Supplementary Information

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

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### TABLE:

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

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Sense primer</th>
<th>Anti-sense primer</th>
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<tbody>
<tr>
<td>G64A</td>
<td>5'-GCTTCCCTCATGGGCTCAGTG</td>
<td>5'-CAGGAGGGAGCGAGATCCAGGTGTC</td>
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<tr>
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<td>G64I</td>
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<td>G64L</td>
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<td>G64R</td>
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<tr>
<td>G64N</td>
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<tr>
<td>G64Q</td>
<td>5'-CAGTCCCTCATGGGCTCAGTG</td>
<td>5'-CAGGAGGGGACAGATCCAGGTGTC</td>
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*Mutated sequences are underlined*
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

**A**

<table>
<thead>
<tr>
<th>Clone #</th>
<th>Mock</th>
<th>WT-V5</th>
<th>G64D-V5</th>
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<tbody>
<tr>
<td>1</td>
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IB: V5

IB: TUBULIN

**B**

<table>
<thead>
<tr>
<th>Clone #</th>
<th>Mock</th>
<th>WT-V5</th>
<th>ΔFLA-V5</th>
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<td>1</td>
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<td>3</td>
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</table>

IB: V5

IB: TUBULIN

**C**

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.
**Fig S3.** Increased dimers of G64D mutant protein in the presence of proteosome 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).
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.
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 ΔFLA-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

**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.

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 μM MG132. After fixation and permeabilization, the cells were stained with the monoclonal antibody 35B11, followed by goat-anti-mouse-Alexa 488.
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**

**A.** HeLa cells stably expressing indicated expression plasmids were treated with DMSO (blue line) or 10 µM MG132 (red line) for 6 h, followed by incubation with 0.1 µM 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 µM MG132 for 6 h and analyzed the luciferase activity. Data are shown as mean ± s.e.m. (Student’s t-test).

**Fig S9.** MG132 restored the intracellular Zn homeostasis.

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**Additional Notes:**
- **Supplementary Figure 9**
- **MG132**
- **MT1**
- **PhRL-TK plasmids**
- **Student’s t-test**

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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 μM 17AAG, 10 μM DBeQ, or 10 μM MG132 in the presence of CHX for 6 h. Total cell lysates were subjected to western blotting using an anti-V5 antibody.
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 μM 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 ± 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 ΔFLA-V5. RT-qPCR analysis for CHOP and BIP was performed as described in A. Data are representative of three experiments, and shown as mean ± s.e.m. (Student’s t-test). N.S.: not significant; MG: MG-132

C. Protein expression levels of CHOP, BIP, IRE1α, PDI, PERK, Ero1-Lα, and calnexin in 293T cells expressing WT-V5, G64D-V5, or ΔFLA-V5. The cells were treated with 0.5 μM MG132 for 6 h. The cell lysate was subjected to SDS-PAGE and analyzed by western blotting using antibodies to the indicated proteins.
REFERENCE: