Supplementary information

Figure Legends

Fig.S1 HDAC6 is involved in DSB repair. (A-B) HeLa cells were transfected with Flag-HDAC6 or an empty plasmid for 48 h. They were then exposed to 10 Gy irradiation (IR) and allowed to recover under normal conditions for the indicated times, before a comet assay was performed. Representative images (A) and data analysis (B) are shown. (**C-D**) HCT116 cells were transfected with Flag-HDAC6 or an empty plasmid for 48 h. All cells were treated with VP16 (10 μM) for 1 h and washed three times with fresh medium. All samples were then counted and seeded for colony formation assays. For the control (Ctr) groups, 500 cells were seeded in each plate, while for the VP16-treated group, 5,000 cells were seeded in each plate. All data represent the means ± SD. **(E)** Whole cell lysates were extracted from HCT116 wild-type (HDAC6-WT) cells or two CRISPR clones, HDAC6-KO$^{\#}$1 and HDAC6-KO$^{\#}$2; HDAC6 protein levels were then analyzed by western blotting. **(F-G)** HeLa cells were transfected with HDAC6 siRNA or an siRNA negative control (NC) for 48 h. All cells were treated with VP16 (10 μM) for 1 h and washed three times with fresh medium. All samples were then counted and seeded for colony formation assays. For the control (Ctr) groups, 500 cells were seeded in each plate, while for the VP16-treated group, 5,000 cells were seeded in each plate**.** All data represent the means ± SD (n=3 for C-D, F-G; **P*<0.05, ***P*<0.01, ****P*<0.001).

Fig.S2 HDAC6 interacts with H2A/H2A.X in response to DSBs, inhibiting H2A/H2A.X ubiquitination. (**A**) HeLa cells were transfected with Flag-H2A, Flag-H2B, or empty plasmids for 48 h. Chromatin fractions were subjected to immunoprecipitation with an anti-Flag antibody and analyzed by western blotting. (**B-C**) Bacterially purified His-HDAC6 fragments were co-incubated with recombinant H2A.X. (B) The schematic structure of HDAC6. DAC1: deacetylase domain 1 (DAC1); DAC2: deacetylase domain 2 (DAC2); ZnF: zinc finger ubiquitin-binding domain. Western blotting was performed to detect GST-H2A.X protein levels (C), and CBB staining was performed to detect His or His-tagged proteins. (**D**) HDAC6-WT or HDAC6 KO HCT116 cells were transfected with Flag-H2A.X and HA-ub, with or without Myc-HDAC6 for 48 h. The Flag-H2A.X proteins were immunoprecipitated from the whole cell lysates and analyzed by western blotting to detect changes in ubiquitination status. (**E**) HDAC6 WT or HDAC6 KO HCT116 cells were transfected with Flag-H2A and HA-ub, with or without Myc-HDAC6, for 48 h. The cells were then exposed to 10 Gy irradiation (IR) and released for 1 h. The Flag-H2A proteins were immunoprecipitated from the whole cell lysates and analyzed by western blotting to detect change in ubiquitination status. (**F**) HeLa cells were transfected with Flag-HDAC6 or an empty plasmid for 48 h. The cells were then exposed to 10 Gy (IR) and released for 1 h. Histone fractions and whole cell lysates were analyzed by western blotting.

Fig.S3 HDAC6 is associated with the H2A/H2A.X ubiquitination signaling cascade in DSB repair. (**A**) HeLa cells were transfected with Myc-HDAC6 or an empty plasmid for 48 h and then treated with VP16 (20 μM) for 2 h before whole cell lysate and chromatin fractions were analyzed by western blotting. (**B-C**) HeLa cells were transfected with Flag-HDAC6 or empty plasmids for 48 h and then exposed to 10 Gy IR and released for 1 h. The cells were then fixed and immunostained with the indicated antibodies. Representative images (B) and statistical analyses (C) are shown. All data represent the means ± SD (n=100, **P*<0.05, ***P*<0.01, ****P*<0.001).

