Natural variation in \textit{CDC28} underlies morphological phenotypes in an environmental yeast isolate

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ABSTRACT

Morphological differences among individuals in a species represent one of the most striking aspects of biology, and a primary aim of modern genetics is to uncover the genetic basis for morphological variation. In a survey of meiosis phenotypes among environmental isolates of *Saccharomyces cerevisiae*, we observed an unusual arrangement of meiotic spores within the spore sac in an Ivory Coast isolate. We mined population genomic data to identify *CDC28* as the major genetic determinant of meiotic and budding cell shape behaviors in this strain. Molecular genetic methods confirmed the role of the Ivory Coast variant of *CDC28* in the arrangement of spores after meiosis, in the shape of budding cells in rich medium, and in the morphology of filamentous growth during nitrogen limitation. Our results shed new light on the role of CDC28 in yeast cell division, and our work suggests that with the growing availability of genomic data sets in many systems, *a priori* prediction of functional variants will become an increasingly powerful strategy in molecular genetics.
INTRODUCTION

Natural genetic variation in morphology has given rise to Darwin’s “endless forms most beautiful” across the tree of life, and the search for the molecular basis of morphological variation remains a major focus of evolutionary genetics. To this end, unbiased genome-wide mapping is often used to find loci that underlie trait variation within species. However, the potential for high cost and limited statistical power of genome-wide analyses has led many researchers to test for causal DNA variants only in a subset of candidate genes, those predicted from prior knowledge about the trait of interest (NEALE and SAVOLAINEN 2004; RISCH 2000; YOO et al. 2009). Classically, the candidate gene approach has met with varied degrees of success, owing to limited genotypic data and lack of detailed functional information for many genes and pathways (HIRSCHHORN and DALY 2005; MCCARTHY et al. 2003; TABOR et al. 2002; TODD 2006).

Recently, increases in the availability of genome sequences and functional genomic resources have renewed interest in hypothesis-driven molecular genetics, in which functional variants can be predicted \textit{a priori} from DNA sequence (MORENO et al. 2008; WANG and MARCOTTE 2010).

The cell morphology and growth habit of budding yeast have been the target of genetic studies in laboratory strains (JORGENSEN et al. 2002; MOSELEY and NURSE 2009; OHYA et al. 2005; PRUYNE et al. 2004) and environmental isolates (FIDALGO et al. 2006; GRANEK and MAGWENE 2010; NOGAMI et al. 2007; YVERT et al. 2003), but the genetic
underpinnings of many naturally occurring yeast morphologies remain unknown. In the search for the genetic basis of varying traits in yeast, a recently reported population genomic data set (Liti et al. 2009) established a powerful test bed for the candidate gene approach. In the present work, we undertook a proof of concept for hypothesis-driven genetic dissection of natural variation, using yeast sporulation as a model system. Upon observing an unusual arrangement of meiotic spores in an environmental yeast isolate, we set out to mine genome sequence data and test predictions about the underlying causal basis of this developmental phenotype.

MATERIALS AND METHODS

Reciprocal hemizygote strain construction: All strains used are listed in Table 1. The Ivory Coast (NCYC110) and oak (YPS606) wild isolates were obtained from the National Center for Yeast Collections (NCYC) in both diploid HO and haploid ho form. Ivory Coast x oak hybrid strains (YHL118 and YHL121) were generated from single-cell matings of the haploid ho strains from the two parental backgrounds to each other. These hybrid strains were subsequently used to make reciprocal hemizygotes by deleting one allele of CDC28 with a URA3 cassette. The CDC28 locus was sequenced in each transformant to verify that only one allele was present and to identify the allele. CDC28 hemizygotes were generated from homozygous diploid parent backgrounds via the same method.
**Genomic analysis:** Whole-genome alignments for a panel of genetically divergent strains, including the Ivory Coast and oak isolates, were retrieved from the *Saccharomyces* Genome Resequencing Project database (Liti et al. 2009). The aligned sequences for the open reading frames of annotated protein-coding genes were analyzed for the presence of nonsynonymous single nucleotide polymorphisms that were unique to the Ivory Coast strain.

