Molecular basis of CTCF binding polarity in genome folding

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Current models propose that boundaries of mammalian topologically associating domains (TADs) arise from the ability of the CTCF protein to stop extrusion of chromatin loops by cohesin. While the orientation of CTCF motifs determines which pairs of CTCF sites preferentially stabilize loops, the molecular basis of this polarity remains unclear. By combining ChIP-seq and single molecule live imaging we report that CTCF positions cohesin, but does not control its overall binding dynamics on chromatin. Using an inducible complementation system, we find that CTCF mutants lacking the N-terminus cannot insulate TADs properly. Cohesin remains at CTCF sites in this mutant, albeit with reduced enrichment. Given the orientation of CTCF motifs presents the N-terminus towards cohesin as it translocates from the interior of TADs, these observations explain how the orientation of CTCF binding sites translates into genome folding patterns.
Mammalian chromosomes are partitioned into topologically associating domains (TADs), which mediate processes ranging from transcriptional regulation to antigen loci recombination. CTCF binding creates TAD boundaries and controls the segmental insulation of chromosome domains. The effect of CTCF on chromosome folding is thought to arise from its ability to block loop extrusion by cohesin proteins and modulate their genomic positioning. Since the proposal that cohesin could enlarge chromatin loops progressively, cohesin complexes have been directly observed extruding DNA loops actively in vitro and found to accumulate at CTCF-binding sites in vivo. Intriguingly, cohesin-dependent chromatid loops preferentially engage pairs of CTCF sites with convergent motif orientation, and, inverting one CTCF motif can lead to repositioning of the corresponding DNA loop. The molecular basis of this polarity, and of how CTCF constrains cohesin mobility, remains however to be explored.

Here, we investigate the molecular basis for CTCF-binding polarity in genome folding. Combining cohesin ChIP-seq and single-molecule imaging in live cells, we observe that although CTCF localizes cohesin at its binding sites, it does not control overall binding or dynamics of cohesin on chromatin, supporting experimentally that CTCF positions cohesin by blocking its translocation. Using an inducible complementation system, we found that CTCF mutants lacking the N terminus are unable to insulate TADs properly, in spite of normal binding to cognate CTCF sites. Cohesin remained at CTCF sites in this N-terminus mutant, albeit with reduced enrichment. Through systematic truncations of the N terminus, we uncovered several regions important for genome folding and discovered a short protein motif that is both necessary and sufficient to recruit the PDS5A subunit of cohesin in a three-hybrid system. The PDS5A-interacting region of CTCF is distinct from the N-terminal region recently reported to interact with RAD21–SA2 in vitro and required for cohesin enrichment at CTCF sites. This CTCF motif displays homology with the PDSS-binding domain of both WAPL and its competitors SORORIN and HASPIN. Nevertheless, by comparing small mutations within the N terminus, both in isolation and in combination, we show that the recently described RAD21–SA2 interaction domain of CTCF, which also displays homology to WAPL, accounts for most of the functions of the CTCF N terminus in genome folding. Given that the orientation of the CTCF DNA motif presents the CTCF N terminus toward cohesin as it translocates from the interior of TADs, these observations provide a molecular explanation for how the polarity of CTCF-binding sites determines the genomic distribution of chromatin loops.

**Results**

**CTCF positions cohesin without controlling its overall binding or dynamics.** Two nonexclusive models may account for both localization of cohesin at CTCF sites and directional DNA looping. Cohesin could load at CTCF-binding sites, downstream of the motif, and initiate loop extrusion unidirectionally. Alternatively, cohesin could load throughout TADs and translocate bidirectionally as it extrudes DNA loops, only stopping when it encounters CTCF sites in the proper orientation.

To test these models, we measured the impact of depleting CTCF on cohesin binding and positioning on chromosomes. As previous studies using inducible CTCF knockout reported that cohesin still displayed ChIP-seq peaks at 80% of initial sites even after 10 days, we sought to achieve more efficient depletion. Using a mouse embryonic stem cell (mESC) line in which CTCF can be degraded by the auxin-inducible degron (AID) system, we observed near-complete disappearance of the cohesin ring subunit RAD21 by ChIP-seq from its initial position at CTCF peaks (Fig. 1a, b). However, spike-in calibration revealed that a RAD21 antibody pulled down an identical amount of chromatin in the absence of CTCF (Fig. 1c). Thus, while cohesin no longer accumulates at CTCF sites in the absence of CTCF, it still associates with chromatin, indicating that it must be redistributed away from CTCF sites—supporting the translocation-and-block model of loop extrusion.

To directly visualize how loss of CTCF may affect cohesin dynamics and association with DNA, we performed single-molecule tracking of RAD21 in WT (Supplementary Fig. 1a–g) and CTCF–AID mESCs (Fig. 1d–g) by targeting both Rad21 alleles with a HaloTag. As previously reported, 60% of RAD21 molecules were bound to chromatin (Fig. 1i). Depleting CTCF did not affect this fraction, nor the distribution of diffusion coefficients or the anomalous diffusion exponent of RAD21 (Fig. 1f–i). Cell-cycle and sister-chromatid cohesion were not a confounding effect in these imaging modalities (see “Methods”), since we obtained similar results in each single-cycling mESC (Supplementary Fig. 1g, and in noncycling astrocytes (Fig. 1f–g). However, CTCF depletion led to a modest but reproducible increase in the number of quickly diffusing molecules (\(\log D_{\text{fast}} < 0\)), in both cycling and noncycling cells (Fig. 1f–g). These fast-diffusing molecules were nevertheless not completely free, since they diffused more slowly than unbound cohesin (\(\log D_{\text{fast}} > 0\)), as estimated from imaging cells blocked in early M phase by means of a 6-h depletion of SORORIN (Supplementary Fig. 1h–o). Such a role for CTCF in controlling the diffusion of a small subset of cohesin molecules is in line with recent FRAP experiments, showing that CTCF can stabilize longer-lived RAD21 molecules. Taken together with the spike-in ChIP-seq, our results refute the idea that CTCF promotes bulk loading of cohesin and supports a mechanism whereby CTCF acts by blocking translocating cohesin.

