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Supplemental information

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Supplemental material

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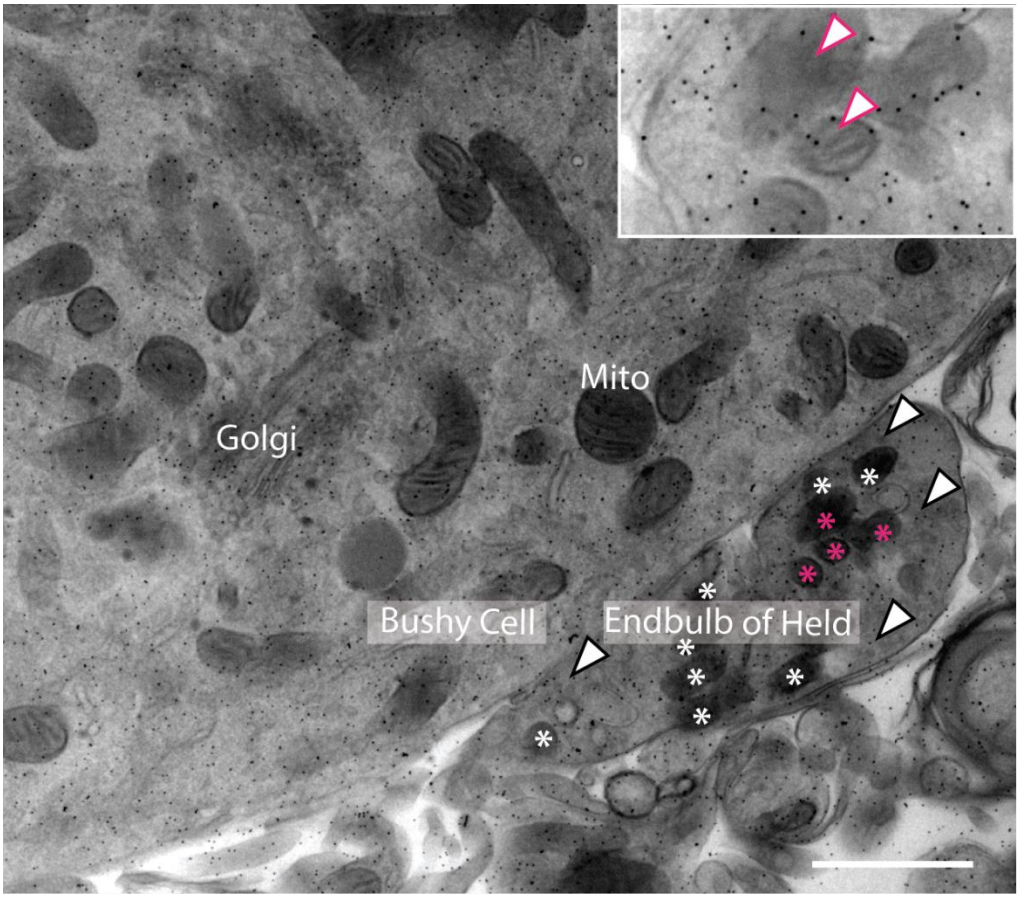

Figure S1: Selection criteria of endbulbs of Held, related to Fig. 4,5. Example endbulb of Held of a P10 wt mouse projecting onto a bushy cell (BC). The image is taken from a 250 nm-semithin section prepared for electron tomography already covered with gold as fiducials. The selection in this kind of images was done based on the morphology of the endbulb terminal. Only large presynaptic terminals with visible vesicle clusters (white arrowheads) were selected for mitochondria analysis. The BC can be clearly distinguished

from other cells by its size, typically other organelles next to mitochondria (Mito) such as the Golgi apparatus (Golgi) are visible. In the example endbulb, the mitochondria (in the inset arrowheads point to identifiable cristae) are marked by asterisks. The pink asterisks label the mitochondria that also appear in the inset. Scale bar 700 nm.

