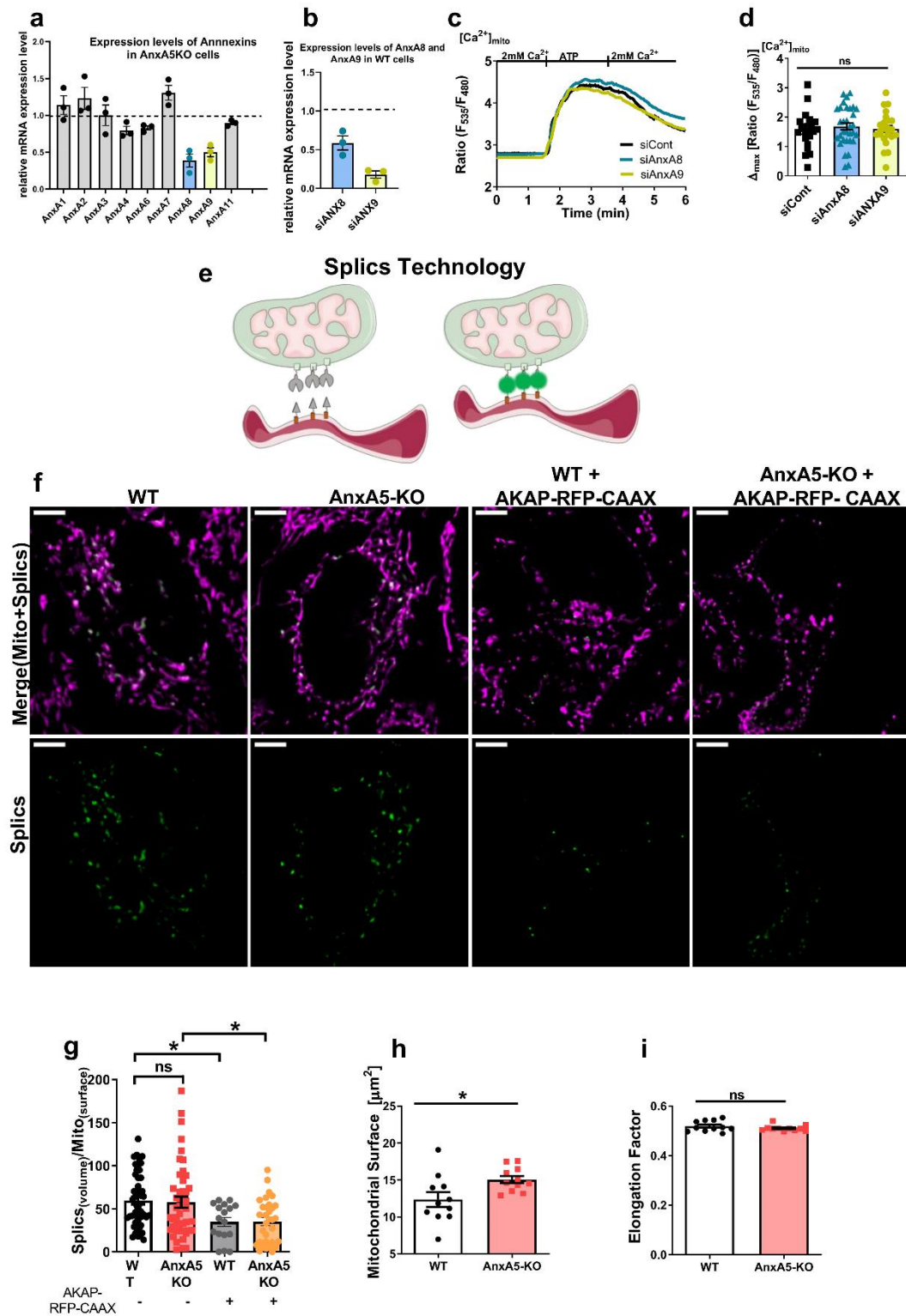


**Appendix for Annexin A5 controls VDAC1-dependent
mitochondrial Ca²⁺ homeostasis and determines cellular
susceptibility to apoptosis**

