Appendix A.

Riboregulator Methods and Materials

All references are in Chapter 2.

Plasmid construction, cell strains, reagents. Standard molecular biology techniques were employed to construct all plasmids.[42] Four different plasmid constructs were generated by cloning into the pRS314-Gal and pRS316-Gal shuttle plasmids.[43] Genes and antiswitch constructs were cloned into multi-cloning sites, downstream of a GAL1 promoter. These plasmids contain an *E. coli* origin of replication (f1) and selection marker for ampicillin resistance, as well as a *S. cerevisiae* origin of replication (CEN6-ARSH4) and selection markers for tryptophan (TRP1-pRS314) and uracil (URA3-pRS316) biosynthetic genes in order to select cells harboring these plasmids in synthetic complete media supplemented with the appropriate amino acid dropout solution.[42] In the first plasmid system, pTARGET1, yEGFP was cloned into the multi-cloning site and is located between a GAL1 promoter and ADH1 terminator. In the second plasmid system, pSWITCH1, various antiswitches were cloned between two hammerhead ribozymes which are located between a GAL1 promoter and ADH1 terminator. In the third plasmid system, pTARGET2, a P_{GAL}-Venus-$ADH1_{term}$ construct was cloned downstream of the P_{GAL}-yEGFP-$ADH1_{term}$ construct in pTARGET1. Therefore, pTARGET2 produces two target transcripts when induced with galactose. In the fourth system, pSWITCH2, a P_{GAL}-antiswitch-$ADH1_{term}$ construct was cloned downstream of the P_{GAL}-antiswitch-$ADH1_{term}$ construct in pSWITCH1. Therefore, pSWITCH2 produces two antiswitch constructs when induced by the presence of galactose. Two sets of plasmids, pTARGET1 and pSWITCH1 or pTARGET2 and pSWITCH2, were transformed into *S. cerevisiae* simultaneously and maintained with the appropriate nutrient selection pressure. In these

two plasmid sets, expression of antiswitch constructs and their targets was induced upon the addition of galactose to the media. Oligonucleotide primers were purchased from Integrated DNA Technologies. All genes and antiswitches were PCR amplified in a Dyad PCR machine (MJ Research) with Taq DNA polymerase (Roche). The *yegfp* gene was obtained from pSVA15,[35] and the *venus* gene was obtained from pCS2/Venus[38]. All antiswitch sequences were obtained using custom oligonucleotide design.

All plasmids were constructed using restriction endonucleases and T4 DNA ligase from New England Biolabs. Plasmids were screened by transforming into an electrocompetent *E. coli* strain, DH10B (Invitrogen; F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80d*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K λ^- *rps*L *nup*G), using a Gene Pulser Xcell System (BioRAD) according to manufacturer's instructions. Subcloning was confirmed by restriction analysis. Confirmed plasmids were then transformed into the wild-type W303α *S. cerevisiae* strain (*MAT*α *his3-11,15 trp1-1 leu2-3 ura3-1 ade2-1*) using standard lithium acetate procedures.[44] *E. coli* cells were grown on Luria-Bertani media (DIFCO) with 100 μg/ml ampicillin (EMD Chemicals) for plasmid selection, and *S. cerevisiae* cells were grown in synthetic complete media (DIFCO) supplemented with the appropriate dropout solution (Calbiochem). Plasmid isolation was done using Perfectprep Plasmid Isolation Kits (Eppendorf).

Protein expression assays. Yeast cells were inoculated into synthetic complete media supplemented with the appropriate drop out solution and sugar source (2% raffinose, 1% sucrose) and grown overnight at 30 °C. Cells were back diluted into fresh media to an OD_{600} of 0.1 and grown at 30 °C. For assaying antiswitch activity, this fresh media

contained appropriate concentrations of theophylline (Sigma), caffeine (Sigma), tetracycline (Sigma), or water (negative control), and expression was induced to a final concentration of 2% galactose, or an equivalent volume of water was added (noninduced control). After growing for 3 hours, the GFP and Venus levels were assayed on a Safire (Tecan) fluorescent plate reader set to the appropriate excitation (GFP- 485 nm; Venus- 515 nm) and emission (GFP- 515 nm; Venus- 508) wavelengths. For assaying the antiswitch temporal response, cells were back-diluted into fresh media containing 2% galactose. After growing in inducing media for 3 h, theophylline or water was added and fluorescence was monitored over time. Fluorescence was normalized for cell number by dividing relative fluorescence units (RFUs) by the OD_{600} of the culture.