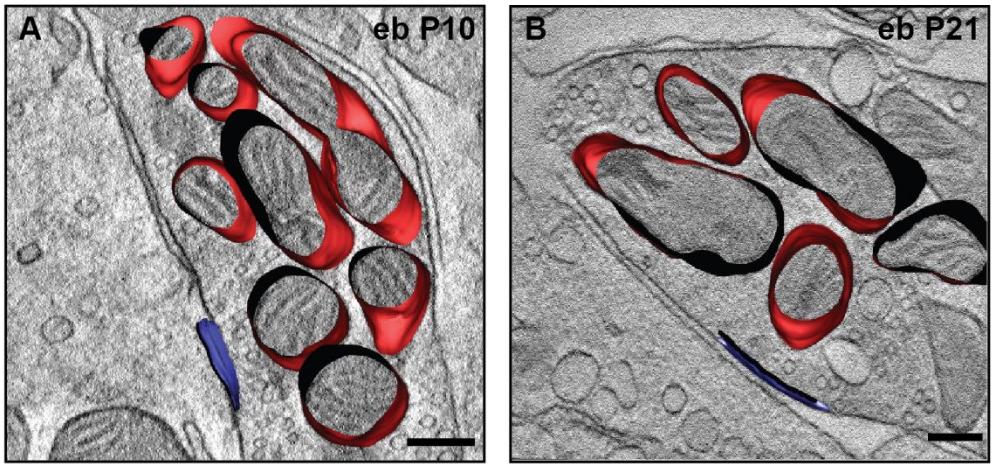

Figure S2: **Mitochondria show a variety of cutting planes, related to Fig. 4,5.** 3D model with the z-view of a tomogram showing presynaptic mitochondria (red) within an endbulb (eb) terminal of a P10 **(A)** and a P21 **(B)** wt mouse. The AZ membrane is shown in blue. Both images show the diversity of mitochondrial sizes and shapes and illustrates that just a portion of the mitochondria can be analyzed with electron tomography of 250 nm slices. Scale bars 200 nm.

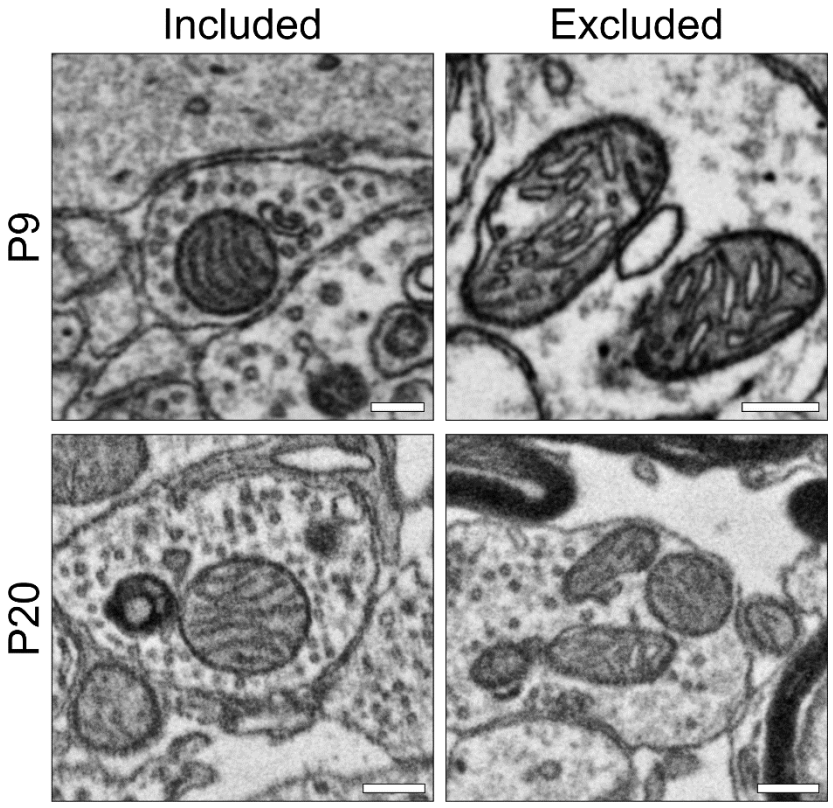

Figure S3: Selection criteria of mitochondria for crista membrane analyses, related to Fig 3. Left: SEM images of presynaptic mitochondria display a homogenous morphology of the mitochondrial outer membrane and no sign of perturbations of the crista membrane architecture in both, P9 and P20 animals. Mitochondria fulfilling these quality criteria were included for the structural analyses of inner mitochondrial architecture. **Right:** Mitochondria display irregular morphologies of the mitochondrial outer membrane and the cytoplasm seemed partially washed out. Since we believe that specifically these features represent embedding artifacts, we excluded this category for any further analysis. We selected mitochondria where crista membranes could be segmented over the entire volume of the mitochondrion. Scale bars 200 nm.

