Cohesin ATPase activities regulate DNA binding and coiled-coil configuration

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The cohesin complex is required for sister chromatid cohesion and genome compaction. Cohesin coiled coils (CCs) can fold at break sites near midpoints to bring head and hinge domains, located at opposite ends of coiled coils, into proximity. Whether ATPase activities in the head play a role in this conformational change is yet to be known. Here, we dissected functions of cohesin ATPase activities in cohesin dynamics in Schizosaccharomyces pombe. Isolation and characterization of cohesin ATPase temperature-sensitive (ts) mutants indicate that both ATPase domains are required for proper chromosome segregation. Unbiased screening of spontaneous suppressor mutations rescuing the temperature lethality of cohesin ATPase mutants identified several suppressor hotspots in cohesin that located outside of ATPase domains. Then, we performed comprehensive saturation mutagenesis targeted to these suppressor hotspots. Large numbers of the identified suppressor mutations indicated several different ways to compensate for the ATPase mutants: 1) Substitutions to amino acids with smaller side chains in coiled coils at break sites around midpoints may enable folding and extension of coiled coils more easily; 2) substitutions to arginine in the DNA binding region of the head may enhance DNA binding; or 3) substitutions to hydrophobic amino acids in coiled coils, connecting the head and interacting with other subunits, may alter conformation of coiled coils close to the head. These results reflect serial structural changes in cohesin driven by its ATPase activities potentially for packaging DNAs.

Significance

Cohesin is a heteropentameric protein complex consisting of two structural maintenance of chromosomes (SMC) subunits and three non-SMC subunits. The two SMC subunits form a heterodimer with an ATPase head and hinge that are connected by long coiled coils. Isolation of ATPase head and hinge are suppressed by ATPase defects was performed. Locations and properties of mutant alleles reflect how ATPase activities could be compromised by structural adaptation. ATP-driven conformational changes may enhance DNA anchoring by the head, alter interactions of coiled coils at the head with other subunits for DNA to go through, and fold/extend coiled coils near break sites around midpoint to bring together DNA elements from each other.

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The authors declare no competing interest.

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Results

Isolation of Cohesin ATPase Temperature-Sensitive Mutants. Multiple in vitro ATPase activity experiments indicated that mutations in the signature motif or D loop of ATP-binding cassette (ABC) ATPase domains caused lower or loss of ATPase activity (10, 31–34). To understand how ATPase domains regulate cohesin dynamics, we selected four consecutive amino acids in the highly conserved signature motif and one leucine residue in the D loop of both Psm1/SMC1 and Psm3/SMC3 ATPase domains (indicated by red arrowheads) for targeted random mutagenesis in fission yeast Schizosaccharomyces pombe (S. pombe) (Fig. 1A). Synthetic oligonucleotides were designed for each of the 10 amino acids to introduce all kinds of potential single amino acid substitutions. After screening ~2,200 integrants, we obtained five ts mutants (Fig. 1B). Four (Psm1-L1132T, Psm3-L1097P, Psm3-S1098A, and Psm3-G1100A) of the five responsible mutations were located in signature motifs (or C motifs) and the other one (Psm1-L1166N) in the Psm1 D loop (Fig. 1C). Temperature sensitivity of psm3-S1098A and psm1-L1166N was confirmed by reintegrating the responsible mutations into wild-type strain. Both signature motifs of the head domains are located at the dimeric interface and the signature motif of one SMC subunit forms the ATP binding site together with the Walker A motif of the other SMC subunit (11). All mutations in signature motifs of Psm3/SMC3 (L1097P, S1098A, and G1100A) and Psm1/SMC1 (L1132T) would destabilize the ATP binding surface by steric hindrance or local structural changes, resulting in decreased ATPase activity.

We reintegrated the Psm3-S1098A ts mutation into a strain, in which histone H2A was tagged with GFP, and α-tubulin Atb2 and a spindle pole body protein Sid4 were tagged with GFP (35). Mitotic chromosome segregation defects were observed at restrictive temperature (36°C) (Fig. 1D). Then, psm3-S1098A and psm1-L1166N mutant cells, cultured at the restrictive temperature (36°C) and the permissive temperature (26°C), were observed under fluorescent microscopy after DAPI staining. Approximately 60% of the analyzed mitotic cells in the cohesin ATPase ts mutants exhibited chromosome missegregation phenotypes at restrictive temperature, while the frequency is less than 20% at the permissive temperature (Fig. 1E). These results indicate that activities from both cohesin ATPase domains are required for cohesin to fulfill its functions in proper chromosome segregation.

