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FOOTNOTES

We greatly appreciate the gift of the expression plasmids for human RPA, p11d-tRPA, from Dr. Marc

S. Wold (University of Iowa College of Medicine, Iowa City, Iowa) and mouse E1 expression vector RLC from Dr. Hideyo Yasuda (School of Life Science, Tokyo University of Pharmacy, and Life Science, Tokyo, Japan). The work was supported by grants-in aid for Scientific Research (A) and (B) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

The abbreviations used are: PCNA, proliferating cell nuclear antigen; RFC, replication factor C; pol, polymerase; RPA, replication protein A; EDTA, ethylenediaminetetraacetic acid; NP-40, Nonidet P-40; SDS-PAGE, sodium dodecyl sulfate poly-acrylamide gel electrophoresis; IR, ionizing radiation.

FIGURE LEGENDS

Figure 1. Accumulation of RFC-complex in chromatin fraction and modification of RFC2 following treatment of 293A cells with DNA damaging agents.

A. 293A cells transfected with a Flag epitope-tagged form of each subunit of RFC and RLCs were irradiated with UV (lane 3 and 9) or -ray (lane 6 and 12), or treated with DMSO (lane 1, 4, 7 and 10), MMS (lane 2 and 8) or HU (lane 5 and 11) for 8 h. Cell extracts recovered from transfected cells were then separated into chromatin (Chromatin; lanes 7-12) and soluble (Sup; lanes 1-6) fractions and analyzed by Western blotting with anti-Flag. Cell extracts recovered from RFC4-transfected cells were also analyzed by Western blotting with anti-tubulin or anti-histone H3 (lowest two blots).

B. 293A cells were irradiated with UV (lane 3) or -ray (lane 5), or treated with DMSO (lane 1), MMS (lane 2) or HU (lane 4) for 8 hr. Cell extracts recovered from transfected cells were then separated into chromatin (Chromatin) and soluble (Sup) fractions and analyzed by Western blotting either with anti-RFC1, anti-RAD17 or anti-RFC2. The arrowheads indicate the position of molecular weight marker (kDa).

C. 293A cells transfected with pCDNA3.RFC2-HA were treated with the indicated dose of MMS for 8 h. Chromatin fractions from the resulting cells were analyzed by immunoblotting with anti-RFC2 or anti-PCNA. The arrowheads indicate the position of molecular weight marker (kDa).

D. 293A cells transfected with pCDNA3.RFC2-HA were treated with 0.85 mM MMS for 1 hr (lanes 2-5) or UV-irradiated at 254 nm with 30 Jm^{-2} (lanes 6-9), and then incubated for the indicated times. Chromatin fractions were prepared and analyzed by Western blotting with anti-RFC2, anti-PCNA. Cells treated with DMSO (lane 1) are shown as control. The arrowheads indicate the position of molecular weight marker (kDa).

E. 293A cells transfected with pCDNA3.RFC2-HA were treated with various genotoxic agents.

Chromatin fractions were prepared and analyzed by Western blotting with anti-RFC2 or anti-PCNA. The arrowheads indicate the position of molecular weight marker (kDa).

Figure 2. RFC2 monoubiquitylation in response to DNA damaging agents is RAD18-dependent.

A. Lysates from RFC2-HA and Flag-Ubiquitin co-transfected 293A cells were analyzed by immunoprecipitation and Western blotting. pCDNA3.RFC2-HA was co-transfected either with pCDNA.Flag-Ubiquitin (lanes 4 and 8) or empty vector (lanes 3 and 7) in 293A cells. The following day, cells were treated with MMS for 8 h and then cell extracts were recovered. Cell extracts were immunoprecipitated with anti-RFC2 antibody. The resulting immune complexes were recovered using protein-A/G agarose and detected by immunoblotting with anti-RFC2 antibody (lanes 1-4) or anti-Flag antibody (lanes 5-8). Asterisks show nonspecific bands.

B. Western blot of lysates from 293A cells over-expressing hRAD18. pCDNA3. RFC2-HA was co-transfected either with pCAGGSh.RAD18 (lane 3) or empty vector (lane 2) in 293A cells. Chromatin fractions were prepared and analyzed by Western blotting with anti-RFC2 (lower panel) or anti-PCNA (upper panel). The arrowheads indicate the position of molecular weight marker (kDa).

C. Western blot of lysates from HCT116 cells (WILD) or *RAD18*-deficient HCT116 cells (*RAD18*$^{-/-}$). HCT116 cells transfected either with empty vector or pCAGGS.hRFC2 were treated with 0.85 mM MMS for 8 h. Chromatin fractions from the resulting cells were analyzed by immunoblotting with anti-RFC2 antibody. The arrowheads indicate the position of molecular weight marker (kDa).

Figure 3. DNA damage independent monoubiquitylation of hRFC2-D228A.