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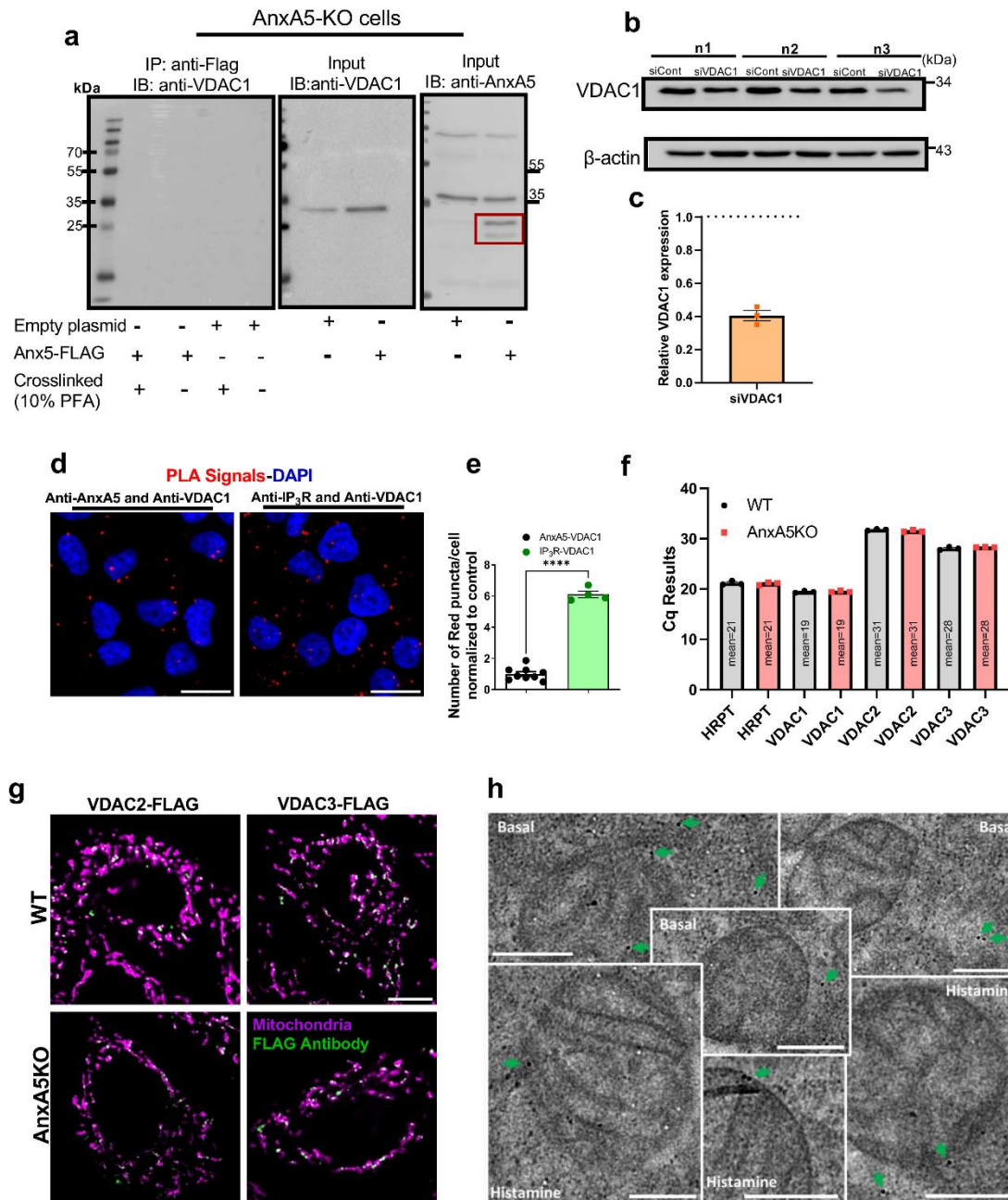
Appendix Fig. S1



Appendix Figure S1. AnxA5 depletion does not affect mitochondria-ER contact sites.

