Retroviral integration, the process of covalently inserting viral DNA into the host genome, is a point of no return in the replication cycle. Yet, strand transfer is intrinsically iso-energetic and it is not clear how efficient integration can be achieved. Here we investigate the dynamics of strand transfer and demonstrate that consecutive nucleoprotein intermediates interacting with a supercoiled target are increasingly stable, resulting in a net forward rate. Multivalent target interactions at discrete auxiliary interfaces render target capture irreversible, while allowing dynamic site selection. Active site binding is transient but rapidly results in strand transfer, which in turn rearranges and stabilizes the intasome in an allosteric manner. We find the resulting strand transfer complex to be mechanically stable and extremely long-lived, suggesting that a resolving agent is required in vivo.
**Results**

**Supercoiling drives efficient formation of long-lived STCs.** To study PFV integration, we assembled CI intasomes from purified integrase and mimetics of 3′ processed viral DNA ends. Using supercoiled plasmid DNA as a target (Fig. 1b, c), we can distinguish unreacted molecules (supercoiled) and half-site (open circular) or full-site (linear) reaction products in both AFM and gel images (Supplementary Fig. 1). Samples prepared by incubating pBR322 plasmids with 10 nM wild-type (WT) CI in reaction buffer and direct deposition for AFM imaging resulted in ~71% of the complexes were linearized and ~13% converted to the open circular topology, implying that full-site integration is the dominant reaction (Fig. 1f, g).

To complement the AFM data, we incubated supercoiled DNA with CI intasomes assembled on Atto532-labeled viral DNA mimetics and separated reaction products before and after deproteination by gel electrophoresis (Fig. 1h and Supplementary Fig. 1). Reaction products analyzed under native conditions show a marked increase of an open circular form associated with the labeled viral DNA, compared with unreacted plasmid samples (Fig. 1h, right). Chemical deproteination of the reacted sample yields linearized (full-site) products with incorporated viral DNA, at the expense of the open circular fraction, indicating that STCs allow torsional relaxation, while remaining stably bound, in agreement with our AFM data and with results obtained previously for HIV integration. In our gel data, relaxed topoisomers that would be expected for a reversible reaction are not detected, suggesting that strand transfer is essentially irreversible. Notably, strand transfer in supercoiled targets features an ~2-fold higher yield as compared with topologically relaxed targets (Fig. 1i), despite similar levels of TCCs seen in AFM data (25 ± 5% and 23 ± 5% formed on relaxed and supercoiled plasmids, respectively; errors reflect \( \pm \text{SD} \)). The twofold lower strand-transfer yield for relaxed vs. supercoiled plasmids suggest an integration yield ≤50% for relaxed DNA, close to the expectation of an iso-energetic reaction, indicating that the release of supercoiling free energy (estimated in Methods section) drives the integration reaction forward.

**DNA binding at auxiliary interfaces governs target capture.** We next used AFM to probe the geometry of CI, TCC, and STC intasomes. High-resolution topographs revealed intasomes with ellipsoidal shapes (Fig. 2a) and a long axis of 19 ± 2 nm (mean ± SD of the full width at half maximum (FWHM) height; \( n = 82 \)), in quantitative agreement with the dimensions obtained via small angle X-ray scattering of PFV intasomes in solution (Fig. 2b, see Methods).

Brief (30 s) incubation of CI with linear target DNA predominantly yields complexes bound to a single target DNA segment (Fig. 2c). Binding occurs in distinct geometries: apical binding with the DNA across the short axis of the ellipsoid near the outer integrase monomers and longitudinal binding with the DNA along the long axis of the intasome (Fig. 2c, d and Supplementary Fig. 3). Surprisingly, exclusive binding to the active site, which runs across the short axis through the intasome’s two-dimensional (2D)–projected center of mass, was not observed. K168E mutant intasomes with reduced positive charge at the secondary grooves of the outer integrase dimer interfaces exhibited a ~3-fold reduced affinity for apical target binding (Fig. 2c, d and Supplementary Fig. 3), suggesting that the apical binding interfaces involve the secondary grooves, which were previously found to be critical for in vivo infectivity.