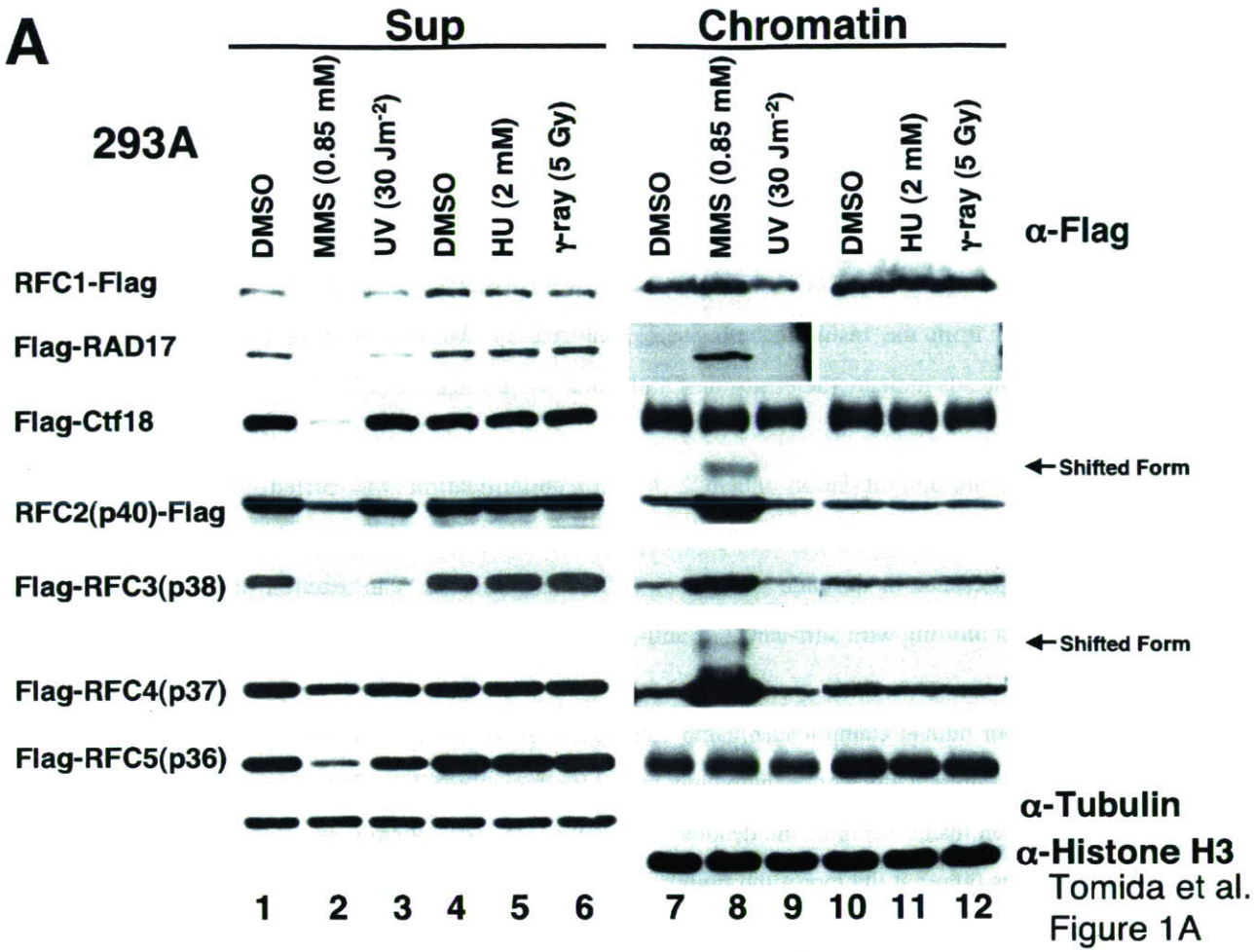
A. Schematic diagram and tertiary model of human RFC2 showing the location of D228 and the sequences of the surrounding regions. Corresponding sequences for *S. cerevisiae* RFC2(p40) and mouse RFC2 homologues are also shown. The conserved Sensor 2 helix is represented by box and the location of the conserved SRC motif is indicated as arrow. D228 of hRFC2, shown in red, corresponds to *S. cerevisiae* D201, which shows synthetic-lethality with mutation in Rpa-1(*rfa1*-Y29H). There are seven conserved RFC boxed numbered consecutively from N-terminus to C-terminus.

B. 293A cells were transfected with expression vectors encoding wild-type (lanes 2 and 6), D228N (lanes 3 and 7) or D228A (lanes 4 and 8) forms of hRFC2-HA. 24 hr after transfection cells were harvested and separated into chromatin (lanes 5-8) and soluble fractions (lanes 1-4) then immunoblotted with anti-RFC2 or anti-PCNA antibody. The arrowheads indicate the position of molecular weight marker (kDa).

