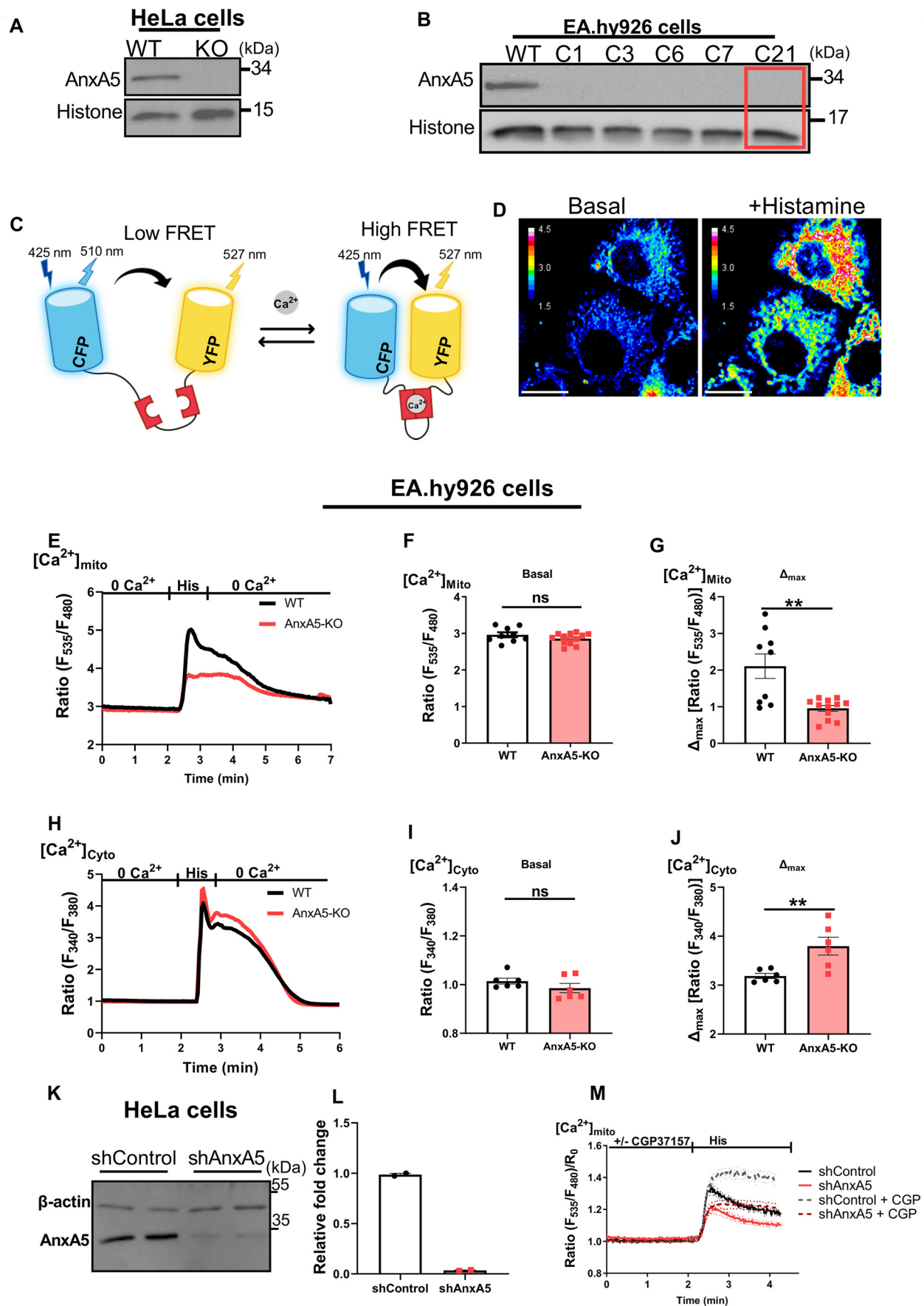


Expanded View Figures

Figure EV1. Characterization of AnxA5-KO cells.

