Danielsen et al., http://www.jcb.org/cgi/content/full/jcb.201106152/DC1

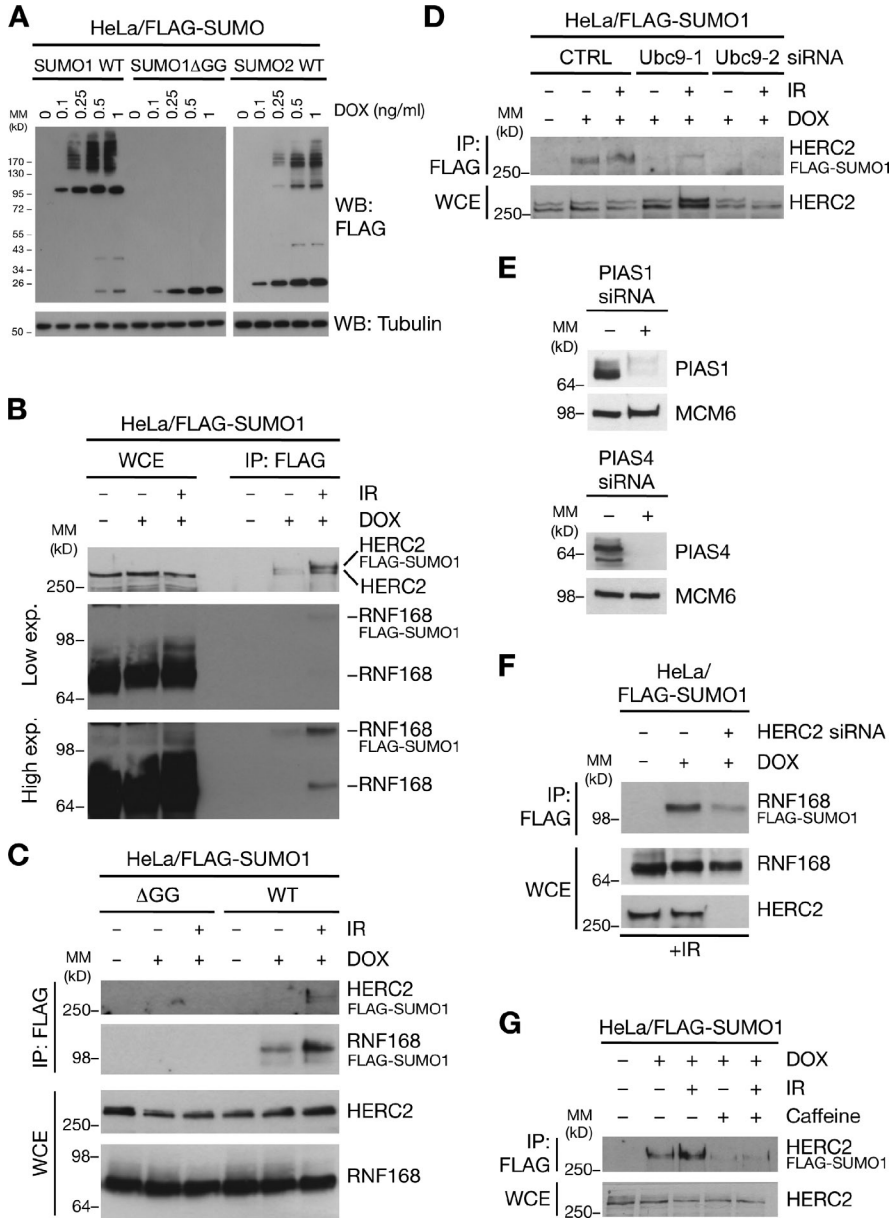

Figure S1. **Conjugation of SUMO1 to HERC2 and RNF168.** (A) Characterization of stable HeLa/FLAG-SUMO cell lines. Cells were treated with the indicated concentrations of doxycycline (DOX) for 24 h, harvested, and processed for immunoblotting with FLAG and tubulin (loading control) antibodies. MM, molecular mass; WB, Western blot. (B) HeLa/FLAG-SUMO1 cells were left untreated or induced to express FLAG-SUMO1 by treatment with doxycycline for 24 and then subjected to 10 Gy IR where indicated and harvested 1 h later. Cells were lysed under denaturing conditions, and protein SUMOylation was analyzed by immunoblotting of FLAG IPs with the indicated antibodies. exp., exposure; WCE, whole-cell extract. (C) HeLa/FLAG-SUMO1 WT or ΔGG cells were treated and processed as in A. The ectopic FLAG-SUMO1 WT and ΔGG proteins were expressed at comparable levels (not depicted). (D) HeLa/FLAG-SUMO1 cells were treated with control (CTRL) or Ubc9 siRNAs for 48 h, induced or not with doxycycline for an additional 24 h, and then treated with IR where indicated and harvested 1 h later. HERC2 SUMOylation was analyzed as in B. The Ubc9 siRNA sequences used were Ubc9-1 (5′-UCGAACCACCAUUAUUUCACCCGAA-3′) and Ubc9-2 (5′-GCUCAAGCAGAGGCCUACACGAUUU-3′). (E) Knockdown efficiency of PIAS1 and PIAS4 siRNAs. U2OS cells were transfected with control (−) or PIAS1 or PIAS4 siRNAs for 48 h as indicated, and the knockdown efficiency of the siRNAs was assessed by immunoblotting of total cell extracts. (F) Reduced SUMOylation of RNF168 in HERC2-depleted cells. HeLa/FLAG-SUMO1 cells were transfected with control or HERC2 siRNAs for 48 h and subsequently induced or not with doxycycline for an additional 24 h. Cells were then exposed to 10 Gy IR and harvested 1 h later. Cells were lysed under denaturing conditions, and protein SUMOylation was analyzed by immunoblotting of FLAG IPs with the indicated antibodies. (G) HeLa/FLAG-SUMO1 cells were treated as in A, except that where indicated, 10 mM caffeine was added to the medium 30 min before IR exposure.

