense elements are in boldfaced type. The sequences that were used to prepare riboprobes hybridizing with mRNA transcribed from either Watson or Crick strand on the Northern blot in A are underlined.

Mapping UTRs of sense and antisense transcripts

We found that *CSU/ASU* loci in both of the examined strains 3153A and CAF4-2 produced multiple sense or anti-

Mutational analysis demonstrates that ASU ORFs are not critical for the Sou⁺⁺ phenotype

ASUs interact with CSUs at the phenotypic and the transcriptional levels

The plasmids with extra *ASUs*, pEA238 and pEA162, were tested on sorbose medium in multiple spot assays, as described above. We found that these plasmids substantially diminished the dominating *Sou*⁻ repressive phenotype of corresponding *CSU* elements. As exemplified in Figure 3E with the plasmid pEA162, an extra *ASU53* resulted in the growth, which was almost indistinguishable from the control

growth, Sou⁺. Consistently, an extra *ASU51* resulted in either intermediate growth between no growth and control growth, i.e., neither Sou⁻ nor Sou⁺ occurred, or high-frequency large Sou⁺ colonies (Figure S4A). We emphasize that the Sou⁺ colonies never occurred, when region A, i.e., *CSU51/ASU51*, was co-overexpressed alone with *SOU1*. This variability presumably occurred due to the highly expressed and thus strong *CSU51* (see above). Also, see Figure S4, A and B for the control growths on glucose medium.

To substantiate the finding of the interaction between *ASUs* and *CSUs*, we integrated one or two copies of *ASU53*, vectors pEA249 or pEA254, respectively, in the *LEU2* locus on Ch7 in the strain CAF4-2. The control integration was with no *ASU53*, pAK156 (Table S1). We have previously demonstrated that integration in *LEU2* did not interfere with the Sou phenotype (Wang *et al.* 2004). The proper integration of pAK156, pEA249, and pEA254 was verified by PCR amplifications with a pair of primers, one for the *LEU2* gene, AF237U, and another for the lacZ N-terminal sequence of integration vector, M-13FU (Table S2). All three amplicons had the expected sizes of ~2.0 kb, 3.8 kb, and 6.2 kb, respectively, while no amplicon was produced with genomic DNA from the control strain, SC5314. The presence of *ASU53* in corresponding amplicons was confirmed with primers AF110 and AF111.

We prepared three batches of total RNA from three independent cultures of each construct. We then synthesized cDNA and used it for PCR amplifications with gene specific primers (Table S2). Reactions were set in duplex with control genes and were conducted with different numbers of cycles followed by quantitation (*Materials and Methods*). See Figure S5 for the examples of amplicons that were quantitated. As expected, the amount of the *ASU53* transcript increased, consistent with the increase of the copy number of *ASU53*, as compared to the control strain with two regular chromosome copies carrying the integration vector with no extra copies of *ASU53* (Table 1). Introduction of one extra copy of *ASU53* increased the *ASU53* transcript to 1.4, 1.5, and 1.6, resulting in an average of 1.5 ± 0.1 ; introduction of two extra copies of *ASU53* increased the *ASU53* transcript to 1.7, 1.9, and 1.9, resulting in an average of 1.8 ± 0.1 . We then asked whether the amount of the *CSU53* transcript changed. We found a copy-dependent inhibition of *CSU53* by *ASU53*. In the presence of one extra copy, the *CSU53* transcript was 0.6, 0.7, and 0.8, resulting in an average of 0.7 ± 0.1 , whereas in the presence of two extra copies, the *CSU53* transcript was 0.5, 0.5, and 0.6, resulting in an average of 0.5 ± 0.1 (Table 1).

The effect of chromosomal extra copies of *ASU51* on the expression of the corresponding *CSU51* was not analyzed, because of a large difference in the expression of the two genes.

SOU1* is upregulated by *ASU53* and downregulated by *CSU51* or *CSU53

We next determined the effect of genomic extra *ASUs* on the metabolic *SOU1* expression. RT-PCR amplifications were

Table 1 Expression changes of *CSU53* and *SOU1* in the presence of genomic extra copies of *ASU53*

Gene	One extra <i>ASU53</i>	Mean $\pm$ SD	Two extra <i>ASU53s</i>	Mean $\pm$ SD
<i>ASU53</i>	1.4, 1.5, 1.6	1.5 ± 0.1	1.7, 1.9, 1.9	1.8 ± 0.1
<i>CSU53</i>	0.6, 0.7, 0.8	0.7 ± 0.1	0.5, 0.5, 0.6	0.5 ± 0.1
<i>SOU1</i>	1.6, 1.8, 1.9	1.8 ± 0.2	2.6, 2.6, 2.7	2.6 ± 0.1

Expression change for each gene was determined by the ratio of the amount of transcript in the integration construct having one or two extra copies of *ASU53s* vs. the control integration construct having no extra *ASU53*.

conducted and analyzed as above. In the presence of one or two extra copies of *ASU53*, the amount of the *SOU1* transcript increased in a copy-dependent fashion (Table 1). In the presence of one extra copy, the *SOU1* transcript was 1.6, 1.8, and 1.9, resulting in an average of 1.8 ± 0.2 , whereas in the presence of two extra copies, the *SOU1* transcript was 2.6, 2.6, and 2.7, resulting in an average of 2.6 ± 0.1 .