(A) Immunoblots show the AnxA5 expression in WT and AnxA5-KO in HeLa and (B) EA.hy926 cells (Experiments are performed in Clone 21 indicated as a red rectangle). Uncropped blots are provided in the Source data. Panel (A) shows a redisplay of content from Fig. EV2J. (C) Graphical representation of genetically encoded FRET-based mitochondrial matrix targeted Ca^{2+} sensor (4mtD3cpv). (D) Representative image of HeLa cells transfected with (4mtD3cpv). The cells have been pseudocolored to represent mitochondrial Ca^{2+} levels as a ratio under basal (left panel) conditions or upon histamine stimulation (left panel) (Scale bar = 5 μm). (E) Average time courses of the 100 μM histamine-induced $[\text{Ca}^{2+}]_{\text{Matrix}}$ responses in WT (black) and AnxA5-KO (red) in EA.hy926 cells measured in Ca^{2+} -free buffer (containing 100 μM EGTA). (F) Bar graphs show the basal $[\text{Ca}^{2+}]_{\text{Matrix}}$ and (G) histamine-induced maximum $[\text{Ca}^{2+}]_{\text{Matrix}}$ levels in WT (black) and AnxA5-KO (red). Data points represent the mean $\pm$ SEM ($n_{\text{WT}} = 9/6$; $n_{\text{AnxA5-KO}} = 12/6$). The p -value for (G) is $p = 0.0011$ (** $p < 0.01$). (H) Mean time courses of the histamine-induced $[\text{Ca}^{2+}]_{\text{Cyto}}$ responses in WT (black) and AnxA5-KO (red) in EA.hy926 cells measured in Ca^{2+} -free buffer (containing 100 μM EGTA). (I) Bar graphs show the basal $[\text{Ca}^{2+}]_{\text{Cyto}}$ and (J) histamine-induced maximum $[\text{Ca}^{2+}]_{\text{Cyto}}$ levels in WT (black) and AnxA5-KO (red). Data points represent the mean $\pm$ SEM ($n_{\text{WT}} = 101/6$; $n_{\text{AnxA5-KO}} = 88/6$). The p -value for (J) is $p = 0.0094$ (** $p < 0.01$). (K) Representative Immunoblot shows the expression level of AnxA5 transfected either with shControl or shAnxA5. Uncropped blots are provided in the Source Data. (L) Bar graph represents immunoblot analysis of AnxA5 expression as mean $\pm$ SEM ($n_{\text{shControl}} = 3$; $n_{\text{shAnxA5}} = 3$). (M) Average time courses of the 100 μM histamine-induced $[\text{Ca}^{2+}]_{\text{Matrix}}$ responses in the presence (dashed lines) and absence (solid lines) of CGP37157 in shControl (black) and shAnxA5 (red) in HeLa cells. Data points represent the mean $\pm$ SEM ($n_{\text{shControl}} = 29/3$; $n_{\text{shAnxA5}} = 28/4$; $n_{\text{shControl-CGP37157}} = 56/3$; $n_{\text{shAnxA5-CGP37157}} = 43/4$). Significant differences were assessed with the two-tailed unpaired Student's t -test (** $p < 0.01$ and ns: not significant). Source data are available online for this figure.



