

Supplementary Information

Molecular pathogenesis of Spondylocheiroidysplastic Ehlers-Danlos syndrome caused by mutant ZIP13 proteins

Bum-Ho Bin, Shintaro Hojyo, Toshiaki Hosaka, Jinhyuk Bhin, Hiroki Kano, Tomohiro Miyai, Mariko Ikeda, Tomomi Kimura-Someya, Mikako Shirouzu, Eun-Gyung Cho, Kazuhisa Fukue, Taiho Kambe, Wakana Ohashi, Kyu-Han Kim, Juyeon Seo, Dong-Hwa Choi, Yeon-Ju Nam, Daehee Hwang, Ayako Fukunaka, Yoshio Fujitani, Shigeyuki Yokoyama, Andrea Superti-Furga, Shiro Ikegawa, Tae Ryong Lee and Toshiyuki Fukada

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TABLE:

Supplementary Table 1. Primers used for the construction of G64 mutants

Mutant	Sense primer	Anti-sense primer
G64A	5'- <u>GCT</u> TCCCTCATGGTGGGGCTCAGTG	5'-CAGGAGGGAGCAGATCCAGGTGTC
G64C	5'- <u>TGT</u> TCCCTCATGGTGGGGCTCAGTG	5'-CAGGAGGGAGCAGATCCAGGTGTC
G64S	5'- <u>TCT</u> TCCCTCATGGTGGGGCTCAGTG	5'-CAGGAGGGAGCAGATCCAGGTGTC
G64I	5'- <u>ATT</u> TCCCTCATGGTGGGGCTCAGTG	5'-CAGGAGGGAGCAGATCCAGGTGTC
G64L	5'- <u>CTT</u> TCCCTCATGGTGGGGCTCAGTG	5'-CAGGAGGGAGCAGATCCAGGTGTC
G64E	5'- <u>GAG</u> TCCCTCATGGTGGGGCTCAG	5'-CAGGAGGGAGCAGATCCAGGTGTC
G64R	5'- <u>AGAT</u> CCCTCATGGTGGGGCTCAG	5'-CAGGAGGGAGCAGATCCAGGTGTC
G64N	5'- <u>AACT</u> CCCTCATGGTGGGGCTCAGTGG	5'-CAGGAGGGAGCAGATCCAGGTGTC
G64Q	5'- <u>CAGT</u> CCCTCATGGTGGGGCTCAGTGG	5'-CAGGAGGGAGCAGATCCAGGTGTC
*Mutated sequences are underlined		

FIGURES:

Supplementary Figure 1

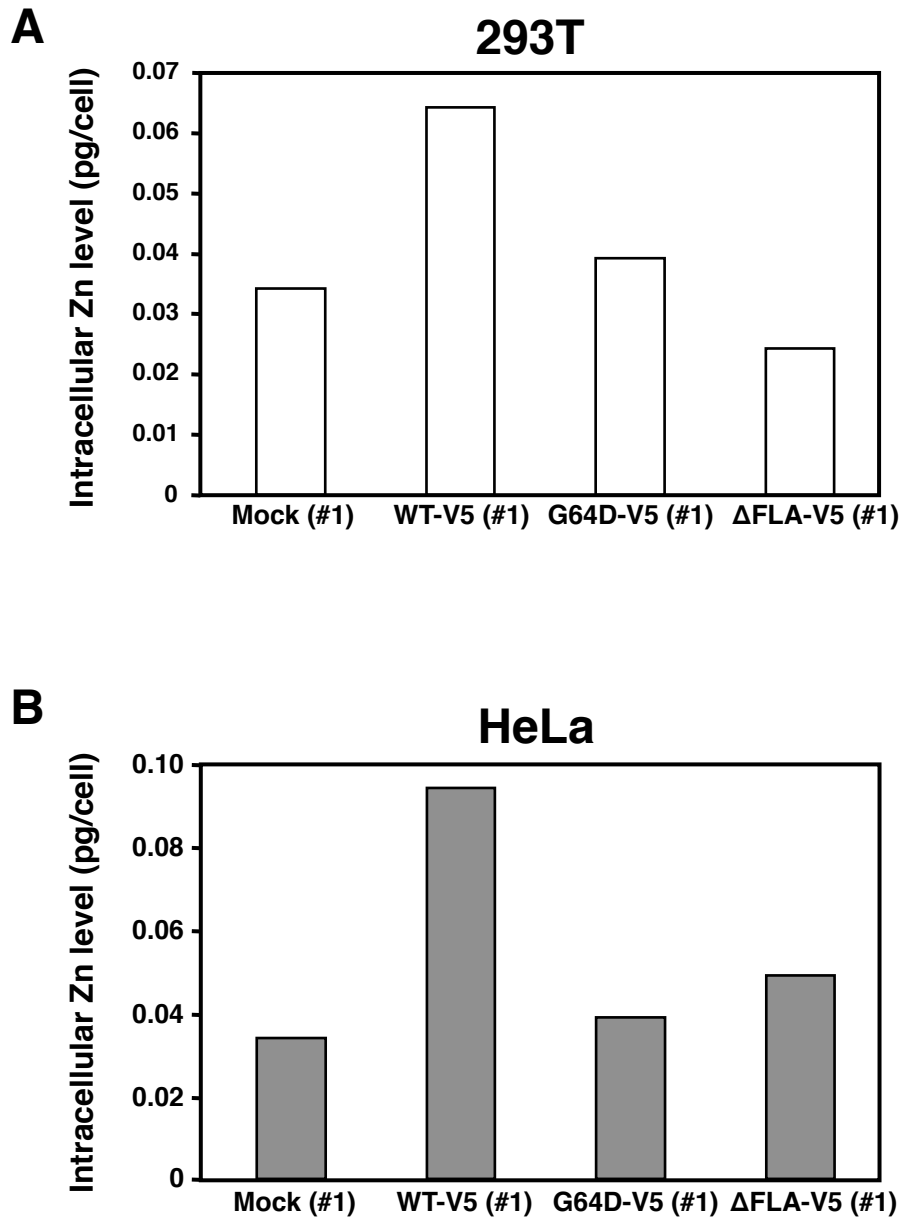


Fig S1. Intracellular Zn level in wild-type or mutant ZIP13 expressing cell lines.

