Supplementary Materials

UvrD double mutant protein expression, purification and labeling

UvrD double mutant proteins were overexpressed in *E. coli* BL21(DE3) ΔUvrD containing pGroESL (gift from C. Frieden, Washington University) in Terrific Broth (supplemented with Kanamycin (50 μg/ml), chloramphenicol (35 μg/ml) and tetracycline (12.5 μg/ml)). Terrific Broth (1 L) was inoculated with 15 mL of a fresh overnight culture (LB broth + 50 μg/ml Kan + 35 μg/ml Chl + 12.5 μg/ml Tet) grown from a single colony. Cells were grown at 37°C until OD_{600}~1.0, followed by a shift to 25°C and addition of IPTG to 0.2 mM to induce expression, followed by continued incubation for 3 hours at 25°C. The decrease in temperature was necessary to improve solubility of the double Cys mutant proteins (DM-1B/2B and DM-2A/2B) which have lower solubility than wtUvrD.

All purification steps were carried out at 4°C, essentially as described for wtUvrD,[43] but with the following modifications. The cleared supernatant of the lysate from original 40 g cell pellet was added to 20 ml of Ni-NTA agarose resin, equilibrated with Buffer A + 10 mM imidazole and packed in an open BioRad Econo-column (2.5 × 10 cm). The Ni-NTA agarose resin was incubated for about 1 hour with slow rocking from end to end at 4°C to maximize interaction of the 6xHis tagged proteins with the resin. The mixture was poured into a 50 ml plastic tube and centrifuged for 5 min at 2000 rpm to sediment the Ni-NTA agarose. The supernatant containing unbound protein was carefully poured off from the tube. 40 ml of Buffer A + 10 mM imidazole was added to the slurry and after gentle mixing, the resin was centrifuged

(5 min, 2000 rpm) and the supernatant again discarded. This washing step was repeated three times. After the last washing the resin was resuspended with Buffer A + 10 mM imidazole and packed into an Bio-rad Econo-column (2.5 × 10 cm) and washed with at least three column volumes of the same buffer, followed by washing to baseline with Buffer A + 25 mM imidazole. The column was eluted with a gradient from Buffer A + 40 mM imidazole to Buffer A + 200 mM imidazole (8 column volumes). Column fractions (8 ml**)** were analyzed by SDS polyacrylamide gels (12.5%) and the fractions containing the UvrD mutant proteins were pooled. The pooled fractions contained several contaminating proteins based on the polyacrylamide gel and thus the sample was subjected to further purification using a Heparin column and ds-DNA and ss-DNA cellulose columns as described for wtUvrD.[43] The final pooled protein fractions were concentrated with a Hitrap Q FF column (GE Health Care) and then dialyzed against storage buffer, flash frozen with liquid nitrogen and stored at -80°C. Protein concentration was determined spectrophotometrically in 20 mM Tris (pH 8.3 at 25 °C), 200 mM NaCl, and 20% (v/v) glycerol, using an extinction coefficient of $\varepsilon_{280} = 1.06 \times 10^{-5}$ $M^{-1}cm^{-1}$.[43] The final protein pool was judged to ~98% pure based on polyacrylamide gel electrophoresis. The final yield was about 10 mg from 40 g frozen cells.

The mutant proteins, DM-1B/2B and DM-2A/2B, were labeled stochastically using equimolar amounts of Cy3 and Cy5 after being bound to a Histrap HP column (GE Health Care) as follows. The protein was slowly loaded onto the Histrap column (1 mL), followed by washing with Buffer B (at least 10 column volumes). Then, the column was washed slowly with Buffer B + 20 mM 2-mercaptoethanol (10 column volumes) to reduce any disulfide bonds that might have

formed. The column was then washed with 15 column volumes of Buffer B to remove 2-mercaptoethanol. Cy3 mono maleimide and Cy5 mono maleimide (Amersham Biosciences, Piscataway, NJ) were dissolved in N,N-Dimethylformamide (DMF) at a 1:1 ratio. The dye solution (about 1 mg/mL) in Buffer B was then loaded onto the column at a 3-fold excess over the protein concentration in Buffer B. The dye solution flow-through was collected and reloaded onto the column slowly three to five more times to maximize contact of the proteins with the dyes in order to increase the labeling efficiency. The labeling reaction was carried out in dark overnight (about 15 hours) at 4°C with slow rotation of the Histrap HP column on a shaker. Excess free dye was washed out with 5 column volumes of Buffer B and 5 column volumes of Buffer B + 20 mM imidazole. The labeled protein was eluted with Buffer B + 150 mM imidazole.

