

AFM-based force spectroscopy unravels stepwise formation of the DNA transposition complex in the widespread Tn3 family mobile genetic elements

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Received December 21, 2022; Revised March 03, 2023; Editorial Decision March 21, 2023; Accepted March 23, 2023

ABSTRACT

Transposon Tn4430 belongs to a widespread family of bacterial transposons, the Tn3 family, which plays a prevalent role in the dissemination of antibiotic resistance among pathogens. Despite recent data on the structural architecture of the transposition complex, the molecular mechanisms underlying the replicative transposition of these elements are still poorly understood. Here, we use force-distance curve-based atomic force microscopy to probe the binding of the TnpA transposase of Tn4430 to DNA molecules containing one or two transposon ends and to extract the thermodynamic and kinetic parameters of transposition complex assembly. Comparing wild-type TnpA with previously isolated deregulated TnpA mutants supports a stepwise pathway for transposition complex formation and activation during which TnpA first binds as a dimer to a single transposon end and then undergoes a structural transition that enables it to bind the second end cooperatively and to become activated for transposition catalysis, the latter step occurring at a much faster rate for the TnpA mutants. Our study thus provides an unprecedented approach to probe the dynamic of a complex DNA processing machinery at the single-particle level.

INTRODUCTION

Transposable elements or ‘transposons’ are widely diverse genetic elements that can propagate from one location to another within the genome of their host (1). This movement is mediated by ‘transposases’, specialized recombination enzymes that cut and join DNA molecules using different mechanisms (1,2). Transposase genes represent the most abundant and ubiquitous annotated genes in genomes, which reflects the evolutionary impact of transposable elements throughout the living world, being responsible for deleterious mutations and diseases, but also the creation of new genes, the establishment of complex regulatory networks, and the exchange of useful phenotypic traits through horizontal gene transfer (3,4). As such, transposable elements play a prominent role in the emergence of new pathogens and the dissemination of antimicrobial resistance among bacteria (5).

In particular, transposons of the wide-spread Tn3-family pose a permanent threat to human health, being continuously involved in the dispersal of new resistances, including against last-chance antibiotics such as carbapenems and colistin (5,6). The effectiveness of these transposons relies on their ability to copy themselves and their passenger genes through replicative transposition every time they move (5). The transposases (TnpA) of Tn3-family transposons form a distinct subgroup of the structurally diverse ‘DDE/D’ transposase/integrase superfamily that cleave and reseat DNA using a triad of acidic residues (DDE or DDD) embedded in a conserved RNaseH-like catalytic domain (7,8). In the case of the Tn3-family, the strand transfer intermediate resulting from the joining of the transposon 3'-ends to the target DNA is processed by the host replication

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machinery to generate two new copies of the transposon. However, despite the biological and societal impact of Tn3-family transposons, the molecular mechanism whereby TnpA interacts with the DNA partners to assemble the active transposition complex and catalyze the reaction remains poorly understood (9).

Our recent work on the Tn3-family member Tn4430 has opened up new avenues toward the understanding of this mechanism (10,11). Biochemical studies identified plausible steps during transposition complex assembly and activation (Figure 1A). These steps were identified by comparing the wild-type TnpA of Tn4430 (TnpA^{WT}) with deregulated TnpA mutants (TnpA^{S911R} and the triple mutant TnpA^{AW24R/A174V/E740G}, hereafter referred to as TnpA^{3X}) that had previously been isolated for their impairment in target immunity, a yet poorly understood TnpA-dependent mechanism whereby Tn3-family transposons avoid inserting multiple times into the same target (7,12). TnpA^{S911R} and TnpA^{3X} displayed promiscuous target selectivity *in vivo* (7,10), promoting transposition into an immune target at a higher frequency than TnpA^{WT}, and proved to be hyperactive in different biochemical assays *in vitro*. TnpA^{WT} was shown to specifically bind to a DNA fragment carrying the terminal inverted repeat (IR) of Tn4430 to form a single-end complex (SEC), whereas TnpA^{S911R} and TnpA^{3X} spontaneously and cooperatively assembled a paired-end complex (PEC) containing two IR ends. Formation of the PEC correlated with activation of DNA cleavage at the transposon ends by TnpA (7,10,12). TnpA^{WT} can only form the PEC on precleaved ends *i.e.* when the initial step of transposition is bypassed, whereas the hyperactive mutants TnpA^{S911R} and TnpA^{3X} can overcome the activation barrier for PEC assembly on both cleaved and un-cleaved (intact) ends (10). Together, the data suggest that transposition is controlled at an early stage of transposition complex assembly, and that activation of TnpA involves a conformational change that promotes the formation of a stable synaptic complex (PEC) to trigger DNA cleavage at both transposon ends (Figure 1A). Since the hyperactive TnpA mutants were identified for their loss in target immunity, we propose that activation of transposition is normally controlled by the target.

This scenario is now supported by high-resolution cryo-EM structures of the TnpA^{WT} apoprotein and the PEC assembled by TnpA^{S911R} onto two transposon ends before and after DNA cleavage and strand transfer (13). These structures reveal that unbound TnpA^{WT} essentially forms tightly packed dimers, whereas TnpA^{S911R} PEC adopts an expanded ‘V’ shaped organization in which both transposon ends make extended specific and non-specific interactions with each protomer of the TnpA dimer and converge to a central position occupied by the juxtaposition of both active sites. The apoform of TnpA dimer is in a closed and inactive conformation with the catalytic domain being incorrectly and incompletely folded, while in the PEC, binding to DNA frees up the space needed to assemble the RNaseH-like domain and properly align the catalytic residues. The TnpA protomer bound ‘in cis’ to one transposon end orient its active site ‘in trans’ toward the scissile phosphate of the other end. In the context of the dimer, this crisscross (‘cis-trans’) arrangement of TnpA protomers largely contributes to the stability of the PEC, while provid-

ing the mechanistic basis for coordinating catalysis at both transposon ends (13).

Although these snapshots provide unprecedented structural insights into the mechanism of TnpA activation, the dynamics of the transition between the inactive and active conformer and its contribution to the control of Tn4430 transposition remained unexplored. Here, we applied force-distance (FD) curve-based atomic force microscopy (AFM) (14) to characterize the formation of transposition intermediates at the single particle level. To this end, we engineered DNA molecules containing one or two Tn4430 IR ends and compared the binding of TnpA^{WT} with the previously characterized TnpA^{S911R} and TnpA^{3X} mutants. We extracted the kinetics and thermodynamics of TnpA-DNA interactions and probed conformational changes taking place between the different TnpA-DNA complexes. The findings provide new quantitative insights into Tn4430 step-wise transposition pathway with a resolution of the reaction kinetics that would be difficult to reach using conventional biochemical methods, thus constituting an inspiring approach for other studies in the field.

MATERIALS AND METHODS

Bacterial strains, plasmids and oligonucleotides

The strains, plasmids, and oligonucleotides used in this study are listed in *SI Appendix*, Supplementary Tables S1 and S2, respectively.

TnpA expression and purification

TnpA proteins fused to a cMyc-His₆ epitope tag at their C terminus were expressed in *Escherichia coli* TOP10 cells under the control of the pAra promoter (*SI Appendix*, Supplementary Table S1). TnpA derivatives were purified using the thermal shift-induction procedure to increase the yield of soluble proteins followed by nickel column chromatography as described (7,11). After purification, proteins were washed with the lysis buffer [50 mM Tris-HCl (pH 8.0), 500 mM NaCl and 20% (vol/vol) glycerol], and concentrated using an Amicon Ultra-0.5 mL 50 K filter (Merck). For cryo-EM experiments, ~350 µL of concentrated sample were applied to a Superose 6 Increase 10/300 GL column equilibrated in 50 mM HEPES (pH 7.9), 200 mM NaCl, and 100 mM L-Arg HCl.

Cryo-EM grids preparation, imaging and processing

Freshly gel filtered sample (TnpA^{S911R} or TnpA^{WT}) was used for grid preparation or in case of TnpA^{S911R} and TnpA^{3X} were mixed with a 4–10-fold excess of IR48 and subjected to a second gel filtration to remove unbound substrate. The grids were prepared by applying 5 µL of the sample on glow-discharged Quantifoil R2/1 mesh 300 or UltrA-foil R1.2/1.3 mesh 300 grids at concentrations of ~0.15 and ~0.6–1 mg/ml, respectively. After 60 s incubation, the excess of the sample was removed by blotting from one side for 2.3 s at 80–90% relative humidity and plunge frozen in liquid ethane using a Cryoplunge 3 System (Gatan).

