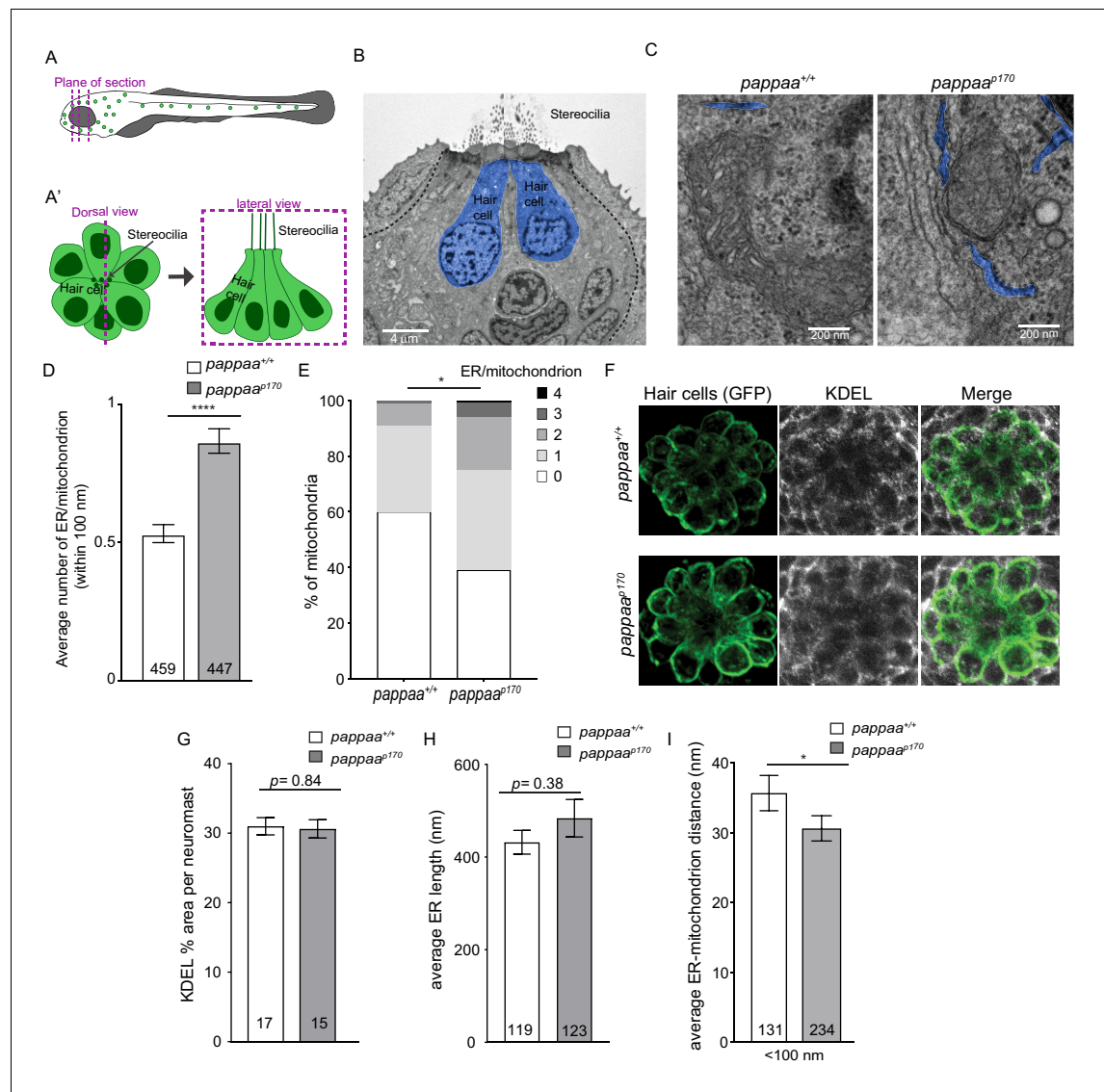


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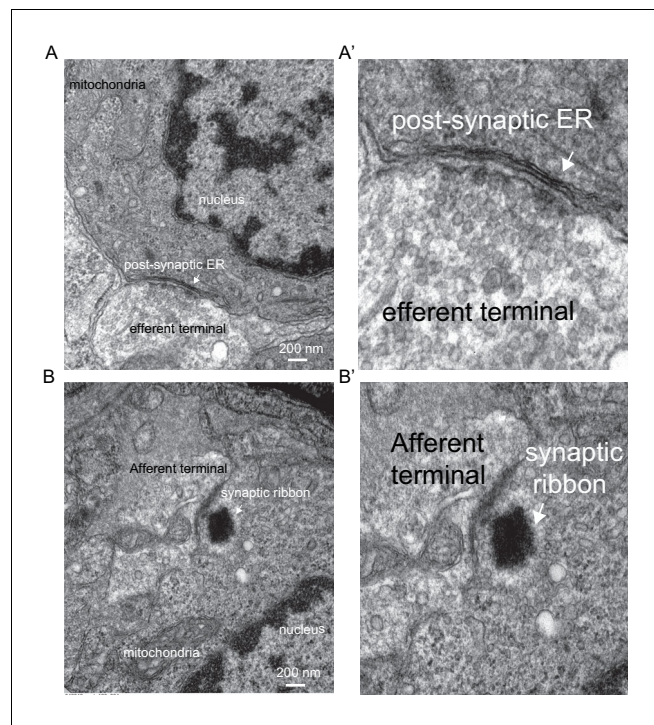
## Figures and figure supplements

Pregnancy-associated plasma protein-aa regulates endoplasmic reticulum–mitochondria associations

