

Copper ionophore elicits calpain-dependent paraptosis coincident with proteotoxic stress

Apiwit Sae-Fung^{1, 2}, and Bengt Fadeel^{1, *}

¹Division of Molecular Toxicology, Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden; ²Graduate Program in Clinical Biochemistry and Molecular Medicine, Department of Clinical Chemistry, Faculty of Allied Health Sciences, Chulalongkorn University, Bangkok, Thailand. *E-mail: bengt.fadeel@ki.se

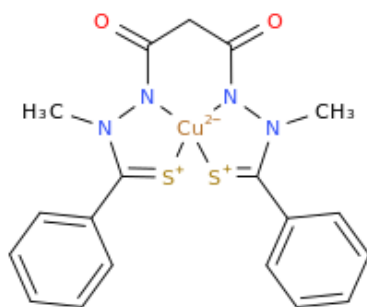


Figure S1. The copper coordination complex formed by elesclomol (Es) through binding to Cu(II) *via* the thiocarbonyl and hydrazino groups. From: PubChem (<https://pubchem.ncbi.nlm.nih.gov>) (Kim et al., Nucleic Acids Res. 2025;53(D1):D1516-D1525).

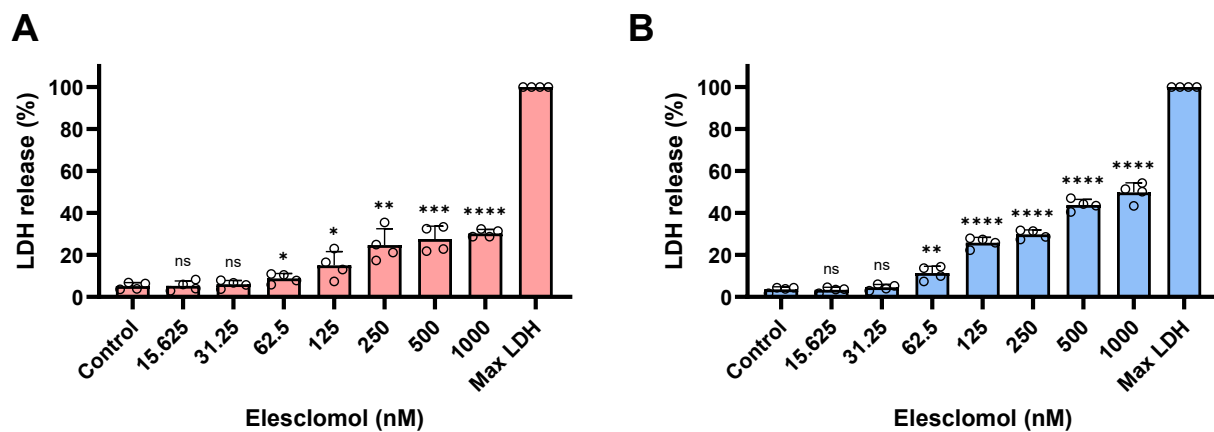


Figure S2. The fibrosarcoma cell line HT-1080 (A) and normal fibroblast cell line BJ (B) were exposed to Es/Cu at the indicated concentrations for 24 h and cell viability was determined by using the LDH release assay while metabolic activity was determined using the Alamar Blue assay (Figure 1A).