Using supercoiled DNA as a target, AFM images revealed WT intasomes predominantly interacting with several DNA strands, indicating that multiple auxiliary interfaces must be engaged (Fig. 2e, f and Supplementary Fig. 3). Interestingly, although complexes bound to three target DNA segments (referred to as branched complexes) occur transiently at short incubation times, longer incubation predominantly yields complexes bound to two target segments (termed bridging complexes; Fig. 2e, f and Supplementary Fig. 3). Control AFM measurements with...
intasomes under non-reactive conditions and ensemble kinetic analysis suggest that branched complexes, stabilized by auxiliary DNA-binding interfaces, constitute on-pathway intermediates during target capture (Supplementary Figs. 3 and 4). Further, AFM imaging of gel-purified STCs demonstrate that auxiliary sites remain engaged in the STC (Fig. 2g and Supplementary Fig. 2).

The exit-angle distributions of DNA in branched and bridging complexes are well-defined, suggesting conserved folding (Fig. 2h, i). Based on high-resolution structural information and our

**Fig. 1 Topology and yield of strand transfer intermediates.**

- **a** Reaction schematic of PFV strand transfer with crystal structures of nucleoprotein intermediates (PDB 3l2r, 3os0, 3os1).
- **b** AFM image of untreated supercoiled plasmids. **c** Relative occurrence of supercoiled (SC), open circular (OC), and linear (LIN) topologies in untreated plasmid samples ($n_{tot} = 214$; errors are $\sqrt{n/n_{tot}}$). **d** AFM image of supercoiled plasmids incubated with CI (10 nM; 4 h, 37 °C). **e** Relative occurrence of different free DNA topologies and nucleoprotein complexes, in samples of supercoiled plasmid incubated with cleaved intermediate (10 nM; 4 h, 37 °C) ($n_{tot} = 518$; errors are $\sqrt{n/n_{tot}}$). **f** AFM image of sample after incubation with CI and subsequent deproteination. **g** Relative occurrence of DNA topological forms in deproteinated samples of supercoiled plasmid incubated with CI ($n_{tot} = 100$; errors are $\sqrt{n/n_{tot}}$). The linearized fraction increases significantly after deproteination compared with the other two conditions ($p < 0.0001$ in both cases). **h** Gel electrophoresis of untreated plasmids (first lane), and native (second lane) and deproteinated (third lane) reaction products of supercoiled pBR322 and intasomes assembled on Atto532-labeled viral DNA mimetics (left: ethidium bromide stain; right: Atto532 dye). **i** Ratio of full-site product to total DNA as a function of incubation time, deduced from electrophoretic separation of reaction mixtures of plasmids with different supercoiling density and CI intasome (25 nM), followed by Sybr Gold staining. Error bars are SD from two independent repeats. Source data are provided as a Source Data file.
AFM data, we propose a minimal low-resolution model of the DNA folding in the TCC and STC (Fig. 2j). The ellipsoidal intasome has the active site positioned centrally along the minor axis. The apical auxiliary interfaces correspond to the secondary grooves involving residues K168 located parallel to the left and right of the active site on the same face of the complex (Fig. 2j). The stability and long lifetime of the STC (Fig. 1) combined with the observation that K168E intasomes exhibit faster topological relaxation and complex disassembly after reaction with supercoiled targets as compared with WT intasomes (Supplementary Fig. 2) strongly indicates that the single strand breaks generated at the active site are mechanically shielded by the apical interfaces. To protect the DNA at the active site, the same target DNA segment must bind both apical interfaces, suggesting an S-shaped or wrapped path of the DNA (Fig. 2j). Indeed, AFM images of purified STCs are consistent with an S-shaped DNA path and the apical interfaces, and the active site remaining bound (Fig. 2g and Supplementary Fig. 2). The paths of the DNA segments entering and exiting the intasome complex require one more DNA segment to be bound (Fig. 2h, i), which we assign to the longitudinal interface (Fig. 2j and Supplementary Fig. 2).

**Real-time observation demonstrates a dynamic target search.** Building on the mesoscale structural data obtained via AFM imaging, we next developed a multiplexed MT assay to probe integration dynamics in real time. Target DNA molecules are tethered between a flow cell surface and magnetic beads, and can be stretched and supercoiled using external magnets. Plectoneme formation upon supercoiling decreases the tether extension.

