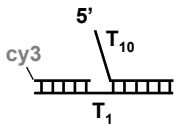
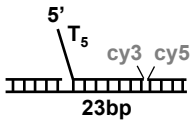
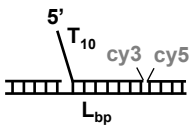
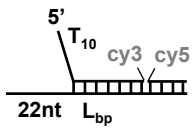
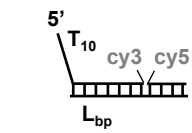
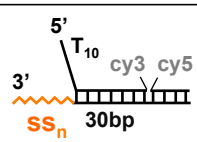
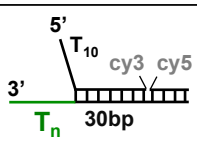
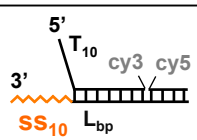
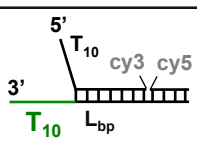
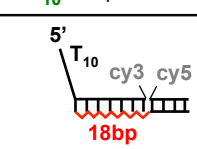
**Table S1.** Sequences (5'-3') of the oligonucleotides used in this work.

| | |
|---|---|
| P | CCGCCGCGGAACTTATTAGTG |
| $T_1$ | GTGACGGTGTGTGGGTGTGAATCTCACTAATAAGTTCCGCGGCGG |
| $D_{18}$ | $T_{10}$GATTCACACCCACACACC |
| $D_{23}$ | $T_{10}$GATTCACACCCACACACCGTCAC |
| $D_{30}$ | $T_{10}$GATTCACACCCACACACCGTCACCATCGAC |
| $D_{40}$ | $T_{10}$GATTCACACCCACACACCGTCACCATCGACTCAAGCAATC |
| $T_2$ | TGGCGACGGCAGCGAGGCGTGACACACCCACACACCGAATCTCACTAATAAGTTCCGCGGCGG |
| $C_{23}$* | $T_5$GATTCGGTGTGTGGGTGTGTCAC-Cy3 |
| $H_{18}$ | GCCTCGCTGCCGTCGCCA |
| $H_{18}$* | CY5-GCCTCGCTGCCGTCGCCA |
| $D_{18}$* | $T_{10}$GATTCACACCCACACACC-Cy3 |
| $D_{23}$* | $T_{10}$GATTCACACCCACACACCGTCAC-Cy3 |
| $D_{30}$* | $T_{10}$GATTCACACCCACACACCGTCACCATCGAC-Cy3 |
| $D_{40}$* | $T_{10}$GATTCACACCCACACACCGTCACCATCGACTCAAGCAATC-Cy3 |
| $S_{22}B_{18}H_{18}$ | TGGCGACGGCAGCGAGGCGGTGTGTGGGTGTGAATCTCACTAATAAGTTCCGCGGCGG |
| $S_{22}B_{23}H_{18}$ | TGGCGACGGCAGCGAGGCGTGACGGTGTGTGGGTGTGAATCTCACTAATAAGTTCCGCGGCGG |
| $S_{22}B_{30}H_{18}$ | TGGCGACGGCAGCGAGGCGTCGATGGTGACGGTGTGTGGGTGTGAATCTCACTAATAAGTTCCGCGGCGG |
| $S_{22}B_{40}H_{18}$ | TGGCGACGGCAGCGAGGCGATTGCTTGAGTCGATGGTGACGGTGTGTGGGTGTGAATCTCACTAATAAGTTCCGCGGCGG |
| $B_{18}H_{18}$ | TGGCGACGGCAGCGAGGCGGTGTGTGGGTGTGAATC |
| $B_{23}H_{18}$ | TGGCGACGGCAGCGAGGCGTGACGGTGTGTGGGTGTGAATC |
| $B_{30}H_{18}$ | TGGCGACGGCAGCGAGGCGTCGATGGTGACGGTGTGTGGGTGTGAATC |
| $B_{40}H_{18}$ | TGGCGACGGCAGCGAGGCGATTGCTTGAGTCGATGGTGACGGTGTGTGGGTGTGAATC |
| $T_{22}B_{30}H_{18}$ | TGGCGACGGCAGCGAGGCGTCGATGGTGACGGTGTGTGGGTGTGAATCT$_{22}$ |
| $T_{10}B_{30}H_{18}$ | TGGCGACGGCAGCGAGGCGTCGATGGTGACGGTGTGTGGGTGTGAATCT$_{10}$ |
| $T_{10}B_{23}H_{18}$ | TGGCGACGGCAGCGAGGCGTGACGGTGTGTGGGTGTGAATCT$_{10}$ |
| $T_5B_{30}H_{18}$ | TGGCGACGGCAGCGAGGCGTCGATGGTGACGGTGTGTGGGTGTGAATCT$_5$ |
| $S_{16}B_{30}H_{18}$ | TGGCGACGGCAGCGAGGCGTCGATGGTGACGGTGTGTGGGTGTGAATCTCACTAATAAGTTCCG |
| $S_{10}B_{30}H_{18}$ | TGGCGACGGCAGCGAGGCGTCGATGGTGACGGTGTGTGGGTGTGAATCTCACTAATAA |
| $S_{10}B_{23}H_{18}$ | TGGCGACGGCAGCGAGGCGTGACGGTGTGTGGGTGTGAATCTCACTAATAA |
| $S_5B_{30}H_{18}$ | TGGCGACGGCAGCGAGGCGTCGATGGTGACGGTGTGTGGGTGTGAATCTCACT |
| $R_{18}H_{18}$ | TGGCGACGGCAGCGAGGCrGrGrUrGrUrGrUrGrGrGrUrGrUrGrArArUrC |