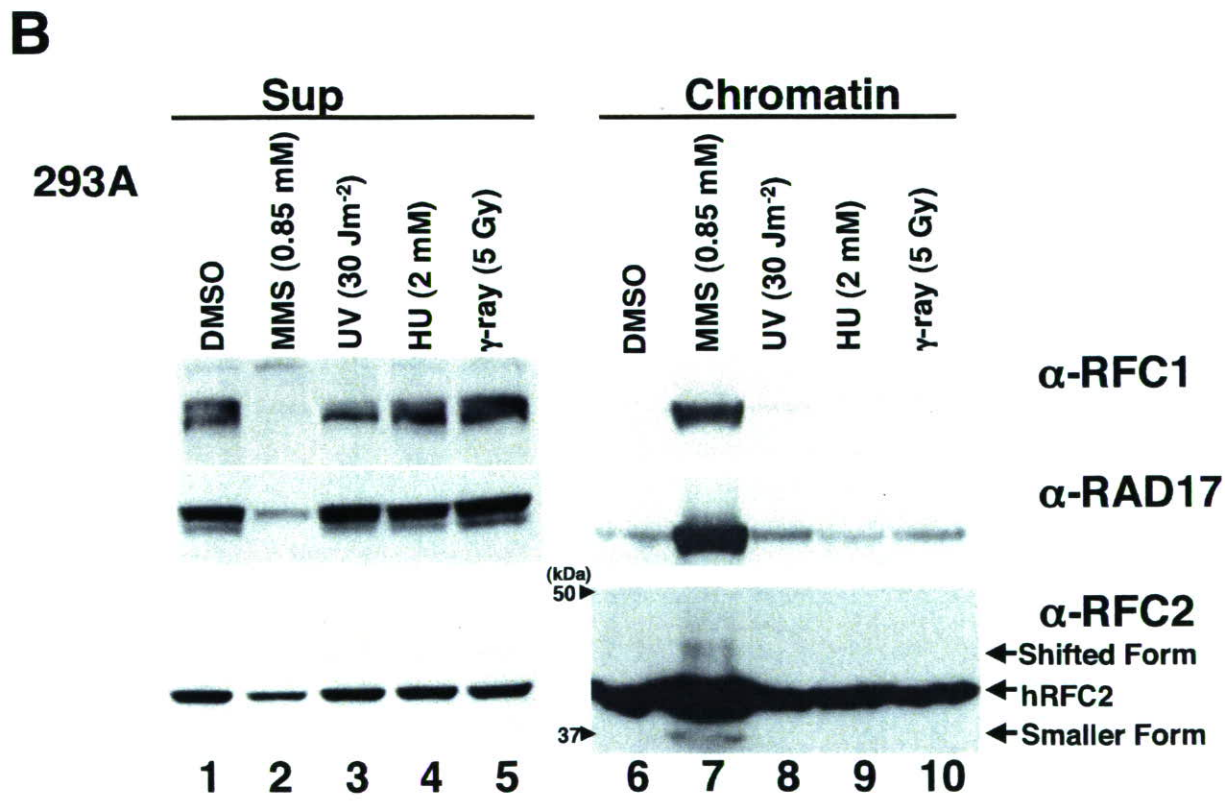
C. Western blot of lysates from HCT116 cells (WILD) or *RAD18*-deficient HCT116 cells (*RAD18*$^{-/-}$). HCT116 cells transfected with pCAGGS.hRFC2(D228) were treated with 0.85 mM MMS for 8 h. Chromatin fractions from the resulting cells were analyzed by Western blotting with anti-RFC2 antibody. The arrowheads indicate the position of molecular weight marker (kDa).

Figure 4. *In vitro* mono-ubiquitylation of RFC2. *In vitro* ubiquitylation was carried out by mixing RFC with mouse E1, RAD18-RAD6A complex, ubiquitin and singly primed single stranded M13 mp18 DNA in the presence or absence of RPA or PCNA as indicated. The reaction products were analyzed by Western blotting with anti-RFC2 or anti-PCNA antibody.

Figure 5. A model for human clamp-loader/clamp complex. Ribbon (RFC2) and wire (Ca-trace, RFC1, RFC3-5 and PCNA) representations of the homology model for human RFC1-5 / PCNA complex. The five subunits of each clamp loader complex are denoted. The colors for each subunits are as follows with the helical collar domains (gray) at the top of the figure; RFC1, pink; RFC2, navy; RFC3, red; RFC4, green; RFC5, orange; PCNA, gold. The side-chain atoms of Asp228 of RFC2 are indicated as balls in cyan. A. A side view of the clamp-loader/clamp complex in which RFC2 is in the front. B. Views from the DNA-interacting pore of the clamp loader subunits. Domain I and II of AAA+-domain and 14 and 15 of RFC2 are indicated.



