**Table S1.** Summary of three different types of DNA unzipping assays, all of which are at near base pair resolution. Related to STAR Methods.
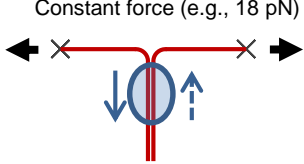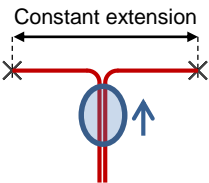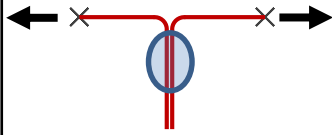
| | Applications | Method | Place used (with trapping mode) | References |
|---|---|---|---|---|
| **unzipping tracker** | Real-time tracking of DNA translocase without tagging | Constant force (e.g., 18 pN) | Fig.1, Fig. S1B (single trap) | This study |
| **unzipping staller** | Real-time stalling of DNA translocase without tagging | Constant extension | Fig. S6 (single trap) <br><br> Fig. 4, Fig. S4A, Fig. 5, Fig. S5A, Fig. S6 (dual trap) | This study |
| **unzipping mapper** | Mapping both locations and strengths of interactions within a DNA-protein complex | Constant loading rate (e.g., 8-16 pN/s) | Figs. 2B-D, Fig. 3, Fig. S2C, Fig. S3, Fig. S4B, Fig. 6, Figs.S7A-B (single trap) <br><br> Fig. 2A, Figs. S2A-B & S2D (dual trap) | This study. Brennan et al., 2016 Li et al., 2015 Jin et al., 2010 Hall et al., 2009 |

**Table S2.** Summary of translocation properties of Mfd. Experiments were carried out under a constant unzipping force of 18 pN. Shown are means and SEMs with the total numbers of traces indicated in parentheses. Within measurement uncertainties, Mfd's mean distance, time, and speed are similar under 2 mM ATP and 1 mN NTPs when translocating either away from ("unzip") or towards ("rezip") the DNA fork. Related to Figures 1 and 2.

|  | mean distance (bp) | mean time (s) | mean speed (bp/s) |
|---|---|---|---|
| **2 mM ATP (unzip)** | 200 ± 37 (48) | 28.2 ± 5.5 (48) | 7.2 ± 0.4 (48) |
| **2 mM ATP (rezip)** | 247 ± 47 (53) | 44.5 ± 8.3 (53) | 5.8 ± 0.2 (53) |
| **1 mM NTPs (unzip)** | 223 ± 49 (25) | 38.4 ± 11.4 (25) | 7.3 ± 0.5 (25) |
| **1 mM NTPs (rezip)** | 218 ± 57 (29) | 39.2 ± 11.0 (29) | 6.5 ± 0.5 (29) |

**Table S3.** Oligonucleotides used in this study. All oligos were purchased from Integrated DNA Technologies (IDT). 'HEX' indicates the HEX fluorophore labeled at the 5'-end. Related to Key Resources Table and STAR Methods.

| | |
|---|---|
| pRL574_rfaQ ops_27bpafterA20_F | GGTAGCGTGCTTTTTTCCTCTAGAGTGCTTGGCGAAC |
| pRL574_rfaQ ops_27bpafterA20_F | GCCCCATGGATGAGGCTAATCCCCCGTGTCCCTCTC |
| pRL574_rfaQ ops_276afterA20_F | GGTAGCGTGCTTTTTTCCGTCTTCTTTGACTCCGACAAAGG |
| pRL574_rfaQ ops_276afterA20_R | GCCCCATGGATGAGGCTACCGGACTACGGTGCAGCT |
| pRL574_F_2kb | GCTGGCGAAAGGGGGATGT |
| pRL574_R_2kb_AlwNI_CTA | GGAACAGCTACTGGGAGTCTCAAGGAAGCCGTATTCG |
| pRL574_F_1kb_inversed | TGGGGTCGAGGTGCCGTAAAG |
| pRL574_R_1kb_inversed_AlwNI | GGAACAGCTACTGCCAGGAACCACGGTAAGGGATG |
| pRL574_ops_275aA20_4kb_F_DraIII | GCAACACCTAGTGATGTGCTGCAAGGCGATTA |
| pRL574_ops_275aA20_4kb_R | AGACTTAACGGTGAGCATTTCCT |
| new_pRL574_F_DraIII | GACACCTAGTGAATTCGAGCTCGGTACC |
| new_P1_R_pRL574 | GGAAACAGCTATGACCATGATTAC |
| PRL574_F_AlwNI_CTA | CGCAGCTACTGTAAAACGACGGCCAGTGAAT |
| PRL574_R_800afterA20_BsaI | GATGGTCTCACGGTGATCCAGATCGTTGGTGAAC |
| Mfd_R685A_fwd | CGAAATGATCTCCGCTTTCCGCAGCGCCAAAG |
| Mfd_R685A_rev | CTTTGGCGCTGCGGAAAGCGGAGATCATTTCG |
| Mfd_N817A_fwd | GGACAGGTTTATTATCTCTACGCTGATGTGGAAAACATTCAG |
| Mfd_N817A_rev | CTGAATGTTTTCCACATCAGCGTAGAGATAATAAACCTGTCC |
| 40-mer DNA_oligo 1 | HEX- AATGTCAGGCCGGAATAACTTCCTATAATGCGCCACCACT |
| 40-mer DNA_oligo 2 | AGTGGTGGCGCATTATAGGAAGTTATTCCGGCCTGACATT |

**Table S4.** DNA unzipping templates used in this study. +1 indicates transcription start site (TSS). +20 indicates the last nucleotide incorporated into the transcript of a TEC paused at +20 position. Related to STAR Methods.

| Name | Place used | Description |
|---|---|---|
| 4.4 kb, co-directional | Figures 1, S1C, 2B-D, S3 | unzipping ↑↓  +1 ATCGAGAGGGACACGGGGGA +20  312 bp  Total length: 4434 bp |
| 1.2 kb, co-directional | Figures 2A, 6A-D, S7A-B | unzipping ↑↓  +1 ATCGAGAGGGACACGGGGGA +20  329 bp  Total length: 1180 bp |
| 1.1 kb, reversed | Figure 6A-B | +1 ATCGAGAGGGACACGGGGGA +20  380 bp  unzipping ↑↓  Total length: 1105 bp |
| 2.0 kb, reversed | Figures 4, 5, S4, S5A, S6 | +1 ATCGAGAGGGACACGGGGGA +20  1600 bp  unzipping ↑↓  Total length: 1980 bp |
| 4.1 kb, co-directional, *ops* 276 bp after +20 | Figures 3C, 3E, 3F | unzipping ↑↓  +1 ATCGAGAGGGACACGGGGGA +20  389 bp  276 bp  *rfaQ ops*: GGCGGTAGCG<u>T</u>  Total length: 4118 bp |
| 1.2 kb, co-directional, *ops* 27 bp after +20 | Figures 3B, 3D, 3F | unzipping ↑↓  +1 ATCGAGAGGGACACGGGGGA +20  329 bp  27 bp  *rfaQ ops*: GGCGGTAGCG<u>T</u>  Total length: 1214 bp |