Micrographs were collected at 300 kV on a CRYO ARM 300 (JEOL) electron microscope at a nominal magnification of 60 000 and corresponding pixel size of approximately 0.76 Å. The images were recorded using a

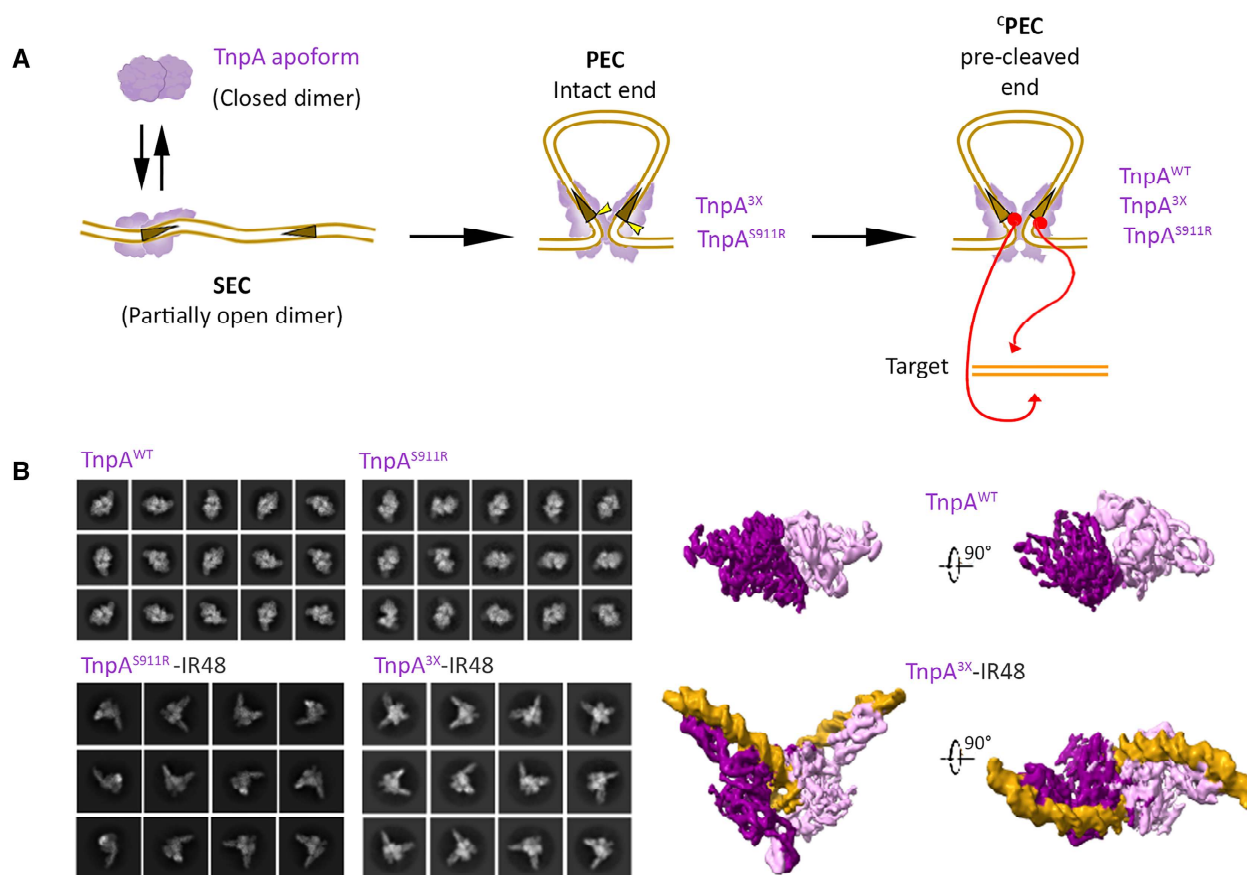


Figure 1. Tn4430 synaptic complex assembly and activation. (A) Model for Tn4430 transposition pathway. TnpA (purple) primarily binds to the transposon IR end (brown triangle) to assemble a single-end complex (SEC). TnpA undergoes an activating conformational change to assemble a paired-end complex (PEC) where it is competent for DNA cleavage (yellow arrowheads). Cleavage exposes a 3'OH group at both transposon ends, which are then transferred (red arrows) to staggered positions of target DNA. In vitro, TnpA^{WT} can only form PEC on pre-cleaved ends (cPEC), while the hyperactive TnpA^{S911R} and TnpA^{3X} mutants spontaneously assemble PEC and cPEC on both intact and pre-cleaved ends. (B) Cryo-EM data showing 2D class averages for TnpA^{WT} and TnpA^{S911R} apoproteins and for the TnpA^{S911R}-IR48 and TnpA^{3X}-IR48 PEC complexes (left panel). Medium resolution models for TnpA^{WT} apoform (4.4 Å) and TnpA^{3X}-IR48 PEC (6.8 Å) showing the dimeric structure of TnpA (right panel). Monomers are colored in light and dark purple and the IR48 DNA fragments containing the 38-bp IR end of Tn4430 plus 5 bp of inner and outer flanking DNA are colored in yellow.

K3 detector (Gatan) operating in correlative-double sampling (CDS) mode. The microscope illumination conditions were set to spot size 6, alpha 1, and the aperture diameters for the condenser and objective apertures were 100 and 150 μm , respectively. The energy filter slit was centered on the zero-loss peak with a slit width set to 20 eV. Coma-corrected data acquisition (15) was used to acquire between 6 and 25 micrographs per stage position using SerialEM v3.0.8 (16). Each micrograph was recorded as a movie of 61 frames over a 3 s exposure time and at a dose rate of $11 \text{ e}^- \text{ pixel}^{-1} \text{ s}^{-1}$ (corresponding to a dose rate per frame of $0.6 \text{ e}^- \text{ Å}^{-2}$) and total exposure dose of approximately $60 \text{ e}^- \text{ Å}^{-2}$.

Initial data processing was performed on-the-fly using RELION.IT (17). Dose-fractionated movies were subjected to motion correction and dose weighting using the Motion-Corr2 (18). The dose-weighted aligned images were used for estimation of the contrast transfer function (CTF) using CTFIND-4 (19). An in-house script was used to plot the calculated parameters, visualize the results, and select the micrographs for further processing (13). The aligned

and dose-weighted images were imported into cryoSPARC v3.1.0 (20), and CTF was calculated using Patch CTF. Particle selection was performed using a blob or template-based picker followed by several rounds of 2D classification. The 2D classification, *ab initio* reconstruction and 3D refinement were performed using cryoSPARC. The data collection parameters are summarized in SI Appendix, Supplementary Table S4.

Functionalization of AFM tips

OMCL-TR400PB-1 cantilevers (Olympus) were used to probe the interaction between TnpA^{WT}, TnpA^{3X} and TnpA^{S911R} and the DNA molecule containing one or two transposon ends. To functionalize the AFM tips, the cantilevers were rinsed with ethanol, dried under a gentle stream of nitrogen, cleaned for 15 min by UV and ozone treatment (Jetlight), and incubated overnight in a polyethylene glycol (PEG) solution made by mixing 0.1 mM HS-(CH₂)₁₁-EG₃-OH with 0.1 mM HS-(CH₂)₁₁-EG₃-NTA in a final ratio (v/v) of 99:1. Then, they were rinsed with ethanol,

dried with nitrogen, and immersed for 30 min in 50 mM NiCl_2 before using (21).

The tips were functionalized with the TnpA proteins through their His₆ tag. The cantilevers were placed on Parafilm (Bemis NA), incubated with 50 μl of a TnpA protein solution (0.1 mg/ml) for 1 h, and washed three times with DNA binding buffer [50 mM HEPES and 100 mM NaCl].

Preparation of DNA-coated model surfaces

DNA molecules containing one or two transposon ends were immobilized using click chemistry as described (22,23). Briefly, gold-coated surfaces were rinsed with ethanol, dried under a gentle stream of nitrogen, cleaned for 15 min by UV and ozone treatment (Jetlight), and incubated overnight in a solution made by mixing 0.1 mM HS-(CH₂)₁₁-EG₃-OH with 0.1 mM HS-(CH₂)₁₁-EG₅-N₃ solutions in a final ratio (v/v) of 95:5. Then, they were rinsed with ethanol and dried with nitrogen. Immediately afterward, a 30 μl aliquot containing 30 μM of the one-end or two-end substrate (with a terminal or internal alkyne group, respectively), 1 M ascorbic acid, and 0.3 M CuSO₄ was deposited on the surfaces and left to incubate overnight in the dark at 4°C. Finally, the surfaces were washed three times with DNA binding buffer.

Force-distance curve-based AFM on model surfaces

The acquisition of force-distance curves by AFM (FD curve-based AFM) on model surfaces was performed in DNA binding buffer at room temperature, with functionalized OMCL probes (Olympus, spring constants from 0.027 to 0.053 N m⁻¹ calculated using thermal tune) (24). FD curve-based AFM consists in approaching and retracting the AFM tip from the surface in the vertical direction while recording the force. A Force-Robot 300 (JPK, Bruker, Germany) was used. Areas of 25 μm^2 (~1024 measurements) were scanned, the approach and retraction speeds of the tip were fixed at 1 $\mu\text{m/s}$, the hold or contact time was 200 ms, the ramp size was set to 300 nm and the setpoint force at 250 pN, with a resolution of 32 × 32 pixels.

Dynamic force spectroscopy (DFS) analysis was performed using a constant approach speed of 1 $\mu\text{m/s}$ and variable retraction speeds (0.1, 0.2, 1, 2 and 10 $\mu\text{m s}^{-1}$), and kinetic on-rate was estimated by measuring the binding probability (BP; i.e. the fraction of curves showing binding events) at different hold or contact time (i.e. the time the tip is in contact with the surface; 100, 200, 400, 600, 800, 1000 and 1200 ms). Force curves were analyzed on the data processing software from JPK Data processing (version 6.1.149). To identify peaks corresponding to adhesion events between proteins linked to the PEG spacer and the DNA molecule of the model surface, the retraction curve before bond rupture was fitted with the worm-like chain model for polymer extension (25,26).

Origin software (OriginLab) was used to display the results in DFS plots, to fit histograms of rupture force distributions for distinct loading rate ranges, and to apply various force spectroscopy models to extract the number of bonds mediating interaction between substrate and ligand, k_{off} and x_u (27–29).

For kinetic on-rate analysis, BP was determined at a certain hold time (t). Those data were fitted and K_D calculated as described (30,31). In brief, the relationship between interaction time (τ) and BP is described by the following equation:

$$\text{BP} = A \left[1 - \exp \left(\frac{-(t - t_0)}{\tau} \right) \right] \quad (1)$$

where A is the maximum BP and t_0 is the lag time. Origin software was used to fit the data and extract τ . In the next step, k_{on} was calculated with the following equation (30), where r_{eff} is the radius of the sphere, n_b the number of binding partners, and N_A the Avogadro constant:

$$k_{\text{on}} = \frac{4\pi r_{\text{eff}}^3 N_A}{3n_b \tau} \quad (2)$$

The effective volume $V_{\text{eff}} [(4\pi r_{\text{eff}}^3)/3]$ represents the volume in which the interaction can take place. In our study, V_{eff} can be approximated by a sphere with a radius of ~54 nm including, the PEG linker (~3 nm), the TnpA protein (~14 nm), and the linear one-end DNA substrate (~37 nm considering that the bp distance in dsDNA is 0.34 nm and the nt distance in ssDNA is 0.68 nm, respectively).