Tomida et al.
Figure 1A



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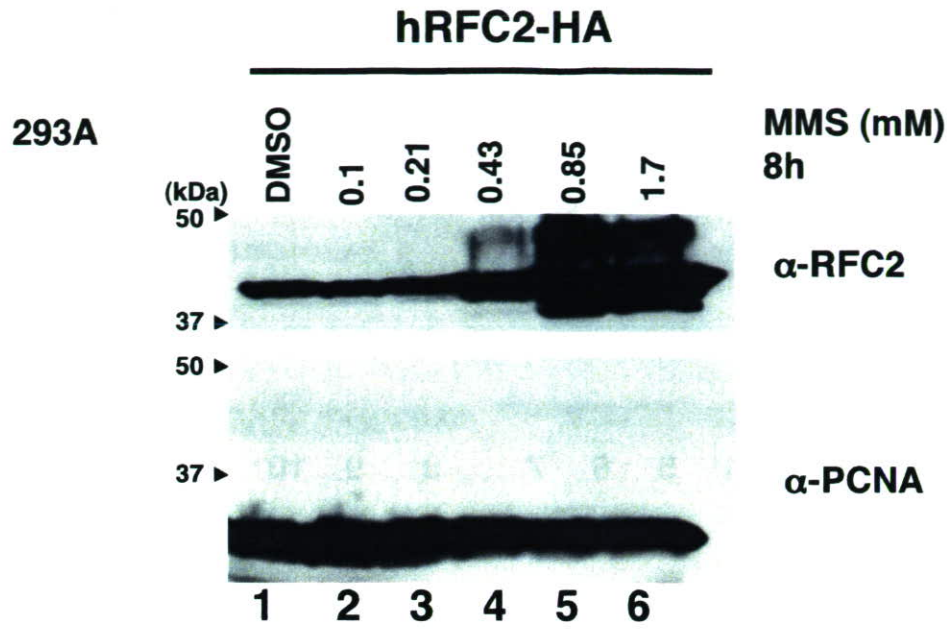

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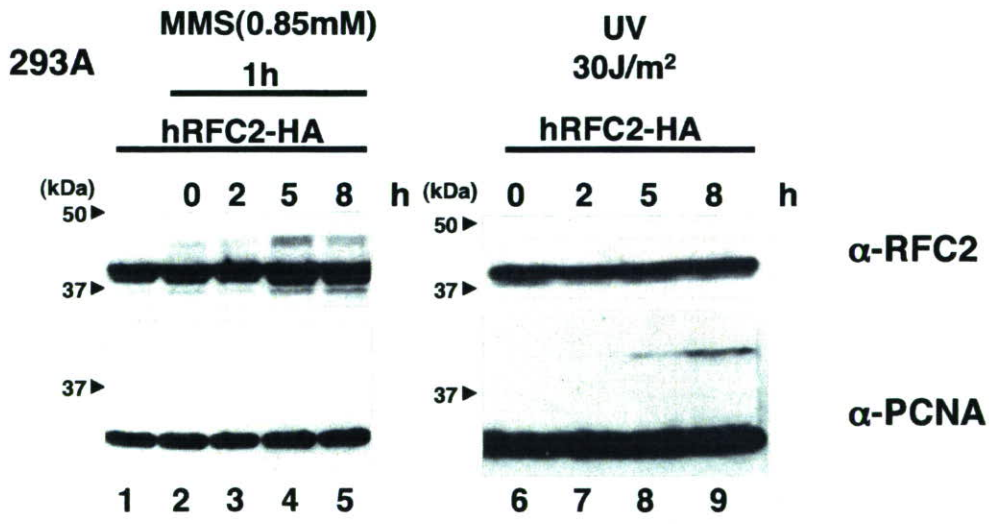
D



