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PLoS GENETICS

A Tradeoff Drives the Evolution of Reduced Metal Resistance in Natural Populations of Yeast

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Abstract

Various types of genetic modification and selective forces have been implicated in the process of adaptation to novel or adverse environments. However, the underlying molecular mechanisms are not well understood in most natural populations. Here we report that a set of yeast strains collected from Evolution Canyon (EC), Israel, exhibit an extremely high tolerance to the heavy metal cadmium. We found that cadmium resistance is primarily caused by an enhanced function of a metal efflux pump, *PCA1*. Molecular analyses demonstrate that this enhancement can be largely attributed to mutations in the promoter sequence, while mutations in the coding region have a minor effect. Reconstruction experiments show that three single nucleotide substitutions in the *PCA1* promoter quantitatively increase its activity and thus enhance the cells' cadmium resistance. Comparison among different yeast species shows that the critical nucleotides found in EC strains are conserved and functionally important for cadmium resistance in other species, suggesting that they represent an ancestral type. However, these nucleotides had diverged in most *Saccharomyces cerevisiae* populations, which gave cells growth advantages under conditions where cadmium is low or absent. Our results provide a rare example of a selective sweep in yeast populations driven by a tradeoff in metal resistance.

Citation: Chang S-L, Leu J-Y (2011) A Tradeoff Drives the Evolution of Reduced Metal Resistance in Natural Populations of Yeast. PLoS Genet 7(3): e1002034. doi:10.1371/journal.pgen.1002034

Editor: Jianzhi Zhang, University of Michigan, United States of America

Received November 18, 2010; **Accepted** February 9, 2011; **Published** March 31, 2011

Funding: This work was supported by Academia Sinica of Taiwan, National Science Council of Taiwan (grant no. NSC99-2321-B-001-031), and the Human Frontier Science Program (grant no. RGY53/2007). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Unicellular microorganisms are often challenged by fluctuating environmental conditions. Especially for those organisms having limited mobility, adaptation to such environmental stresses is critical for survival of their populations. However, mutations beneficial for survival in one environment may impose a cost under other conditions [1,2]. Cells need to fine-tune the evolved gene function or regulation in order to maintain an optimal physiology under a range of conditions. It is important to understand how cells adapt to novel or adverse environments. Such information may not only allow us to dissect the factors affecting evolution of organisms, but may also provide us some insights into pathway or functional network flexibility (or evolvability) of the cell. To address this issue, identifying the mutations responsible for the adaptive phenotypes is the most direct approach, and yet it remains challenging even in simple organisms such as *E. coli*. Moreover, even if a mutation is identified, detailed population and phylogeny data are required in order to deduce the evolutionary trajectory of adaptive traits.

Experimental evolution represents a simplified approach since it allows scientists to follow the evolutionary history of populations exposed to known selective pressures. Several adaptive mutations in microorganisms have been discovered and characterized at the molecular level from laboratory experimental evolution, adding greatly to our understanding of adaptive evolution [1,3–9]. On the other hand, studies related to natural adaptation are more complicated. Although the mechanistic basis or phylogeny of adaptive traits have been revealed in several previous studies [10–15], systematic approaches dealing with both aspects are still rare [16–18].

Metal ions such as copper, iron, zinc, potassium and sodium are essential nutrients involved in a broad range of biological processes [19,20]. These essential metals function as catalysts for biochemical reactions, stabilizers of protein structures or cell walls, or regulators of intracellular osmotic balance. Despite their importance, unbalanced metal concentrations can cause deleterious effects, sometimes leading to programmed cell death [21], and thus represent a double-edged sword. It is important for cells to tightly regulate homeostasis of these metal ions.

In natural environments, cells often encounter other nonessential metal ions. Some of them such as cadmium, lead and arsenic are highly toxic to cells. Toxicity often occurs through the displacement of essential metals from their native binding sites or through ligand interactions, resulting in altered structural conformations or interference with biochemical reactions [22]. These metal ions can induce the generation of reactive oxygen species and cause damages to various cellular components [23–25]. Organisms have evolved several different mechanisms to cope with metal induced stresses, including specific metal transporters, metal sequestration proteins or compartments, and different detoxification enzymes [12,22,26,27]. These various systems often cooperate with each other to quickly respond to variations in environmental metal concentrations, indicating the importance of metal ion balance to cells.

Author Summary

Understanding the genetic and molecular bases of adaptive mutations allows us to gain insight into how new biological functions evolve. In natural populations, examples in which adaptive mutations are characterized at the molecular level are still rare. We studied wild yeast strains isolated from Evolution Canyon (EC), Israel, that exhibit an extremely high tolerance to the heavy metal cadmium. We found that high cadmium resistance was mainly caused by DNA sequence changes in the promoter of a metal transport gene, *PCA1*.These mutations increase *PCA1* gene expression, thus leading to a more efficient cadmium pump-out. Comparison among different yeast species shows that the critical nucleotides found in EC strains are conserved and functionally important for cadmium resistance in other species, suggesting that they represent an ancestral type. When the *PCA1* sequence and the cadmium resistance in different *S. cerevisiae* populations collected globally were compared, we found that most populations carried weak *PCA1* alleles and had a low cadmium tolerance. Since cells carrying the strong *PCA1* allele grow slowly under low-cadmium conditions, it is likely that the tradeoff between cadmium resistance and growth rate drives the evolution of reduced cadmium tolerance in most *S. cerevisiae* populations.

In this study, we observed that a subset of diploid yeast strains collected from different locations of the EC could tolerate a heavy metal, cadmium, to a level unseen in most known yeast strains. We found that the cadmium-resistant phenotype is primarily caused by regulatory changes in the *PCA1* gene, which encodes a P-type ATPase required for cadmium efflux [28,29]. By performing functional assays and phylogenetic analyses, we show that *PCA1* has experienced several rounds of selective adaptation during yeast evolution. More strikingly, we observe that a weak *PCA1* allele spread to most *S. cerevisiae* populations, probably due to a tradeoff between metal resistance and fitness under low cadmium conditions.

Results/Discussion

One subset of EC yeast strains is highly resistant to cadmium

Evolution Canyon is an east-west-oriented canyon at Lower Nahal Oren, Israel. It originated 3–5 million years ago and is believed to have experienced minimal human disturbance [30,31]. In contrast to other wild yeast, the strains collected from EC are often polyploid and most of them are heterothallic [32,33]. Previous studies have revealed high allelic diversity among EC yeast strains [32,33]. To assess whether these strains also carry specific adaptive phenotypes, we performed a panel of phenotypic assays including cell growth under several stress conditions. Only diploid strains were included in this study since triploid and tetraploid strains are less amenable to further genetic analyses. The results showed that a subset of EC strains (EC9, 10, 35, 36, 39, 57, and 58) was resistant to a very high concentration of cadmium (0.8 mM $CdCl_2$), while all other strains analyzed were unable to grow on plates containing 0.2 mM $CdCl_2$ (Figure S1A).

