Structural and Functional Comparison of the RING Domains of two p53 E3 ligases, Mdm2 and Pirh2

Supplementary Material:

Table 1. The list of human E2s used in the *in vitro* ubiquitylation assays.

Figure 1. The MS analysis of p53 ubiquitylation by Pirh2 and Mdm2. In vitro reaction products were subjected to SDS-PAGE, and high MW products analyzed by LC-ESI-MS/MS. The ubiquitylation conjugation sites within p53 C-terminus in the reaction catalyzed by Pirh2. A. The numbers of observed spectra at the indicated sites. B. The observed peptides of each reaction components in the Pirh2 and Mdm2 catalyzed reactions. C. Average p53 coverage per MS analysis.

Figure 2. The *in vitro* ubiquitylation assays with a panel of representative E2s. A mixture of ATP, E1, His-Ub were reacted with a panel of E2s in the absence of an E3. An antibody against the His6-tag was used to detect polyubiquitylation. Cdc34 was found to autoubiquitylate in the absence of an E3, but does not support Pirh2 or Mdm2 mediated ubiquitylation as seen in supplementary figure 3 and 4. Asterisk represents the core domain of the respective E2.

Figure 3. Selection of E2s by Pirh2 and Mdm2. A mixture of ATP, E1, His-Ub and E3 (GST-Pirh2 or His-Mdm2) were reacted with a panel of E2s as indicated. Left panel: autoubiquitylation activity of Pirh2 was evaluated by blotting with an antibody against GST. Right panel, autoubiquitylation activity of Mdm2 was evaluated by blotting with an antibody against Mdm2.

Figure 4. Selection of E2 conjugating enzymes by Pirh2 and Mdm2 in mediating p53 ubiquitylation. P53 ubiquitylation mediated by Pirh2 (left) or Mdm2 (right) using a library of E2s as indicated. GST-Pirh2 (E3) and His-Mdm2 (E3) were subjected to *in vitro* ubiquitylation assay in the presence of ATP, E1, E2, His-tagged ubiquitin and p53 as indicated. After the reaction, the samples were resolved by SDS-PAGE and detected by western blot. Ubiquitylated p53 was detected using monoclonal antibody against p53 (PAB1801).

Figure 5. Chemical shift perturbation of Pirh2 RING domain upon binding to different E2s. (A) The residues with chemical shift changes of 0.05 ppm or greater induced upon binding to UBE2D2 are colored on the surface representation of Pirh2 RING domain. The most shifted residues from the first zinc binding sites I147, C148 are colored in yellow, the residues from the helix α1 T171, Y173, E174, M176 in magenta and the residues from the second zinc binding sites Y181, M185 and L187 in cyan. (B-F) Composite chemical shift changes versus residue number for Pirh2 RING domain (residue 138-189) upon binding to UBE2D2 (B), UBE2D3 (C), UBE2D1 (D), UBE2D4 (E) and UBE2E2 (F). The values shown were calculated by using the equation $\Delta_{comp} = [\Delta\delta_{HN}^2 + (\Delta\delta_N/5)^2]^{1/2}$.

Figure 6. The HSQC spectrum of Mdm2 417- 484 indicating that deletion of the C-terminal seven residues affects the Mdm2 RING domain structure.