**Linkage analysis:** A homothallic hybrid was constructed by mating single spores of *HO* wild-type Ivory Coast and oak strains to each other. This hybrid (YHL047) was sporulated and 24 tetrads were dissected. One homothallic segregant from each tetrad was sequenced at *CDC28* and analyzed for sporulation and mitotic growth phenotypes. Sporulation morphology was quantified as the proportion of linear tetrads observed by microscopy, with >600 tetrads analyzed in each sample. Linkage to *CDC28* was assessed by a Wilcoxon signed-rank test to compare segregant phenotypes associated with the two *CDC28* alleles. The percent variance due to *CDC28* genotype was calculated as the $R^2$ of the linear regression of the segregant phenotypes to their *CDC28* genotypes.

**Sporulation:** Cultures were grown for at least five days on solid minimal sporulation media (Amberg et al. 2005), prior to imaging at 40X magnification.
Mitotic growth: Cultures were grown to mid-log phase (OD600 ~ 0.7) in liquid rich media (Amberg et al. 2005), prior to imaging at 40X magnification.

Pseudohyphal growth: Cultures were grown overnight on solid rich media. Cells from a single colony were suspended in water and diluted to an OD600 of 0.01 before plating on nitrogen-limited media with 1% butanol to induce filamentous growth (Gimeno et al. 1992). Colonies were imaged at 10X after four days of growth.

RESULTS

In wild-type yeast, meiosis is induced by nutrient limitation, and the four haploid spores resulting from meiotic divisions form a structure called a tetrad. We surveyed meiotic phenotypes in a panel of fully sequenced, genetically diverse yeast isolates which fall into well-defined phylogenetic populations (Liti et al. 2009). Upon starvation, all strains exhibited tetrads with a tetrahedral shape (Figure S1), except for NCYC110, an Ivory Coast isolate, in which we frequently observed an alternate form where the spores were arranged linearly in the ascus or spore sac (Figure 1A). This observation was particularly striking given the tetrahedral tetrad form in DBVPG6044 (Figure S1), an isolate from Guinea which is only 4.8 x 10^{-5} percent divergent from the Ivory Coast strain across protein-coding regions. Linear tetrads have previously been observed in laboratory yeast, but only rarely (Lindsey et al. 2010; Piccirillo and Honigberg 2010;
THOMAS and BOTSTEIN 1987), and were used to deduce the first centromere linkage in yeast (HAWTHORNE 1955).

To identify candidate alleles underlying the naturally occurring linear tetrad form of the Ivory Coast isolate, we searched population genomic sequence data (LITI et al. 2009) for nonsynonymous coding variants unique to this strain. Of the six genes harboring such variants (CDC28, PAU3, SEC27, SIW14, RAS2 and TRM44), we considered CDC28 to be the best candidate based on its known role in cell morphology. CDC28 is a cyclin-dependent kinase that regulates polarization during cell division (LEW and REED 1993). CDC28 knockouts are inviable and conditional loss-of-function mutations confer both meiotic and mitotic defects (BENJAMIN et al. 2003; GIAEVER et al. 2002; KITAZONO et al. 2003; KITAZONO and KRON 2002), but various CDC28 point mutations give rise to enhanced polarized growth (AHN et al. 2001; EDGINGTON et al. 1999). Furthermore, a laboratory strain null for a CDC28 binding partner, CLB2, phenocopied the tetrad morphology of the Ivory Coast isolate (Figure 1). The Ivory Coast strain harbored a unique serine-to-phenyalanine amino acid change at residue 79 in the CDC28 sequence, an amino acid forming part of a region known in the human homolog, Cdk1 (Cdc2), to be critical for Cdk-cyclin interactions (JEFFREY et al. 1995; OTYPEKA et al. 2006). On this basis, we hypothesized that the Ivory Coast allele of CDC28 was a genetic determinant of the linear tetrad phenotype.
To test this hypothesis, we first crossed the Ivory Coast strain to YPS606, a strain isolated from the exudate of North American oak trees, which has high sporulation efficiency (GERKE et al. 2006) and normal tetrad form (Figure 1). We collected haploid recombinant progeny from this cross and quantified the proportion of linear tetrads in each progeny strain. The results, shown in Figure 2, revealed strong linkage between the linear tetrad phenotype and genotype at *CDC28* (Wilcoxon \( p = 3.5 \times 10^{-5} \)).