Spontaneous Mutations Rescuing ATPase Temperature-Sensitive Mutants. Spontaneous suppressor screening is a powerful and unbiased tool to identify second mutation (suppressor mutation) that can bypass the lethality (functional defects) caused by the temperature-sensitive mutation. To reveal the functional effects of cohesin ATPase domains, spontaneous suppressor screens were performed for three cohesin ATPase ts mutants (psm3-S1098A, psm1-L1132T, and psm1-L1166N) at restrictive temperature (Fig. 2A) (36). Next-generation sequencing identified a number of suppressor mutations in genes encoding either SMC subunit of cohesin (Psm1/SMC1 or Psm3/SMC3). They are presented in Table S1 and their relative locations in Psm1/SMC1 and Psm3/SMC3 were shown in Fig. 2B and C. From the data we realized that suppressor mutations are enriched in cohesin ATPase head domains, head coiled-coil junctions, and some regions of coiled coils.

Localization of potential coiled-coil domains in Psm1/SMC1 and Psm3/SMC3 was predicted by MARCOIL (37) and are shown as coiled-coil probability (Fig. 2B and C). Locations with reduced coiled-coil probability scores indicate potential break sites in Psm1/SMC1 and Psm3/SMC3 coiled coils (red arrowheads in Fig. 2B and C). Surprisingly, suppressor mutations of psm3-S1098A and psm1-L1166N that are mapped to coiled coils of Psm1/SMC1 and Psm3/SMC3 are enriched in or close to these break sites. Therefore, the cohesin ATPase activities seem to have relevant functions in regulating coiled-coil dynamics at break sites around midpoints.

Comprehensive Targeted Suppressor Identification. To understand the properties of the suppressor mutations and how they affect cohesin dynamics to compensate for cohesin ATPase defects, several regions (Psm3-HCJ, Psm3-CCN, Psm1-CCN, and Psm3-BS) that are enriched in suppressor mutations, but are not in cohesin ATPase domains, were selected for comprehensive targeted suppressor screening using saturation mutagenesis (Fig. 2D and E). Locations of Psm3-HCJ, Psm3-CCN, Psm1-CCN, and Psm3-BS in cohesin were illustrated (Fig. 2D and E). Psm3/SMC3 head-coiled coil junction (Psm3-HCJ, amino acids 95–130) may bind DNA (Fig. 2E). Psm3/SMC3 coiled coil emerging from N-terminal head domain (Psm3-CCN, amino acids 181–200) and Psm1/SMC1 coiled coil emerging from N-terminal head domain (Psm1-CCN, amino acids 179–202) interact with Rad21N and Mis4C, respectively (Fig. 2E), and a Psm3/SMC3 break site in N-terminal coiled coil (Psm3-BS, amino acids 295–350) is far from the cohesin ATPase head domain (Fig. 2D).

In a previous study, a spontaneous suppressor screen for a ts mutant rad21-I67F, which contains a I67F mutation at the Rad21 N-terminal domain that was predicted to destabilize the interaction between Rad21N and Psm3 coiled coil, identified mutations in cohesin ATPase domains (30). Here, we crossed psm3-G1100A and psm1-L1132T, isolated in this study, with rad21-I67F and found that they can rescue the rad21-I67F ts mutant partially (SI Appendix, Fig. S1A). Therefore, loss of cohesin ATPase activity rescues the rad21-I67F ts mutant. We proposed that suppressors of cohesin ATPase ts mutants (psm3-S1098A and psm1-L1166N) may mimic the effects of cohesin ATPase activities, while suppressors of rad21-I67F may mimic cohesin ATPase defects (SI Appendix, Fig. S1B).

In summary, targeted suppressor screens followed the procedure described in SI Appendix, Fig. S1C and targeted sequencing identified 455 single amino acid substitutions in these hotspots from ~1,600 revertants isolated at restrictive temperatures (SI Appendix, Fig. S1D).

