Adaptation to diverse nitrogen-limited environments by deletion or extrachromosomal element formation of the *GAP1* locus

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To study adaptive evolution in defined environments, we performed evolution experiments with *Saccharomyces cerevisiae* (yeast) in nitrogen-limited chemostat cultures. We used DNA microarrays to identify copy-number variation associated with adaptation and observed frequent amplifications and deletions at the *GAP1* locus. *GAP1* encodes the general amino acid permease, which transports amino acids across the plasma membrane. We identified a self-propagating extrachromosomal circular DNA molecule that results from intrachromosomal recombination between long terminal repeats (LTRs) flanking *GAP1*. Extrachromosomal DNA circles (*GAP1*^{circle}) contain *GAP1*, the replication origin *ARS1116*, and a single hybrid LTR derived from recombination between the two flanking LTRs. Formation of the *GAP1*^{circle} is associated with deletion of chromosomal *GAP1* (*gap1Δ*) and production of a single hybrid LTR at the *GAP1* chromosomal locus. The *GAP1*^{circle} is selected following prolonged culturing in L-glutamine–limited chemostats in a manner analogous to the selection of oncogenes present on double minutes in human cancers. Clones carrying only the *gap1Δ* allele were selected under various non-amino acid nitrogen limitations including ammonium, urea, and allantoin limitation. Previous studies have shown that the rate of intrachromosomal recombination between tandem repeats is stimulated by transcription of the intervening sequence. The high level of *GAP1* expression in nitrogen-limited chemostats suggests that the frequency of *GAP1*^{circle} and *gap1Δ* generation may be increased under nitrogen-limiting conditions. We propose that this genomic architecture facilitates evolvability of *S. cerevisiae* populations exposed to variation in levels and sources of environmental nitrogen.

adaptive evolution | genome plasticity | intrachromosomal recombination | double minute | retrotransposon

Adaptive evolution is the process by which a population becomes better suited to its environment through mutation and selection. The pace at which a population adapts to a novel environment is determined by the rate at which beneficial mutations arise in the population. In both experimental and natural populations, mutations underlying adaptive evolution include nucleotide changes (1, 2), transposition events (3, 4), and copy-number variants including gene amplifications and deletions (5, 6). Although the relative importance of these different classes of genomic variation for adaptive evolution is not known, their spontaneous rates differ dramatically. In the single-celled eukaryote *Saccharomyces cerevisiae* (budding yeast) the rate of point mutation is 10^{-9}–10^{-10} per base pair/generation (7), whereas rates of retrotransposition are estimated to be on the order of 10^{-8} events/generation (8). Gene deletion events are estimated to occur at a very high rate of around 10^{-5} events/generation (9). Conversely, gene amplification events, which are much more difficult to assay accurately, have been estimated to occur at rates as low as 10^{-10} events/generation (10).

Repetitive elements often result in higher rates of deletion, translocation, and duplication. In the yeast genome, repetitive DNA at the ribosomal DNA (rDNA) (11) and telomeric (12) loci facilitates the formation of extrachromosomal circular DNA. Besides rDNA and telomeric repetitive elements, eukaryotic genomes carry a large number of dispersed repeats, including paralogues, tRNA genes, and transposons. Transposon sequences facilitate a higher rate of recombination than other repetitive elements in the genome (13), and their ability to transpose and rearrange is a driving force in the evolution of eukaryotic genomes (14). In yeast, long terminal repeat (LTR)-containing retrotransposons (Ty1–Ty5) are the dominant class of transposable elements. Ty retrotransposons are typified by two ORFs flanked by tandem LTRs. Ty elements are purged from the genome via intrachromosomal recombination, leaving a scar of a solo LTR and producing an extrachromosomal circle as an intermediate in the excision process (15). As a result of this process, the *S. cerevisiae* genome contains ≈300 solo LTRs dispersed throughout the genome. Given sufficient sequence similarity, solo LTRs remain potential substrates for intrachromosomal recombination.

We have identified a class of extrachromosomal circles that arise upon homologous recombination between solo retrotransposon LTRs flanking the general amino acid permease gene *GAP1*. The resulting circular DNA elements contain *GAP1*, the active replication origin *ARS1116*, and a recombination product of the Ty1 solo LTRs YKRCδ11 and YKRCδ12. The extrachromosomal circle is the product of an intrachromosomal recombination event that results in a solo hybrid LTR at the chromosomal locus. Whereas wild-type yeast carry the chromosomal *GAP1* allele, retention of the extrachromosomal *GAP1* circle (*GAP1*^{circle}) is selected under long-term L-glutamine limitation in chemostats. Deletion of *GAP1* (*gap1Δ*) and loss of the resultant circular DNA are selected under various other nitrogen-limiting conditions in chemostats including ammonium, urea, and allantoin limitation. Thus, a single recombination event can generate two different *GAP1* alleles that confer selective advantages under different nitrogen-limiting conditions. We suggest that this particular genomic architecture has been retained to facilitate evolvability of *S. cerevisiae* populations exposed to dramatically varying nitrogen conditions. We also provide evidence that the *GAP1*^{circle} is able to reintegrate at the original locus, suggesting that *GAP1*^{circle} formation is genetically reversible.