Protein Name	Synonyms	Gene Accession #
E2A	UBC2, HHR6A, RAD6A	NM_003336
E2B	UBC2, HR6B, HHR6B, RAD6B, E2-17K	NM_003337
E2C	UBCH10	NM_007019
E2D1	SFT, UBCH5, UBC4/5, UBCH5A, E2(17)KB1	NM_003338
E2D2	UBCH5B, UBC4	NM_003339
E2D3	UBC4/5, UBCH5C, MGC5416, MGC43926, E2(17)KB3	NM_003340
E2D4	HBUCE1	NM_015983
E2E2	UBCH8, FLJ25157	NM_152653
E2E3	UBCH9, UBCM2	NM_006357
E2F	NCE2	NM_080678
E2G1	UBE2G	NM_003342
E2G2	UBC7	NM_003343
E2H	UBC8, UBCH, UBCH2, E2-20K	NM_003344
E2I	UBC9	NM_003345
E2J1	UBC6p, CGI-76, NCUBE1, HSPC153, HSPC205, HSU93243, MGC12555	NM_016021
E2J2	NCUBE2, PRO2121	NM_194315
E2K	HIP2, LIG, HYPG, UBE2K	NM_005339
E2L3	E2-F1, L-UBC, UBCH7, UbcM4	NM_198157
E2L6	RIG-B, UBCH8, MGC40331	NM_004223
E2M	UBC12, hUbc12, UBC-RS2	NM_003969
E2N	UBCH-BEN, UBC13, MGC8489	NM_003348
E2O	E2-230K, FLJ12878, KIAA1734	NM_022066
E2Q1	GTAP, UBE2Q, NICE-5, PRO3094	NM_017582
E2Q2		NM_173469
CDC34	UBE2R1, E2-CDC34	NM_004359
E2R2	UBC3B, CDC34B, FLJ20419, MGC10481	NM_017811
E2S	E2-EPF	NM_014501
E2T	PIG50, HSPC150	NM_014176
E2U	MGC35130, RP4-636O23.1	NM_152489
E2V1	CIR1, UEV1, CROC1, UBE2V, UEV-1, UEV1A, CROC-1	NM_021988
E2V2	MMS2, UEV2, EDPF1, UEV-2, DDVIT1, EDAF-1, EDPF-1, DDVIT-1	NM_003350
E2W	FLJ11011	NM_001001481
E2Z		NM_023079
TSG101	TSG10, VPS23	NM_006292
FLJ25076	UBE2Q like	XM_059689

Supplementary Table 1: The human E2s used in the *in vitro* ubiquitylation assays with Pirh2 and Mdm2 RING domain. For each entry, the Genbank nucleotide sequence accession number is provided to facilitate retrieval of E2 nucleotide sequence data.