**Systematic evaluation of CTCF domains in chromosome folding.** We next investigated how CTCF mediates TAD insulation. Mutational analysis of CTCF is challenging because CTCF is essential for long-term cell survival, and mutations altering CTCF protein stability or CTCF binding will de facto alter cohesin positioning and TAD folding—since insulation of TADs relates quantitatively to CTCF levels. To overcome these obstacles, we used a complementation system where inducible CTCF cDNA transgenes are stably targeted in CTCF−/− mice by means of a 6-h depletion of SORORIN (Supplementary Fig. 1h–o). Such a role for CTCF in controlling the diffusion of a small subset of cohesin molecules is in line with recent FRAP experiments, showing that CTCF can stabilize longer-lived RAD21 molecules. Taken together with the spike-in ChIP-seq, our results refute the idea that CTCF promotes bulk loading of cohesin and suggests a mechanism whereby CTCF acts by blocking translocating cohesin.
CTCF N(1–265) mediates chromatin folding into TADs. We proceeded to establish an additional 12 stable cell lines, each harboring a different mutated CTCF cDNA, leaving the core of the DNA-binding domain intact (central zinc-finger (ZF) array)—Fig. 2a and Supplementary Fig. 2d). Several CTCF mutants failed to rescue TAD insulation to the extent expected from their expression levels (Fig. 2b). Deletion of the entire N-terminal domain ΔN(1–265) had the most impact (Fig. 2c, d). Within the N terminus, multiple subregions participate to the ability of CTCF to insulate TADs (Fig. 2b): ΔN(1–89) triggered a mild but detectable insulation defect, while ΔN(179–265) had a more pronounced effect. ΔN(264–288), which overlaps one RNA-binding region and ZF1, as well as mutation of the ZF1 itself (H288R), also led to insulation defects and is characterized further in a parallel study28.

As for the C-terminal domain, while the single ΔC(577–736) clone analyzed affected insulation, expression of the mutant in vitro data25, and encompasses the C-terminal internal RNA-binding region, RBR, (Supplementary Figs. 2d and 3a)26,27. ΔC (577–614) is expressed at around 60% of the level of the full-length transgene, confirming that the region contributes to CTCF stability (Supplementary Fig. 3b). ΔC(577–614) displayed lower DNA binding by ChIP-seq (Supplementary Fig. 3e–g) and rescued insulation as expected based on its expression level (Fig. 2b and Supplementary Fig. 3c, d). Furthermore, ΔC (577–614) co-immunoprecipitated with the cohesin subunit SA2 from nuclear extracts (Supplementary Fig. 3h), in line with other studies26,27. C(577–614) is therefore dispensable for connecting CTCF and cohesin in vivo, and appears to contribute minimally to TAD folding beyond promoting CTCF binding (it is possible that our 5C assay did not detect subtle changes at the subset of micro-C peaks recently reported to change in this mutant)27. Another domain must therefore mediate cohesin blocking and overall directional loop retention by CTCF.
protein was very low. Given that two other tiling deletions ΔC (577–614) and ΔC(615–736) expressed at higher levels did not disrupt TAD insulation noticeably, we conclude that the N terminus is the most potent domain of CTCF for insulating TADs.

CTCF N(1–265) participates in retaining cohesin at CTCF sites. To understand the pronounced chromatin-folding defects in ΔN(1–265), we measured binding of transgenic CTCF and endogenous Rad21 by ChIP-seq. Deleting the entire N terminus did not alter CTCF binding, as indicated by FLAG pulldown (Fig. 3). RAD21 enrichment at FLAG–CTCF peaks remained detectable in the ΔN(1–265) mutant, but was reduced twofold (Fig. 3). Therefore, proper retention of cohesin at CTCF sites requires N(1–265), indicating that the CTCF N terminus either participates in inhibiting cohesin translocation (thereby promoting insulation) or—nonexclusively—protects blocked cohesin from unloading (thereby bolstering 5C peaks between CTCF sites). These observations are in line with a parallel study concluding that the N terminus is required for RAD21 occupancy at CTCF sites.

Given that deleting the CTCF N terminus led to milder insulation defects than complete CTCF depletion, and that deleting the C terminus had little-to-no effect, the ZF array mediates some degree of insulation and must therefore participate in halting cohesin translocation. The ZF domain confers to CTCF an unusually long residence time for a transcription factor a s in halting cohesin translocation. The ZF domain confers to CTCF a long residence time for a transcription factor.

CTCF N(13–33) can recruit PDS5A via a motif shared with WAPL and SORORIN. Our results suggested that N(1–265) may contain one region (possibly more given Fig. 2b) able to interact directly or indirectly with cohesin and alter its behavior during loop extrusion. To test this hypothesis, we tethered CTCF to a LacO array (or the nuclear periphery, Supplementary Fig. 4) and monitored the recruitment of transiently overexpressed cohesin subunits by fluorescent three-hybrid (F3H) (Fig. 4a). The only cohesin subunit recruited by CTCF in this assay was PDS5A (Fig. 4b, c), ΔN(1–265) completely abrogated PDS5A recruitment, as did the smaller ΔN(13–33) (Fig. 4d). Conversely, fusing CTCF N(13–33) to eGFP was sufficient to elicit PDS5A recruitment (Fig. 4e).