Results

Diverse *GAP1* Alleles Are Recovered from Nitrogen-Limited Populations. To study adaptive evolution of yeast in nitrogen-limited environments, we propagated isogenic populations of prototrophic haploid yeast in chemostats for several hundred generations. In

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these experiments, a single nitrogen source was present at a growth-limiting concentration (Table 1) while all other media components were present in excess. Steady-state populations of $\approx 10^{10}$ cells were established and maintained under continuous culture conditions for a period of about 2 mo. Samples were recovered from the population at regular intervals, and upon completion of the evolution experiment we isolated clones from each population for analysis.

Previous studies have reported a high frequency of gene amplifications and gross chromosomal rearrangements in strains that have undergone adaptive evolution in nutrient-limited environments (5, 16, 17). Therefore, we performed array comparative genomics hybridization (aCGH) using a DNA microarray with probes spaced every ~400 bases to determine whether adaptation to nitrogen limitation is associated with similar genomic variation. In our sample of 21 clonal isolates from 8 independent populations analyzed using aCGH, we observed a high frequency of copy-number variant alleles at the *GAP1* locus (8 of 21 clones; Table 1). These alleles include large-scale amplifications involving many contiguous genes in addition to *GAP1* in clones adapted to L-glutamine limitation (Fig. 1*A*) and L-glutamate limitation (Fig. 1*B*) and deletion alleles in several populations that appeared to include only *GAP1* (Fig. 1*C*). Examination of the likely boundaries of the two amplification alleles did not show evidence of repeated sequences, and these alleles were not examined further.

The *gap1* deletion allele appeared to be flanked by Ty1 solo LTRs YKRCδ11 and YKRCδ12 (Fig. 1*D*), which are 82% sequence-identical and in a tandem orientation. To precisely map the breakpoints of this deletion event, we hybridized genomic DNA to a high-density overlapping tiling array (average probe spacing ~4 bp). We mapped the deletion of *GAP1* to an ≈4.2-kb region spanning nucleotide coordinates 513174–517424 on chromosome XI that lie within the Ty1 solo LTRs YKRCδ11 and YKRCδ12 (Fig. 1*D*).

Loss-of-function mutations in *GAP1*, through either point mutation or deletion, result in resistance to the toxic amino acid D-histidine. We measured the fraction of *gap1* mutants in each population by determining the proportion of D-histidine-resistant (D-His^R) cells using a plate assay. Loss of GAP1 function ranged from 15% to 94% in experimentally evolved populations (Table 1), indicating that one or more *gap1* clones had rapidly increased in frequency in several nitrogen-limiting conditions. To investigate the fitness effect of different *GAP1* alleles, evolved strains were mixed with the ancestral strain and their relative fitness was measured using competitive growth rate assays. *GAP1* chromosomal amplification alleles are associated with higher fitness in clones adapted to L-glutamine and L-glutamate (Table 1). *GAP1* deletion alleles (*gap1Δ*) are associated with higher fitness in clones recovered from ammonium, urea, and allantoin limitation; however, *gap1Δ* clones are not significantly fitter under L-glutamine and L-glutamate limitation (Table 1). This is surprising, given that D-His^R cells are recovered at significant frequency in both these populations.

GAP1 Deletion Occurs via a Homologous Recombination Event. To determine the molecular basis of *GAP1* deletion alleles, we designed PCR primers distal to the two LTRs and sequenced the deletion product in *gap1Δ* strains. These strains included one clone obtained from long-term ammonium limitation and four independent spontaneous *gap1Δ* alleles recovered by selection for resistance to D-histidine. Sequencing confirmed the presence of an intact solo LTR in all five clones that is a hybrid of the LTRs YKRCδ11 and YKRCδ12. We resolved cross-overs to regions of sequence identity of fewer than 20 bases (Fig. 2). The identification of a single LTR containing clearly defined breakpoints is consistent with homologous recombination underlying these deletion events.

To determine the rate of *GAP1* loss-of-function mutations, we performed a Luria–Delbrück fluctuation analysis by testing cultures for acquisition of resistance to D-histidine. We estimate that the rate of mutations inactivating *GAP1* is 3.1×10^{-7} mutations per generation. This mutation rate is similar to other loci of similar target size in the genome (7) and suggests that under normal laboratory conditions—that is, growth in rich media—the rate of *GAP1* loss-of-function mutation through either mutation or recombination is not increased relative to other loci in the genome.

Detection of Circular Extrachromosomal *GAP1*. We noticed that an origin of DNA replication (*ARS1116*) lies proximal to the 3′ end of the *GAP1*-coding region. We hypothesized that deletion of *GAP1* via LTR-mediated homologous recombination could result in a self-replicating extrachromosomal DNA molecule. To test for the existence of this circular DNA, we purified total DNA from clonal isolates recovered from L-alanine, L-glutamine, and ammonium sulfate limitations and used a PCR assay designed to produce a unique fragment only upon circularization of the *GAP1* locus (Fig. 3*A*). In addition, we used PCR primers that unambiguously detect the presence of full-length *GAP1*, the *gap1Δ* allele, and the chromosomal location of *GAP1* (Fig. 3*A*). Whereas the parental strain contains the full-length *GAP1* at the chromosomal locus (Fig. 3*B*, lanes 1 and 4), D-His^R clones recovered from long-term ammonium limitation contain the *GAP1* deletion (Fig. 3*B*, lane 6). DNA from a representative clone from the L-glutamine–limited evolution experiment produces a positive PCR product for the predicted circular product, substantiating the existence of the extrachromosomal *GAP1*^{circle} (Fig. 3*B*, lane 11). Using PCR amplification, we de-