Competition-binding assays

To monitor the effect of a second transposon end (IR end-2; see *SI Appendix*, Supplementary Figure S5A and Supplementary Table S2) on TnpA-one end interaction, BP was measured before and after tip incubation with 12.5, 25 and 50 μM of competitor DNA. A map (25 μm^2 , ~1024 measurements) was first recorded as described above in the absence of competitor DNA and then after injection of increasing amounts of free competitor IR end-2 DNA into the AFM chamber.

RESULTS

Dimeric structure of the free and DNA bound TnpA

The finding that Tn4430 TnpA essentially forms dimeric particles in cryo-EM datasets (13) contradicts our previous estimations based on gel filtration chromatography (GFC) and dynamic light scattering (DLS) analysis suggesting that TnpA was a monomer in solution (10). However, the DLS data were somewhat ambiguous supporting an ‘extended’ configuration of the protein with an apparent size between that of a monomer and a dimer (10). Since it was necessary to clarify the molecular organization of TnpA to interpret the AFM results, we decided to revisit the data by measuring the structural properties of the purified protein with greater accuracy using small-angle X-ray scattering (SAXS) coupled to gel filtration chromatography (GFC-SAXS) (*SI Appendix*, Supplementary Figure S1 and Supplementary Table S3).

The GFC-SAXS profile of TnpA^{WT} shows a major symmetric peak with a nearly constant radius of gyration (R_g) across the elution profile, indicating the absence of significant interparticle interactions (*SI Appendix*, Supplementary Figure S1A–C). Further analysis of SAXS data yielded

R_g (from Guinier plot) and maximal dimension of particles (D_{max}) values of 46 ± 5 and 160 ± 10 Å, respectively, whereas the molecular mass estimation was ~270–300 kDa (*SI Appendix*, Supplementary Table S3), which is closer to the expected mass of a dimer (232 kDa) than that of a monomer. These values are in good agreement with the results obtained for proteins of a similar size, e.g. the tetrameric pyruvate decarboxylase (ID: SASDAX2) which gives an R_g of 39 Å and a D_{max} of 110 Å for a MW of 244 kDa (32). The apparent overestimation of TnpA MW could reflect some degree of flexibility or extended conformation in solution. To check this, we performed a dimensionless Kratky (dKratky) plot analysis to compare SAXS data obtained for TnpA^{WT} with the data obtained for a globular (BSA) or highly flexible (hTau40wt) protein (*SI Appendix*, Supplementary Figure S1D). The results thus suggest that TnpA^{WT} exists as a rather compact molecule in solution. This confirmed by cryo-EM structures obtained for apo TnpA^{WT} in the presence or absence of DNA ((13), Figure 1B), showing a tightly packed, 2-fold symmetric dimer in which both protomers are interlocked by a double dimerization interface involving separate domains of the protein (13). Apart from some low-density regions protruding at the protein surface, the cryo-EM 3D reconstruction of unbound TnpA^{WT} exhibits a good overall fit to the SAXS experimental data (supplemental Figure S1E and G). The dimeric organization of TnpA is maintained in the cryo-EM structures of PEC obtained for the hyperactive mutant TnpA^{S911R} (13). However, compared to apo TnpA^{WT}, the DNA-bound TnpA^{S911R} dimer exhibits a substantial rearrangement of the protein domains to accommodate the two transposon ends and adopt its active configuration (13).

To see whether these observations can apply to other TnpA variants, cryo-EM data were collected from TnpA^{S911R} in apo form and from TnpA^{3X} in complex with 48-bp long transposon ends (Figure 1B, Supplementary Figure S2 and Supplementary Table S2). A small dataset was recorded for apo TnpA^{S911R}, which was sufficient to obtain 2D class averages showing shape and size similarity with apo TnpA^{WT}, suggesting that TnpA^{S911R} forms an inactive dimer in apo state. Likewise, 6.1-Å 3D reconstruction calculated for TnpA^{3X}-IR48 complex revealed a dimeric structure superimposable to that of TnpA^{S911R}-IR48 PEC, indicating that the mutations do not change the organization or conformation of the complexes (Figure 1B).

Together with the GFC-SAXS analysis, the cryo-EM data support the conclusion that free TnpA wild-type and hyperactive mutants exists as a ‘closed’ dimer in solution, and that PEC formation involves extensive deployment of the dimer to bind tightly two transposon ends in an active form. No evidence of SEC complex could be found in the TnpA^{S911R}-IR48 and TnpA^{3X}-IR48 cryo-EM datasets, which is consistent with DNA binding by the two mutant TnpAs (S911R and 3X) being highly cooperative, showing much higher affinity for two transposon ends than for one [(10), see also below]. Only a few particles corresponding to singly bound proteins were observed when TnpA^{WT} was imaged in the presence of an IR-containing DNA fragment (13). However, compared to free protein, the fraction of particles corresponding to putative SEC conformation was too

low for calculating 3D reconstructions, which likely reflects the low stability of SEC under conditions used for cryo-EM (13).

TnpA binding to a single transposon end

According to previous biochemical studies (10), SEC formation appears to be a intermediate stage in the assembly of the transposition complex, with the subsequent structural transition leading to PEC being a rate-limiting step in the transposition process (Figure 1A). We have therefore used AFM force spectroscopy [refer to recent reviews (14,32) for further details] to evaluate the formation and stability of SEC by comparing the binding strength (i.e. rupture force) of TnpA^{WT}, TnpA^{3X} and TnpA^{S911R} to DNA substrates containing a single Tn4430 end (one-end substrate). Using ‘click’ chemistry (22,23), short linear DNA molecules carrying an alkyne reactive group at their 5’ end (*SI Appendix*, Supplementary Figure S3A and Supplementary Table S2) were covalently immobilized in an oriented manner onto gold surfaces coated with alkanethiols terminated with polyethyleneglycol (PEG) with a small fraction of them (5%) harboring an azide group (PEG-N₃) (Figure 2A). These model surfaces were imaged by AFM, and the thickness of the grafted layer was validated by a scratching experiment, revealing a deposited layer of ~1.5 and 2 nm for PEG/PEG-N₃ and PEG/PEG-N₃/one end, respectively (*SI Appendix*, Supplementary Figure S3).

To study the binding of TnpA to DNA, purified TnpA^{WT}, TnpA^{3X} and TnpA^{S911R} proteins were attached in an oriented manner to AFM tips functionalized with Ni²⁺-NTA groups through the His-tag present at the C-terminus (21).

In the force spectroscopy mode, the tip is approached toward and retracted from the sample in a controlled manner. The cantilever deflection as well as the cantilever displacement are recorded and then converted into a force *versus* distance curve (FD curve) (Figure 2B). Upon retraction, an adhesion event can be observed and then appears as a peak towards negative forces. The height of this peak corresponds to the adhesion force or binding force (14,33). More precisely, in the force-volume (contact) AFM mode, the cantilever is raster scanned over the sample following a pixel matrix. For each pixel, the tip is approached and retracted from the surface (14,33). In our experiments, we recorded adhesion events on a 5 µm × 5 µm area with a 32 × 32 pixels map (or 1024 force curves).

FD curves were recorded to probe the binding strength of the three TnpA variants to the one-end DNA substrate (Figure 2A, B). For a contact time between the tip and the surface the surface of 200 ms, specific adhesion events were observed in $10.7 \pm 4.5\%$ (mean ± S.D., $N = 18845$), $7.7 \pm 4.1\%$ ($N = 22548$) and $5.7 \pm 3.7\%$ ($N = 14336$) of the retraction FD curves for TnpA^{WT}, TnpA^{3X} and TnpA^{S911R}, respectively (Figure 2C).

To confirm the specificity of these interactions, independent control experiments were performed using (i) a surface functionalized with a nonspecific DNA substrate in which all the positions of the 38-bp IR end were substituted (*SI Appendix*, Supplementary Table S2), (ii) a surface-functionalized only with the PEG/PEG-N₃ mix (without DNA) and (iii) an AFM tip functionalized only with the