We next used a co-overexpression system to address whether *CSU51* or *CSU53* controls *SOU1* expression. Northern blot analyses were carried out with independently prepared batches of total RNA that was extracted from CAF4-2 cells transformed with the replicative plasmids pEA105 or pCA135 (Figure 4A) co-overexpressing *SOU1* with *CSU53*, as well as with the plasmid pAK65 (Figure 4B), co-overexpressing *SOU1* with *CSU51* (see also Figure 2, diagrams 3 and 4). Note that *CSUs* are represented by the natural configurations *CSU/ASU*, because *CSUs* cannot be separated from *ASUs*. However, in these natural configurations, *CSUs* dominate *ASUs* (see above). As exemplified in Figure 6, the co-overexpression with each *CSU* gene clearly diminished the amount of the *SOU1* transcript, as compared to the control pCA88, which overexpressed *SOU1*, but lacked *CSU*. Specifically, *SOU1* was downregulated 0.55-, 0.63-, 0.33-, and 0.36-fold, resulting in an average of 0.47 ± 0.15 by *CSU53* in four independent experiments, as well as 0.18- and 0.29-fold by *CSU51* in two independent experiments. As expected from the phenotypes and transcript abundances (see above), *CSU51* repressed *SOU1* more strongly than *CSU53*.

Discussion

We found that the previously identified *CSU51* and currently identified *CSU53* each contain a genetic element, *ASU*, which is embedded in *CSU* in the opposite orientation. *ASUs* are manifested in several ways. They produce antisense transcripts that are, however, significantly less abundant than the corresponding *CSU* sense transcripts. Extra copies of *ASU* in the genome lead to an decrease or increase of the transcript of the regulatory *CSU* or the metabolic *SOU1*, respectively. *ASUs* are also manifested at the phenotypic level. A copy of *ASU* overexpressed from the low copy number plasmid, possesses a distinct, albeit weak phenotype, enhancing the growth on sorbose. This is, presumably due to a combined action of the plasmid and chromosomal copies

Figure 6 Amount of the *SOU1* transcript is inversely related to the amount of *CSU* transcript. Co-overexpression of *SOU1* with *CSU51* from a low copy number plasmid (Figure 2, diagram 3) decreases the *SOU1* transcript, while increasing, as expected, the *CSU51* transcript, as determined with Northern blot analysis. The recipient CAF4-2 cells carry the following plasmids: (1) negative control pRC2312, an empty vector; (2) positive control pCA88 overexpressing *SOU1*; and (3) pAK65 co-overexpressing *SOU1* with region A carrying a natural *CSU51/ASU51* configuration in which *CSU51* always dominates *ASU51*. Hybridization signals were obtained with total RNAs and dsDNA probes prepared from *SOU1* or *CSU51*, as indicated. Normalization for the amounts of loaded RNA was done with the same blot using dsDNA probe prepared from 18S rDNA. Note the decrease of the *SOU1* signal from pAK65, as compared to pCA88.

of *ASU* on the chromosomal copies of *CSU*. Consistently, a copy of *ASU* co-overexpressed from plasmid with the corresponding natural *CSU/ASU* configuration greatly diminishes the *CSU* repressive phenotype.

The interaction between *CSUs* and *ASUs* is thus clearly revealed at the transcription and at the phenotypic levels. It is also clear that the levels of the *CSU* and *ASU* transcripts are in a reverse relationship. Conversely, the expression of metabolic *SOU1* on Ch4 directly depends on the transcription of *ASUs* and reversely depends on the transcription of negative regulatory *CSUs*. It is reasonable to suggest that *ASU* elements act as modulators repressing *CSUs*, because the phenotype of *CSUs* dominates and because the *ASU* transcripts are less abundant and are completely imbedded in the sense transcripts. Although not specifically addressed, both *CSU* sense and *ASU* antisense transcripts are expected to be produced in the same cell, as this is required for their interaction. The complementarity of sense/antisense elements is also a strong indication of their interaction. As discussed in *Results*, we obtained preliminary evidence that, as expected for the complementary sequences, sense and antisense RNA interact. This is because *ASU* phenotype is manifested when the sequence upstream to the *CSU* ORF is abrogated, thus greatly interfering with the *CSU* transcription, but not when the *Csu* protein is mutated, leaving the *CSU* transcription normal.

All sense *CSU* and antisense *ASU* transcripts contain ORFs and are also capped and polyadenylated, which is indicative of translation. However, while both *CSU* ORFs are essential for the *Sou*[−] repressive function, *ASU* ORFs are not essential for the *Sou*⁺⁺ enhancing function, as clearly demonstrated by mutational analysis. This could be interpreted, as *Asu*

proteins are not produced or, alternatively, are not relevant for the *Sou* phenotype. In this respect, noncoding RNA can be capped and polyadenylated; furthermore, some RNAs can function both as mRNA and as noncoding RNA (Callahan and Butler 2008; Dinger *et al.* 2008; Rapicavoli and Blackshaw 2009). Future studies should establish whether proteins are translated from *ASUs* and, if so, how they function. Currently, we consider that *ASU* transcripts are implicated with the *Sou* phenotype as noncoding RNAs.

Our current model proposes that interactions between three kinds of elements *CSU*, *ASU*, and *SOU1* are based on the transcript ratios. *ASUs* repress *CSUs* that, in turn, repress *SOU1*. Because the amount of *CSU* transcript diminishes, when corresponding *ASU* is overexpressed, a plausible scenario is that the complementary *CSU* and *ASU* RNAs form dsRNA molecules that are subsequently degraded.

