Nepenthesin from monkey cups for Hydrogen/Deuterium Exchange Mass Spectrometry

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Supplementary Methods

Horticulture of Nepenthes Plants

Plants *N. rafflesiana, N. ampularia, N. mirabilis, and N.globosa* were purchased from Kheen's Carnivores (http://www.keehnscarnivores.ca/). The plants were potted with wood bark, perlite, peat moss and humus mix (40, 35, 10, 5% respectively) and grown under a 14 hour light cycle in a terrarium. The terrarium was 36" x 18" x 24" (width, depth, height) and was of sufficient size to accommodate 6-8 pots (8"). Light was supplied with 2x4 Phillips Mini Twister Daylight Compact Fluorescent Bulbs (23 Watts). Plants were watered frequently with deionized water and the humidity was maintained between 60% and 80%. Waterings were applied at the soil level, taking care to minimize addition of water to the pitchers. Temperature was between 26-28°C, slightly lower in the dark. Every other week, the plants were fed with frozen drosophilae, 1 or 2 in every pitcher (left picture), and the fluid harvested the following week (right picture).

Nepenthes pitcher fluid was collected with a 1 ml plastic pipette. Crude pitcher fluid was filtered through a .22µm filter and then concentrated 80X. The concentrated fluid was acid-activated with 100 mM Glycine HCl (pH 2.5) for a minimum of 3 hours. Any peptides resulting from activation of the enzyme and fluid-protein digestion were washed away by 3 dilution-concentration cycles using 100 mM Glycine-HCl (pH 2.5) in an Amicon Ultra Centrifugal Unit (10kDa cutoff), prior to use in HDX-MS workflows.

Proteins: cloning and purification

Full-length human XRCC4 isoform 2 (Genebank accession number: NP_003392) was amplified from a HeLa cell cDNA library as described previously (1) and expressed in *E. coli* as a GST-fusion protein using pGEX-6P-1 vector (GE Healthcare). Proteins were purified over glutathione sepharose and GST tags were removed as previously described (2). To further purify XRCC4, protein-containing fractions were diluted to 50 mM KCl with buffer containing 50 mM Tris-HCl, pH 8.0, 5% (v/v) glycerol, 0.2 mM EDTA plus 1 mM DTT, 1 mM PMSF, 0.2 μg/ml leupeptin and 0.2 μg/ml pepstatin, loaded on to a 5 ml Heparin column (GE Healthcare) and eluted using a salt gradient of 50 to 750 mM KCl over 55 minutes at 1 ml/min. Fractions containing a single band of 55 kDa corresponding to XRCC4 were pooled and concentrated using a Vivaspin 30 kDa concentrator (GE Healthcare).

XRCC4 (aa1-200) was generated from full length XRCC4 using the following primers (written 5'>3') then expressed and purified as above:

5'-X4(1-200): CGGGATCCATGGAGAGAAAAATAAGCAGAATC
3'-X4(1-200): CCCCCCGGGTTAATTTAATAATTTATTATGCAAACTTCTG

Full-length human XLF (Genebank accession number: NM_024782) was amplified from a HeLa cell cDNA library as described previously (Yu et al., 2008) and subcloned into pGEX-6P-1 vector (GE Healthcare), expressed, purified and further purified by Heparin column as above.

Human DNA ligase IV BRCT domain (aa631-911) (Gene bank accession number: NP_996820) was amplified from a human HeLa cell cDNA library and subcloned into pGEX-6P-1 vector (GE Healthcare), expressed, purified and further purified by Heparin column as above. The primers used to amplify Human DNA ligase IV BRCT domain (aa631-911) were as follows (written 5'>3'):

5’-BRCT: CGGGATCCGCCCCAAAGATGAAGAAA
3’-BRCT: CCGCTCGAGTTAAATCAAATACTGGTTTTCTTCT

References

1. Yu, Y., Wang, W., Ding, Q., Ye, R., Chen, D., Merkle, D., Schriemer, D., Meek, K., and Lees-Miller, S. P. (2003) DNA-PK phosphorylation sites in XRCC4 are not required for survival after radiation or for V(D)J recombination. *DNA Repair* 2, 1239-1252.