Because chromosomal rearrangement has been suggested to be involved in adaptive evolution [17,34,35], we first examined the karyotype of 14 diploid EC strains. Pulsed-field gel electrophoresis (PFGE) analysis revealed that these EC strains comprised three major karyotypes, EC-C1, EC-C2 and EC-C3 (with some minor deviations)(Figure S1B). Interestingly, all cadmium-resistant strains belong to EC-C1, suggesting that the metal-resistant phenotypes have already evolved before the EC-C1 populations split. Therefore, we chose to use mainly one strain (EC9) from EC-C1 for subsequent genetic analyses.

The cadmium-resistant phenotype is caused by a mutant allele of *PCA1* (*PCA1-C1*)

We performed a genetic analysis to assess how many genetic loci are involved in the cadmium-resistant phenotype. A haploid Cd-resistant clone (EC9-8) was crossed with two Cd-sensitive strains (EC13 from EC-C2 and lab strain S288C). Both hybrid diploids were Cd resistant, indicating that the Cd-resistant phenotype was dominant. The hybrid diploids were then induced to sporulate, and their haploid segregants were examined for their cadmium tolerance. These segregrants showed a 2:2 (resistant to sensitive) segregation pattern, indicating that the Cd-resistant phenotype was primarily controlled by a single genetic locus.

To screen for the gene responsible for cadmium resistance, a genomic DNA library constructed from EC9 genomic DNA was transformed into Cd-sensitive cells. All Cd-resistant colonies carried plasmids containing *PCA1*. Sequencing the *PCA1* allele (*PCA1-C1*) of the EC-C1 strains revealed many mutations in both promoter and protein-coding regions as compared with the *PCA1* sequences from other strains (Table S1). Next, we directly tested whether *PCA1-C1* alone is able to improve the cadmium tolerance of cells. Plasmids carrying *PCA1-C1* were transformed into three Cd-sensitive strains (S288C, SK1, and YJM789) in which the *PCA1* gene had been deleted. As shown in Figure 1A, the different strains carrying *PCA1-C1* all exhibited a level of cadmium resistance close to the level of EC-C1. By contrast, when the *PCA1* alleles from Cd-sensitive strains were transformed into an EC9 *pca1*Δ mutant, the transformants remained cadmium sensitive (Figure 1B). Finally, we sequenced the *PCA1* alleles of the segregants obtained from the previous genetic analysis and confirmed that all Cd-resistant segregants carry the *PCA1-C1* allele.

A previous study by Adle and co-workers has shown that the PCA1-dependent cadmium resistance is mainly a consequence of active cadmium export (efflux) [29]. To determine whether cadmium efflux is higher in cells containing PCA1-C1, cells carrying *PCA1-C1* or *PCA1-SK1* were pretreated with cadmium, washed to remove extracellular cadmium, resuspended in fresh media, and collected at different time points to measure the cellular cadmium content using inductively coupled plasma-atomic emission spectroscopy (ICP-AES). The *PCA1-SK1* allele from the SK1 strain was chosen for comparison because this allele is phylogenetically more related to *PCA1-C1* based on an analysis of the corresponding ORF sequences (Figure S2D) and because it is a cadmium-sensitive allele lacking the G970R mutation (which abolishes the activity of Pca1) present in other laboratory strains. Indeed, cells carrying *PCA1-C1* could reduce the intracellular cadmium concentration more quickly than cells carrying *PCA1-SK1*. These data indicate that cells containing *PCA1-C1* have a very efficient cadmium efflux (Figure 1C).

Mutations in both the promoter and coding regions of *PCA1-C1* contribute to cadmium resistance

In order to understand how *PCA1-C1* has evolved a high cadmium resistance, chimeric proteins with regions from *PCA1-C1* and *PCA1-SK1* were constructed and assayed for their ability to complement cadmium sensitivity of the *pca1*Δ mutant. We found that swapping the promoters drastically affected cadmium resistance (Figure 2A, compare C1 with H6 and SK1 with H3), whereas

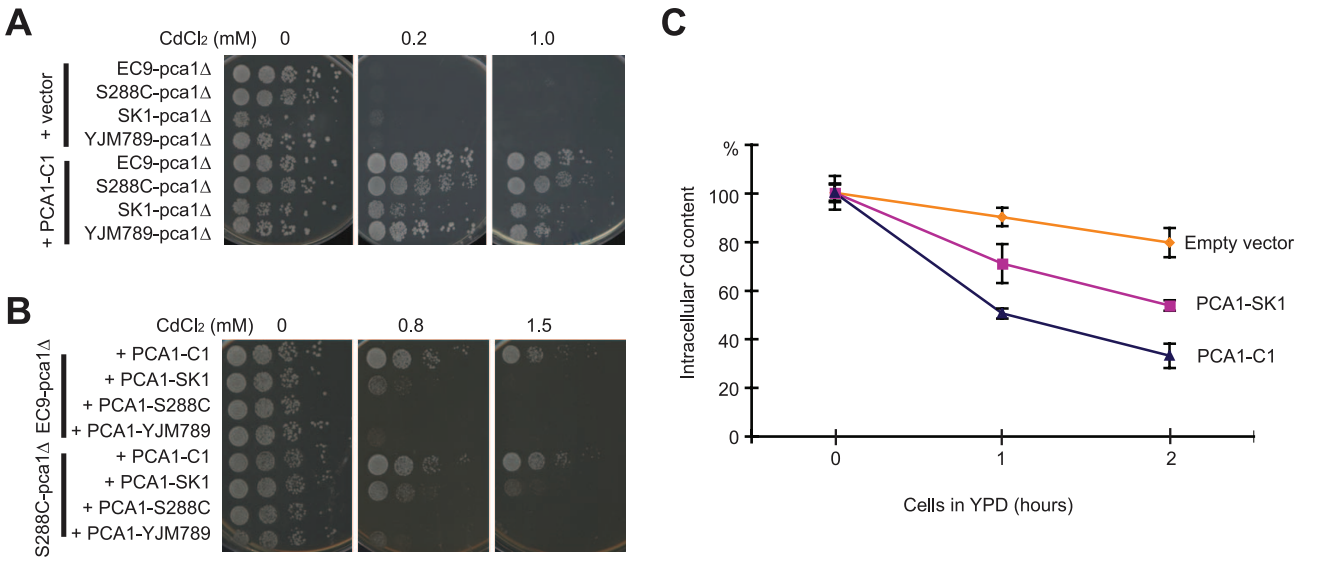

Figure 1. High cadmium resistance is mainly caused by a mutant allele of *PCA1* (*PCA1-C1*). (A) *PCA1-C1* isolated from EC-C1 strains enables the Cd-sensitive strains (S288C, SK1, and YJM789) to tolerate high levels of cadmium. A plasmid containing *PCA1-C1* was transformed into different strains, in which the wild-type copy of *PCA1* had been deleted. Cells were then grown in YPD media overnight, serially diluted, and plated on YPD plates containing different concentrations of $CdCl_2$. (B) *PCA1-C1* is a main contributor of the Cd-resistant phenotype in EC9. *PCA1* alleles from different strains were cloned into plasmids and transformed into either EC9 or S288C *pca1*Δ mutants. Only cells carrying *PCA1-C1* were able to tolerate high levels of cadmium. (C) PCA1-C1-containing cells have a stronger cadmium efflux. Cells carrying *PCA1-C1* or *PCA1-SK1* were pretreated with 0.4 mM $CdCl_2$ for 1 h, washed to remove extracellular cadmium, resuspended in fresh media, and collected at different time points to measure cellular cadmium content using inductively coupled plasma-atomic emission spectroscopy (ICP-AES). Data represent the mean ± s.e.m. of three biological replicates.
doi:10.1371/journal.pgen.1002034.g001

