Discovery of an Unconventional Centromere in Budding Yeast Redefines Evolution of Point Centromeres

Highlights

- A new type of point centromere has been identified in budding yeast \textit{N. castellii}
- Its DNA sequence and evolutionary origin are different from other point centromeres
- \textit{N. castellii} centromeres are bound by CBF3 that recognizes other point centromeres
- Contrary to the conventional view, point centromeres can change rapidly in evolution

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In Brief

All known point centromeres share common DNA sequences and a single evolutionary origin. Kobayashi et al. have identified a new type of point centromere in budding yeast \textit{N. castellii}. Its DNA sequence and evolutionary origin are different from other point centromeres. Discovery of the new centromere redefines the evolution of point centromeres.
Discovery of an Unconventional Centromere in Budding Yeast Redefines Evolution of Point Centromeres

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SUMMARY

Centromeres are the chromosomal regions promoting kinetochore assembly for chromosome segregation. In many eukaryotes, the centromere consists of up to mega base pairs of DNA. On such “regional centromeres,” kinetochore assembly is mainly defined by epigenetic regulation [1]. By contrast, a clade of budding yeasts (Saccharomycetaceae) has a “point centromere” of 120–200 base pairs of DNA, on which kinetochore assembly is defined by the consensus DNA sequence [2, 3]. During evolution, budding yeasts acquired point centromeres, which replaced ancestral, regional centromeres [4]. All known point centromeres among different yeast species share common consensus DNA elements (CDEs) [5, 6], implying that they evolved once and stayed essentially unchanged throughout evolution. Here, we identify a yeast centromere that challenges this view: that of the budding yeast Naumovozyma castellii is the first unconventional point centromere with unique CDEs. The N. castellii centromere CDEs are essential for centromere function but have different DNA sequences from CDEs in other point centromeres. Gene order analyses around N. castellii centromeres indicate their unique, and separate, evolutionary origin. Nevertheless, they are still bound by the ortholog of the CBF3 complex, which recognizes CDEs in other point centromeres. The new type of point centromere originated prior to the divergence between N. castellii and its close relative Naumovozyma dairenensis and disseminated to all N. castellii chromosomes through extensive genome rearrangement. Thus, contrary to the conventional view, point centromeres can undergo rapid evolutionary changes. These findings give new insights into the evolution of point centromeres.

RESULTS AND DISCUSSION

All known point centromeres have a common DNA sequence, known as CDEI, II, and III (Figures 1A and 1B) [5, 6]. Among different budding yeast species, the order of orthologous genes is generally conserved around the centromeres (i.e., in synteny) [11]. These indicate that the point centromeres themselves are orthologous. However, there are peculiar exceptions to this view: Naumovozyma castellii (N. castellii) (previously called Saccharomycaces castellii) and its close relative N. dairenensis belong to the clade of budding yeasts expected to have point centromeres [3, 7], but no CDEI,II,III-like sequences are found at the loci expected from synteny (Figure 1A; see Figure S1D) [6, 12]. They may use a novel type of point centromere or have re-acquired regional centromeres during their evolution. Here, we aim to identify the centromere in N. castellii.

Kinetochore Components Show Single-Peak Localization on Each Chromosome of N. castellii by Chromatin Immunoprecipitation

In Saccharomyces cerevisiae (S. cerevisiae) and other budding yeasts, their point centromeres are recognized by the CBF3 complex, which binds the CDEII DNA consensus [5, 13]. The CBF3, consisting of Ndc10, Cep3, Ctf13, and Skp1 proteins, is exclusively found in budding yeasts with point centromeres. In S. cerevisiae, the CBF3 and other kinetochore components show a bi-lobed pattern on the metaphase spindle and segregate following movement of the spindle poles during anaphase [14] (Figure 1C, top). Despite an apparent lack of CDEII,III-containing centromeres, the N. castellii genome encodes orthologs of the CBF3 components [10, 15]. In N. castellii, Ndc10 and Cep3 proteins showed the kinetochore-like localization pattern, similar to S. cerevisiae (Figure 1C, bottom). The same localization pattern was found for Ndc80 (Figure 1C, bottom), an outer kinetochore component [5]. Thus, Ndc10, Cep3, and Ndc80 might indeed be N. castellii kinetochore components.