2. Hammel, M., Yu, Y. P., Fang, S. J., Lees-Miller, S. P., and Tainer, J. A. (2010) XLF Regulates Filament Architecture of the XRCC4.Ligase IV Complex. *Structure* 18, 1431-1442.

Supplementary Data

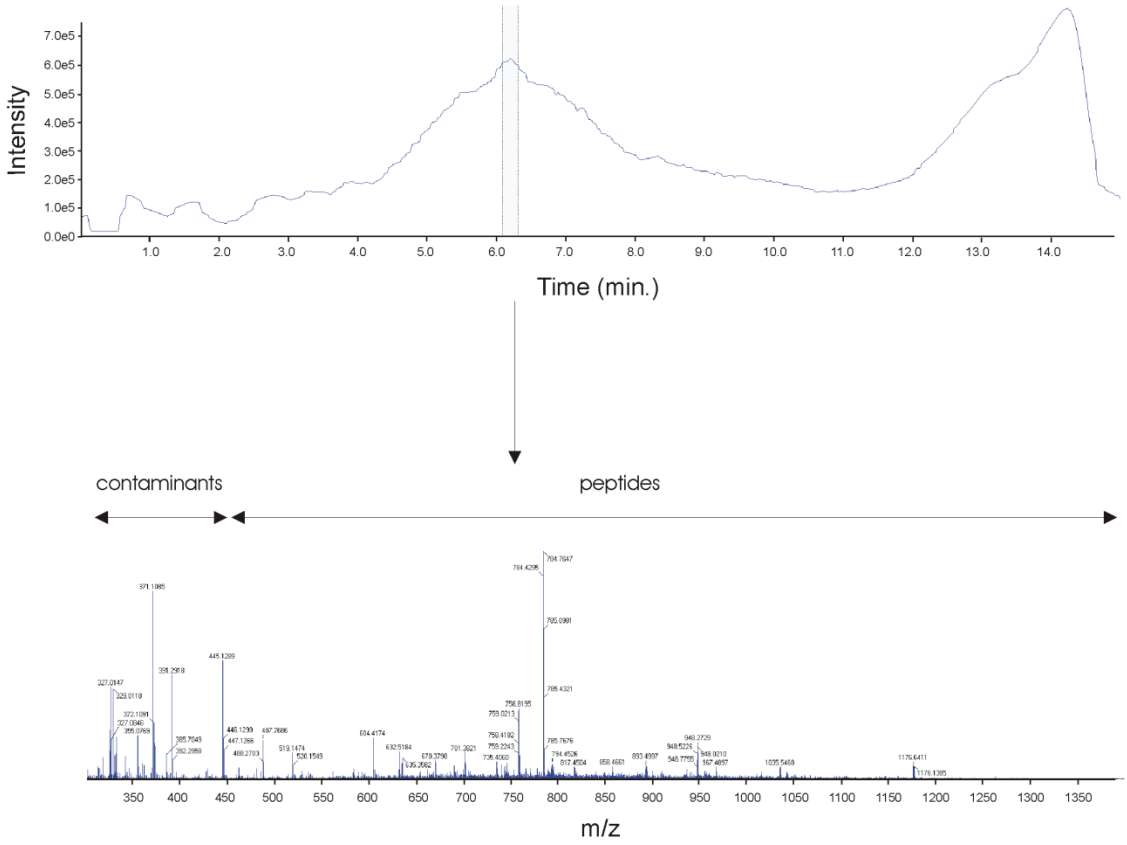

Figure S1. **Autolysis profile of concentrated nepenthesin fluid**. Analysis represents a loading of 11X the nepenthesin used in the typical digestions described in the manuscript. Chromatogram represents the main peptidic region (from m/z 450-1400) although most of the peaks in this mass range have not been confirmed to be peptides. Chromatogram intensities are 30X lower than conventional digests described in the manuscript. Mass spectrum shows that most intense background ions in the peptidic region are roughly the same intensity as low-abundance LC solvent contaminations.