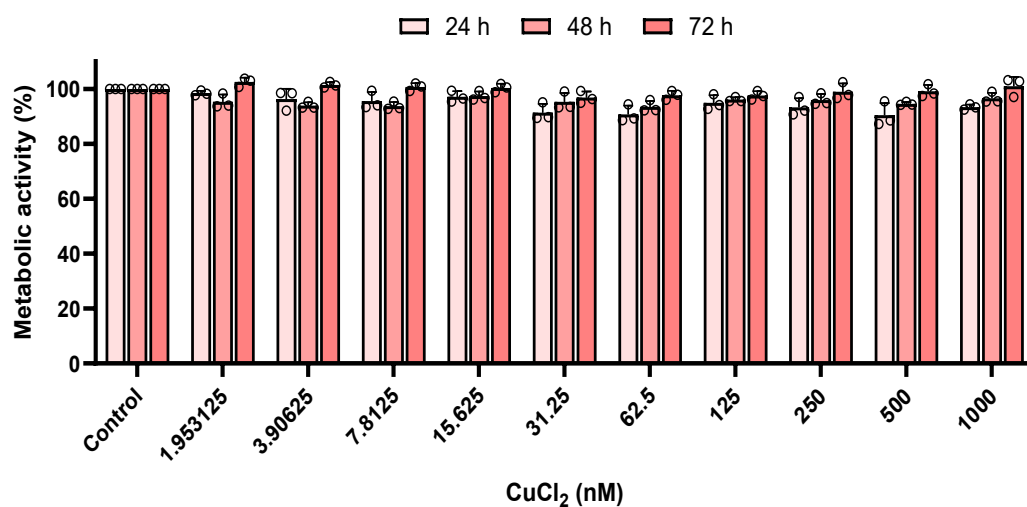


Figure S3. The fibrosarcoma cell line HT-1080 was exposed to CuCl_2 alone for 24, 48, and 72 h, and metabolic activity was determined using the Alamar Blue assay.

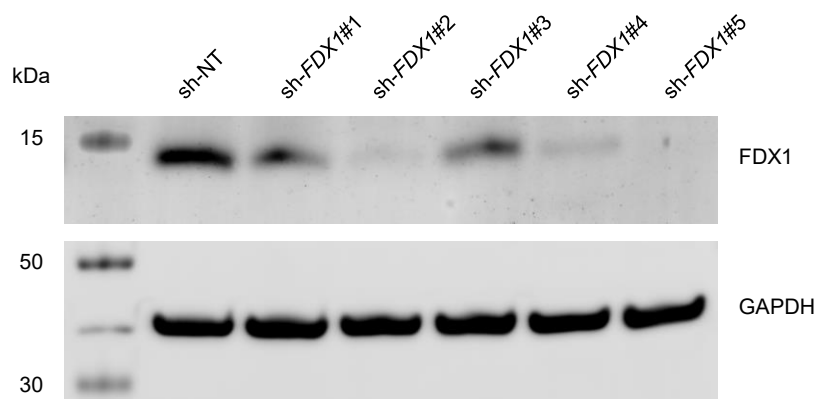


Figure S4. Gene silencing of *FDX1*. The expression of FDX1 in HT-1080 cells transduced with non-targeting (NT) shRNA and five different *FDX1*-targeting shRNAs was determined by Western blot. GAPDH was used as a loading control.