To identify N. castellii centromeres, we added epitope tags to Ndc10, Cep3, and Ndc80 at their original loci, carried out chromatin immunoprecipitation followed by high-throughput DNA sequencing (ChiP-seq), and analyzed in reference to the
Figure 1. Kinetochore Proteins Show Single-Peak Localization on Each Chromosome of *N. castellii*

(A) A clade of budding yeast species carries point centromeres with common consensus DNA sequences CDEI, II, and III (pink), except for *N. castellii* and *N. dairrenensis* (red rectangle). Phylogenetic tree of budding yeast species is taken from [6, 7] with modification. Horizontal length in the tree is proportional to the change in genomic DNA sequences. The WGD (black oval) is estimated to have occurred approximately 100 million years ago [8], and the *N. castellii/N. dairrenensis* divergence is about half this age.

(legend continued on next page)
annotated \textit{N. castellii} genome sequence [15, 16]. We also carried out ChIP-seq for Cse4, a centromere-specific histone H3 variant. Crucially, Ndc80 ChIP-seq gave a distinct single peak at an intergenic region on each of ten chromosomes (Figures 1D and S1A). Cse4, Ndc10, and Cep3 gave peaks at the same intergenic regions as Ndc80 and gave one, two, and six additional peaks, respectively (Figures 1D and S1A–S1C). The chromosomal regions, where Ndc80 showed accumulation (together with Ndc10, Cep3, and Cse4), may serve as the centromeres in \textit{N. castellii}. On this assumption, we tentatively named them \textit{N. castellii} CEN1–10, or NcCEN1–10 for short, on chromosomes 1–10, respectively.

**Most \textit{N. castellii} CENs Are Not at Conserved Syntenic Locations on Chromosomes, Compared with Locations of Other Point Centromeres**

We compared the order of orthologous genes between \textit{S. cerevisiae}, \textit{N. castellii}, and an ancestor. This ancestor is evolutionarily positioned prior to the whole-genome duplication (WGD), which occurred during the evolution of budding yeasts [3] (Figure 1A), and its genome was constructed using bioinformatics [17]. Between \textit{S. cerevisiae} and the ancestor, the gene order across the centromeres is conserved, including the centromeres themselves [11, 17] (Figure S1D). Thus, chromosomal positions of the centromeres did not change during evolution from the ancestor to \textit{S. cerevisiae}. The gene order across the majority of the ancestral centromeric regions (excluding the centromeres themselves) is also conserved without rearrangement on \textit{N. castellii} chromosomes (Figure S1D). However, \textit{N. castellii} CEN locations are not syntenic to the CENs in the ancestor (Figures S1D and S1E). This suggests that centromeres “disappeared” from these ancestral centromeric loci during the \textit{N. castellii} evolution. In fact, ancestral CEN4 and \textit{N. castellii} CEN10 make the only centromere pair whose surrounding orthologs show complete synteny (Figures S1D and S1E). Conversely, \textit{N. castellii} CEN1–9 are not located in regions of conserved gene order when compared to \textit{S. cerevisiae} or the ancestor (Figure 1E).

More specifically, the synteny along \textit{N. castellii} chromosomes (relative to those of \textit{S. cerevisiae} and the ancestor) is disrupted at the positions of NcCENs. It is therefore likely that \textit{N. castellii} CEN1–9 have been positioned, at least partly, by genome rearrangement during evolution, rather than by de novo centromere formation between the existing genes.