Table 1. Variation of *GAP1* genotypes and fitness in populations subjected to long-term nitrogen limitation in chemostat cultures

Nitrogen source	Generations	*gap1* (%)	*GAP1*^{circle} (%)	Genotypes of selected clones	Relative fitness of clone (gen^{-1})
Ammonium*	400	70	0	RB17 *gap1Δ*†	1.09
L-glutamine*	250	15	12	RB10 *gap1Δ*†	0.99
				CDG23-1 *GAP1* amplification†	1.28
				CDG23-2 *GAP1* amplification†	1.14
				CDG23-3 *GAP1* amplification†	1.31
				RB410 *gap1Δ GAP1*^{circle}	n.d.
				RB411 *gap1Δ GAP1*^{circle}	n.d.
				RB413 *gap1Δ GAP1*^{circle}	n.d.
				RB414 *gap1Δ GAP1*^{circle}	n.d.
L-alanine	250	94	0		
L-glutamate	250	15	n.d.	CDG25-2 *gap1Δ*†	0.93
				CDG25-3 *GAP1* amplification†	1.44
Urea	250	100	n.d.		
Allantoin	250	15	n.d.	CDG29-2 *gap1Δ*†	1.64

n.d., no data.

*Two independent populations were propagated under ammonium and L-glutamine limitation. A single population was analyzed for each of the four additional nitrogen limitations.

†Genotype determined by array comparative genomics hybridization.

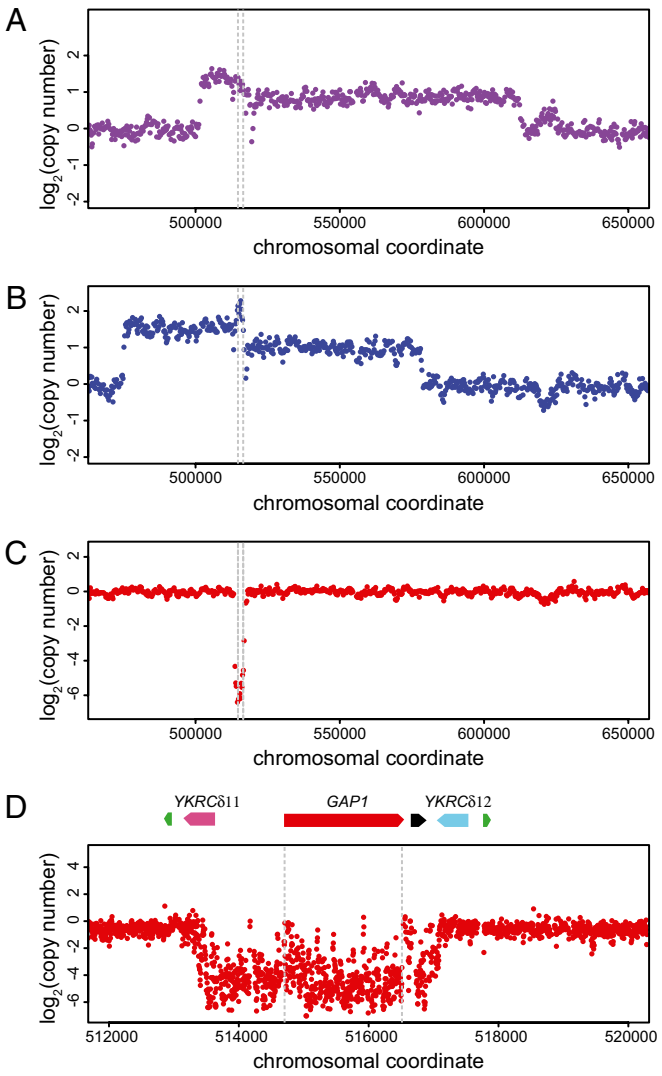

Fig. 1. Gene amplification and deletion alleles at the *GAP1* locus identified by array comparative genomic hybridization. We identified frequent copy-number polymorphisms at the *GAP1* locus in clones subjected to long-term adaptation in diverse nitrogen-limited chemostats. These included unique amplification alleles in clones adapted to L-glutamine (*A*) and L-glutamate (*B*). Each of these amplification alleles is ~100 kb and includes *GAP1* (bounded by gray dashed lines in all plots) and several flanking genes. In addition, we identified deletion events that include only the *GAP1* gene in several clones, including one adapted to allantoin limitation (*C*). We hybridized DNA from the allantoin-adapted clone to an overlapping tiling microarray (*D*) and determined that the deletion event is bounded by two flanking long terminal repeats: YKRCδ11 and YKRCδ12. The locus also includes an origin of replication (black) and two tRNA genes (green) that lie distal to the LTRs.

termined that the clone containing the $GAP1^{circle}$ has a full-length copy of *GAP1* and a deletion at the chromosomal *GAP1* locus (Fig. 3*B*, lanes 9 and 10). Interestingly, our PCR assay also provides evidence that a chromosomal copy of *GAP1* is contained in at least some of the cells containing the $GAP1^{circle}$ (Fig. 3*B*, lane 12).