**Table S2.** Substrates used in this work.

| | Oligonucleotides (Table S1) | Figure |
|---|---|---|
|  | P + $T_1$ + $D_{23}$ | 1, 2a |
|  | P + $T_2$ + $C_{23}$* + $H_{18}$* | 2c |
|  | **$L_{18}$**: P + $S_{22}B_{18}H_{18}$ + $D_{18}$* + $H_{18}$*<br>**$L_{23}$**: P + $S_{22}B_{23}H_{18}$ + $D_{23}$* + $H_{18}$*<br>**$L_{30}$**: P + $S_{22}B_{30}H_{18}$ + $D_{30}$* + $H_{18}$*<br>**$L_{40}$**: P + $S_{22}B_{40}H_{18}$ + $D_{40}$* + $H_{18}$* | S2, 3 |
|  | **$L_{18}$**: $S_{22}B_{18}H_{18}$ + $D_{18}$* + $H_{18}$*<br>**$L_{23}$**: $S_{22}B_{23}H_{18}$ + $D_{23}$* + $H_{18}$*<br>**$L_{30}$**: $S_{22}B_{30}H_{18}$ + $D_{30}$* + $H_{18}$*<br>**$L_{40}$**: $S_{22}B_{40}H_{18}$ + $D_{40}$* + $H_{18}$* | 2d, 3, 4, 6b, S3, S4, S6 |
|  | **$L_{18}$**: $B_{18}H_{18}$ + $D_{18}$* + $H_{18}$*<br>**$L_{23}$**: $B_{23}H_{18}$ + $D_{23}$* + $H_{18}$*<br>**$L_{30}$**: $B_{30}H_{18}$ + $D_{30}$* + $H_{18}$*<br>**$L_{40}$**: $B_{40}H_{18}$ + $D_{40}$* + $H_{18}$* | 3, 7b, S7 |
|  | **$SS_{16}$**: $S_{16}B_{30}H_{18}$ + $D_{30}$* + $H_{18}$*<br>**$SS_{10}$**: $S_{10}B_{30}H_{18}$ + $D_{30}$* + $H_{18}$*<br>**$SS_5$**: $S_5B_{30}H_{18}$ + $D_{30}$* + $H_{18}$* | 5, 6a, 7a, S3, S4b |
|  | **$T_{22}$**: $T_{22}B_{30}H_{18}$ + $D_{30}$* + $H_{18}$*<br>**$T_{10}$**: $T_{10}B_{30}H_{18}$ + $D_{30}$* + $H_{18}$*<br>**$T_5$**: $T_5B_{30}H_{18}$ + $D_{30}$* + $H_{18}$* | 5, 6a, 7a, S3a, S4b |
|  | **$L_{23}$**: $S_{10}B_{23}H_{18}$ + $D_{23}$* + $H_{18}$*<br>**$L_{30}$**: $S_{10}B_{30}H_{18}$ + $D_{30}$* + $H_{18}$* | 5b |
|  | **$L_{23}$**: $T_{10}B_{23}H_{18}$ + $D_{23}$* + $H_{18}$*<br>**$L_{30}$**: $T_{10}B_{30}H_{18}$ + $D_{30}$* + $H_{18}$* | 5b, S4b |
|  | $R_{18}H_{18}$ + $D_{18}$* + $H_{18}$* | S7 |