swapping the region between amino acids 207 and 1216 had a mild effect (C1 vs. H1 and H2 vs. H3). Four nonsynonymous mutations are present in this coding region: N223, T358, T363, and G365. Previous studies have identified a few domains important for the stability or function of Pca1 [29,36]. However, none of these four mutations are located within these functional domains.

To assess whether the different levels of cadmium resistance resulted from differences in promoter strength, we fused the *PCA1-C1* or *PCA1-SK1* promoters to a luciferase reporter and assayed the luciferase activity of these constructs. The expression driven by the *PCA1-C1* promoter was about four-fold higher than that driven by the *PCA1-SK1* promoter in the absence and presence of cadmium treatments, suggesting that mutations in the *PCA1-C1* promoter increased the degree of cadmium resistance by increasing the *PCA1* gene expression without destroying its regulation (Figure 2B). We also performed quantitative PCR to determine the level of *PCA1* mRNA in EC9 and SK1 strains. The data were consistent with the results from the luciferase reporter gene assay.

The increase in *PCA1-C1* expression is mainly caused by three point mutations in the promoter region

By comparing the promoter sequences (including 600 bp upstream of the initiation codon) of *PCA1-C1* and *PCA1-SK1*, we observed 18 single nucleotide polymorphisms (17 single nucleotide substitutions and one 1-bp deletion) and one 10-bp insertion (Table S1A). Because altered *PCA1-C1* expression plays a key role in enhancing cadmium resistance, we sought to understand how this gene evolved and the degree to which changes in its promoter contribute to expression differences. To address the latter issue, we fused chimeric promoters with regions from *PCA1-C1* and *PCA1-SK1* promoters to a luciferase reporter and assayed their expression levels. We found that only the region immediately upstream of the initiation codon (−213 to −1) contributed significantly to the enhanced gene expression (Figure 2B, p5-2). Only 6 of the 18 single nucleotide polymorphisms are present in this region. To determine which mutations led to the enhanced promoter activity, we introduced the *PCA1-C1* version of each of these sites into the *PCA1-SK1* promoter and assayed the reporter gene expression. Only mutations in three nucleotides (*PCA1-SK1* to *PCA1-C1*: −97T > C, −148T > G, and −159G > T) had obvious effects (Figure 2C; Table S1A).

To confirm that the changed expression is important for the cadmium resistance, we introduced the same mutations into the *PCA1-SK1* allele and then measured the cadmium resistance of cells carrying these mutant alleles. The expression level of the *PCA1-SK1* mutants was indeed correlated with the cadmium resistance (Figure 2D). When all three mutations were combined together into a single mutant clone (*PCA1-SK1+2/5/6*), cells carrying this clone were as resistant to cadmium as the cells carrying *PCA1-H3*, in which the *PCA1-C1* promoter is fused to the *PCA1-SK1* ORF. This result demonstrated that the enhanced cadmium resistance of EC-C1 strains is mainly caused by three single nucleotide substitutions in the promoter of *PCA1-C1*. Currently, it is still unclear how *PCA1* transcription is regulated. Although we could not identify any transcription factor binding motif in the sequences where the critical mutations (−97C, −148G and −159T) are located, it is quite possible that these regions contain some of the regulatory elements of *PCA1*.

Cadmium resistance is an ancestral phenotype

To determine whether the Cd-resistant phenotype is specific to EC-C1 strains, we examined the cadmium sensitivity of two closely related species, *S. paradoxus* and *S. mikatae*. Two *S. mikatae* strains and 28 *S. paradoxus* strains isolated from different niches were