In summary, identification of an additional *CSU53* confirms the previous evidence of multiple *CSUs* on Ch5. The inverse relationship between *CSU51* or *CSU53* transcripts on one hand and *SOU1* transcript on the other hand is consistent with the previous proposal that *CSUs* are negative regulators of *SOU1* (Kabir *et al.* 2005) that determine the regulatory role of Ch5 copy number. Discovery of *ASU* positive elements that counteract *CSU* negative elements reveals an unanticipated layer of complexity in the negative regulation of growth on sorbose.

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Supporting Information

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Transcriptional Regulatory Circuitries in the Human Pathogen *Candida albicans* Involving Sense–Antisense Interactions

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File S1

Supporting Information

General strategy to create mutation in ORF of *CSU53*, *ASU53*, and *ASU51*.

Mutations of *CSU53* ORF were generated within an entire region 135, which is carried on plasmid pCA135. Mutations of *ASU53* ORF or *ASU51* ORF were generated within, respectively, the portion pEA104 from region 135, Figure 3B, or the portion pEA243 from region A, Figure 6. For the plasmids and their inserts, see also Table S1. Region 135, the portion pEA104 from region 135 or the portion pEA243 from region A were PCR amplified and each amplicon was individually sub-cloned in the plasmid pUC19 or pCR-Blunt (Invitrogen Corp., Carlsbad, CA). See Table S2 for the primers. The resulting plasmids were used as templates to create different frame-shift or stop-codon mutations with Stratagene's QuickChange Site Directed Mutagenesis Kit (La Jolla, CA) according to the manufacturer recommendations. Mutations were confirmed by sequencing and corresponding portions finally sub-cloned into the low copy number replicative plasmid pCA88 for further examinations.

Frame-shift mutations in *CSU53* ORF. Plasmids pEA201, pEA158, and pEA227.

Plasmid pEA227 carries the mutation, which was created by two adding an extra G between +65 and +66 with primers CSU53-1MF and CSU53-1MR, respectively. Plasmid pEA201 carries the mutation, which was created by adding two extra G between +232 and +233 with primers CSU53F and CSU53R. Finally, plasmid pEA158 carries the mutation, which was created by replacing T of +112 by G, as well as by introducing an extra G between +111 and +112 with primers AF52 and AF53.

Frame-shift mutation in *ASU53* ORF. Plasmid pEA232.

A frame-shift mutation was created in *ASU53* ORF by adding one extra C between +3 and +4 and another extra C between +6 and +7 using primers ASU53-FM1 and ASU53-FM2, thus, resulting in plasmid pEA232.

Stop-codon mutation in *ASU53* ORF. Plasmid pEA205.

A stop-codon was created in *ASU53* ORF by replacing the T with A at position +12 using primers ASU53-MF and ASU53-MR, thus, resulting in plasmid pEA205.

Frame-shift mutation in *ASU51* ORF. Plasmid pEA243.

A frame-shift mutation was created in *ASU51* ORF by adding an extra T and an extra G between +38 and +39 using primers A51AGE1F and A51AGE1R, thus, resulting in plasmid pEA243.

Opposite orientation of *ASU51*. Plasmids pEA209 and pEA236.

A PCR product of approximately 400 bp was amplified from the plasmid pAK65 in two ways with two pairs of primers. The pair of primers AF217 and AF224 contained, respectively, a *Bam*H1 and a *Pst*1 restriction site in their sequences. The amplicon was sub-cloned into pCA88, which was digested with *Bam*H1 and *Pst*1, resulting in plasmid pEA209 ($\leftarrow$ *ASU51*, $\leftarrow$ *SOU1*). Similarly, the pair of primers AF228 and AF229 containing, respectively, a *Pst*1 and a *Bam*H1 restriction site produced an amplicon, which, upon sub-cloning into pCA88, resulted in plasmid pEA236 ($\rightarrow$ *ASU51*, $\leftarrow$ *SOU1*). See Table S2 for the primers and plasmids.

Opposite orientation of *ASU53*. Plasmids pEA104 and pEA234.

A PCR product of approximately 2.1 kb was amplified from the plasmid pCA135 using two pairs of primers AF29 and AF30, as well as AF226 and AF227. Similarly to above, the presence of a *Bam*H1 and *Pst*1 restriction site in the primer sequences determined the amplicon orientation in plasmid pEA104 ($\leftarrow$ *ASU51*, $\leftarrow$ *SOU1*), as well as plasmid pEA236 ($\rightarrow$ *ASU53*, $\leftarrow$ *SOU1*). See Table S2 for the primers and plasmids.

***CSU51* and *ASU51* combined together on the plasmid pEA238.**

A PCR product of approximately 400 bp encompassing *ASU51* ORF, as well as 256 bp of upstream region and 44 bp of downstream region, was amplified from the plasmid pAK65 by using primers AF217 and AF229 as *Bam*H1 – *Bam*H1 fragment and sub-cloned into pAK65 at *Bam*H1 site as *Bam*H1 – *Bam*H1 fragment. This insert is similar to the insert of pEA209, Figure 6 thus, resulting in plasmid pEA238.