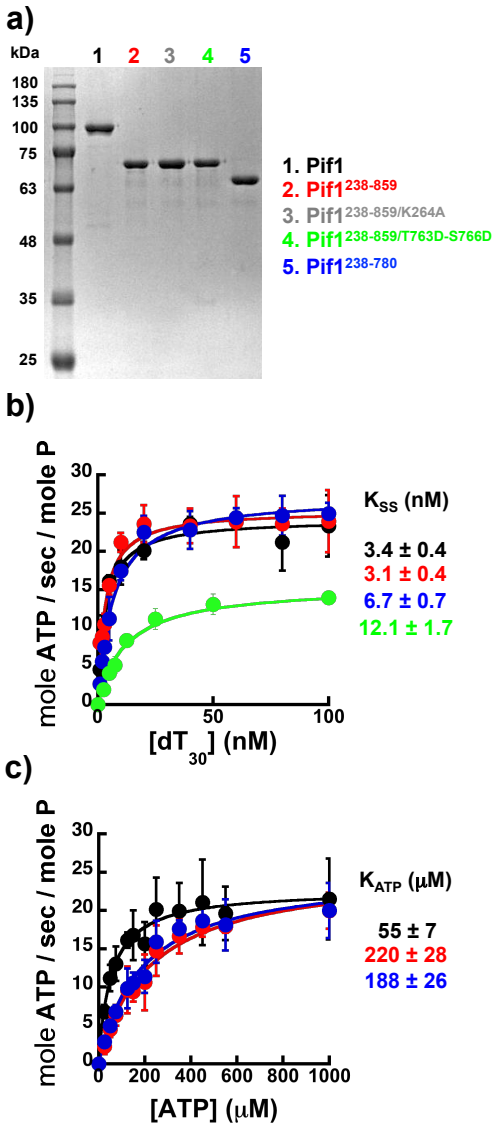



**Figure S1. a)** SDS-PAGE of the different Pif1 constructs stained with Coomassie. **b,c)** ATPase activity of the different Pif1 constructs as function of ssDNA or ATP concentration using an NADH-coupled assay. The experimental conditions used are the same as the ones used to monitor unwinding activity (see main text).

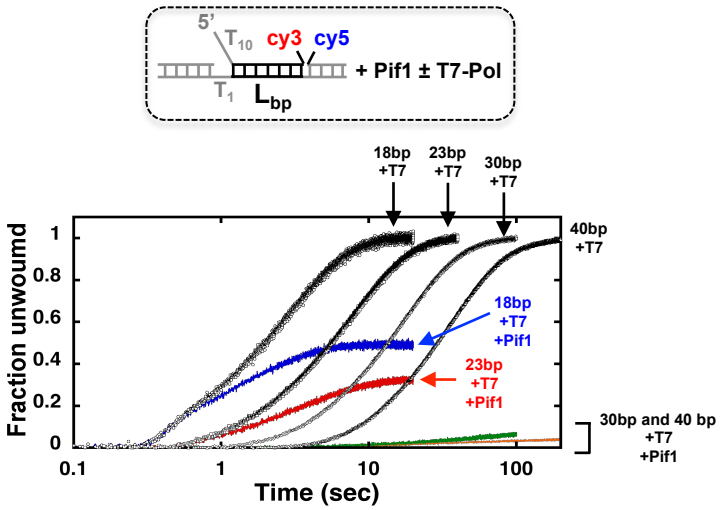



**Figure S2.** Stopped-flow FRET-based assay with DNA containing 5'-d$T_{10}$ flap and different lengths of the dsDNA region to be strand displaced/unwound. The reactions were started by mixing 100 mM dNTPs and pre-formed complexes of 20 nM DNA with 15 nM Pif1 in the absence or presence of 20 nM T7-Pol (all concentrations are after mixing).