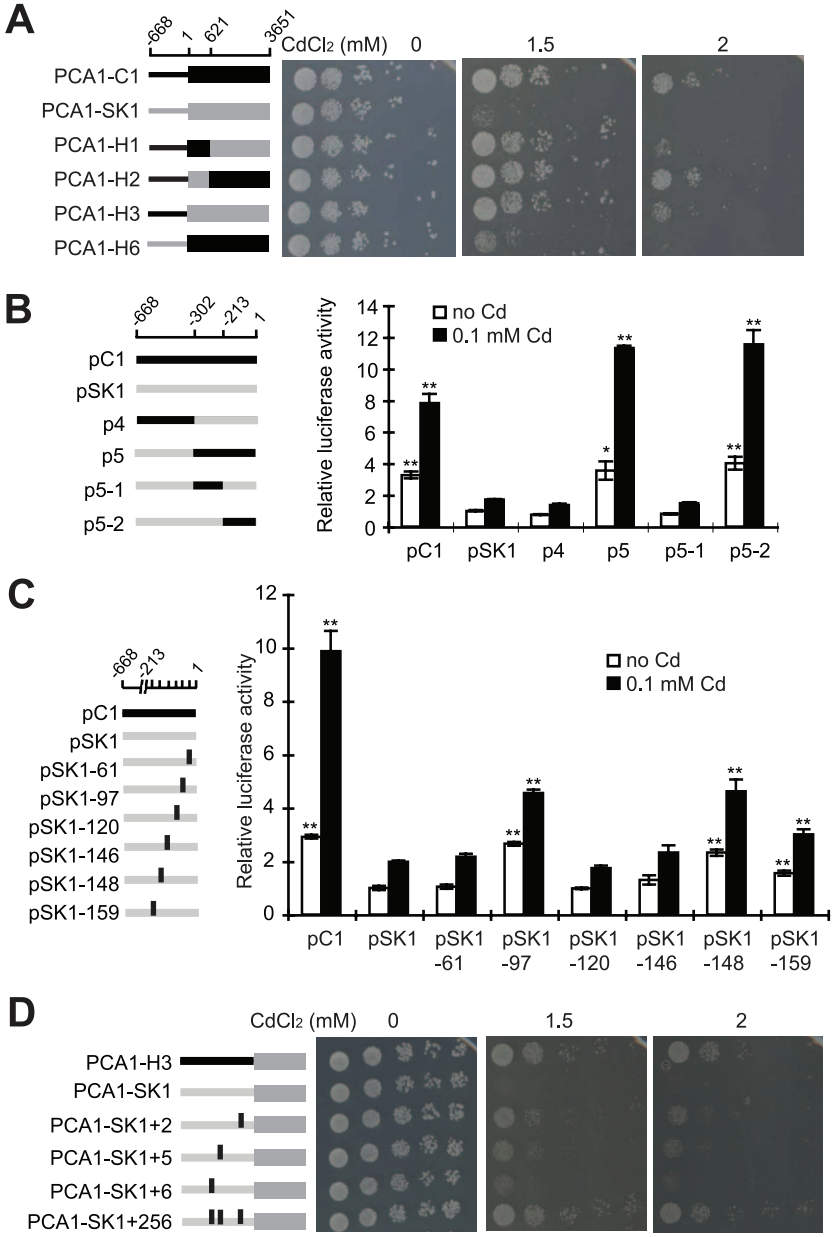

Figure 2. Three nucleotides in the *PCA1-C1* promoter contribute largely to the enhanced *PCA1-C1* function. The thin horizontal line represents the *PCA1* promoter and the box represents the coding region. The number 1 indicates the first bp of the coding region. (A) Both the promoter and coding region between amino acids 207 and 1216 contribute to the enhanced *PCA1-C1* function; however, the promoter has a major effect. Different chimeric *PCA1* constructs are shown in the left panel. Regions derived from EC9 and SK1 are shown in black or grey, respectively. Different hybrid *PCA1* plasmids were transformed into an EC9 *pca1*Δ mutant, and the transformants were assayed for their cadmium tolerance. (B)(C) The activity of the *PCA1-C1* promoter is significantly higher than that of the *PCA1-SK1* promoter. Essentially, three point mutations give rise to this enhanced activity. Hybrid promoters were constructed by fusing different regions of *PCA1-C1* and *PCA1-SK1* promoters to a luciferase reporter. The different constructs were then transformed into *pca1*Δ mutant cells to test their expression (B). The region between 1 and 213 bp upstream of the initiation codon is critical for the enhanced *PCA1-C1* promoter activity. There are six mutations (five single nucleotide substitutions and one 1-bp deletion) in this region as compared with the *PCA1-SK1* promoter. Each individual mutation was introduced into the *PCA1-SK1* promoter and tested for its effects on the promoter activity (C). Three of them (bp −97, −148 and −159) were found to have obvious effects. The numbers indicate the nucleotide positions where mutations were introduced. Data represent the mean ± s.e.m. of three biological replicates. Asterisks indicate that the expression level of the mutant allele was significantly different from the pSK1 allele (*, $p<0.01$; **, $p<0.001$, two-tailed *t*-test). (D) The expression level of *PCA1* is correlated with the cadmium-resistant level of cells. The critical mutations were introduced into the *PCA1-SK1* allele individually or together and tested for their effects on the cadmium resistance of cells.
doi:10.1371/journal.pgen.1002034.g002

tested [37]. Although both species could not tolerate, unlike EC-C1, a high level of cadmium (2.0 mM $CdCl_2$), they exhibited much higher Cd resistance (0.4–0.8 mM $CdCl_2$) than the other *S. cerevisiae* strains (Figure S3). To confirm that the medium level of cadmium resistance in *S. paradoxus* was also mediated through *PCA1*, we cloned the *S. paradoxus PCA1* gene (*Sp-PCA1*) into a plasmid, transformed the resulting vector into *S. cerevisiae pca1*Δ mutant cells, and then assayed the transformants for cadmium

sensitivity. The result showed that *Sp-PCA1* was able to generate a medium level of cadmium resistance in *S. cerevisiae pca1*Δ mutants (Figure S3B). Consistent with these results, deletions of *PCA1* in *S. paradoxus* strains resulted in a Cd-sensitive phenotype (Figure S3C).

Interestingly, comparison between *S. cerevisiae*-, *S. paradoxus*-, and *S. mikatae-PCA1* promoter sequences revealed that those critical residues (−97C, −148G and −159T) identified in the previous experiment are conserved between EC-C1 strains, *S. paradoxus* (28 strains) and *S. mikatae* (2 strains)(Figure 3). When we mutated these nucleotides of *Sp-PCA1* (−100C, −149G and −162T) to non-EC-C1 *Sc-PCA1* sequences, the mutant allele became cadmium sensitive (Figure S3D), indicating that these nucleotides were also critical for the function of *Sp-PCA1*. Since both *S. paradoxus* and *S. mikatae* can tolerate a medium level of cadmium, it is likely that this phenotype represents an original phenotype of the common ancestor of *S. cerevisiae*, *S. paradoxus*, and *S. mikatae*, which has been lost in most *S. cerevisiae* populations (Figure S4).

Loss of cadmium resistance provides a fitness advantage under cadmium-free conditions in *S. cerevisiae*

If the common ancestor of *S. cerevisiae* and *S. paradoxus* was cadmium resistant, why did most *S. cerevisiae* populations become cadmium sensitive? By comparing the promoter sequences of *PCA1* from EC-C1, EC-C2, EC-C3, and 38 other *S. cerevisiae* strains (collected from various habitats on different continents; see [37]), a striking pattern was revealed: most *S. cerevisiae* strains carry a weak *PCA1* promoter similar to the one in the SK1 strain (Figure 4A). Hence, reduced promoter strength accounts for the cadmium-sensitive phenotype observed in these strains (Figure S4). The only exceptions are EC-C1, UWOPS87_2421, and UWOPS83_787_3. Interestingly, the *PCA1* promoters of UWOPS87_2421 and UWOPS83_787_3 also contain the mutations critical for cadmium resistance (−97C and −159T in UWOPS87_2421 and −97C in UWOPS83_787_3), and both strains show cadmium-resistant phenotypes (Table S1).

A previous study showed that cells overexpressing *PCA1* in a medium without cadmium suffered reduced fitness [38]. To determine whether expression of *PCA1-C1* also imposes a high fitness cost on cells, we conducted a competition assay to measure the fitness of cells containing *PCA1-C1*, *PCA1-SK1* or *PCA1-S288C*. Plasmids carrying either *PCA1-C1*, *PCA1-SK1* or *PCA1-S288C* were transformed into S288C *pca1*Δ mutants. The resulting transformants were then mixed with a reference strain carrying a green fluorescent protein-tagged Pgk1 protein and grown in a medium without cadmium. The results showed that cells containing *PCA1-C1* had a lower fitness than cells containing *PCA1-SK1* or *PCA1-S288C* ($p<0.001$, two-tailed *t*-test), suggesting a tradeoff between high Cd resistance and the fitness of cells under Cd-free conditions (Figure 4B). It is possible that *Sc-PCA1* was selected to reduce the fitness cost, thus resulting in lower Cd resistance if most *S. cerevisiae* cells were constantly living in environments containing low levels of cadmium. Alternatively, *Sc-PCA1* might have been selected, at a cost of reduced Cd resistance, to enhance other activities. Previous studies have suggested that Pca1 is also involved in copper resistance [38]. However, we found that cells carrying *PCA1-C1* or *PCA1-SK1* showed a similar level of copper resistance, indicating that the mutations in *PCA1-C1* are specific to cadmium resistance (Figure S5).