***CSU53* and *ASU53* combined together on the plasmid pEA162.**

A PCR product of approximately 1.7 kb portion of region 135 encompassing *ASU53* ORF with 1505 bp of 5'-end and 102 bp of 3'-end was PCR amplified from the plasmid pCA135 with primers AF128 and AF129 as *Bam*H1 – *Bam*H1 and sub-cloned into pCA88 at the site *Bam*H1 as *Bam*H1 – *Bam*H1, thus, creating plasmid pEA160. A PCR product of approximately 2.5kb portion of region 135 encompassing *CSU53* ORF with 1400 bp of 5'-end and 100 bp of 3'-end and also having *ASU53* in natural configuration, was similarly amplified with primers AF32 and AF134 as *Pst*1 – *Pst*1 fragment and sub-cloned into pEA160 at the *Pst*1 site as *Pst*1 – *Pst*1, thus, creating plasmid pEA162.

Figure S1 Analysis of region 135 resulting in the identification of *CSU53* and the *CSU53/ASU53* configuration. Examples of spot assay for growth on medium containing L-sorbose as a sole carbon source. Control growth on glucose medium, SD, is also shown. Approximately 5×10^5 cells per spot were plated in duplicates. The spotted cells of CAF4-2 expressed various plasmids, as indicated. The names of the plasmids correspond to the inserted portions of region 135, as indicated on the schematics in Figure 3B. Important genes or sequences carried on each plasmid are indicated in parenthesis. The Sou phenotypes conferred by plasmids are indicated. The Sou phenotypes are, as follows: the control Sou[–] lack of growth due to an empty vector pRC2312, the control Sou⁺ growth due to pCA88, which expresses the metabolic *SOU1*; the repression Sou[–] lack of growth due to pEA135, which co-over expresses *SOU1* with region 135; the Sou^{–*} lack of growth with multiple well growing Sou⁺ colonies, which accumulate in course of prolonged incubation; and the Sou⁺⁺ enhanced growth. *CSU53*** indicates that the portion encompassing the *CSU53* gene also contains the *ASU53* gene, which is embedded in *CSU53*.

Figure S2 Mutational analysis of the *CSU* or *ASU* ORFs demonstrates that the putative *Csu* proteins are implicated in the *Sou* phenotype (A), while the putative *Asu* proteins are not (B), as exemplified with the *CSU53/ASU53* locus. (A) Spot assay for growth on sorbose of cells carrying the plasmids pEA227, pEA201, and pEA158, each containing a region 135 with a different frame-shift mutation of *CSU53* ORF (cartoon 5 in Figure 2 and Figure 3B). Note that region 135 with any of the above mutations in *CSU53* lost the *Sou*⁻ repression phenotype in favor of the control growth on sorbose due to *SOU1*, *Sou*⁻ → *Sou*⁺. (B) Spot assay for growth on sorbose of cells carrying the plasmids pEA232 or pEA205 with, correspondingly, frame-shift or stop codon mutation in the *ASU53* ORF (cartoon 7 in Figure 2 and Figure 3B). Note that the mutations do not affect the original *Sou*⁺⁺ phenotype. For more details of the phenotypes of control and other plasmids see the legend of Figure 3A. Also shown are control growths on the glucose medium.

Figure S3 Over expression of *ASU53* (A) or *ASU51* (B) confers the enhanced, *Sou*⁺⁺, growth on sorbose medium that is independent of the insert orientation on a plasmid. Shown are examples of spot assays for growth on sorbose of the cells carrying, respectively, plasmids pEA104 or pEA234 over expressing *ASU53* in opposite orientation and pEA209 or pEA236 expressing *ASU51* in opposite orientation. Insert orientation is indicated by an arrow. See the legend of Figure 3A for more details.

Figure S4 ASUs and CSUs interact, as shown at the phenotypic level with the co-over expression system. (A) and (B) Examples of spot assays for growth on sorbose of the cells carrying, respectively, plasmid pEA238 (*SOU1 CSU51 ASU51*) or pEA162 (*SOU1 CSU53 ASU53*). Each of these plasmids co-over expresses three essential genes: (i) *SOU1*; (ii) *CSU* in a natural configuration *CSU5/ASU5*; and (iii) *ASU*. Notice that pEA238 carries an entire region A encompassing *CSU51* in a natural configuration *CSU51/ASU51*, while pEA162 carries a portion from region 135 encompassing *CSU53* in a natural configuration *CSU53/ASU53*, which is designated *CSU53***. For the plasmid types, see, respectively, cartoons 8 and 9 in Figure 2. Note that either one of natural configurations *CSU/ASU* confers the Sou⁻ repressive phenotype, because of the dominant *CSU* (Results). Also included is the general negative and positive control for growth due to the plasmid pRC2312 and pCA88, respectively. Also included is growth due to co-over expression with *SOU1* of a single element, either a *CSU/ASU* or an *ASU*, as indicated in parenthesis. The corresponding plasmids serve as the additional repressive Sou⁻ (pAK65 in A or pEA105 in B) or the additional Sou⁺⁺ (pEA209 in A or pEA104 in B) controls. Note that the presence of either an extra *ASU51* or an extra *ASU53* diminished the Sou⁻ repressive phenotype of a corresponding *CSU*. Specifically, the plasmid pEA238 in A conferred either the intermediate confluent growth designated Sou^{+/-} (A, top) or the high frequency Sou⁺ colonies designated Sou^{-*} (A, bottom), instead of the repressive Sou⁻ phenotype. See also section “*CSUs* and *ASUs* interact among themselves at the phenotypic and transcriptional levels”. Furthermore, the plasmid pEA162 in B conferred the confluent growth, which is almost equal to the Sou⁺ growth due to control pCA88 and is designated Sou^{+/-}. It should be noted that sorbose plates in A are presented after five days of incubation. This enabled the control growth due to pCA88 to catch up with enhanced growth due to pEA238 (see section “Identification of new elements, *ASUs*, which are associated with *CSUs*, and which act opposite to *CSUs* by enhancing the growth on sorbose” for explanations). Also shown is growth of the control spots on glucose medium.