Cellular Zn level was measured by ICP-AES in representative **(A)** 293T (**Fig 5**) and **(B)** HeLa clones (**Fig E2**) stably expressing Mock, wild-type (WT-V5), G64D mutant (G64D-V5), or ΔFLA mutant (ΔFLA-V5) vector.

Supplementary Figure 2

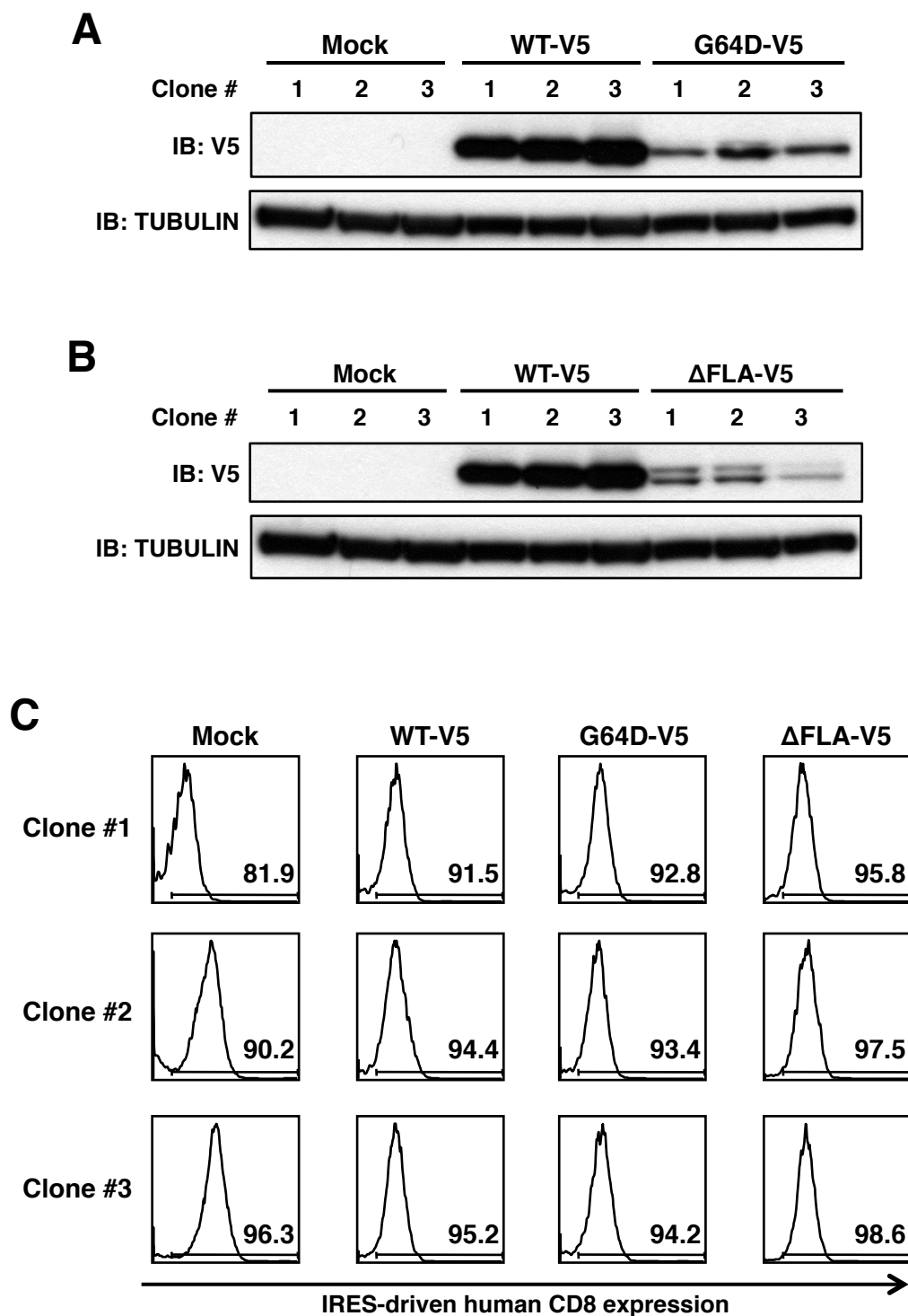


Fig S2. Decreased protein levels of the SCD-EDS pathogenic mutants of ZIP13 in stably expressing HeLa cells.

A. Protein levels of the G64D mutant ZIP13. Cell lysates of three representative HeLa clones stably expressing C-terminally V5 epitope-tagged WT-V5 or G64D-V5 ZIP13, were analyzed by western blotting using an anti-V5 antibody.

B. Protein levels of the ΔFLA mutant ZIP13. Cell lysates of three representative HeLa clones stably expressing WT-V5 or ΔFLA-V5 were analyzed by western blotting using an anti-V5 antibody.