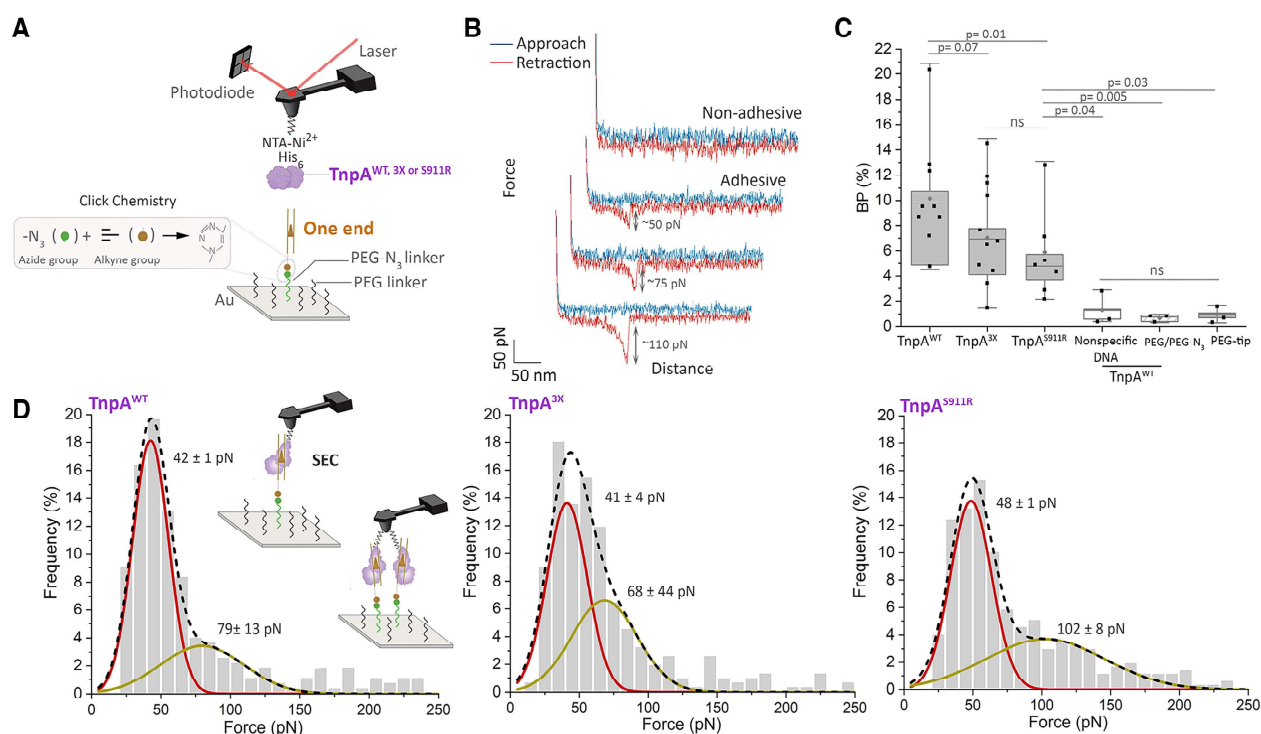


Figure 2. Probing of TnpA binding to linear DNA containing a single transposon end by AFM-based force spectroscopy. (A) AFM set up. The interactions between TnpA and DNA are monitored by AFM on model surfaces. DNA molecules containing one copy of Tn4430 IR end are covalently attached to a PEG-coated surface using click chemistry (22,23) by reacting the alkyne group at the 5' end of the DNA substrate with polyethyleneglycol linkers harboring an azide group (PEG-N₃). The AFM tip is functionalized by PEG linkers to which Ni²⁺-loaded NTA (Ni²⁺-NTA) is bound. TnpA contains a His₆-tag, that specifically binds to the Ni²⁺-NTA ligands. (B) Representative force-distance curves showing either non-adhesive or specific adhesive interactions. The rupture force measured in each case is indicated to the right of the curves. (C) Box plot of AFM-measured binding probability (BP) between tips tips functionalized with TnpA^{WT}, TnpA^{3X} or TnpA^{S911R} as indicated, and surfaces grafted with DNA molecules containing one transposon end (contact time, 200 ms). The binding frequencies between TnpA^{WT} and the nonspecific DNA molecule or solely the PEG/PEG-N₃ layer are shown as a specificity control. The binding probability measured with a TnpA-free polyethylene glycol (PEG) tip and the one-end surface gives the background level of the experimental setup. The horizontal line within the box indicates the median, the boundaries of the box indicate the 25th and 75th percentile, and the whiskers indicate the highest and lowest values of the results. The gray square in the box indicates the BP mean of a specific area of 25 μm² scanned two times (~2048 measurements). For all experiments, data are representative of at least *N* = 3 independent experiments. P values were determined by a two-sample t-test with R software (version 3.1.1). (D) Frequency distributions of rupture forces between TnpA^{WT}, TnpA^{3X} and TnpA^{S911R} and the one-end substrate. The total number of binding events was of 275, 311 and 380 for TnpA^{WT}, TnpA^{3X} and TnpA^{S911R}, respectively. The red curve corresponds to SEC while the light brown curve probably corresponds to two interactions established in parallel. The most probable interaction force and its error are shown for each curve (force ± standard error). The dashed line corresponds to the sum of the red and light brown curves.

PEG thiol. As expected, TnpA did not bind significantly to non-specific DNA nor PEG, and the binding frequency was also significantly reduced when the AFM tip was functionalized with the PEG linker only (without TnpA) (Figure 2C). Finally, a competition assay was performed by injecting increasing amounts of a free DNA molecule carrying the Tn4430 IR end sequence within the AFM reaction chamber (SI Appendix, Supplementary Figure S5A, B). For all three TnpA proteins (TnpA^{WT}, TnpA^{3X} and TnpA^{S911R}), a reduction of the binding probability (BP) between the immobilized partners was observed as a function of the concentration of the competitor DNA (Supplementary Figure S5C–E). Altogether, these controls confirm the specificity of the interactions and the critical importance of the IR sequence in the formation of SEC.

Next, we extracted the binding strength from the individual FD curves and analyzed the force distribution of the interactions established between the one-end substrate and

the three TnpA proteins. The binding forces were displayed as a histogram for each TnpA variant and we observed a peak in the range 40–50 pN in each case (Figure 2D). We attributed this interaction to the formation of the SEC. In addition, at a lower frequency, a second population of interactions is present in the range 70–100 pN, which likely indicates the formation of two interactions established in parallel (Figure 2D), presumably between two different TnpA molecules attached to the AFM tip and two DNA fragments present on the surface. Consistent with this interpretation, the relative frequency of this double interaction increased with the density of the one-end substrate attached to the surface (SI appendix, Supplementary Figure S4).

These results show that TnpA^{WT}, TnpA^{3X} and TnpA^{S911R}, can specifically bind to a single transposon end to form the SEC, consistent with previous DNA footprint results showing that TnpA^{WT} and the mutants interacts all with the same inner part of the IR (10).

Exploring the kinetic properties of SEC formation

To characterize the binding properties of the interactions occurring between TnpA and the one-end substrate, the binding strength of the complex was probed at various force loading rates (LR), i.e. by increasing the force acting on the bond over time, which enables to extract kinetic parameters (34) (Figure 3). To explore a wide spectrum of loading rates, the retraction speed was varied upon recording FD curves. The loading rate was calculated from the slope of the force vs time curve just before the rupture, as previously described (35). The ‘force-LR’ data points were plotted in so called dynamic force spectroscopy (DFS) plots, for each TnpA-one end system (Figure 3B–D). As predicted by the Bell-Evans theory (27,28), the unbinding force increased with the logarithm of the LR (gray data point). To precisely quantify this relationship, bond strengths were analyzed through distinct discrete ranges of LRs, plotted as force histograms, and further fitted with a multipeak Gaussian distribution (36). This analysis allowed us to determine for each discrete range of LR the most probable unbinding force for the single (red data point in the DFS plots of Figure 3B–D) or for multiple interactions (brown data point in the DFS plots of Figure 3B–D).

To extract the kinetics parameters of the interaction between the TnpA molecules and the one-end substrate, we approximated the binding free-energy landscape as a simple two-state model in which the bound state is separated from the unbound state by a single energy barrier (Figure 3A). Following this assumption, the Bell-Evans model (27,28) was used to fit the single-rupture event, providing access to the kinetics parameters (Figure 3B–D, solid line). First, the width of the energy barrier (x_u) was extracted from the slope of the fit. We obtained the following x_u values: 0.74 ± 0.05 nm, 0.79 ± 0.09 nm and 0.67 ± 0.11 nm for TnpA^{WT}, TnpA^{3X} and TnpA^{S911R}, respectively. These values confirm that the bonds are similar for the three systems and are characteristic of a stiff bonding network, clearly indicating an all or none unbinding mechanism rather than a gradual ‘unpeeling’ or sliding of the protein along the DNA (37). The kinetic off-rate (k_{off}) or dissociation rate obtained from the intersection of the fit (at loading rate, LR = 0) yielded k_{off} values of 0.20 ± 0.06 s⁻¹, 0.10 ± 0.10 s⁻¹ and 0.10 ± 0.14 s⁻¹ for TnpA^{WT}, TnpA^{3X} and TnpA^{S911R}, respectively (Figure 3B–D). These values are within the range of those reported for the LexA repressor, a well characterised DNA binding protein (which controls approximately 31 genes involved in the SOS response of *Escherichia coli*), as previously determined by AFM-based single-molecule force spectroscopy (37). The LexA repressor showed dissociation rate constants of 0.048 s⁻¹ and 0.13 s⁻¹ for the *recA* and *yebG* operons, respectively (37).

Next, the predictive Williams-Evans model (29) allowed us determine whether the multiple bonds are correlated or not, i.e. if all the bonds are equivalent or if the first binding event induces an allosteric modulation of the second binding event. To this end, we analyzed the higher force observed on the DFS plot. Since the second peaks, observed at each loading rate, (brown data point in the DFS plots of Figure 3B–D) fit well with the Williams-Evans prediction, those bonds are predicted to originate from uncorrelated

bonds loaded in parallel (Figure 3B–D, dashed line), confirming that under our experimental conditions two SEC complexes can be simultaneously probed as suggested from the force distribution histograms (Figure 2D).