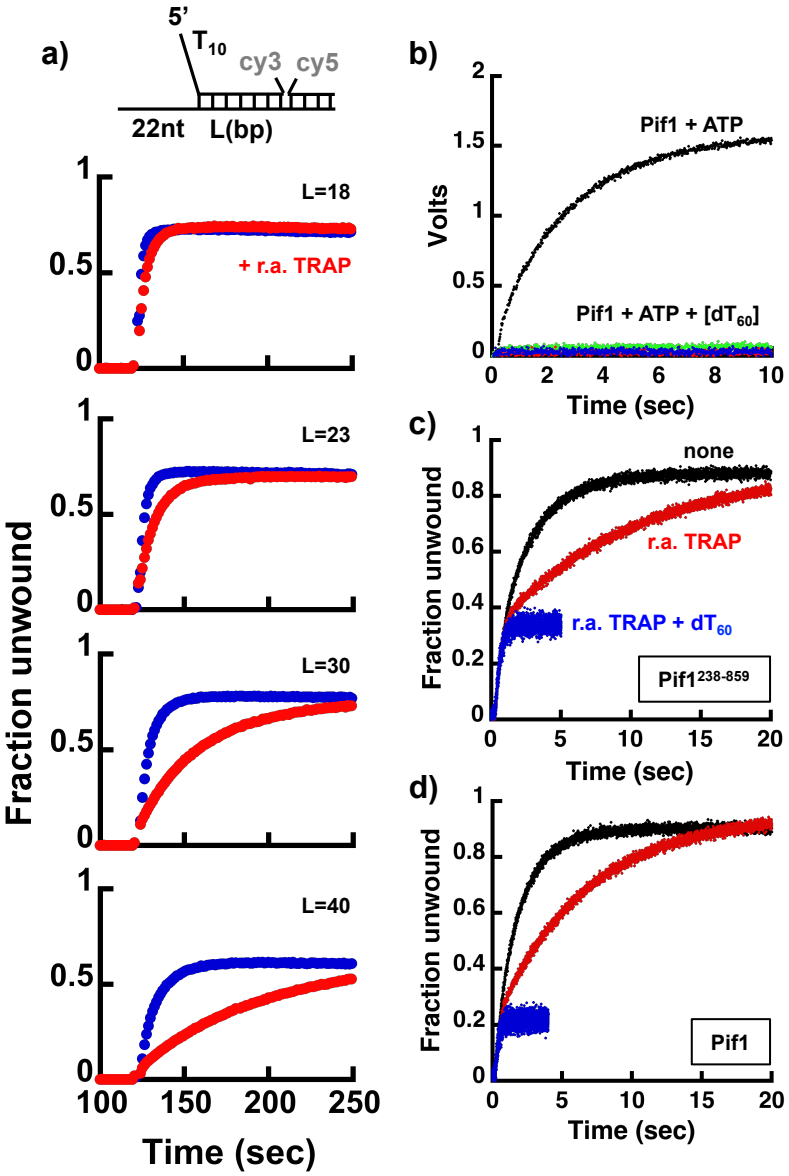



**Figure S3. a)** Same experiments as in Figure 3, panels 5-8 (see main text) but performed with full-length Pif1. **b)** Efficacy of $dT_{60}$ as a protein trap. Stopped-flow FRET-based unwinding experiments were performed by mixing 15nM Pif1 either with 0.5 ATP or with 0.5mM ATP and 100-1000nM of $dT_{60}$. **c,d)** Expanded time scale for the data shown in Figure 4c and 4f in the main text.