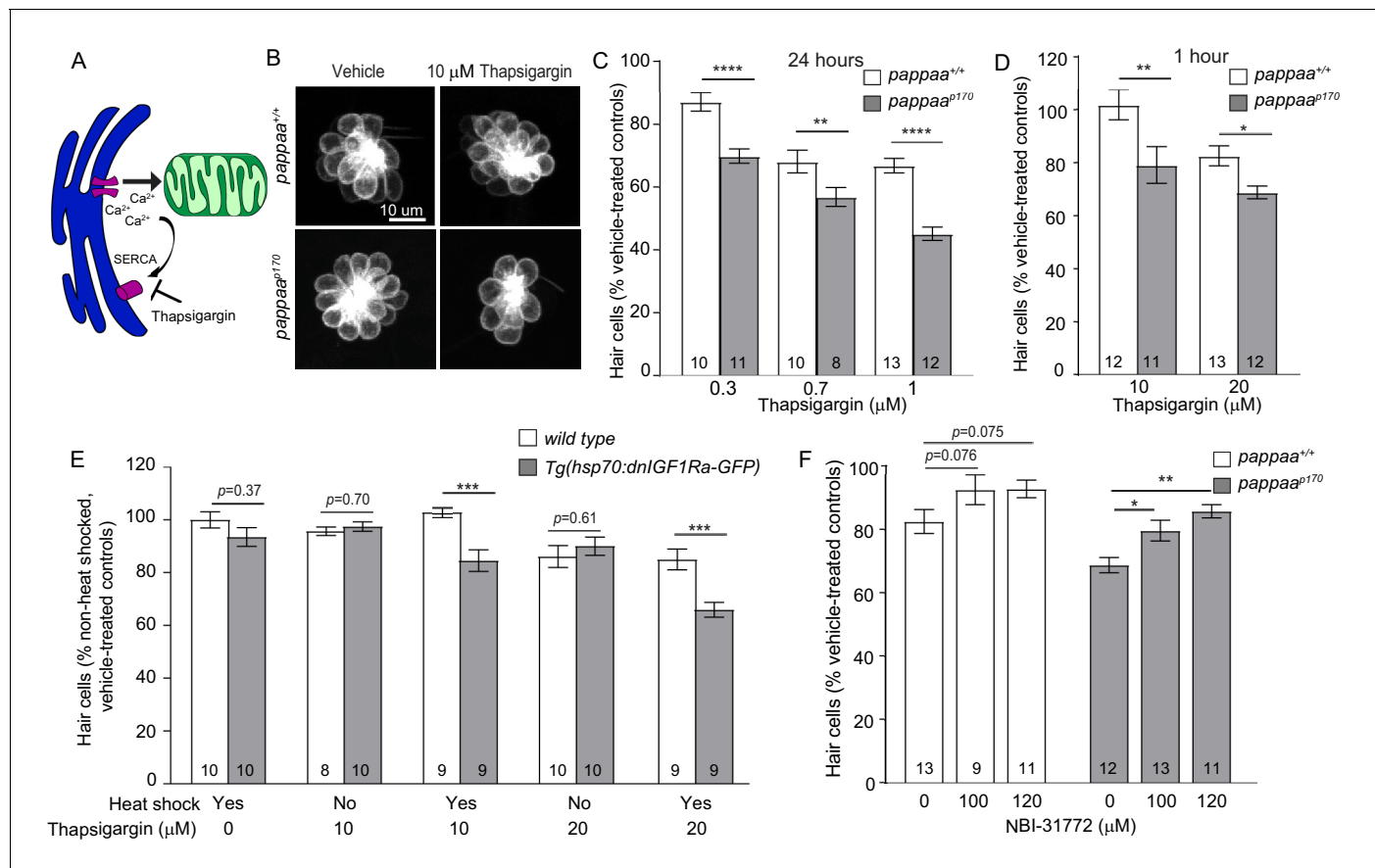
**Mroj Alassaf and Mary C Halloran**



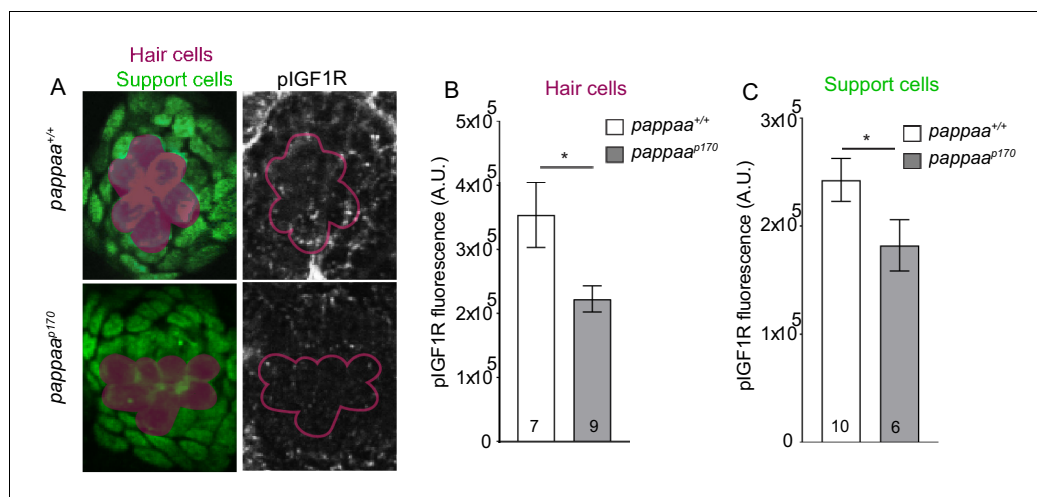
**Figure 1.** Pappaa regulates ER-mitochondria associations. (A–A') Schematic of zebrafish lateral line hair cells. (A) The dotted lines represent EM plane of section. (A') Schematic of a dorsal view (left) and lateral view (right) of a single neuromast. (B) Representative EM section of lateral line neuromast taken along the apical-basal axis of lateral line hair cells (blue) in 5 dpf larva. Scale bar = 4  $\mu$ m. (C) Representative EM images of ER-mitochondria associations in wild-type and *pappaa* hair cells. ER is pseudo colored in blue. Scale bar = 200 nm. (D) Mean number of ER tubules within 100 nm of mitochondria. \*\*\*\* $p < 0.0001$  t-test, Mann-Whitney correction. N = 459 mitochondria (wild type) and 447 mitochondria (*pappaa*<sup>p170</sup>) collected from six larvae/genotype. Error bars = SEM. (E) Percentages of mitochondria associated with 0, 1, 2, 3, or 4 ER tubules. \* $p < 0.05$  chi-square test. N = 459 mitochondria (wild type) and 447 mitochondria (*pappaa*<sup>p170</sup>) collected from six larvae/genotype. (F) KDEL immunolabeling in 5 dpf wild-type and *pappaa*<sup>p170</sup> *brn3c:mGFP*-labeled hair cells. (G) Mean percentage of area covered by KDEL immunolabeling per neuromast. Unpaired t-test with Welch correction revealed no significant difference between groups  $p = 0.84$ . N = 15–17 larvae/genotype (shown at base of bars), 1–3 neuromasts/larva. Total number of neuromasts included in the analysis = 35 (wild type) and 31 (*pappaa*<sup>p170</sup>) neuromasts from two experiments. (H) Mean length of ER tubules. t-test with Mann-Whitney correction found no significant difference. N = 119 ER tubules (wild type) and 123 ER tubules (*pappaa*<sup>p170</sup>) collected from six larvae/genotype. (I) Mean distance between the ER and mitochondria that are within 100 nm of each other. \* $p < 0.05$  t-test, Mann-Whitney correction. N = 131 ER-mitochondria associations (wild type) and 234 ER-mitochondria associations (*pappaa*<sup>p170</sup>) collected from six larvae/genotype. Error bars=SEM.