**Fig. 2** Auxiliary DNA-binding interfaces are engaged in TCCs and STCs. a AFM images of CI intasomes. b Atomistic model of PFV intasome solution structure (21). Purple spheres indicate residues K168. Viral DNA mimetics (red) are not visible in our AFM data. c Early TCCs formed on linear pBR322 DNA reveal longitudinal and apical binding geometries. d Polar plots of entry and exit angles with respect to the intasome long axis for apical and longitudinal binding modes, and relative occurrence in WT and K168E intasomes. e AFM image of intasomes incubated briefly (2 min) with supercoiled plasmid DNA, depicting a branched complex as found in ~50% of early complexes. f AFM image of a bridging complex that dominates (~80%) the population of complexes at longer (>45 min) incubation. g AFM image of a gel-purified STC. h Polar plot of exit angles in branched complexes. i Polar plot of exit angles in bridging complexes. j Model for target DNA (blue) folding in TCC and STC intasomes (yellow dotted contour). Viral DNA mimetics are not shown. Source data are provided as a Source Data file.
compared with torsionally relaxed molecules. In our assay, typically ~50 magnetic beads tethered by a single double-stranded DNA molecule are selected for tracking per field of view (Fig. 3a). We first record extension traces of supercoiled DNA and then introduce CI intasomes (10 nM) into the flow cell, which results in ~30% of DNA tethers (296 out of 934 tethers overall) exhibiting signatures in their extension that deviate from the bare DNA behavior. Consequently, we expect that tether interactions predominantly (~90%) involve a single intasome, in agreement with our AFM observations.

We first investigated intasomes under conditions that allow target capture yet inhibit strand transfer, by using either intasomes carrying the A188D mutation that blocks the active site (either via the A188D mutation or addition of Raltegravir), or by using Ca²⁺ site, by adding the strand-transfer inhibitor Raltegravir, or by intasomes carrying the A188D mutation that blocks the active target capture yet inhibit strand transfer, by using either

\[
\text{Introduce A188D intasome}
\]

\[
\text{Introduce WT intasome, Ca}^2⁺
\]

\[
\text{Target capture}
\]

\[
\text{Dynamic DNA bridging}
\]

\[
\Delta t_{TCC} = 3.0 ± 0.5 \text{ s}
\]

\[
\Delta t_{TCC} = 6.9 ± 1.3 \text{ s}
\]

**Fig. 3** Magnetic tweezers assay reveals target capture dynamics. **a** A typical field of view depicting ~50 beads used for tracking (yellow) and reference beads (blue). **b** DNA tethers are supercoiled using external magnets and exhibit extension fluctuations with SD \(\sigma_z\). Target capture reduces \(\sigma_z\) via DNA bridging. Transient interface unbinding repositions intasomes thereby affecting extension. **c** Time trace of supercoiled target DNA extension before and after binding A188D CI intasomes (blue: raw data at 58 Hz, yellow: 1 Hz smoothed data). **d** Enlarged region of **c** highlighting the onset of dynamic bridging (green dotted line) and coinciding \(\sigma_z\) reduction calculated with a 0.5 s moving window (red lines: trace fitted using step-finding algorithm). **e** Exponential fit of \(\Delta t_{TCC}\) distribution yields a lifetime of auxiliary interfaces \(\tau_{TCC} = 3.0 ± 0.5 \text{ s}\). (Error is 95% CI; \(n = 724\)). **f** Time trace of supercoiled DNA extension and response to binding WT CI in Ca²⁺ buffer. **g** Enlarged region of **f** shows the onset of dynamic bridging and \(\sigma_z\) reduction (green dotted line). **h** Exponential fit of \(\Delta t_{TCC}\) distribution with lifetime \(\tau_{TCC} = 6.9 ± 1.3 \text{ s}\). (Error is 95% CI; \(n = 750\)). Source data are provided as a Source Data file.
indicative of complete intasome dissociation. Thus, target capture is virtually irreversible owing to multivalent binding at auxiliary interfaces. The \( \sim \) seconds lifetime of binding to (at least) one auxiliary interface and the active site allows dynamic integration site selection, while preserving continuous contact between intasome and target DNA for extended periods of time.

**Strand transfer stabilizes apical auxiliary interfaces.** On introducing catalytically competent intasomes, the dynamic and rapid changes in tether extension level, seen consistently with catalytically inactive intasomes (Fig. 3), are only seen transiently. Instead, we see two additional signatures in the MT extension traces (Fig. 4a-c). A first signature is rapid stepwise increases in extension that are released in steps due to transient apical interface unbinding. Extension plateaus quantify dwell times (Fig. 4a, b). The stepwise pattern is explained by our model for the STC (Fig. 2i), wherein apical interface and target DNA for extended periods of time.