Removal of the His tag from the proteins was carried out by thrombin digestion (Thrombin Protease, Novagen, Madison, WI) overnight at 4 °C in Buffer B containing 2.5 mM $CaCl_2$ (final concentration), using 2 unit of thrombin per 1 mg of 6×His-tagged protein. The digested protein was separated from undigested 6×His-tagged proteins by loading the protein solution onto a Histrap column equilibrated with Buffer B + 10 mM imidazole. The column was washed with Buffer B + 20 mM imidazole to remove any protein without the His-tag. Fractions containing the digested protein were pooled and adjusted to conductivity of Buffer C + 150 mM NaCl by slowly adding Buffer C. The protein was then loaded onto a ssDNA cellulose column (5 mL) pre-equilibrated with Buffer C + 75 mM NaCl and eluted with a 10 column volume gradient from 100 mM NaCl to 1 M NaCl in Buffer C. The concentrations of the UvrD mutants and the

extent of fluorophore labeling were determined spectrophotometrically by obtaining absorbance spectra over the range from 250 nm to 750 nm. Molar extinction coefficients of 150,000 $M^{-1}cm^{-1}$ at 550 nm for the Cy3 dye and 250,000 $M^{-1}cm^{-1}$ at 650 nm for the Cy5 dye were used to calculate the fluorophore concentration on the protein. The absorbance at 280 nm was used to calculate the protein concentration after correcting for the absorbance of the dye at 280 nm (approximately 5% of the absorbance at 650 nm for Cy5 and approximately 8% of the absorbance at 550 nm for Cy3).

We observed no apparent preferential labeling of either protein with the donor (Cy3) or acceptor (Cy5) fluorophores for any of the two Cysteine residues. Therefore, the double labeled proteins will have a statistical distribution of double (50% Cy3 and Cy5) and uniformly labeled populations (25% Cy3 and 25% Cy5). In general, the final labeling efficiency was around 90%. The proteins were stored at -20 °C in storage minimal buffer.

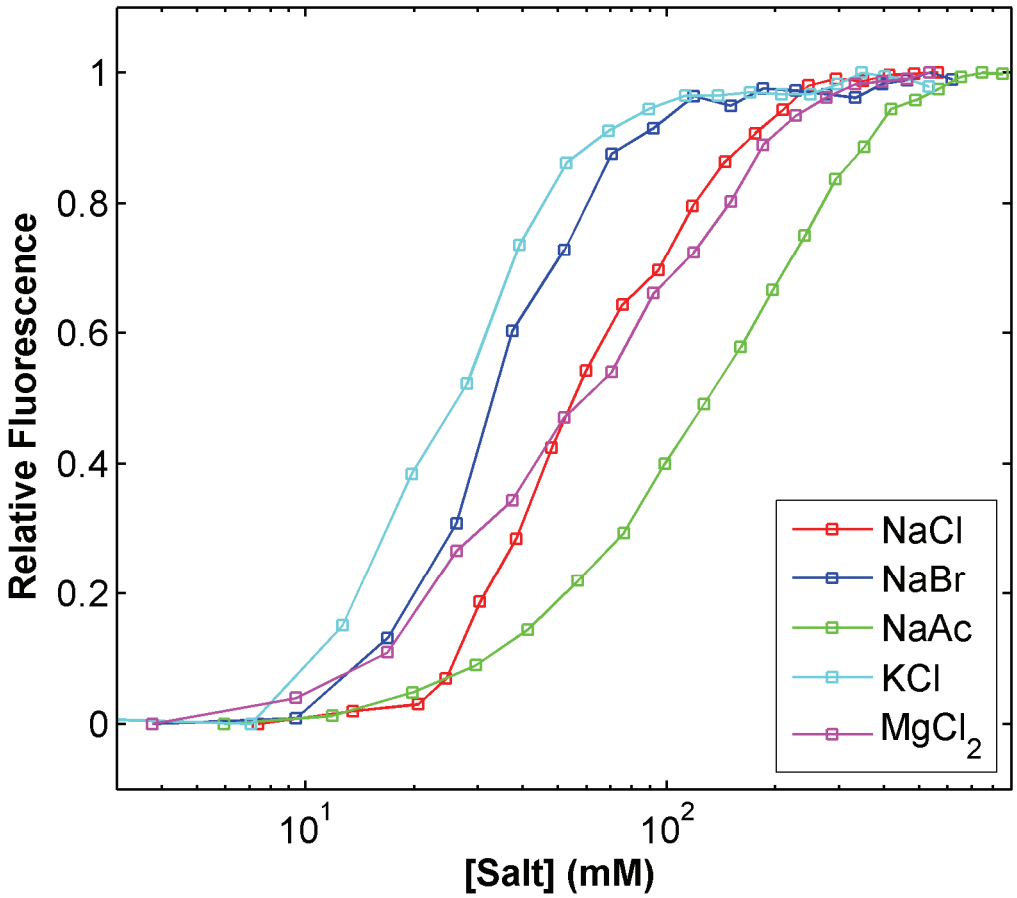

Figure S1. Salt effect on 2B sub-domain rotational conformation. Normalized fluorescence changes of acceptor (open squares and continual lines, excitation at 515 nm/emission at 670 nm) when UvrD(DM-1B/2B) labeled with Cy3/Cy5 was titrated with NaCl (red), NaBr (blue), $NaCH_3CO_2$ (green), KCl (cyan) and $MgCl_2$ (magenta), respectively.

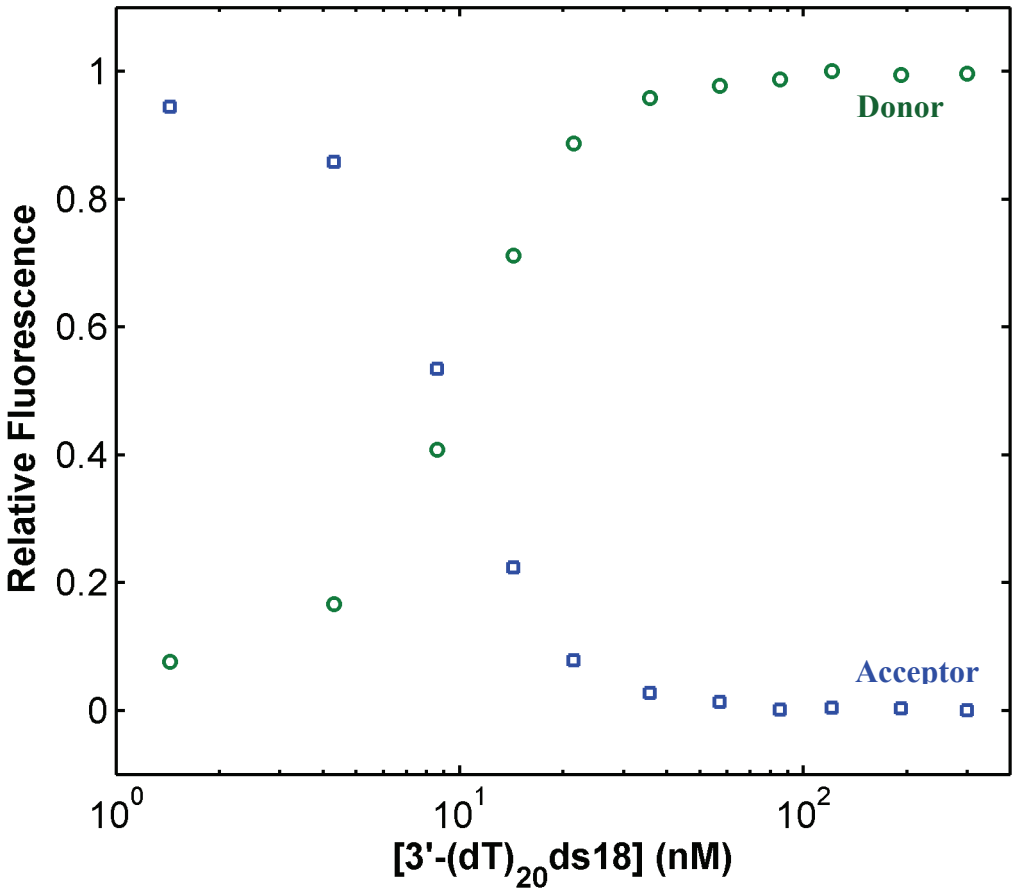

Figure S2. Titration of DM-1B/2B with a partial DNA duplex with a 3′-ssDNA tail. The cartoon shows the changes of the relative Cy3 (donor, open circles) and Cy5 (acceptor, open squares) fluorescence intensities of DM-1B/2B (20 nM) upon titration with 3-′$(dT)_{20}$ds18 in buffer T_{20}.