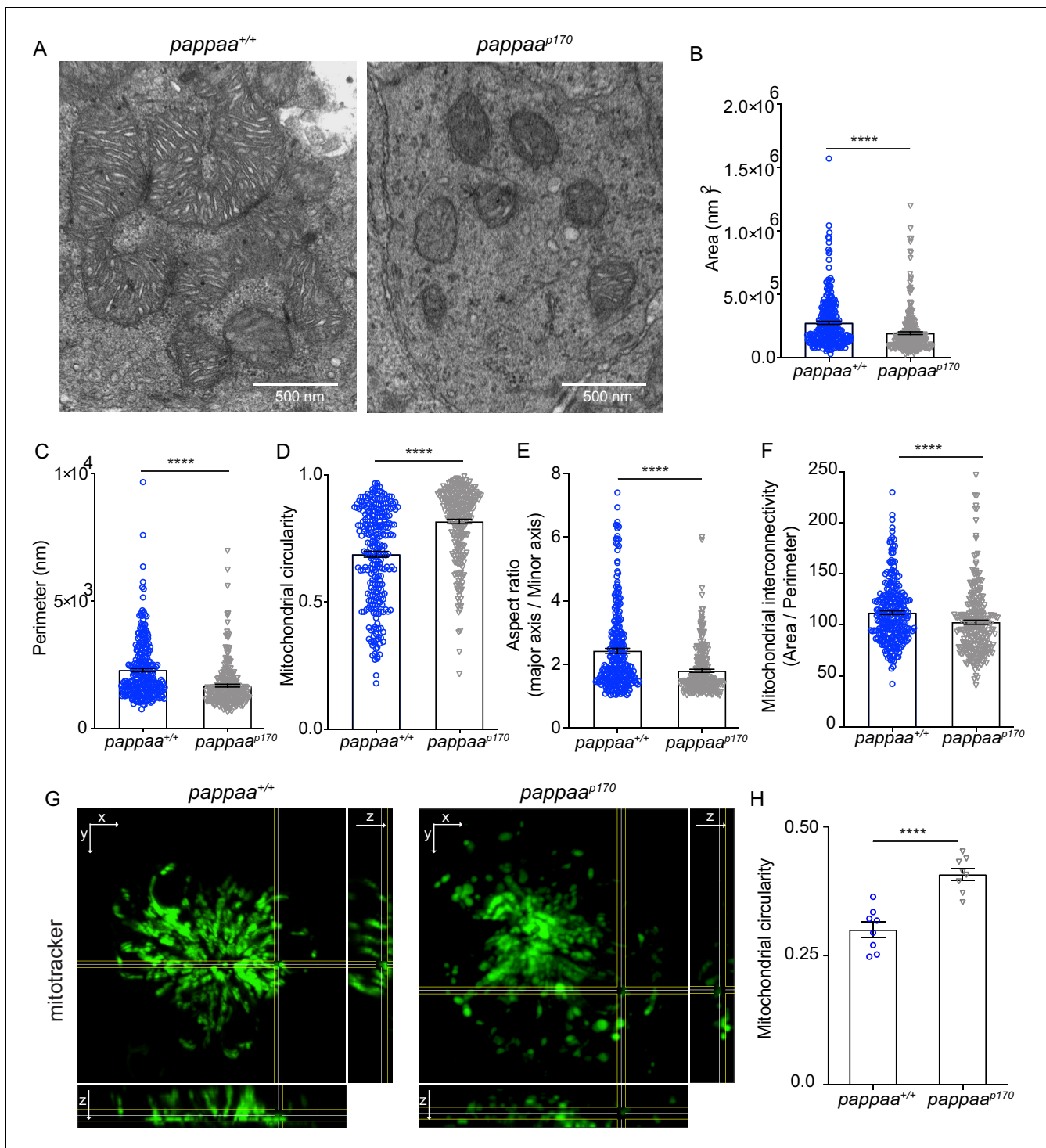
**Figure 1—figure supplement 1.** Representative EM images of (A–A') an efferent contact showing the post-synaptic ER (arrow) and afferent (B–B') contact identified by the synaptic ribbon (arrow).