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**Fig. 4** Real-time observation of strand transfer and apical interface stability in STCs. **a** Scheme depicting signatures of strand transfer. Top: binding and reaction near plectoneme end loops minimally affect \( \sigma_t \) but enables supercoil release through apical interface unbinding (green arrow) and rotational relaxation (black arrow). Bottom: binding near the origin of the plectoneme suppresses \( \sigma_t \), strand transfer quenches dynamic bridging by intasome-target anchoring. **b** Extension time-traces of supercoiled target DNA reacting with intasome, depicting stepwise extension increments (\( \Delta \Gamma_{int} \)) and extension hopping followed by a stable extension level (\( \Delta \Gamma_{int} \)). **c** Extension time-trace of supercoiled target DNA reacting with WT intasome. External magnets introduce supercoils that are released in steps due to transient apical interface unbinding. Extension plateaus quantify dwell times \( \Delta \Gamma_{Apic}^{\text{STC}} \), step sizes \( \Delta \Gamma \) quantify extension increments. **d** In the plectonemic regime, the number of turns released per unbinding event \( \Delta \Gamma \) is proportional to \( \Delta \sigma_t \). **e** \( \Delta \Gamma \) distribution (kernel density estimate; bandwidth 0.2 turns). Inset: Fourier transformation after subtracting an exponential background. **f** \( \Delta \Gamma \) distribution on the sign of \( \Delta \sigma_t \) in TCCs and on the sign of the torque \( \Gamma_{STC} \) applied to STCs (red data points are mean lifetime \( \Gamma_{Apic}^{\text{STC}} \) and error bars are 95% CI as obtained from an exponential fit; Supplementary Fig. 7). Significance calculated using two-sample Kolmogorov-Smirnov test (\( \Gamma_{Apic}^{\text{STC}}+ = 227; \Gamma_{Apic}^{\text{STC}} = 288; \Gamma_{Apic}^{\text{STC}}- = 412, \Gamma_{Apic}^{\text{STC}}- = 444 \)). Source data are provided as a Source Data file.
DNA to the intasome on strand transfer that stops the dynamic target search (Fig. 4a, c). Establishing a stable extension level is typically fast (<1 min; Fig. 4c and Supplementary Fig. 6) and irreversible in all observations.

The extent of fluctuation reduction observed in the traces is anti-correlated with the total extension increase by stepwise supercoil release, which can be understood from the variable positions of binding and reaction along the plectoneme (Supplementary Fig. 5). DNA binding close to the plectoneme end loop allows large extension increments via supercoil release but features minimal \( \sigma_z \) reduction (Fig. 4a, top, and Supplementary Fig. 5). Conversely, binding near the plectoneme origin significantly reduces extension fluctuations \( \sigma_z \) but allows relaxation of few or no supercoils (Fig. 4a, bottom, and Supplementary Fig. 5).

We can controllably introduce new supercoils by rotating the magnets. For reacted DNA tethers, new supercoils are released in a stepwise manner, independent of the position of intasome binding and reaction (Fig. 4d). The time-traces of stepwise supercoil relaxation provide information on apical interface binding kinetics. We exploit the linear relation between extension and linking difference in the plectonemic regime (Fig. 4e) to convert step heights \( \Delta z \) to number of supercoils \( \Delta L_k \) released per dissociation event\(^{25,26} \). The probability distribution of \( \Delta L_k \) exhibits maxima at integer values (Fig. 4f), implying that apical interfaces involve specific contacts with the target DNA. The number of supercoils removed per event is exponentially distributed, indicative of a mechanism where the interface has a fixed probability of reforming every turn\(^{25} \). Large supercoil relaxation steps (\( \Delta L_k > 10 \)) occur significantly more frequently for K168E intasomes compared with WT (\( p < 0.0001 \); two-sample \( t \)-test; Supplementary Fig. 7), highlighting the stabilizing contribution of the secondary grooves at the outer dimer interface.