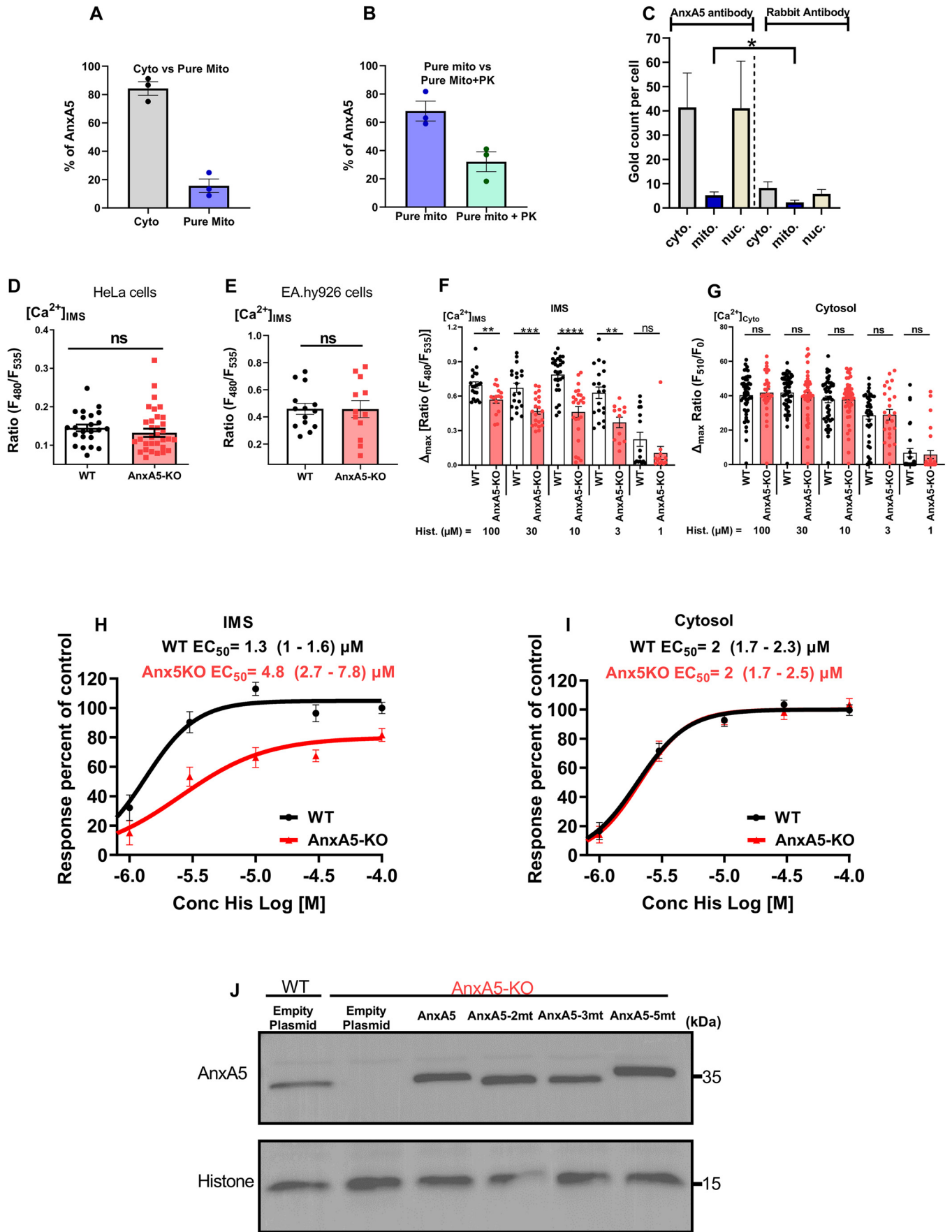


Figure EV2. AnxA5 localizes on and within the mitochondria and regulates IMS Ca^{2+} signaling.