Approaching to the free-energy landscape of binding

Assuming that SEC can be approximated by pseudo-first-order kinetics, the kinetic on-rate (k_{on}) was estimated from our single-molecule force spectroscopy experiments by monitoring the binding probability (BP) between the different pairs of partners (wild-type, S911R or 3X TnpAs with the single end substrates) at various contact times (Figure 3E–G). k_{on} values were extracted by taking the effective concentration as the number of binding partners (TnpA-one end) within an effective volume V_{eff} accessible under free-equilibrium interaction. V_{eff} can be approximated by a sphere whose radius includes the linker, the TnpA protein, and the linear one-end DNA substrate (see equations (1) and (2) in materials and methods) (30). The binding frequency followed a logarithmic growth with the contact time for all three TnpAs (Figure 3E–G). The values extracted for interaction time were 0.44 ± 0.06 s, 0.58 ± 0.27 s and 0.41 ± 0.10 s, which led to a k_{on} of 3.94×10^5 M⁻¹ s⁻¹, 3.00×10^5 M⁻¹ s⁻¹ and 4.22×10^5 M⁻¹ s⁻¹ for TnpA^{WT}, TnpA^{3X} and TnpA^{S911R} complexes, respectively. Finally, the dissociation constant K_D , calculated as the ratio between k_{off} and k_{on} yielded values around ~ 508 nM, 333 nM, and 236 nM for the TnpA^{WT}, TnpA^{3X} and TnpA^{S911R} complexes, respectively (Figure 3E–G). For single-molecule interactions, the bond lifetime can be directly related to the inverse kinetic off-rate (lifetime = k_{off}^{-1}), resulting here in a lifetime of 5 s for TnpA^{WT} and 10 s for both hyperactive mutants. The sub-micromolar affinities confirm the specificity of the complexes established between the different TnpA proteins and the one-end substrate (38).

TnpA binding to two transposon ends and PEC formation

According to our model, PEC formation is a critical step of the Tn4430 transposition pathway during which the TnpA dimer bound to a single transposon end undergoes a conformational change that allows it to capture the opposite end of the transposon and to adopt an active configuration (Figure 1A). We believe that this is a rate-limiting step in the activation pathway, since TnpA^{WT} hardly forms the PEC under standard conditions, whereas for the hyperactive TnpA^{3X} and TnpA^{S911R} mutants, PEC formation is highly cooperative (10,13). However, so far, the dynamics of PEC formation is poorly understood due to the lack of direct quantitative information.

To this end, a specific DNA substrate containing inversely oriented Tn4430 IR ends was assembled by annealing three complementary oligonucleotides (SI Appendix, Supplementary Figure S6A, and Supplementary Table S2). The duplex regions corresponding to the IR ends are separated by a 20-nt single-stranded DNA linker to allow for enough flexibility to pair both transposon ends inside the PEC based on structural data (Figure 1B) (13). Restriction digest followed by gel electrophoresis showed that $\sim 100\%$ of the DNA substrates assembled correctly (SI Appendix, Supplementary

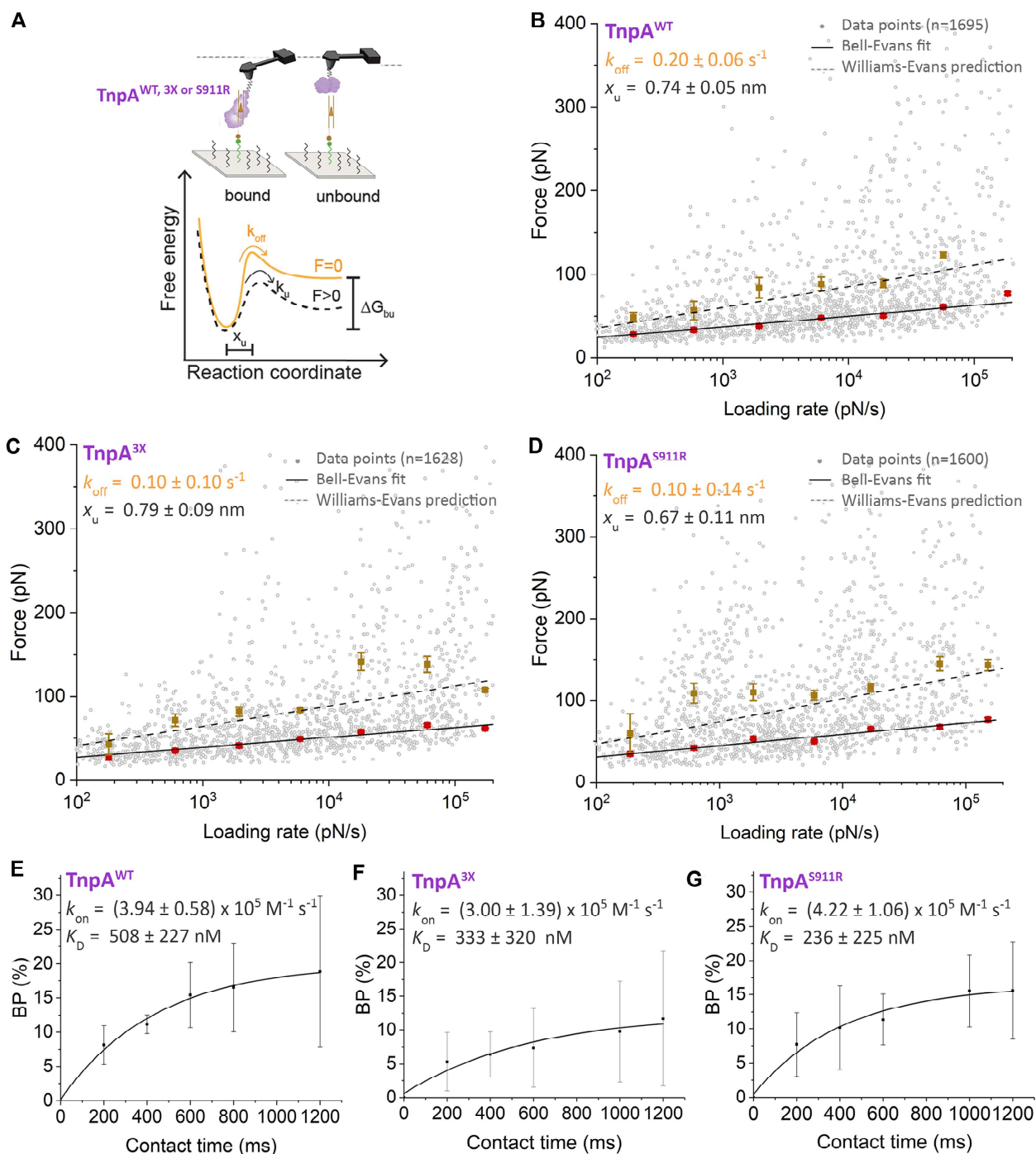


Figure 3. Dynamics of SEC. (A) The binding of TnpA^{WT}, TnpA^{3X} or TnpA^{S911R} is probed on a surface coated with one-end DNA molecules. Bell-Evans model describes a ligand-receptor bond as a simple two-state model. The bound state is separated from the unbound state (distance between the two states, x axis) by an energy barrier located at distance x_u . k_{on} and k_{off} represent the transition rate under force and transition at thermal equilibrium, respectively. (B–D) Dynamic force spectroscopy (DFS) plots showing the distribution of the rupture forces as a function of the loading rates measured either between TnpA^{WT} (B), TnpA^{3X} (C) or TnpA^{S911R} (D) and the one-end-coated surface. Data corresponding to single interactions are fitted with the Bell-Evans model (black solid line). The red squares correspond to the most probable unbinding force for each discrete range of LR (for a single interaction). Dashed lines represent the predicted binding forces for two simultaneous uncorrelated interactions (Williams-Evans model), with the brown squares being the highest and most probable unbinding force for each discrete range of LR. Error bars indicate standard deviations (SD) of the mean values. Experiments were reproduced at least five times with independent tips and samples. (E–G) The binding probability (BP) is plotted as a function of the contact time for TnpA^{WT} (E), TnpA^{3X} (F) or TnpA^{S911R} (G) on the one end-coated surface, and data points were fitted using a least-squares fit of a monoexponential growth. One data point belongs to the BP from one map acquired at $1 \mu\text{m/s}$ retraction speed for the different contact times. Experiments were reproduced at least three times with independent tips and samples. Error bars indicate s.d. of the mean values.

Figure S6B). The central position of the DNA linker was substituted with an alkyne group-containing dU nucleotide to anchor the substrate to azide-modified AFM surfaces by click chemistry (22,23) (Figures 2A and 4B). This model surface was imaged by AFM, and the thickness of the grafted layer was validated by a scratching experiment, revealing a deposited layer of 2 nm (*SI Appendix*, Supplementary Figure S6C, D). Purified TnpA proteins were attached to the AFM tip as described above for the one-end experiments.

Conformational dynamics occurring during PEC formation were analyzed by FD curve-based AFM by tracking the binding strength between the various transposases and the substrate with either one end (Figure 4A) or two ends (Figure 4B). To follow the dynamics, the contact time between the binding partners was progressively increased from 100 to 1200 ms, to provide sufficient time for binding and conformational transition (*SI Appendix*, Supplementary Figures S7–S9).

While only two populations are observed with the one-end substrate (Figure 4A), a third population at higher forces appeared in the histograms for the two-end substrate (Figure 4B) and the three TnpA variants. As evidenced above, an interaction appears with an average force of ~ 40 pN corresponding to SEC formation (Figure 4A, B, scheme I). A second sub-population with a force of ~ 70 pN was observed confirming the possibility of forming two SEC in parallel with both the one-end and two-end substrates (Figure 4A, B, scheme II). Interestingly, only in the experiments with the two-end substrate, a third population around ~ 120 pN was observed. Since this third population only appears with the two-end substrate, we attributed it to the formation of a complex between a single TnpA dimer and both transposon ends of the substrate, thus mimicking PEC formation (Figure 4B, scheme III). For the three TnpAs, the strength of interactions measured for the third peak is higher than that measured for parallel interactions between two TnpA dimers and two independent ends, supporting the idea that PEC formation involves a larger network of contacts between TnpA and DNA than SEC. The dynamics of PEC formation was analyzed taking into account the area below green curve (subtracting the shared area with the light brown and red curves) for every contact time (Figure 4C). We observed that PEC formation by TnpA^{WT} follows pseudo-first-order association kinetics with a interaction time (τ) of ~ 156 ms. By contrast, for the TnpA^{3X} and TnpA^{S911R} hyperactive mutants, the kinetic analysis showed that complex III formation occurred at a much faster rate (τ of 2 ms and 17 ms, respectively), at the limit of time resolution of the experiment.