It is unclear why cells carrying *PCA1-C1* have a lower fitness under Cd-free conditions. Since Pca1 is not a highly abundant protein, the fitness reduction is unlikely due to the energy cost for producing extra amounts of the Pca1 protein. *PCA1* belongs to a

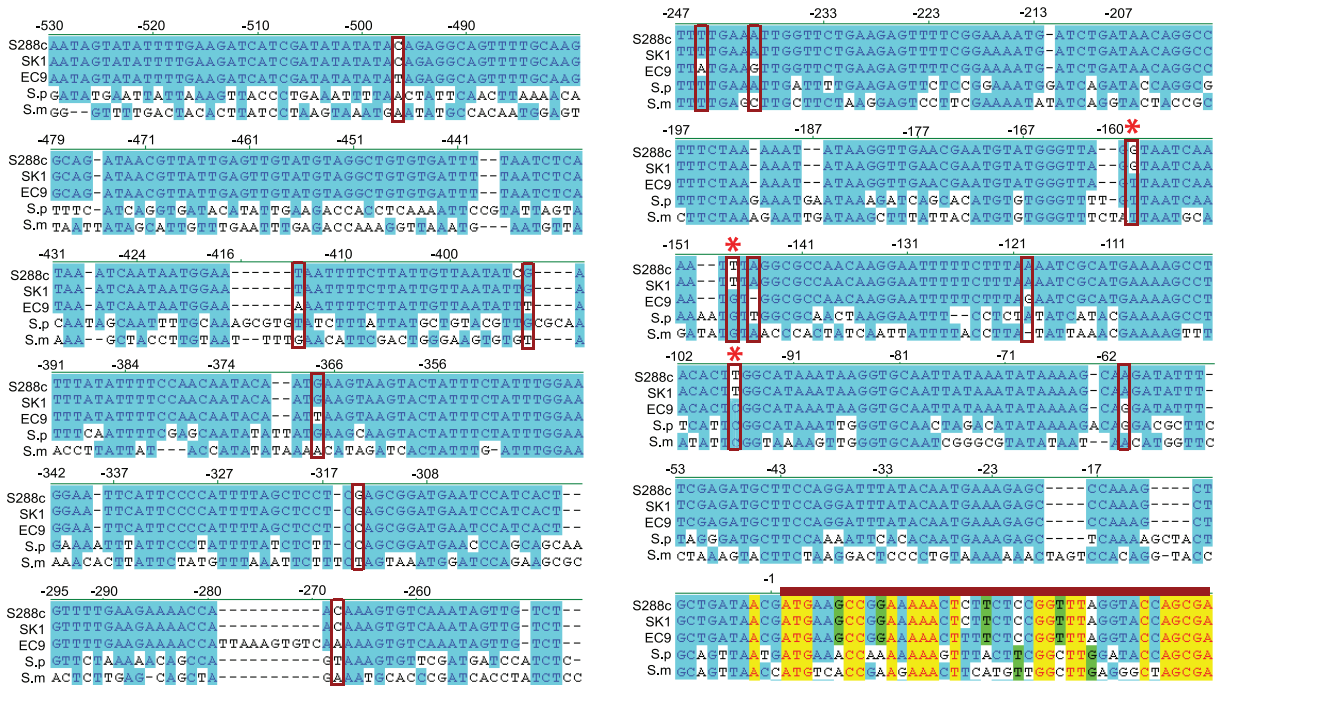

Figure 3. Sequence alignment of *PCA1* promoters from different species. Numbers indicate the nucleotide position upstream of the initiation codon in *PCA1-C1* (from the EC9 strain). Red boxes highlight the nucleotides with polymorphisms between *PCA1-C1* and *PCA1-SK1*. Red asterisks indicate the critical mutations that change the promoter strength. S288C, SK1, and EC9 are chosen as representative strains of *S. cerevisiae*. Only one *S. paradoxus-PCA1* promoter sequence (S.p) is shown in the alignment since the specific nucleotides mentioned in the main text are all conserved between different *S. paradoxus* isolates (see also Figure S7). S.m, *S. mikatae*.
doi:10.1371/journal.pgen.1002034.g003