Fig.S4 HDAC6 regulates the H2A/H2A.X ubiquitination signaling cascade in an RNF168-dependent manner during DSB repair. (**A**) HeLa cells were co-transfected with Flag-H2A, Myc-HDAC6, or an empty plasmid for 48 h and exposed to 10 Gy irradiation (IR) and released for 1 h. Chromatin fractions were subjected to immunoprecipitation with an anti-Flag antibody followed by western blotting. (**B-C**) HeLa cells were transfected with HDAC6 or negative control (NC) siRNA for 12 h and then transfected with GFP-RNF168 for 48 h before laser micro-irradiation. Images were captured every 10 s for 200 s, and the IR path signal intensity was calculated. (**D-E**) HeLa cells were co-transfected with GFP-RNF8 and mCherry-HDAC6 or an empty plasmid for 48 h before laser micro-irradiation. Images were captured every 10 s for 220 s, and the IR path signal intensity was calculated. (**F**) Various recombinant His-HDAC6 constructs and GST-RNF168 were subjected to *in vitro* ubiquitination assays in the presence of ATP, E1 (UBE1), E2 (UbcH5c), ub (ubiquitin), or H2A/H2B dimer, as indicated. Western blotting was performed with the indicated antibodies (His-HDAC6 WT: wild-type, DC1: DAC1 domain, DC2: DAC2 domain, ZnF: ZnF-UBP domain). The data represent the means ± SD.

Fig.S5 Nuclear HDAC6 is displaced from chromatin and degraded via the proteasome in response to DSBs. (**A**) HCT116 or U2OS cells were treated with etoposide (VP16) at increasing doses for 2 h. Whole cell lysate and chromatin fractions were analyzed by western blotting, with β-actin and H3, respectively, used as loading controls. (**B**) HCT116 cells were exposed to 10 Gy irradiation (IR) and released for the indicated times before nuclear and chromatin fractions were analyzed by western blotting, with Lamin-B1 and H3, respectively, used as loading controls. (**C**) HeLa cells were exposed to 10 Gy IR and released for the indicated times before the chromatin fractions were analyzed by western blotting. (**D-E**) HeLa cells were treated with laser micro-irradiation. After being fixed, the cells were immunostained with the indicated antibodies. Representative images are shown (D) and the intensity was calculated (E). (**F**) HCT116 cells were exposed to 10 Gy IR and released for the indicated times. The total RNA was extracted, and HDAC6, p21, and GAPDH relative gene expression levels were analyzed by real-time PCR. (**G**) HCT116 cells were co-transfected with Flag-HDAC6 and HA-ub for 48 h and treated with MG132 (10 μM) for 1 h and then etoposide (VP16, 20 μM) for an increasing time. The nuclear fractions were subjected to immunoprecipitation with an anti-Flag antibody and analyzed by western blotting. (**H**) Cells were co-transfected with Flag-HDAC6 wild-type (WT) or mutant (K116R)

for 48 h and treated with MG132 (10 μM) for 1 h and then etoposide (VP16, 20 μM) for 2 h. The nuclear fractions were subjected to immunoprecipitation with an anti-Flag antibody and analyzed by western blotting.

Fig.S6 RNF168 directly interacts with HDAC6 and mediates DSB-induced nuclear HDAC6 degradation. (**A-B**) Bacterially purified GST-RNF168 fragments were co-incubated with recombinant HDAC6. (A) The schematic structure of RNF168. RING: RING domain; UDM1: ub-dependent DSB recruitment module 1; UDM2: ub-dependent DSB recruitment module 2. Western blotting was performed to detect HDAC6 protein levels (B), and CBB staining was performed to detect GST or GST-tagged proteins. The asterisks indicate the corresponding protein bands. (**C-D**) Bacterially purified His-HDAC6 fragments were co-incubated with recombinant RNF168. (C) The schematic structure of HDAC6. DC1: deacetylase domain 1 (DAC1); DC2: deacetylase domain 2 (DAC2); ZnF: zinc finger ubiquitin-binding domain. Western blotting was performed to detect RNF168 protein levels (D), and CBB staining was performed to detect His or His-tagged proteins. The asterisks indicate the corresponding protein bands. (**E**) HeLa cells were transfected with RNF168 or negative control (NC) siRNAs and treated with etoposide (VP16, 20 μM) for 2 h before the whole cell lysate, nuclear, and chromatin fractions were analyzed by western blotting. (**F**) Whole cell lysates were extracted from HCT116 wild-type (RNF168-WT) cells or the two CRISPR clones, RNF168-KO$^{\#}$1 and RNF168-KO$^{\#}$2; they were then analyzed by western blotting to detect RNF168 protein levels. (**G**) HeLa cells were co-transfected with Flag-HDAC6 wild-type (WT) or mutant (K116R) and different HA-ubiquitin constructs (WT: wild-type ubiquitin; K48R: K48-mutant ubiquitin; K63R: K63-mutant ubiquitin), with or without Myc-RNF168, for 48 h. Then, the cells were treated with MG132 (10 μM) for 3 h, the nuclear fractions were subjected to immunoprecipitation with an anti-Flag antibody, and western blotting was performed to detect changes in ubiquitination status.

Fig.S7 RNF168-mediated nuclear HDAC6 degradation is beneficial for DSB repair and cell survival. (A-B) pEJ5-GFP U2OS (A) and DR-U2OS (B) cells were transfected with an HDAC6-specific inhibitor (ricolinostat) and subjected to NHEJ (A) and HR (B) assays, respectively.