C. The hCD8 expression level as an indicator of the amount of transfected plasmid DNA (pMX-WT-IRES-hCD8, pMX-G64D-IRES-hCD8, or pMX-ΔFLA-IRES-hCD8). Three representative HeLa clones stably expressing WT-V5, G64D-V5, or ΔFLA-V5, were analyzed by flow cytometry using an APC-conjugated anti-hCD8 antibody. Histograms were gated on hCD8-positive cells.

Supplementary Figure 3

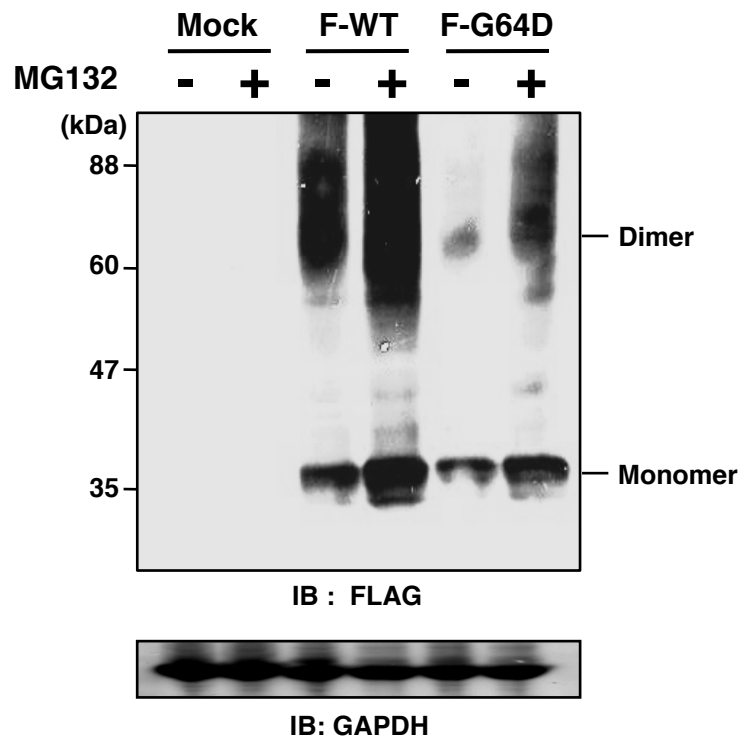


Fig S3. Increased dimers of G64D mutant protein in the presence of proteasome inhibitor MG132. The dimer formation of ZIP13 was analyzed by western blotting under non-reducing conditions using the lysates of HT1080 cells expressing N-terminally 3xFLAG-tagged wild-type (F-WT) and G64D mutant (F-G64D) ZIP13 (Bin et al, 2011).