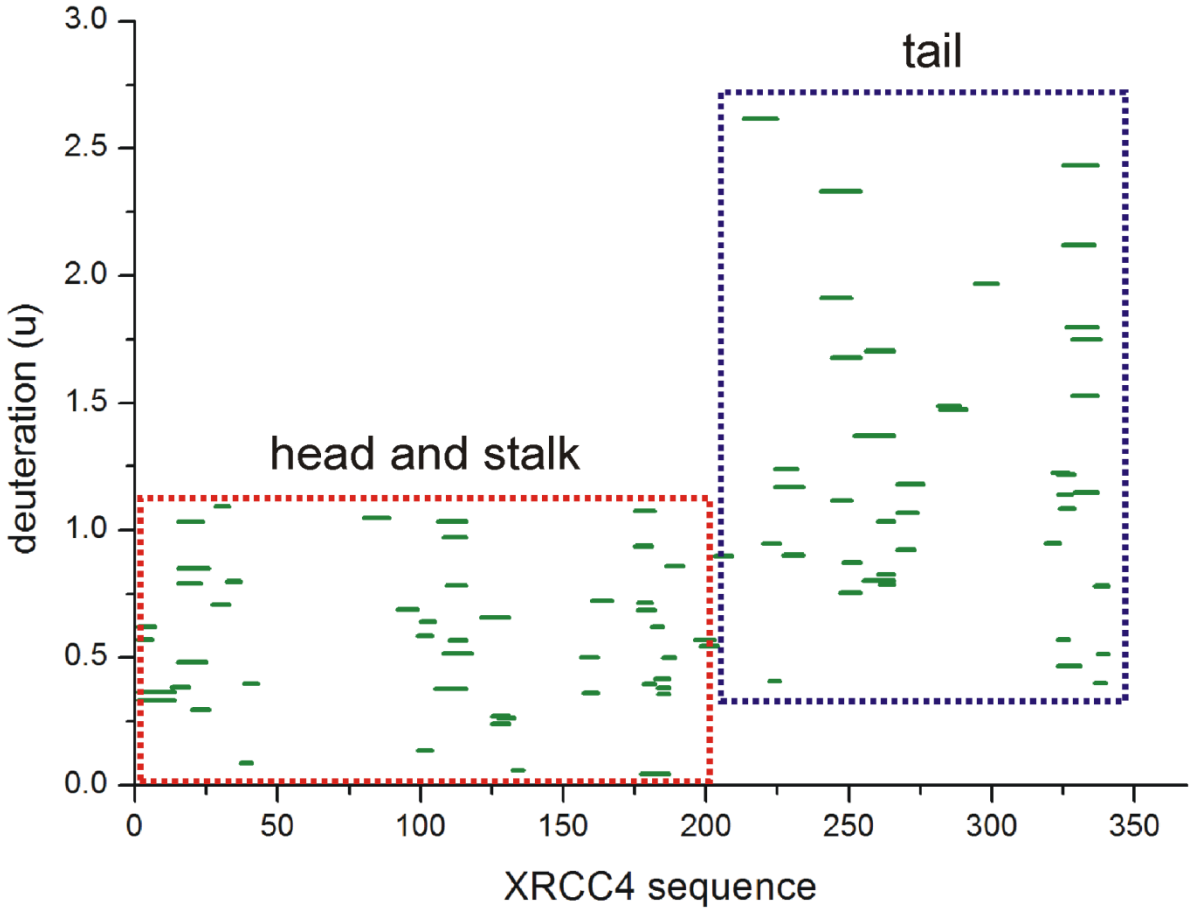

Figure S2. Deuteration map of unbound, full length XRCC4 dimer. The relatively low average level of deuteration in residues 1-207 highlight the stability of the head and stalk regions in the dimer, whereas the higher average level of deuteration in residues 208-341 demonstrate the intrinsic disorder of the C-terminal tail. XRCC4 was truncated to remove this disordered region (at residue 200) on this basis. This deuteration map was built using a nepenthesin digestion, under a single set of conditions (2 min digestion at 10°C). Deuteration is expressed in mass units (u) relative to the unlabeled protein, with green horizontal line length and position representing peptide length and position in sequence.