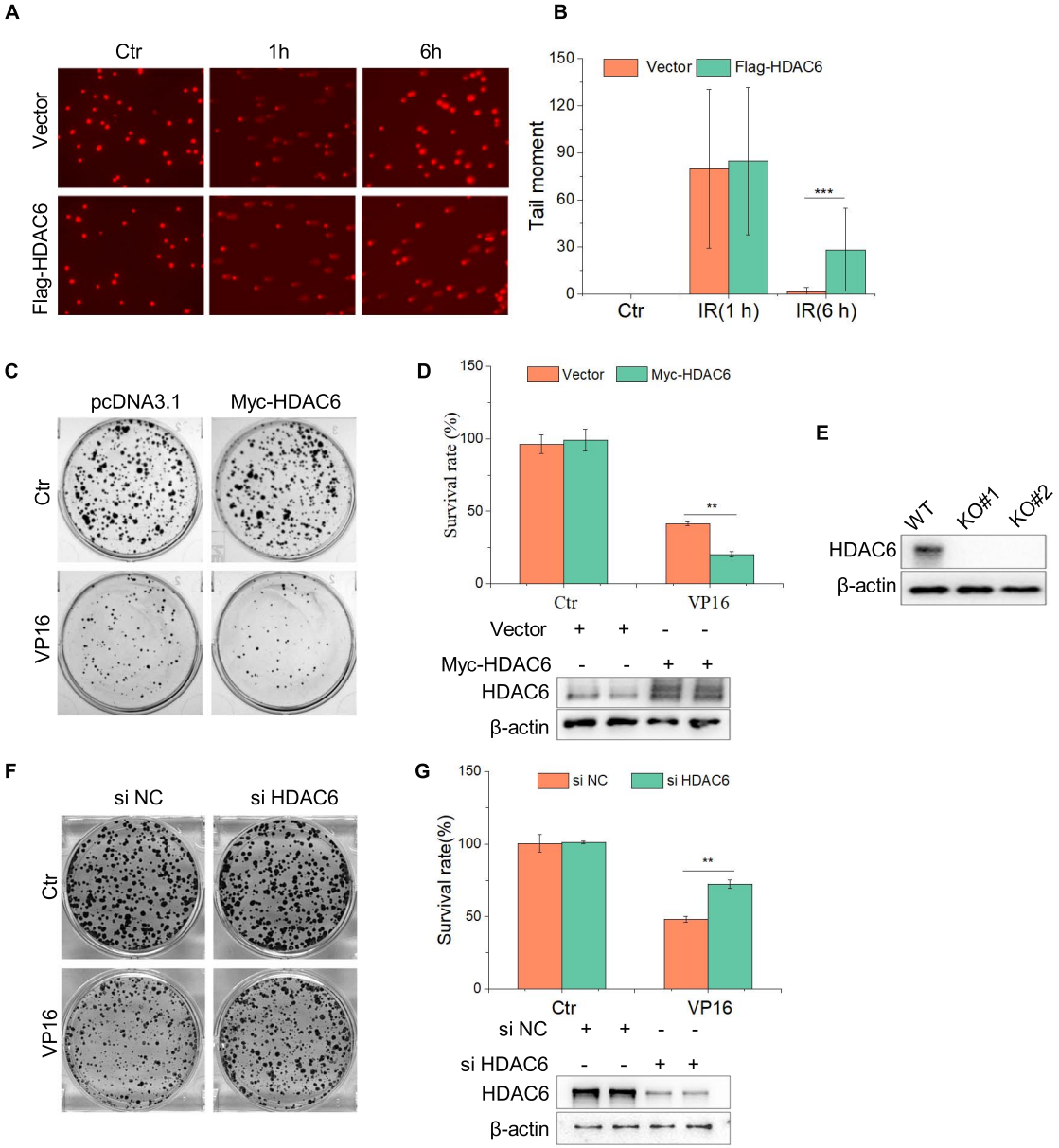


Fig.S1 HDAC6 is involved in DSB repair.