(A) Bar graphs show the calculated percentage localization of AnxA5 in cytosol versus pure mitochondria, (B) pure mitochondria versus pure mitochondria + proteinase K (PK) treatment in mitochondria isolated from HeLa cells. Data points represent the mean $\pm$ SEM in HeLa cells ($n = 3$). (C) Bar graphs show the distribution of gold particles in cytosol, mitochondria, and the nucleus using AnxA5 antibody or rabbit antibody as a negative control. Data points represent the mean $\pm$ SEM in HeLa ($n_{\text{AnxA5 antibody}} = 13/3$; $n_{\text{Rabbit antibody}} = 15/3$). The p -value is $p = 0.0337$ ($*p < 0.05$). (D) Bar graphs show the basal $[\text{Ca}^{2+}]_{\text{IMS}}$ levels in WT (black), AnxA5-KO (red), and Rescue (blue) in HeLa and (E) EA.hy926 cells. Data points represent the mean $\pm$ SEM in HeLa ($n_{\text{WT}} = 23/7$; $n_{\text{AnxA5-KO}} = 34/8$; $n_{\text{Rescue}} = 30/9$) and in EA.hy926 cells ($n_{\text{WT}} = 14/6$; $n_{\text{AnxA5-KO}} = 12/6$). (F) Bar graphs show a histamine-induced maximum $[\text{Ca}^{2+}]_{\text{IMS}}$ and (G) $[\text{Ca}^{2+}]_{\text{cyto}}$ elevation in WT (black) and AnxA5-KO (red) HeLa cells measured in Ca^{2+} -free buffer. The p -values for (F), from left to right, are: $p = 0.0037$ ($**p < 0.01$), $p = 0.0002$ ($***p < 0.001$), $p < 0.0001$ ($****p < 0.0001$), $p = 0.0014$ ($**p < 0.01$), and $p = 0.1758$ (ns). (H) Concentration-response curve of histamine (1, 3, 10, 30, 100 μM) shows maximum $[\text{Ca}^{2+}]_{\text{IMS}}$ and (I) $[\text{Ca}^{2+}]_{\text{cyto}}$ rise in WT (black) and AnxA5-KO (red) cells measured in Ca^{2+} -free buffer. The values were calculated from panels (F) and (G). Data points represent the mean $\pm$ SEM in IMS ($n_{\text{WT-100 } \mu\text{M-Hist}} = 19/4$; $n_{\text{AnxA5-KO-100 } \mu\text{M-Hist}} = 14/5$; $n_{\text{WT-30 } \mu\text{M-Hist}} = 19/5$; $n_{\text{AnxA5-KO-30 } \mu\text{M-Hist}} = 20/5$; $n_{\text{WT-10 } \mu\text{M-Hist}} = 26/6$; $n_{\text{AnxA5-KO-10 } \mu\text{M-Hist}} = 23/6$; $n_{\text{WT-3 } \mu\text{M-Hist}} = 19/6$; $n_{\text{AnxA5-KO-3 } \mu\text{M-Hist}} = 11/6$; $n_{\text{WT-1 } \mu\text{M-Hist}} = 14/3$; $n_{\text{AnxA5-KO-1 } \mu\text{M-Hist}} = 11/3$) and in the cytosol ($n_{\text{WT-100 } \mu\text{M-Hist}} = 50/5$; $n_{\text{AnxA5-KO-100 } \mu\text{M-Hist}} = 33/4$; $n_{\text{WT-30 } \mu\text{M-Hist}} = 50/5$; $n_{\text{AnxA5-KO-30 } \mu\text{M-Hist}} = 40/5$; $n_{\text{WT-10 } \mu\text{M-Hist}} = 46/4$; $n_{\text{AnxA5-KO-10 } \mu\text{M-Hist}} = 46/4$; $n_{\text{WT-3 } \mu\text{M-Hist}} = 39/4$; $n_{\text{AnxA5-KO-3 } \mu\text{M-Hist}} = 25/4$; $n_{\text{WT-1 } \mu\text{M-Hist}} = 30/5$; $n_{\text{AnxA5-KO-1 } \mu\text{M-Hist}} = 28/5$). (J) Representative immunoblots show the expression of AnxA5 in WT and AnxA5-KO cells transfected either with an empty plasmid (in WT and AnxA5-KO cells) or with AnxA5, AnxA5-2mt, AnxA5-3mt, and AnxA5-5mt (in AnxA5-KO cells). Uncropped blots are provided in the Source Data. Significant differences were assessed using either one-way ANOVA with Tukey's multiple comparison tests or Kruskal-Wallis test (ns: not significant) and with the two-tailed unpaired Student's t -test or Kolmogorov-Smirnov ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$, and ns: not significant). Source data are available online for this figure.