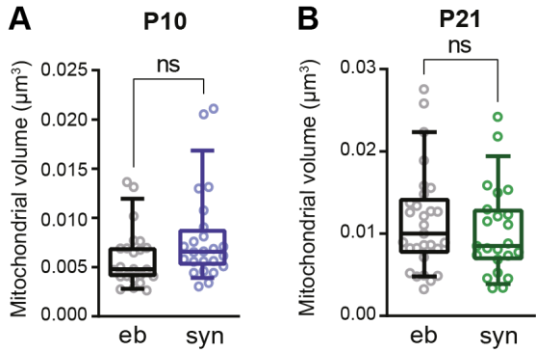

Figure S4: **Volumes of endbulb and non-endbulb terminals are comparable, related to Fig. 4.** The volumes of the mitochondria in which the CMs could be segmented are not significantly different between endbulbs (eb) and non-endbulbs (syn) at P10 **(A)** and P21 **(B)**. Box and whisker plots present median, lower/upper quartiles, 10–90th percentiles. ns, not significant. eb P10 (*N* = 3; *n* = 23) in black; syn P10 (*N* = 3; *n* = 24) in blue; eb P21 (*N* = 3; *n* = 29) in black and syn P21 (*N* = 3; *n* = 23) in green. (*N*: number of animals; *n*: number of mitochondria. For values and statistics, see Tables S3 and S4.