To confirm the presence of the $GAP1^{circle}$, we performed a Southern blot analysis on complete genomic DNA digested with NspI using a probe that hybridizes to the entire *GAP1* ORF (Fig. 3*C*). As Nsp1 cuts within *GAP1* (Fig. 3*A*), we expected to identify two bands of 3,231 and 1,929 bases in clones carrying a chromosomal version of *GAP1*. We confirmed the detection of the expected bands and the absence of any hybridization signal in a *gap1Δ* strain (Fig. 3*C*). NspI is expected to linearize the $GAP1^{circle}$, which is predicted to be 4,252 bp. We confirmed the presence of a band of this size in four different $GAP1^{circle}$ clones (Fig. 3*C*). Consistent with our PCR assay, we also detect the presence of the chromosomal *GAP1* allele in clones containing the $GAP1^{circle}$. One explanation for the presence of the $GAP1^{circle}$ and the corresponding chromosomal deletion in addition to the chromosomal copy of *GAP1* in these haploid cells is the occurrence of reintegration events that restore the *GAP1* chromosomal allele. Alternatively, the circular element may arise through an initial duplication of the *GAP1* locus by unequal sister chromatid exchange and subsequent generation of a $GAP1^{circle}$ from one of the duplicated copies.

To determine whether the $GAP1^{circle}$ contains a functional copy of *GAP1*, a *gap1Δ* strain was transformed with DNA from the plasmid fraction recovered from a $GAP1^{circle}$ clone. As GAP1 is the sole transporter of L-citrulline, we were able to select transformants on minimal citrulline medium. We recovered cells able to grow on L-citrulline with reasonably high frequency. These transformants were subsequently tested for their ability to grow on D-histidine (Fig. 3*D*). Whereas *gap1Δ* cells grow on D-histidine, the parental *GAP1* and the *gap1Δ* strain transformed with the $GAP1^{circle}$ are unable to grow on D-histidine. This confirms that the $GAP1^{circle}$ contains a functional copy of *GAP1*.

To gain insight into the frequency of the $GAP1^{circle}$, PCR was performed on 192 descendants from each of L-alanine-, L-glutamine-, and ammonium-limited cultures. Twelve percent of the clones from an L-glutamine limitation experiment gave rise to the $GAP1^{circle}$-specific PCR product, whereas one clone from ammonium limitation and none of the clones from L-alanine limitation experiments gave rise to this product.

We sequenced the putative cross-over site in both the $GAP1^{circle}$ and *gap1Δ* alleles for the four $GAP1^{circle}$ clones from the L-glutamine–limited culture analyzed by Southern blot. All $GAP1^{circle}$ strains carried one hybrid LTR on the $GAP1^{circle}$, suggesting that the circle had formed through recombination between YKRCδ11 and YKRCδ12. Sequence analysis indicated that the four $GAP1^{circle}$ alleles contained different cross-over sites within the $GAP1^{circle}$ (Fig. 4*A*). Sequence analysis of corresponding *gap1Δ* alleles in these four clones confirmed the presence of a single hybrid LTR at the chromosomal locus in all four clones. The cross-over site within the *gap1Δ* alleles was found at the same site in all four of the investigated clones (Fig. 4*A*). The cross-over sites in the $GAP1^{circle}$ are as close as 1–15 bases and as far as 148–170 bases from the chromosomal cross-over site (Fig. 4*A*). The precise location of the cross-over site is a function of the site of the initial double-strand DNA break and the extent of resection and branch migration (Fig. 4*B*). The identification of different cross-over sites in the $GAP1^{circle}$ of the four clones indicates that the $GAP1^{circle}$ alleles had arisen by four independent recombination events in the same L-glutamine–limited culture. We attribute the identical cross-over site in the *gap1Δ* alleles to chance, as alternate *gap1Δ* breakpoints are identified in strains containing only the *gap1Δ* (Fig. 2).

Discussion

We have identified a genomic architecture at the *GAP1* locus that facilitates gene deletion and generation of an extrachromosomal circular DNA species. A single intrachromosomal recombination event between the solo LTRs YKRCδ11 and YKRCδ12 generates a circular self-replicating DNA element containing a functional *GAP1* gene and results in a *gap1Δ* deletion on chromosome XI. Three lines of evidence suggest that this event occurs through homologous recombination: (*i*) the uneven breakpoints between YKRCδ11 and YKRCδ12 in the $GAP1^{circle}$ and *gap1Δ* alleles, which is consistent with variable DNA resection and branch migration resolving a double-strand DNA break during homologous recombination; (*ii*) the identification of a single hybrid LTR in the $GAP1^{circle}$ and *gap1Δ* alleles; and (*iii*) the fact that LTR pairs flanking Ty retrotransposons are excised from the genome through homologous recombination (18). Retrotransposons and retroviruses can be excised from eukaryotic genomes by homologous recombination, leaving an extrachromosomal circle with one LTR (15, 19). The formation of the $GAP1^{circle}$ containing a single LTR is likely to follow the same mechanism.