a Bar graphs show the mRNA expression level of the Annexin family (AnxA1, AnxA2, AnxA3, AnxA4, AnxA6, AnxA7, AnxA8, AnxA9, and AnxA11) in AnxA5-KO cells. **b** Bar graphs show the Knock-down efficiency of AnxA8 and AnxA9 in HeLa cells. Data points represent the mean $\pm$ SEM (n=3) **c** Average time courses of the 100 μ M ATP-induced $[Ca^{2+}]_{Matrix}$ responses in WT (black), si-AnxA8 (light blue), and si-AnxA9 (light yellow) in HeLa cells measured in Ca^{2+} (2 mM) containing buffer. **d** Bar graphs show the ATP-induced maximum $[Ca^{2+}]_{Matrix}$ level in WT (black), si-AnxA8 (light blue), and si-AnxA9 (light yellow) in HeLa cells. Data points represent the mean $\pm$ SEM (nWT = 22/6; n si-AnxA8 = 32/8; nsi-AnxA9 = 23/6). **e** The graphical illustration of the SPLICS sensor. **f** Representative confocal images of WT and AnxA5-KO HeLa cells were captured, stained with MTR-CMX (magenta), and expressing ER-mitochondria contact site sensor SPLICS (green) alone or together with AKAP-RFP-CAAX (tags mitochondria to sub-plasma membrane region). **g** Bar graphs show the ratio of SPLICS volume to mitochondrial surface area in WT (black), AnxA5-KO (red), WT + AKAP-RFP-CAAX (gray), and AnxA5-KO + AKAP-RFP-CAAX (orange) in HeLa cells. Data points represent the mean $\pm$ SEM (nWT = 49/4; nAnxA5-KO = 45/4; nWT+ AKAP-RFP-CAAX = 17/5; nAnxA5-KO+AKAP-RFP-CAAX = 32/5). **h** Bar graphs show the mitochondrial surface area [μm^2] and **i** elongation factor in WT (black) and AnxA5-KO (red) HeLa cells. Data points represent the mean $\pm$ SEM (nWT = 91/11; nAnxA5-KO = 88/11). Significant differences were assessed using either one-way ANOVA with Tukey's multiple comparison tests or Kruskal–Wallis test (* $p < 0.05$, and ns: not significant) and with the unpaired Student's t-test or Kolmogorov-Smirnov test (* $p < 0.05$, and ns: not significant).