DISCUSSION

Transposition, like other complex protein-DNA transactions, is a carefully orchestrated process involving multiple partners. In particular, transposition catalyzed by DDE/D transposases proceeds through a series of DNA cleavages and strand transfer reactions fusing the transposon ends to a new target locus (2). Since these reactions are potentially harmful to the host, it is important that each step be irreversible and coordinated with each other. Directionality is

generally thought to result from energetically favorable conformational changes taking place in the transposition complex to rearrange and adequately position the partners at each step of the reaction (39–43). During replicative transposition of Tn3 family transposons, 4 DNA strands are broken and two are reformed, which also largely contributes to the favorable net change in free energy driving the overall reaction.

In many cases, initial activation of transposition results from the formation of an oligomeric nucleoprotein complex in which both transposon ends are brought together by the transposase. In the simplest situation referred to as ‘synapsis-by-protein-dimerization’ (S-PD), the transposase separately binds as a monomer to each transposon end and then oligomerizes to assemble the synaptic complex (44). Detailed GFC-SAXS analysis performed in the present work demonstrates that TnpA is a dimer in solution, refuting the original hypothesis of a monomeric transposase, dimerizing upon binding to its substrate (10). The reasons why former estimations were misleading is unclear, but they likely resulted from the relative imprecision of the methods used and a poorer quality of the protein sampled than those used here.

Furthermore, data obtained by cryo-EM for TnpA^{WT} and hyperactive TnpA mutants unambiguously demonstrate the dimeric structure of both the free and DNA-bound transposases and suggest that they remain dimeric throughout the transposition pathway (Figure 1B) (13). Together with previous biochemical data, the finding that the TnpA transposase of Tn4430 forms a stable dimer in solution indicates that it uses a conceptually different ‘synapsis-by-naked-end-capture’ (S-NEC) mechanism in which an inactive TnpA dimer first binds to a single transposon end to form the SEC and then undergoes a conformational change to capture the other end and adopt the active PEC configuration (44) (Figures 1 and 5).

Here, we have used FD-curve based AFM to decipher the dynamics of Tn4430 transposition complex formation at the single particle level by comparing the DNA binding properties of TnpA^{WT} with those of the two hyperactive variants TnpA^{3X} and TnpA^{S911R}, which are thought to recapitulate two key intermediates of the assembly pathway. We took advantage of click chemistry (22,23) to assemble and decorate the AFM surfaces with specifically designed one-end and two-end DNA substrates allowing us to collect high throughput data for both the SEC and PEC with high selectivity and reproducibility, an approach that could readily apply to any other protein-DNA system.

The results show that TnpA^{WT} and both hyperactive mutants specifically bind to a spatially constrained (i.e. non-diffusible) single transposon end with quite similar kinetic and thermodynamic parameters, indicating that the determinants for SEC formation are essentially the same for the three proteins. The measured force of interaction (~ 45 pN) and the deduced sub-micromolar affinities are typical of specific bimolecular associations such as between a protein-ligand and its receptor or an enzyme with its substrate (45–47), which is consistent with [(OP)2-Cu+] footprint patterns showing that within the SEC, both the wild-type and the mutant TnpAs protect the same ~ 30 -bp internal region by of Tn4430 38-bp IR sequence by contacting both DNA

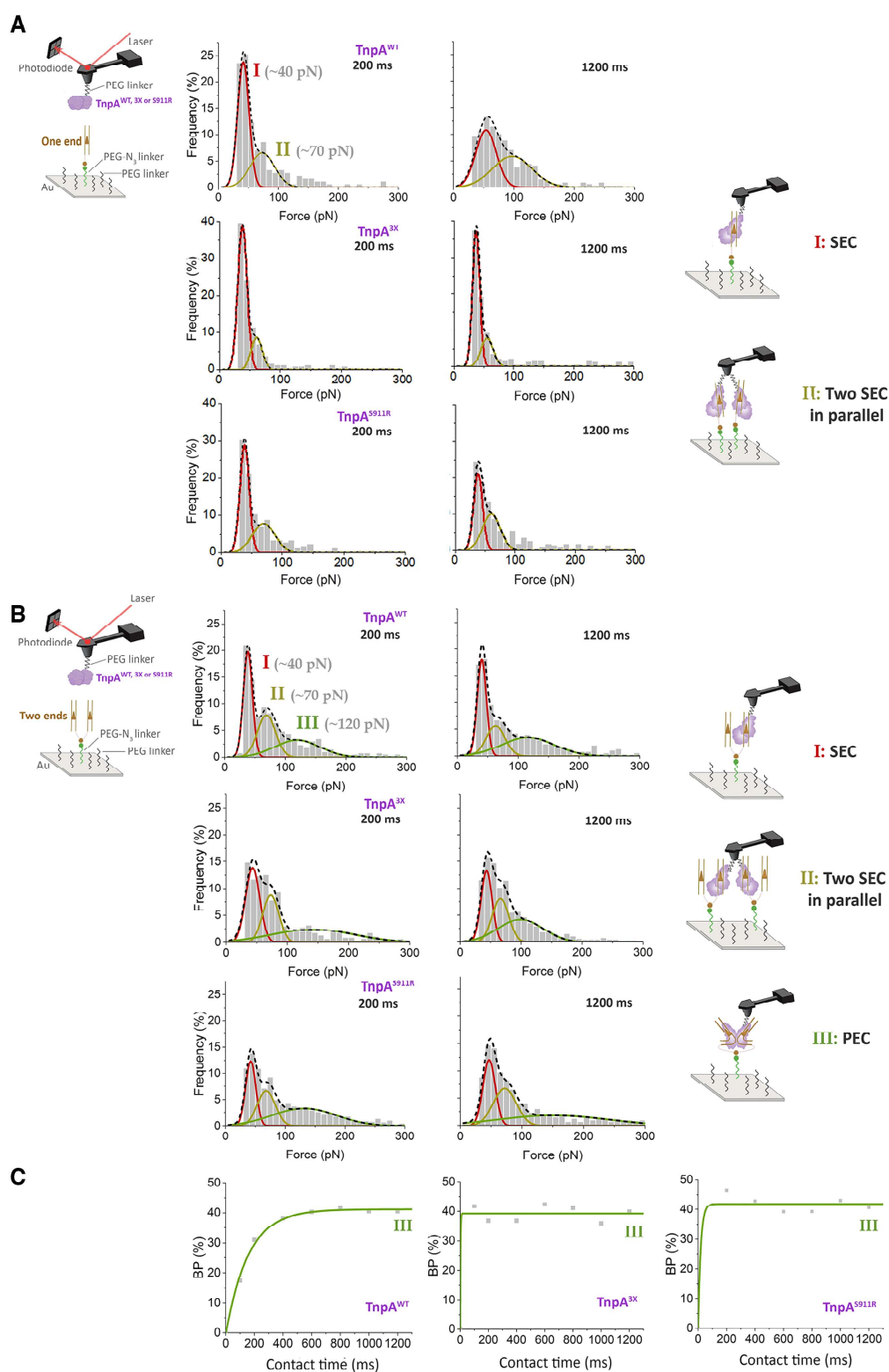


Figure 4. Probing PEC formation. (A and B) Binding of TnpA to surface coated with one-end molecules is compared to TnpA binding to two transposon ends. Frequency distributions of rupture forces at 200 ms and 1200 ms of contact time between either TnpA^{WT}, TnpA^{3X} or TnpA^{S911R}, and the one-end substrate (A) or the two-end substrate (B) were extracted from data presented in *SI Appendix*, Supplementary Figures S7–S9. I, II or III populations are shown by red, light brown or green curves respectively, and their corresponding interpretation is schematized to the right of each panel. (C) Binding probability (BP) as a function of contact time for the third population (III).

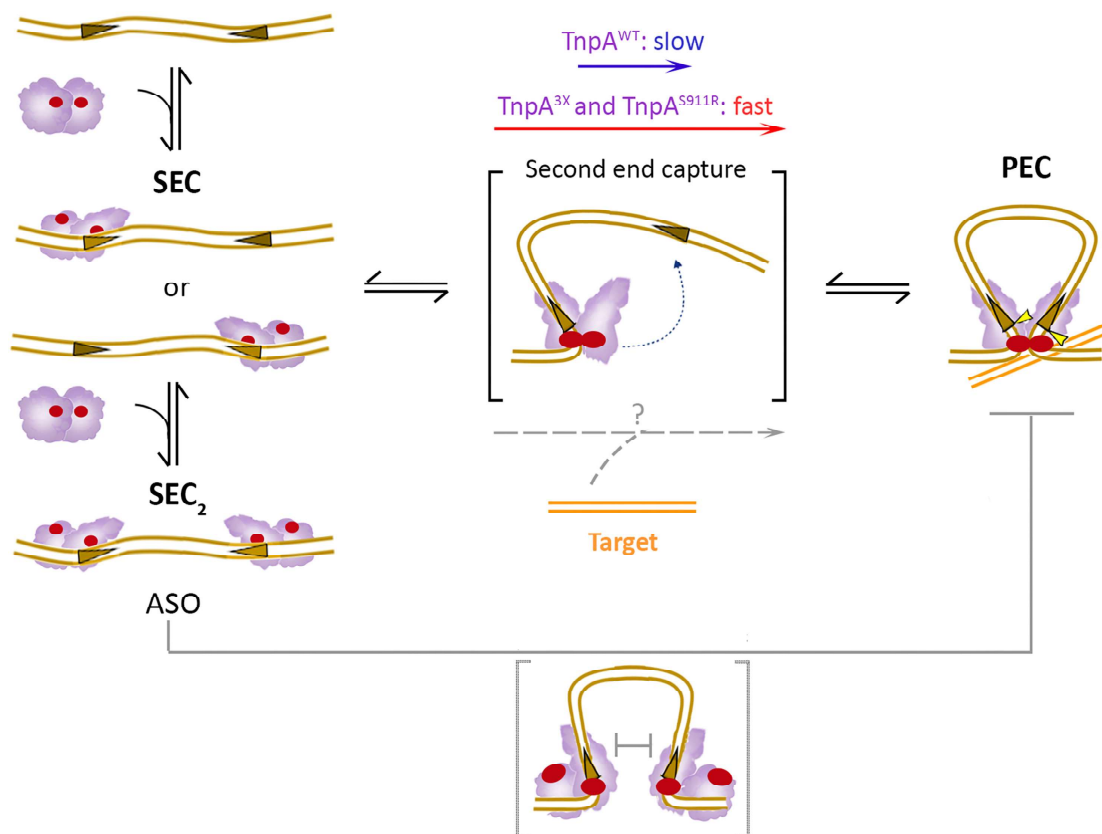


Figure 5. Model for Tn4430 transpososome activation pathway. Synapsis-by-naked-end-capture (S-NEC) mechanism in which an inactive TnpA dimer first binds to a single transposon end to form the inactive SEC and then undergoes a conformational change to capture the other end and adopt the active PEC configuration. ASO: assembly site occlusion. See text for further details.

strands (10). In the absence of high-resolution data for the SEC, we speculate that these contacts correspond to a subset of protein-DNA interactions established in ‘cis’ between one TnpA protomer and one IR end in the PEC structures (13).