Supplementary Figure 4

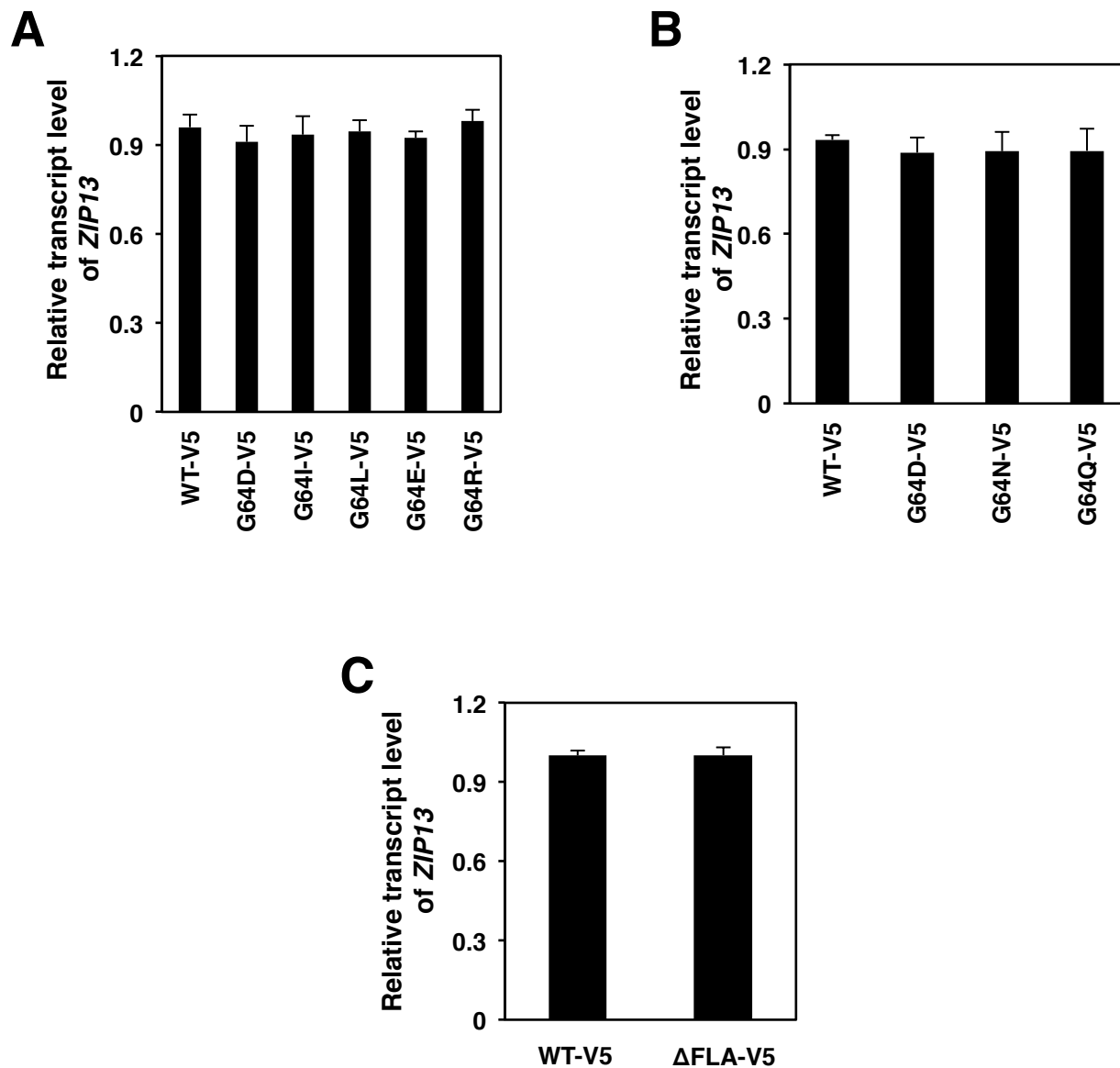


Fig S4. Equivalent ZIP13 mRNA expression levels in cells transiently expressing wild-type ZIP13 (WT-V5), G64 mutants, and ΔFLA mutant (ΔFLA-V5).

A. The transcript levels of mutant constructs encoding ZIP13-V5 with various amino acids at position 64 were comparable to that of wild type.

B. The transcript levels of the mutant constructs encoding G64N-V5 and G64Q-V5 were comparable to that of wild type.

C. The transcript level of the mutant construct encoding ΔFLA-V5 was comparable to that of wild type.

Supplementary Figure 5

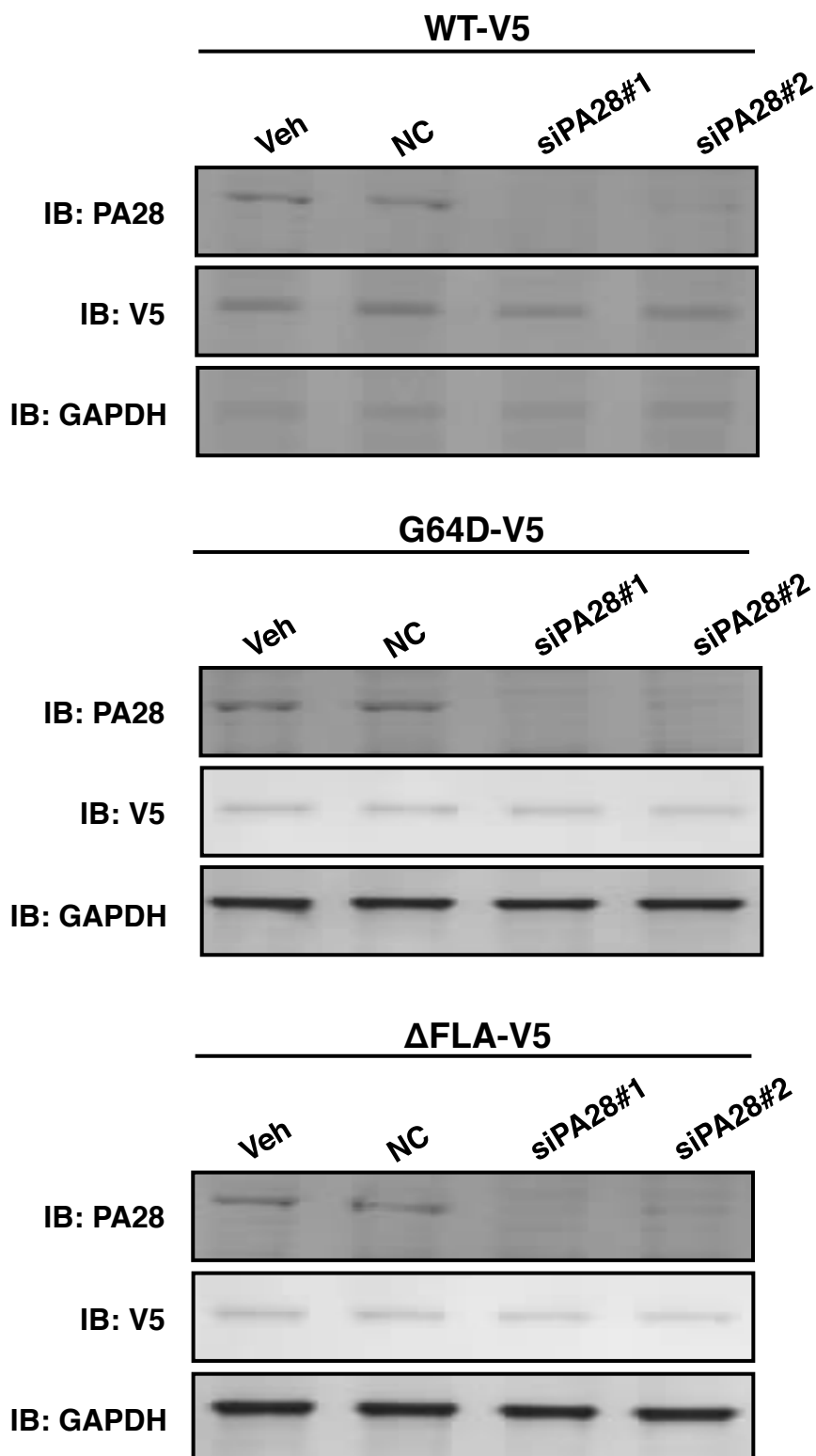


Fig S5. The 20S proteasome is not significantly involved in the degradation of SCD-EDS pathogenic ZIP13 mutants.