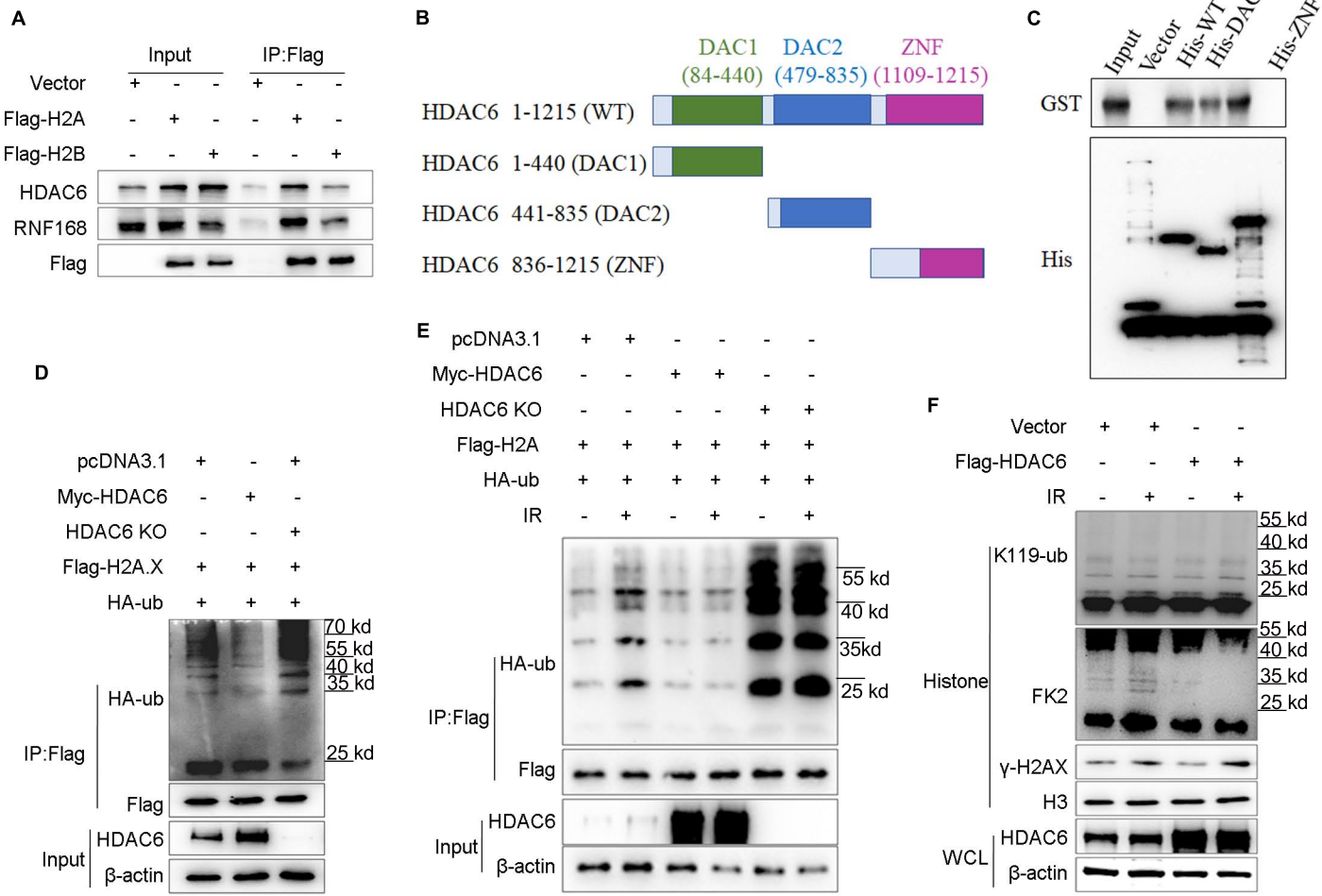


Fig.S2 HDAC6 interacts with H2A/H2A.X in response to DSBs, inhibiting H2A/H2A.X ubiquitination.

