

The ATF6 β -calreticulin axis promotes neuronal survival under endoplasmic reticulum stress and excitotoxicity

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Supplementary information

Supplementary tables (Table S1-S3)

Table S1 Differentially expressed genes in *Atf6b*^{-/-} brain (p<0.05)

Downregulated genes in <i>Atf6b</i> ^{-/-} brain					Upregulated genes in <i>Atf6b</i> ^{-/-} brain				
Gene	WT	<i>Atf6b</i> ^{-/-}	log2 (fold-change)	p-value	Gene	WT	<i>Atf6b</i> ^{-/-}	log2 (fold-change)	p-value
<i>Atf6b</i>	31.633	7.66511	-2.04505	5.00E-05	<i>Gm47283</i>	13.354	43.6105	1.70741	5.00E-05
<i>Rsph3a</i>	11.5898	6.87324	-0.753794	0.03625	<i>Igf2</i>	19.0054	39.9251	1.07089	5.00E-05
<i>Slc4a1ap</i>	14.1345	8.47099	-0.738618	0.00545	<i>Ptgds</i>	452.8	942.637	1.05783	5.00E-05
<i>Calr</i>	203.013	123.236	-0.720147	5.00E-05	<i>Mgp</i>	20.9161	35.9567	0.781646	5.00E-05
<i>Tagap1</i>	10.7218	7.09068	-0.596551	0.00155	<i>Hspa5</i>	121.782	195.426	0.682327	0.00145
<i>Zfp46</i>	16.4713	11.5521	-0.511808	0.0306	<i>Igfbp2</i>	16.5051	26.1894	0.666073	2.50E-04
<i>Ddx3y</i>	12.4869	8.78594	-0.507145	0.00365	<i>Pacrg</i>	15.6079	24.531	0.652331	0.0077
<i>Thsd4</i>	11.2284	8.18176	-0.456674	0.0046	<i>Fxyd5</i>	14.7043	22.6957	0.626184	0.00605
<i>Nkain1</i>	10.2744	7.63411	-0.428519	0.027	<i>Pdia4</i>	16.5207	24.6939	0.579886	3.00E-04
<i>Pla2g7</i>	84.9424	64.1773	-0.404422	0.00865	<i>Tmem181b-ps</i>	50.8316	72.4126	0.510517	0.0072
<i>Col6a1</i>	33.9319	25.7853	-0.396092	0.01075	<i>H2-D1</i>	12.1692	17.213	0.500263	0.0353
<i>Calb2</i>	41.538	31.5872	-0.395091	0.012	<i>Neat1</i>	34.358	48.3574	0.493091	0.0025
<i>Fam107a</i>	134.643	102.774	-0.389658	0.01485	<i>Ly6a</i>	18.7354	26.0661	0.47641	0.0085
<i>Trpm3</i>	10.1718	7.77531	-0.387606	0.01675	<i>Cars</i>	17.0994	23.7093	0.471512	0.04035
<i>Sgk1</i>	25.7941	19.7465	-0.385444	0.0236	<i>H2-K1</i>	16.9189	23.4385	0.470241	0.03755
<i>Slc4a4</i>	25.3338	19.5091	-0.376918	0.0152	<i>Myl9</i>	11.9739	16.2991	0.444894	0.01375
<i>Mt2</i>	244.703	188.703	-0.374911	0.01655	<i>Apod</i>	85.8839	116.502	0.439901	0.0043
<i>Gpr17</i>	13.7092	10.7602	-0.349437	0.0296	<i>Psrl</i>	10.3895	13.8884	0.418754	0.03945
<i>Mal</i>	75.9335	59.7091	-0.346785	0.0185	<i>Bex1</i>	34.0072	44.8394	0.398926	0.0186
<i>Slit3</i>	13.5417	10.8053	-0.325672	0.0341	<i>Fxyd7</i>	47.639	61.2804	0.363284	0.02985
<i>Col25a1</i>	19.4255	15.5616	-0.319957	0.04735	<i>Egr1</i>	19.154	24.279	0.342066	0.0258
<i>AC149090.1</i>	300.785	242.572	-0.310319	0.04235	<i>Dnajb11</i>	24.9619	31.5182	0.336456	0.03615
					<i>Atf4</i>	60.6921	76.4419	0.332855	0.0253
					<i>Wfs1</i>	22.6167	28.3747	0.327215	0.04035
					<i>P4hal</i>	19.794	24.6951	0.319167	0.04445
					<i>Rgs7</i>	23.7278	29.4115	0.309803	0.04605
					<i>Stmn1</i>	146.103	179.343	0.295739	0.048