The siRNA targeting PA28, which induces the 20S proteasome, did not affect the protein expression of C-terminally V5 epitope-tagged WT-V5, G64D-V5, or Δ FLA-V5 ZIP13 in 293T cells. The siRNAs were transfected into 293T cells stably expressing each type of ZIP13 protein. Seventy-two hours post-transfection, the cells were harvested and subjected to western blotting using an anti-PA28 or anti-V5 antibody.

Supplementary Figure 6

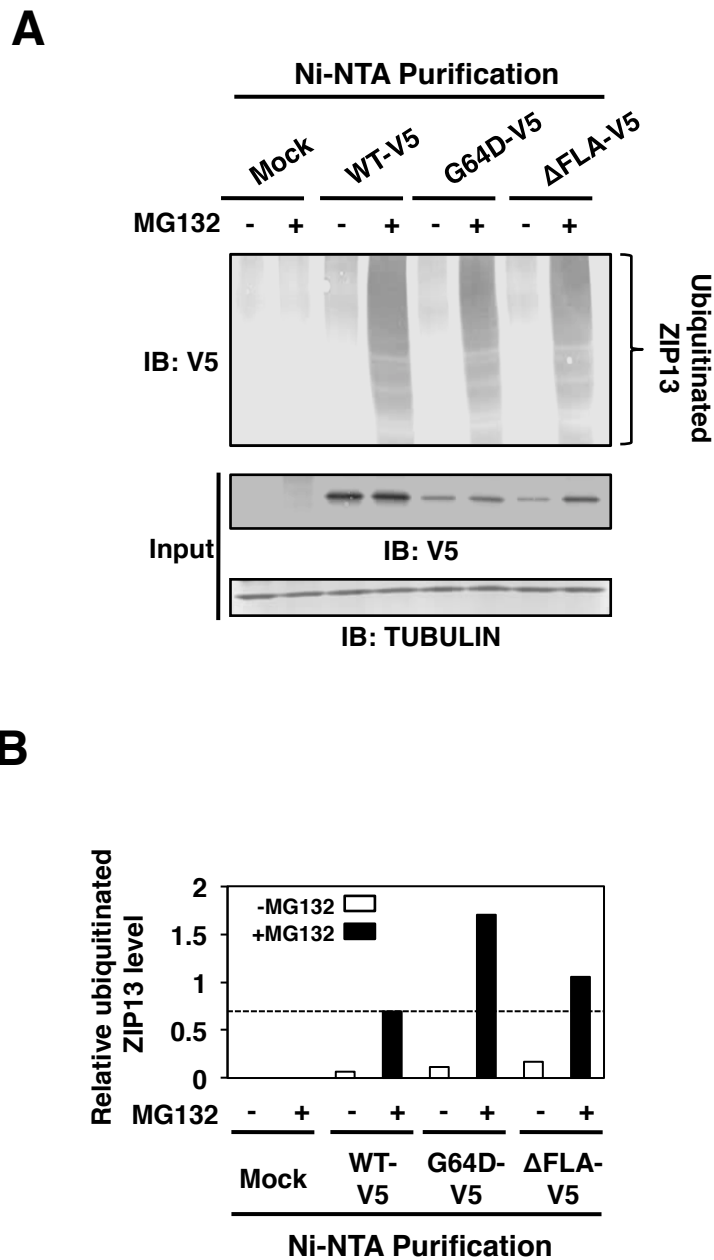


Fig S6. Degradation of the SCD-EDS pathogenic ZIP13 mutants involves ubiquitination.

A. Detection of ubiquitinated ZIP13 proteins. 293T cells co-expressing 6x histidine-tagged ubiquitin and WT-V5, G64D-V5, or ΔFLA-V5 were treated with 10 μM MG132 for 6 h, lysed with denaturing buffer, and purified by Ni-NTA agarose. The ZIP13 proteins in the purified samples were then subjected to western blotting. Ubiquitinated ZIP13 proteins were detected using an anti-V5 antibody.

B. The ubiquitinated/normal protein ratio for the wild-type and mutant ZIP13 proteins were shown.

Supplementary Figure 7

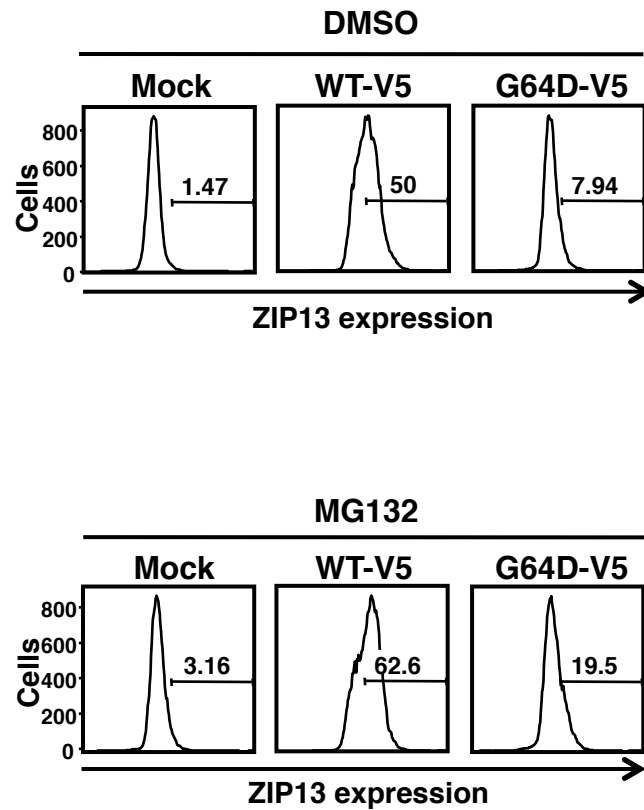


Fig S7. Intracellular flow cytometric analysis for exogenous ZIP13 expression.