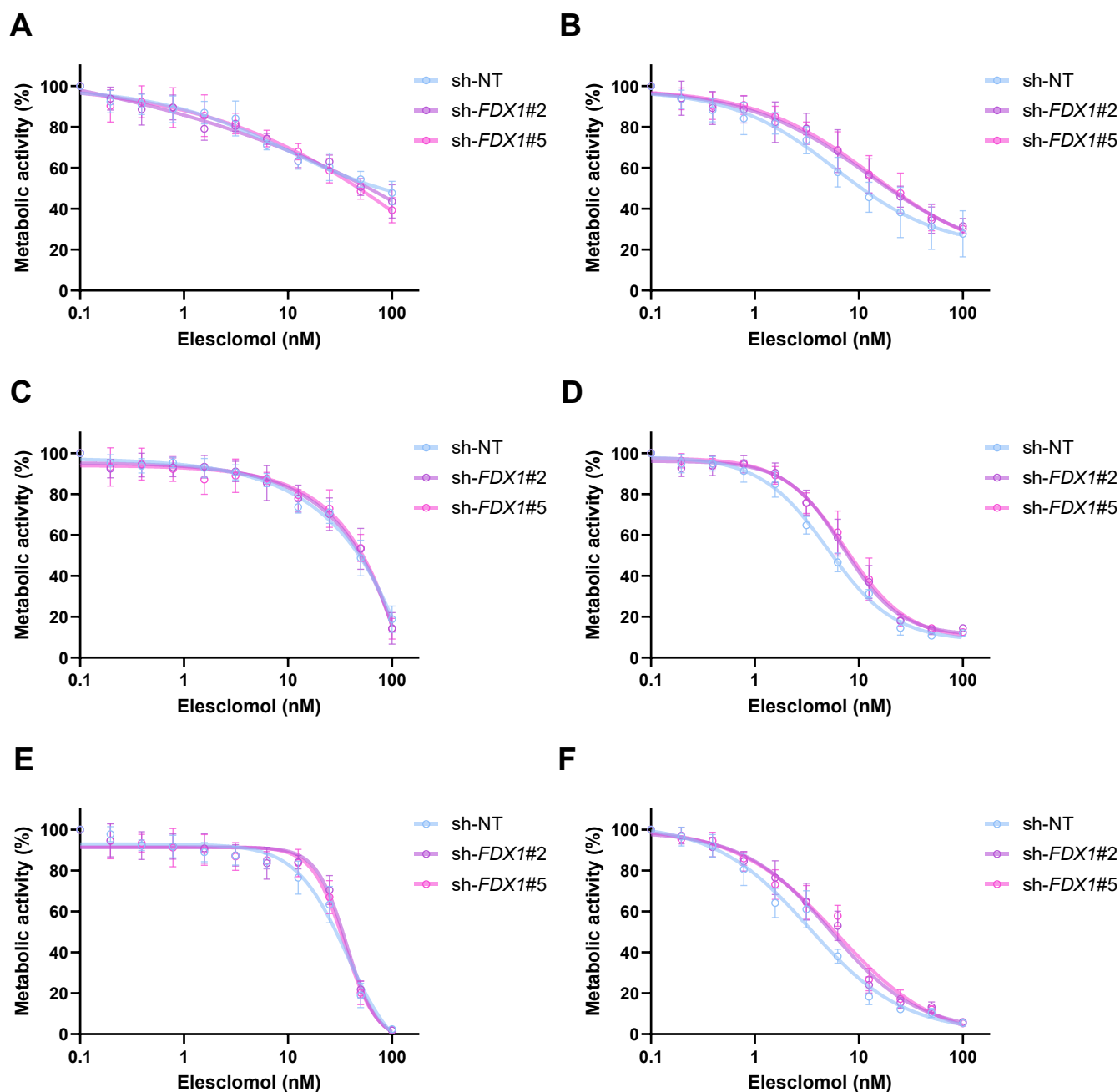


Figure S5. Metabolic capacity of HT-1080 cells with or without silencing of FDX1 were exposed to Es/Cu for 24 h (A, B), 48 h (C, D), and 72 h (E, F). Cells were maintained in medium supplemented with glucose (A, C, E) or galactose (B, D, F). Data shown are mean values $\pm$ S.D. (n=3).