Table S2 Expression of genes encoding molecular chaperones in the ER in WT and *Atf6b*^{-/-} mouse brains

Gene	WT	<i>Atf6b</i> ^{-/-}	log ₂ (fold-change)	p-value
<i>Calr</i>	203.013	123.236	-0.720147	5.00E-05
<i>Hspa5</i> (GRP78)	121.782	195.426	0.00145	0.00145
<i>Hsp90b1</i> (GRP94)	110.637	128.981	0.1398	0.1398
<i>Hyou1</i> (GRP170/ ORP150))	26.6409	27.9665	0.07006	0.66575
<i>Pdia3</i> (ERp57)	71.9795	69.394	0.7614	0.7614
<i>Pdia4</i> (ERp72)	16.5207	24.6939	0.0003	0.0003
<i>Pdia6</i> (ERp5)	54.1244	67.2112	0.312422	0.0606
<i>Pdia10</i> (ERp44)	15.0019	15.8719	0.0813263	0.7464
<i>Hsp40</i> (Dnajb11)	24.9619	31.5182	0.336456	0.03615
<i>Canx</i>	103.499	102.51	-0.0138629	0.94155

Table S3 List of qRT-PCR primers used in this study

Gene (mouse)	Primer sequences (5'-3')	
	Forward	Reverse
<i>Atf6b</i>	AACAGGAAGTTGTCTGCATCAT	CTGCTCATCCGAGGAGAGACATG
<i>Atf6a</i>	ACAGCTGCCTAACCATGTG	AGCGATATCCGAACCCATAC
<i>Calr</i>	ATAAAGGGCTGCAGACAAGC	CCACAGTCGATATTCTGCTC
<i>Canx</i>	AGCAGAGATGGCATGATGCT	GTTGAGGCTCATGATGGACA
<i>Hsp90b1</i> (GRP94)	AAGCCCTCAAGGACAAGAT	TGCCAGACCATCCATCCATACTGA
<i>Hspa5</i> (GRP78)	ATGGTATTCTCCGAGTGACA	GCTTTCCAGCCATTCAATCT
<i>Fos</i>	CTGCAGCCAAGTGCCGGAATC	GGCAATCTCAGTCTGCAACGC
<i>Fosb</i>	TTTTCCCGGAGACTACGACTC	GTGATTGCGGTGACCGTTG
<i>Bdnf</i>	ACAAGGCAACTTGGCCTACC	AGCAGCTCTTCGATGACGTG

Supplementary figure legends

Figure S1. Expression of *Atf6a* and *Atf6b*. Expression of *Atf6a* (A, B, D) and *Atf6b* (C) mRNAs. Total RNA was isolated from the indicated samples and qRT-PCR was performed. HPC: hippocampus, Cx: cerebral cortex, SC: spinal cord. A, n=5 mice. B, n=4 experiments. C, n=4 (male) and n=3 (female) mice. D, n=4 experiments. Data are shown as mean ± SEM. *p

< 0.05, **p < 0.001, ***p < 0.001 by a one-way ANOVA followed by the Tukey test.

Figure S2. Read alignments of RNA-sequencing for *Atf6b*. RNA-sequencing read alignments from the hippocampus of WT (upper row) and *Atf6b*^{-/-} (lower row) mice are illustrated by Integrated Genomics Viewer (<http://software.broadinstitute.org/software/igv/>). The snapshot shows an enlarged view for these alignments within the *Atf6b* region. The gray bar chart at the top of each alignment (Coverage track) shows the depth of reads at each locus, with the number of reads ranging from 0 to 500. The exons of *Atf6b* gene are indicated on the bottom.

Figure S3. Expression of molecular chaperones in the ER and promoter activities for CRT and GRP78. A, Expression of molecular chaperones in the ER in WT, *Atf6a*^{-/-} and *Atf6b*^{-/-} hippocampi. Total RNA was isolated from the CA3 region of the hippocampus and qRT-PCR was performed. n=5–6 mice. Data are shown as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 by a one-way ANOVA followed by the Tukey test. B, C, Reporter assays using WT and *Atf6b*^{-/-} MEFs. B, The CAT ELISA and luciferase assay were performed using cells transfected with the mouse CRT promoter pCC1 (left) and the human CRT promoter huCRT (right), respectively. C, The luciferase assay were performed using cells transfected with the human GRP78 promoter huGRP78, n=6. Data are shown as mean ± SEM. **p < 0.01, ***p < 0.001 between two genotypes and ###p < 0.01 compared to normal conditions by a two-way ANOVA followed by the Bonferroni test.