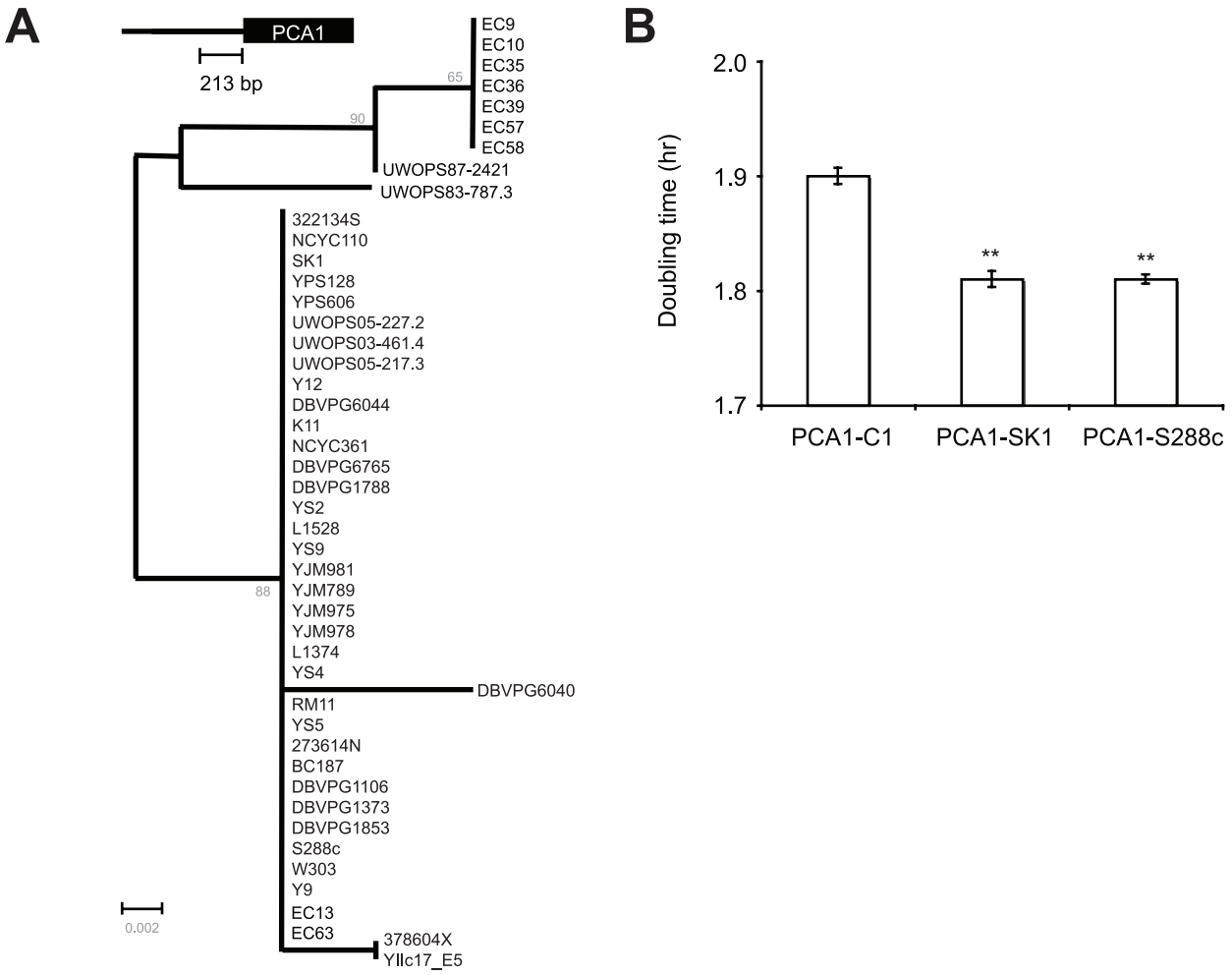

Figure 4. A selective sweep has occurred in *S. cerevisiae* populations probably due to a tradeoff in metal resistance. (A) A phylogenetic tree based on the *PCA1* promoter sequences (including 213 bp upstream of the initiation codon) shows that most *S. cerevisiae* strains contain similar *PCA1* promoter sequences. (B) Cells carrying *PCA1-C1* have a lower fitness as compared to *PCA1-SK1*- or *PCA1-S288C*-containing cells ($p<0.001$, two-tailed *t*-test). Cells were pre-adapted to a non-cadmium medium for one day and those in the early log phase were used for the competitive relative fitness assays in a non-cadmium medium. Data represent the mean ± s.e.m. of five biological replicates.
doi:10.1371/journal.pgen.1002034.g004

P-type ATPase family whose members have been shown to transport metal ions such as cadmium, copper, zinc, cobalt, and lead [39,40]. Hence, the fitness cost of *PCA1-C1* under non-cadmium conditions may result from a depletion of essential metal ions caused by enhanced *PCA1* expression. In *S. cerevisiae*, it has been shown that Pca1 exports cadmium, not copper [28]. However, a study by Adle et al. has also demonstrated that Pca1 affects copper balance by chelating copper ions in a manner analogous to metallothionine [29]. Thus, it is possible that the high expression of *PCA1-C1* depletes copper or other unidentified vital metal ions by metal sequestration or by metal exportation.

In nature, *S. paradoxus* and *S. cerevisiae* were found to occasionally coexist in the same ecological niches [41]. We have shown that most *S. cerevisiae* strains have lost the ancestral cadmium-resistant phenotype probably due to its fitness cost. Why do *S. paradoxus* populations still maintain this phenotype? Using the aforementioned competitive fitness assay, we found that *S. paradoxus* cells carrying either wild-type or low-expression alleles of *Sp-PCA1* exhibit similar fitness under Cd-free conditions (Figure S6). These data suggest that *S. paradoxus* has evolved other mechanisms to offset the fitness cost of high *PCA1* expression.

Evolution of *PCA1* in *S. cerevisiae* populations

In EC-C1 cells, the increase in the expression of *PCA1-C1* was caused by three nucleotide substitutions in the promoter that were also shared by the *S. paradoxus* and *S. mikatae PCA1* genes. Horizontal gene transfer between different species of yeast has been observed in previous studies [42,43]. One possible explanation for the high Cd resistance of EC-C1 strains is that the ancestor of EC-C1 strains acquired a *S. paradoxus PCA1* allele through a horizontal gene transfer event, and that the transferred *Sp-PCA1* function was reinforced later on by natural selection in EC-C1 strains. If that was the case, we would expect to see that the sequence of *PCA1-C1* is more similar to that of *Sp-PCA1* than to sequences of *PCA1* alleles from other *S. cerevisiae* strains. Phylogenetic analyses using the *PCA1* coding or promoter sequences, however, showed that the distance between *Sp-PCA1* and *PCA1-C1* is farther than that between *Sp-PCA1* and other *S. cerevisiae PCA1* alleles, suggesting that *PCA1-C1* was not derived from *Sp-PCA1* (Figure 3 and Figure S7). Moreover, we can rule out the possibility that gene conversion of a small region between *Sc-PCA1* and *Sp-PCA1* has occurred in the ancestor of EC-C1 strains. At alignment of *PCA1* promoter sequences, we found that even in

the region containing the critical nucleotides several nucleotides (−99, −112, −115, −119, −124, and −137) were shared by all *S. cerevisiae* strains, but did not exist in *S. paradoxus* strains (Figure 3); it should be noted, however, that these sequences were also conserved in all 28 *S. paradoxus* strains.

We have shown that, unlike *S. paradoxus* and *S. mikatae*, most *S. cerevisiae* strains except for EC-C1 and two other strains (UWOPS87_2421 and UWOPS83_787_3) are highly sensitive to cadmium. Intriguingly, we found that sequences of the *PCA1* promoter in Cd-sensitive strains showed high identity (Table S1A). When population data were analyzed, a dramatic decrease in the frequency of DNA polymorphisms was observed in this region, inconsistent with the phylogenetic relationship observed in both upstream and downstream regions (Figure 4A and Figure S2; Tajima's $D = -1.94$, $p<0.05$). This result suggests that the cadmium-sensitive phenotype did not evolve independently in different strains. Instead, it was caused by a selective sweep of a weak *PCA1* promoter in *S. cerevisiae* populations. A selective sweep of nonfunctional aquaporin alleles in *S. cerevisiae* populations has been reported recently [44]. However, unlike the aquaporin case, the selective sweep of *S. cerevisiae PCA1* is mainly caused by a single allele and covers a wider range of populations. In addition, the *PCA1* allele involved in the sweep is still functional. In *S. cerevisiae* populations, only the *PCA1* alleles from S288C and W303 have lost the function completely due to a mutation (R970G) in the catalytic domain [28,29]. Expression of *PCA1-SK1* is upregulated when cells sense environmental cadmium and deletion of *PCA1* in other Cd-sensitive *S. cerevisiae* strains makes mutant cells at least 20-fold more sensitive to cadmium.

The reduced cadmium resistance in most *S. cerevisiae* strains is probably a result of regulatory fine-tuning that allows cells to maintain a certain level of cadmium efflux activity, without compromising their fitness, under normal conditions. Such an 'optimized' *PCA1* might explain why this weak allele spread so efficiently to different *S. cerevisae* populations. On the other hand, the EC-C1 strains maintained and even reinforced the ancestral Pca1 activity, probably due to constant selection in their living environments. We measured the soil cadmium concentrations at the collection sites of Evolution Canyon using inductively coupled plasma-atomic emission spectroscopy (see Materials and Methods) and found that they ranged from 2.5 to 4.2 ppm, which is about 17–28-fold higher the median soil cadmium concentration in Europe [45]. It is intriguing that some cadmium-sensitive strains (EC-C2 and EC-C3) were isolated from the same areas as the EC-C1 strains. We found that the *PCA1* sequences of EC-C2 and EC-C3 strains are more closely related to those in the European isolates than to those in EC-C1 strains (Figure 4A and Figure S2). One possible explanation is that these cadmium-sensitive strains arrived in Evolution Canyon more recently and have not yet adapted to the environment. An in-depth study combining genomics and population distribution of the EC strains will help us address this issue.