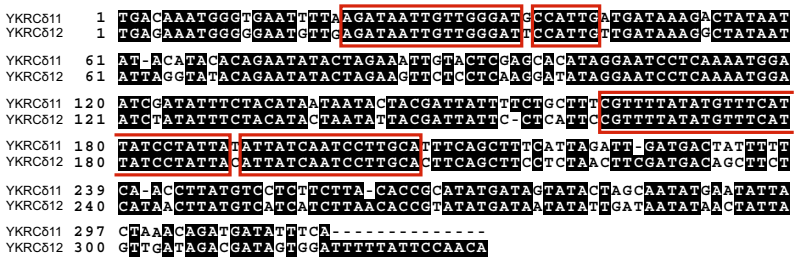

Fig. 2. Homologous recombination breakpoints in the two LTRs mediating intrachromosomal recombination at the *GAP1* locus. Sequences of YKRδ11 (upper strand) and YKRδ12 (lower strand) are aligned, and identity between the two sequences is indicated in black. Recombination breakpoints in five different clones were identified on the basis of sequence differences between the two LTRs and resolved to the level of the regions defined by red-bounding boxes. The hybrid LTRs contain YKRδ11 5′ to the breakpoint and the YKRδ12 sequence in the subsequent 3′ region.

The different alleles of *GAP1* have selective advantage under different nitrogen conditions (Table 1). The deletion allele *gap1Δ* is selected under long-term ammonium, allantoin, and urea limitation, whereas retention of the *GAP1*^{circle} and large-scale genome amplification events that include *GAP1* are found following long-term selection in L-glutamine and L-glutamate limitation. The strong selective advantage associated with increased *GAP1* copy number in L-glutamine and L-glutamate limitation conflicted with our initial observation of *gap1Δ* alleles in L-glutamine and L-glutamate limitation. This was resolved by the identification of the *GAP1*^{circle} in these populations, the generation of which produces a *gap1Δ* allele. The high frequency of the *GAP1*^{circle} allele in