HeLa stable clones expressing WT-V5 or G64D-V5 were treated with DMSO or 10 μ M MG132. After fixation and permeabilization, the cells were stained with the monoclonal antibody 35B11, followed by goat-anti-mouse-Alexa 488.

Supplementary Figure 8

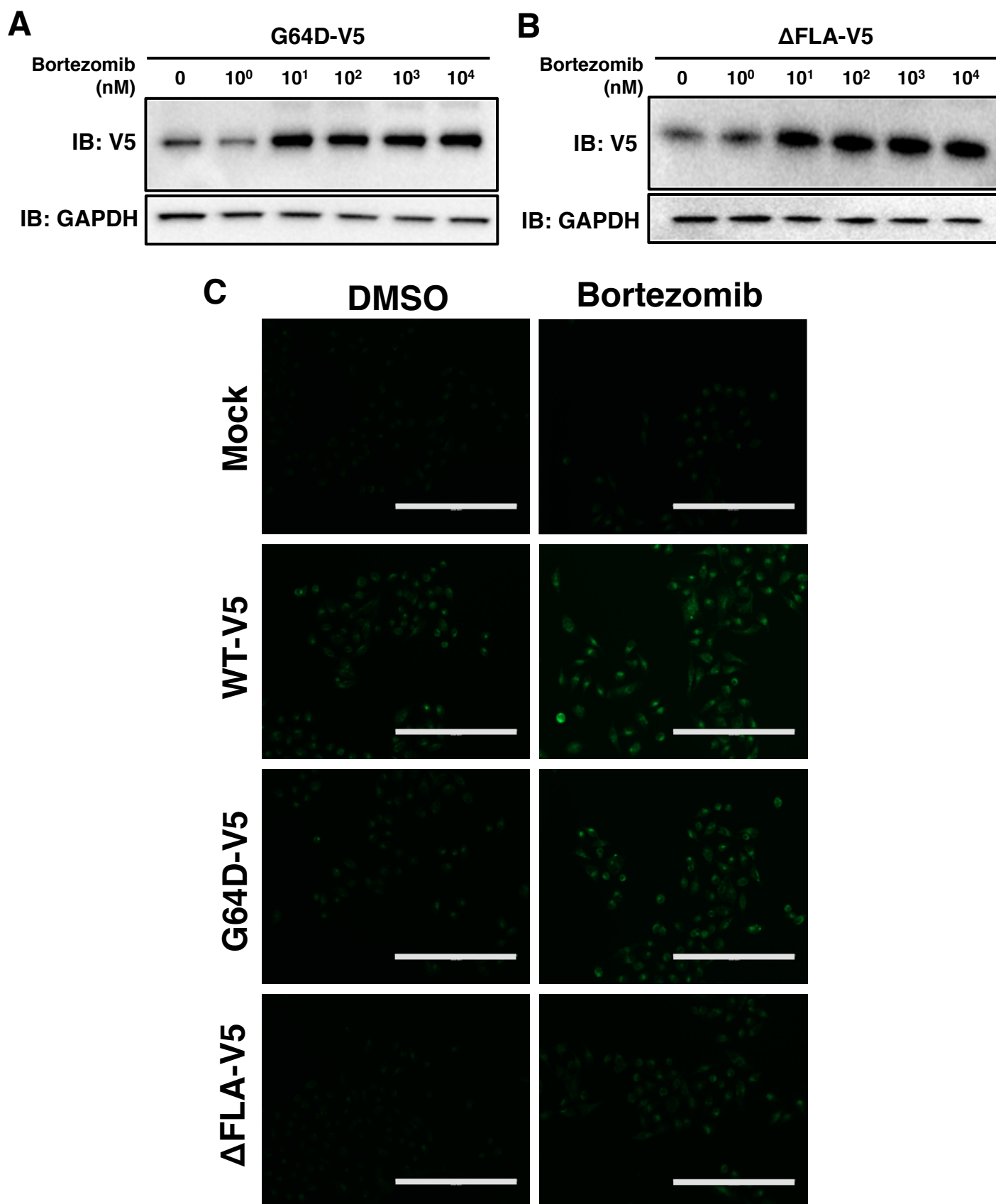


Fig S8. Bortezomib restored the mutant ZIP13 proteins and the intracellular Zn homeostasis.

A. 293T cells stably expressing G64D-V5 were treated with Bortezomib at the indicated concentrations, followed by western blotting using an anti-V5 antibody.

B. 293T cells stably expressing ΔFLA-V5 were treated with Bortezomib at the indicated concentrations, followed by western blotting using an anti-V5 antibody.

C. HeLa cells stably expressing WT-V5, G64D-V5, or ΔFLA-V5, were treated with 10 nM Bortezomib for 6 h. The intracellular Zn level was monitored by Fluozin-3 in combination with Zn pyrithione treatment. Bars, 200 μm.