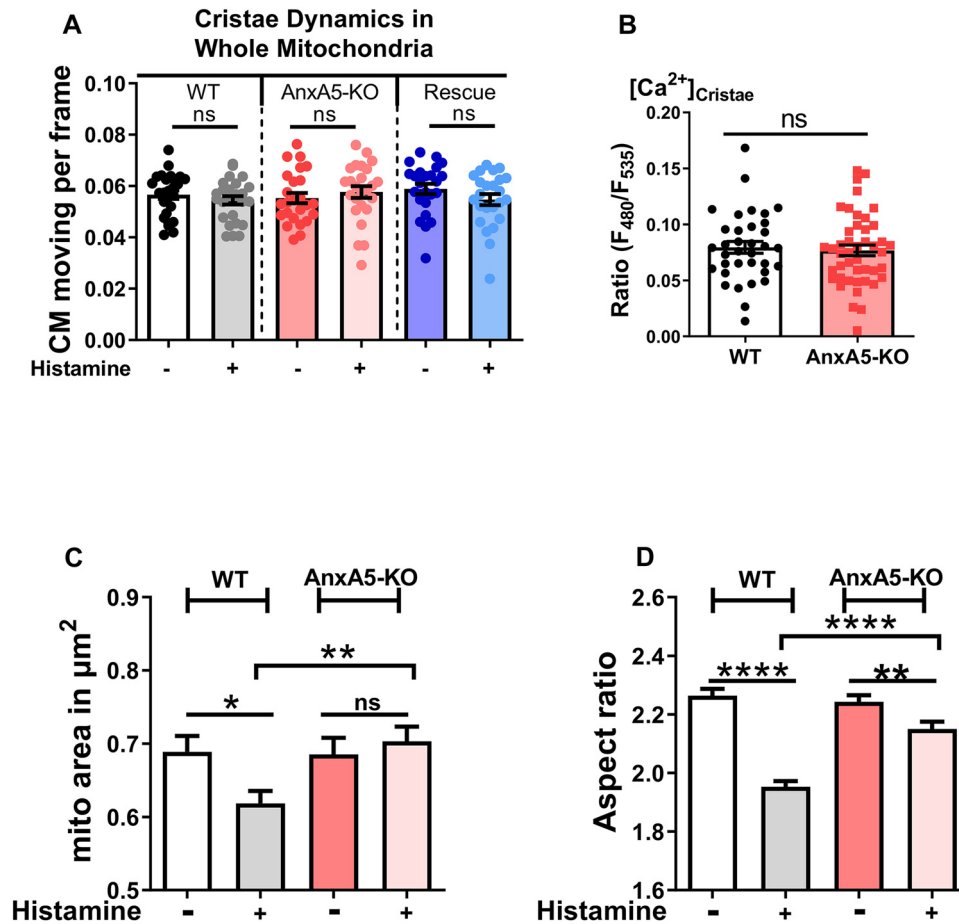


Figure EV3. AnxA5 contributes to Ca²⁺-induced remodeling of mitochondrial morphology.

(A) Bar graph shows cristae membrane movements per frame in the whole mitochondria under basal and upon IP₃-induced [Ca²⁺]_{ER} release in WT (black), AnxA5-KO (red), and Rescue (blue) in HeLa cells. Data points represent the mean ± SEM ($n_{WT} = 26/6$; $n_{AnxA5-KO} = 25/6$; $n_{Rescue} = 24/6$). (B) Bar graph shows the basal [Ca²⁺]_{Cristae} levels in WT (black) and AnxA5-KO (red) cells. Data points represent the mean ± SEM ($n_{WT} = 35/6$; $n_{AnxA5-KO} = 44/6$). (C) Bar graphs show the mitochondrial area and (D) aspect ratio in WT and AnxA5-KO before and 90 s after [Ca²⁺]_{ER} release. Data points represent the mean ± SEM ($n_{WT} = 84/9$; $n_{AnxA5-KO} = 88/9$). The *p*-values, from left to right, are for (C): $p = 0.0109$ (* $p < 0.05$), $p = 0.0015$ (** $p < 0.01$) and $p = 0.5559$ (ns); and for (D): $p < 0.0001$ (**** $p < 0.0001$), $p < 0.0001$ (**** $p < 0.0001$), and $p = 0.0066$ (** $p < 0.01$). Significant differences were assessed using either one-way ANOVA with Tukey's multiple comparison tests or the Kruskal-Wallis test and with the two-tailed unpaired Student's *t*-test (* $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$, and ns: not significant). Source data are available online for this figure.