Figure S5 Expression changes of genomic *CSU53* and *SOU1* due to integration of one or two extra copies of *ASU53* into genome of the strain CAF4-2, as indicated. Also included is the control CAF4-2 strain with integration of a control vector, as indicated, having no extra copy of *ASU53*. See MATERIAL AND METHODS for the RT-PCR method. Shown are the agarose gels with three PCR amplicons for the test or control strain. Each amplicon was generated with different number of cycles that increase from left to right. Amplifications for test and control gene for normalization, as indicated, were performed in the same tube. The PCR reactions for the control strain were prepared with the same master mix and run in parallel with reactions for the test strain. Note that one representative gel for one out of three independent experiments for each test gene is presented. See MATERIAL AND METHODS for quantitation and Table 1 for the quantitation results.

Table S1 LIST of plasmids

Name	Short description	Phenotype of transformed cells on sorbose plates	Reference
pRC2312	Replicative, low copy number, carries <i>URA3</i> and <i>LEU2</i>	Sou ⁻	Cannon <i>et al.</i> , 1992
pCA88	<i>SOU1</i> in pRC2312	Sou ⁺	Wang <i>et al.</i> , 2004
pAK65	Region A of 0.95 kb in pCA88	Sou ⁻	Kabir <i>et al.</i> , 2005
pCA135	Region 135 of 4.3 kb in pCA88	Sou ⁻	Kabir <i>et al.</i> , 2005
pAK156	Integrative, pUC19 based, carries <i>URA3</i> and <i>LEU2</i> , was constructed from pAK45 (Kabir and Rustchenko, 2005). 1.8 kb fragment carrying <i>LEU2</i> was amplified from <i>C. albicans</i> genomic DNA and inserted between <i>Pst</i> I and <i>Hind</i> III sites.	Sou ⁻	This work
pEA249	Integrative, a 1.8 kb fragment carrying <i>ASU53</i> inserted between <i>Bam</i> HI and <i>Sma</i> I sites of pAK156 .	Sou ⁻	This work
pEA254	Same as pEA249, but two 1.8 kb fragments each carrying an <i>ASU53</i> inserted between <i>Bam</i> HI and <i>Sma</i> I sites.		

Plasmids derived in this work by sub-cloning various inserts into pCA88*

pEA104	<i>ASU53</i> with 1.5 kb upstream region**	Sou ⁺⁺
pEA105	<i>CSU53/ASU53</i> natural configuration with ~1.4 kb upstream region of <i>CSU53</i> **	Sou ⁻
pEA130	508 bp of region 135	Sou ⁺
pEA132	1.1 kb portion of region 135	Sou ⁺
pEA133	600 bp portion of region 135	Sou ⁺
pEA140	1.1 kb portion of region 135	Sou ⁺
pEA141	1.0 kb portion of region 135	Sou ⁺
pEA142	<i>ASU53</i> with 900 bp upstream region	Sou ⁺
pEA143	<i>CSU53/ASU53</i> natural configuration with 0.5 kb and 1.1 kb of <i>CSU53</i> or <i>ASU53</i> upstream regions, respectively	Sou ^{-*}
pEA144	<i>CSU53/ASU53</i> natural configuration with ~2.1 kb upstream region of <i>CSU53</i> and ~1.1 kb upstream region of <i>ASU53</i>	Sou ⁻

pEA145	600 bp portion of region 135	Sou ⁺
pEA146	1.1 kb portion of region 135	Sou ⁺
pEA155	<i>ASU53</i> with 500 bp upstream region	Sou ⁺⁺
pEA156	<i>ASU53</i> with 1.0 kb upstream region	Sou ⁺⁺
pEA158	Region 135 with frame-shift mutation at +112 of <i>CSU53</i>	Sou ⁺
pEA162	<i>CSU53/ASU53</i> natural configuration from pEA105 and an extra <i>ASU53</i> from pEA104	Sou ^{-*}
pEA183	<i>ASU51</i> with 256 bp upstream region	Sou ⁺
pEA201	Region 135 with frame-shift mutation at +233 of <i>CSU53</i>	Sou ⁺
pEA205	<i>ASU53</i> with 1.5 kb upstream region and stop codon at +12	Sou ⁺⁺
pEA209	<i>ASU51</i> with 256 bp upstream and 43 bp downstream region	Sou ⁺
pEA219	<i>ASU51</i> with 650 bp upstream and 37 bp downstream region	Sou ⁺⁺
pEA221	<i>ASU51</i> with 650 bp upstream and 43 bp downstream region	Sou ⁺
pEA227	Region 135 with frame-shift mutation at +66 of <i>CSU53</i>	Sou ⁺
pEA232	Same as pEA104, but frame-shift	Sou ⁺⁺

	mutation in <i>ASU53</i> at +4 and +7	
pEA234	Same as pEA104, but <i>ASU53</i> is differently orientated toward <i>SOU1</i>	Sou ⁺⁺
pEA236	Same as pEA209, but <i>ASU51</i> is differently orientated toward <i>SOU1</i>	Sou ⁺⁺
pEA238	<i>CSU51/ASU51</i> natural configuration from pAK65 and an extra <i>ASU51</i> from pEA209	Sou ^{-*}
pEA240	<i>ASU51</i> with 20 bp upstream region	Sou ⁺
pEA243	Same as pEA209, but frame-shift mutation in <i>ASU51</i>	Sou ⁺⁺
pEA261	2,158 bp portion of region 135	Sou ⁺

* Inserts are sub-cloned in pCA88 as *Bam*H1 – *Pst*1, *Pst*1 – *Bam*H1, *Bam*H1 – *Bam*H1, or *Pst*1 – *Pst*1 fragments.