Supplementary Figure 9

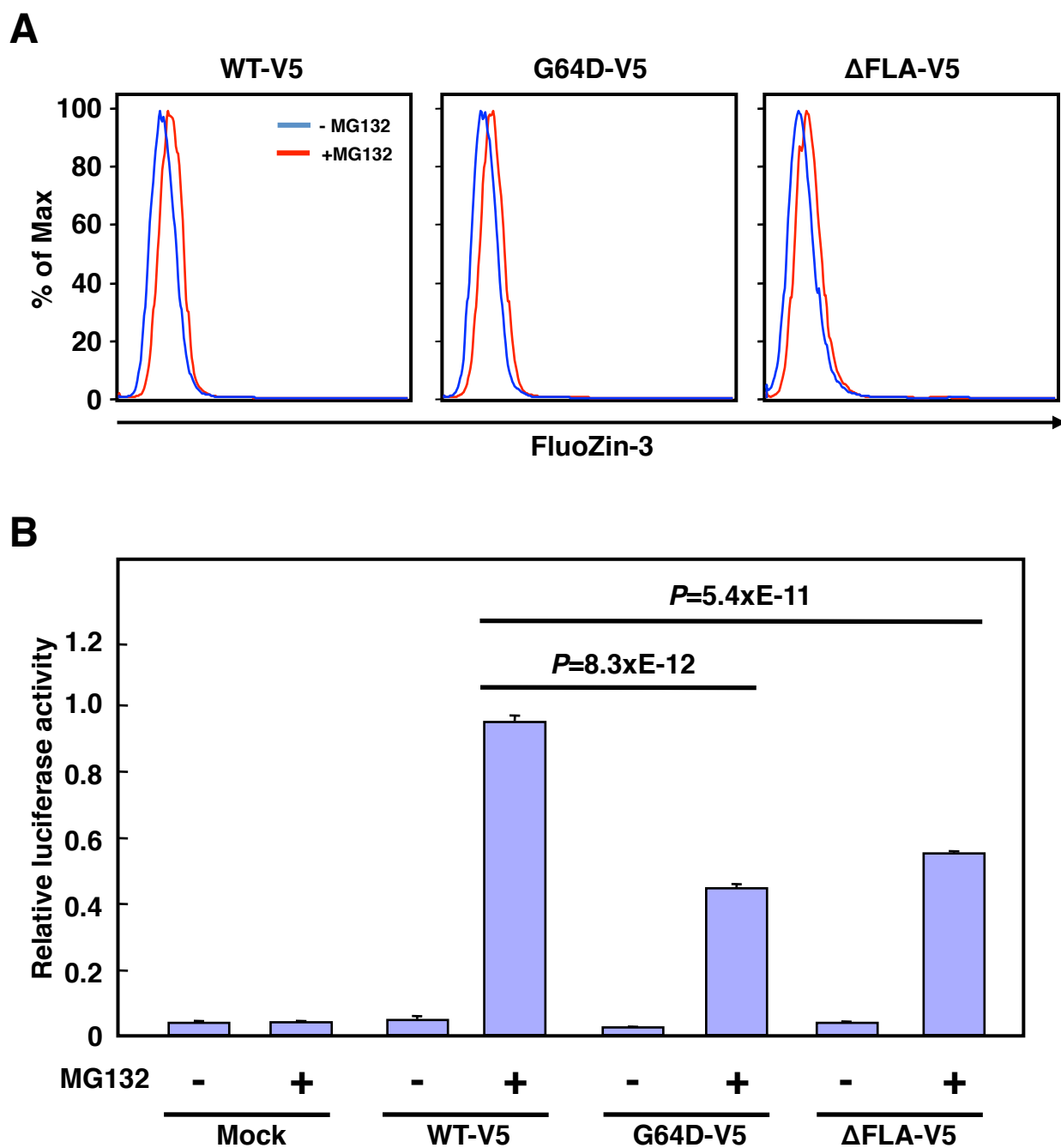


Fig S9. MG132 restored the intracellular Zn homeostasis.

A. HeLa cells stably expressing indicated expression plasmids were treated with DMSO (blue line) or 10 μ M MG132 (red line) for 6 h, followed by incubation with 0.1 μ M of FluoZin-3 and 7AAD. 7AAD-negative populations were subjected to flow cytometric analysis.

B. HeLa cells stably expressing indicated expression plasmids were transiently transfected with *MT1* promoter reporter and phRL-TK plasmids, followed by incubation of 10 μ M MG132 for 6 h and analyzed the luciferase activity. Data are shown as mean \pm s.e.m. (Student's t-test).

Supplementary Figure 10

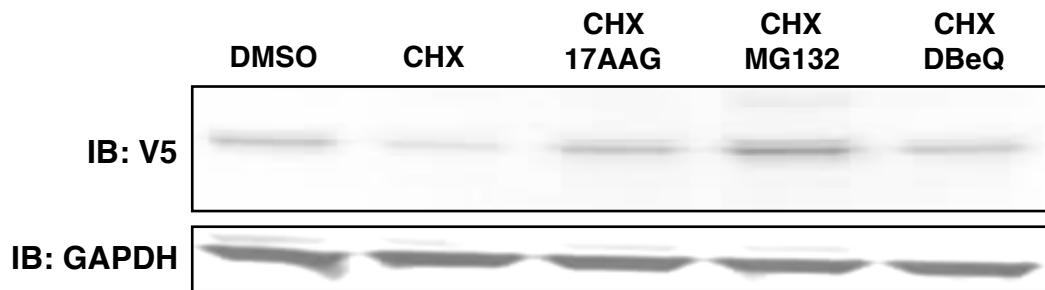


Fig S10. HSP90 inhibitor treatment restores the G64D mutant ZIP13 protein level.

Treatment with an HSP90 inhibitor, 17-(Allylamino)-17-demethoxygeldanamycin (17AAG), restored the protein level of G64D-V5 in the presence of CHX. 293T cells stably expressing G64D-V5 were treated with 10 μ M 17AAG, 10 μ M DBeQ, or 10 μ M MG132 in the presence of CHX for 6 h. Total cell lysates were subjected to western blotting using an anti-V5 antibody.