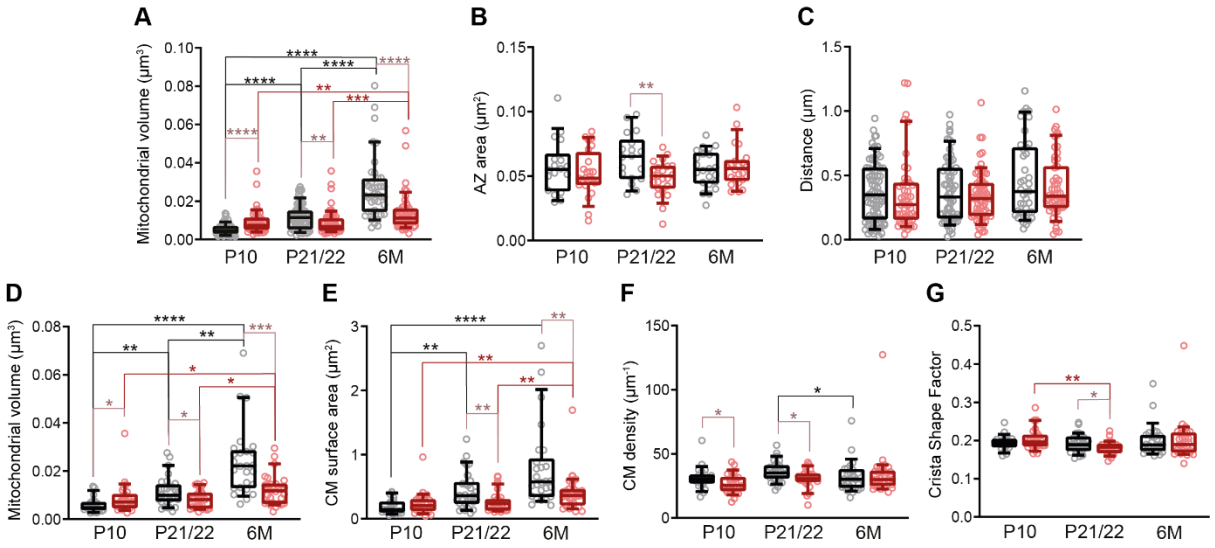

Figure S5: Otoferlin lacking animals exhibit smaller mitochondria in endbulbs of Held, related to Fig. 5. **(A)** Comparing all mitochondria, their mean reconstructed volume increases upon development in both, wt and *Otof*$^{-/-}$. While mitochondria are larger in *Otof*$^{-/-}$ endbulbs at P10, their volumes are smaller at P22 and 6M compared to wt. **(B)** The sizes of the AZ areas reconstructed in the tomograms are comparable between the age groups. However, AZs of P22 *Otof*$^{-/-}$ endbulb synapses are significantly smaller compared to wt. **(C)** The shortest distance of the organelles to the AZ membrane is similar between endbulbs of all analyzed age groups and genotypes. **(D)** The volume of the mitochondria in which the CMs could be reconstructed increases upon development in wt and from P10 to 6M in *Otof*$^{-/-}$ endbulbs. **(E)** The sum of surface areas of CMs follows the same pattern. **(F)** The CM density (CM surface area/ mitochondrial volume) decreases from P21 to 6M in wt while it is unchanged in *Otof*$^{-/-}$ mice during development. Comparing genotypes, the CM density is smaller in mitochondria of *Otof*$^{-/-}$ endbulbs compared to wt at P10 and P21/22. **(G)** The crista shape factor (CM surface area/CM lumen volume) indicates no change in the organization of the cristae in wt but points to more tubular shaped cristae at P22 compared to P10 in *Otof*$^{-/-}$ mice. Box and whisker plots present median, lower/upper quartiles, 10–90th percentiles. Indicated are all significant changes. ns, not significant; **p* < 0.05; ****p* < 0.001, *****p* <0.0001. wt P10 (*N* = 3; *n* = 82/23), wt P21 (*N* = 3; *n* = 65/29), wt 6M (*N* = 3; *n* = 40/26) in black. *Otof*$^{-/-}$ P10 (*N* = 3; *n* = 48/27), *Otof*$^{-/-}$ P22 (*N* = 3; *n* = 67/29), *Otof*$^{-/-}$ 6M (*N* = 3; *n* = 52/29) in red. (*N*: number of animals; *n*: number of mitochondria (all/with segmentation of CMs). For details about values and statistics, see Tables S5-S9.

Parameter	**syn P10**	**syn P21**	***p*-value**
Mitochondrial volume (all; µm³)	0.0077 ± 0.0045 (0.0063)	0.0099 ± 0.0050 (0.0087)	**p* = 0.0167

AZ area (µm^2)	0.0469 ± 0.0165 (0.0444)	0.0546 ± 0.0165 (0.0521)	p = 0.1825
Distance (Mitochondrion – AZ membrane; µm)	0.2518 ± 0.1522 (0.2123)	0.2942 ± 0.1509 (0.2375)	p = 0.2638
Mitochondrial volume (µm^3)	0.0080 ± 0.0047 (0.0066)	0.0103 ± 0.0055 (0.0085)	p = 0.0577
CM surface area (µm^2)	0.2322 ± 0.1543 (0.1908)	0.3028 ± 0.1520 (0.2642)	*p = 0.0424
CM density (µm^{-1})	28.66 ± 5.13 (27.55)	30.42 ± 5.95 (31.12)	p = 0.2782
Crista Shape Factor	0.1852 ± 0.0173 (0.1843)	0.1957 ± 0.0197 (0.1966)	p = 0.0606

Table S1. Quantitative analysis of tomograms of wt mitochondria within non-endbulb terminals (syn), related to Fig. 1. Data are presented as mean ± SEM (median in brackets). Normal distribution was assessed with the Jarque-Bera test and equality of variances was assessed with the *F*-test in normally distributed data. *t*-test was used to determine statistical significance of normally distributed data. For non-normally distributed data Wilcoxon Rank test was used. *p*-value > 0.05: not significant, **p*-value < 0.05.