Supplementary Figure 1

A. p53 C-terminal conjugation sites (number of spectra oberved)

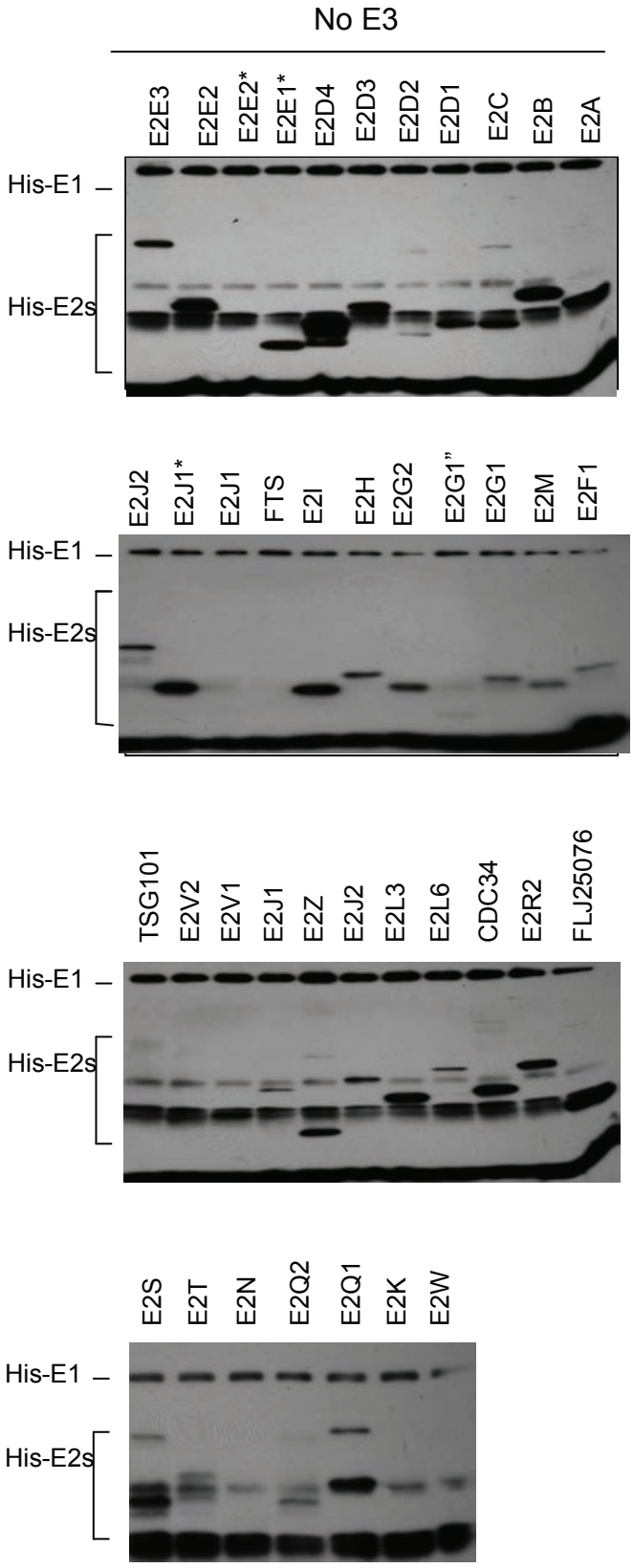
	K370	K382	K386
Pirh2	4	10	53

B. Peptide Totals (15 mass spectrometry runs each)

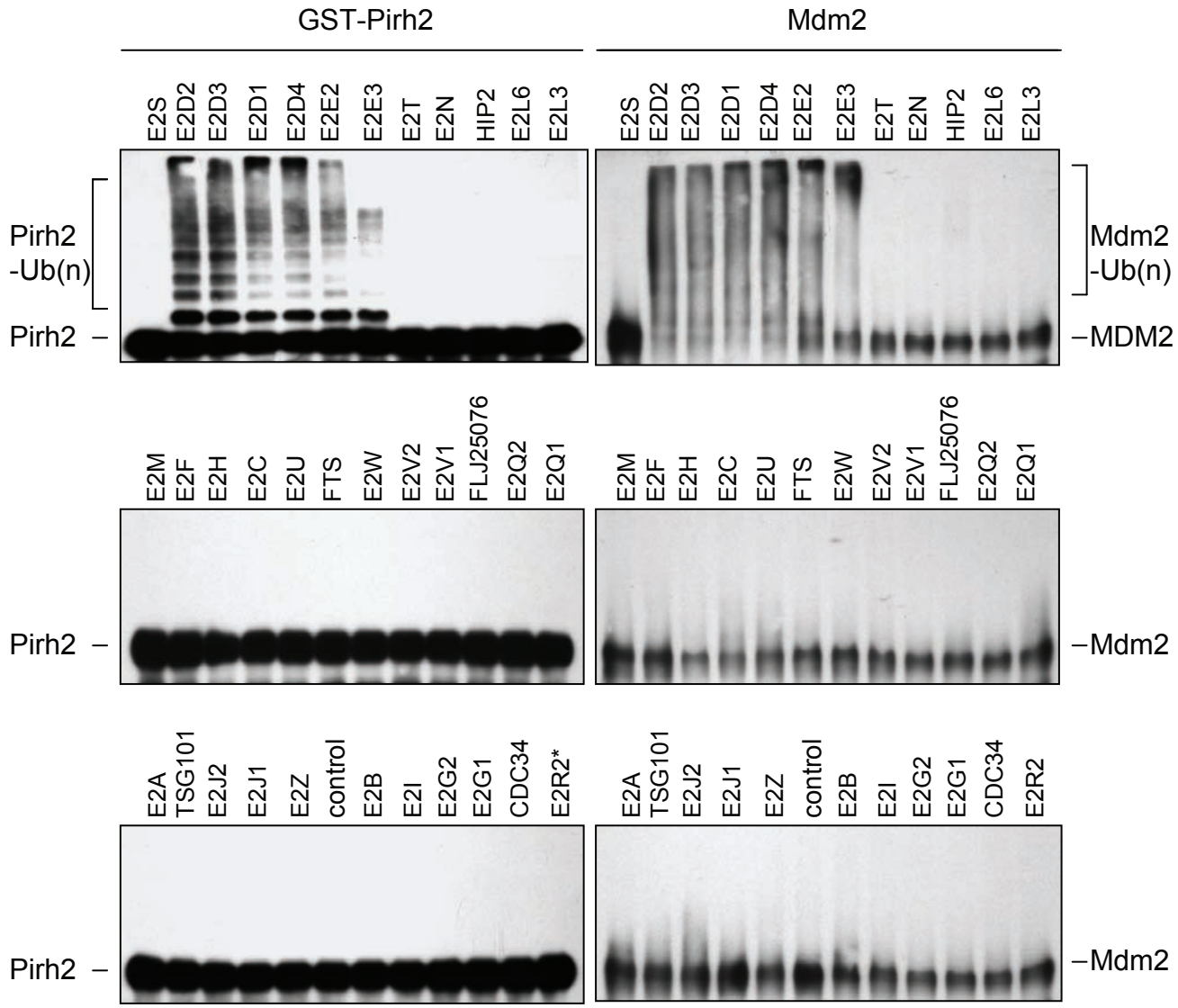
	Pirh2	Mdm2
p53	5889	5989
E1	10357	11010
E2	109	79
Mdm2	9	7124
Pirh2	1059	0
ubiquitin	4427	12005