However, the SEC appears to be a metastable intermediate of PEC assembly, with an estimated lifetime being comprised between 5 s for TnpA^{WT} and 10 s for TnpA^{3X} and TnpA^{S911R}, respectively. As discussed below, reversible binding of TnpA to the transposon ends is important since it gives the possibility to regulate the initial step of transposition prior to proceeding further by forming the PEC or not (Figure 5). Intermediate transition states between SEC and PEC cannot be excluded but could not be evidenced by AFM, suggesting that if they exist, they must be extremely short-lived (below the temporal resolution limit of AFM).

Most remarkably, kinetic analysis performed with a two-end substrate showed that PEC formation by TnpA^{3X} and TnpA^{S911R} occurs at a much faster rate than with TnpA^{WT}, faster than can be measured using this method (Figure 4C). This result is consistent with previous EMSA titration experiments showing that PEC formation by TnpA^{3X} and TnpA^{S911R} is highly cooperative, with the SEC being not or barely detectable even at low protein-DNA ratios (10), and with cryo-EM datasets showing that incubation of both deregulated TnpA mutants with IR DNA substrates al-

most exclusively gives PEC (Figure 1B) (13). The higher force required to disrupt the PEC compared to two independent SEC (i.e. ~120 pN versus ~70–80 pN) is consistent with DNA footprint analyses and high-resolution structural data showing that TnpA binding into the PEC makes extended contacts with the outer part of the transposon end and the flanking DNA to activate the transposon cleavage sites (10,13). A substantial proportion of these interactions are mediated in ‘trans’ with each TnpA protomer bound in ‘cis’ to one transposon end making specific and non-specific contacts with the other end, thereby contributing to the overall cohesion of PEC. Non-specific interactions with the outer flanking DNA generate a sharp bend at the transposon cleavage sites corresponding to hypersensitive positions in the DNA footprint pattern (10). DNA bending is required to expose the scissile phosphate from each transposon end into the catalytic domain of the partner TnpA protomer across the dimer. Thus, together the results support the conclusion that SEC to PEC transition is a rate-limiting step in the Tn4430 S-NEC assembly pathway, and that specific mutations in TnpA^{3X} and TnpA^{S911R} have lowered the energy barrier for PEC formation, making the hyperactive mutants more prone than TnpA^{WT} to undergo the required conformational change to capture both transposon ends and become catalytically active (Figure 5) (13). Supporting this conclusion, mapping of the

hyperactivating mutations onto the TnpA structure reveals that they are all susceptible to enhance the flexibility of the protein by destabilizing the inter-domain interface that maintains the apoform in a locked (i.e. closed) conformation and/or by stabilizing the activated (i.e. open) PEC configuration (13). In particular, the S911R substitution is localized in a so called ‘switch’ helix adjacent to the catalytic domain and is proposed to contribute to proper folding of the RNaseH-like domain upon DNA binding, while the positions of the three amino-acid substitutions of TnpA^{3X} (W24R, A174V and E740G) suggest a dual role in the deployment of the TnpA DNA binding domains (W24R and A174V) and the assembly of the catalytic domain (E740G), which may explain their additive effects on TnpA activity (7,10,13).

What then promotes activation of TnpA under the wild-type situation? Since TnpA^{3X} and TnpA^{S911R} were initially isolated for their impairment in transposition immunity, they are less demanding than TnpA^{WT} concerning target selection, being able to transpose at a higher frequency into non-permissive DNA molecules (7). We, therefore, propose that SEC to PEC transition is normally controlled by the target, with permissive targets providing a positive signal to induce the formation of PEC and/or immune targets preventing the activating conformational change (Figure 5). This target activation checkpoint would ensure that all partners are brought together before triggering transposition and provide a rationale for target immunity and the behavior of immunity-defective TnpA mutants. The presence of a proper target may either promote SEC to PEC transition or stabilize the PEC in the active configuration (Figure 5). We favor the latter possibility since TnpA^{WT} was shown to form the PEC on the geometrically favorable two-end substrate (Figure 4B and Supplementary Figure S7B), albeit with lower efficiency than TnpA^{3X} and TnpA^{S911R}.

The metastability of SEC and the implication of a putative activation mechanism to stabilize the PEC should also provide a means to counteract a process known as ‘assembly site occlusion’ (ASO) that is proposed to act as an autoregulation mechanism to limit the activity of transposons, mostly from eukaryotes, which like Tn4430 transpose via the S-NEC pathway (44). In this mechanism, termed ‘overproduction inhibition’ (OPI), increasing expression of the transposase dimer resulting from the genomic expansion of the element gradually saturate the transposon ends, thereby creating a barrier for PEC assembly. There is currently no evidence for an overproduction regulatory mechanism acting on Tn4430 transposition *in vivo*. *In vitro*, the PEC assembled by the hyperactive TnpA^{3X} and TnpA^{S911R} mutants was only shown to dissociate into SECs when a DNA substrate was incubated with over-saturating concentrations of the protein (10). We propose that under favorable conditions for active transposition complex assembly (e.g. in presence of an appropriate target), stabilization of the PEC would displace the doubly bound SEC₂ state, thereby pushing the reaction forward (Figure 5). To validate this model, our current work aims at characterizing which properties of the target are involved in integration site discrimination by Tn4430 and how they contribute to transposition activation at the functional and structural level.

DATA AVAILABILITY

All study data are included in the article and/or supporting information. The AFM data have been deposited in Figshare (<https://figshare.com/articles/figure/Fernandez.Source.Data.xlsx/20032232>). The SAXS data were deposited in the small-angle scattering biological data bank under accession code SASDMR5. The cryo-EM maps of the TnpA^{3X}-IR48 and TnpA^{WT} have been deposited with accession codes EMD-15213 and EMD-15218.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We thank Gopinath Muriganandam for his help with SAXS data collection. The authors are grateful for beam time and excellent beamline support at SWING beamline of SOLEIL Synchrotron.

Author contributions: M.F., B.H. and D.A. conceived the project, planned the experiments, and analyzed the data. M.F. conducted the AFM experiments. C.S. and S.D. provided technical support in protein purification and AFM manipulation, respectively. A.V.S., R.G.E. and Y.L. conducted and analyzed the SAXS and Cryo-EM experiments. M.F., B.H. and, D.A. wrote the draft manuscript. All authors read and approved the manuscript.

FUNDING

Université catholique de Louvain; Fonds National de la Recherche Scientifique (F.R.S-FNRS) [PDR T.0070.16 to D.A., CDR J.0096.20 to B.H.]; Fonds Wetenschappelijk Onderzoek (FWO) [G.0266.15N to R.G.E.]; M.F. and D.A. are postdoctoral fellow and Research Associate at the FNRS, respectively. Funding for open access charge: FNRS.

Conflict of interest statement. None declared.

REFERENCES

1. Craig, N. (2015) A moveable feast: an introduction to mobile DNA. In: *Mobile DNA III*. pp. 3–39.
2. Hickman, A.B. and Dyda, F. (2016) DNA transposition at work. *Chem. Rev.*, **116**, 12758–12784.
3. Aziz, R.K., Breitbart, M. and Edwards, R.A. (2010) Transposases are the most abundant, most ubiquitous genes in nature. *Nucleic Acids Res.*, **38**, 4207–4217.
4. Wells, J.N. and Feschotte, C. (2020) A field guide to eukaryotic transposable elements. *Annu. Rev. Genet.*, **54**, 539–561.
5. Partridge, S.R., Kwong, S.M., Firth, N. and Jensen, S.O. (2018) Mobile genetic elements associated with antimicrobial resistance. *Clin. Microbiol. Rev.*, **31**, 1–61.
6. Brandt, C., Viehweger, A., Singh, A., Pletz, M.W., Wibberg, D., Kalinowski, J., Lerch, S., Müller, B. and Makarewicz, O. (2019) Assessing genetic diversity and similarity of 435 KPC-carrying plasmids. *Sci. Rep.*, **9**, 11223.
7. Lambin, M., Nicolas, E., Oger, C.A., Nguyen, N., Prozzi, D. and Hallet, B. (2012) Separate structural and functional domains of Tn4430 transposase contribute to target immunity. *Mol. Microbiol.*, **83**, 805–820.
8. Majorek, K.A., Dunin-Horkawicz, S., Steczkiewicz, K., Muszewska, A., Nowotny, M., Ginalski, K. and Bujnicki, J.M. (2014) The RNase H-like superfamily: new members, comparative structural