Parameter	P9		P20		*p*-value
Mitochondrial occupation (%)	55.30 ± 2.26 (55.30)		79.95 ± 2.05 (79.95)		***p* = 0.0079
	P9 no Mito	**P9 Mito**	**P20 no Mito**	**P20 Mito**	***p*-value**
Bouton volume (µm³)	0.5543 ± 0.2515 (0.5023)	1.266 ± 1.074 (0.951)	1.202 ± 1.022 (1.007)	2.947 ± 2.481 (2.161)	P9 no Mito vs. P9 Mito: ***p* = 0.0011 P20 no Mito vs. P20 Mito: **p* = 0.0222 P9 no Mito vs. P20 no Mito: **p* = 0.0182 P9 Mito vs. P20 Mito: **p* = 0.0409
PSD volume (µm³)	0.0050 ± 0.0040 (0.0032)	0.0069 ± 0.0047 (0.0061)	0.0121 ± 0.0151 (0.0096)	0.0113 ± 0.0074 (0.0118)	P9 no Mito vs. P9 Mito: *p* = 0.1612 P20 no Mito vs. P20 Mito: *p* = 0.4994 P9 no Mito vs. P20 no Mito: ***p* = 0.0052 P9 Mito vs. P20 Mito: *p* = 0.0549
SV number	364.4 ± 146.1 (339.5)	639.1 ± 574.2 (450.5)	483.1 ± 298.7 (482.0)	846.3 ± 349.9 (874.0)	P9 no Mito vs. P9 Mito: *p* = 0.0615 P20 no Mito vs. P20 Mito: ***p* = 0.0049 P9 no Mito vs. P20 no Mito: *p* = 0.1585 P9 Mito vs. P20 Mito: **p* = 0.0150
Mitochondrial volume (µm³)		0.0520 ± 0.0235 (0.0516)		0.1592 ± 0.0947 (0.1218)	****$p < 0.0001$
CM surface area (µm²)		0.8845 ± 0.3754 (0.8014)		4.537 ± 2.79 (3.614)	****$p < 0.0001$
CM density (µm⁻¹)		18.85 ± 7.42 (17.85)		29.16 ± 9.53 (27.08)	***p = 0.0002

Table S2. Quantitative analysis of FIB-SEM data of wt P9 and P20 mitochondria, related to Fig. 2,3. Data are presented as mean ± SEM (median in brackets). Normal distribution was assessed with the Jarque-Bera test and equality of variances was assessed with the *F*-test in normally distributed data. *t*-test was used for normally distributed data, or, when not normally distributed, the Wilcoxon rank test was used to determine statistical significance. *p*-value > 0.05: not significant, **p*-value < 0.05, **p-value < 0.01, ***p*-value < 0.001, ****p*-value < 0.0001.

Parameter	eb P10	syn P10	*p*-value
Mitochondrial volume (all; µm³)	0.0052 ± 0.0027 (0.0045)	0.0077 ± 0.0045 (0.0063)	***p = 0.0007
AZ area (µm²)	0.0573 ± 0.0209 (0.0552)	0.0469 ± 0.0165 (0.0444)	p = 0.0987
Distance (Mitochondrion – AZ membrane; µm)	0.3723 ± 0.2316 (0.3482)	0.2518 ± 0.1522 (0.2123)	**p = 0.0018
Mitochondrial volume (µm³)	0.0060 ± 0.0030 (0.0048)	0.0080 ± 0.0047 (0.0066)	p = 0.0772
CM surface area (µm²)	0.1915 ± 0.1120 (0.1443)	0.2322 ± 0.1543 (0.1908)	p = 0.2806
CM density (µm^{-1})	31.25 ± 8.61 (30.15)	28.66 ± 5.13 (27.55)	p = 0.1681
Crista Shape Factor	0.1937 ± 0.0182 (0.1930)	0.1852 ± 0.0173 (0.1843)	p = 0.0972

Table S3. Quantitative analysis of tomograms of P10 wt mitochondria within endbulb (eb) and non-endbulb terminals (syn), related to Fig. 4, S4. Data are presented as mean ± SEM (median in brackets). Normal distribution was assessed with the Jarque-Bera test and equality of variances was assessed with the *F*-test in normally distributed data. *T*-test was used to determine statistical significance of normally distributed data. For non-normally distributed data Wilcoxon Rank test was used. *P*-value > 0.05: not significant, ***p*-value < 0.01, ****p*-value < 0.001.

Parameter	eb P21	syn P21	*p*-value
Mitochondrial volume (all; µm³)	0.0116 ± 0.0064 (0.0115)	0.0099 ± 0.0050 (0.0087)	p = 0.2034
AZ area (µm²)	0.0643 ± 0.0186 (0.0652)	0.0546 ± 0.0165 (0.0521)	p = 0.1221
Distance (Mitochondrion – AZ membrane; µm)	0.3801 ± 0.2431 (0.3318)	0.2942 ± 0.1509 (0.2375)	p = 0.1983
Mitochondrial volume (µm³)	0.0115 ± 0.0062 (0.0100)	0.0103 ± 0.0055 (0.0085)	p = 0.3747
CM surface area (µm²)	0.4375 ± 0.2901 (0.3605)	0.3028 ± 0.1520 (0.2642)	p = 0.1115
CM density (µm^{-1})	36.16 ± 7.99 (35.14)	30.42 ± 5.95 (31.12)	**p = 0.0046
Crista Shape Factor	0.1927 ± 0.0235 (0.1892)	0.1957 ± 0.0197 (0.1966)	p = 0.6241