C. Average p53 coverage per analysis: 43.8%. Range 36-53%

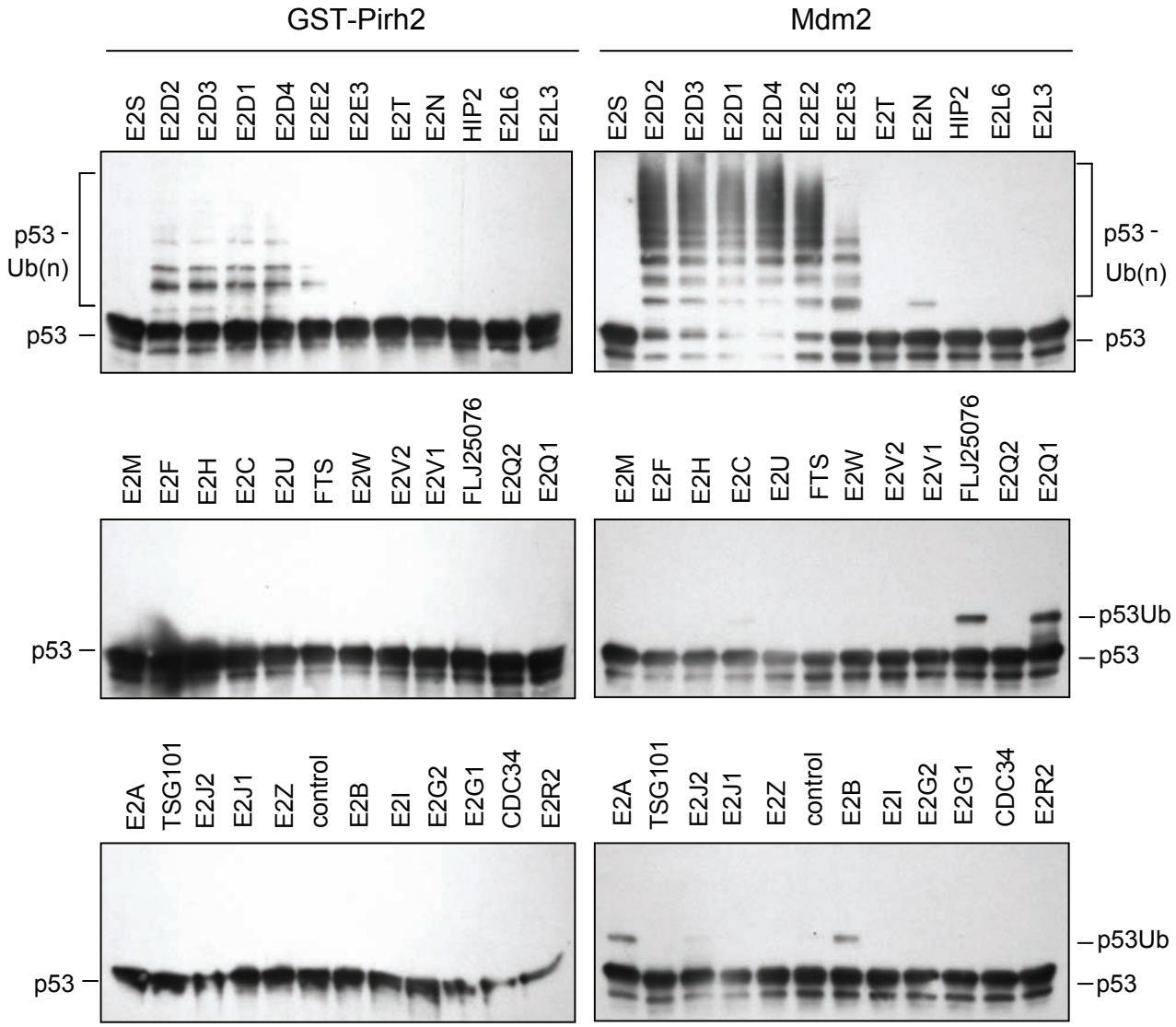
Supplementary Figure 2