Consistent with the phenotypes of the parent strains, the Ivory Coast allele of *CDC28* was associated with a high proportion of linear tetrads relative to the effect of the oak allele (Figure 2). Interestingly, segregants that bore the Ivory Coast allele at *CDC28* did not all phenocopy the Ivory Coast parent: the frequency of linear tetrads varied across this strain set, reflecting the action of additional modifier loci segregating in the cross (Figure 2). In contrast, across segregants bearing the oak allele at *CDC28*, proportions of linear tetrads were tightly distributed near zero (Figure 2). In an analysis of all segregants, 73% of variance in the linear tetrad phenotype was explained by the *CDC28* locus. We conclude that the *CDC28* locus is the major genetic determinant of this trait in the cross, with additional epistatic modifiers manifesting only in the presence of the Ivory Coast allele of *CDC28* that together give rise to the phenotype of the Ivory Coast parent strain.

To confirm the identity of *CDC28* as the major causative gene underlying the linear tetrad phenotype, we generated reciprocal hemizygotes (STEINMETZ et al. 2002) for *CDC28* in a diploid hybrid formed from a mating between the Ivory Coast and oak
parents. This strategy produced two hybrid strains genetically identical to one another at all loci except \textit{CDC28}, at which one hemizygote strain bore only the Ivory Coast allele and the other strain only the oak allele. Inducing meiosis in the wild-type Ivory Coast x oak hybrid yielded a low proportion of linear tetrads (Figures 1 and 3), indicating that the genetic determinants of the linear tetrad phenotype in the Ivory Coast strain act in a largely recessive manner. Consistent with predictions from linkage analysis, the hemizygote bearing only the Ivory Coast allele of \textit{CDC28} had a relatively high proportion of linear tetrads when sporulated, and linear tetrads were rare in sporulated cultures of the hemizygote bearing the oak allele of CDC28 (Figure 3). We conclude that \textit{CDC28} is a major causative gene for the high frequency of linear tetrads in the Ivory Coast strain.

The evidence for dominance by the oak allele of \textit{CDC28} in the genetics of the linear tetrad trait (Figure 3) suggested that the Ivory Coast allele at this locus was likely hypomorphic. However, given that the oak x Ivory Coast hybrid strain produced a modest proportion of linear tetrads when sporulated (Figures 1 and 3), we further hypothesized that there was a subtle effect of haploinsufficiency at \textit{CDC28} in the hybrid relative to the oak parent. Under this model, we expected that a reduction in \textit{CDC28} dose would also give rise to an increased frequency of linear tetrads in either parental strain background. To test this notion, we deleted one copy of \textit{CDC28} in the oak homozygous parent and, separately, in the Ivory Coast parent. In each case, the hemizygote produced more linear tetrads than did its respective homozygote (Figure 3),
with a more dramatic effect in the Ivory Coast background. Thus, the functional dose of CDC28 correlated inversely with the proportion of linear tetrads; the Ivory Coast allele of CDC28 acted as a partial loss of function, and haploinsufficiency in CDC28 in either strain increased the frequency of linear tetrads. Notably, the hemizygote bearing a given allele in the parental background did not phenocopy the respective hemizygote in the hybrid (Figure 3), consistent with the evidence from linkage analysis (Figure 2) for additional variant loci as modifiers to CDC28.

In laboratory yeast, experimentally inducing elongated morphology during mitotic growth gives rise to a linear tetrad phenotype upon sporulation (LINDSEY et al. 2010; THOMAS and BOTSTEIN 1987), and a laboratory strain null for the CDC28 binding partner CLB2 grows as elongated cells in rich medium (AHN et al. 1999). We hypothesized that the natural variant of CDC28 in the Ivory Coast strain would also confer elongated cell shape in mitotically budding cells. Consistent with this prediction, the Ivory Coast parent and hemizygotes bearing the Ivory Coast allele of CDC28 showed elongated cell morphology during mitotic growth, in contrast to the round cells of the oak parent and the hemizygotes with the oak allele of CDC28 (Figure 4). Furthermore, in the progeny of the cross between the oak and Ivory Coast strains, segregants with hyperpolarized cells in rich media also produced linear tetrads when sporulated (Figure S2). Interestingly, in contrast to our analysis of tetrads (Figure 3), we found no evidence for haploinsufficiency in CDC28 dose as a determinant of cell shape: oak homozygotes with a single copy of CDC28 still grew as round cells, as did the wild-type hybrid between the oak and Ivory Coast strains (Figure 4). We conclude that the Ivory Coast
allele of \textit{CDC28} is sufficient to confer elongated cell morphology, reflecting a relationship between hyperpolarized mitotic growth and linear tetrads during sporulation.