Table S4. Quantitative analysis of tomograms of P21 wt mitochondria within endbulb (eb) and non-endbulb terminals (syn), related to Fig. 4, S4. Data are presented as mean ± SEM (median in brackets). Normal distribution was assessed with the Jarque-Bera test and equality of variances was assessed with the *F*-test in normally distributed data. *T*-test was used to determine statistical significance of normally

92 distributed data. For non-normally distributed data Wilcoxon Rank test was used. *P*-value > 0.05: not
93 significant, ***p*-value < 0.01.

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Parameter	wt P10	wt P21	wt 6M	*p*-value
Mitochondrial volume (all; µm³)	0.0052 ± 0.0027 (0.0045)	0.0116 ± 0.0064 (0.0115)	0.0269 ± 0.0165 (0.0231)	wt P10 vs. wt P21: ****$p < 0.0001$ wt P10 vs. wt 6M: ****$p < 0.0001$ wt P21 vs. wt 6M: ****$p < 0.0001$
AZ area (µm²)	0.0573 ± 0.0209 (0.0552)	0.0643 ± 0.0186 (0.0652)	0.0555 ± 0.0143 (0.0550)	$p = 0.2825$
Distance (Mitochondrion – AZ membrane; µm)	0.3723 ± 0.2316 (0.3482)	0.3801 ± 0.2431 (0.3318)	0.4669 ± 0.3071 (0.3745)	$p = 0.2813$
Mitochondrial volume (µm³)	0.0060 ± 0.0030 (0.0048)	0.0115 ± 0.0062 (0.0100)	0.0241 ± 0.0144 (0.0221)	wt P10 vs. wt P21: **$p = 0.0037$ wt P10 vs. wt 6M: ****$p < 0.0001$ wt P21 vs. wt 6M: **$p = 0.0018$
CM surface area (µm²)	0.1915 ± 0.1120 (0.1443)	0.4375 ± 0.2901 (0.3605)	0.7937 ± 0.6359 (0.5745)	wt P10 vs. wt P21: **$p = 0.0016$ wt P10 vs. wt 6M: ****$p < 0.0001$ wt P21 vs. wt 6M: $p = 0.0596$
CM density (µm⁻¹)	31.25 ± 8.61 (30.15)	36.16 ± 7.99 (35.14)	31.99 ± 12.22 (29.55)	wt P10 vs. wt P21: $p = 0.0610$ wt P10 vs. wt 6M: $p > 0.9999$ wt P21 vs. wt 6M: *$p = 0.0492$
Crista Shape Factor	0.1937 ± 0.0182 (0.1930)	0.1927 ± 0.0235 (0.1892)	0.2004 ± 0.0411 (0.1885)	$p = 0.9304$

Table S5. Quantitative analysis of tomograms of wt mitochondria, related to Fig. 5, S5. Data are presented as mean ± SEM (median in brackets). Normal distribution was assessed with the Jarque-Bera test and equality of variances was assessed with the *F*-test in normally distributed data. Statistical significance of normally distributed data was determined by one-way ANOVA test followed by Tukey´s's test. For non-normally distributed data, Kruskal–Wallis (KW) test followed by Dunn's test was used. *p*-value > 0.05: not significant, **p*-value < 0.05, ***p*-value < 0.01, ****p*-value < 0.001, **** *p*-value < 0.0001.