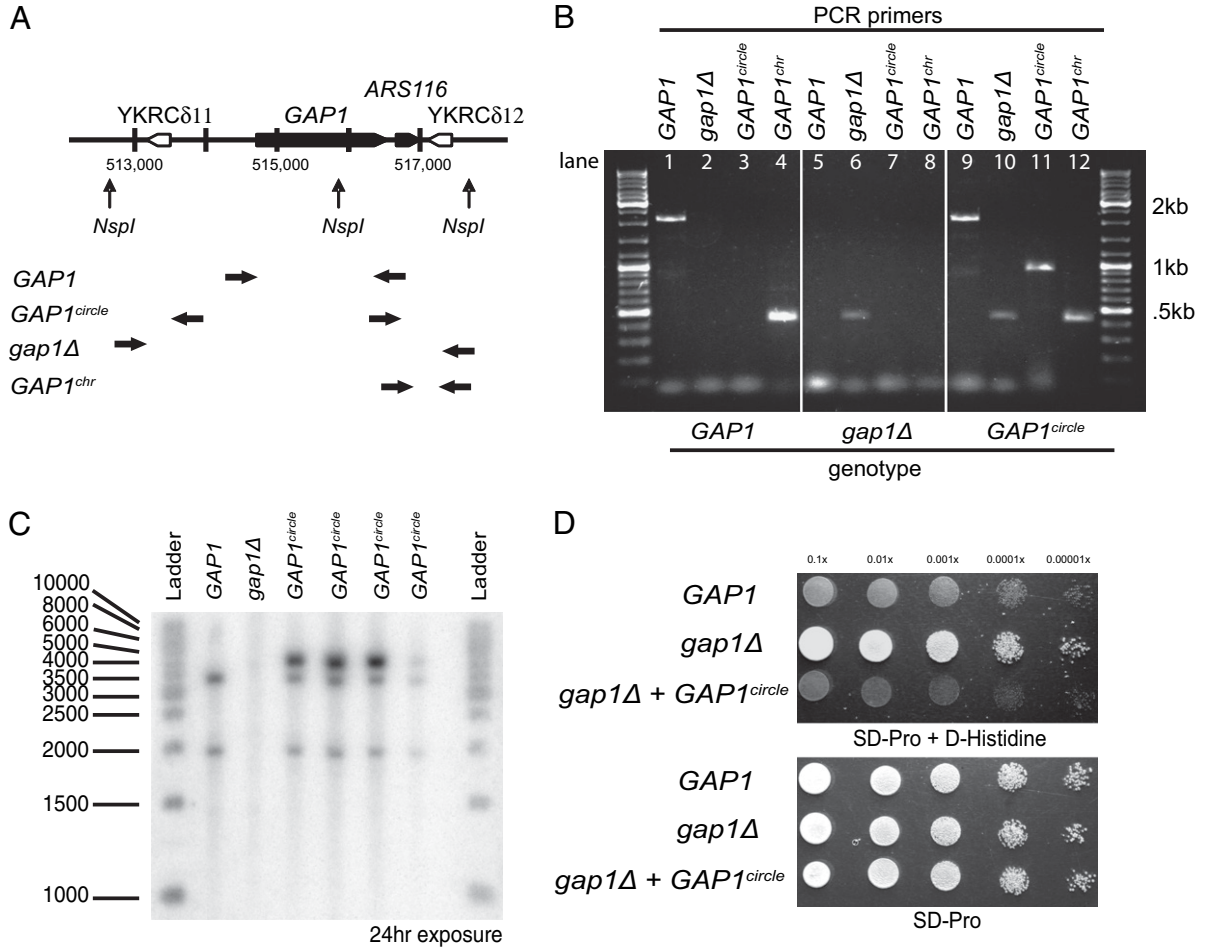

Fig. 3. Test for the presence of a circular extrachromosomal molecule containing a functional *GAP1*. (*A*) Schematic representation of the wild-type chromosomal allele of *GAP1*. Horizontal arrows represent annealing sites for PCR primers used for verification of the wild-type *GAP1* ORF, *GAP1*^{circle}, *gap1Δ*, and the chromosomal presence of *GAP1*. NspI restriction sites are indicated. (*B*) Agarose gel electrophoresis of PCR products obtained from three different genotypes of *GAP1* (indicated below image). Wild-type *GAP1* results in PCR amplification of the ORF and chromosomal fragment (lanes 1 and 4); deletion of *GAP1* results in the deletion product only (lane 6); the presence of the *GAP1*^{circle} results in a PCR product using primers that face the opposite direction in a chromosomal context (lane 11). In addition, PCR products corresponding to the ORF and deletion allele are detected (lanes 9 and 10). We also find evidence for the chromosomal location of *GAP1* in a clone containing the *GAP1*^{circle} (lane 12). (*C*) Southern blot analysis of the three different *GAP1* genotypes. Two bands of expected size 3,213 bp and 1,929 bp are detected for the clone carrying the wild-type *GAP1* allele. No signal is detected for the clone with the *gap1Δ* allele. The predicted *GAP1*^{circle} is 4,252 bp and contains a single NspI site. We detect a single band corresponding to the expected size of the linearized product in four *GAP1*^{circle} clones (Table 1). In addition, we detect bands corresponding to the wild-type *GAP1* locus in all four *GAP1*^{circle}-containing strains. (*D*) Growth of *GAP1*, *gap1Δ*, and *gap1Δ* transformed with the *GAP1*^{circle} on minimal media containing proline and D-histidine. The presence of a wild-type *GAP1* allele confers sensitivity to D-histidine, whereas loss of *GAP1* (*gap1Δ*) results in resistance. Introduction of the plasmid DNA recovered from a *GAP1*^{circle} strain into a *gap1Δ* strain confers sensitivity, confirming that the *GAP1*^{circle} contains a functional copy of *GAP1*. All genotypes are able to grow in minimal media containing proline. Tenfold dilutions from an overnight culture are shown.

populations adapted to L-glutamine limitation (Table 1) suggests that retention of the *GAP1*^{circle} confers a strong fitness advantage. Self-replication of the *GAP1*^{circle} is likely to be enabled by the presence of the replication origin, *ARS1116*, which has previously been shown to be an active replication site (20). The *GAP1*^{circle} is lost after a few generations in rich media, suggesting that the *GAP1*^{circle} confers a strong selective disadvantage under nitrogen-rich conditions.

GAP1 encodes a plasma membrane transporter for all 20 common amino acids (21). Expression of *GAP1* mRNA is normally repressed under nitrogen-rich conditions and, when the gene is expressed, the protein is sorted to the plasma membrane only under particular conditions (22). Under nitrogen-limiting conditions in the chemostat, *GAP1* mRNA is expressed at high levels regardless of the nitrogen source (23). Selective advantage of clones carrying *GAP1*^{circle} or *GAP1* amplifications in L-glutamine and L-glutamate limitation is likely due to increased uptake of the limiting nutrient through increased GAP1 transport capacity. Selective advantage of the *gap1Δ* in ammonium-, urea-, and allantoin-limited populations may be due to two causes. First, the expression of *GAP1* in nitrogen-limited chemostats is high, but the inability of GAP1 to transport these nitrogen-containing molecules may create an unfavorable genetic load. Alternatively, as transport is driven by the electrochemical gradient across the plasma membrane, it is possible that GAP1 exports amino acids when the gradient of amino acids between cytosol and cell exterior is steeper than that of the electrochemical gradient. Recent data have shown that amino acids are excreted under particular conditions (24) consistent with this possibility. The loss and reimport of amino acids would generate a futile cycle, with a concomitant reduction in cellular fitness. We are currently unable to distinguish between these two underlying causations, although their relative importance can be experimentally tested.

Nitrogen availability plays a unique role in determining commitment to major processes in yeast such as meiosis and sporulation and differentiation into pseudohyphae. Sufficient nitrogen levels are also required for commitment to the cell-division cycle. Although the natural history of yeast is the subject of speculation, it seems likely that in its natural habitat yeast experience extreme fluctuations in nitrogen availability and source. We speculate that the genomic architecture at the *GAP1* locus has been retained through evolution to facilitate adaptation to diverse nutrient-limited environments. It is interesting to note that intrachromosomal recombination between tandem repeats is stimulated by transcription of the intervening sequence (9). This is likely to be true for the *GAP1* locus, and therefore poor nitrogen environments that result in increased *GAP1* expression might increase the frequency at which *gap1Δ* and *GAP1*^{circle} alleles are generated via intrachromosomal recombination. The generation of diverse *GAP1* alleles of strong and opposing phenotypic effect at a high frequency may provide a population-level benefit in fluctuating environments akin to phase switching or phenotypic switching in bacteria.