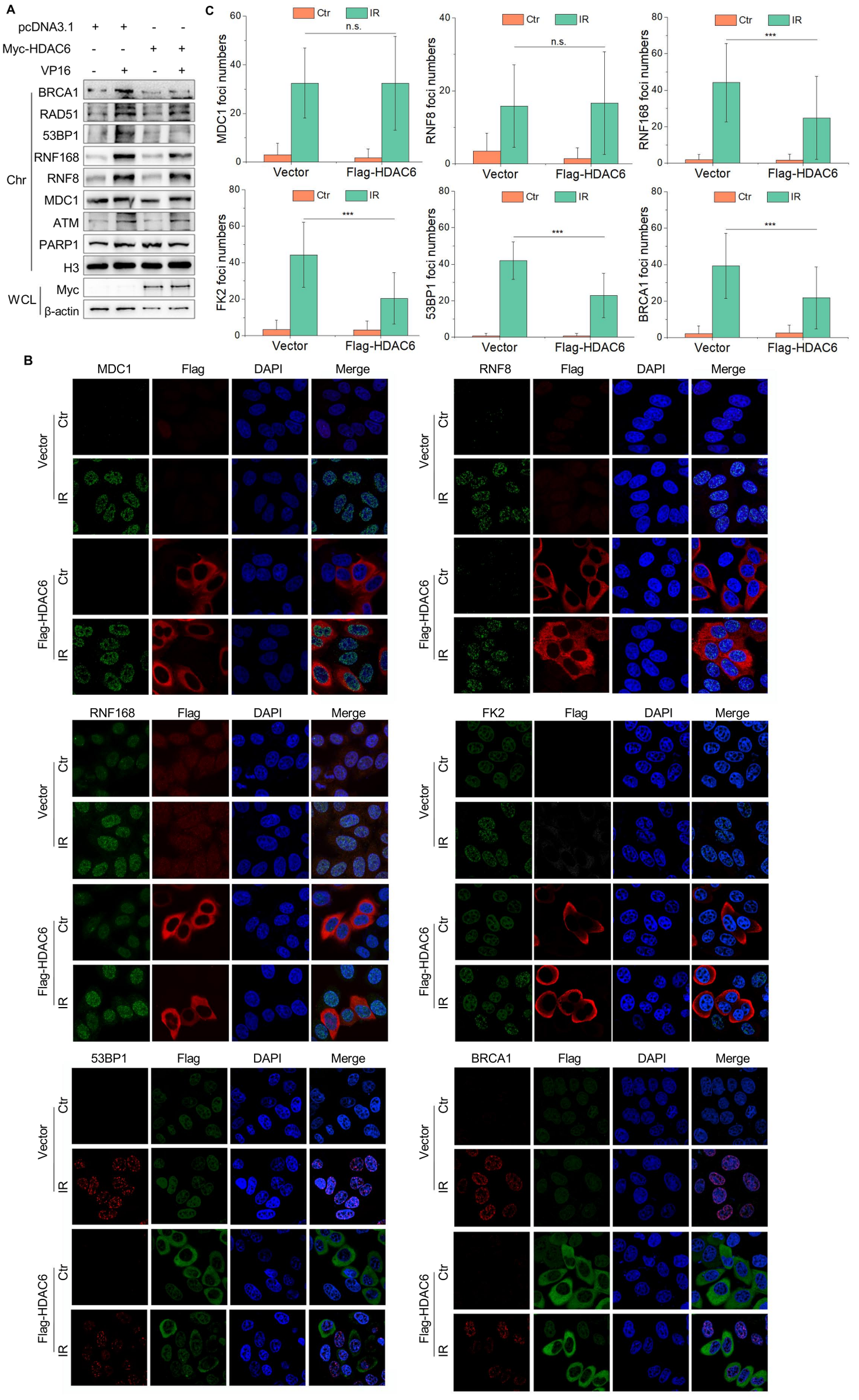


Fig.S3 HDAC6 is associated with the H2A/H2A.X ubiquitination signaling cascade in DSB repair.