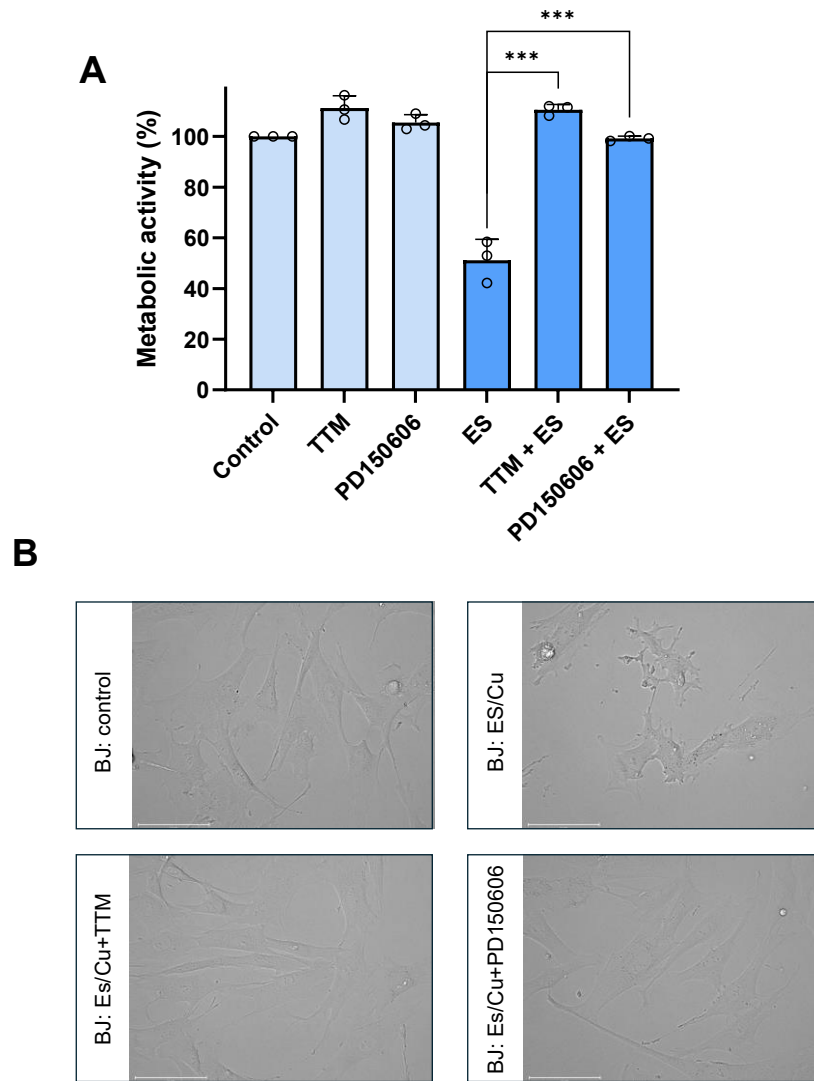


Figure S6. (A) BJ cells were exposed to Es/Cu for 24 h in the presence and absence of the copper chelating compound TTM (10 μ M) or the calpain inhibitor PD150606 (100 μ M) and metabolic capacity was determined by using the Alamar Blue assay. Data are shown as mean values $\pm$ S.D. (n=3). *** $p < 0,005$. (B) BJ cells exposed as detailed above were visualized under the light microscope. The morphological changes elicited by Es/Cu were blocked by TTM and PD150606.