**Candidate Centromere Regions Show Dynamic Behaviors Expected for Functional Centromeres in \textit{N. castellii} Cells**

We addressed whether \textit{N. castellii} CENs show expected localizations of functional centromeres in cells. If \textit{N. castellii} CENs promote kinetochore assembly, spindle microtubules should attach and apply forces on these chromosome regions. In \textit{S. cerevisiae}, such forces cause separation of sister chromatids up to 10 kb from centromeres on the metaphase spindle [18–21]. To investigate this in \textit{N. castellii}, we inserted tet operators at 2–4 kb from NcCEN9 (CEN9L–4) and NcCEN10 (CEN10R–2; Figure 2A). As controls, we also inserted the tet operators on chromosome arms (CEN9L–238 and CEN10R–214). CEN9L–238 is also positioned at 4 kb right of the locus corresponding to the ancestor CEN7, based on synteny. The tet operators were bound by TetR-GFP fusion proteins [22] and visualized as small GFP dots (Figure 2B). In G1 phase, GFP dots at CEN9L–4 and CEN10R–2 localized in the vicinity of the spindle pole body (SPB), whereas those at CEN9L–238 and CEN10R–214 were at a larger distance from the SPB (Figure 2C). In metaphase, GFP dots at CEN9L–4 and CEN10R–2 located near the axis defined by two SPBs (Figure 2D) and often showed two signals, indicative of sister chromatid separation (Figure 2E). In contrast, the GFP dots at CEN9L–238 or CEN10R–214 did not separate until early anaphase (Figures 2E and 2F), whereas the GFP dots at CEN9L–4 and CEN10R–2 moved immediately after SPB segregation during anaphase (Figure 2B, top). These behaviors of GFP dots at CEN9L–4 and CEN10R–2 are similar to those at the \textit{S. cerevisiae} centromeres [18–21] and consistent with NcCENs indeed being functional centromeres in \textit{N. castellii}.

\textit{N. castellii} CENs Include Unique Consensus DNA Elements, which Are Crucial for Minichromosome Propagation

We compared DNA sequences at NcCEN1–10, which include Ndc80-enriched regions in ChIP-seq (Figure 1D). Approximately in the middle of the enriched regions, we identified NcCEN consensus DNA sequences (Figures 3A and S2A). In particular, two short DNA elements showed very high similarity in all ten NcCENs at positions 20–26 and 45–52 in Figure 3A, which we name NaCDEI and NaCDEII (Naumovozyma consensus DNA element), respectively. In addition, other regions showed similarity or common AT- or GC-rich areas among NcCENs. The overall
The consensus within the 70-bp sequence at positions 20–89 in Figure 3A is unique to NcCENs and found at no other regions in the N. castellii genome. Remarkably, the consensus found at N. castellii CENs is very different from the consensus of other point centromeres (CDEI, II, and III; Figure 1B).

We evaluated the activity of candidate centromeres on mini-chromosomes in N. castellii cells. If minichromosomes were able to undergo both DNA replication and mitotic segregation, they are stably propagated during cell proliferation. DNA replication origins have not yet been identified in N. castellii, and we investigated the propagation of pRS306 and pRS316 plasmids, on which an S. cerevisiae centromere and replication origin are absent and present, respectively [23]. pRS306 and pRS316 were maintained at high copy number in N. castellii cells (>70 per cell; see Figure 3E). Yeast cells accumulate minichromosomes with high copy number if their replication occurs normally but segregation is inefficient [25]. We reasoned that, in N. castellii cells, an S. cerevisiae centromere does not promote minichromosome segregation efficiently, but its replication is supported, even without an S. cerevisiae replication origin (Figures S2B–S2D). Notably, addition of NcCEN1 to pRS306 caused a marked reduction in its copy number to 3–5 per cell (see Figure 3E; NcCEN1 WT) and the formation of many more yeast colonies (Figure 3B; NcCEN1 1,173 bp). Addition of NcCEN10 showed similar effects (Figure 3B; NcCEN10 589 bp), whereas addition of a control, chromosome arm DNA fragment (A5960–5970) had no such effect (see Figures 3D and 3E). Thus, N. castellii CENs are able to facilitate minichromosome propagation and yeast colony formation, presumably by promoting mitotic segregation.