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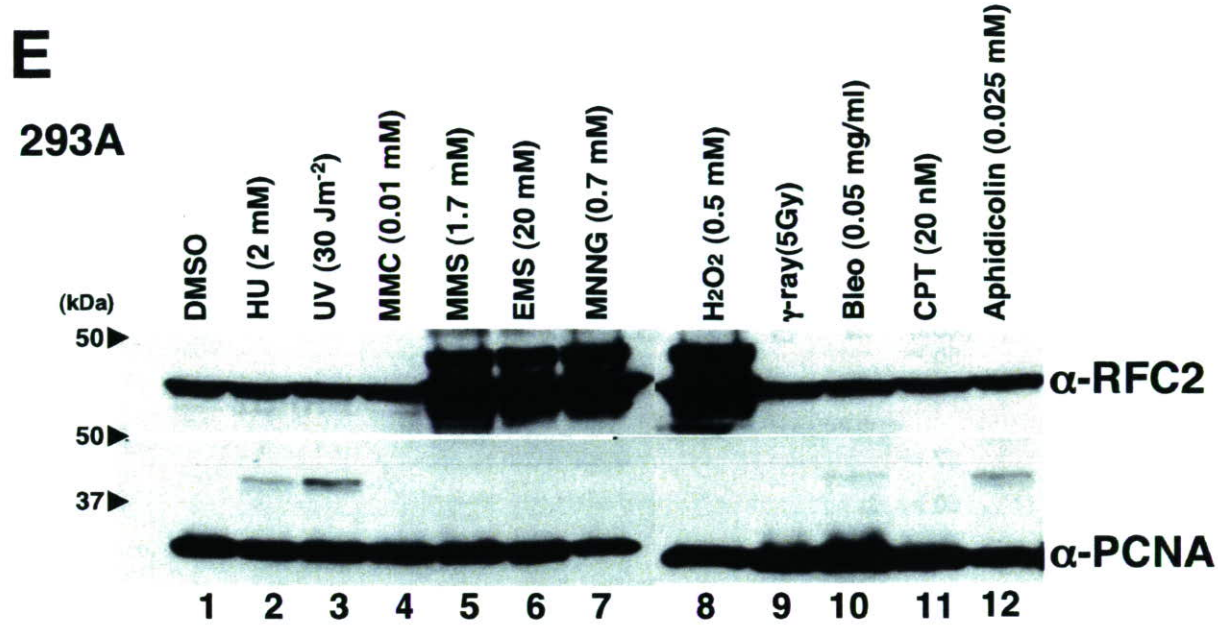

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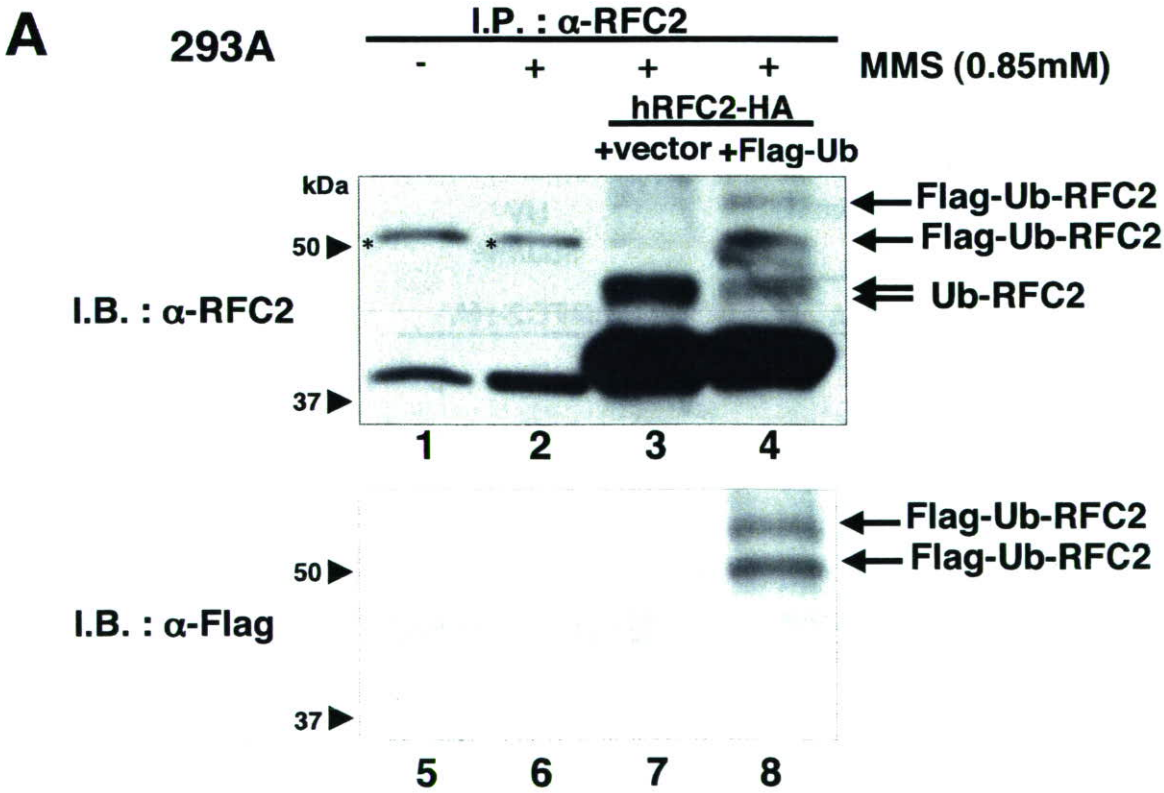