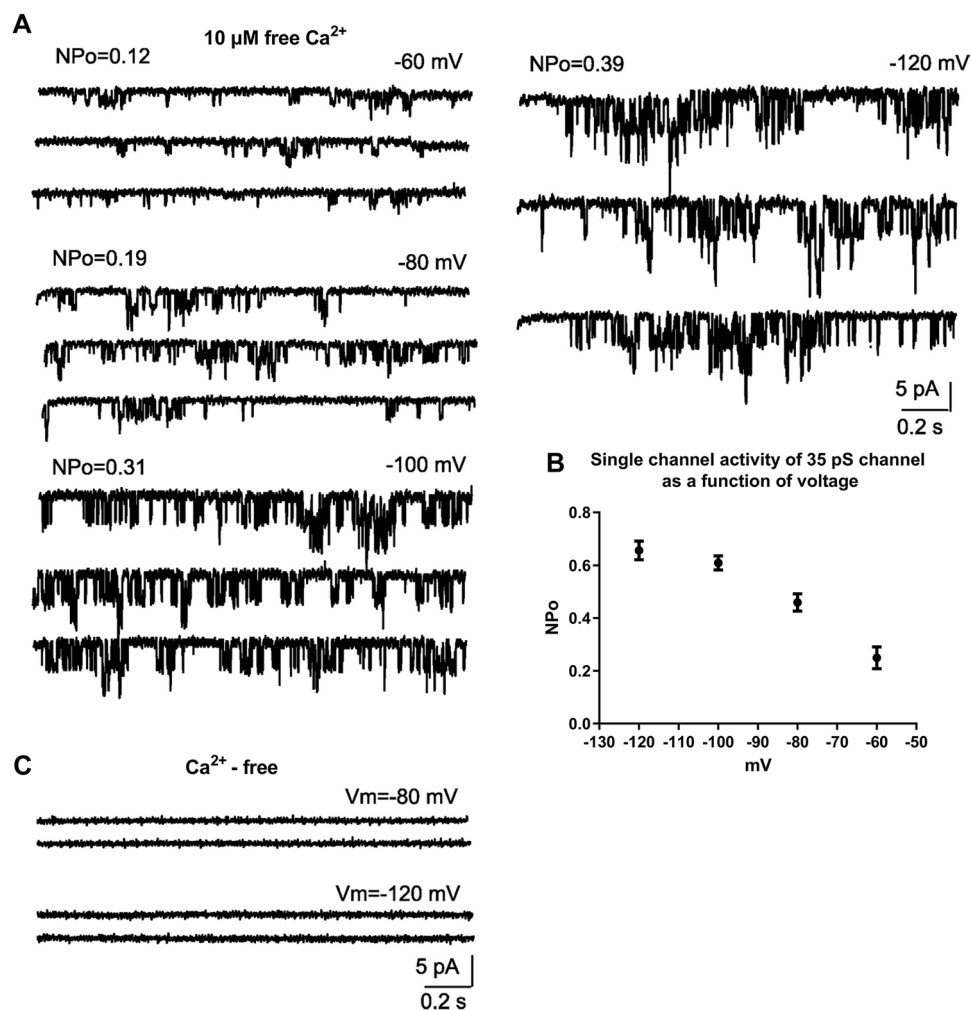
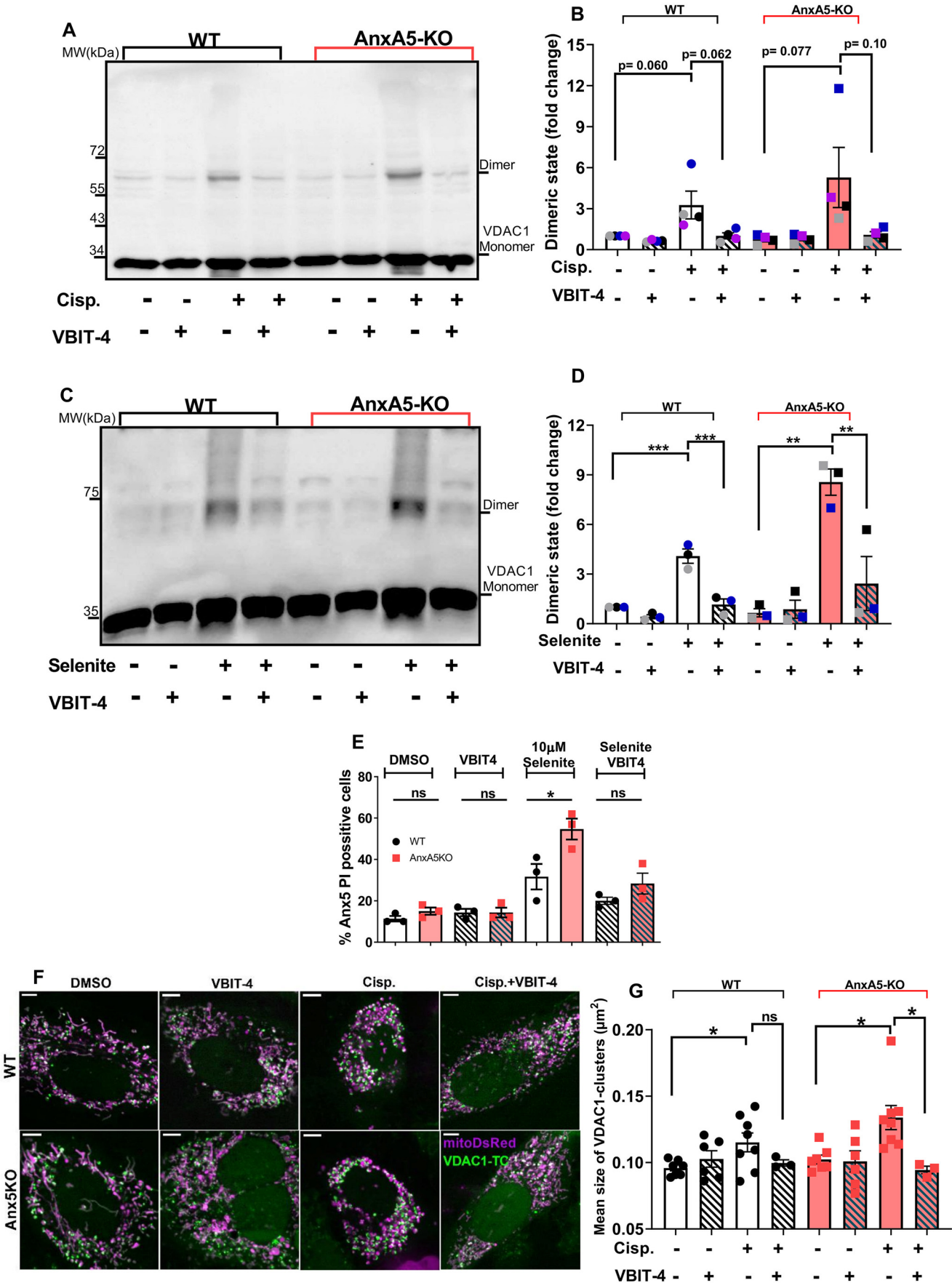


Figure EV4. Characterization of the 35 pS channel at the OMM.