We evaluated whether stepwise supercoil removal is affected by (i) the sign of DNA supercoiling on which the TCC is assembled \( \Delta L_k^{\text{TCC}} \) and (ii) the sign of the supercoils introduced by the external magnets after strand transfer (and consequently the torque on the STC \( I^{\text{STC}} \)). We find that the rebinding probability is independent of \( \Delta L_k^{\text{TCC}} \) (Fig. 4g and Supplementary Fig. 7), both for the removal of positive and negative supercoils. In contrast, a larger mean number of supercoils removed per step \(< \Delta L_k \) is observed under positive compared with negative \( I^{\text{STC}} \) (Fig. 4g and Supplementary Fig. 7). Furthermore, relaxation traces allow quantification of the dwell times of individual plateaus, which report on the apical interface lifetime \( \tau_{\text{apic}^{\text{STC}}} \). Dwell times are exponentially distributed in all cases (Supplementary Fig. 7). The effect of supercoil chirality follows a similar pattern to the step size distributions: there is no significant difference in lifetime \( \tau_{\text{apic}^{\text{STC}}} \) between TCCs assembled on positively or negatively supercoiled target DNA for the removal of supercoils of either sign (Fig. 5h). In contrast, there are significant differences in lifetime for removal of positive supercoils compared with negative supercoils. Larger average step sizes, corresponding to a lower rebinding probability per turn, and shorter average dwell times for the removal of positive compared with negative supercoils both suggest that a positive torque \( I^{\text{STC}} \) destabilizes the apical interface more than a negative torque. Overall, the apical interface lifetime after reaction in the STCs \( (\tau_{\text{apic}^{\text{STC}} } \sim 90 \text{s}, \text{depending on the applied torque } I^{\text{STC}} ) \) is at least 20-fold longer than the lifetime of the auxiliary interfaces before reaction in the TCC \( (\tau_{\text{apic}^{\text{TCC}} } = 3.0 \pm 0.5 \text{s}; \text{error is } 95\% \text{ CI} ) \) implying stabilization of target DNA–intasome interactions beyond the active site after strand transfer.

### Forced disassembly of the STC

Remarkably, in our MT assay at low forces \( (F = 0.5 \text{ pN}; 223 \text{ reacted tethers}) \), we never observed release of the DNA-tethered beads after reaction (Fig. 4), which would be expected for disassembly of the STC after completion of the integration reaction. The high stability of the STC in the MT is consistent with the results from AFM imaging (Fig. 1e) and gel electrophoresis (Fig. 1i), which also suggest a very stable and long-lived STC. To directly quantify the mechanical stability of the STC, we use the MT’s ability to apply large and precisely calibrated stretching forces and subject STCs to varying levels of tension. Upon applying high tension \( (F \geq 30 \text{ pN}) \), tether extensions immediately increase due to elastic DNA stretching and removal of plectonemes, but beads remain tethered for \( \sim \)hours before the eventual bead release that we interpret as STC disassembly (Fig. 5a). Interestingly, tethers that exhibited strongly reduced extension fluctuations at low force, indicative of STC formation near the plectoneme origin, initially show increased extension on increasing tension to values much smaller than the expected DNA contour...
Discussion

Using single-molecule assays, we have directly probed the PFV integration landscape (Fig. 6) in real time. Our data reveal that target capture and engagement of the active site is the rate-limiting step of integration (Supplementary Fig. 4) caused by the requirement for strong target deformation, yet irreversible, owing to multivalent interactions at auxiliary interfaces that collectively warrant a high affinity for the target (Fig. 3). Importantly, individual interactions with the target at auxiliary interfaces and the active site are sufficiently weak to enable dynamic site selection. Nevertheless, on supercoiled targets, site selection is limited and quickly followed by strand transfer (Supplementary Fig. 6).

The chemistry of strand transfer is intrinsically iso-energetic, yet our data indicate a high (~85%) yield when the target is supercoiled (Fig. 1), in stark contrast with intasomes bound to topologically relaxed targets that achieve ≤50% conversion (Fig. 1). Mutant intasomes with significantly lower binding energy react with yields similar to the WT (Supplementary Fig. 2). Accordingly, we propose that the release of supercoiling free energy (~100 k_B T; Methods) following the generation of single-strand breaks on strand transfer is the main driving force for shifting the transesterification equilibrium forward.

