Supplemental Methods for:

The genome organization of *Neurospora crassa* at high-resolution uncovers principles of fungal chromosome topology

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Materials and Methods

Culture conditions, crosslinking, and isolation of Neurospora spheroplasts

Wild type (WT) *Neurospora crassa* strains (N150; 74-OR23-1V and N3752; 74-OR23-1VA) were used for all genome organization experiments. Crosslinked chromatin used for *in situ* Hi-C was isolated from spheroplasts of Neurospora cultures, performed essentially as described (Galazka *et al.* 2016; Klocko *et al.* 2016), with several slight protocol modifications.

To isolate spheroplasts containing crosslinked chromatin, 25 mL cultures containing 1x Vogel's medium + 1.5% sucrose were inoculated with $1.25x10^8$ conidia, (harvested from ~six baby slants by vortexing with water; conidia were washed twice prior to use) and grown at 32 °C with shaking at 200 revolutions per minute (RPM) for ~four hours until ~90% of conidia had germinated, and growth tubes were ~4x the length of the initial conidia. Methanol-free formaldehyde was added to a final concentration of 1% and cultures were incubated 10 minutes at room temperature (RT; ~25°C) with shaking at 100 RPM for crosslinking; 1 M Tris-HCl [pH 8 at 25°C] (Hoffman *et al.* 2015) was added to a final concentration of 125 mM and cultures were incubated at RT for 10 minutes, shaking at 100 RPM, to quench the reaction. Conidia were harvested by centrifugation (4000 RPM, 3 min) and resuspended gently in 20 mL spheroplasting buffer (1 M sorbitol, 100 mM KPO_4 [pH 7.5]) with β-mercaptoethanol added to 30mM. Conidia were pelleted again and resuspended in 20 mL spheroplasting buffer with β-mercaptoethanol added to 1mM. VinoTaste (Novozymes; powder dissolved in water to a stock solution of 50 mg/mL) was added to 50mg and strains

were digested at 30°C, shaking at 100 RPM, for 60 min. Spheroplasts were harvested by centrifugation (3500 RPM, 2 min) and pellets were washed twice with 20 mL Spheroplasting buffer, followed by three washes of the buffer of the 1x restriction enzyme to be used for digestion, either *Mse*I buffer (50 mM NaCl, 10 mM Tris-HCl [pH 7.9 at 25°C], 10 mM $MgCl_2$, 100 µg/mL bovine serum albumin [BSA]) or *Dpn*II buffer (100 mM NaCl, 50 mM Tris-HCl [pH 7.9 at 25°C], 10 mM $MgCl_2$, 100 µg/mL BSA). Washed pellets were resuspended in 3.5 mL of the same restriction enzyme buffer, split into four – 1 mL aliquots in 1.7 mL Eppendorf tubes, pelleted (10,000 rpm, 5 min), whereupon the supernatant was removed, and spheroplast pellets were stored at -80°C.

Hi-C library construction

Hi-C libraries were generally constructed as previously described (Lieberman-Aiden *et al.* 2009; Galazka *et al.* 2016; Klocko *et al.* 2016), but the protocol was adjusted to generate ligation products in the nucleus (*in situ* Hi-C) (Rao *et al.* 2014; Tanizawa *et al.* 2017), which more accurately reflects *in vivo* genomic contacts; the protocol was also refined for efficient use of reagents. All enzymes detailed below are from New England Biolabs (NEB), unless otherwise indicated.

To begin, the concentration of genomic DNA per spheroplast aliquot was determined by resuspending one aliquot per culture replicate in 200 µL decrosslinking buffer (50 mM Tris-HCl [pH 8], 5 mM sodium ethylenediaminetetraacetic acid [Na EDTA], 0.5% [w/v] sodium dodecyl sulfate [SDS]) plus 100 mg proteinase K (Invitrogen) and incubated at 65°C for 16 hours. The volume was then increased to a total of 500 µL using TE buffer (10 mM Tris-HCl [pH 8], 1 mM Na-EDTA), and 40 mg RNase A (Invitrogen) was added. Aliquots were incubated at 37 °C for 30 min, and genomic DNA was extracted with twice 25:24:1 phenol:chloroform:isoamyl alcohol (25:24:1; ThermoFisher cat# AC327115000), once with chloroform, and precipitated with 0.1 volume sodium acetate (pH 5.2) and 1 volume isopropanol, centrifuged (10 minutes, 13k rpm), and the resulting DNA pellet was washed once with 70% (v/v) ethanol, dried for 10 min in a Savant SpeedVac (ThermoFisher, Model # DNA120-115), and resuspended in 50 µL TE. The total DNA concentration per pellet was determined by quantifying 2 µL of the total resuspension of genomic DNA (50 µL) using a Qubit 3.0 HS DNA quantification kit (ThermoFisher cat# Q33217).

Spheroplasts containing 3.5 µg of genomic DNA were resuspended in 270 µL of 1x restriction enzyme buffer (either *Dpn*II or *Mse*I, above; double digests used *Dpn*II buffer) and 50 µL hydrated glass beads were added (Sigma-Aldrich #G1145, Acid washed, 150-212 µm). Spheroplasts were lysed by vortexing tubes for 30 seconds followed by

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30 seconds on ice; five total cycles were performed. Following the settling of the beads, 270 µL of supernatant (and cell debris, if possible) were moved to a new tube. SDS was added to 0.625% final concentration (30 µL of 6.25% stock), tubes incubated at 62 °C for 7 minutes, and immediately placed on ice. Triton X-100 was added to a final concentration of 1% [33 µL of 10% (v/v)] and 10x *Dpn*II or *Mse*I buffer was added to account for added volumes of SDS, Triton and enzymes. Genomic DNA in chromatin was then individually digested with 200 U of either *Dpn*II or *Mse*I (for single enzyme datasets), or 100 U of both *Dpn*II and *Mse*I (200 U total) for double digests, for 16 hours at 37°C while nutating.