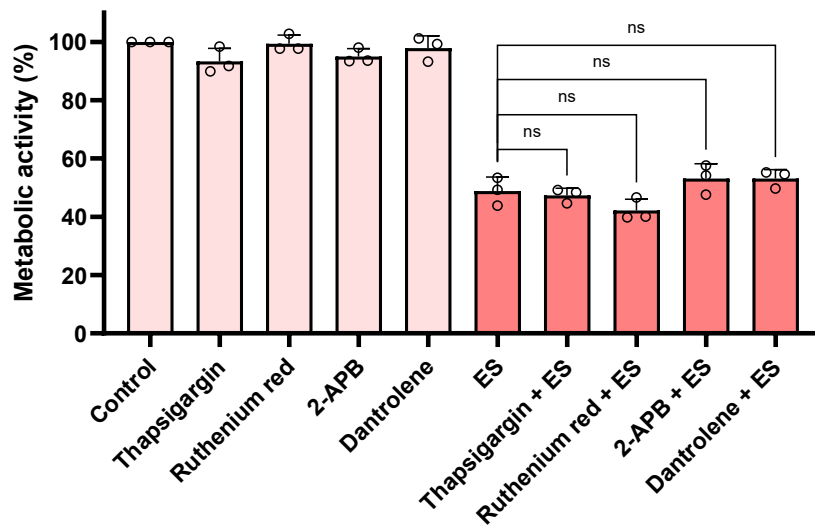


Figure S7. Exploring the role of intracellular calcium stores using pharmacological inhibitors. HT-1080 cells were exposed to Es/Cu (50 nM) for 24 h in the presence or absence of thapsigargin (40 nM), ruthenium red (5 μ M), 2-APB (10 μ M), or dantrolene (50 μ M), and metabolic activity was determined using the Alamar Blue assay. Thapsigargin inhibits the sarcoplasmic/endoplasmic reticulum calcium-ATPase pump, ruthenium red and dantrolene block ryanodine receptors, 2-APB is an inositol trisphosphate (IP_3) receptor antagonist. For results using DIDS, an inhibitor of VDAC1 oligomerization, refer to Figure 6. Data are shown as mean values $\pm$ S.D. (n=3). ns, not significant.

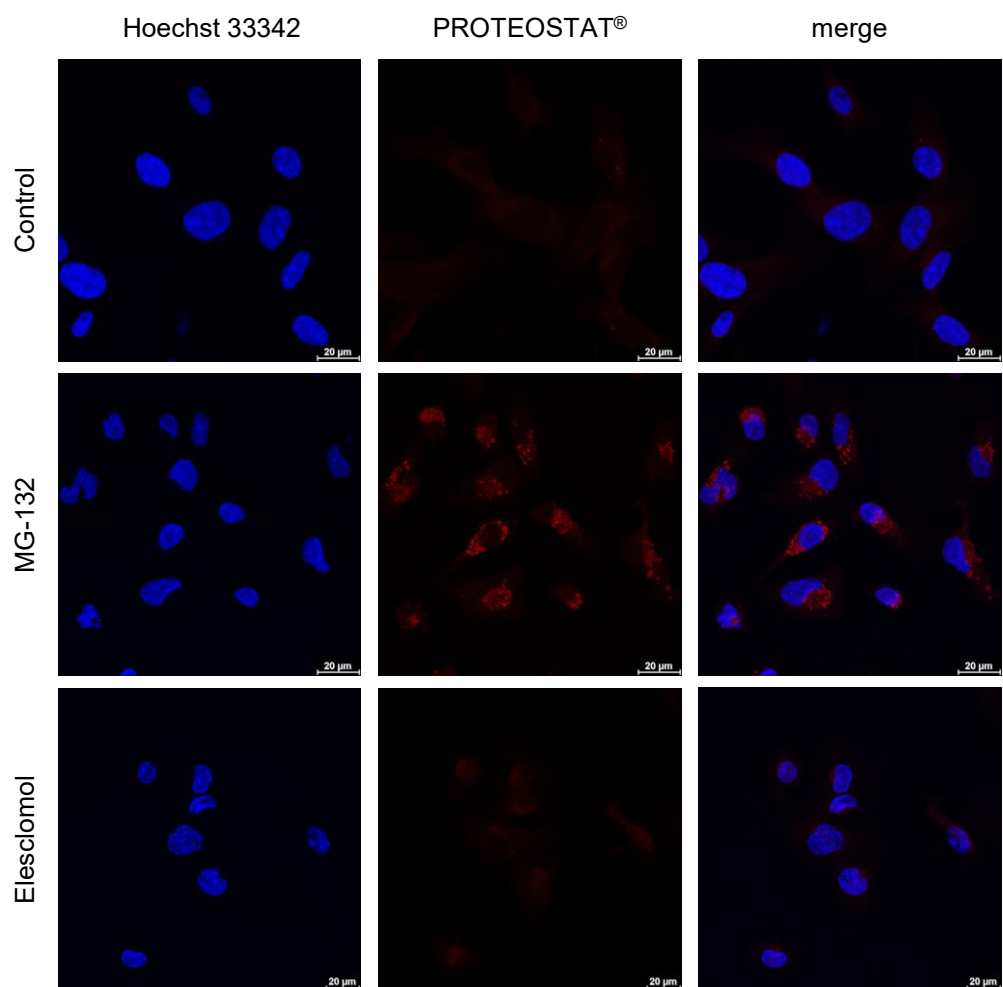


Figure S8. HT-1080 cells were exposed to MG-132 (500 nM) or Es-Cu (50 nM) for 16 h, and protein aggregation (i.e., aggresome formation due to the aggregation of misfolded proteins) was determined using the PROTEOSTAT® reagent (red). Cell nuclei were counterstained with Hoechst (blue).