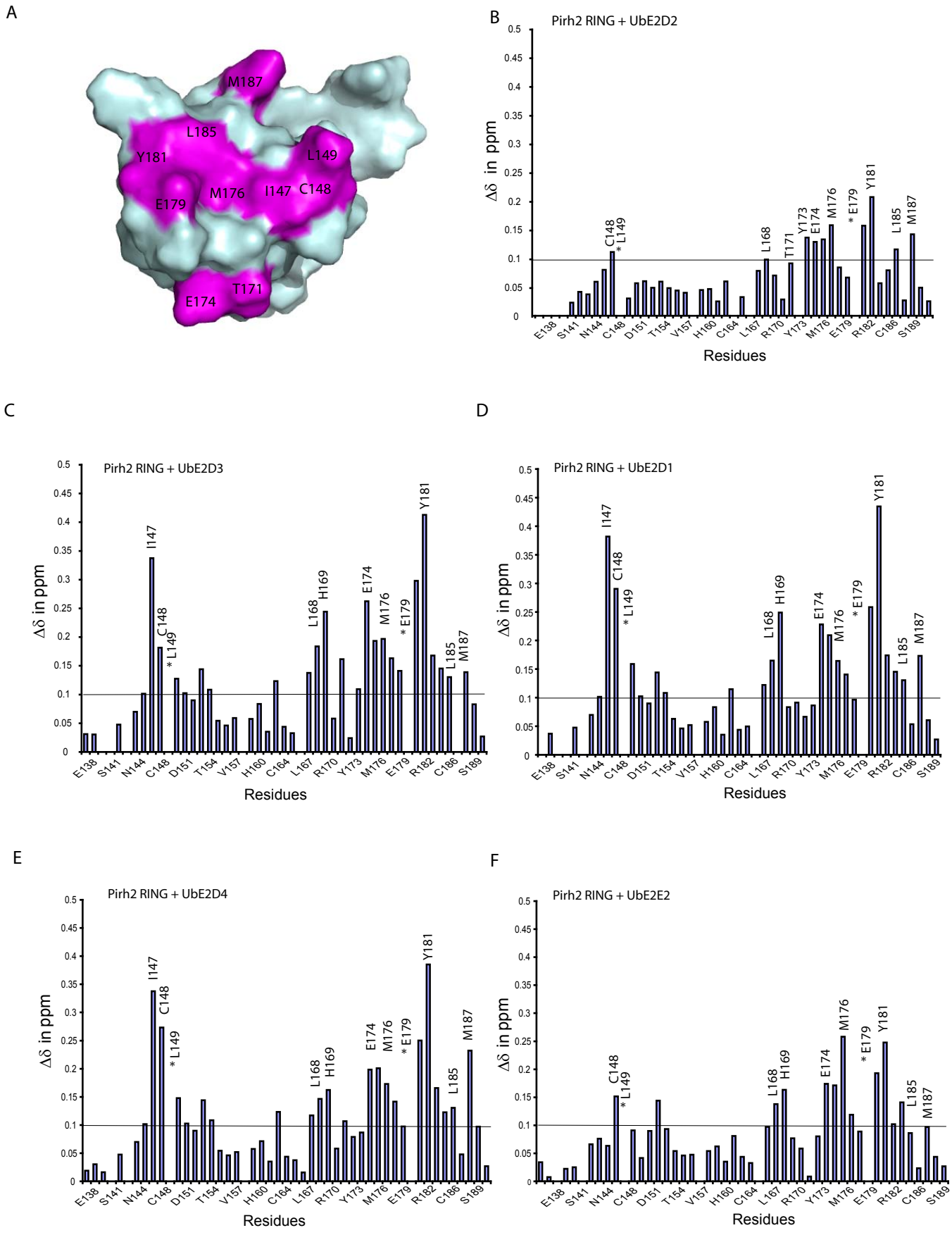
Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5



Supplementary Figure 6