**Figure 2.** *pappaa*<sup>p170</sup> hair cells are more sensitive to disruption in ER-mitochondria calcium signaling. (A) Thapsigargin increases calcium concentration at the ER-mitochondria junction by blocking the SERCA pump and inhibiting calcium uptake by the ER. (B) Representative images of *brn3c:mGFP*-labeled hair cells from vehicle or 10  $\mu$ M thapsigargin-treated larvae. (C) Mean percentage of surviving hair cells following 24 hr treatments with thapsigargin starting at 4 dpf. To calculate hair cell survival percentage, hair cell number post-drug treatment was normalized to mean hair cell number in vehicle treated larvae of the same genotype. \*\*p < 0.01, \*\*\*\*p < 0.0001, two-way ANOVA, Holm-Sidak post-test. N = 8–13 larvae per group (shown at base of bars), three neuromasts/larva were analyzed. Total number of neuromasts included in the analysis = 24 (wild type; vehicle-treated), 24 (*pappaa*<sup>p170</sup>; vehicle-treated), 30 (wild type; 0.3  $\mu$ M thapsigargin), 33 (*pappaa*<sup>p170</sup>; 0.3  $\mu$ M thapsigargin), 30 (wild type; 0.7  $\mu$ M thapsigargin), 24 (*pappaa*<sup>p170</sup>; 0.7  $\mu$ M thapsigargin), 39 (wild type; 1  $\mu$ M thapsigargin), 36 (*pappaa*<sup>p170</sup>; 1  $\mu$ M thapsigargin). (D) Mean percentage of surviving hair cells following 1 hr treatment with thapsigargin at 5 dpf. \*p < 0.05, \*\*p < 0.01, two-way ANOVA, Holm-Sidak post-test. N = 8–13 larvae per group (shown at base of bars), three neuromasts/larva from two experiments were analyzed. Total number of neuromasts included in the analysis = 60 (wild type; vehicle-treated), 60 (*pappaa*<sup>p170</sup>; vehicle-treated), 36 (wild type; 10  $\mu$ M thapsigargin), 33 (*pappaa*<sup>p170</sup>; 10  $\mu$ M thapsigargin), 39 (wild type; 20  $\mu$ M thapsigargin), 36 (*pappaa*<sup>p170</sup>; 20  $\mu$ M thapsigargin). (E) Mean percentage of surviving hair cells following induction of *dnIGF1R* expression. To calculate hair cell survival percentage, hair cell number after 1 hr treatments with thapsigargin was normalized to mean hair cell number in non-heat-shocked, vehicle-treated larvae of the same genotype. \*\*\*p < 0.001 two-way ANOVA, Holm-Sidak post-test. N = 8–10 larvae per group (shown at base of bars), three neuromasts per larva. Total number of neuromasts included in the analysis = 30 (wild type; non-heat-shocked, vehicle-treated), 27 (*dnIGF1Ra*; non-heat-shocked, vehicle-treated), 30 (wild type; heat-shocked, vehicle-treated), 30 (*dnIGF1Ra*; heat-shocked, vehicle-treated), 24 (wild type; non-heat-shocked, 10  $\mu$ M thapsigargin), 30 (*dnIGF1Ra*; non-heat-shocked, 10  $\mu$ M thapsigargin), 27 (wild type; heat-shocked, 10  $\mu$ M thapsigargin), 27 (*dnIGF1Ra*; heat-shocked, 10  $\mu$ M thapsigargin), 30 (wild type; non heat-shocked, 20  $\mu$ M thapsigargin), 30 (*dnIGF1Ra*; non-heat-shocked, 20  $\mu$ M thapsigargin), 27 (wild type; heat-shocked, 20  $\mu$ M thapsigargin), 27 (*dnIGF1Ra*; heat-shocked, 20  $\mu$ M thapsigargin). (F) Mean percentage of surviving hair cells following co-treatment with NBI-31772 and 20  $\mu$ M thapsigargin. To calculate hair cell survival percentage, hair cell counts after treatment were normalized to hair cell number in vehicle treated larvae of the same genotype. \*p < 0.05, \*\*p < 0.01. Two-way ANOVA, Holm-Sidak post-test. N = 9–13 larvae per group (shown at base of bars), three neuromasts per larva. Total number of neuromasts included in the analysis = 24 (wild type; vehicle-treated), 24 (*pappaa*<sup>p170</sup>; vehicle-treated), 39 (wild type; 20  $\mu$ M thapsigargin), 36 (*pappaa*<sup>p170</sup>; 20  $\mu$ M thapsigargin), 27 (wild type; 20  $\mu$ M thapsigargin + 100  $\mu$ M NBI-31772), 39 (*pappaa*<sup>p170</sup>; 20  $\mu$ M thapsigargin + 100  $\mu$ M NBI-31772), 33 (wild type; 20  $\mu$ M thapsigargin + 120  $\mu$ M NBI-31772), and 33 (*pappaa*<sup>p170</sup>; 20  $\mu$ M Thapsigargin + 120  $\mu$ M NBI-31772).