Sequence alignment revealed that CTCF N(13–33) contains a KTYQR motif highly analogous to the known PDS5-binding domains of WAPL, SORORIN, and HASPIN (Fig. 4g). Alanine substitution of CTCF KTYQR abrogated PDS5A recruitment by F3H. Reciprocally, alanine substitution of the APEAP motif in PDS5, known to bind WAPL and SORORIN, also abrogated its recruitment by CTCF in F3H (Fig. 4h). Altogether, this indicates that CTCF binds the same region in PDS5 as SORORIN and WAPL. This is especially interesting, given that SORORIN binding to PDS5 through this region is known to shield PDS5 from the releasing activity of WAPL, thereby opposing cohesin unloading. Our observations raise the possibility that CTCF might act similarly.

It remains unclear at this stage why CTCF cannot recruit PDS5B in F3H, in spite of the region around the APEAP motif being highly similar between PDS5A and PDS5B. Human and
mouse CTCF 13–33 are 100% identical (Supplementary Fig. 4), with extremely high conservation throughout the protein, including the N terminus, up to fishes. Supporting our observations with mouse orthologs, we observed that human CTCF also recruits human PDS5A, and much more efficiently than human PDS5B (Supplementary Fig. 4). It is possible that a remote segment unique to PDS5B interferes with its recruitment than human PDS5B (Supplementary Fig. 4e). It is possible that a remote segment unique to PDS5B interferes with its recruitment of ectopic Hi–C peaks mediated by most but not all effects of CTCF in mouse 26–28, these findings prompted us to explore further how CTCF can block cohesin and stabilize DNA loops. First, because binding of PDS5 and NIPBL to the cohesin ring is mutually exclusive, CTCF may prevent NIPBL from promoting ATP hydrolysis and cohesin translocation, thereby blocking cohesin at CTCF sites. Second, CTCF may interfere with completion of the unloading process, employing its N terminus to disconnect PDS5A from the cohesin unloader WAPL. These observations also offer insight as to why depleting PDS5A and PDS5B interferes with its recruitment by CTCF.

CTCF N(13–33) does not mediate the effects of the N(13–33) did not trigger obvious growth defects, and chromosome folding was very mildly affected by 5C (Fig. 5b), across 4 replicates of 4 clonal cell lines. The CTCF N terminus was recently discovered to bind RAD21–SA2 in vitro via amino acids N(226–230), and the Y226A/F228A mutation triggers almost complete loss of Hi–C peaks between TAD boundaries 19. Given that N(226–230) can compete out a WAPL-binding site on RAD21–SA2 in vitro 19, we explored whether N(226–230) might compensate the deletion of the N(13–33) region, which we show also has the potential to compete out WAPL binding (this time to PDS5A, Fig. 4). We therefore leveraged our inducible rescue system to mutate N(13–33) either alone or in combination with N(226–230). We also assessed the impact of these N-terminal mutations relative to either deletion of the entire N terminus or complete loss of CTCF, using both 5C (Fig. 5a) and Hi–C (Fig. 5c, d).

Metagenalyses of TAD insulation and loops genome-wide by Hi–C, using boundaries previously identified by ultra-deep sequencing 36, enabled us to use shallow sequencing across seven genotypes in replicate (Fig. 5c, d, Supplementary Fig. 5). In line with these experiments, the CTCF N(226–228) mediated most but not all effects of CTCF in TAD folding. Importantly, the CTCF–PDS5A axis mediated by N(13–33) cannot account for all functions of the N(1–265) region in TAD folding, since ΔN(1–89) exhibits only partial insulation defects (Fig. 2b).

Fig. 3 The CTCF N terminus participates in but is not strictly required for cohesin positioning at CTCF sites. a, ChIP–seq track snapshot, b, c density plots, d, e scatterplots, and f fraction of reads in peak (FRIP) scores indicates that RAD21 is still detected at CTCF peaks 3 in cells expressing CTCF ΔN(1–265), albeit with a twofold reduced enrichment compared to the full-length CTCF transgene. CTCF ChIP–seq data were obtained from ref. 3.
with our 5C data, deleting N(13–33) did not reduce insulation or Hi–C peak strength, even in combination with Y226A/F228A. We conclude that the PDS5A-interacting domain of CTCF is dispensable for chromosome folding as monitored by our assays. It remains possible that the CTCF–PDS5A interaction is relevant for pathways we have not assayed. Of note, Y226A/F228A alone exhibited similar Hi–C defects as the entire N-terminal deletion, which itself retained more insulation than full CTCF depletion—consistent with our 5C analyses shown in Fig. 2. We conclude that the N(226–228) region is the most potent domain of CTCF in genome folding. Future experiments will address whether the other disruptive N-terminal truncations detected in Fig. 2b and other studies20,28,41,42 alter the function of this domain.