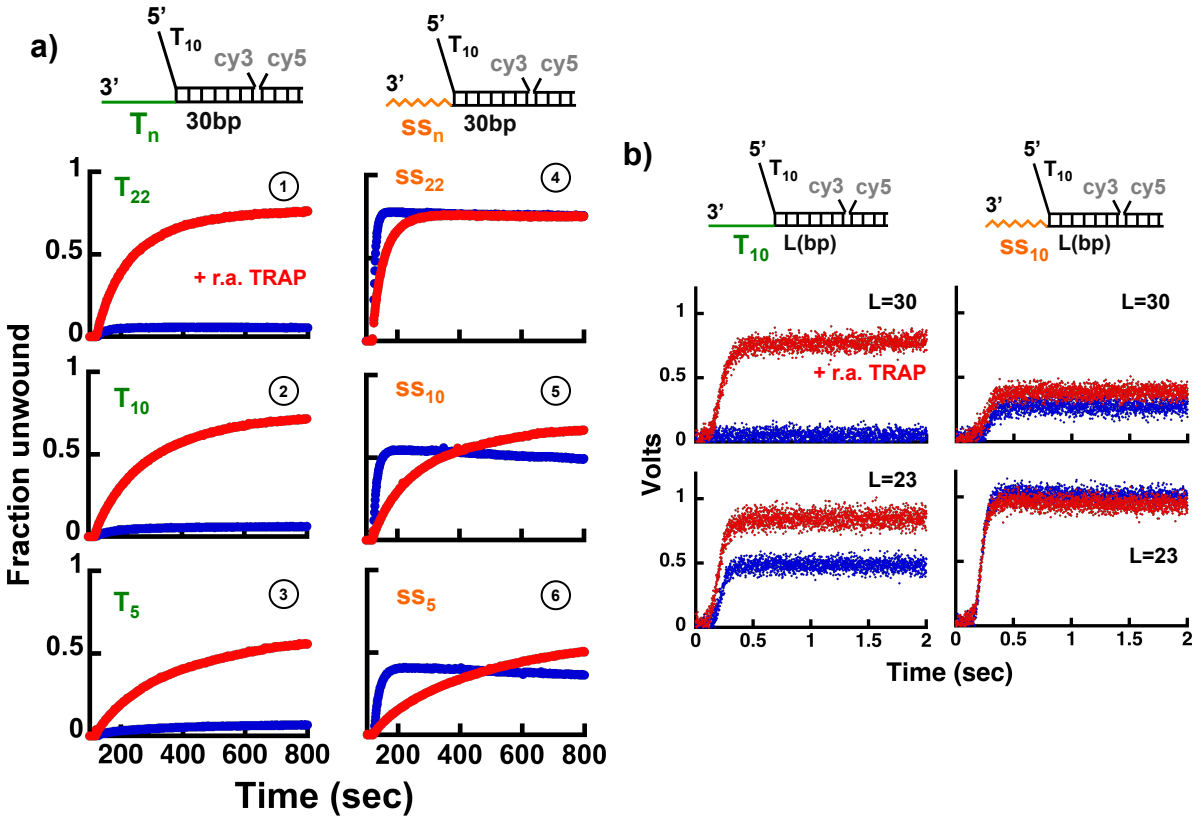



**Figure S4. a)** Same experiments as in Figure 5a in the main text but using full-length Pif1. **b)** Experiments as in Figure 5b in the main text performed with full-length Pif1.