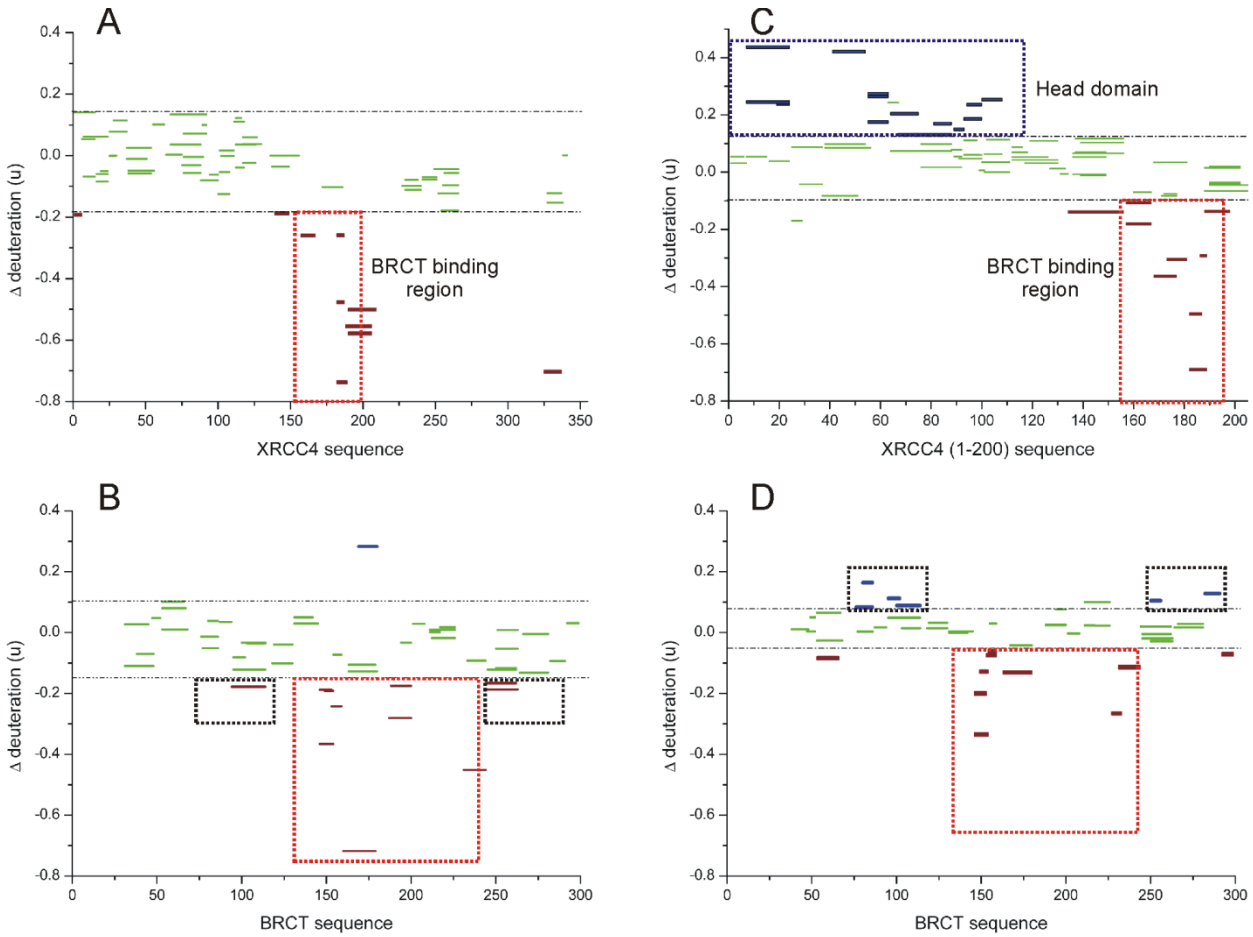

Figure S3. Pepsin-based deuteration maps of the XRCC4-BRCT interaction. Full-length XRCC4 bound to the tandem BRCT domains, showing (A) the deuteration map for full length XRCC4 and (B) the deuteration map for the tandem BRCT domains. Truncated XRCC4(1-200) bound to the tandem BRCT domains, showing (C) the deuteration map for truncated XRCC4 and (D) the deuteration map for the tandem BRCT domains. Changes in deuteration are expressed in mass units (u) relative to the free form of the protein. Green horizontal lines represent statistically insignificant changes in deuteration, bounded by a 95% confidence interval (dashed black lines). Blue horizontal line represent deprotection and red horizontal lines represent protection from exchange. The red boxes (A,C) mark the known binding interface on the stalk (residues 154-195), and as is shown, most of the reductions in deuteration occur in this region. The strong increase in deuteration observed in (C), in the blue box, represents a destabilization of the head domain only in the truncated form. This truncated XRCC4, and not the full length protein, self-polymerizes at the head domain (Hammel *et al.