**Discussion**

Altogether, our data reveal the importance of the N-terminus portion of the CTCF protein in stabilizing cohesin at CTCF-binding sites, providing a molecular explanation for how CTCF-binding site polarity instructs chromosome folding (Fig.6 and Supplementary Fig. 6). The inducible degron-based genetic complementation approach presented here allowed comparing the effect of either mutating or acutely depleting CTCF, in a context where endogenous CTCF is not present. Our conclusions are in line with two recent studies that used distinct strategies to ascertain the importance of the N terminus for cohesin retention and genome folding. Li et al.19 introduced a point mutation at the endogenous locus (Y226A/F228A) without comparing to full CTCF-Cohesin fluorescent 3-hybrid a b c d e f g h

![Fluorescent three-hybrid setup testing the ability of CTCF to recruit cohesin subunits in BHK-LacO cells. c PDS5A is the only subunit recruited by CTCF by F3H. Each data point corresponds to the Pearson correlation between the green (CTCF) and red (cohesin subunits) channels at one GFP-positive LacO array. High values denote high colocalization of CTCF and cohesin subunits at the array. Boxplots indicate the first and third quartile and median. d Deleting CTCF N(13–33) prevents PDS5A recruitment in F3H. e, f N(13–33)-eGFP is sufficient to recruit PDS5A by F3H. g CTCF N(23–27) aligns with the known PDS5-binding region of WAPL, SORORIN, and HASPIN reported to interact with the APEAP motif of PDS5. h Mutation of the APEAP motif of PDS5A prevents its recruitment by CTCF in the F3H assay. Alanine substitution of CTCF N(23–27) prevents PDS5A recruitment by CTCF.

Significance for future studies and technological advancement

The CTCF protein is a key player in genome folding and regulation of cohesin recruitment. By understanding the role of the N-terminus in stabilizing cohesin, we can develop new strategies to manipulate chromosome structure at specific loci. This knowledge could be exploited in cancer research, as altered chromosome folding is a hallmark of cancer. Additionally, the inducible degron-based genetic complementation approach offers a powerful tool for studying protein function in living cells, which can be translated into the development of novel therapeutic strategies.
**Fig. 5 5C and Hi-C analysis of N-terminal mutations of CTCF.**

**a** Summary of 5C experiments in stable mESC lines harboring CTCF transgenes with N-terminal mutations and treated with dox and auxin as in Fig. 2b. Colored data points are reproduced from Fig. 2b for comparison.

**b** TAD insulation analysis from 5C data obtained on cells with region N(13–33) from endogenous CTCF alleles. Each point is the average insulation measured in one 5C replicate and notch marks the median.

**c** Hi-C in stable mESC lines expressing CTCF transgenes. Aggregate insulation scores are depicted next to aggregate heatmaps of select genotypes. Each point is the average insulation measured in one Hi-C replicate and notch marks the average.

**d** Same data as in **b** for aggregate peak analysis.
With CTCF N terminus

Extrusion of DNA loop

CTCF binding pauses cohesin N-terminus blocks translocation and protects from unloading

Looping between convergent CTCF sites is stabilized

Without CTCF N terminus

Extrusion of DNA loop

CTCF binding pauses cohesin

CTCF ΔN-term fails to fully block cohesin translocation

Insulation is lost

CTCF ΔN-term fails to protect halted cohesin from unloading

Loop is released

**Fig. 6 Summary model for the role of the CTCF N terminus in chromosome folding.** Upon encountering a bound CTCF site, cohesin halts, irrespective of motif orientation. Because of the nonpalindromic nature of the CTCF DNA motif, the effect of the CTCF N terminus on cohesin retention and DNA loop stabilization is polarized to one side of CTCF-binding site. Altogether, these events result in pairs of interacting TAD boundaries being preferentially populated by CTCF motifs in convergent orientation. Upon deleting the N terminus of CTCF, cohesin occupancy is diminished but still detectable, indicating that cohesin still pauses upon encountering bound CTCF sites. Loss of cohesin occupancy may reflect either or both decreased ability of truncated CTCF to block cohesin (leading to insulation defects) and decreased ability of truncated CTCF to protect halted cohesin from unloading (leading to loss of the DNA loop). See Supplementary Fig. 6.

Depletion of CTCF, and Pugacheva et al. complemented a cell line where CTCF binding is disabled at a subset of sites interspersed between unaffected sites.

The importance of the CTCF N terminus draws support from evolutionary data: while the ZF domain of CTCF is highly conserved across bilateria, vertebrate and invertebrate N termini are highly divergent overall. In *Drosophila*, CTCF-binding sites also overlap cohesin ChIP-seq peaks (Supplementary Fig. 7), but do not exhibit motif orientation bias at domain borders and do not anchor Hi–C peaks. This reinforces the notion that, while the conserved ZF domain is an impediment to cohesin translocation, the mammalian N terminus is required to fully retain cohesin and stabilize chromatin loops as they appear by Hi–C. While the CTCF N terminus is highly conserved across mammals, it is highly divergent from that of its paralog BORIS/CTCFL. BORIS does not interact with PDS5A (Fig. 4d), lacks homology to the RAD21–SA1-interaction domain in CTCF, and does not share the functions of CTCF in genome architecture.

Altogether, our observations also explain why TAD boundaries are preferentially populated by pairs of CTCF sites with binding sites in a convergent orientation, and why inverting a CTCF site impairs chromatin interactions, in spite of leaving cohesin ChIP enrichment unchanged. Indeed, orientation of the CTCF motif ensures that cohesin translocating from the inner portion of the ZF array, CTCF is unable to rescue TAD folding, indicating that oriented presentation of the N terminus is crucial (Fig. 2b). Finally, our observations also provide insight as to why depleting WAPL triggers accumulation of DNA loops between non-convergent CTCF sites: the unloading complex is necessary to release loops held by cohesin at CTCF sites, even when cohesin halts by encountering the C-terminal pole of CTCF-binding site (Supplementary Fig. 4). This would account for the cohesin traffic jam at CTCF motifs in divergent orientation in cells devoid of WAPL. In summary, our results point toward additional functions of CTCF beyond cohesin blocking, namely protecting from unloading, and pave the way for further mechanistic dissection of the process.