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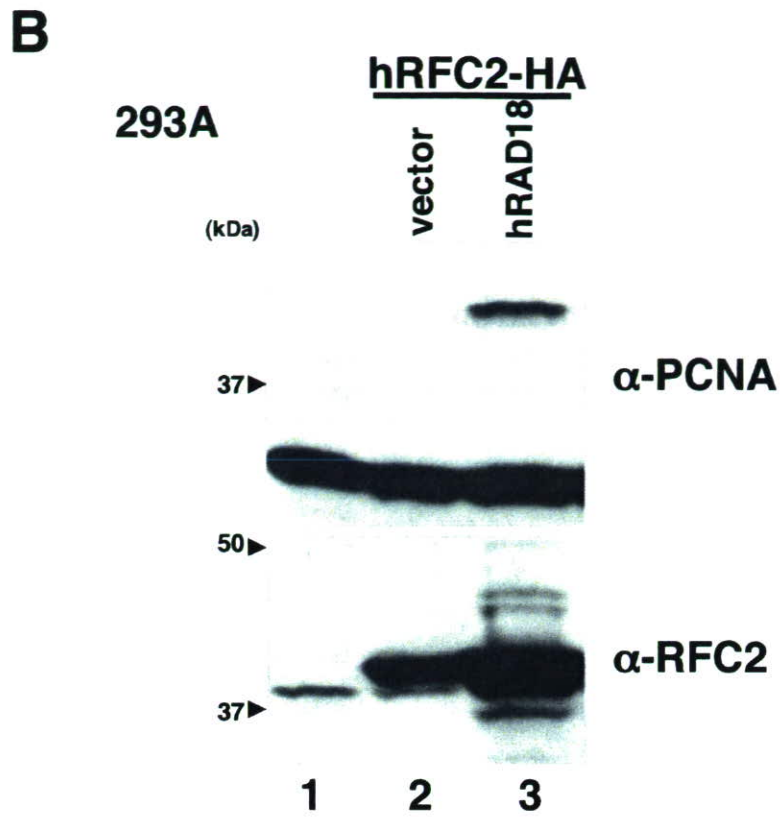

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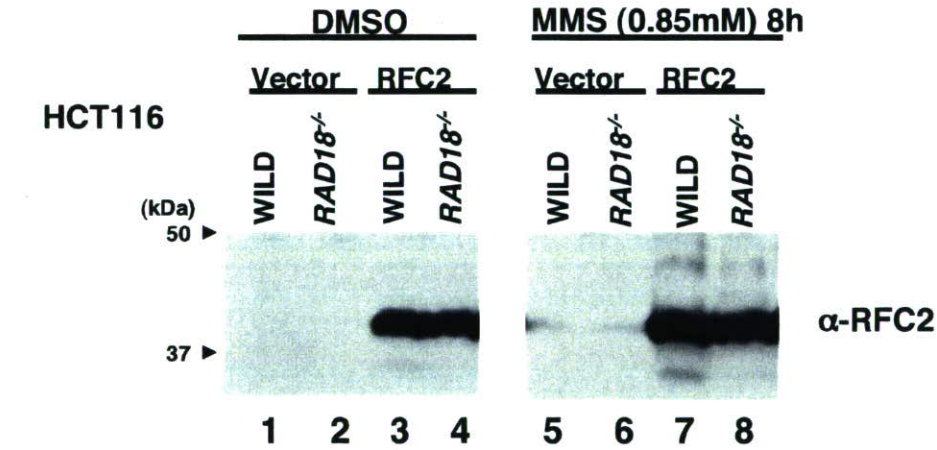