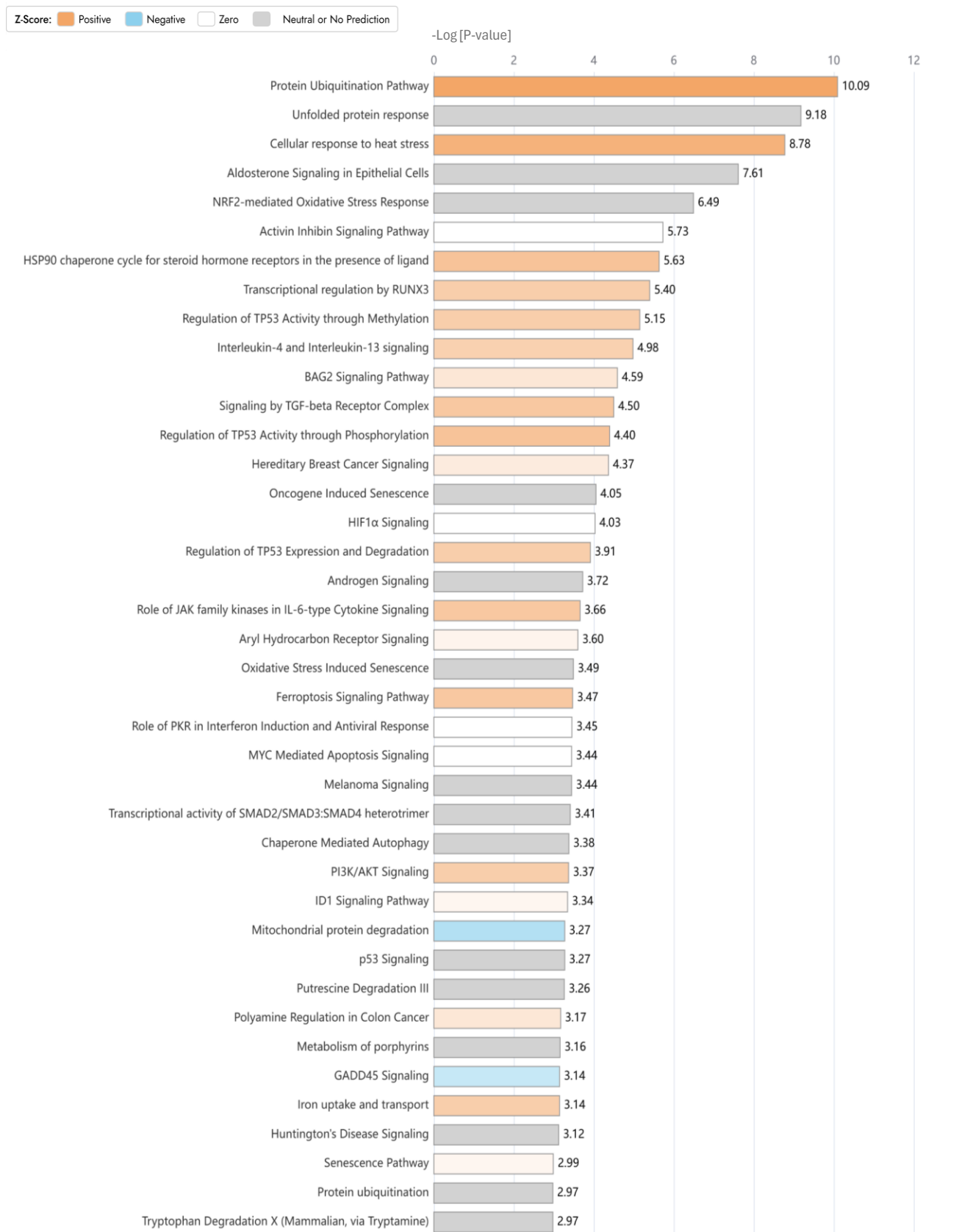


Figure S9. Canonical pathway analysis of differentially expressed proteins in Es/Cu-exposed samples. Data were analyzed by using QIAGEN IPA (<https://digitalinsights.qiagen.com/IPA>). The bar chart represents the top-40 signaling and metabolic pathways. A positive z-score (orange) denotes pathway activation, and a negative z-score (blue) denotes pathway inhibition.