Many strains of yeast, when starved for nitrogen, undergo unipolar budding, which generates filaments of linked cells (Gimeno et al. 1992). Given the pronounced filamentous phenotype under nitrogen limitation in a laboratory strain mutant for CLB2, the binding partner of CDC28 (Ahn et al. 1999), we hypothesized that the Ivory Coast allele of CDC28 would drive a similar phenotype. In accord with this expectation, the Ivory Coast isolate, when starved for nitrogen, grew as long, branching filaments composed of elongated cells growing from the colony edge, whereas the oak strain showed a more modest response with non-branching filaments composed of round cells (Figure 5). Reciprocal hemizygote analysis confirmed that the Ivory Coast allele of \textit{CDC28} was sufficient to confer highly branched, elongated filaments under nitrogen limitation (Figure 5). Again, we observed no evidence for haploinsufficiency of \textit{CDC28} dose as a determinant of filamentous morphology: hemizygous strains generated from the homozygous parent backgrounds resembled their respective parents, and the wild-type hybrid between the oak and Ivory Coast strains resembled the oak parent (Figure 5). Thus, the Ivory Coast allele of \textit{CDC28} underlies the dramatic filamentation behavior in this environmental isolate, mirroring its effects on tetrad and single-cell morphology.

DISCUSSION
Many variations in yeast cell form and growth habit have been reported in environmental isolates (Casalone et al. 2005; Cavalieri et al. 2000; Dengis et al. 1995; Graneck and Magwene 2010; Nogami et al. 2007; St’Ovicek et al. 2010), with the molecular basis known for only a small subset of these traits. We have shown that natural genetic variation at a single essential gene, CDC28, can lead to striking changes in yeast cell shape during meiotic and mitotic division. The rod-like tetrads, elongated cell shape, and hyperfilamentous growth of the environmental Ivory Coast isolate studied here can be attributed to variation in CDC28. Previous reports have implicated loss of function in CDC28/CLB2 activity in hyperpolarized cell division and filamentous growth (Ahn et al. 1999; Ahn et al. 2001; Edgington et al. 1999; Lew and Reed 1993), but temperature-sensitive mutations available in laboratory strains have not been well-suited to the study of CDC28 loss of function under starvation conditions. By contrast, our ability to map starvation-induced sporulation and filamentation phenotypes to CDC28 highlights the utility of analysis of natural genetic variation when drawing relationships between genes and phenotypes.

The molecular mechanism by which the Ivory Coast allele of CDC28 impacts tetrad and cell morphology remains an open question. The phenotypic similarity between the Ivory Coast strain and the Δclb2 lab strain, and the S79F change in the former in a known cyclin-interaction domain of CDC28, suggest that the Ivory Coast allele attenuates the binding of CDC28 to CLB2. We favor a model in which elongated cell shape is the proximate cause of the linear tetrad morphology and dramatic filamentation phenotype.
attributable to the Ivory Coast allele, in light of the relationship between these traits when cell shape is manipulated in the laboratory (Lindsey et al. 2010; Thomas and Botstein 1987). With respect to sporulation, one compelling idea for the underlying mechanism is that, although meiotic cell division is ordered in yeast (Hawthorne 1955), spores arrange passively in the ascus as a function of cell shape before meiosis (Piccirillo and Honigberg 2010). Among the most notable aspects of the Ivory Coast allele of CDC28 are its incomplete penetrance and its sensitivity to genetic modifiers, for which the Ivory Coast strain’s unique coding alleles of the morphology regulator RAS2 and other genes represent strong candidates. As such, our data indicate that the effect of the Ivory Coast variant of CDC28 on linear tetrad formation is highly sensitized both to stochastic variation in cell shape and to genetic differences between yeast strains.