** See Figures 4A and B for the insert positions within region 135 or region A, respectively.

Table S2 LIST of primers

Name	Used for plasmid	Sequence
Analysis of region 135 containing <i>CSU53</i> and <i>ASU53</i>		
AF29 (F)	pEA104	5'–TCG GAA TCC ATA GAT TTC GAC TAT TTT TCT–3'
AF30 (R)		5'–TCG CTG CAG CAA TAT TGT CCA AAA AGA AGG–3'
AF31 (F)	pEA105	5'–TCG GGA TCC TAA GCA GTA GGT TCA TTT GTC–3'
AF32 (R)		5'–TCG CTG CAG TGA TAT GTA CGT TTT TCT GTG–3'
AF72 (F)	pEA130	5'–TCG GGA TCC CTA CTA CTA CAT CAT TCT ATA–3'
AF75 (R)		5'–TCG CTG CAG AGC TAC CAA CCA AGG TGC TAA–3'
AF76 (F)	pEA132	5'–TCG GGA TCC AGT AAT ATT ATT ATT TTT TCT–3'
AF88 (R)		5'–TCG GGA TCC AAC AAT TGA CTT TTC AAC CTA –3'
AF89 (F)	pEA133	5'–TCG GGA TCC ATT GGT GAG AAG GAC AAT TAT–3'
AF79 (R)		5'–TCG GGA TCC ATG AAA TGA TAG TTA AAA ATT–3'
AF92 (F)	pEA140	5'–TCG GGA TCC TTC ACT ATC AAA TCC TCT AAC–3'
AF93 (R)		5'–TCG CTG CAG CAA AGC ATA TCT TCA AAA ACA–3'
AF92 (F)	pEA141	5'–TCG GGA TCC TTC ACT ATC AAA TCC TCT AAC–3'
AF94 (R)		5'–TCG CTG CAG ATT GGA TAC CAT TAA AAC AAG–3'
AF31a (F)	pEA142	5'–TCG GGA TCC ATA ACT GTA GTG GTA GAA CCT–3'
AF95 (R)		5'–TCG CTG CAG CAT CAA AGT GAG AAT GCA ATT–3'
AF92 (F)	pEA143	5'–TCG GGA TCC TTC ACT ATC AAA TCC TCT AAC–3'
AF74 (R)		5'–TCG CTG CAG CGC AAT TAT TTT TTT CTT TTG–3'
AF92 (F)	pEA144	5'–TCG GGA TCC TTC ACT ATC AAA TCC TCT AAC–3'
AF54 (R)		5'–TCG GGA TCC GGA TCT ACC ACT ACT GAG ACC–3'
AF96 (F)	pEA145	5'–TCG GGA TCC AAA ATG CTG CCG GTG AAG TAG–3'
AF97 (R)		5'–TCG CTG CAG TAT CAA AGT ATG GTA ACG AGT–3'
AF98 (F)	pEA146	5'–TCG GGA TCC ATC TTA CGA TGA TGT ATA GAA–3'
AF99 (R)		5'–TCG CTG CAG AAT GAT TTT TGT AAA AAA GTA–3'
AF72 (F)	pEA155	5'–TCG GGA TCC CTA CTA CTA CAT CAT TCT ATA–3'
AF95 (R)		5'–TCG CTG CAG CAT CAA AGT GAG AAT GCA ATT–3'
AF92 (F)	pEA156	5'–TCG GGA TCC TTC ACT ATC AAA TCC TCT AAC–3'
AF95 (R)		5'–TCG CTG CAG CAT CAA AGT GAG AAT GCA ATT–3'
AF134 (F)	pEA162	5'–TCG CTG CAG TAT ATA ACT GTA GTG GTA GAA–3'
AF32 (R)		5'–TCG CTG CAG TGA TAT GTA CGT TTT TCT GTG–3'
AF226 (F)	pEA234	5'–TCG CTG CAG GGA TCT TAC GAT GAT GTA TAG AAA TG–3'
AF227 (R)		5'–TCG GGA TCC GTG TCA ATA TTG TCC AAA AAG AAG G–3'