To determine the minimum DNA sequence carrying a centromere activity, we examined the ability of several DNA fragments within NcCEN1 and NcCEN10 to support minichromosome
propagation (Figure 3B). The 70-bp sequence at position 20–89 in Figure 3A was essential for centromere activity, and additional 20- to 40-bp sequences around it facilitated the activity. The 20- to 40-bp sequences are not particularly similar among the NcCENs (Figure 3A), but their AT richness may contribute to their centromere activity (Table S1) as does the AT-rich CDEII in other budding yeasts [13]. Subsequently, we addressed whether the consensus DNA elements NaCDEI and NaCDEII are important for centromere activity. Three base-pair mutations within the NaCDEI (21–23a and 24–26c) and NaCDEII (45–47t) of NcCEN1 (Figure 3C) showed substantial decreases in yeast colony formation (Figure 3D) and high copy numbers of minichromosomes (>70 per cell), indicative of inefficient segregation (Figure 3E). Three base-pair control mutations between NaCDEI and NaCDEII (32–34c and 37–39g) showed similar numbers of yeast colonies to wild-type NcCEN1 (Figure 3D) and maintained low copy numbers of minichromosomes (3–5 per cell; Figure 3E). Thus, NaCDEI and NaCDEII are important for the centromere activity.

Using these assays, we next evaluated requirement of RNAi for centromere activity in N. castellii. This pathway is present in some budding yeasts, including N. castellii [26], and required for centromere activity in fission yeast [27]. The centromere activity for minichromosome propagation was still normal without the RNAi pathway in N. castellii (Figures S2E and S2F).
Figure 4. Consensus DNA Elements in *N. castellii* CENs Are Important for the Centromere Function in the Context of Authentic Chromosomes

(A) *NcCEN1* wild-type (WT; 1,173 bp; Figure 3B) and its mutants (1,173 bp with 21–23a, 24–26c, and 45–47t; Figure 3C) were inserted at 214 kb right of *NcCEN10* on chromosome 10 (orange oval in diagram) in haploid *N. castellii* cells (T11605). Karyotypes of individual clones were analyzed (representative examples are shown here) by pulsed field gel electrophoresis (PFGE), followed by Southern blots with ProbeL and ProbeR; see positions of the probes in diagram. Table shows the number of clones that did, or did not, show breakage of chromosome 10 (shortening detected by ProbeL and/or ProbeR; Figure S3A).

(B) *p* < 0.0001

(C) CHIP-qPCR

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<th>Chromosome 10</th>
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(D) Whole genome duplication

(E) Conventional point centromere with *CDEI*, *II* and *III* (8 like fragments)

Unconventional point centromere with *NaCDEI*, *II* and *III* (6 like fragments)

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Consensus DNA Elements in N. castellii CENs Promote CBF3 Binding and Facilitate Centromere Activity on Authentic Chromosomes

We next addressed whether the centromere DNA elements identified above are also crucial for centromere activity on authentic chromosomes. When an additional active centromere is inserted into a yeast chromosome, it causes chromosome breakage between the original and the newly inserted centromere [28, 29]. We employed this procedure to assess the centromere activity in N. castellii. We inserted wild-type and mutated NccENT1s on the chromosome 10 arm (Figure 4A, diagram) and analyzed breakage of this chromosome using pulsed field gel electrophoresis (PFGE), followed by Southern blotting (Figure 4A). After insertion of a wild-type NccENT1, all of 13 randomly chosen clones showed breakage of chromosome 10 (Figures 4A and S3A). By contrast, no such breakage was observed after insertion of NccENT1 carrying mutations at NaCDEI and NaCDEII (21–23a, 24–26c, and 45–47t). Next, we visualized the intracellular localization of wild-type and mutated NccENTs, inserted on chromosome 10. Wild-type NccENT1 on chromosome 10 was near SPBs during telophase to G1 (Figure S3B, left) and on the metaphase spindle with frequent sister separation (Figures 4B and S3B, right). However, mutated NccENT1 (21–23a, 24–26c, and 45–47t) did not show such behavior. Thus, NaCDEI and NaCDEII are crucial for centromere activity on authentic chromosomes.