The evolutionary pressures driving variation in yeast morphology and growth habit in the wild are as yet almost entirely unknown, although several morphological characters are well-characterized targets of artificial selection in beer and wine yeasts (Verstrepen et al. 2003). Given the origin of the Ivory Coast strain as an isolate from ginger beer (Liti et al. 2009), its elongated cell and tetrad forms may be associated with a fitness benefit in the wild or during domestication; alternatively, these phenotypes, and the variant in the essential cell cycle regulator CDC28, may represent a rare genetic defect destined to be eliminated by purifying selection. In either case, our discovery of CDC28 as the major determinant underscores the power of combining dense population genomic sampling with a candidate gene/candidate network approach. With the continued
growth of population genomic sequence compendia, and increasing knowledge of gene function and genetic interactions, this strategy holds promise for application to many organisms and traits.

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FIGURE AND TABLE LEGENDS

TABLE 1.— Yeast strains used.

FIGURE 1.— Linear tetrad morphology observed in an Ivory Coast isolate of S. cerevisiae. Each panel shows a micrograph of yeast culture taken after five days of growth on solid minimal sporulation media. Examples of linear tetrads are indicated with arrowheads. (A) Ivory Coast and North American oak strains. (B) Σ1278b laboratory strain bearing a deletion in CLB2. (C) Ivory Coast x oak hybrid.
FIGURE 2.— The linear tetrad phenotype links to the $CDC28$ locus. Each column reports a distribution of proportions of linear tetrads across cultures of homozygote, homothallic yeast strains grown for five days of growth on solid minimal sporulation media. For each distribution, the median is reported as a thick horizontal line, the 25% quantile is shown as a box, and the extremes are shown as thin horizontal bars. Parent: homozygotes derived from wild isolates (Liti et al. 2009); distributions represent sporulation phenotypes across three biological replicates. Segregants, Ivory Coast allele: F1 progeny from a cross between Ivory Coast and oak strains bearing the Ivory Coast allele of $CDC28$ ($n = 12$). Segregants, oak allele: F1 progeny bearing the oak allele of $CDC28$ ($n = 12$).

FIGURE 3.— Natural variation in $CDC28$ is causative for the linear tetrad phenotype. (A) Proportion of linear tetrads in hemizygote and wild-type strains. Each column reports data from three biological replicates (parental wild-type strains) or independently generated isogenic strains (hybrids and hemizygotes). Bar heights report means, and error bars represent one standard deviation. (B) Micrographs of $CDC28$ reciprocal hemizygote strains in the oak x Ivory Coast hybrid background. (C) Micrographs of $CDC28$ hemizygote strains in homozygous oak and Ivory Coast parental backgrounds. Examples of linear tetrads are indicated with arrowheads. All micrographs were taken after five days of growth on solid minimal sporulation media.

FIGURE 4. — $CDC28$ genotype affects cell shape during mitotic growth. Each panel shows a representative micrograph of culture grown to mid-log phase in rich media. (A)
Ivory Coast and North American oak strains. (B) Oak x Ivory Coast wild-type hybrid strain. (C) CDC28 reciprocal hemizygotes in the oak x Ivory Coast hybrid background. (D) CDC28 hemizygotes in homozygous oak and Ivory Coast parental backgrounds.

FIGURE 5.— CDC28 genotype affects morphology of the filamentous growth response. Each panel represents a representative micrograph of colonies after four days of growth on solid nitrogen limitation media. (A) Ivory Coast and North American oak strains. (B) Oak x Ivory Coast wild-type hybrid. (C) CDC28 reciprocal hemizygotes in the oak x Ivory Coast hybrid background. (D) CDC28 hemizygotes in homozygous oak and Ivory Coast parental backgrounds.
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WORKS CITED


A

Ivory Coast

Oak

B

Σ1278b Δclb2

Ivory Coast x Oak

C
Ivory Coast parent

segregants

Ivory Coast allele

oak allele

oak parent

Genotype at CDC28

Proportion of linear tetrads