After strand transfer, STCs do not disassemble spontaneously (Figs. 1 and 4). We used the ability of MT to apply high tension and to quantitatively challenge STC stability. STC lifetimes were found to decrease exponentially with applied force and extrapolation yields a zero-force lifetime of ~8 months (Fig. 5), much longer than the retrival replication cycle (~1 day). As STC resolution is the key towards establishing a functional provirus, our data strongly suggest the necessity of enzymatic STC resolution in vivo. Interestingly, the lifetime of auxiliary DNA-binding interfaces is significantly increased in STCs compared with TCCs. In addition, the stability of apical interfaces in STCs is ~2-fold higher under negative as compared with positive torque, whereas initial supercoil chirality of the target does not affect target capture (Fig. 4). Taken together, our data imply that strand transfer triggers chiral conformational changes that stabilize and rearrange the intasome at apical interfaces, i.e., well beyond the active site. We hypothesize that these conformational changes might signal strand transfer completion to engage cellular machinery involved in STC resolution and repair.

The finding that PFV intasomes employ auxiliary-binding sites for modulating the barriers to integration raises the question how the topology of higher-order intasomes governs integration of pathogenic retroviruses, most notably HIV. The single-molecule assays developed in this work are expected to be particularly useful to further unravel the complexity of this important class of molecular machines.

Methods

Integrate expression and purification. BL21 Escherichia coli cells (Agilent Technologies) were grown to OD_{600} = 0.9–1.0 at 29°C prior to the addition of 0.25 mM IPTG and 50 μM ZnCl₂ for 4 h at 25°C. Cells were collected and stored at −80°C. Protein purification started with the resuspending the BL21 E. coli cells in 25 ml HSB buffer (50 mM Tris-HCl pH 7.4, 500 mM NaCl). Afterwards, 0.5 mM phenylmethylsulfonyl fluoride, 1 U/10 mL DNase, and 1 mg/mL lysozyme were added, and the mixture was incubated for 10–15 min before sonication (6 × 30 s or until homogenized). The lysate was cleared by centrifugation for 45 min at 18,000 r.p.m. Before loading the lysate on the Ni-Sepharose beads, the beads were washed and HSB supplemented with 20 mM imidazole. The cleared lysate was incubated for 5 h at 4°C with Ni-Sepharose. After lysis flow through the bed, the beads were washed with five-column volumes and HSB supplemented with 20 mM imidazole. Protein was eluted with HSB supplemented with 200 mM imidazole and 10 mM dithiothreitol (DTT). The concentration of the eluted protein was determined by measuring the absorbance at 280 nm (ε_{280} = 58,110 M⁻¹ cm⁻¹, MW = 47,485 Da). The fractions containing PFV integrase were pooled and digest overnight at 4°C with HRV3C protease during dialysis to HSB supplemented with 10 mM DTT (1:100 w/w ratio of protease: integrase). Afterwards, the protein was cleared from the His-tag by the use of a Ni²⁺ and additionally a GSH column. The protein was dialyzed against HSB supplemented with 5 mM DTT and 10% glycerol.

PFV intasome assembly and purification. Intasome assembly and purification was performed according to published protocols [10]. Briefly, PFV integrase (120 μM) was equilibrated with synthetic DNA duplex 5′-TGGCGAAATTCCATGACA-3′ and 5′-ATTGTGATCATGCGACAA-3′ (20 μM) in 50 mM BisTris propane-HCl buffer supplemented with 500 mM NaCl (pH 7.45, 500 μL). For assembly of fluorescently labeled intasomes, we used the modified oligo 5′-5ATTO532N/TGGCGAAATTCCATGACA-3′. The mixture was then dialyzed for 18 h at 18°C against 200 mM NaCl, 2 mM DTT, 20 mM BisTris propane-HCl, pH 7.45. Next, a further dialysis step for 3 h to the dialysate was performed. Finally, 120 mM NaCl were added and the sample was incubated on ice for 1 h. Size-exclusion gel chromatography (Superdex 200 10/300 GL column) coupled to an ÄKTA Purifier system (GE Healthcare) was employed to fractionate the assembly, with mobile phase 20 mM NaCl, 25 mM BisTris propane-HCl, pH 7.45 at 1 mL/min, 4°C. The concentration of the intasome was determined spectrometrically using molar extinction coefficients ε_{280} = 626,329 M⁻¹ cm⁻¹ and ε_{260} = 841,491 M⁻¹ cm⁻¹.
DNA plasmids for AFM and bulk integration assays. Negatively supercoiled circular DNA plasmids (pUC19, 2686 bp, pBR322: 4361 bp, M13mp18 RF I DNA: 7249 bp) were obtained commercially (New England Biolabs, Ipswich, MA, USA). To construct the 1800 bp plasmid, commercial pUC19 plasmid (New England Biolabs, Ipswich, MA, USA) was linearized with two primers: FW pUC19 (EcoRV) (5′-GATATCCGTTTTAAGACGCGGC-3′) and REV pUC19 (BspQI) (5′-GCTCTTGCCATAGCCTAGTTGGC-3′). The linearization reaction was carried out via PCR amplification with a DNA polymerase Master Mix, Thermo Fisher Scientific, Inc., Waltham, MA, USA). One nanogram DNA was used as template for the PCR reaction. The PCR program was: 98 °C, 2 min, 30 cycles (98 °C, 10 s, 55 °C, 10 s, 72 °C, 30 s), 72 °C 5 min. After the PCR reaction was completed, the product was analyzed on a 1% (w/v) agarose gel. The PCR product was directly used in a ligation reaction supplemented with DpnI and T4 Polynucleotide Kinase (PNK; New England Biolabs, Ipswich, MA, USA). DpnI was used to remove all initial DNA template and T4 PNK was used to phosphorylate its use in the generation of positively supercoiled DNA, as originally reported by its sequence.