Phenotypic studies in budding yeast have suggested that resistance to various metal ions in different yeast strains is quite diverse [16,37,46]. A recent genomic analysis of both promoter and coding regions of three *S. cerevisiae* strains also indicates that metal ion transporter genes are significantly enriched in the gene group showing signatures of positive selection [47]. Our data with *PCA1* provide a clear example how a metal transporter gene evolves after experiencing various types of selection that occurred at both inter- and intra-species levels.

In the present study, we found that mutations in the regulatory and coding regions both contribute to the adaptive phenotype. However, the mutations in the regulatory region have a more profound effect as compared to those in the coding region. Recent studies in a variety of organisms have suggested that regulatory changes are critical for adaptive evolution [46,48–53]. It is possible that the promoter is more flexible to accommodate functional changes since it has less structural constraints than the coding region [54]. Our results showed that, by fine-tuning the *PCA1* gene expression, Cd tolerance and cell growth could be dramatically affected in natural yeast isolates, further emphasizing the importance of regulatory changes in evolution.

Materials and Methods

Strains and genetic procedures

EC diploid strains consist of *S. cerevisiae* collected from an east-west-oriented canyon (Evolution Canyon) at Lower Nahal Oren, Israel [32,55]. Our strain numbers are the same as the numbers shown in Figure 2 of reference 26. In brief, EC3, 5, 23, 33, 34, 35 and 36 were isolated from the south-facing slope (SFS), EC7, 9, 10, 39, 40, 45 and 48 were isolated from the valley bottom (VB), and EC13, 14, 57, 58, 59, 60 and 63 were isolated from the north-facing slope (NFS). *S paradoxus*, *S. mikatae* and other *S. cerevisiae* strains were obtained from the collections of Dr. Duncan Greig (University College London, UK) and Dr. Edward Louis (University of Nottingham) [37]. Substitutive and integrative transformations were carried out by the lithium acetate procedure [56]. Media, microbial, and genetic techniques were as described [57].

Karyotyping of EC strains

A total amount of $\sim 2\times10^8$ yeast cells was used for plug preparation. Cells were washed with 1 ml EDTA/Tris (50 mM EDTA, 10 mM Tris-HCl, pH 7.5) and transferred into EDTA/Tris containing 0.13 mg/ml zymolyase (Seikagaku America Inc., St. Petersburg, FL). The cell mixtures were incubated for 30 s at 42°C and then embedded in low melting point agarose (Sigma-Aldrich, St. Louis, MO). The agarose plugs were then incubated at 37°C overnight for zymolyase digestion. After digestion, the agarose plugs were placed in LET solution (0.5 M EDTA, 10 mM Tris-HCl (pH 7.5), 2 mg/ml protease K, and 1% N-lauroylsarcosine) at 50°C overnight. This step was repeated three times. The plugs were transferred to EDTA/Tris solution and dialyzed four times for 1 h at 37°C. Yeast chromosomes were separated in 0.7% agarose gels by pulsed-field gel electrophoresis (PFGE) using a Rotaphor Type V apparatus (Biometra, Göttingen, Germany). Electrophoresis was performed for 48 h at 13°C in 0.5× TBE buffer at a fixed voltage of 120 V and an angle of 115°, with pulse time intervals of 30 s.

Genomic DNA library screening

To construct an EC9 genomic DNA library, yeast genomic DNA was extracted using the Qiagen Genomic-Tip 100/G kit (Qiagen, Valencia, CA), digested with restriction enzymes, and ligated into a yeast vector pRS416 as described [57]. To screen for Cd-resistant genes, a Cd-sensitive lab strain (S288C) was transformed with the EC9 genomic DNA library and plated on cadmium-containing plates (0.4 mM $CdCl_2$). Plasmids isolated from the Cd-resistant colonies were sequenced to identify the insert-containing genes.

Functional assay

To measure the intracellular cadmium concentration, log-phase cells carrying different alleles of *PCA1* were pretreated with 0.4 mM $CdCl_2$ for 1 h, washed with PBS containing 10 mM EDTA, resuspended in rich medium without cadmium, and then

collected at indicated time points. Collected cells were immediately washed with PBS containing 10 mM EDTA. Total intracellular cadmium levels were measured by inductively coupled plasma-atomic emission spectroscopy (ICP-AES).

Quantitative PCR

After cadmium treatment (0.1 mM $CdCl_2$) for 2 h at 28°C, total RNA was isolated from cells using the Qiagen RNeasy Midi Kit (Qiagen, Valencia, CA). First-strand cDNA was synthesized for 2 h at 37°C using the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA). A 20-fold dilution of the reaction products was then subjected to real-time quantitative PCR using gene-specific primers, the SYBR Green PCR master mix, and an ABI-7000 sequence detection system (Applied Biosystems). Data were analyzed using the built-in analysis program.

Reporter assay

To construct the luciferase reporter plasmids, different promoter regions (up to 668 bp upstream of the start codon) of *PCA1-C1* and *PCA1-SK1* were amplified by PCR. The luciferase coding region (from *Renilla reniforms*) was also amplified by PCR. The PCR fragments were co-transformed with pRS416 digested with XhoI and SacI into the lab strain S288C. The genomic DNA of Ura+ colonies was isolated and transformed into component *E. coli* cells. The plasmids from ampicillin-resistant clones were isolated and sequenced. The constructed luciferase reporter plasmids were transformed into an EC9 *pca1*Δ mutant.

Yeast cells carrying different luciferase reporter plasmids were treated with 0.1 mM of $CdCl_2$ for 2 h. After the treatment, 0.5×10^7 cells were harvested for detection of the luciferase activity on a luminometer (PE Victor3 luminometer plus autojector, Perkin Elmer, Waltham, MA). To the test samples, 100 µl of 1 µM substrate (coelenterazine) was added. Following a 5 second equilibration time, luminescence was measured with a 10 second integration time.

Competitive relative fitness assay

We measured the fitness of the testing strains by competing them against a reference strain expressing PGK1::GFP in CSM-URA media at 28°C. The testing cells and reference cells were inoculated in the CSM-URA medium individually and acclimated for 24 h. The cells were subsequently diluted and refreshed in new media for another 4 h. The reference and testing cells were then mixed at a 1:1 ratio, diluted into fresh medium at a final cell concentration of 5×10^3 cells/ml, and allowed to compete for 21 h, which represents about 12 generations of growth. The ratio of the two competitors was quantified at the initial and final time points using a fluorescence activated cell sorter (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ). Five independent replicates for each fitness measurement were performed.