Parameter	*Otof*$^{-/-}$ P10	*Otof*$^{-/-}$ P22	*Otof*$^{-/-}$ 6M	*p*-value
Mitochondrial volume (all; µm^3)	0.0091 ± 0.0061 (0.0074)	0.0089 ± 0.0060 (0.0069)	0.0137 ± 0.0098 (0.0112)	*Otof*$^{-/-}$ P10 vs. *Otof*$^{-/-}$ P22: $p > 0.9999$ *Otof*$^{-/-}$ P10 vs. *Otof*$^{-/-}$ 6M: $^{**}p = 0.0019$ *Otof*$^{-/-}$ P22 vs. *Otof*$^{-/-}$ 6M: $^{***}p = 0.0001$
AZ area (µm^2)	0.0526 ± 0.0181 (0.0484)	0.0485 ± 0.0135 (0.0501)	0.0576 ± 0.0165 (0.0559)	$p = 0.1706$
Distance (Mitochondrion – AZ membrane; µm)	0.3549 ± 0.2824 (0.2724)	0.3391 ± 0.1953 (0.3200)	0.4079 ± 0.2341 (0.3385)	$p = 0.2823$
Mitochondrial volume (µm^3)	0.0088 ± 0.0063 (0.0071)	0.0082 ± 0.0038 (0.0082)	0.0122 ± 0.0063 (0.0118)	*Otof*$^{-/-}$ P10 vs. *Otof*$^{-/-}$ P22: $p > 0.9999$ *Otof*$^{-/-}$ P10 vs. *Otof*$^{-/-}$ 6M: $^{*}p = 0.0262$ *Otof*$^{-/-}$ P22 vs. *Otof*$^{-/-}$ 6M: $^{*}p = 0.0246$
CM surface area (µm^2)	0.2364 ± 0.1769 (0.2044)	0.2534 ± 0.1468 (0.2322)	0.4058 ± 0.2915 (0.3620)	*Otof*$^{-/-}$ P10 vs. *Otof*$^{-/-}$ P22: $p > 0.9999$ *Otof*$^{-/-}$ P10 vs. *Otof*$^{-/-}$ 6M: $^{**}p = 0.0019$ *Otof*$^{-/-}$ P22 vs. *Otof*$^{-/-}$ 6M: $^{**}p = 0.0087$
CM density (µm^{-1})	26.76 ± 7.50 (25.33)	30.97 ± 7.10 (30.71)	34.19 ± 19.09 (29.90)	KW: $p = 0.0375$ Dunn's: *Otof*$^{-/-}$ P10 vs. *Otof*$^{-/-}$ P22: $p = 0.0539$ *Otof*$^{-/-}$ P10 vs. *Otof*$^{-/-}$ 6M: $p = 0.1142$ *Otof*$^{-/-}$ P22 vs. *Otof*$^{-/-}$ 6M: $p > 0.9999$
Crista Shape Factor	0.2027 ± 0.0276 (0.1966)	0.1813 ± 0.0171 (0.1825)	0.2008 ± 0.0541 (0.1898)	*Otof*$^{-/-}$ P10 vs. *Otof*$^{-/-}$ P22: $^{**}p = 0.0039$ *Otof*$^{-/-}$ P10 vs. *Otof*$^{-/-}$ 6M: $p = 0.5406$ *Otof*$^{-/-}$ P22 vs. *Otof*$^{-/-}$ 6M: $p = 0.1684$

Table S6. Quantitative analysis of tomograms of *Otof*$^{-/-}$ mitochondria, related to Fig. 5, S5. Data are presented as mean ± SEM (median in brackets). Normal distribution was assessed with the Jarque-Bera test and equality of variances was assessed with the *F*-test in normally distributed data. Statistical significance of normally distributed data was determined by one-way ANOVA test followed by Tukey's's test. For non-normally distributed data, Kruskal–Wallis (KW) test followed by Dunn's test was used. *p*-value > 0.05: not significant, $^{*}p$-value < 0.05, **p-value < 0.01, $^{***}p$-value < 0.001.

Parameter	wt P10	*Otof*$^{-/-}$ P10	*p*-value
Mitochondrial volume (all; µm^3)	0.0052 ± 0.0027 (0.0045)	0.0091 ± 0.0061 (0.0074)	$^{****}p < 0.0001$
AZ area (µm^2)	0.0573 ± 0.0209 (0.0552)	0.0526 ± 0.0181 (0.0484)	$p = 0.4450$
Distance (Mitochondrion – AZ membrane; µm)	0.3723 ± 0.2316 (0.3482)	0.3549 ± 0.2824 (0.2724)	$p = 0.6090$
Mitochondrial volume (µm^3)	0.0060 ± 0.0030 (0.0048)	0.0088 ± 0.0063 (0.0071)	$^{*}p = 0.0339$
CM surface area (µm^2)	0.1915 ± 0.1120 (0.1443)	0.2364 ± 0.1769 (0.2044)	$p = 0.3897$