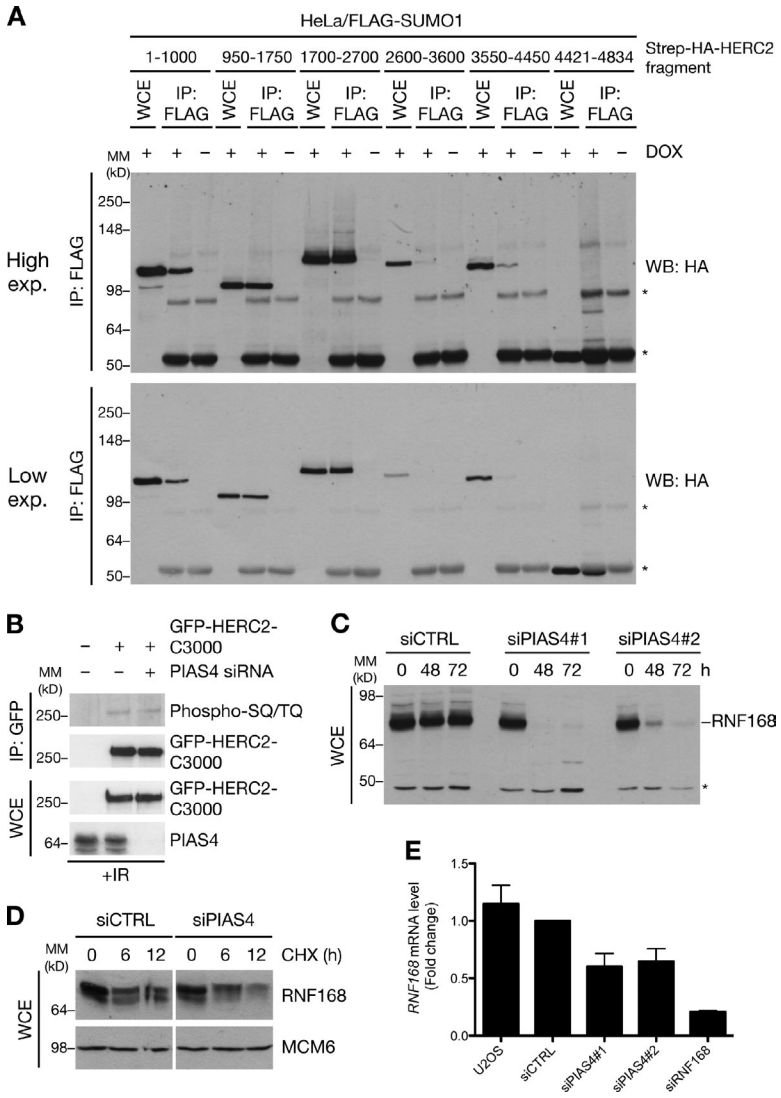

Figure S2. **SUMOylation and phosphorylation of HERC2 and impact of PIAS4 on RNF168 expression and stability.** (A) SUMOylation of overlapping HERC2 fragments. HeLa/FLAG-SUMO1 cells were transfected with doxycycline (DOX)-inducible constructs encoding Strep-HA–tagged versions of the indicated regions of HERC2 for 24 h and induced to express FLAG-SUMO1 and the transfected constructs or not by addition of doxycycline for an additional 24 h. Cells were lysed under denaturing conditions, subjected to FLAG IP, and immunoblotted with HA antibody. Discernible SUMOylated products are primarily evident in FLAG IPs from cells expressing Strep-HA-HERC2 (4421–4834). Note that in several cases, the unmodified Strep-HA-HERC2 fragments were immunoprecipitated nonspecifically by the FLAG resin. Asterisks denote bands corresponding to FLAG IgH cross-reacting with the HA antibody. exp., exposure; MM, molecular mass; WB, Western blot; WCE, whole-cell extract. (B) Extracts of HEK293T cells transfected with control (−) or PIAS4 siRNAs for 48 h and with the GFP-HERC2-C3000 construct (Fig. 4 A) for an additional 24 h were subjected to GFP IP followed by immunoblotting. (C) U2OS cells were transfected with control (CTRL) or PIAS4 siRNAs and harvested at the indicated times after transfection. Cell lysates were processed for immunoblotting with RNF168 antibody. The asterisk indicates a nonspecific band that serves as an internal loading control. (D) Depletion of PIAS4 reduces RNF168 half-life. U2OS cells transfected with control or PIAS4 siRNAs for 20 h were incubated in the presence of cycloheximide (CHX) for the indicated times to inhibit protein synthesis. Cell lysates