SUPPORTING INFORMATION

Direct Correlation of DNA Binding and Single Protein Domain Motion via Dual Illumination Fluorescence Microscopy

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Supplemental Methods

Evaluation of the labeling efficiency of XPD helicase:

We developed a single-molecule assay to estimate the XPD labeling efficiency, which tolerates the presence of trace amounts of unincorporated Cy3–Hydrazide dye (Figure S1a-c). The assay monitors the 5'→3' translocation of XPD on ssDNA via FeS-mediated quenching of a Cy5 fluorescent dye incorporated at the 3'-end of an oligonucleotide[1, 2]. Cy3-labeled XPD was immobilized on surface of the microscope slide as described in the main methods section, and freely diffusing Cy5-labeled ssDNA molecules were flown in the imaging chamber in buffer containing 2 mM ATP, 50 mM Tris-HCl (pH 7.5), 2 mM DTT and 3 mM $MgCl_2$, oxygen scavenging system and Trolox. After few minutes of fluorescence imaging with green (532 nm) laser excitation to pinpoint labeled XPD molecules, the excitation was switched to red (640 nm) laser to monitor translocation of immobilized XPD molecules on the freely diffusing Cy5-labeled ssDNA. Fluorescence intensity time trajectories were classified into three categories: (1) trajectories showing Cy3-labeled XPD molecules during green laser excitation and translocation events associated with appearance and gradual quenching of Cy5 signal during the second phase of the experiment (molecules in this group were classified as "labeled and active"); (2) trajectories showing Cy3-labeled XPD molecules, but no translocation events during the observation window ("labeled but inactive" group); and (3) trajectories showing only Cy5-associated translocation events, but no Cy3 signal (this group contains unlabeled XPD molecules that we classify as "active but unlabeled"). It is important to note that molecules which are both unlabeled and inactive are not detected by this single-molecule assay. Upper limit of the labeling efficiency (P_{label}) can be calculated as follows:

$$P_{label} = \frac{N(1) + N(2)}{N(1) + N(2) + N(3)} * 100\% \ ,$$

where N(i) is the number of molecules of group "i". Although, P_{label} represents an upper limit of labeling efficiency, it closely approximates the actual value since the fraction of inactive XPD molecules is insignificant. This assumption is made because the wild type and Cy3-labeled XPD forms showed comparable helicase activity (Figure 2c) and from previous stoichiometric titrations we know that our purification scheme yields 100% active XPD [3, 4].

Exclusion of surface effects:

To rule out the possibility that surface-tethering may affect XPD activity, we carried out the control experiments whereby translocation of XPD is followed via a single-molecule fluorescence quenching assay established previously in our lab[1, 2]. We compared the translocation activity of freely diffusing wild type XPD helicase to that of the biotinylated, surface-tethered XPD. Both immobilized and freely diffusing forms of XPD were free from aldehyde-tag motif and any fluorescent label. In the case of the freely diffusing wild type helicase, a 5'-end biotinylated ssDNA substrate (labeled with Cy3 at the 3'-end) was immobilized on the microscope slide via biotin-neutravidin linkage (Figure S2a). In the case of biotinylated XPD tethered to the surface, an identical (except for the absence of the biotin moiety) Cy3-labeled ssDNA substrate was freely diffusing in the imaging buffer (Figure S2b). Length and sequence of the two Cy3-labeled ssDNA substrates were the same (Table S1). Concentration of both forms of XPD was 150 pM, and concentration of both forms of ssDNA was 100 pM. Translocation of XPD helicase on fluorescently labeled ssDNA was monitored by fluorescence imaging under green laser (532 nm) TIR illumination at data acquisition rate of 33 frames per second. Translocation activities of freely diffusing and immobilized forms of XPD were evaluated by comparing respective distributions of the durations of the translocation events (Figure S2c & d).