As shown earlier, CBF3 components Ndc10 and Cep3 bind NccENTs (Figures 1D and S1C). To address whether this binding requires NaCDEs, we used ChIP followed by qPCR (ChIP-qPCR; Figure 4C). Both Ndc10 and Cep3 showed enrichment at the original NccENT1 and wild-type NccENT1 inserted on chromosome 10, but not at the mutated NccENT1 on chromosome 10 (Figure 4C). Thus, the consensus DNA elements within NccENT are required for CBF3 binding. Furthermore, we found both Ndc10 and Cep3 are essential genes in N. castellii (Figure S3C), as is expected if they have central roles in recognizing NccENTs.

How can the CBF3 complex, which recognizes standard CDE I,II,III-type CENs, also bind NccENTs despite the different DNA sequences? We investigated the evolutionary conservation of Ndc10 and Cep3, the CBF3 components recognizing consensus DNA elements in budding yeasts [5, 13]. A putative DNA-binding domain of Cep3 is conserved between N. castellii and other budding yeasts [30]. By contrast, the core DNA-binding domain of Ndc10 showed a more-rapid change during evolution of N. castellii, compared with other budding yeasts with standard CENs (Figures S3D–S3F). Such a rapid change may have happened to adapt to the new type of point centromere.

N. dairenensis, a Close Relative of N. castellii, Has N. castellii-like Consensus DNA Elements at Its Candidate Centromere Regions

We next aimed to identify candidate centromeres in N. dairenensis, a close relative to N. castellii (Figure 1A). Based on the annotated N. dairenensis genome [16], we found that the orders of orthologous genes around most N. castellii CENs are conserved on N. dairenensis chromosomes (Figure S4A). Crucially, at the corresponding intergenic regions, we identified CDEs that are very similar to NaCDEI, II found at N. castellii CENs (Figures 4D and S4B). It is likely that these regions serve as N. dairenensis centromeres, but we could not test this prediction because of a lack of molecular genetics methods in N. dairenensis. This analysis revealed a third sequence element with evolutionary conservation (NaCDEIII; Figure 4D), but mutation analysis showed that it is not essential for centromere function in N. castellii (Figure S4B legend). N. dairenensis Ndc10 showed evolutionary changes, similarly to N. castellii Ndc10 (Figures S3D–S3F). In conclusion, N. castellii CENs and N. dairenensis candidate centromeres have very similar consensus DNA elements (Figure 4D). The new type of centromere CDEs (NaCDEI, II, and III) originated prior to the branching point of N. castellii and N. dairenensis in evolution (Figure 4E).

Conclusions

We have identified centromeres in the budding yeast N. castellii. We conclude that they make point centromeres, because (1) consensus DNA elements are found among all ten centromeres, (2) these DNA elements are important for the centromere activity, and (3) a short DNA fragment (110 bp) containing the consensus DNA elements is sufficient for centromere function. Crucially, the consensus centromere DNA elements are very different from those in other known point centromeres, highlighting the N. castellii centromere as the first unconventional, i.e., non-CDE I,II,III-type, point centromere (Figure 4E). The gene order analyses give the following insights: first, most N. castellii centromeres are not located in intergenic regions orthologous to those containing standard CDEI,II,III-type point centromeres in other species (Figure S1D), although these two are often in close proximity (Figure S4C). This indicates that these N. castellii centromeres did not descend from standard point centromeres at their individual chromosome regions. Second, at most N. castellii centromeres, synteny is disrupted when compared with the

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ancestral budding yeast genome (Figure 1E). This can be explained if N. castellii centromeres were propagated to all chromosomes during evolution through extensive genome rearrangement. The origin of the N. castellii centromere is still elusive, but it may have been propagated and superseded the conventional point centromeres.

**ACCESSION NUMBERS**

DNA sequences have been deposited under the following accession codes: ChIP-seq of Ndc80-6xHA, DDBJ: DRA002836; Ndc10-6xHA, DDBJ: DNA sequences have been deposited under the following accession numbers.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, four figures, and four tables and can be found with the article online at http://dx.doi.org/10.1016/j.cub.2015.06.023.

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