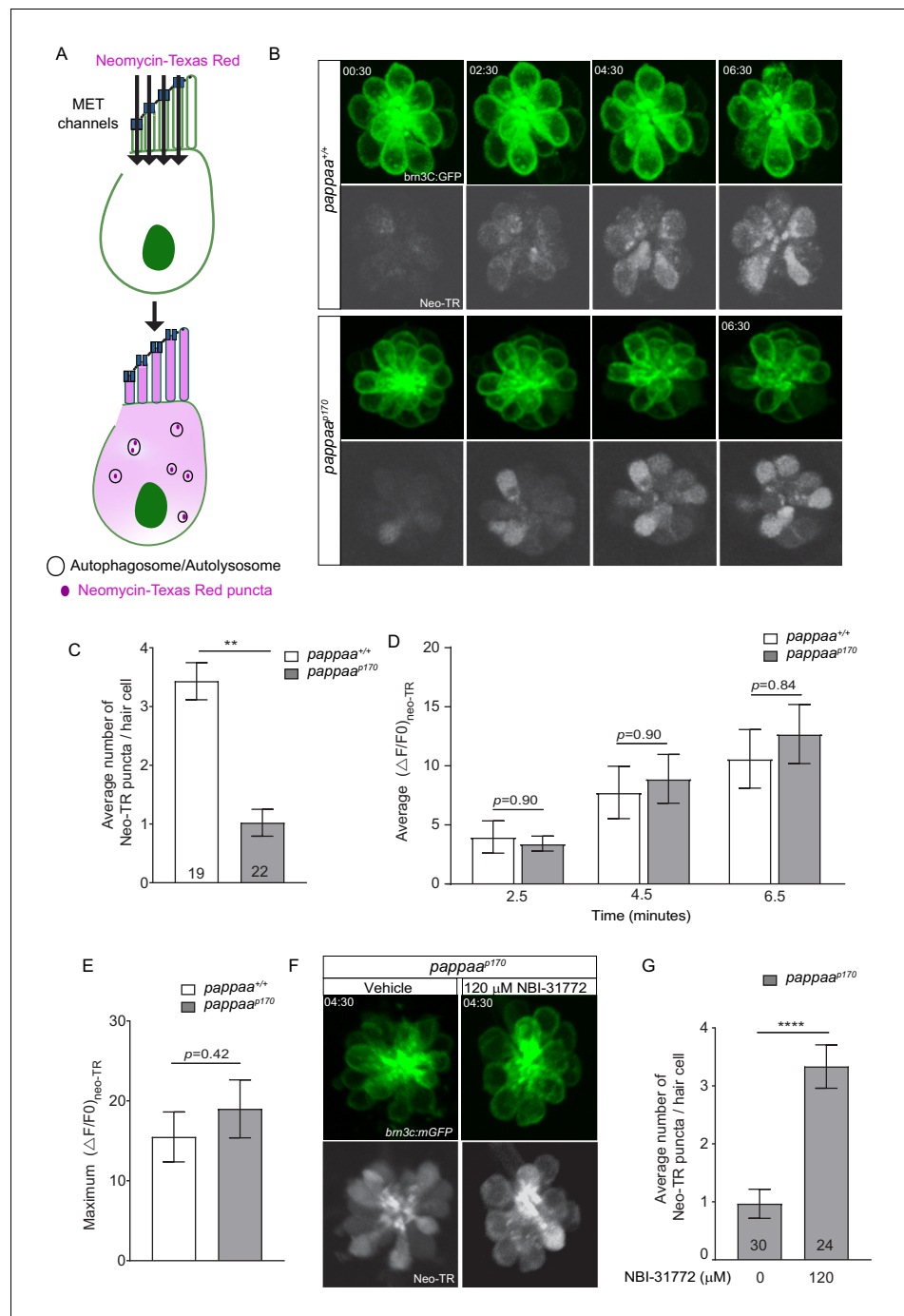


**Figure 2—figure supplement 1.** Anti-pIGF1R immunolabeling. (A) *brn3c:mGFP*-labeled hair cells (magenta) and SOX2 labeled support cells (green) immunostained with anti-pIGF1R antibody (white). (B) Mean pIGF1R fluorescence from Z-stack summation projections of *brn3c:mGFP*-labeled hair cells. \* $p < 0.05$  t-test, Mann–Whitney correction. N = 7–9 larvae per group, 3–4 neuromast/larva. Total number of neuromasts included in the analysis = 23 (wild type) and 27 (*pappaa*<sup>P170</sup>). (C) Mean pIGF1R fluorescence from Z-stack summation projections of 10 randomly selected support cells per neuromast. \* $p < 0.05$  t-test, Mann–Whitney correction. N = 6–10 larvae per group, 2–4 neuromast/larva. Total number of neuromasts included in the analysis = 26 (wild type) and 21 (*pappaa*<sup>P170</sup>). Error bars=SEM.