To construct the 1800 bp plasmid, commercial pUC19 plasmid (New England Biolabs, Ipswich, MA, USA) was generated starting from topologically relaxed pBR322 (see above) for 45 min. One microliter of the reaction was used to transform DH5α (containing 100 ng of DNA) and SYBR Gold for 30 min. At the position of the STC, a thin slice of the gel was excised. Gentle squeezing between two glass slides (covered with paraffin) removes the aqueous solution contained within the gel. This solution is mixed 1:1 with high salt buffer (400 mM NaOAc, 25 mM Tris-HCl pH 7.4) and it is directly drop-cast on poly-l-lysine-coated mica for AFM imaging.

Bulk strand-transfer assays. Bulk integration kinetic assays were carried out by mixing plasmid DNA with CI (25 nM for the topology dependence; 10 nM for the strand transfer reaction) (12 M Falcon2, Teledyne Dalsa, Canada) with a field of view 150.PD, PI, Germany) controlled rotation of the magnets. A ×40 oil-immersion objective (UPLFLN x40, Olympus, Japan) was employed to image the beads onto a CMOS sensor camera (12 M Falcon2, Teledyne Dalsa, Canada) with a field of view of 400 μm by 300 μm. Images were recorded at 58 Hz and transferred to a frame grabber (PCIe 1433, NI, USA). A custom-written tracking software analysed the intensity patterns on all the (x,y,z) coordinates of all the beads in real time (Max, 640/647 nm, Lumionix LED Technik GmbH, Germany) was used for illumination. For tracking of the bead z-position, a look-up table (LUT) is required to translate the defocused pattern of the bead to its height. The LUT was generated over a range of 10 μm, with a step size of 100 μm, by moving the objective using a piezo stage (Pievo P-726.1CD, PI, Germany).

DNA constructs and magnetic beads for magnetic tweezers. A 7.9 kb DNA construct, prepared as described in published protocols34,35, was used for all our MT measurements. PCR-generated DNA fragments (~600 bp) labeled with
multiple biotin and digoxigenin groups were ligated to the DNA, to bind magnetic beads and the flow cell surface, respectively. For all measurements, except for those that probe the force stability of the STC, we used 1.0 µm diameter M270 magnetic beads (Life Technologies, USA). For force probing of the STC, we used 2.8 µm diameter M270 magnetic beads (Life Technologies, USA). The DNA construct was attached to the streptavidin-coated beads by incubating 0.5 µl of picomolar DNA stock solution and 2 µl MyOne beads in 250 µl x1 phosphate-buffered saline (PBS) (Sigma-Aldrich, USA) for 5 min. Subsequently, the bead-coupled DNA constructs were introduced into the flow cell. Alternatively, 0.5 µl DNA stock solution and 10 µl M270 beads were incubated in 250 µl 1× PBS for 2 min, prior to their introduction in the flow cell.