Phylogenetic tree construction

The evolutionary history of the *PCA1* ORF (3651 bps), *SUL1* ORF (2580 bps), *PCA1* promoter (213 bps) and *PCA1-SUL1* intergenic region (820 bps) was inferred using the Neighbor-Joining method [58]. Sequences were obtained from previously released data [37]. The percentage of replicate trees in which the associated taxa were clustered together in the bootstrap test (500 replicates) is shown next to the branches, analogous to a previous study [59]. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [60] and are expressed as number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were conducted in MEGA4 [61]. Tajima's D of the PCA1 promoter (213 bp) was calculated by DnaSP V5 [62].

Measurement of soil cadmium concentrations

Soil samples were collected at 7 locations of Evolution Canyon corresponding to the collection sites of the EC yeast strains (3 at the SFS, one at the VB, and 3 at the NFS). Soil cadmium levels were measured by inductively coupled plasma-atomic emission spectroscopy (ICP-AES) using at least 200 g of individual samples.

Supporting Information

Figure S1 A subset of EC diploid strains is highly resistant to heavy metals. (A) Strains EC9, 10, 35, 36, 39, 40, 57 and 58 can tolerate a high level of cadmium (0.8 mM $CdCl_2$). Diploid EC strains were grown in YPD media overnight, serially diluted, and plated on YPD plates containing different concentrations of $CdCl_2$. Plates were incubated at 28°C until colonies were easily detected. Strains EC3, 5, 7, 14, 23, 40 and 48 were excluded from later analyses because these strains either could not sporulate or produced only dead spores. (B) Analysis by pulsed-field gel electrophoresis reveals that the EC strains consist of three major karyotypes: EC cluster 1 (EC-C1) containing seven strains (EC9, 10, 35, 36, 39, 57, and 58), EC cluster 2 (EC-C2) containing six strains (EC13, 33, 34, 45, 59, and 60), and EC cluster 3 (EC-C3) containing only one strain (EC63). All Cd-resistant strains belong to EC-C1. M, yeast chromosomal DNA from a standard laboratory strain.
(EPS)

Figure S2 Phylogenetic analyses of the *PCA1* alleles from different *S. cerevisiae* populations. (A) An illustration showing the upstream gene (*SUL1*), intergenic, promoter and coding regions of *PCA1*. (B) A phylogenetic tree of different *S. cerevisiae* strains based on the *SUL1* coding sequences (2589 bp). (C) A phylogenetic tree based on the *SUL1-PCA1* intergenic sequences (820 bp). (D) A phylogenetic tree based on the *PCA1* coding sequences (3651 bp). When the *SUL1* or *PCA1* coding regions were used, strains could be subdivided into two major groups: the SK1 group containing most of the North American, West African, Malaysian, Sake and EC-C1 strains, and the W303 group containing most European, clinical and laboratory strains. However, this division disappeared when the intergenic or promoter regions of *PCA1* were used to construct the tree (see also Figure 4).
(EPS)

Figure S3 Cadmium resistance of different yeast species. (A) Cadmium resistance of other yeast species. Diploid *S. cerevisiae* (EC9 and SK1), *S. mikatae* (Sm), and *S. paradoxus* (Sp) strains were grown in YPD media overnight, serially diluted, and plated on YPD plates containing different concentrations of $CdCl_2$. (B) Cells carrying *S. paradoxus PCA1* (*Sp-PCA1*) show a medium level of cadmium resistance. *PCA1* alleles from *S. cerevisiae* or *S. paradoxus* (S36.7) strains were cloned into plasmids, transformed into an EC9 *pca1*Δ mutant, and the transformants were assayed for their Cd resistance. (C) Deletion of *PCA1* in *S. paradoxus* strains results in a Cd-sensitive phenotype. (D) The critical nucleotides observed in the *PCA1-C1* promoter are also important for the function of *Sp-PCA1*. When we mutated these nucleotides of *Sp-PCA1* (−100C, −149G and −162T) to non-EC-C1 *Sc-PCA1* sequences, the mutant allele became cadmium sensitive.
(TIF)

Figure S4 Most *S. cerevisiae* strains are cadmium sensitive. Diploid *S. cerevisiae* strains collected from various habitats on different continents were grown in YPD media overnight, serially diluted, and plated on YPD plates containing different concentrations of $CdCl_2$. Only three strains, EC9, UWOPS87_2421, and UWOPS83_787_3 showed high cadmium resistance.
(EPS)

Figure S5 The enhanced *PCA1-C1* function has no effect on copper resistance. The same constructs used in Figure 2A were assayed for their copper resistance. Cells were grown in YPD media overnight, serially diluted, and plated on YPD plates containing different concentrations of $CuCl_2$.
(EPS)

Figure S6 High expression of *Sp-PCA1* does not affect the fitness of *S. paradoxus* under Cd-free conditions. Plasmids carrying either *Sp-PCA1* or *Sp-PCA1(−100, −149, −162)* were transformed into *S. paradoxus pca1*Δ mutants. The resulting transformants were used for competitive relative fitness assays in a non-cadmium medium. Cells carrying *Sp-PCA1(−100, −149, −162)* have a similar fitness as compared to *Sp-PCA1*-containing cells ($p = 0.5$, two-tailed *t*-test). In the *Sp-PCA1(−100, −149, −162)* mutant, the critical nucleotides (−100C, −149G and −162T) were mutated to non-EC-C1 *Sc-PCA1* sequences (see also Figure S3D). Data represent the mean ± s.e.m. of five biological replicates.
(EPS)

Figure S7 Phylogenetic analyses of the *PCA1* alleles from different *S. paradoxus* populations. (A) An illustration showing the upstream gene (*SUL1*), intergenic, promoter and coding regions of *Sp-PCA1*. (B–D) Phylogenetic trees of EC9, SK1 and different *S. paradoxus* strains based on the *SUL1-PCA1* intergenic and promoter sequences (B), the *PCA1* promoter sequences (C), or the *PCA1* coding sequences (D). All three trees showed that the phylogenetic distances between different *S. paradoxus* strains were closer than those between *S. paradoxus* and *S. cerevisiae* strains.
(EPS)

Table S1 EC strains contain multiple mutations in the *PCA1* promoter and coding regions.
(PDF)

Acknowledgments

We thank G. Lang, A. Murray, C.-T. Ting, and members of the Leu lab for helpful discussions and comments on the manuscript. We also thank H. Kuhn for manuscript editing and the FACS Core of IMB for technique help. We thank M.-T. Hsieh, A. Segre, G. Lidzbarsky, E. Nevo, and E. Louis for providing the plasmid and yeast strains.

Author Contributions

Conceived and designed the experiments: S-LC J-YL. Performed the experiments: S-LC. Analyzed the data: S-LC J-YL. Contributed reagents/materials/analysis tools: J-YL. Wrote the manuscript: S-LC J-YL.

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