**Methods**

**Cell culture.** Parental WT mESC E14Tg2a (karyotype 19, XY, 129/Ola isogenic background) and subclones were cultured in DMEM + Glutamax (ThermoFisher cat 10566-016) supplemented with 15% fetal bovine serum (ThermoFisher SH30071.03), 550 µM b-mercaptoethanol (ThermoFisher 21985023), 1 mM sodium pyruvate (ThermoFisher 11360-070), 1× nonessential amino acids (ThermoFisher 12100-147), 550 µM b-mercaptoethanol (ThermoFisher 21985023), and 104 U of Leukemia-inhibitory factor (Millipore ESG1107). Cells were maintained at a density of 0.2–1.5 × 106 cells/cm2 by passage using TrypLE (12563011) every 24–48 h on 0.1% gelatin-coated dishes (Millipore cat ES-006-B) at 37 °C and 7% CO2. The medium was changed daily when cells were not passed. Cells were checked for mycoplasma infection every 3–4 months and tested negative. The CTCF-AID mESCs (full genotype CTCF-AID-eGFP, Tir1(random insertion)) were described as cell line #EN52.9.1 in ref. 3. A full list of the cell lines used and generated in this study, with unique identifiers numbers, can be found in Supplementary Table 1.

To establish neural progenitors and astrocytes, CTCF-AID mESCs were seeded at around 0.1 million cells in a 75-cm2 gelatinized dish in mESC medium. The following day, cells were rinsed twice in 1× phosphate-buffered saline and switched to N2B27 medium (30% DMEM/F12 medium: Gibco 31300-038, 30% Neurobasal medium: Gibco 21103-049, 1× Glutamax Gibco 35050061, 0.5x B27 Gibco 17504-044, 1x N2 Millipore SCN012, and 0.1mM 2-mercaptoethanol) (Thermo Fisher 21985023) and changed daily. After 7 days, cells were detached using TrypLE and seeded on nongelatinized bacterial dishes for suspension culture at 3 million cells per 75 cm2 dish. Cell lines were passed twice weekly. After 2–4 days, floating aggregates were seeded on gelatinized dishes. After 2–4 days, cells were dissociated using Accutase and passaged twice on gelatinized dishes in N2B27 + EGF + FGF and cryopreserved after expansion. For differentiation into quiescent astrocytes, adherent NPC cultures were washed twice with N2B27 and cultured for at least 48 h with N2B27 + 10 ng/mL BMP4 (R&D Systems 314-HP-010).

Schneider’s Drosophila Line 2 (S2) cells were obtained from ATCC and cultured in Schneider’s Drosophila Medium (ThermoFisher 21720001) with 10% heat-inactivated FBS (ThermoFisher SH30071.03) at 28 °C according to the ThermoFisher protocol.
The Baby Hamster Kidney (BHK) LacO clone #2 was used for Fluorescent three-hybrid was created in the laboratory of David Spector and kindly provided by Pierre-Arnaud Deschamps. Homozygous clones were assayed for co-transfection with pCAGGS-FlpO-IRES-puro and the selection cassette. Tiri was then introduced at rs26 using vectors pX330-EN479 (Addgene #86234) and pEN114 (Addgene # 92143). Homozygous clones were identified by PCR.

To delete the nucleotides encoding for CTCF(13–33) from the endogenous allele, we created a targeting vector consisting of 1 kb upstream and downstream of the region to delete clones into pUC19 (Buenaulavector pENT15). We co-transfected this plasmid together with the sgRNA vector pX459–EN238 (derived from Dr. M. J. Chomber, pX459–2A-puro, Addgene #52989—see Supplementary information). We used the Neon system (ThermoFisher) using a 100-µL tip with 1 million cells at 1400 V, 10 ms, and 3 pulses with 15 µg of pEN715 and 5 µg of pX459–EN238. One day later, puromycin was added at 1 µg/mL. One day later, cells were split for limiting dilution in 10-cm plates with puromycin. Starting 1 day later, the medium was changed daily without puromycin. Single colonies were picked manually and grafted by PCR. Deletions were also confirmed from cDNA generated from the selected clones, and that no WT CTCF cDNA was produced by these cells. These mutant cells did not exhibit noticeable growth defects.

The list of cell lines generated in this study and the corresponding CRISPR sgRNAs can be found in Supplementary Data 1.

ChIP-seq Preparation of spike-in chromatin from S2 cells—cells that were detached from culture dishes by splashing them gently but thoroughly with culture medium, and transferred to a 15-ml conical tube before spinning at 1000g for 3 min. Cells were resuspended at 106 cells/ml in complete 24-well culture medium at room temperature. After 7 days, 270 µl of 3X Formaldehyde (Electron Microscopy Sciences) was added, and cells were left agitating for 5 min @ RT. Then, 510 µl of 2.5 M glycine (final concentration 125 mM) was added, and cells were left agitating for 5 min @ RT, then spun at 1000g for 2 min. 4 C. Fixed cells were washed once in 1 ml of cold 1X PBS–0.125 μg/mL glycine, and spun at 1000g for 3 min, 4 C. Cells were used for sonication without prior freezing, as we noticed that snap freezing dramatically altered shearing efficiency. Fresh cell pellets were resuspended in 1 ml of Cell lysis buffer (20 μM Tris HCl, pH 8.0, 85 mM KCl, 0.5% GEPAL, and 1X Halt protease inhibitors, ThermoFisher P78425) and incubated on ice for 10 min. Nuclei were pelleted by spinning at 2500g for 5 min at 4°C and lysed in 50 mM Tris HCl, pH 8.0, 10 mM EDTA, 1% sodium dodecyl sulfate, and 1X Halt protease inhibitors for 30 min on ice. Chromatin was sheared using a Covaris S220 ultrasonicator 5 Duty cycle, 5 intensity, and 200 cycles/burst for 7 min. Debris was pelleted by centrifugation at 1500g for 5 min. The supernatant was transferred into a new tube, and glyceral was added at 10% final concentration before freezing at −80 °C as single-use aliquots. For each ChIP experiment, 600 ng of Drosophila chromatin (as estimated from the amount of DNA retrieved after reverse cross-linking) was used in combination with sonicated chromatin obtained from 10 million S2 cells.