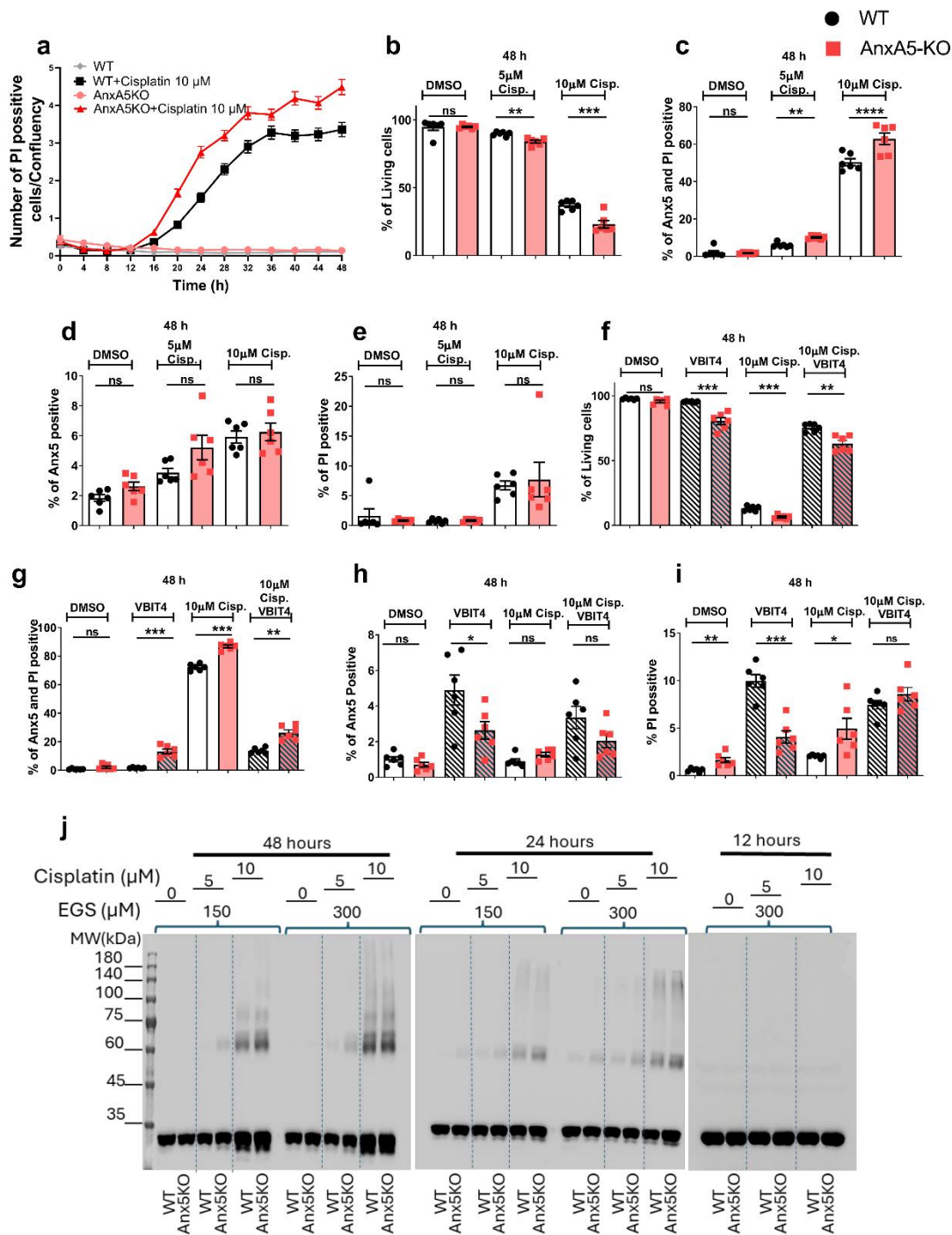
Appendix Fig. S2



Appendix Figure S2. AnxA5 localizes near VDAC1, and ER Ca²⁺ release induces its accumulation on the OMM. **a** Immunoblots show the AnxA5-KO HeLa cells that were transfected either with AnxA5- FLAG or with empty plasmid (set of cells were fixed with 10% PFA as described in methods). AnxA5 was immunoprecipitated from whole cell lysate using the FLAG tag. Co-immunoprecipitation of VDAC1 was checked by immunoblotting with VDAC1-antibody. Uncropped blots are provided in the Source Data. **b** Immunoblots show the expression level of VDAC1 transfected either with siNeg or siVDAC1. **c** Bar graph represents immunoblot analysis of VDAC1 expression as mean $\pm$ SEM (nsiControl =3; nsiVDAC1 =3). **d** Representative image of PLA assay indicating the protein-protein proximity between AnxA5-VDAC1 (left panel) and IP3R- VDAC1 (right panel) in HeLa

cells (scale bar = 10 μ m). **e** Bar graph shows the ratio of total PLA signals to the number of cells. Data points represent the mean $\pm$ SEM (nAnxA5-VDAC1 =2; nIP3R-VDAC1 =2). **f** Bar graph represents the cycle threshold (Cq) values of HRPT1, VDAC1, VDAC2, and VDAC3 genes in WT and AnxA5KO cells, shown as mean $\pm$ SEM (nWT = 3; nAnxA5KO = 3). **g** Representative confocal images of WT and AnxA5-KO HeLa cells expressing FLAG-tagged VDAC2 and VDAC3 (green), stained with MTR-CMX (magenta), were captured (Scale bars = 10 μ m). **h** Representative immunoelectron micrographs of mitochondria from HeLa cells (Scale bars = 200 nm). Individual gold particles (indicated by green arrows) represent the localization of AnxA5 under basal conditions (upper images) or 20 seconds after histamine stimulation (lower images). This panel shows a redisplay of content from Figure 3B.

Appendix Fig. S3



Appendix Figure S3. AnxA5-KO cells are more susceptible to cisplatin-induced apoptosis. **a** Time course of cisplatin-induced cell death in WT (gray), WT + cisplatin (black), AnxA5-KO (light red), and AnxA5-KO + cisplatin (red) cells. Cells were treated with 10 μ M cisplatin, stained with 1 μ g/ml PI, and imaged every 4 hours using the CellCyteX microscopy system in an incubator. Data points represent the mean $\pm$ SEM. (nWT =2; nAnxA5-KO =2). **b** Bar graphs show the percentage of living cells, **c** late apoptosis, **d** early apoptosis, and **e** necrosis in WT (black) and AnxA5-KO (red) HeLa cells upon 48 h DMSO, 5 μ M, and 10 μ M cisplatin treatment. Data points represent the mean $\pm$ SEM (nWT =6; nAnxA5-KO =6). **f** Bar graphs show the percentage of living cells, **g** late apoptosis, **h** early apoptosis, and **i** necrosis in WT (black) and AnxA5-KO (red) cells upon 48 h DMSO, 20 μ M VBIT-4, 10 μ M cisplatin, and cisplatin + VBIT-4 treatment. Data points represent the mean $\pm$ SEM (nWT =6; nAnxA5-KO =6). **j** Immunoblots showing monomeric and dimeric levels of VDAC1 in WT and AnxA5-KO cells treated with 5 μ M and 10 μ M cisplatin for 12, 24, and 48 hours. Two different concentrations of the crosslinking reagent EGS (ethylene glycol bis(succinimidyl succinate)), 150 μ M and 300 μ M, were used to more effectively capture the dimerization levels of VDAC1. Significant differences were assessed using the unpaired Student's t-test or Kolmogorov-Smirnov test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, and ns: not significant).