were immunoblotted with RNF168 and MCM6 antibodies. (E) Effect of PIAS4 depletion on *RNF168* mRNA levels. U2OS cells were left untreated or transfected with indicated siRNAs. RNA was retrieved using the RNeasy mini kit (QIAGEN) 72 h later, and cDNA was synthesized using the SuperScript III reverse transcription kit and oligo$(dT)_{20}$ primers (Invitrogen). *RNF168* and *Actin* (reference gene) mRNA levels were measured using a quantitative PCR instrument (Mx3005P; Agilent Technologies). The primer sequences used were actin (forward 5′-CTCCCCGGGCTGTATTCC-3′ and reverse 5′-CCTCTCTTGCTCTGGGCCTC-3′) and RNF168 (forward 5′-TCCAGTTACACCCAAGTCTGAA-3′ and reverse 5′-GAGGCTGACCCAACCTGAGA-3′). Fold changes in *RNF168* mRNA levels were calculated with the comparative C_T method (ddC_T), using actin as a reference gene. Results depict the mean of two independent experiments.

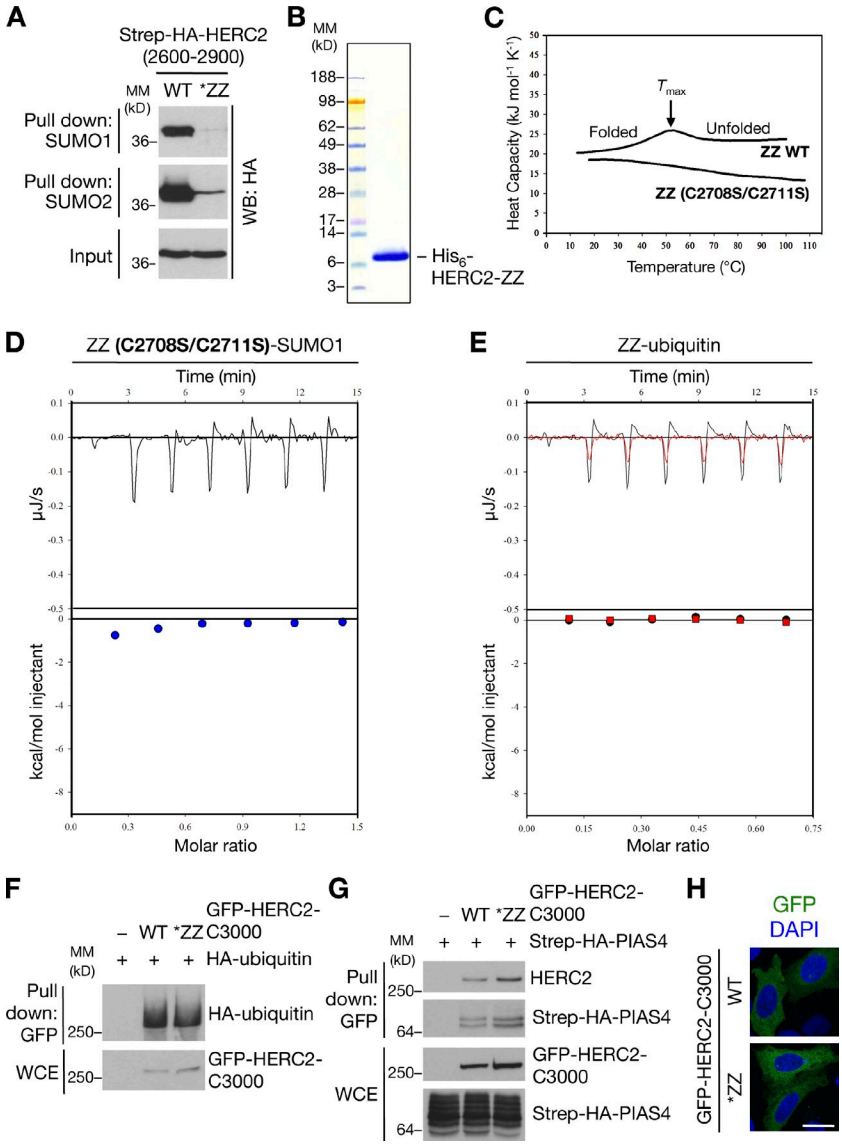

Figure S3. **Characterization of the HERC2 ZZ domain.** (A) HEK293T cells were transfected with the indicated Strep-HA-HERC2(2600–2900) constructs and harvested 24 h later, and cell extracts were incubated with SUMO1 or SUMO2 agarose. Bound complexes and inputs were analyzed by immunoblotting with HA antibody. MM, molecular mass; WB, Western blot. (B) Coomassie-stained gel of the HERC2 ZZ domain, purified as described in Materials and methods. (C) Differential scanning calorimetry analysis of the HERC2 ZZ domain. Thermal unfolding of Zn^{2+}-reconstituted HERC2 ZZ domains (WT or *ZZ mutated) was monitored on a VP–differential scanning calorimetry capillary cell microcalorimeter (MicroCal). Scans were performed from 10 to 100°C at a scan rate of 90°C/h, and the data were corrected by subtracting the buffer baseline. The peak at 52°C (indicated by T_{max}) for the WT ZZ domain is characteristic for a cooperative unfolding transition, indicating that the protein is folded at lower temperatures. Unlike