**Figure 3.** Pappaa loss causes mitochondrial fragmentation. (A) Representative EM images of mitochondria in lateral line hair cells in 5 dpf wild-type and *pappaa*<sup>p170</sup> larvae. (B–F) Mean mitochondrial (B) area, (C) perimeter, (D) circularity, (E) aspect ratio, and (F) interconnectivity in 5 dpf wild-type and *pappaa*<sup>p170</sup> lateral line hair cells. \*\*\*\*p < 0.0001 t-test, Mann–Whitney correction. N = 272 mitochondria (wild type) and 262 mitochondria (*pappaa*<sup>p170</sup>) collected from six larvae/genotype. (G) Representative images of 5 dpf wild-type and *pappaa*<sup>p170</sup> lateral line hair cells loaded with the vital mitochondrial dye, Mitotracker. Images are maximum intensity projection through neuromast in xy view, with cross sections of yz plane shown at right and xz plane shown at bottom. (H) Mean mitochondrial circularity measured from Z-stack max intensity projections of wild-type and *pappaa*<sup>p170</sup> lateral line hair cells. \*\*\*\*p < 0.0001 t-test, Welch correction. N = 8 larvae per group (shown at base of bars), one neuromast/ larva. Error bars=SEM. See Videos 1 and 2.



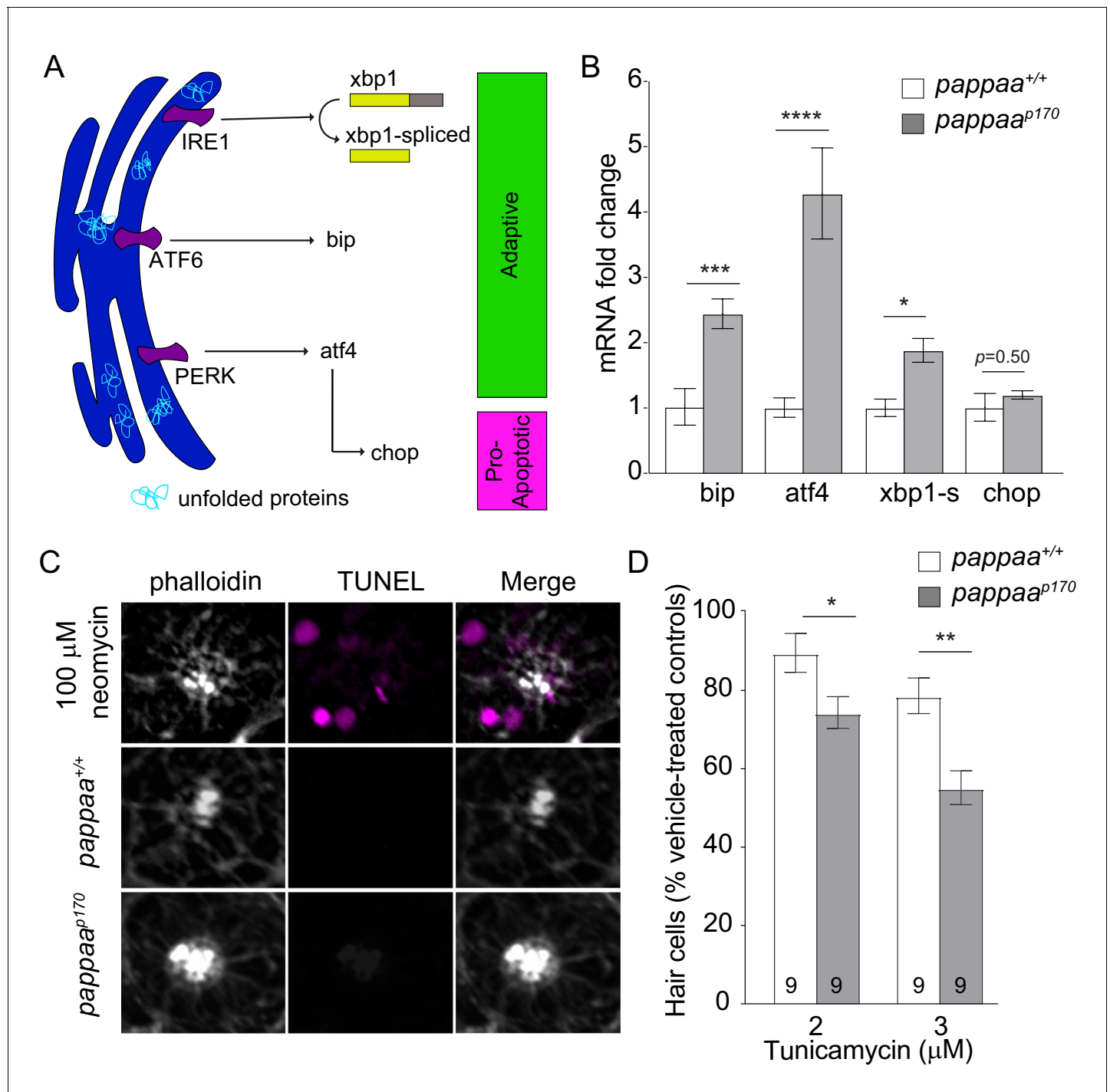


**Figure 4.** Pappaa regulates neomycin-induced autophagy. (A) Schematic showing cell entry and autophagy of neomycin-Texas Red. (B) Representative time lapse images of *brn3C:mGFP*-labeled neuromast hair cells (green) at 5 dpf following exposure to 10  $\mu$ M neomycin-Texas Red (white). (C) Mean number of neomycin-Texas Red puncta/hair cell in wild-type and *pappaa*<sup>p170</sup> larvae at 5 dpf. \*\* $p < 0.01$  t-test, Mann-Whitney correction. N = 19 hair cells (wild type) and 22 hair cells (*pappaa*<sup>p170</sup>) collected from four larvae/genotype. (D) Mean neomycin-Texas Red  $\Delta F/F_0$  at 2.5, 4.5, and 6.5 min post-exposure. Multiple t-test with Holm-Sidak correction found no significant difference. N = 22 hair cells (wild type) and 22 hair cells (*pappaa*<sup>p170</sup>) collected from four larvae/genotype. (E) Maximum change in neomycin-Texas Red fluorescent intensity across treatment time. Unpaired t-test with Mann-Whitney correction found no significant difference. N = 22 hair cells (wild type) and 22 hair cells (*pappaa*<sup>p170</sup>) collected from four larvae/genotype. See **Videos 1** and **2**. (F) Representative time lapse images of vehicle or 120  $\mu$ M NBI-31772 treated *brn3C:mGFP*-labeled neuromast hair cells (green) of *pappaa*<sup>p170</sup> larvae at 5 dpf following exposure to 10  $\mu$ M neomycin-Texas Red (white). (G) Average number of neomycin-Texas Red puncta/hair cell in *pappaa*<sup>p170</sup> larvae at 5 dpf following exposure to 10  $\mu$ M neomycin-Texas Red (white) with or without 120  $\mu$ M NBI-31772. \*\*\*\* $p < 0.0001$  t-test, Mann-Whitney correction. N = 30 hair cells (Vehicle) and 24 hair cells (120  $\mu$ M NBI-31772) collected from four larvae/genotype. Figure 4 continued on next page

*Figure 4 continued*