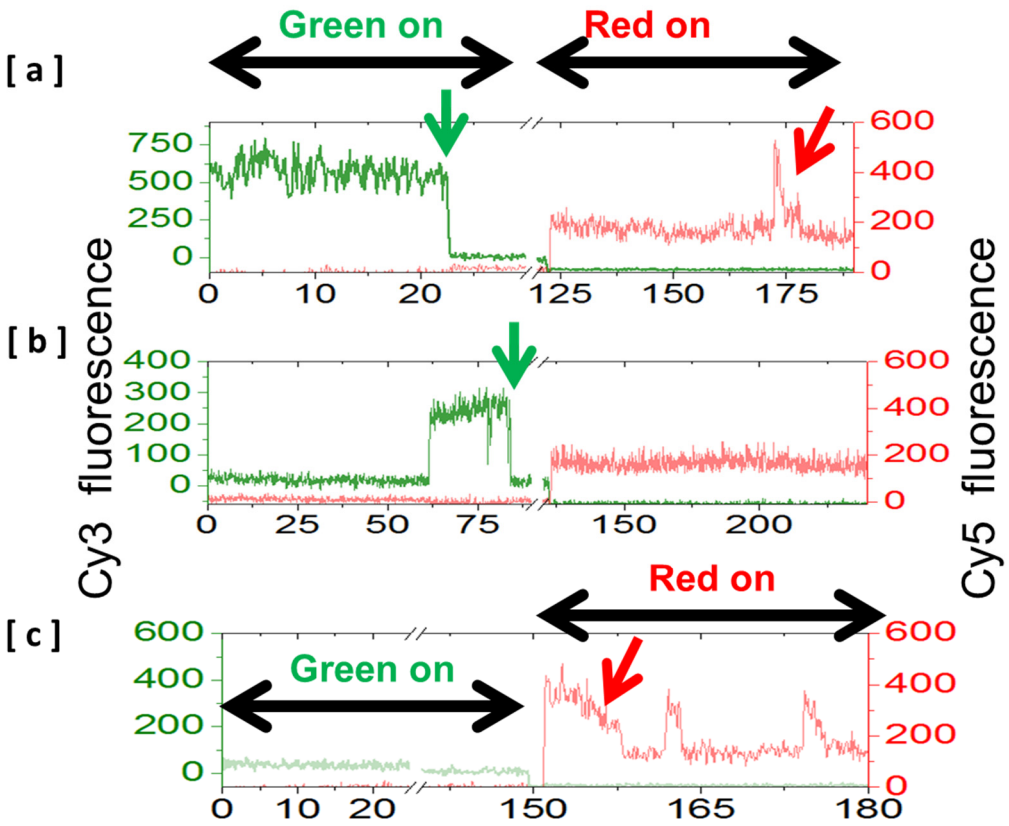

Figure S1. Labeling efficiency of Cy3-labeled XPD. Representative single-molecule fluorescence trajectories for the molecules in categories 1 (Figure S1-a), 2 (Figure S1-b) and 3 (Figure S1-c) observed in the FeS-mediated fluorescence quenching single-molecule translocation assay (see Supplemental methods). In all of the traces shown, green laser was switched on for sorting out labeled and unlabeled XPD molecules, then switched off before switching on the red laser to monitor translocation events via FeS cluster-mediated quenching of the Cy5 fluorescent dye attached at the 3'-end of a 37-nt long ssDNA. In all trajectories, green arrow indicates the irreversible photobleaching of Cy3 and red arrow indicates binding of an ssDNA and start of a translocation event observed as gradual quenching of Cy5 fluorescence. Data acquisition time was 100 ms per frame. Green traces are from Cy3 (attached to ARCH domain of XPD) and red traces are from Cy5 (attached at 3'-end of ssDNA).