We compared suppressor mutations of psm3-S1098A in Psm3-BS obtained independently from spontaneous suppressor screening and targeted suppressor screening. Five of the six spontaneous suppressors mapped in Psm3-BS were identified in targeted suppressor screening too (SI Appendix, Fig. S2A). In addition, two independent targeted suppressor screens were performed in Psm3-CCN for comprehensiveness (SI Appendix, Fig. S2B). Suppressor mutations identified in the first and second screens are highly overlapped (SI Appendix, Fig. S2C). Therefore, the results supported suppressions of the ts mutants as indeed caused by the suppressor mutations identified in the targeted suppressor screens performed in this study.

Suppressor Mutations in the Head Lie in a DNA Binding Domain. Psm3-HCJ binds DNA and interacts with Rad21N (Fig. 3A) (11). Single amino acid substitutions in Psm3-HCJ that were identified as suppressors of psm3-S1098A or rad21-I67F, were shown in Fig. 3B. Indeed, suppressor mutations of psm3-S1098A and rad21-I67F were not overlapped at all. Among
psm3-S1098A suppressors, arginine (R) was frequently observed in mutant alleles. Substitutions to arginine in psm3-S1098A suppressors lie in a region of the head close to DNA in the cohesin complex; therefore, they may enhance the DNA binding ability of Psm3-HCJ by establishing new electrostatic interactions with DNA (Fig. 3C and D).

Four amino acid residues in Psm3-HCJ (D107, S117, K118, and T119) make direct contact with DNA (Fig. 3B). Among rad21-I67F suppressors, Psm3-K118 is frequently mutated to other amino acids (Fig. 3B), as 12 distinct single amino acid substitutions were obtained at Psm3-K118. Lys118 of Psm3/SMC3 is the unique residue in Psm3-HCJ that forms a salt bridge with DNA (Fig. 3E); the suppressor mutations of rad21-I67F at Psm3-K118 would result in less interaction of Psm3-HCJ with DNA.

Many Suppressors in Coiled Coils Near the Head Lie in or near Regions Associated with Other Subunits. Psm1-CCN interacts with the Mis4/SCC2/NIPBL C terminus and Psm3-CCN interacts with the Rad21/SCC1 N terminus (Fig. 4A) (11). Spontaneous suppressors of Psm1/SMC1 ATPase ts mutants (psm1-L1132T and psm1-L1166N) were enriched in Psm1-CCN, while spontaneous suppressors of rad21-I67F were enriched in Psm3-CCN (Fig. 2B and C). Single amino acid substitutions, identified from targeted saturation mutagenesis, as suppressors of psm1-L1166N in Psm1-CCN or of rad21-I67F in Psm3-CCN are presented (Fig. 4B and C).

To understand hydrophobicity tendency of mutated residues, we counted the frequency of each of the 20 amino acids in mutant alleles involved in the single amino acid substitutions.
Compared to hydrophilic amino acids, hydrophobic amino acids are enriched in mutant alleles in both Psm1-CCN (Fig. 4D) and Psm3-CCN (Fig. 4E).

Suppressors of psm1-L1166N in Psm1-CCN locate at the positions facing either Mis4/SCC2/NIPBL or Psm1 CCC, and these suppressors seem to affect the interaction with Mis4/SCC2/NIPBL directly or indirectly through changing intra-association between CCN and CCC of Psm1/SMC1 (Fig. 4F). Suppressors of the rad21-I67F ts mutant in Psm3-CCN locates at positions either having van der Waals interactions with Rad21N or forming tight interactions with Psm3 C-terminal coiled coil (Fig. 4G). These suppressors in Psm3-CCN are predicted to repair the improper subunit interaction between Rad21/SCC1 and Psm3/SMC3 caused by I67F mutation.

How cohesin ATPase activities regulate interaction between coiled coils emerging from the head with associated non-SMC subunits is still unclear. Cohesin ATPase activities may induce structural changes at the head, which alters coiled coils’ orientations at the head and affects non-SMC subunits’ association with coiled coils at the head (Fig. 4H).

