Supplementary Methods

Construction of Fluorescently Marked Strains

Fluorescently tagged strains were constructed by integrating plasmids pGS62, pGS63, and pGS64 into FY2. These plasmids were constructed from YIplac211 (Gietz and Sugino, 1988) in the following fashion: YIplac211 was digested with *Hind*III, blunt-ended with Klenow polymerase, and then digested with *Sph*I, making it compatible with *Eco*RV and *Sph*I cutting sites. A 157 bp region from the dubious ORF, *YBR209W*, was amplified by PCR, digested with *Eco*RV and *Sph*I, then cloned into this vector. The resulting plasmid was digested with *Eco*RI, blunt-ended with Klenow, then digested with *Kpn*I; into this was then inserted a DNA fragment containing a 161 bp region from the 5' region of *YBR209W* digested with *Eco*RV and *Kpn*I. *Eco*RI and *Sph*I were then used to cut this plasmid in the middle of the *YBR209W* region and into this was then inserted a fragment of DNA containing the *ACT1* promoter amplified from pRB2138 (Doyle and Botstein, 1996), coupled with either Green Fluorescent Protein (GFP) amplified from pFA6a-GFPS65T-HisMX6 (Wach et al., 1997), Yellow Fluorescent Protein (YFP) amplified from pDH5 (http://depts.washington.edu/yeastrc/), or a red fluorescing protein (DsRED1.T1) amplified from pTY24 (http://depts.washington.edu/yeastrc/); each was followed by the *ACT1* terminator amplified from pRB2132 (Doyle and Botstein, 1996). These inserts were generated using fusion PCR followed by digestion with *Eco*RI and *Sph*I. The resulting plasmids were linearized with *Hind*III and transformed into FY2 to integrate at the 5' region of *YBR209W* using a lithium acetate protocol (Gietz et al., 1995). Integration was confirmed by PCR. The resulting strains were GSY1136

(YBR209W::YIplac211-*ACT1*p-GFP-*ACT1*t), GSY31137 (YBR209W::YIplac211-*ACT1*p-YFP-*ACT1*t), and GSY1135 (YBR209W::YIplac211-*ACT1*p-DsRED.T1.N1-*ACT1*t).

FACS error estimation

The error of FACS measurements were determined by mixing approximately equal numbers of parental strains expression GFP, YFP, or DsRED, followed by FACS analysis. Three different mixtures were generated, and each mixture was measured 3 times by the FACS. The results are shown in Supplementary Figure 1. The error of FACS measurement is less than 1%.

Fluor bias

The potential biases in the different fluorescent proteins were tested by serial batch transfer experiments with equal numbers of parental strains expressing GFP, YFP, or DsRED in the starting population. Approximately 10,000 cells were serially transferred every 24 hours in 200 μl of 0.25% glucose minimum medium aerating on a roller drum at 30 °C. The cells undergo approximately 10 generations per 24 hour period. The results for three replicate experiments are shown in Supplementary Figure 2. No significant bias in fitness was observed between the three fluors from the serial batch transfer experiments.

Allelic frequency test

The primers used for the population allelic frequencies were tested for their ability to estimate the proportion of each allele in a mixture. The wild-type genomic DNA was mixed in with the genomic DNA of M1, M3, M4, or M5 (M2 was not used since it contains an identical mutation as in M4) to generate mixtures with the following percentages of wild-type genomic DNA: 100, 95, 90, 80, 20, 10, 5, 0. Each series was tested at least twice. The allele frequency is calculated using the following equations:

$$\text{frequency of wild-type allele} = 1/(2^{(\text{Ct wild-type} - \text{Ct mutant})} + 1)$$

$$\text{frequency of mutant allele} = 1 - \text{frequency of wild-type allele}$$

The results are shown in Supplementary Figure 3A. For the estimation of the *HXT6/7* frequency in the population, M4 genomic DNA was mixed in with a parental genomic DNA to generate mixtures with the following percentages of wild-type genomic DNA: 100, 95, 90, 80, 70, 60, 50, 40, 30, 20, 10, 5, and 0. Each series was repeated 3 times. The calculation of allelic frequency in the mixture was estimated by using a copy number of 8 for the *HXT6/7* allele in M4. The following formula was used to estimate the frequency of the *HXT6/7* allele:

$$\text{frequency of wild-type HXT6/7 allele} = -(2^{(\text{Ct wildtype} - \text{Ct mutant})} - 8)/7$$

$$\text{frequency of mutant } \textit{HXT6/7} \text{ allele} = 1 - \text{frequency of wild-type } \textit{HXT6/7} \text{ allele}$$

The results are shown in Supplementary Figure 3B. Note, the measurement of the *HXT6/7* mutant frequency was not as accurate as for the other mutations, and thus the estimation of the *HXT6/7* amplification frequency in the population is only a rough estimate.

Gene Expression Data

SAM analysis (Tusher et al., 2001) found a total of 393 genes to be significantly induced or repressed in at least one of the adaptive clones assayed. As expected, the nonsense mutation in *MTH1* in adaptive mutant M1 resulted in an increased expression of *HXT1* and *HXT4*, while the genes whose expression was induced in M3 were also enriched for those involved in hexose transport. Even though these genes are known to be affected by the PKA signaling pathway, it is unclear whether the mutation in *IRA1*, which results in a conservative substitution, was the direct cause of their differential expression. Disruption of *RIM15* has previously been shown to affect genes involved in stress response (*HSP12*, *HSP26*, *SSA1*, *CTT1*Reinders et al., 1998). Indeed, these genes were repressed in M4, indicative of a nonfunctional *RIM15*. In addition, the amplification in *HXT6/7* in M4 also resulted in an increase in gene expression of *HXT6*, but not *HXT7*. Surprisingly, even though an amplification was also observed in the region of *HXT6/7* in M5, the increase in *HXT6* gene expression was not as significant. The mutation in *GPB2* in M5 may result in an increase in PKA activity. Comparison of our gene expression data to a published list of genes whose transcript abundances change in response to activation of RAS or GPA2 (Wang et al., 2004), showed that several genes previously found to be induced or repressed by PKA activation were also similarly induced or repressed in M5.

Supplementary methods references

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