**Magnetic tweezers flow cells.** Flow cells were built from two microscope cover-slips (24 × 60 mm, Carl Roth, Germany). To attach the DNA molecules to the flow cell, the bottom cover-slip was first coated with (3-Glycidoxypropytri-methoxysilane (Novabiochem GmbH, Germany). Afterwards, 20 µl of a 5000x diluted stock solution of polystryrene beads (Polyciences, USA) in ethanol (Carl Roth, Germany) was deposited on the silanized slides, slowly dried in a closed container, and baked at 80 °C for 1 min, to serve as reference beads. The top cover-slip was processed using a laser cutter, to produce openings with a radius of 1 mm, to enable liquid exchange. The two cover-slips were glued together by a single layer of melted Parafilm (Carl Roth, Germany), precut to form a ~50 µl channel connecting the inlet and outlet opening of the flow cell. Following flow cell assembly, 100 µg/ml anti-digoxigenin (Roche, Switzerland) in 1× PBS was introduced and incubated for 2 h. To minimize nonspecific interactions, the flow cell was flushed with 800 µl of 25 mg/ml bovine serum albumin (Carl Roth, Germany), incubated for 1 h and rinsed with 1 ml of 1× PBS. The premixed DNA-bead solution was added to the flow cell for 5 min for MyOne beads or 2 min for M270 beads to allow for the digoxigenin–anti-digoxigenin bonds to the surface to form. Subsequently, the flow cell was rinsed with 1× PBS, to flush out unbound beads. Next, the magnet was mounted, which constrains the supercoiling density of the tethers and applies an upward force on the beads. For measurements probing the force dependence of STCs, an additional step involving the application of high (50 pN) tension for 30 min was implemented to remove weakly bound tethers.

**Measurement protocols for magnetic tweezers experiments.** Prior to each measurement, selected beads were tested for the presence of multiple tethers and torsional constraint, by measuring their response to force and torque. The presence of multiple tethers was assessed by introducing MyOne magnetic beads under high tension (F ≥ 5 pN). In the case of a single DNA tether, high tension impedes the formation of plectonemes at negative linking differences. As a result, no height change is observed. In contrast, for the case of multiple tethers, introduction of negative linking differences results in braiding, decreasing the z-extension of the bead. Beads bound by multiple tethers are discarded from further analysis. To assess whether DNA tethers were fully torsionally constrained, positive linking differences are introduced at low force (0.4 pN). In torsionally constrained DNA tethers, this leads to the formation of plectonemes, which decrease the z-extension. In nicked DNA tethers, no linking difference can be induced and the z-extension remains constant on magnet rotation.

Last, to define the state of zero torque (Lk = LB) of individual DNA molecules relative to the position of the rotation motor, rotation–extension curves were constructed before the start of the single-molecule strand-transfer assay.

The single-molecule strand-transfer assay was performed in reaction buffer. For some experiments, Raltegravir (NIBSC labs) was deposited on the silanized slides, slowly dried in a closed container, and baked for our analysis. As a control, we tested the rupture probability distribution in the flow cell via the use of a peristaltic pump (Fig. 4g). In the case of a single DNA tether, high tension (~25 in the tethers at 0.5 pN tension. The difference was only ~2-fold (22.7 ± 0.5 h) and the distributions of lifetimes for the state of zero torque (Lk = LB) is then approximated by

\[ \Delta G = \frac{A}{k_B T} \frac{\theta^2}{2L} \]  

(2)

where A is the bending persistence length (A = 40 nm under the conditions used), \( \theta \) is the bending angle of the DNA, and L is the length over which the DNA is bent. For the DNA in the TCC, we can estimate from the crystal structure \( \frac{\Delta G}{L} = 140 \) for positive torque and \( \frac{\Delta G}{L} = 140 \) for negative torque respectively. We estimate \( \Delta G \approx 0.6 k_B T \) per interface and 1.2 k_B T for the two apical interfaces. This relatively modest difference can be clearly detected in the sensitive single-molecule tweezers assay (Fig. 4g, h), but is too small to be reliably observed in our bulk assay with negatively and positively supercoiled DNA (Fig. 1i).

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

**Data availability**

Data supporting the findings of this manuscript are available from the corresponding authors upon reasonable request. A reporting summary for this Article is available as Supplementary Information file.

The source data underlying Figs. 1b, 2b, 3a, 3e, 4h, 4l, 5b, d and Supplementary Figs. 4d, 6a, 7d, e are provided as a Source Data file.

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Conceptualization, W.V. and J.L. Methodology, W.V. and J.L. Investigation, W.V., T.B., P.K. Resources, T.B., P.W., W.O., L.M., P.N., Z.D.; J.L. Original draft, W.V., J.L. Funding acquisition, W.V., Z.D., J.L. Supervision, W.V. and J.L.

Competing interests

The authors declare no competing interests.

Additional information

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