CM density (μm^{-1})	31.25 ± 8.61 (30.15)	26.76 ± 7.50 (25.33)	*p = 0.0412
Crista Shape Factor	0.1937 ± 0.0182 (0.1930)	0.2027 ± 0.0276 (0.1966)	p = 0.4226

Table S7. Quantitative analysis of tomograms of P10 wt and *Otof*$^{-/-}$ mice, related to Fig. 5, S5. Data are presented as mean ± SEM (median in brackets). Normal distribution was assessed with the Jarque-Bera test and equality of variances was assessed with the *F*-test in normally distributed data. *t*-test was used to determine statistical significance of normally distributed data. For non-normally distributed data Wilcoxon Rank test was used. *p*-value > 0.05: not significant, *p-value < 0.05, ****p-value < 0.0001.

Parameter	**wt P21**	***Otof*$^{-/-}$ P22**	***p*-value**
Mitochondrial volume (all; μm^3)	0.0116 ± 0.0064 (0.0115)	0.0089 ± 0.0060 (0.0069)	**p = 0.0066
AZ area (μm^2)	0.0643 ± 0.0186 (0.0652)	0.0485 ± 0.0135 (0.0501)	**p = 0.0048
Distance (Mitochondrion – AZ membrane; µm)	0.3801 ± 0.2431 (0.3318)	0.3391 ± 0.1953 (0.3200)	p = 0.3966
Mitochondrial volume (μm^3)	0.0115 ± 0.0062 (0.0100)	0.0082 ± 0.0038 (0.0082)	*p = 0.0387
CM surface area (μm^2)	0.4375 ± 0.2901 (0.3605)	0.2534 ± 0.1468 (0.2322)	**p = 0.0036
CM density (μm^{-1})	36.16 ± 7.99 (35.14)	30.97 ± 7.10 (30.71)	*p = 0.0210
Crista Shape Factor	0.1927 ± 0.0235 (0.1892)	0.1813 ± 0.0171 (0.1825)	*p = 0.0378

Table S8. Quantitative analysis of tomograms of P21/22 wt and *Otof*$^{-/-}$ mice, related to Fig. 5, S5. Data are presented as mean ± SEM (median in brackets). Normal distribution was assessed with the Jarque-Bera test and equality of variances was assessed with the *F*-test in normally distributed data. *t*-test was used to determine statistical significance of normally distributed data. For non-normally distributed data, Wilcoxon Rank test was used. *p*-value > 0.05: not significant, *p-value < 0.05, **p-value < 0.01.

Parameter	wt 6M	*Otof*$^{-/-}$ 6M	*p*-value
Mitochondrial volume (all; µm^3)	0.0269 ± 0.0165 (0.0231)	0.0137 ± 0.0098 (0.0112)	****p < 0.0001
AZ area (µm^2)	0.0555 ± 0.0143 (0.0550)	0.0576 ± 0.0165 (0.0559)	p = 0.8708
Distance (Mitochondrion – AZ membrane; µm)	0.4669 ± 0.3071 (0.3745)	0.4079 ± 0.2341 (0.3385)	p = 0.6697
Mitochondrial volume (µm^3)	0.0241 ± 0.0144 (0.0221)	0.0122 ± 0.0063 (0.0118)	***p = 0.0001
CM surface area (µm^2)	0.7937 ± 0.6359 (0.5745)	0.4058 ± 0.2915 (0.3620)	**p = 0.0015
CM density (µm^{-1})	31.99 ± 12.22 (29.55)	34.19 ± 19.09 (29.90)	p = 0.6881
Crista Shape Factor	0.2004 ± 0.0411 (0.1885)	0.2008 ± 0.0541 (0.1898)	p = 0.8936

Table S9. Quantitative analysis of tomograms of 6M wt and *Otof*$^{-/-}$ mice, related to Fig. 5, S5. Data are presented as mean ± SEM (median in brackets). Normal distribution was assessed with the Jarque-Bera test and equality of variances was assessed with the *F*-test in normally distributed data. *t*-test was used to determine statistical significance of normally distributed data. For non-normally distributed data, Wilcoxon Rank test was used. *p*-value > 0.05: not significant, ***p*-value < 0.01, ****p*-value < 0.001, *****p*-value < 0.0001.