**Coiled-Coil Mutations at a Break Site Are Supposed to Affect Coiled-Coil Probability.** Psm3-BS contains eight heptad repeats (designated HR1~HR8). Among the 186 single amino acid substitutions identified in Psm3/BS for psm3-S1098A, 122 mutations locate in the first four heptad repeats (HR1~HR4), while the other 64 mutations locate in the last four heptad repeats (HR5~HR8); therefore, the first four heptad repeats (HR1~HR4) contain many more mutations than the last four heptad repeats (HR5~HR8) (Fig. 5A). HR1~HR4 have much lower coiled-coil probability than HR5~HR8 (Fig. 5B). Mutation frequency and coiled-coil probability in Psm3-BS are inversely correlated.

We compared molecular weights of mutant alleles with their corresponding wild-type alleles and calculated mean relative molecular weights (MWs) for each amino acid position and...
plotted those against their positions in Psm3-BS. Surprisingly, a negative value of mean relative MW was obtained at most of the positions in Psm3-BS (Fig. 5C), indicating that psm3-S1098A suppressors in Psm3-BS tend to replace wild-type amino acids with other amino acids having smaller side chains.

Then coiled-coil probabilities were calculated for each substitution in Psm3-BS using the mutant Psm3/SMC3 protein sequences (37). A mean relative CC probability value at each amino acid position was calculated by comparing coiled-coil probabilities of mutant alleles with the coiled-coil probability of the wild-type allele. Mean relative CC probabilities were plotted against their positions in Psm3-BS (Fig. 5D). Most single amino acid substitutions in Psm3-BS tend to reduce the coiled-coil probability of Psm3-BS.

SI Appendix, Fig. S3A presents a heptad repeat showing how positions a–g appear when viewed from the top of an antiparallel helix. Residues at “a” and “d” are hydrophobic, forming a hydrophobic core between helices. Residues at “e” and “g” are charged residues forming ion pairs (38). We aligned sequences in HR5, HR6, and HR7 according to amino acid positions in a heptad repeat. Numbers of single amino acid substitutions at each position were plotted against their positions (SI Appendix, Fig. S3B). Most single amino acid substitutions occurred at core residues (a, d, e, and g), which are critical in maintaining helix–helix interactions in the coiled coil. The suppressor mutations probably tend to destabilize the interactions.

The suppression of cohesin ATPase ts mutants by mutations in coiled coils at the break site indicates that cohesin ATPase activities may regulate coiled-coil probabilities at break sites. Since break sites in coiled coils are located around midpoints far from the ATPase head, how cohesin ATPase activities regulate coiled-coil probabilities around break sites is still unclear. Cohesin ATPase activities may induce structural changes at the head, which may be propagated to coiled coils and reduce coiled-coil probabilities at break sites. Therefore, cohesin ATPase activities may regulate coiled-coil folding and/or extension (Fig. 5E).

Discussion

The functions of the cohesin complex in sister chromatid cohesion have been well studied (1, 2). The cohesin complex has been proposed to form a ring-shaped structure (39), in which the globular ATPase head domain and hinge domain are 50 nm away in distance, connected by long coiled coils (4, 40); chromosomal DNAs are topologically entrapped in the ring (21, 41, 42). In anaphase, activated Cut1/separase cleaves Rad21/SCC1 to open the cohesin ring to release chromosomal DNAs inside (43–46). Recently, a folded conformation of cohesin molecules about...
25 nm in length was also observed with atomic force microscopy (40, 47) and electron microscopy (20, 28). Coiled coils contain interruptions around their midpoints (27, 28, 48). Cohesin coiled-coils were supposed to fold around midpoints to bring head and hinge domains into proximity (28, 30, 49), and much work has been done to detect coiled-coil folding (28, 50, 51).
Mis4/SCC2/NIPBL and Psc3/SCC3 were supposed to mediate interaction between head and hinge too (11, 15, 21, 52). Coiled coils can align and zip up (53), which is reminiscent of the Mre11-Rad50 complex. Upon DNA binding, the two coiled coils of Rad50 zip up into a rod (54). Cohesin probably held and released DNA through the actions of coiled coils (30, 50). Except for its fundamental role in sister chromatid cohesion, cohesin is also required to shape three-dimensional genome architecture (55). Cohesin organizes the genome via DNA loop extrusion (29, 56, 57). Single-molecule imaging provided direct evidence that the cohesin complex extrudes DNA loops in vitro, and loop formation and extrusion requires cohesin’s ATPase activity (20, 58). In addition, folding and extension of cohesin coiled coils were supposed to have a role in DNA looping (28, 29) and holding sister chromatids together (51). Cohesin coiled coils are supposed to transfer ATP binding/hydrolysis signals generated in the head to the hinge domains (24). Therefore, extension of the folded coiled coils may require energy released from ATP hydrolysis.