Analysis of region A containing *CSU51* and *ASU51*

AF215 (R)	pEA183	5'-TCG CTG CAG CAA TCT TTT TAT TCC ATC AAT TAC ACA T-3'
AF216 (F)		5'-TCG GGA TCC GAA ATG TAA AAT GAG GAA TAG GAA AAA C-3'
AF217 (F)	pEA209	5'-TCG GGA TCC GAA TTA AAT TAA GTC CCA TTG GAA TCA-3'
AF224 (R)		5'-TCG CTG CAG TAT AAT CAA CAA TGC AAT TCA CCA AAG-3'
AF216 (F)	pEA219	5'- TCG GGA TCC GAA ATG TAA AAT GAG GAA TAG GAA AAA C-3'
AF223 (R)		5'-TCG CTG CAG CAA CAA TGC AAT TCA CCA AAG TTA TC-3'
AF216 (F)	pEA221	5'-TCG GGA TCC GAA ATG TAA AAT GAG GAA TAG GAA AAA C-3'
AF224 (R)		5'-TCG CTG CAG TAT AAT CAA CAA TGC AAT TCA CCA AAG -3'
AF228 (F)	pEA236	5'-TCG CTG CAG GAA TTA AAT TAA GTC CCA TTG GAA TCA-3'
AF229(R)		5'-TCG GGA TCC TAT AAT CAA CAA TGC AAT TCA CCA AAG-3'
AF217 (F)	pEA238	5'-TCG GGA TCC GAA TTA AAT TAA GTC CCA TTG GAA TCA-3'
AF229(R)		5'-TCG GGA TCC TAT AAT CAA CAA TGC AAT TCA CCA AAG-3'
AF224 (R)	pEA240	5'-TCG CTG CAG TAT AAT CAA CAA TGC AAT TCA CCA AAG-3'
AF230 (F)		5'-TCG GGA TCC TTA GAA GCA CCA GTA CCG TTA TG-3'
AF241 (F)	pEA261	5'-TCG GGA TCC ATG GAA TAG AAT TGAATG AAG GAG TG-3'
AF242 (R)		5'-TCG GGA TCC CGT AAA TAT TTT GAA GAC GCT GC-3'

Frame-shift mutation in *CSU53* at position +112

AF50 (F)	pEA158	5'-AGA TCT GAT TAA ATC ACT GAT TGG AGA TTG TTT CTC CTA T-3'
AF51 (R)		5'-ATA GGA GAA ACA ATC TCC AAT CAG TGA TTT AAT CAG ATC T-3'

Frame-shift mutation in *CSU53* at position +65

CSU53 -1MF	pEA227	5'-GTT GCT GGG GGT GTT GCT GGT TTG GAT TCG AAG CTT TGT GTT GTC -3'
CSU53 -1MR		5'-GAC AAC ACA AAG CTT CGA ATC CAA ACC AGC AAC ACC CCC AGC AAC -3'

Frame-shift mutation in *CSU53* at position +232

CSU53F	pEA201	5'-GGG GGC CGT TGT TAT TGG TAGGTT GTG CCA AAA ATG GCT ATC AG-3'
CSU53R		5'-CTG ATA GCC ATT TTT GGC ACA ACC TAC CAA TAA CAA CGG CCC CC-3'

Stop codon in *ASU53*

ASU53 MF	pEA205	5'–CCA AGG TGC TAA CTT TGC TAC TTA TTC TAC CAT CAA AGC ATA TCT TC–3'
ASU53 MR		5'–GAA GAT ATG CTT TGA TGG TAG AAT AAG TAG CAA AGT TAG CAC CTT GG–
3'		

Frame–shift mutation in *ASU53*

ASU53-FM1	pEA232	5'–GAA GAT ATG CTT TGA TGC GTA CGA ATA TGT AGC AAA GTT AGC ACC–3'
ASU53-FM2		5'–GGT GCT AAC TTT GCT ACA TAT TCG TAC GCA TCA AAG CAT ATC TTC–3'

Frame–shift mutation in *ASU51*

A51AGE1F	pEA243	5'–GAG TTG GAA CCA CCG GTG CAA CGG TAC CGT TAC C–3'
A51AGE1R		5'–GGT AAC GGT ACC GTT GCA CCG GTG GTT CCA ACT C–3'

Rapid Amplification of cDNA Ends (RACE)

3'- and 5'-RACE outer (F1) and inner (F2) general primers were provided with the kit and gene specific 3'- and 5'-RACE outer (F1) and inner (F2) primers are listed below

CSU53

3'-RACE

53CSU3'F1	5'–GCA TGG CAA ACC AGT ATT GTC GGT–3'
53CSU3'F2	5'–TGA TTT CTG GTG CAG TCG GTC CAT–3'

5'-RACE

5CSU53–F1	5'–TGC AGC ATG AGG AGC GTT TCT GTA–3'
5CSU53–F2	5'–AC AGC ACC AAC ACC AGC AAG GA–3'

ASU53

3'-RACE

53ASU3'F1	5'–CTG GCA CAA GTT AAA GAA ACA CCA–3'
53ASU3'F2	5'–CCA CGG TAT AAA GTA CTG AAA CCT–3'

5'-RACE

5ASU53–F1	5'–TCC GGT CAA AAA CCA CCA GGT–3'
5ASU53–F2	5'–TAA AGG TTT GGG GGC CGT TGT–3'

CSU51
3'-RACE

51*CSU3'*F1

5'-CGC TTC ATT AGC CTT AGT TGC TTC C-3'

51*CSU3'*F2

5'-CAC AAC ACC ACT GGT GCT GCT GCT G-3'

5'-RACE

5*CSU51*-F1

5'-TTG ACA GCA GCA CCG GAA CCA GA-3'

5*CSU51*-F2

5'-CCA GCA GCA GCA CCA GTG GTG TTG T-3'

ASU51

3'-RACE

51*ASU3'*F1

5'-TGG GAA CCA GCA CCA CCA GTA GAG-3'

51*ASU3'*F2

5'-TGG AAC CAC CGG CAA CGG TAC CGT-3'