**RAD21 ChIP-seq** In Figure 1—the first set of RAD21 ChIP-seq was performed in parallel with the CTCF AID-mESC clone EN52.9.1 published in 2017, using 10 mg of antibody Abcam ab992 together with 40 ng of Drosophila melanogaster spike-in chromatin (Active motif 53083) and spike-in antibody (Active motif 61866). These tracks are tagged as “2017 protocol” in Supplementary Data 2 and companion GEO submission of this study. Direct RAD21 and FLAG-ChIP-seq data files in RNA-seq fa and bed format from the RAD21 ChIP-seq in mESCs containing CTCF reverse transgenes, as well as replicates of the parental CTCF–AID line EN52.9.1 post 2017, were prepared with a protocol differing from data in Figure 1 by the lysis and wash buffers. For the full-length transgene, we used the high-expressing clone (EN133.10) to be closest to the expression level of the AN (1–263) clones.

For fixed, mESCs were dissociated using TrypLE and resuspended in 10% PBS in PBS, counted, and adjusted to 1 million cells per ml. Formaldehyde was then added to 1% final concentration followed by 10 min of incubation at room temperature. Quenching was performed by adding 2.5 M glycine–PBS to 0.125 M final concentration. Cells were pelleted by spinning at 2500g for 5 min at 4°C and lysed in 50 mM Tris HCl, pH 8.0, 10 mM EDTA, 1% sodium dodecyl sulfate, and 1X Halt protease inhibitors for 30 min on ice. Chromatin was sheared using a Covaris S220 ultrasonicator 5 Duty cycle, 5 intensity, and 200 cycles/burst for 7 min. Debris was pelleted by centrifugation at 1500g for 5 min. The supernatant was transferred into a new tube, and glyceral was added at 10% final concentration before freezing at −80 °C as single-use aliquots. For each ChIP experiment, 600 ng of Drosophila chromatin (as estimated from the amount of DNA retrieved after reverse cross-linking) was used in combination with sonicated chromatin obtained from 10 million S2 cells.
ChIP-seq analysis. Mapping and peak calling were performed as exactly as described previously\(^5\) using mm9 assembly. Fastq files were trimmed using the fastq-mcf program, aligned to the mm9 reference genome with the bowtie2 software\(^6\). Reads with a mapq score of 30 or greater were retained, using Samtools. Heatmap visualization and integration with RNA-seq was performed using the Easeq version 1.03 software\(^7\). Published\(^8\) CTCF-ChIP-seq peaks in untreated and auxin-treated CTCF-AID mESCs were used to identify total and auxin-dependent CTCF peaks. The fraction of randomly shuffled peak scores was calculated by the proportion of uniquely mapping reads within auxin-sensitive CTCF peaks compared to the total number of uniquely mapping reads, and excluding genomic regions known to display artificial ChIP-seq signal\(^9\) retrieved from https://sites.google.com/site/eraserch/reagents/projects/blocks.

The RAD21 ChIP-seq presented in Fig. 1, and identified as Rad21 in 2017\(^{10}\) protocol in Supplementary Data 2, was generated in parallel with the CTCF
ChIP-seq data published\(^11\) in GEO series GSE98671. We used matching inputs for the analysis as those were generated in parallel (see Supplementary Data 2). These samples were generated using the commercial Active motif spike-in reagents (spike-in chromatin cat#52083 and spike-in antibody cat#61686), where spike-in samples were generated using the commercial Active motif spike-in reagents exactly as described\(^3\) with the same 5C oligonucleotide pool, which corresponds to a single alternating design of 486 Forward and 504 Reverse oligos, spanning 4.5 Mb.

Chromosome-conformation capture carbon copy (SC). SC was performed exactly as described\(^12\) with the same Sc oligonucleotide pool, which corresponds to a single alternating design of 486 Forward and 504 Reverse oligos, spanning 4.5 Mb across mm9 chrX:98373477-103425147. Note that all cells used here are XY with a 100471148–100471148 boundary1 chrX:99431148–99431148, and boundary4 chrX:100671148–100691148, boundary5 chrX:101211148–101231148, and boundary6 chrX:1013211148–103231148.