In this study, we identified single amino acid substitutions in *S. pombe*, which either rescued the cohesin ATPase defects or caused the same defects as cohesin ATPase mutants, through unbiased spontaneous genetic screens followed by targeted comprehensive genetic screens. Distribution and characteristics of the single amino acid substitutions indicate the functional effects of cohesin ATPase activity in cohesin structure, thereby helping us to dissect the conformational changes driven by cohesin ATPase activities: 1) Some of cohesin ATPase mutants’ suppressor mutations were mapped in DNA binding domains in the Psm3/SMC3 head (Psm3-HCJ); mutational analysis indicated that they may enhance cohesin’s interaction with DNA. Therefore, cohesin ATPase activities may cause structural changes at the head to grip DNA tighter (Fig. 3D). The results also suggested that the cohesin head may serve as a DNA

**Fig. 5.** Suppressors around a coiled-coil break site. (A) Data matrix showing single amino acid substitutions in Psm3-BS that rescue the temperature sensitivity of *psm3-S1098A*. The eight heptad repeats predicted by MARCOIL are shown above the primary sequence. (B) Numbers of single amino acid substitutions versus coiled-coil probability. (C) Mean relative molecular weight. (D) Mean relative coiled-coil probabilities. (E) A cartoon exhibiting that cohesin ATPase activities may regulate coiled-coil folding and extension at break sites.
anchor site. 2) Certain suppressor mutations of cohesin ATPase mutants and rad21-I67F were mapped in coiled coils emerging from the head, so that they are supposed to distort interactions between coiled coils and other subunits (Rad21/SCC1 and Mis4/SCC2/NIPBL) associated. One possible interpretation is that ATP-driven conformational changes may open closed coiled coils at the head as illustrated in Fig. 4H. 3) Suppressors in coiled coils around break sites indicate that structural changes may happen at break sites in coiled coils after ATP hydrolysis to reduce coiled-coil probabilities (Fig. 5E).

Taken together, we propose that Mis4/SCC2/NIPBL and DNA may stimulate cohesin ATPase activity (20–22) to drive a series of conformation changes at coiled coils emerging from the head to distort interactions between coiled coils and associated subunits (Rad21/SCC1 and Mis4/SCC2/NIPBL) to open closed coiled coils at the head and to extend coiled coils around break sites. These whole processes may bring DNA elements that are far from each other in linear distance together, which results in DNA loop formation and/or extrusion (Fig. 6).

Materials and Methods

Strains, Plasmids, and Media. The S. pombe haploid wild-type strain 972h was used as the host strain for isolation of Psm1/SMC1 and Psm3/SMC3 ATPase mutants. A pBluescript plasmid containing a hygromycin-resistance antibiotic marker was used for construction of various targeting vectors. Vectors with the Psm1/Psm3 wild-type open reading frame (ORF) integrated upstream of the antibiotic marker and ~500-bp sequences after the Psm1/Psm3 ORF integrated downstream of the antibiotic marker were constructed and used as PCR templates for targeted saturation mutagenesis.

A series of site-directed, PCR-based mutagenesis was then performed to introduce random "NNN" (encoding one amino acid) into the Psm1/Psm3 wild-type ORF to substitute each amino acid of "5SG" in the signature motif (or the conserved "L" in the D loop) of the Psm1/Psm3 ATPase domain (Fig. 1A), using the two-step PCR protocol described in ref. 59 with three modifications: 1) Only the forward primer of the partially complementary primer pairs, designed to introduce random NNN into the Psm1/Psm3 wild-type ORF, contains random NNN in its 3′ protrusion. 2) In the current work, not only the corresponding ORFs, but also the hygromycin-resistant antibiotic marker, and the 500-bp DNA sequences after the corresponding ORFs, were amplified by the PCRs. 3) In order to exclude potential nonspecific PCR bands, the first PCR products were purified by gel extraction instead of PCR cleanup. The final PCR products containing mutated Psm1/Psm3 ORFs, the hygromycin-resistance antibiotic marker, and DNA sequences after the Psm1/Psm3 ORF, were transformed into wild-type strain 972h,−, followed by incubation on YPD agar plates containing hygromycin (500 μg/mL) for 5 d at 26 °C. Colonies (integrants) were picked and streaked on YPD plates to screen for ts mutants. Two copies were prepared for each colony, one copy was incubated at 26 °C and the other at 37 °C. Three days later, growth conditions of each colony at each temperature were compared. Ts mutants that grew at 26 °C, but not at 37 °C, were selected, and their responsible mutations were identified by targeted sequencing of the mutated region in Psm1/Psm3 gene sequences.