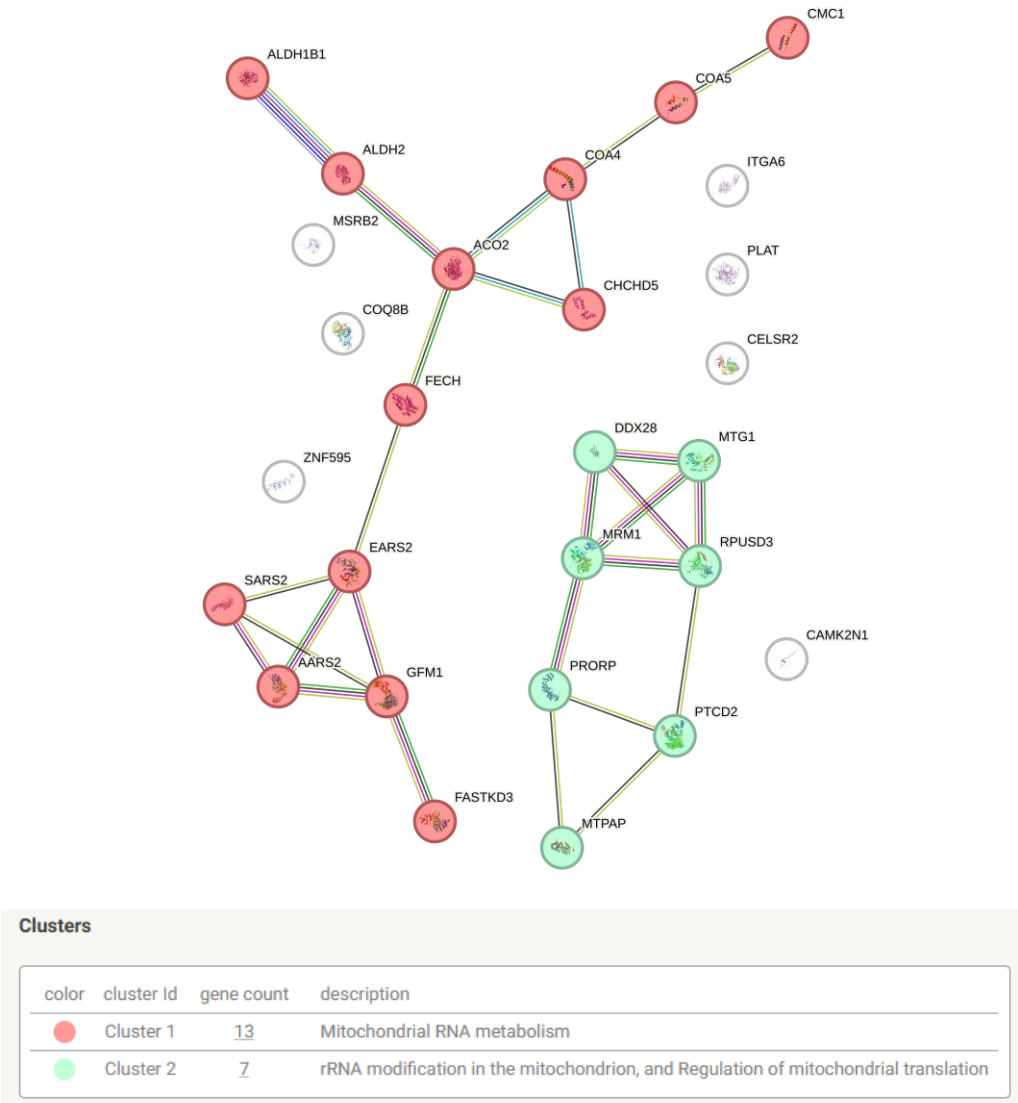


Figure S11. STRING analysis of all the significantly downregulated proteins (n=27) in HT-1080 cells exposed to Es/Cu (50 nM). The description of the two identified clusters is provided below the graph. The analysis was performed using the STRING database, version 12.0 (<https://www.string-db.org>).

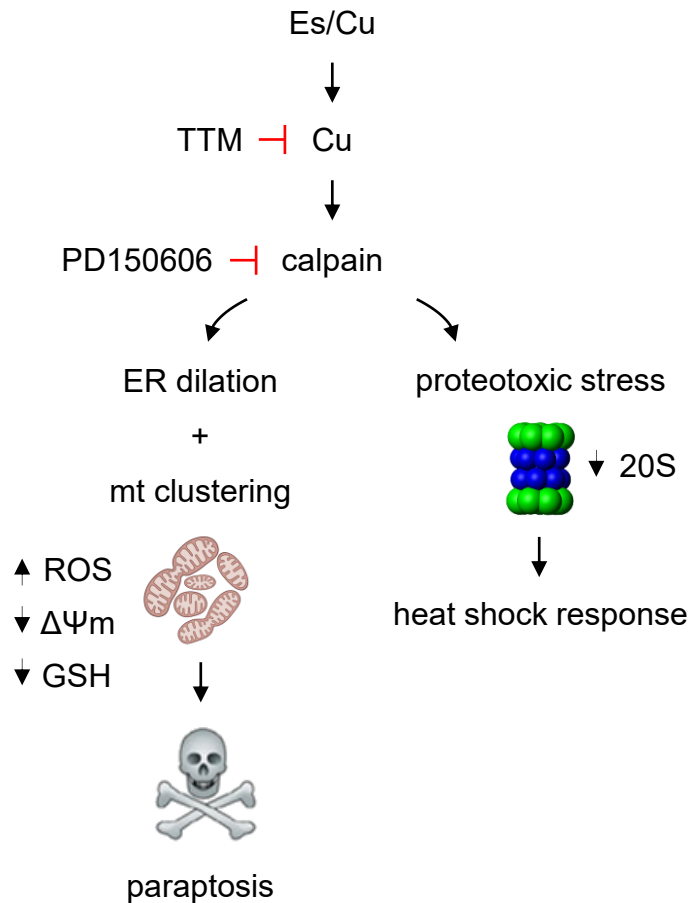


Figure S12. Current model of Es/Cu-triggered cell death. The key finding in the present study is that the copper ionophore elesclomol (Es) triggers calpain-dependent cell death with morphological attributes of *paraptosis* (ER dilation) along with perinuclear mitochondrial clustering albeit in the absence of the chromatin compaction that is typically seen in cells undergoing apoptosis. Es/Cu was also shown to trigger calcium elevation and mitochondrial dysfunction, and DIDS, a non-selective inhibitor of VDAC1, rescued cells from Es/Cu, suggesting the crosstalk between ER and mitochondria (not shown). Furthermore, we have provided evidence of proteasome inhibition in cells exposed to Es/Cu. This could potentially be due to protein misfolding leading to proteasomal insufficiency and/or to a direct inhibitory effect of copper on the proteasome. We could also confirm a heat shock response in exposed cells.