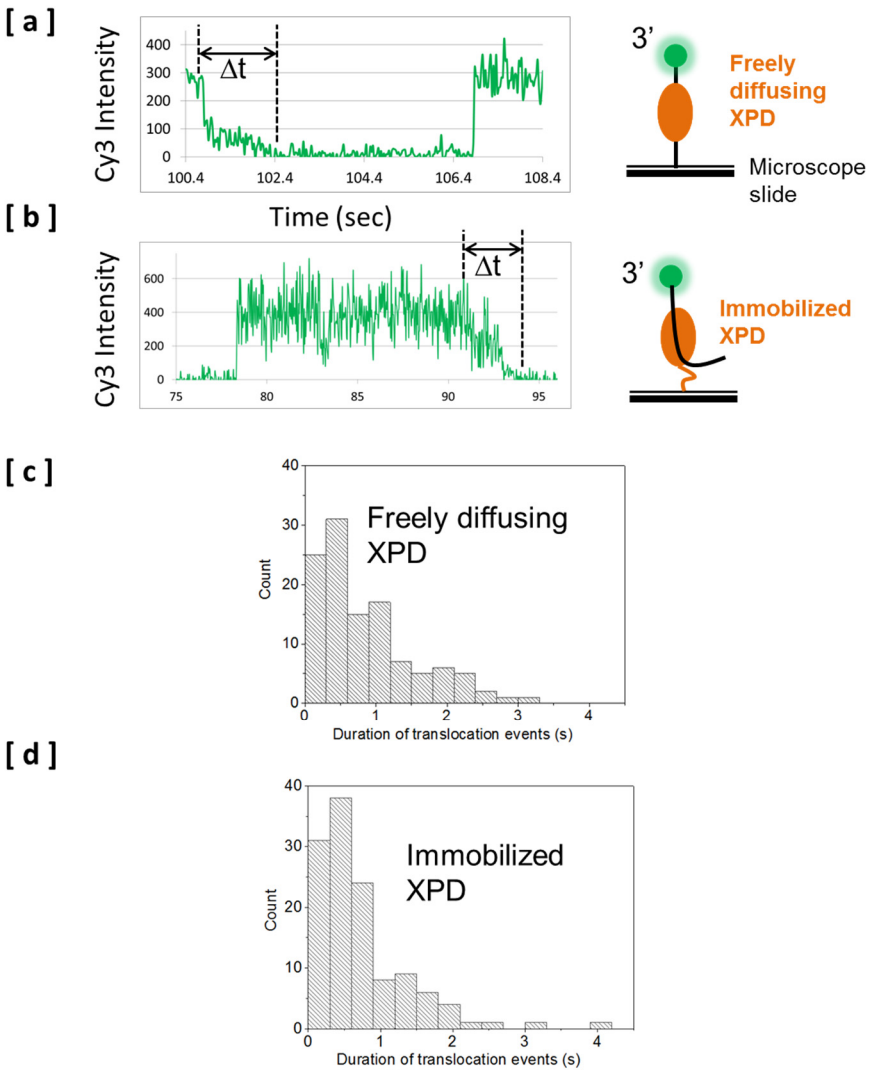

Figure S2. Surface tethering has no effect on XPD translocation activity. (a & b) Representative single-molecule translocation events of freely diffusing and immobilized XPD molecules. (a) In case of freely diffusing form of XPD, we immobilized ssDNA (42-mer) labeled with Cy3 at the 3'-end. XPD translocation along ssDNA resulted in gradual quenching of Cy3 intensity followed by its full recovery when XPD dissociated from the DNA. (b) In case of immobilized form of XPD, we used a freely diffusing Cy3-labeled ssDNA of the same length and sequence as the substrate used in 'a'. Binding of labeled ssDNA to XPD is associated with an abrupt increase in Cy3 fluorescence signal with the translocation event resulting in a gradual quenching of Cy3 intensity, until the signal decreases to baseline level indicating the dissociation of the labeled DNA from the unlabeled immobilized protein. "Δt" is the duration of each translocation event. (c & d) Distributions of durations of translocation events of freely diffusing and immobilized forms, respectively, of XPD helicase.

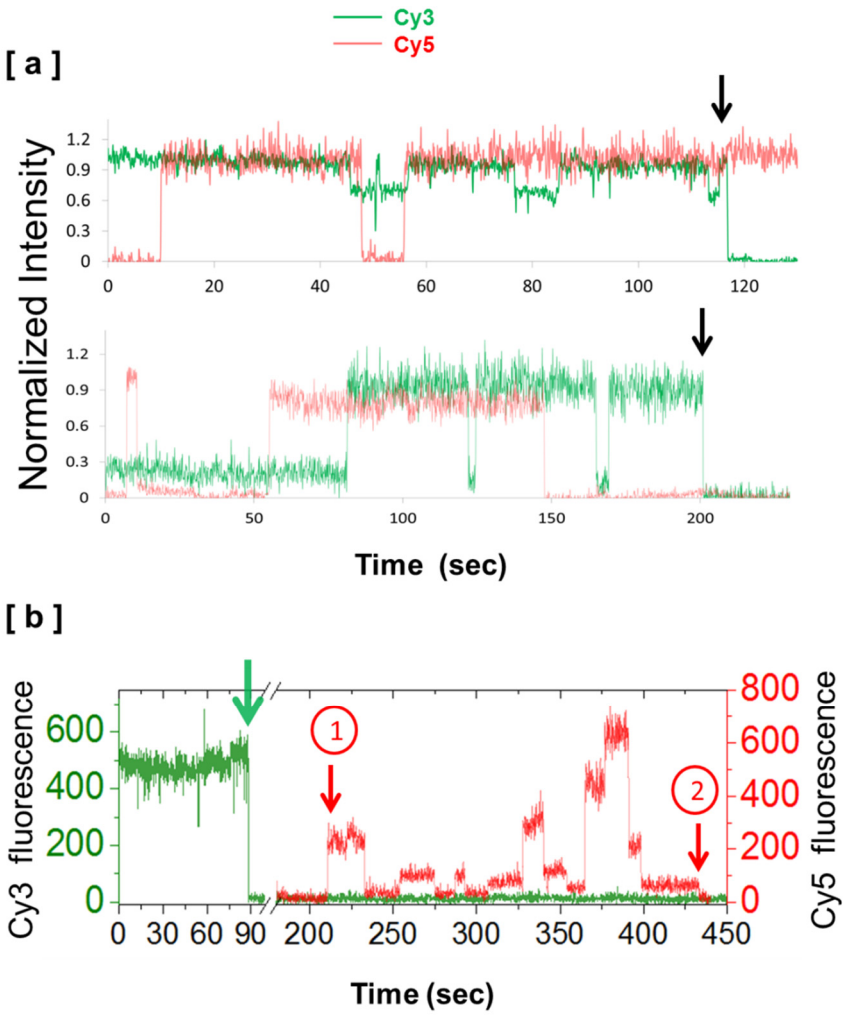

Figure S3. Representative single-molecule fluorescence intensity time trajectories from dual illumination experiment. (a) Representative trajectories showing the dominant type of binding events in the presence of undamaged DNA. The green trace (Cy3) shows an ARCH domain opening and closing and the red trace (Cy5) shows association and dissociation of individual undamaged bubble DNA molecules. Black arrow at the end of each Cy3 trajectory indicates the single irreversible photo-bleaching step. (b) Representative trajectory showing a less frequent 'complex' type of an individual binding event for the CPD-containing bubble DNA. The green arrow points to the irreversible photo-bleaching of Cy3 (green trace). The red arrows labeled with numbers "1" and "2" point to the moments of binding and dissociation, respectively, of the Cy5-labeled bubble DNA. Enhancement in Cy5 fluorescence signal indicates motion of XPD helicase away from the Cy5-labeled 5'-end of the DNA strand which does not have CPD (see Table S1). DNA concentration was 150 pM.

Table S1. Oligonucleotides used in this study.

OLIGONUCLEOTIDES	SEQUENCE (5' - 3')	ASSAY
FP1	5'- ATCAGGATCCGGGTCTTAATGATATTTTTGA - 3'	Cloning
RP1-A	5'- CTTCAATCCTATCATATGACCGCGATGGTGTGCACAGATTATAAGTT TCAATTGGAAAATCCTTTAC -3'	Cloning
FP2-A	5'- GTAAAGGATTTTCCAATTGAAACTTATAATCTGTGCACACCATCGCGG TCATATGATAGGATTGAAG - 3'	Cloning
RP2	5'- TGATCTCGAGTTATTTTGTGTTAGGAAGCAAATTGGATTG - 3'	Cloning
5'-20T-22ss-Cy3-3'	5' – TTTTTTTTTTTTTTTTTTTTGGAATTAAGCTCTAAGCCATCC-(Cy3) - 3'	Surface effect
5'-biotin-20T-22ss-Cy3-3'	5' – (biotin) -TTTTTTTTTTTTTTTTTTTTGGAATTAAGCTCTAAGCCATCC -(Cy3) - 3'	Surface effect
5'-20T-17-Cy5-3'	5' – TTTTTTTTTTTTTTTTTTTTGGAATTAAGCTCTAAGC- (Cy5) – 3	Helicase activity
5'-17ss-20T-3'	5' – GCTTAGAGCTTAATTCCTTTTTTTTTTTTTTTTTTT – 3'	Helicase activity
5'-20T-17-Cy5-3'	5' – TTTTTTTTTTTTTTTTTTTTGGAATTAAGCTCTAAGC- (Cy5) – 3	Labeling efficiency
Bubble-1-A	5'- TTT CCG AAT CTA CTG GTATCT AGG GTT TTT TTT TTT TTT TTT TTT TTG CGT ACATGG ATG GCT TAG AGC ATA -3'	Dual illumination
Cy5 - Bubble-1-B	5'- (Cy5) -TATGCTCTAAGCCATCCATGTACGCTTTTTTTTTTTTTTTTTTTT TTTTCCCTAGATACCAGTAGATTCGGAAA -3'	Dual illumination
TD - Bubble-1-A *	5' – TTTCCGAATCTACTGGTATCTAGGGTTTTTTTTTTT**X**TTTTTTTTTTTT GCGTACATGGATGGCTTAGAGCATA – 3'	Dual illumination

* The oligonucleotide which contains thymidine dimer (bold underlined **X**) at the center of the middle poly-dT region.

References:

(1) Honda, M.; Park, J.; Pugh, R. A.; Ha, T.; Spies, M. *Molecular cell* **2009,** 35, (5), 694-703.
(2) Pugh, R. A.; Honda, M.; Spies, M. *Methods* **2010,** 51, (3), 313-21.
(3) Pugh, R. A.; Honda, M.; Leesley, H.; Thomas, A.; Lin, Y.; Nilges, M. J.; Cann, I. K.; Spies, M. *The Journal of biological chemistry* **2008,** 283, (3), 1732-43.
(4) Pugh, R. A.; Lin, Y.; Eller, C.; Leesley, H.; Cann, I. K.; Spies, M. *Journal of molecular biology* **2008,** 383, (5), 982-98.