(A) Representative single-channel traces showing the 35 pS channel in different voltages in intact mitochondria isolated from the WT HeLa cells (10 μ M free Ca^{2+} in the pipette). (B) Graph shows the mean NPo of the 35 pS channel at -60 , -80 , -100 , and -120 mV. (C) Representative single-channel traces showing no channel activity in intact mitochondria isolated from the WT HeLa cells (Ca^{2+} -free). Data points represent the mean $\pm$ SEM ($n_{\text{WT-60}} = 3$; $n_{\text{WT-80}} = 7$; $n_{\text{WT-100}} = 7$; $n_{\text{WT-120}} = 10$). Source data are available online for this figure.



◀ **Figure EV5. AnxA5 regulates cisplatin/selenite-induced VDAC1 dimerization.**

(A) Representative immunoblot shows monomeric and dimeric VDAC1 levels. Uncropped blots are provided in the Source data. (B) Bar graph shows the quantification of the immunoblot in WT (black) and AnxA5-KO (red) cells upon 24 h DMSO, 20 μ M VBIT-4, 10 μ M cisplatin, and cisplatin+VBIT-4 treatment (each color represents the experiments from the same day). Data points represent the mean $\pm$ SEM ($n_{\text{WT-all}} = 4$; $n_{\text{AnxA5-KO-all}} = 4$). (C) Representative immunoblot shows monomeric and dimeric VDAC1 levels in response to 48-hour DMSO, 20 μ M VBIT-4, 10 μ M selenite, and selenite + VBIT-4 treatment. (D) Bar graph shows the quantification of the immunoblot in panel (C). Data points represent the mean $\pm$ SEM ($n_{\text{WT-all}} = 3$; $n_{\text{AnxA5-KO-all}} = 3$). The p -values, from left to right, are: $p = 0.0002$ ($***p < 0.001$), $p = 0.0003$ ($***p < 0.001$), $p = 0.0017$ ($**p < 0.01$), and $p = 0.0082$ ($**p < 0.01$). (E) Bar graphs show the percentage of late apoptosis in WT (black) and AnxA5-KO (red) cells upon 48 h DMSO, 20 μ M VBIT-4, 10 μ M selenite, and selenite+VBIT-4 treatment. Data points represent the mean $\pm$ SEM ($n_{\text{WT-all}} = 3$; $n_{\text{AnxA5-KO-all}} = 3$). The p -values, from left to right, are: $p = 0.1688$ (ns), $p > 0.9999$ (ns), $p = 0.0448$ ($*p < 0.05$), and $p = 0.1932$ (ns). (F) Representative confocal images of WT and AnxA5-KO HeLa cells, expressing VDAC1-TC (green) and mitoDsRed (red), were captured (Scale bar = 5 μ m). (G) Bar graph shows the quantification of the obtained confocal images indicating VDAC1 cluster size in μm^2 in WT (black) and AnxA5-KO (red) cells upon 12 h DMSO, 20 μ M VBIT-4, 10 μ M cisplatin, and cisplatin+VBIT-4 treatment. Data points represent the mean $\pm$ SEM ($n_{\text{WT-DMSO}} = 7$; $n_{\text{WT-VBIT-4}} = 6$; $n_{\text{WT-cisp.}} = 8$; $n_{\text{WT-cisp.+VBIT-4}} = 3$; $n_{\text{AnxA5-KO-DMSO}} = 7$; $n_{\text{AnxA5-KO-VBIT-4}} = 6$; $n_{\text{AnxA5-KO-cisp.}} = 8$; $n_{\text{AnxA5-KO-cisp.+VBIT-4}} = 3$). The p -values, from left to right, are: $p = 0.0494$ ($*p < 0.05$), $p = 0.2773$ (ns), $p = 0.0126$ ($*p < 0.05$), and $p = 0.0168$ ($*p < 0.05$). Significant differences were assessed using one-way ANOVA with Tukey's multiple comparison tests or with the unpaired Student's t -test ($*p < 0.05$, $**p < 0.01$, $***p < 0.005$, and ns: not significant). Source data are available online for this figure.