Samples were then centrifuged for 3000 rpm for 10 minutes, the supernatant was removed, and the pellets, which contained permeabilized nuclei and chromatin, were resuspended in 83 µL of 1x *Dpn*II or *Mse*I buffer. Sticky ends were blunted in a 100 µL reaction with 30 µM each of dCTP, dGTP, dTTP, Biotin-14-dATP (Invitrogen cat# 19524-016), and 25 U of Klenow (large fragment). Reactions were mixed by pipetting and incubated at 37°C for 60 minutes with nutating. An additional 110 µL of 1x *Dpn*II or *Mse*I buffer was added to the samples (210 µL total chromatin volume), and ligation reactions with 1x T4 Ligation buffer, 1% Triton X-100, 100 µg/mL BSA, and 1675 U of T4 DNA ligase were immediately prepared and incubated at 16 °C for 4 hours. Proteinase K (250 µg total; Gold Biotechnology cat# 97062-238) was added and reactions were decrosslinked at 65°C once overnight (~16 hours) and following the addition of another 250 µg of proteinase K, reactions were decrosslinked a second time for 2 hours. Cooled samples were extracted twice with phenol:chloroform:isoamyl alcohol, the aqueous fraction was transferred to a 2.0 mL microcentrifuge tube, and the volume increased to 500 µL with additional TE buffer. Ligated DNA circles were precipitated by addition of 0.1x volume 3M sodium acetate pH 6.0, 2.5x volumes 100% ethanol, 40 µg glycogen (ThermoFisher cat# R0561), incubation at -80 °C for 30 minutes followed by centrifugation at 13,000 rpm for 15 minutes. Pellets were washed once with 70% ethanol, dried at RT for 10 minutes in a speedvac, and resuspended in 50 µL TE. RNaseA (20 µg; SigmaAldrich cat# R4875) was added and samples were incubated at 37 °C for 30 minutes. An additional 450 µL TE was added, and ligation products were extracted once with 25:24:1 phenol:chloroform:isoamyl alcohol and once with chloroform. The aqueous fraction was transferred to a new 2.0 mL microcentrifuge tube, and ligation products were precipitated (0.1x 3M sodium acetate pH 6.0 and 2.5x 100% ethanol were added, and tubes were incubated at -80 °C for 20 minutes and centrifuged at 13,000 rpm for 10 minutes), washed, dried, resuspended in 25 µL TE/10 (10 mM Tris-HCl [pH 8 at 25 °C], 0.1 mM sodium EDTA) and stored at -20 °C.

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To remove biotin-dATP from unligated DNA ends, 50 µL reactions consisting of 1x NEB buffer 2.1, 100 µM each of dATP and dGTP, and 5 U of T4 DNA polymerase were incubated at 12 °C for 2 hours and quenched with EDTA (final concentration 10 mM), and the reaction volume was increased to 500 µL with TE/10. DNA ligation products were extracted once with 25:24:1 phenol:chloroform:isoamyl alcohol and once with chloroform, transferred to a 2.0 mL microcentrifuge tube, and ethanol precipitated (0.1x volume 3M sodium acetate, pH 6.0 and 2.5x volume 100% ethanol were added, tubes were incubated at -80 °C for 20 minutes, centrifuged at 13,000 rpm for 10 minutes, washed, dried, and DNA pellets were resuspended in 500 µL TE/10). Ligation products were sheared by sonication (Qsonica, Model Q55; amplitude = 30%, 30 seconds sonication, 30 second incubation on ice between pulses). Sheared, biotinylated ligation products were captured on streptavidin magnetic beads (Dynabeads M280, Invitrogen cat# 11205D) equilibrated with 1x BW buffer (5 mM Tris-HCl [pH 8 at 25°C], 0.5 mM Na-EDTA, 1 M NaCl, 0.05% [v/v] Tween-20) at RT for 30 minutes, with pipetting every 10 minutes. Beads were washed on a magnetic rack three times with 1x BW buffer, twice with TE/10, and resuspended in 25 uL TE/10 buffer.

References

Galazka, J. M., A. D. Klocko, M. Uesaka, S. Honda, E. U. Selker *et al.*, 2016 Neurospora chromosomes are organized by blocks of importin alpha-dependent heterochromatin that are largely independent of H3K9me3. Genome Res 26: 1069–1080.

Hoffman, E. A., B. L. Frey, L. M. Smith, and D. T. Auble, 2015 Formaldehyde Crosslinking: A Tool for the Study of Chromatin Complexes. J Biol Chem 290: 26404–26411.

Klocko, A. D., T. Ormsby, J. M. Galazka, N. A. Leggett, M. Uesaka *et al.*, 2016 Normal chromosome conformation depends on subtelomeric facultative heterochromatin in Neurospora crassa. Proc National Acad Sci 113: 15048–15053.

Lieberman-Aiden, E., N. L. van Berkum, L. Williams, M. Imakaev, T. Ragoczy *et al.*, 2009 Comprehensive Mapping of Long-Range Interactions Reveals Folding Principles of the Human Genome. Science 326: 289–293.

Rao, S. S. P., M. H. Huntley, N. C. Durand, E. K. Stamenova, I. D. Bochkov *et al.*, 2014 A 3D Map of the Human Genome at Kilobase Resolution Reveals Principles of Chromatin Looping. Cell 159: 1665–1680.

Tanizawa, H., K.-D. Kim, O. Iwasaki, and K. Noma, 2017 Architectural alterations of the fission yeast genome during the cell cycle. Nat Struct Mol Biol 24: 965–976.