*, (2011) *J. Biol. Chem.* 286, 32638-50). Upon binding to the tandem BRCT domains, the polymer dissociates, consistent with increased deuteration in the head upon BRCT binding. The primary binding interface in the BRCT domains is non-contiguous, and marked with a red box (B,D). The proposed secondary interfacial regions are marked with two black boxes in (B). The same regions are boxed in (D), showing that these regions are destabilized when the XRCC4 tail is absent, and only the stalk binding site is preserved.

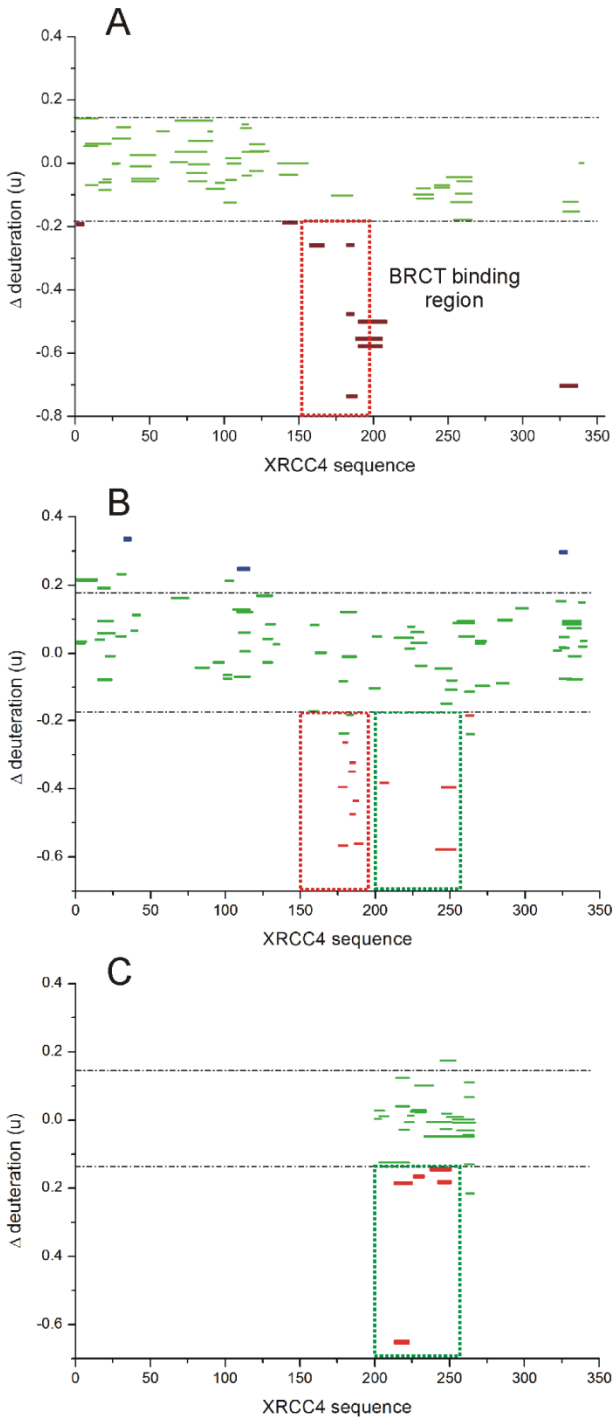

Figure S4. **Deuteration maps of full-length XRCC4 bound to the tandem BRCT domains.** (A) the pepsin-based map, (B) the untargeted nepenthesin map and (C) the targeted nepenthesin map. The red boxes mark the known primary binding site, and the green boxes mark the portions of the C-terminal tail proposed to also bind BRCT, at secondary sites. Line colors and boundaries as in Figure S3.