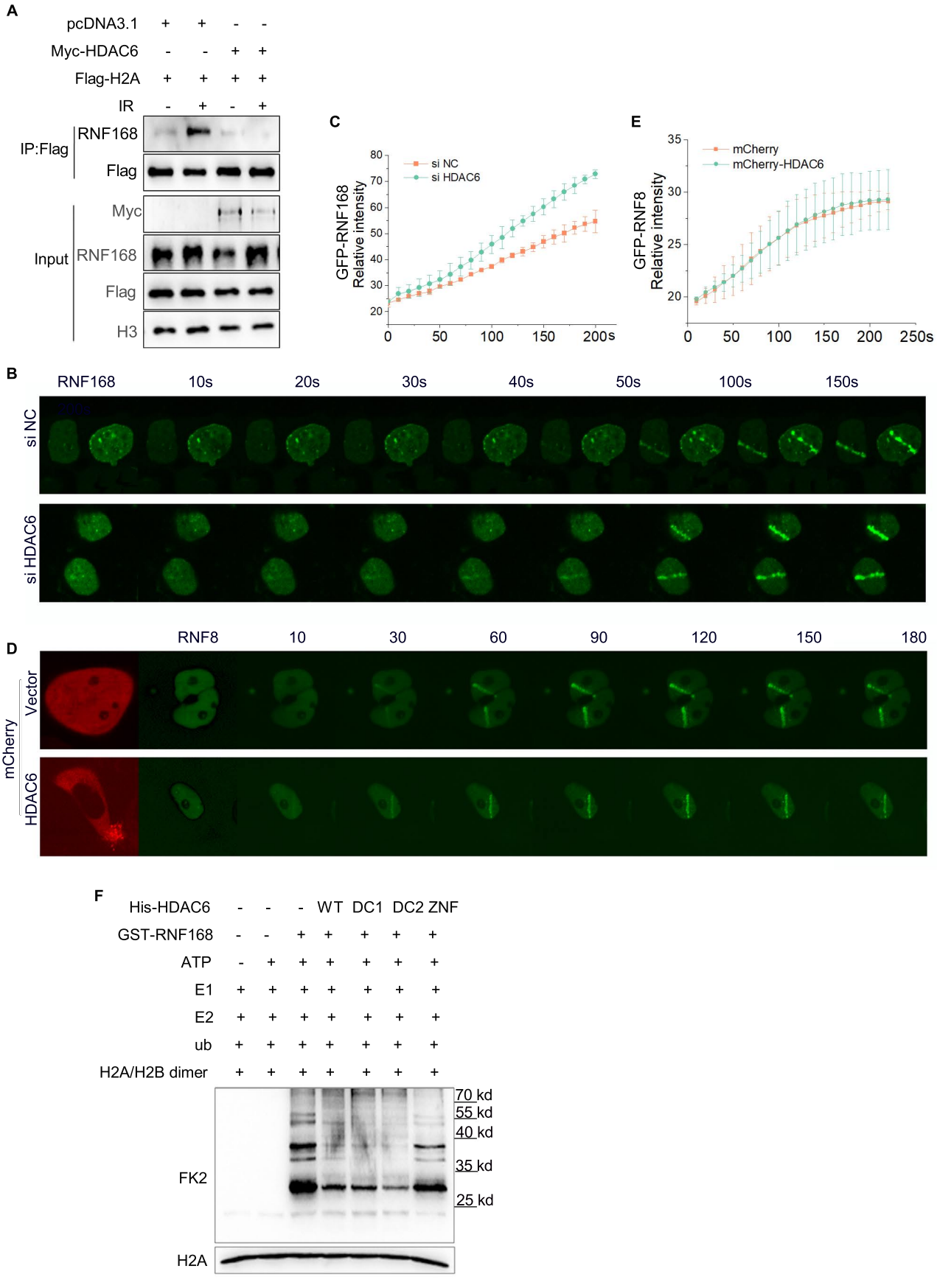
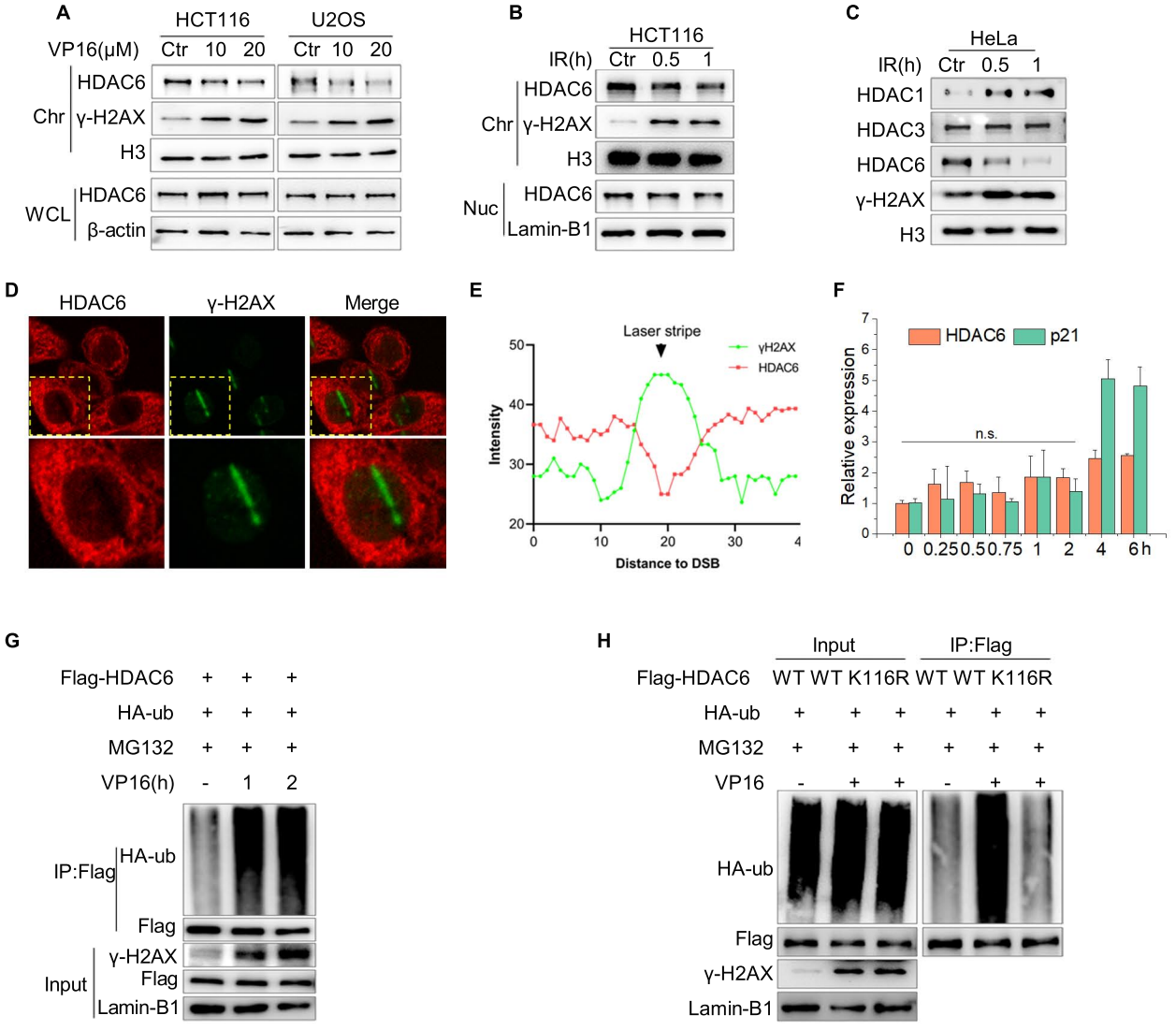