Live single-molecule imaging. Microscopy setup—Single-molecule imaging was performed on an epifluorescence-inverted microscope (IX71, Olympus) in HILO illumination\(^13\). In all, a 500 nm achromatic lens conjugates the slit to the specimen plane to achieve a proper HILO. The lens focuses the excitation beam on the back focal plane of a 150X objective lens (UPlan N 150× TIRF 1.45 NA, O.L., Olympus, France). The lens is mounted on a translation stage together with a metallic mirror that directs the beam to the microscope. Displacement of the translation stage allows a precise positioning of the focused beam at the back focal plane of the objective without influencing the lens-BFP distance. Thanks to this configuration, it is possible to adjust the tilting of the laser beam at the output of the objective and thus the effective thickness of the tilted light-sheet excitation at the specimen. For the complete separation between the excitation and emission, we used a fluorescence cube containing a quad-band dichroic mirror (FF04/499/453/572/Di02-25 x 36, SEMROCK) together with adequate emission filters. The setup is provided with a 561-nm laser (Sapphire 561, Coherent, CA, USA), a 488-nm laser (488LM-200, ERROL, France), and a 405-nm laser (405LM-200, ERROL, France). Lasers were tuned via an acousto-optical tunable filter (AOTF 400-650-TN, A&A Optoelectronic, France) and controlled by a homemade interface in Micromanager v1.42\(^{16}\). The signal was acquired with an EM-CCD camera (iXonEM DMV860DCS-BV, Andor, Ireland) running in frame-transfer mode. Acquisitions—To perform single-molecule-tracking experiments, cells (both mitotic and non-mitotic) were grown on circular petri dishes with glass bottom (MatTek, Part No: PT5G-1.5-14-C) coated with fibronectin (Millipore SAS cat# FC010-5mg). Cells were seeded at a density of 3 × 10^4/cm^2 the day before the experiments, in culture medium based on Fluorobrite DMEM for mESCs (ThermoFisher 13248017). We underline the importance of performing single-molecule imaging in phenol-red free medium to both reduce the background fluorescence and minimize localization errors.
The experiments were performed 20 h (labeled as 1 day) after adding auxin to culture medium. To achieve single-molecule labeling, cells were incubated with 1 PM of antibody probe for 20 min at room temperature (incubation followed by a first rinsing step, 15-min wait, and another rinsing). While waiting for the second rinsing step, cells were incubated with 1 PM Hoechst and consequently washed to minimize the fluorophores unbound in solution. All washings were performed using cell- culture medium; the coverslips treated with auxin were washed with medium. During the last wash, the coverslips were kept at 37 °C and 5% CO₂ with a Takai Hit heating system (INUBGE-PPZ). To locate nuclei, cells were stained with Hoechst 33342 (bisBenzimid H 33342 trihydrochloride, Sigma-Alrich, ref 14533), excited with 405-nm light. The CTCF- GFP protein was endogenously expressed in the nucleus. To track CTCF-Halo-JF594, the sample was excited with the 561-nm laser. At least 5000 frames were recorded in a continuous imaging regime, the laser being controlled by the camera. Laser power was approximately 0.1 kW/cm² and adjusted depending on the exposure time in order to keep the amount of excitation photons constant. To determine the fraction of bound molecules, we acquired images in a continuous imaging regime, the laser being controlled by the camera. Laser power was approximately 0.1 kW/cm² and adjusted depending on the exposure time in order to keep the amount of excitation photons constant. For the analysis of the dynamics (MSD) and the residence time, we acquired videos at a rate of 20 Hz (50 ms).

Quantification of photobleaching—To characterize the photobleaching of the organic dye used for our single-particle-tracking experiments (SPT), we acquired movies in the same imaging conditions of the SPT experiments in terms of laser power and exposure. Cells were stained with the JF594 organic dye at 1 nM for 5 min on ice for 5% formaldehyde (Electron Microscopy Sciences), then rinsed three times with 1× PBS, incubated with 0.5% Triton X in 1× PBS for 5 min and 1 μl/mℓ DAPI, rinsed twice with 1× PBS, and left in 1× PBS for imaging. Typically, 20–40% of cells displayed green fluorescence at the LacO array.

Extended data—The trajectories were acquired as 3D slices on a Zeiss Axio microscope using 405-, 488-, and 561-nm excitation lasers with a 60× oil objective. Images were acquired in jpeg with the JACOg plugin to calculate the Pearson correlation between red and green channels within a 12 × 12 × 8 X × Y × Z box manually placed on each GFP positive LacO array. As recommended in the original FISH protocol, all images were acquired with the same laser settings. Both pemblasts were excluded by filtering out cells with low signal intensity in the red channel. Using different thresholds did not affect the conclusions. For the boxplots presented in Fig. 4 and extended Fig. 4, we used a threshold of 5000 for the red channel (and no threshold for the green channel), in reference to the data in the Source Data file. Boxplots show the results merged over at least 30 Lacto epithelial layers across at least two independent transfections carried on different days, typically.

Flow cytometry. mESCs were dissociated with TrypLE, resuspended in culture medium, spin, and resuspended in 4% FBS–PBS before live flow cytometry on a MACSQuant instrument (MiltenyiBiotec). Dissociation, wash, and flow buffers were supplemented with auxin, when appropriate, to avoid re-expression of the CTCF–AID–eGFP fusion. Analysis was performed using the Flowjo software.

Western blots. mESCs were dissociated, resuspended in culture medium, pelleted, washed in PBS, pelleted again, and kept at −80 °C. In total, 15–20 million cells were used to prepare nuclear extracts. Cell pellets were resuspended in 10 mM HEPES, pH 7.9, 2.5 mM MgCl₂, 0.25 M sucrose, 0.1% NP-40, 1 mM DTT, and 1× HALT protease inhibitors (ThermoFisher) and swelled for 10 min on ice. After centrifugation at 500 g, nuclei were resuspended on ice in 25 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 0.1% NaN₃, 0.25% Triton X-100, 1 mM EDTA, 0.1% DTT, and 250 U benzonase, and incubated on ice for 10 min. Insoluble materials were pelleted by centrifugation at 18,000 g for 10 min at 4 °C, and the supernatant (nuclear extracts) was stored at −80 °C. Protein concentration from supernatants was measured using the Pierce BCA Protein Assay (ThermoFisher).