exposure to 10  $\mu$ M neomycin-Texas Red (white). (G) Mean number of neomycin-Texas Red puncta/hair cell in vehicle or 120  $\mu$ M NBI-31772 treated *pappaa*<sup>p170</sup> larvae at 5 dpf. \*\*\*\*p<0.0001 t-test, Mann-Whitney correction. N = 30 hair cells (Vehicle) and 24 hair cells (120  $\mu$ M NBI-31772) collected from four larvae/ group. Error bars=SEM.





**Figure 5.** Pappaa loss causes ER stress. (A) Schematic of the UPR pathway. The accumulation of unfolded proteins activates the UPR receptors, IRE1, ATF6, and PERK, signifying ER stress. In the early adaptive phase of ER stress, the UPR promotes cell survival through the upregulation of pro-survival factors including *bip*, *atf4*, and *spliced xbp1*. A switch from an adaptive to a pro-apoptotic UPR occurs during the late phase of ER stress in which Chop, a pro-apoptotic transcription factor, is upregulated. (B) Mean fold change in UPR mRNA levels in wild-type and *pappaa*<sup>p170</sup> hair cells at 5 dpf. N = 2–3 technical replicates/gene. \*p < 0.05, \*\*\*p < 0.001, \*\*\*\*p < 0.0001, two-way ANOVA, Holm–Sidak post-test. Error bars = SEM. (C) Representative images of TUNEL staining (magenta) in wild-type and *pappaa*<sup>p170</sup> lateral line hair cells. Stereocilia are counterstained with phalloidin (white). A 30 min treatment with 100  $\mu$ M neomycin was used as positive control (top). (D) Mean percentage of surviving hair cells following a 24 hr treatment with tunicamycin starting from 4 dpf. To calculate hair cell survival percentage, hair cell number post-treatment was normalized to mean hair cell number in vehicle-treated larvae of the same genotype. \*p < 0.05, \*\*p < 0.01, two-way ANOVA, Holm–Sidak post-test. N = 8–10 larvae per group (shown at base of bars), three neuromasts/larva from two experiments were analyzed. Total number of neuromasts included in the analysis = 51 (wild type); Figure 5 continued on next page

Figure 5 continued

vehicle treated), 57 (*pappaa*<sup>P170</sup>; vehicle treated), 27 (wild type; 2  $\mu$ M Tunicamycin), 27 (*pappaa*<sup>P170</sup>; 2  $\mu$ M Tunicamycin), 27 (wild type; 3  $\mu$ M Tunicamycin), 27 (*pappaa*<sup>P170</sup>; 3  $\mu$ M Tunicamycin). Error bars=SEM.