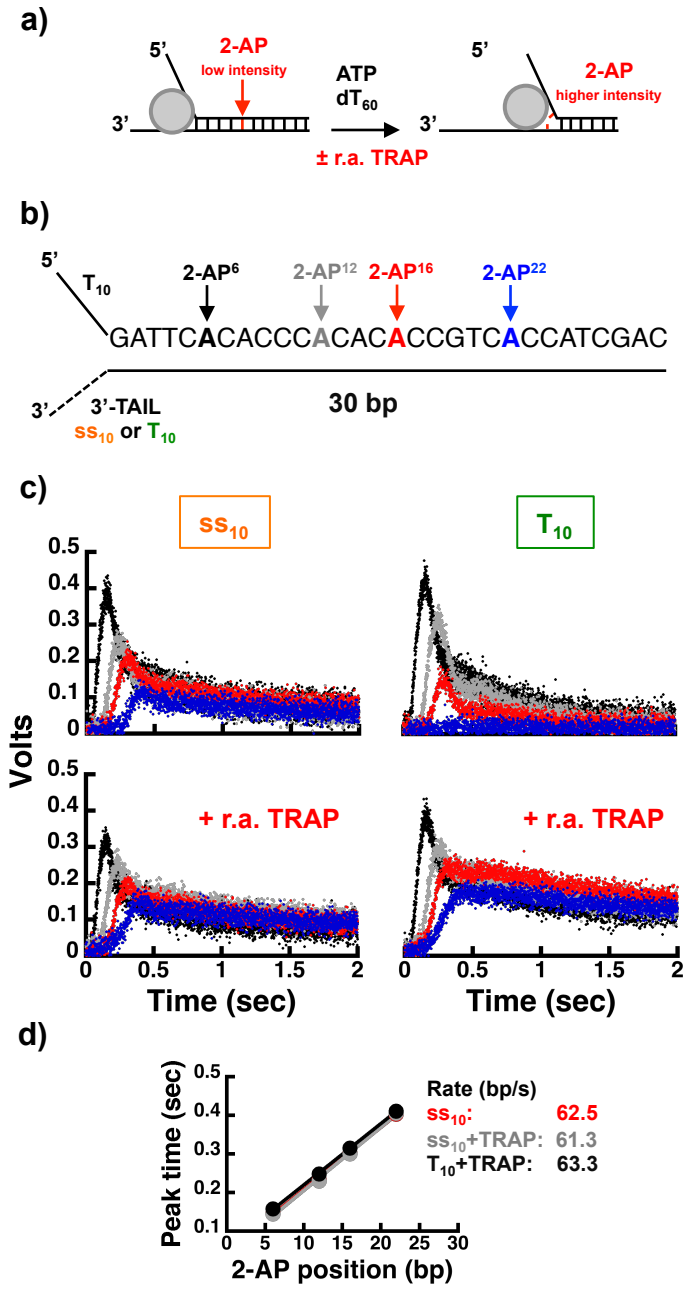



**Figure S5. a)** Schematic of the experiment to monitor opening of a base-pair carrying a 2-aminopurine modified base. **b)** Strands carrying the 2-AP modification at either the 6$^{th}$, 12$^{th}$, 16$^{th}$ or 22$^{nd}$ position were annealed to the complementary strand containing a T10 or a 10 nt 3'tail. **c)** Single turnover experiments with the different 2-AP modified substrates (color coded according to b)) were performed in the absence or presence of the trap to prevent re-annealing. **d)** The rate of unwinding calculated from the peak time in the 2-AP signal is similar to the one determined in Figure 4.