Fig.S4 HDAC6 regulates the H2A/H2A.X ubiquitination signaling cascade in an RNF168-dependent manner during DSB repair.



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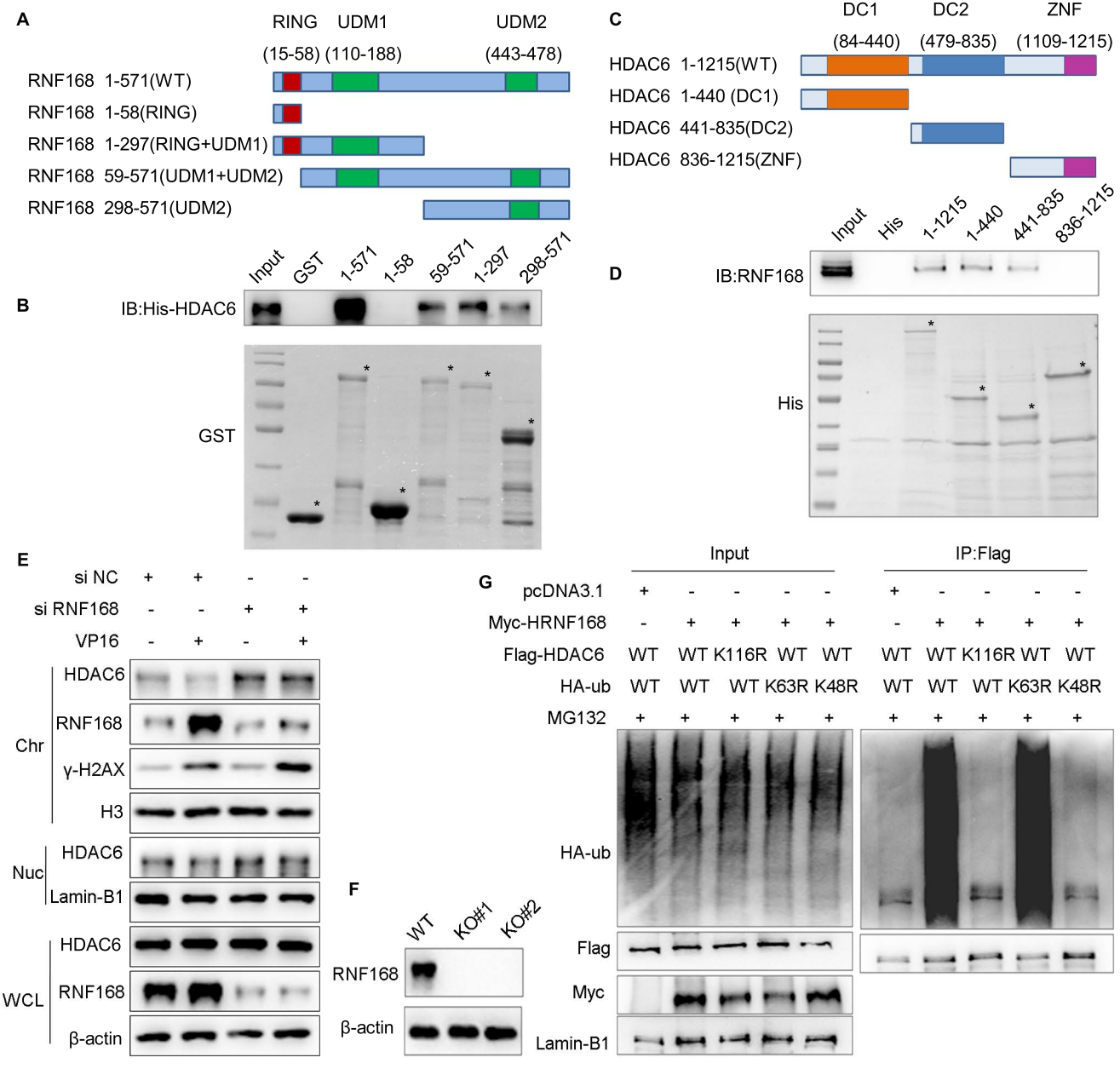