RNA quantification. Yeast cells were grown according to methods detailed in protein expression assays. Total RNA was extracted using standard acid phenol extraction procedures.[45] Briefly, cells were pelleted and frozen in liquid nitrogen. Pellets were resuspended in a 50 mM NaOAc (pH 5.2) and 10 mM EDTA buffer. Cells were lysed by the addition of SDS to a final concentration of 1.6% and equal volume of acid phenol. Solutions were kept at 65 °C with intermittent vortexing for 10 min. Following cooling on ice, the aqueous phase was extracted, and further extraction was carried out with an equal volume of chloroform. RNA samples were ethanol precipitated and resuspended in water. Total RNA was quantified by OD_{260} readings. RNA samples were DNased (Invitrogen) according to manufacturer's instructions. cDNA was synthesized using gene-specific primers and Superscript III reverse transciptase (Invitrogen) according to the manufacturer's instructions. qRT-PCR was performed on this cDNA using an iCycler iQ system (BioRAD). Samples were prepared using the iQ SYBR green supermix and

primer pairs specific for different templates on dilution series of the cDNA, according to the manufacturer's instructions. Data was analyzed using the iCycler iQ software.

***In vitro* antiswitch affinity experiments.** Antiswitch and target sequences were amplified with primers containing a T7 polymerase promoter. RNA was transcribed using Ampliscribe T7 transcription kits (Epicentre) according to manufacture's instructions, except that transcription was carried out at 42 °C, and for gel-shift assays, antiswitches were radiolabeled by the addition of [α-^{32}P]-UTP to the transcription mix. The RNA was purified on a 15% denaturing gel, eluted, ethanol precipitated, and resuspended in water. RNA was quantified by OD_{260} readings. For nuclease mapping, antiswitches were 5' end labeled with fluorescein (Molecular Probes) by incubating 25 μg of RNA with phosphate reactive label in labeling buffer (0.12 M methylimidazole pH 9.0, 0.16 M EDAC) for 4 hours, according to manufacturer's instructions. Labeled RNA was purified by ethanol precipitation and run on a 12% denaturing gel. Fluorescent bands were excised from the gel, eluted into water for 3 hours at 37 °C, and ethanol precipitated.

For gel shift assays, equimolar amounts (5 nM) of radiolabeled antiswitches and target RNA were incubated in varying concentrations of theophylline at room temperature for 30 min in 15 μL buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$). Following the incubation, 10% glycerol was added to the RNA-target-ligand mixtures, and RNA complexes were separated from free RNA by electrophoresis at 125 V on an 8% polyacrylamide gel in 1X Tris-borate buffer at room temperature for several hours. Gels were dried, and antiswitch mobility was imaged on a FX phosphorimager (BioRAD).

For nuclease mapping, fluorescein-labeled antiswitch RNA was resuspended in buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$), denatured at 65 °C for 3 min, and allowed to slow cool to room temperature. Antiswitch RNA was incubated with varying concentrations of theophylline at room temperature for 15 min. RNase T1 (Ambion) was added to the antiswitch-ligand mixture and incubated at room temperature for 15 min. Cleavage products were visualized using laser-induced fluorescence capillary electrophoresis on a P/ACE MDQ machine (Beckman) using a single-stranded nucleic acid analysis kit (Beckman) according to manufacturer's instructions.

RNA free energy calculations. RNA free energy was calculated with RNAstructure version 3.71.[32]