Amplification of genes encoding nutrient transporters has also been observed in yeast populations evolved under glucose (5) and sulfate (16) limitation. In both cases, the amplified transporter genes are found at their endogenous locus. The LTR-mediated generation of a circular autonomously replicating DNA element described in this study is a unique mechanism by which gene amplifications are generated and selected. Three percent of the *S. cerevisiae* genome consists of retrotransposable elements (25), and any genomic fragment bounded by LTRs and containing an autonomously replicating sequence (ARS) has the potential to generate a self-replicating circular element. For example, the closely linked hexose transporters *HXT3*, *HXT6*, and *HXT7* are bound by the LTRs YDRWδ25 and YDRWδ26 and contain two ARSs. We would predict that an extrachromosomal circle containing these genes would confer a selective advantage under hexose-limiting conditions.

A related example in yeast is known in which intact Ty1 elements recombine to form an extrachromosomal circle with a 39-kb fragment of chromosome II (26). The chromosome II element contains the histone genes *HTA2-HTB2*, a centromere, and origins of replication and can be selected for in *HTA1-HTB1/hta1-htb1* heterozygotes. The *HTA2-HTB2* circle is formed with high frequency and

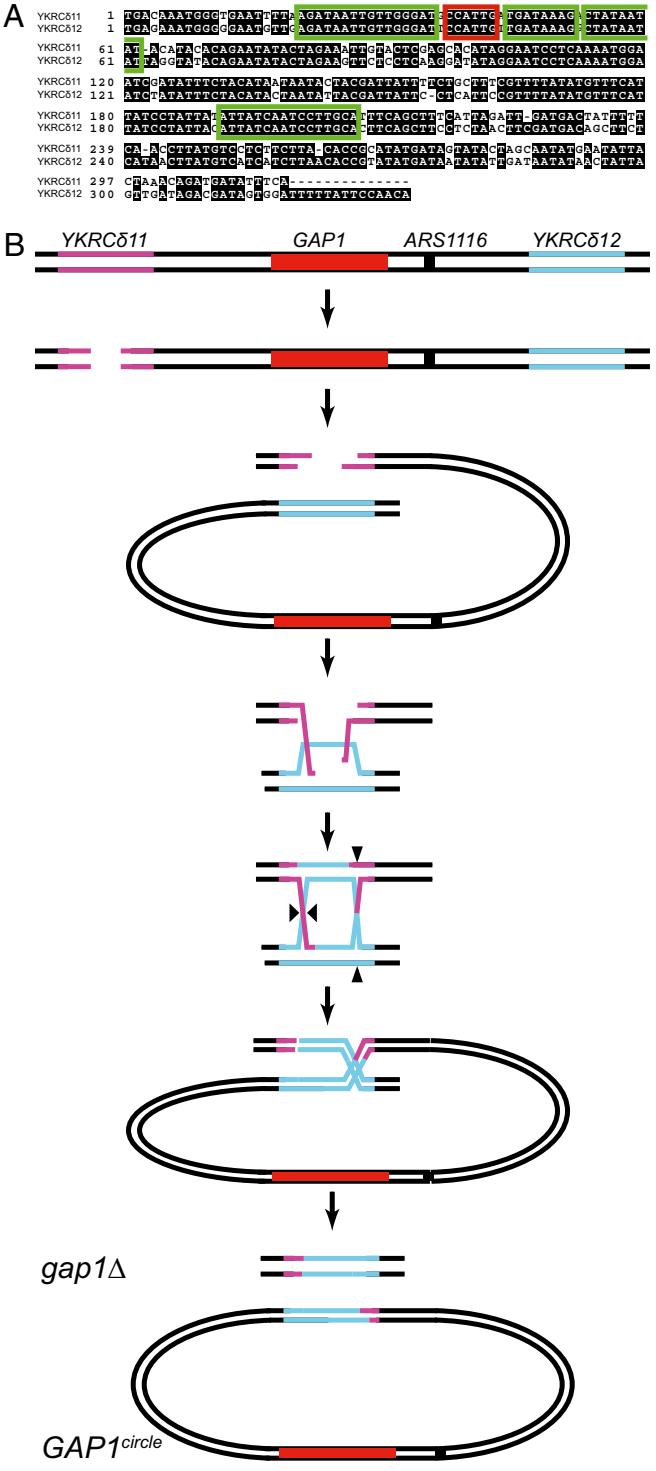

Fig. 4. Recombination sites in the LTRs YKRCδ11 and YKRCδ12 generated by formation of *gap1Δ* and the corresponding *GAP1*^{circle} alleles in four clones (described in Table 1 and Fig. 3). (*A*) Sequences of YKRCδ11 (upper strand) and YKRCδ12 (lower strand) are aligned, and identity between the two sequences is shown in black. The YKRCδ11-YKRCδ12 breakpoint within the *GAP1*^{circle} is shown for four clones (green boxes) together with the corresponding YKRCδ11-YKRCδ12 breakpoint from the chromosomal *gap1Δ* deletions (red box) in each of the same four clones. (*B*) Model of LTR-mediated homologous recombination at the *GAP1* locus resulting in generation of a circular DNA element and a chromosomal deletion. Black triangles indicate the sites of DNA cross-overs.