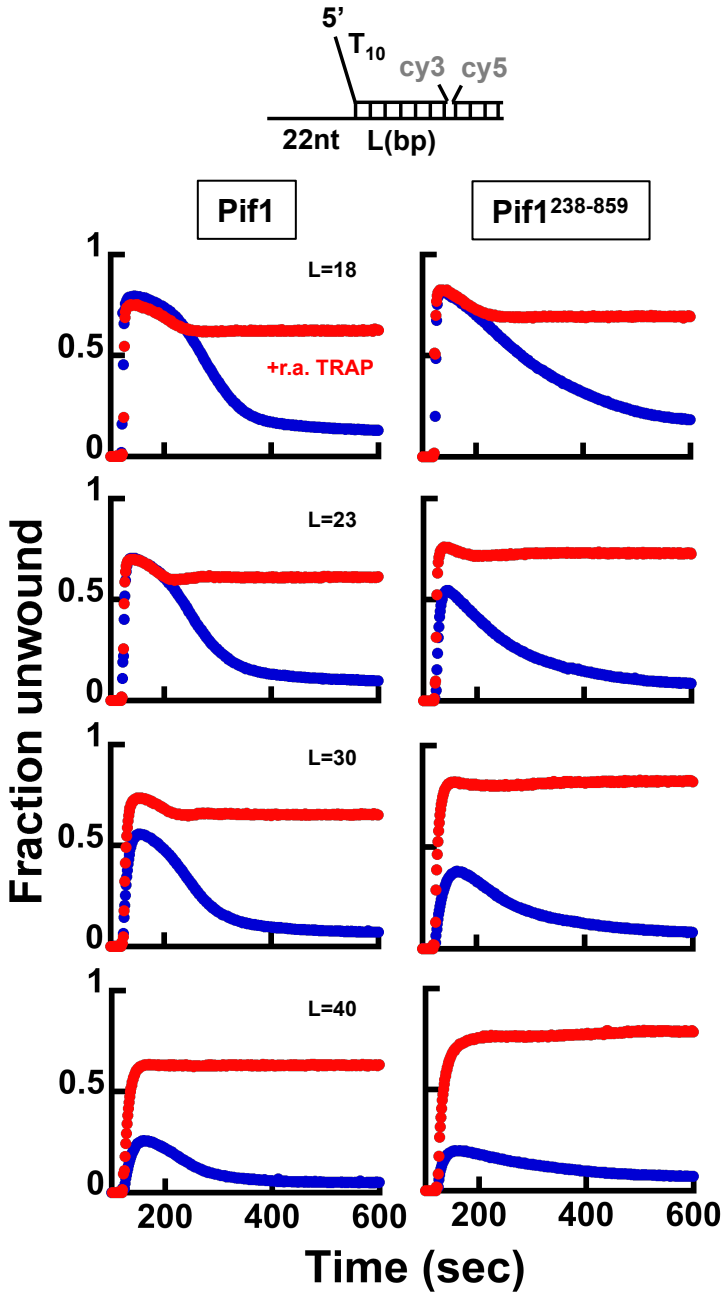



**Figure S6.** Multiple-turnover FRET-based unwinding experiments using 20nM of the indicate substrates and 200nM of either full-length Pif1 (left panels) or Pif1$^{238-859}$ (right panels). The reactions were started as discussed in the main text.

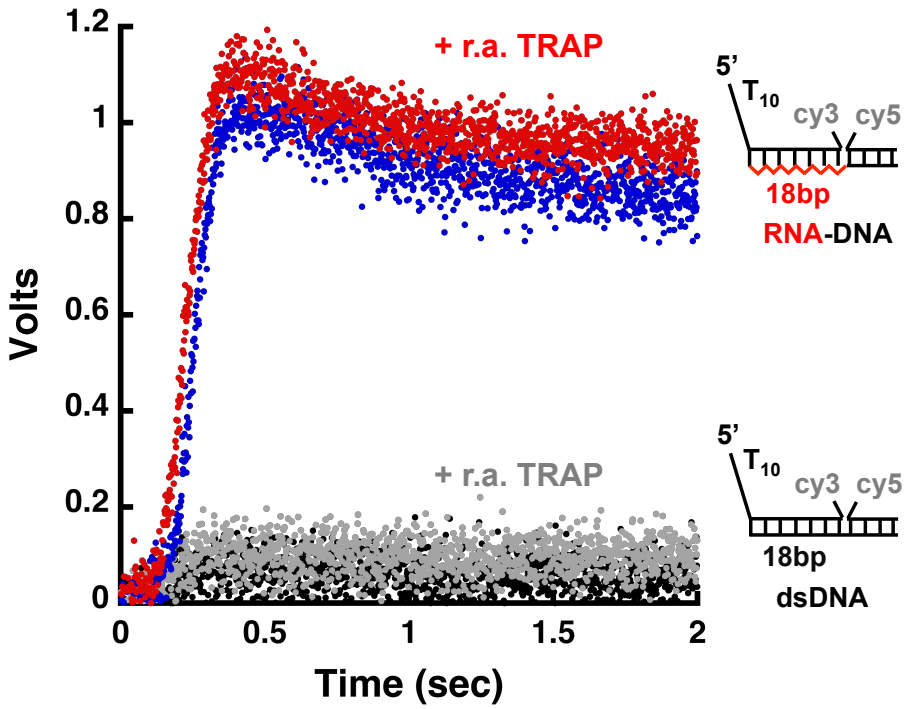



**Figure S7.** Stopped-flow FRET-based unwinding experiments under single-turnover conditions using 20nM of the indicate substrates and 15nM of Pif1$^{238-859}$. The reactions were started by addition of either 0.5mM ATP + 0.5mM $dT_{60}$ (blue or black) or 0.5mM ATP + 0.5mM dT60 + 3.5x of the r.a. TRAP (red or gray).