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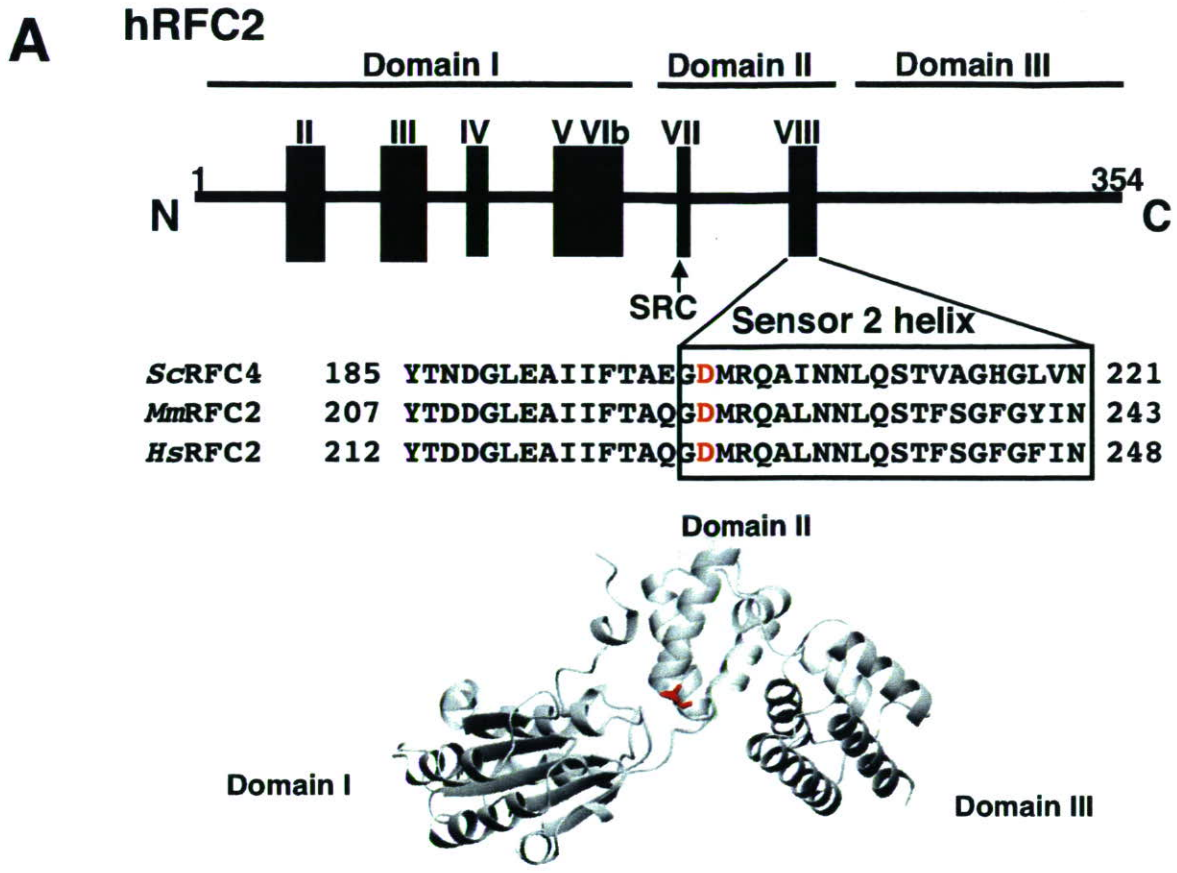

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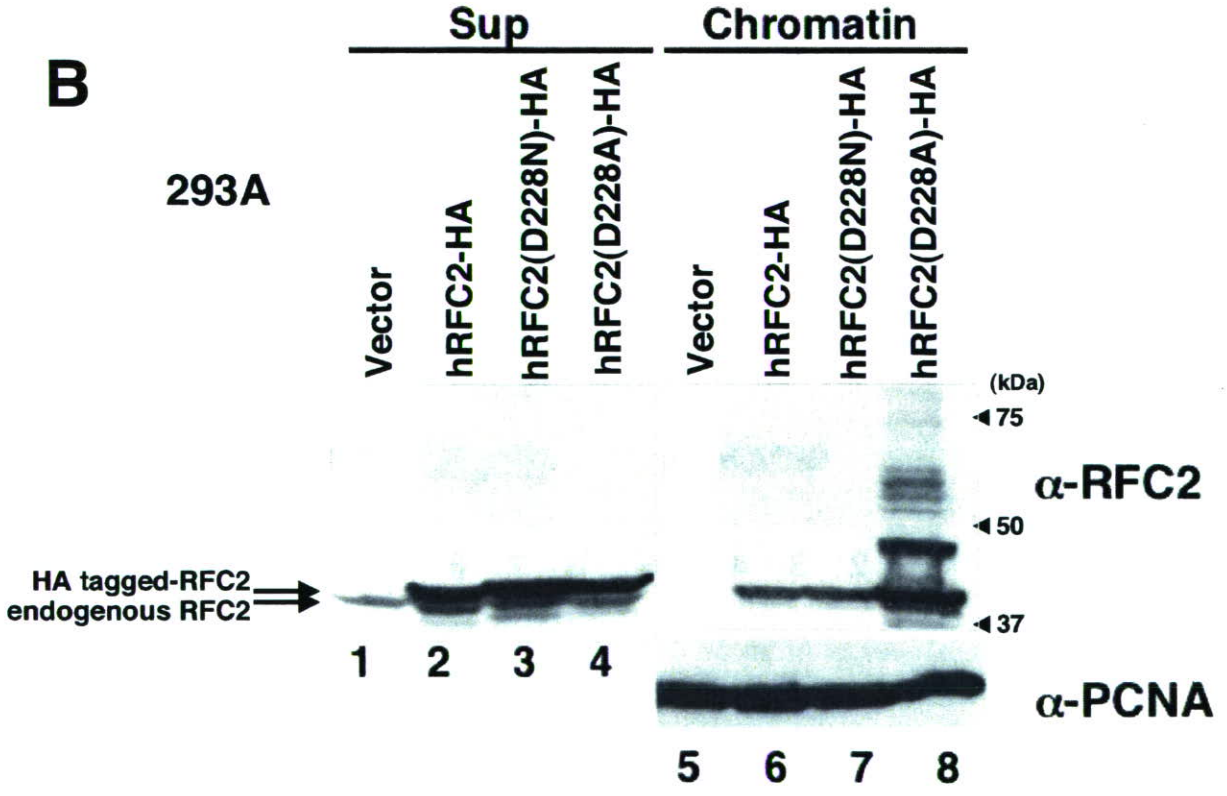