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is thought to be induced in the absence of *HTA1* and *HTB1*. The site within the 7.5-kb Ty1s in which recombination occurs is not known and likely cannot be known due to the sequence identity of the Ty1 elements. By contrast, the sequence differences between YKRCδ11 and YKRCδ12 enable mapping of the intrachromosomal recombination breakpoints with unprecedented resolution.

Eight and a half percent of the human genome consists of LTR elements (27). It is likely that the human genome has the ability to form extrachromosomal self-replicating circles through homologous recombination of LTRs. Extrachromosomal self-replicating circular DNA elements are found in tumor cells, where they are known as double minutes (28). Double minutes carry amplified oncogenes or genes involved in drug resistance such as the epidermal growth factor gene (*EGFR*) (29) and are thought to confer proliferative potential. The discovery of autonomously replicating circular DNA that confers a selective advantage to yeast in evolution experiments is analogous to this genomic phenomenon observed in human cancer cells (17) and provides a potential system for studying genetic and chemical approaches to inhibiting their formation.

Materials and Methods

Strains and Media. Prototrophic haploid *S. cerevisiae* strains CEN.PK113-7D (*MAT*α) and FY4 (*MAT***a**) were used as ancestors for long-term chemostat selection experiments. Descendants from evolution experiments were recovered by plating cells on rich solid media. Defined media were used for all experiments as described (23, 30) with a limiting nitrogen concentration of either 6 mM L-alanine–, ammonium-, and L-glutamine–limited cultures of CEN.PK113-7D) or 800 μM (allantoin-, urea-, L-glutamate–, and L-glutamine–limited cultures of FY4). Minimal proline + D-histidine media were prepared as described (20).

Chemostat Cultivation. Steady-state aerobic chemostat cultures were grown at 30 °C at a dilution rate of 0.2 (CEN.PK113-7D) or 0.12 (FY4) culture volumes per hour. For a subset of cultures, we measured residual nitrogen concentration every 50 generations by ion-exchange HPLC using a Waters 474 scanning fluorescence detector to ensure that cultures were indeed limited for nitrogen throughout the experiment. Populations of an initial size of 10^{10} cells were propagated for at least 250 generations.

DNA Microarray Analysis. Comparative genomics hybridization was performed using Agilent 44k and Affymetrix Yeast Tiling Array 1.0R yeast microarrays as described (16).

Genotyping. The status of the *GAP1* locus was determined using the following PCR primer sets: primer set *GAP1* (5′-TGAGACGATGTTTTTCTC-3′; 5′-TTAGTGCACGGAAATATA-3′) detects a 2.1-kb product corresponding to an intact *GAP1*, primer set *gap1Δ* (5′-GTCATCACTGTTGTTGGG-3′; 5′-AGGCGGCAACAAACGCG-3′) detects the deletion of *GAP1* and produces a 0.5-kb PCR product, and primer set $GAP1^{circle}$ (5′-ACTGCGGCTCCGCTC-3′; 5′-GACCTGTGGAAGGTCG-3′) detects the $GAP1^{circle}$ producing a 0.9-kb PCR product. Primer set $GAP1^{chr}$ (5′-GGCTGTTCAATCTCCGG-3′; 5′-ACTGCGGCTCCGCTC-3′) detects the presence of *GAP1* at its chromosomal location and produces a 0.5-kb product.

Southern Blot. Purified genomic DNA was digested with NspI, separated on a 1.5% native gel, and probed with a ^{32}P-labeled *GAP1* probe. To prepare the probe, 50 ng of purified PCR product generated with primer set *GAP1* (5′-TGAGACGATGTTTTTCTC-3′; 5′-TTAGTGCACGGAAATATA-3′) was labeled radioactively by random priming with [α-^{32}P]dCTP (PerkinElmer) using a Prime-It II Random Primer Labeling Kit (Stratagene) and purified using illustra ProbeQuant G-50 micro columns (GE Healthcare). Southern blots were scanned by PhosphorImager (Molecular Dynamics) and analyzed with conventional Kodak BioMax MS film (PerkinElmer).

Fluctuation Analysis. *GAP1* mutation rates were determined by fluctuation analysis. CEN.PK113-7D cells were inoculated into 200 μL liquid YPD at a concentration leading to 0–1 cell per sample and grown to a saturation of ~10^8 cells/mL. The 14 resultant cell lines were subsequently plated on (*i*) solid YPD to calculate the exact number of cell divisions in each cell line and (*ii*) MP + D-histidine to measure the proportion of *gap1* mutants. Mutation rates were calculated using the P_0 method of Luria and Delbrück (31).

Competitive Growth Assays. Competition experiments were performed by coculturing the strain of interest with the corresponding parental strain in chemostat cultures. We measured the relative abundance of the two strains by determining the fraction of D-His^R cells on plates or the proportion of $GAP1^{circle}$ cells using PCR as described above. For some competition experiments, we competed the unlabeled adapted strain against a fluorescently labeled ancestral strain and measured their relative abundance using a FACS Analyzer (BD Biosciences). Fitness was estimated by determining the slope of the regression of ln(evolved/ancestral) against time in generations (16).

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