Supplementary Figure 11

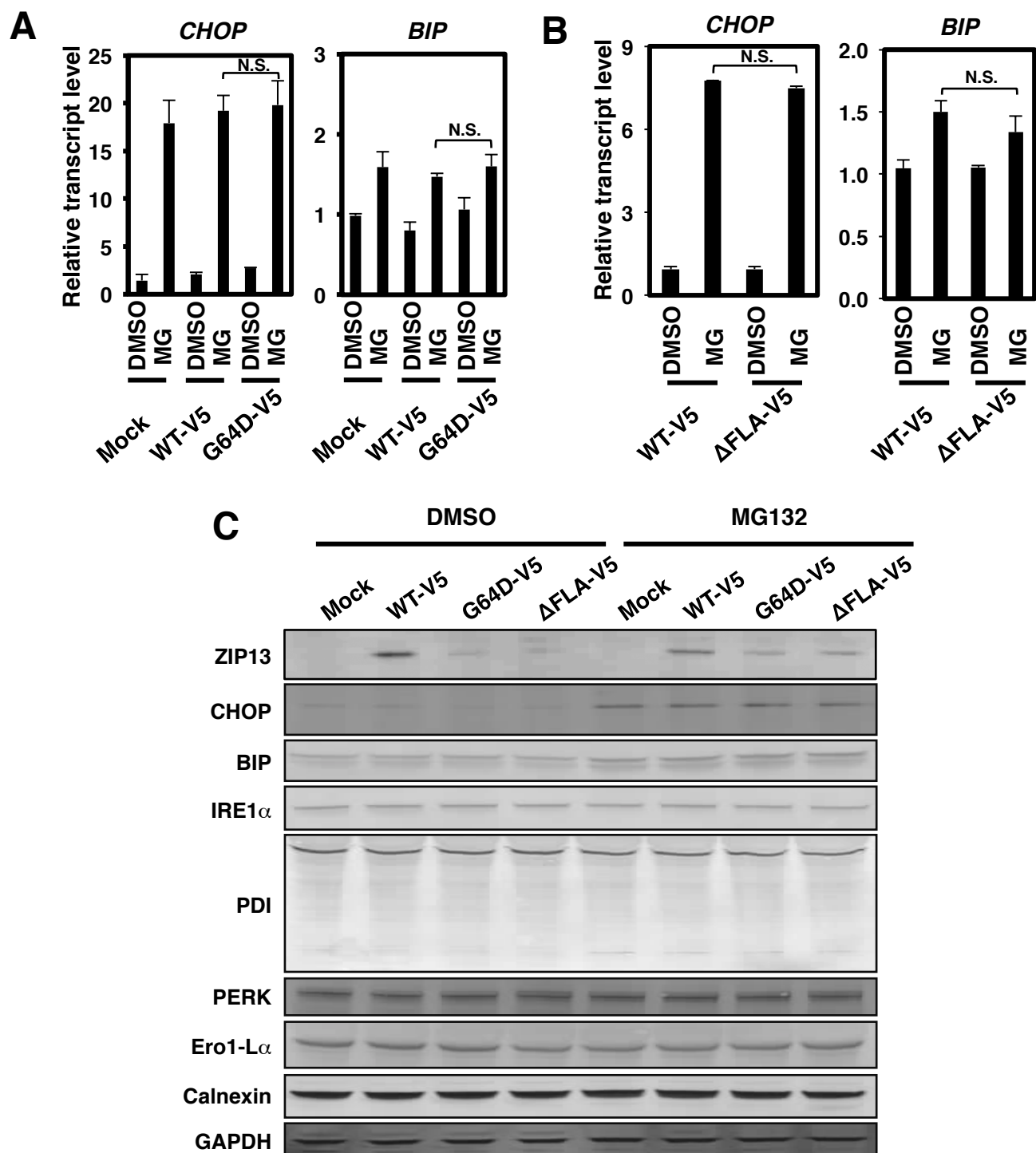


Fig S11. Expression levels of ER stress responsive genes and proteins.

A. The mRNA expression levels of *CHOP* and *BIP* in 293T cells expressing WT-V5 or G64D-V5. Cells were transfected with expression plasmids, and then treated with 0.5 μ M MG132 for 6 h. The mRNA expression levels of *CHOP* and *BIP* were analyzed by RT-qPCR. Data are representative of three experiments, and shown as mean \pm s.e.m. (Student's t-test). N.S.: not significant, MG: MG-132

B. The mRNA expression levels of *CHOP* and *BIP* in 293T cells expressing WT-V5 or Δ FLA-V5. RT-qPCR analysis for *CHOP* and *BIP* was performed as described in **A**. Data are representative of three experiments, and shown as mean \pm s.e.m. (Student's t-test). N.S.: not significant; MG: MG-132

C. Protein expression levels of CHOP, BIP, IRE1 α , PDI, PERK, Ero1-L α , and calnexin in 293T cells expressing WT-V5, G64D-V5, or Δ FLA-V5. The cells were treated with 0.5 μ M MG132 for 6 h. The cell lysate was subjected to SDS-PAGE and analyzed by western blotting using antibodies to the indicated proteins.

REFERENCE:

Bin BH, Fukada T, Hosaka T, Yamasaki S, Ohashi W, Hojyo S, Miyai T, Nishida K, Yokoyama S, Hirano T (2011) Biochemical characterization of human ZIP13 protein: a homo-dimerized zinc transporter involved in the Spondylocheiro dysplastic Ehlers-Danlos syndrome. *J Biol Chem.* 286: 40255-40265.