ZZ WT, the C2708S/C2711S mutant does not show any transition in differential scanning calorimetry, indicating a lack of structural integrity. (D) ITC showing absence of high-affinity binding between the HERC2 ZZ (C2708S/C2711S) domain and SUMO1. Purified SUMO1 was titrated into the ITC sample cell at 10°C containing the recombinant HERC2 ZZ (C2708S/C2711S) domain until saturation was achieved. Heat effects (top) and cumulative heat effects (bottom) of the SUMO1-ZZ (C2708S/C2711S) domain interaction are shown. (E) ITC experiment showing absence of high-affinity binding between the HERC2 ZZ domain and ubiquitin. Purified ubiquitin was titrated into the ITC sample cell at 10 or 25°C containing the folded, recombinant HERC2 ZZ domain until saturation was achieved. Heat effects (top) and cumulative heat effects (bottom) of the ubiquitin-ZZ domain interaction are shown. The solid line represents the single binding site model fit to the experimental data. Red and black experiments were performed at 25 and 10°C, respectively. (F) Mutation of the HERC2 ZZ domain does not affect its autoubiquitylation activity. HEK293T cells were transfected with indicated constructs for 24 h, lysed under denaturing conditions, and subjected to GFP immunoprecipitation followed by immunoblotting with GFP and HA antibodies. WCE, whole-cell extract. (G) Mutation of the ZZ domain in HERC2 does not affect its binding to PIAS4 and HERC2 oligomerization. HEK293T cells were transfected with indicated constructs for 24 h, lysed, and subjected to GFP IP followed by immunoblotting with HERC2, HA, and GFP antibodies. The antibody used to detect endogenous HERC2 recognizes an N-terminal epitope and does not react with GFP-HERC2-C3000. (H) Identical subcellular localization of HERC2-C3000 WT and *ZZ fragments. U2OS cells were transfected with GFP-HERC2-C3000 constructs (Fig. 4 A) for 24 h and were then fixed and stained with DAPI. Bar, 10 µm.