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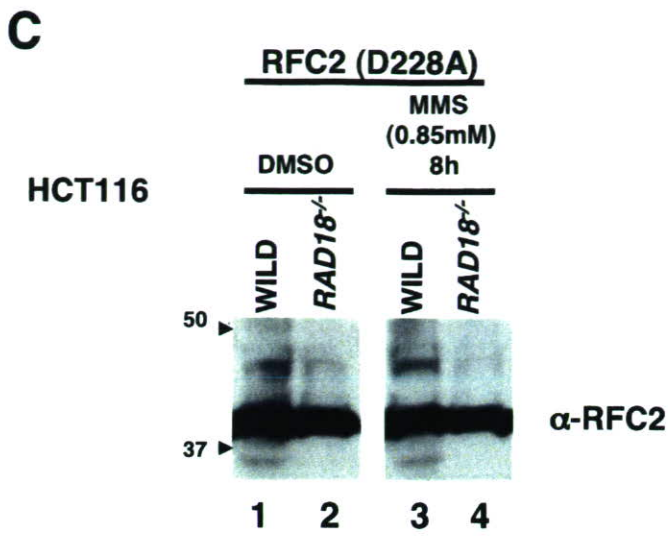

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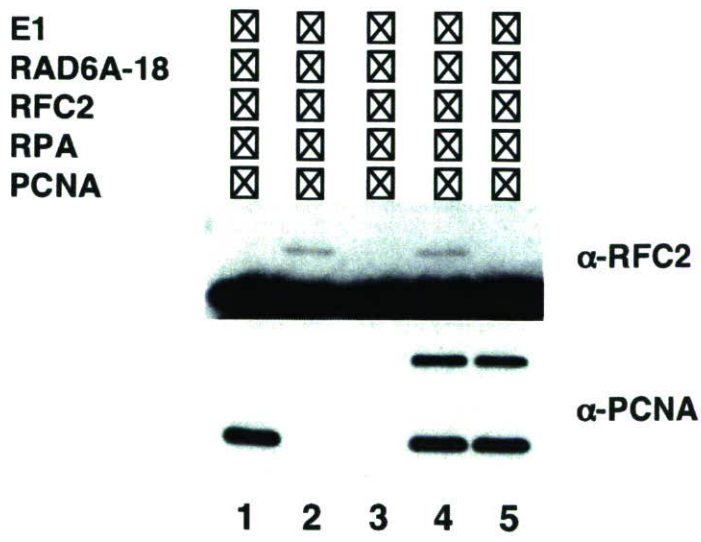

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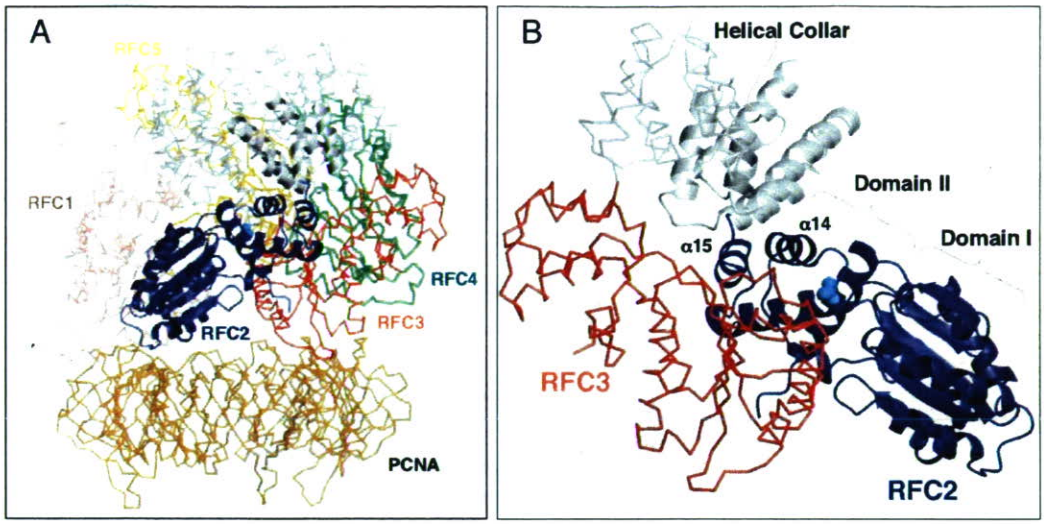

Tomida et al. Figure 5