- analysis and evolutionary classification. *Nucleic Acids Res.*, **42**, 4160–4179.
9. Nicolas, E., Lambin, M., Dandoy, D., Galloy, C., Nguyen, N., Oger, C.A. and Hallet, B. (2015) The Tn3 family of replicative transposons. *Microbiol. Spectr.*, **3**, <https://doi.org/10.1128/microbiolspec.MDNA3-0060-2014>.
 10. Nicolas, E., Oger, C.A., Nguyen, N., Lambin, M., Draime, A., Leterme, S.C., Chandler, M. and Hallet, B.F.J. (2017) Unlocking Tn3-family transposase activity in vitro unveils an asymmetric pathway for transposome assembly. *Proc. Natl. Acad. Sci. U.S.A.*, **114**, 669–678.
 11. Guynet, C., Nicolas, E., Ton-Hoang, B., Bouet, J. and Hallet, B. (2020) First biochemical steps on bacterial transposition pathways. *Methods Mol. Biol.*, **2075**, 157–177.
 12. Nicolas, E., Lambin, M. and Hallet, B. (2010) Target immunity of the Tn3-family transposon Tn4430 requires specific interactions between the transposase and the terminal inverted repeats of the transposon. *J. Bacteriol.*, **192**, 4233–4238.
 13. Shkumatov, A.V., Aryanpour, N., Oger, C.A., Goossens, G., Hallet, B.F. and Efremov, R.G. (2021) Structural insight into Tn3 family transposition mechanism. *Nat. Commun.*, **13**, 6155.
 14. Müller, D.J., Dumitru, A.C., Lo Giudice, C., Gaub, H.E., Hinterdorfer, P., Hummer, G., De Yoreo, J.J., Dufrêne, Y.F. and Alsteens, D. (2021) Atomic force microscopy-based force spectroscopy and multiparametric imaging of biomolecular and cellular systems. *Chem. Rev.*, **121**, 11701–11725.
 15. Efremov, R.G. and Stroobants, A. (2021) Coma-corrected rapid single-particle cryo-EM data collection on the CRYO ARM 300. *Acta Crystallogr. Sect. D Struct. Biol.*, **77**, 555–564.
 16. Mastronarde, D.N. (2005) Automated electron microscope tomography using robust prediction of specimen movements. *J. Struct. Biol.*, **152**, 36–51.
 17. Zivanov, J., Nakane, T., Forsberg, B.O., Kimanius, D., Hagen, W.J.H., Lindahl, E. and Scheres, S.H.W. (2018) New tools for automated high-resolution cryo-EM structure determination in RELION-3. *Elife*, **7**, e42166.
 18. Zheng, S.Q., Palovcak, E., Armache, J.-P., Verba, K.A., Cheng, Y. and Agard, D.A. (2017) MotionCor2 - anisotropic correction of beam-induced motion for improved cryo-electron microscopy. *Nat. Methods*, **14**, 331–332.
 19. Rohou, A. and Grigorieff, N. (2015) CTFFIND4: fast and accurate defocus estimation from electron micrographs. *J. Struct. Biol.*, **192**, 216–221.
 20. Punjani, A., Rubinstein, J.L., Fleet, D.J. and Brubaker, M.A. (2017) CryoSPARC: algorithms for rapid unsupervised cryo-EM structure determination. *Nat. Methods*, **14**, 290–296.
 21. Pfreundschuh, M., Alsteens, D., Wieneke, R., Zhang, C., Coughlin, S.R., Tampé, R., Kobilka, B.K. and Müller, D.J. (2015) Identifying and quantifying two ligand-binding sites while imaging native human membrane receptors by AFM. *Nat. Commun.*, **6**, 8857.
 22. Ebner, A., Wildling, L., Kamruzzahan, A.S.M., Rankl, C., Wruss, J., Hahn, C.D., Hölzl, M., Zhu, R., Kienberger, F., Blaas, D. *et al.* (2007) A new, simple method for linking of antibodies to atomic force microscopy tips. *Bioconjug. Chem.*, **18**, 1176–1184.
 23. Kolb, H.C., Finn, M.G. and Sharpless, K.B. (2001) Click chemistry: diverse chemical function from a few good reactions. *Angew. Chem. Int. Ed.*, **40**, 2004–2021.
 24. Butt, H.-J. and Jaschke, M. (1995) Calculation of thermal noise in atomic force microscopy. *Nanotechnology*, **6**, 1.
 25. Bustamante, C., Marko, J.F., Siggia, E.D. and Smith, S. (1994) Entropic elasticity of λ -phage DNA. *Science*, **265**, 1599–1600.
 26. Rief, M., Gautel, M., Oesterhelt, F., Fernandez, J.M. and Gaub, H.E. (1997) Reversible unfolding of individual titin immunoglobulin domains by AFM. *Science*, **276**, 1109–1112.
 27. Evans, E. and Ritchie, K. (1997) Dynamic strength of molecular adhesion bonds. *Biophys. J.*, **72**, 1541–1555.
 28. Evans, E.A. and Calderwood, D.A. (2007) Forces and bond dynamics in cell adhesion. *Science*, **316**, 1148–1153.
 29. Evans, E. and Williams, P. (2002) Dynamic force spectroscopy. In: *Physics of Bio-molecules and Cells. Physique des biomolécules et des cellules* Ch. Springer, pp. 145–204.
 30. Rankl, C., Wildling, L., Neundlinger, I., Kienberger, F., Gruber, H., Blaas, D. and Hinterdorfer, P. (2011) Determination of the kinetic on- and off-rate of single virus–cell interactions. In: Braga, P. and Ricci, D. (eds). *Atomic Force Microscopy in Biomedical Research. Methods in Molecular Biology*. Vol. **736**, pp. 197–210.
 31. Rankl, C., Kienberger, F., Wildling, L., Wruss, J., Gruber, H.J., Blaas, D. and Hinterdorfer, P. (2008) Multiple receptors involved in human rhinovirus attachment to live cells. *Proc. Natl. Acad. Sci. U.S.A.*, **105**, 17778–17783.
 32. Svergun, D.I., Petoukhov, M.V., Koch, M.H.J. and König, S. (2000) Crystal versus solution structures of thiamine diphosphate-dependent enzymes. *J. Biol. Chem.*, **275**, 297–302.
 33. Lo Giudice, C., Dumitru, A.C. and Alsteens, D. (2019) Probing ligand-receptor bonds in physiologically relevant conditions using AFM. *Anal. Bioanal. Chem.*, **411**, 6549–6559.
 34. Merkel, R., Nassoy, P., Leung, A., Ritchie, K. and Evans, E. (1999) Energy landscapes of receptor–ligand bonds explored with dynamic force spectroscopy. *Nature*, **397**, 50–53.
 35. Alsteens, D., Pfreundschuh, M., Zhang, C., Spoerri, P.M., Coughlin, S.R., Kobilka, B.K. and Müller, D.J. (2015) Imaging G protein-coupled receptors while quantifying their ligand-binding free-energy landscape. *Nat. Methods*, **12**, 845–851.
 36. Alsteens, D., Newton, R., Schubert, R., Martinez-Martin, D., Delguste, M., Roska, B. and Müller, D.J. (2017) Nanomechanical mapping of first binding steps of a virus to animal cells. *Nat. Nanotechnol.*, **12**, 177–183.
 37. Kühner, F., Costa, L.T., Bisch, P.M., Thalhammer, S., Heckl, W.M. and Gaub, H.E. (2004) LexA–DNA bond strength by single molecule force spectroscopy. *Biophys. J.*, **87**, 2683–2690.
 38. Ros, R., Eckel, R., Bartels, F., Sischka, A., Baumgarth, B., Wilking, S.D., Pühler, A., Sewald, N., Becker, A. and Anselmetti, D. (2004) Single molecule force spectroscopy on ligand–DNA complexes: from molecular binding mechanisms to biosensor applications. *J. Biotechnol.*, **112**, 5–12.
 39. Montaño, S.P. and Rice, P.A. (2011) Moving DNA around: DNA transposition and retroviral integration. *Curr. Opin. Struct. Biol.*, **21**, 370–378.
 40. Chalmers, R.M. and Kleckner, N. (1994) Tn10/IS10 transposase purification, activation, and in vitro reaction. *J. Biol. Chem.*, **269**, 8029–8035.
 41. Mizuuchi, K. (1992) Transpositional recombination: mechanistic insights from studies of Mu and other elements. *Annu. Rev. Biochem.*, **61**, 1011–1051.
 42. Harshey, R.M. (2014) Transposable phage Mu Rasika. *Microbiol. Spectr.*, **2**, <https://doi.org/10.1128/microbiolspec.MDNA3-0007-2014>.
 43. Fuller, J.R. and Rice, P.A. (2017) Target DNA bending by the Mu transpososome promotes careful transposition and prevents its reversal. *Elife*, **6**, e21777.
 44. Bouaert, C.C., Lipkow, K., Andrews, S.S., Liu, D. and Chalmers, R. (2013) The autoregulation of a eukaryotic DNA transposon. *Elife*, **2**, e00668.
 45. Evans, E.A. and Ritchie, K. (1999) Strength of a weak bond connecting flexible polymer chains. *Biophys. J.*, **76**, 2439–2447.
 46. Grubmüller, H., Heymann, B. and Tavan, P. (1996) Ligand binding: molecular mechanics calculation of the streptavidin–biotin rupture force. *Science*, **271**, 997–999.
 47. Schwesinger, F., Ros, R., Strunz, T., Anselmetti, D., Güntherodt, H.J., Honegger, A., Jermutus, L., Tiefenauer, L. and Plückthun, A. (2000) Unbinding forces of single antibody–antigen complexes correlate with their thermal dissociation rates. *Proc. Natl. Acad. Sci. U.S.A.*, **97**, 9972–9977.