Suppressor Screening, Next-Generation Sequencing, and Suppressor Identification. The psm1-L1132T, psm1-L1166N, and psm3-S1098A strains were inoculated into YPD medium and cultured overnight. The 1 × 10⁷, 5 × 10⁷, and 1 × 10⁸ cells were spread onto five YPD agar plates for psm1-L1132T, psm1-L1166N, and psm3-S1098A, respectively (2 × 10⁶ cells/plate for psm1-L1132T, 1 × 10⁷ cells/plate for psm1-L1166N, and 2 × 10⁶ cells/plate for psm3-S1098A). The YPD agar plates were then incubated at restrictive temperatures (37 °C for psm1-L1132T, 36 °C for psm1-L1166N, and 37 °C for psm3-S1098A) for 4–6 d for colonies to grow up. In total, 240, 320, and 320 survivor colonies were picked up for psm1-L1132T, psm1-L1166N, and psm3-S1098A, respectively. They were streaked on YPD agar plates for 4 d, and then stored in −80 °C.

Next-generation sequencing and suppressor identification followed the suppressor screening protocols described in ref. 36 with two modifications: 1) In the previous study, revertants (survivor strains) were divided into groups and each group contained 10 revertants. Genomic DNAs from each of the 10 survivor strains were extracted and then equal amount of genomic DNAs from each of the 10 survival strains in the group were mixed together as a genomic DNA pool for next-generation sequencing. In the current study, we first together equal amount of cells from each of the 10 strains in the group, then genomic DNAs of the pooled cells were extracted for next-generation sequencing. 2) A DNaseq platform with paired-end (2 × 150 bp) runs was used instead of the Illumina HiSeq 2000 sequencing system.

Targeted Saturation Mutagenesis Followed by Suppressor Screen. A pBluescript plasmid containing a nourseothricin sulfate (or clonNAT) resistance antibiotic marker was used for construction of targeting vectors. Vectors with corresponding ORFs integrated upstream of the antibiotic marker and ~500-bp sequences after the corresponding ORFs integrated downstream of the antibiotic marker were constructed and used for saturation mutagenesis. A series of site-directed PCR-based mutagenesis was then performed to introduce random NNN (encoding one amino acid) into the corresponding ORFs to replace each, but only one amino acid in the targeted regions. Equal amounts of each PCR product were mixed together to generate a mutation library for each targeted region. These mutation libraries were then transformed into corresponding ts mutants, streaked onto YPD plates containing clonNAT (200 μg/mL) and incubated at the restrictive temperature for 6 d. Revertants, each containing a single amino acid substitution in the targeted regions, were isolated. Their responsible single amino acid substitutions were identified by targeted sequencing of the mutated regions.
The relative molecular weight of every single amino acid substitution in Psm3-BS, which was identified as a suppressor mutation of the Psm3/SMC3 ATPase ts mutant psm3-S1098A, was calculated using the formula: Relative molecular weight = molecular weight of mutant amino acid – molecular weight of wild-type allele. Mean relative molecular weight at every amino acid position in Psm3-BS was then calculated as the sum of relative molecular weights of all single amino acid substitutions identified at that position divided by the number of single amino acid substitutions at the position.

Mean relative coiled-coil probability at each amino acid position in Psm3-BS was calculated in a similar way as mean relative molecular weight. The coiled-coil probability of every Psm3/SMC3 mutant with a single amino acid substitution in Psm3-BS was then calculated as the sum of relative coiled-coil probabilities of every single amino acid substitution identified at that position divided by the number of single codons of the mutant amino acid.

**Data Availability.** The sequencing data reported in this paper have been deposited in the National Center for Biotechnology Information BioProject database (https://www.ncbi.nlm.nih.gov/bioproject, accession no. PRJNA846538).

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