5'-RACE

5*ASU51*-F1

5'-GCC TTA GTT GCT TCC ATC AAC GC -3'

51*ASU5'*F2

5'-ACA ACA CCA CT GGT GCT GCT GCT -3'

Riboprobes

CSU53

AF166

5'-TAA TAC GAC TCA CTA TAG GGA TGG AGC ATT GGT TAA TGG ACC GAC TG-3'

AF167

5'-GTA AGA CTT TCC TTG CTG GTG TTG GTG CTG G-3'

ASU53

AF168

5'-TAA TAC GAC TCA CTA TAG GGG TA A GAC TTT CCT TGC TGG TGT TGG TGC TGG-3'

AF169

5'-CAG TCG GTC CAT TAA CCA ATG CTC CAT-3'

CSU51

AF170

5'-TAA TAC GAC TCA CTA TAG GGA CCA GTG ACG GAG TTG ACA GC A GC-3'

AF171

5'-GCA ATT CAC CAA AGT TAT CGC TTC ATT AGC C-3'

ASU51

AF172

5'-TAA TAC GAC TCA CTA TAG GGG GCT AAT GAA GCG ATA ACT TT G GTG AAT TGC-3'

AF173

5'-GCT GCT GTC AAC TCC GTC ACT GGT-3'

RT-PCR determination of relative amounts of *CSU*/*ASU* transcripts

cDNA

CSU53, C53-RT

5'-TGA CAG CTT CAT ACA CTG TGA ATA CCA-3'

<i>ASU53</i> , A53-RT	5'-GCT GTT GAT TTC GTT GCT GGG GGT GTT GCT GG-3'
<i>CSU51</i> , C51-RT	5'-GTT TCT TCT TAT AAT GTA TTG TTG TGC ACA TC-3'
<i>ASU51</i> , A51-RT	5'-ATT CAC CAA AGT TAT CGC TTC ATT AGC-3'
<i>PGK1</i> , PGK-RT	5'-AAC GAC ACC GTA TTT CTT AGC AAC AGT-3'
<i>CDC6</i> , CDC6-RT	5'-TAT TTC TGG TCC ACC AGG GAC GG-3'

PCR

CSU53 or *ASU53*

AC53-FR1	5'-TGC AGC ATG AGG AGC GTT TCT G-3'
AC53-FR2	5'-ACT TTC CTT GCT GGT GTT GGT GCT G-3'

CSU51 or *ASU51*

AC51-FR1	5'-GGT ACC GTT GCC GGT GGT TCC AA-3'
AC51-FR2	5'-GCA GCA CCA ACA GCA GCT AAA GCA-3'

PGK1

PGK-F	5'-GCC CAC TCC TCT ATG GTT GGT CTC G-3'
PGK-R	5'-TGA AGG CCA TAC CAC CAC CAA CA-3'

CDC6

CDC6-F	5'-CCT TCA ACT TCA CCT TCA ATT CCA-3'
CDC6-R	5'-CCG TCC CTG GTG GAC CAG AAA TA-3'

PCR verification of plasmid integrations that carry *ASU53*

M-13U-F	5'-ACG CCA GCG TTT TCC CAG TCA CGA CG-3'
AF237U-R	5'-GAG CAT CAT CTG GAA GCG GGA CGC C-3'

RT-PCR determination of transcriptional changes due to genomic extra copies of *ASU53*

cDNA synthesis with gene-specific primers

<i>ASU53</i> cDNA	5'-CCA TTG GAC ATT CCA AAA TAC AGA AAC GCT-3'
<i>CDC6</i> cDNA	5'-CAC CGT AAG AGT GGT AGT AGT AGC AGC AGT-3'
<i>CSU53</i> cDNA	5'-TGA CAG CTT CAT ACA CTG TGA ATA CCA-3'
<i>EMP24</i> cDNA	5'-AAC TGA CCA CCA TTT GAC TCT GGA GT-3'
<i>SOU1</i> cDNA	5'-ATG GAC ATG TAT AAC CCC CAT CAA CAG CA-3'
<i>TPK2</i> cDNA	5'-ATG ACG AAT CAC CAA CCC CAG CTG TGA-3'

PCR

<i>ASU53</i> SQ-F	5'-GTA GAA TAT GTA GCA AAG TTA GCA-3'
<i>ASU53</i> SQ-R	5'-TGC TGC ATA TCT TAT TGT CAA GGA-3'

CDC6SQ-F	5'-TCC AAC TAA AAC ACC AAC CAA ATC C-3'
CDC6SQ-R	5'-TTG GAA TTG AAG GTG AAG TTG AAG G-3'
CSU53SQ-F	5'-ACT TTC CTT GCT GGT GTT GGT GCT G-3'
CSU53SQ-R	5'-TGC AGC ATG AGG AGC GTT TCT G-3'
EMP24SQ-F	5'-AGC TCA TAA TGT GTT GAT TGC TCC A-3'
EMP24SQ-R	5'-CGG TTT GTC CTT GAG GTG AAT TG-3'
SOU1SQ-F	5'-TGG TGC TGA TGT TGC CAT CTG GT-3'
SOU1SQ-R	5'-TCA GTC CAT GCA ACT CCA GCA TT-3'
TPK2SQ-F	5'-TCA GTC CAT GCA ACT CCA GCA TT-3'
TPK2SQ-R	5'-TCA GTC CAT GCA ACT CCA GCA TT-3'