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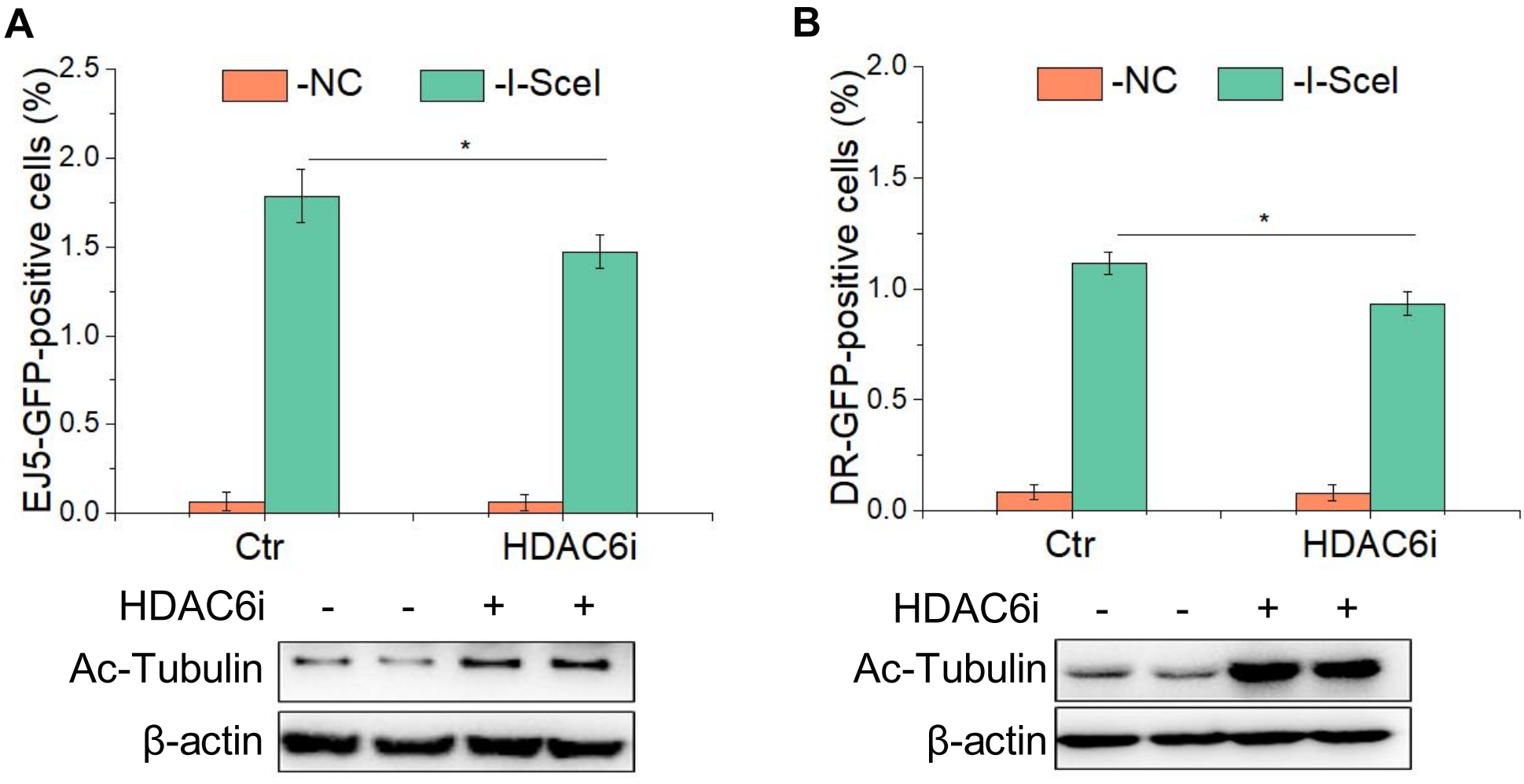

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