For CTCF Western blot in Extended Fig. 2, 40 μg of nuclear extracts were loaded per lane. Samples were mixed with Laemmli buffer and 2.5% beta-mercaptoethanol, then loaded on a Bolt 4–12% Bis–Tris Plus gel (ThermoFisher). Gels were wet-transferred onto PVDF membranes in transfer buffer (25 mM Tris-Base, 192 mM Glycine, and 10% Methanol) for 3 h at 80 V. Membranes were blocked for 2 h with Odyssey blocking buffer (Li-Cor cat. 927-40000) and subsequently incubated with primary antibody overnight at 4 °C (1:1000 anti-CTCF C-terminus Millipore 61311 and 1:2000 anti-TBP Abcam ab51841) in Odyssey blocking buffer. Membranes were washed three times in TBS–0.1% Tween, 5–10 min per wash, and were incubated with secondary antibody (1:1000 1× Halt anti-mouse and 1× Halt anti-rabbit Cell Sign #7074 and #7076). Blots were washed 3 times for 5–10 min in TBS–0.1% Tween. CTCF blot used Amscher ECL Prime Western Blotting Detection Reagent (GE RPN2236) and TBP blot used Amscher ECL Western Blotting Detection Kit (GE RPN2108) forHRP activation. Blots were then exposed onto X-ray films for different exposure times.

Co-immunoprecipitation. mESCs were dissociated, resuspended in culture medium, pelleted, washed in PBS, pelleted again, and kept at −80 °C. In total, 15–20 million cells were used for protein extraction. Pellets were thawed on ice and lysed in 10 mM Tris at pH 7.9 at 4 °C, 1.5 mM MgCl₂, 10 mM KCl, 0.2% IGEPAH CA-630, and 1× Halt protease inhibitors (ThermoFisher 78429) by incubating for 15 min on ice. Nuclei were pelleted by centrifugation at 14,500 g for 15 min at 4 °C and resuspended in 100 μl of 20 mM Tris at pH 7.9 at 4 °C, 25% glycerol, 400 mM NaCl, 1.5 mM MgCl₂, 10 mM EDTA, 250 U benzonase, and 1× Halt protease...
inhibitors, and incubated on an orbital shaker for 60 min at 4 °C. Insoluble materials. Insoluble materials were precipitated by centrifugation at 18,000g for 4°C for 10 min, and the supernatant (nuclear extracts) was diluted to 200 mM NaCl final by adding 100 μL of 20 mM Tris at pH 7.9 at 4 °C, 25% glycerol, 1.5 mM MgCl2, 10 mM EDTA, and 1× Halt protease inhibitors. Protein concentration from supernatants was measured using the Pierce Coomassie Plus assay kit (ThermoFisher) and the concentration was adjusted to 1 mg/mL. In all, 3% input was set aside, and 500 μL of nuclear extracts were used for immunoprecipitation by adding 4 μg of anti-SAI antibody (Abcam ab4457) and incubating for 3 h by rotation at 4°C. In the meantime, 25 μL Protein G beads (ThermoFisher) were washed twice with the 200 mM NaCl IP buffer and blocked for 1 h by adding 0.5% BSA final (Gemini 700-1000). After blocking, beads were rinsed twice in the 200 mM NaCl IP buffer, resuspended in 25 μL of IP buffer, and added to the lysates for 1 h at 4°C under rotation. Beads were then collected on a magnetic stand, rinsed three times with 200 μL of NaCl IP buffer, resuspended in 100 μL in 100 μL IP-CL buffer containing 1× Laemmli buffer (Biorad 1610737) and incubated at 95°C for 5 min. Beads were then collected and discarded, and eluates were loaded equally on four separate 12% acrylamide gels (Biorad). Proteins were transferred onto PVDF membranes using the iBlot system (ThermoFisher) Program 0 for 8 min. Membranes were incubated at least 30 min with Odyssey blocking buffer (Li-Cor) and incubating for 3 h at 4°C. After incubation, membranes were washed 3 times for 5 min in 1× PBS-0.1% Tween-20 at room temperature, incubated with secondary antibodies (Goat Anti-Rabbit 680RD and Donkey Anti-Mouse 800CW (Li-Cor), 1:10,000) in Odyssey blocking buffer with 0.1% Tween-20 and 0.01% SDS for 1 h at room temperature, washed 3 times, and analyzed on a Li-Cor imaging system. Panels were mounted using ImageJ preserving linearity.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The data that support this study are available from the corresponding authors upon reasonable request. Sequencing data presented in Figs. 2, 3, and 5 are available on Gene Expression Omnibus GEO GSE150688. We used the following publicly available datasets: GEO GSE98671, UniproKB Q61164. Source data are provided with this paper

Code availability
Scripts used to analyze imaging or genomic data are available upon request. Cootools analysis software available at https://github.com/open2c/cootools.

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Author contributions
E.P.N. designed the study with G.F., L.A.M., M.D., and B.G.B. E.P.N. created the transgenic cell lines, performed and analyzed ChIP-seq, 5C, immunofluorescence, and co-immunoprecipitation, and designed and analyzed the F3H. L.C. performed and analyzed single-molecule imaging with help of B.H., A.L.S., and A.C. G.F. analyzed 5C data and helped in the design of the study. K.S., A.N., and A.U. provided the support for cloning and tissue culture, and K.S. performed and analyzed the F3H as well as Western blots. V.K. performed the ChIP-seq experiments. K.S.P. provided the support for bioinformatic analyses. E.P.N. wrote the paper with input from all authors, particularly G.F. E.P.N. directed the project with B.G.B.

Competing interests
The authors declare no competing interests.

Additional information
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