Figure S4. Expression of Ca²⁺-indicator proteins in the primary hippocampal neurons. A, After transfection of cDNAs for each Ca²⁺-indicator protein, cells were fixed and subjected to immunocytochemical staining with GFP antibody. n=50–60 cells in each condition from two independent experiments. Data are shown as mean ± SEM. Scale bar: 10 μm.

Figure S5. ER stress-induced death in the primary hippocampal neurons. Nuclear localization of EthD-1 in Figure 4A is shown by the images with single fluorescence for Hoechst 33342 and EthD-1. Scale bar: 10 μm.

Figure S6. Protection of Neuro2a cells by ATF6β. A, Gene silencing of *Atf6b* in Neuro 2a cells. Neuro 2a cells were transfected with ATF6β-siRNA1, ATF6β-siRNA2, or control-siRNA, and qRT-PCR was performed after 24 h. n=4 experiments. Data are shown as mean ± SEM. ***p < 0.001 by a one-way ANOVA followed by the Tukey test. B, C, LIVE/DEAD viability assay (B) and immunocytochemistry (C). Neuro 2a cells transfected with ATF6β-siRNA1, ATF6β-siRNA2, or control-siRNA were incubated for 48 h and treated with Tg (20 nM) or Tm (5 μg/ml). Cell survival/death and apoptosis were evaluated by the LIVE/DEAD viability assay at 36 h (B) and immunocytochemical staining for cleaved caspase-3 at 24 h after stimulation (C), respectively. n=3 in (B) and n=4 in (C). Data are shown as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 by a two-way ANOVA followed by the Bonferroni test. Scale bar: 15 μm.

Figure S7. Protection of the primary hippocampal neurons by treatment with Ca²⁺/ER

stress-modulating compounds. A, WT and *Atf6b*^{-/-} hippocampal neurons were treated with Tm (1 µg/ml) together with BAPTA-AM (5 µM), 2-APB (2 µM), or salubrinal (5 µM). Cell death was evaluated by immunocytochemical staining for cleaved caspase-3. Typical images related to Figure 5C are shown. Scale bar: 20 µm. B, WT and *Atf6b*^{-/-} hippocampal neurons were treated with BAPTA-AM (5 µM) or 2-APB (2 µM) alone. Cell death was evaluated by immunocytochemical staining for cleaved caspase-3. n=3 experiments. Data are shown as mean ± SEM. ***p < 0.001 by a two-way ANOVA followed by the Bonferroni test. Scale bar: 20 µm. C, WT and *Atf6b*^{-/-} hippocampal neurons were treated with Tm (1 µg/ml) together with different concentrations of salubrinal (5-75 µM). Cell death was evaluated by immunocytochemical staining for cleaved caspase-3. n=3 experiments. Data are shown as mean ± SEM. *p < 0.05, ***p < 0.001 between two genotypes and # p < 0.05, ## p < 0.01, ### p < 0.001 compared to normal conditions by a two-way ANOVA followed by the Bonferroni test. Scale bar: 20 µm.

Figure S8. Neuroprotection by ATF6β in KA-injected mice and expression of *Calr* in *Calr*^{+/-} mice. A, Brain sections containing the CA3 area of the hippocampus from WT and *Atf6b*^{-/-} mice were subjected to Nissl staining. The right graph depicts the number of surviving CA3 neurons. n=6-8 mice. Data are shown as mean ± SEM. **p < 0.01, ***p < 0.001 by a two-way ANOVA followed by the Bonferroni test. Scale bar: 100 µm. B, Total RNA was isolated from WT and *Calr*^{+/-} brains including the CA3 region of the hippocampus and qRT-PCR analysis of *Calr* and *Canx* was performed. n=4 mice. Data are shown as mean ± SEM. *p < 0.001 by the Mann Whitney-U test. C, Protein extracts from WT and *Calr*^{+/-} brains

including the CA3 region of the hippocampus were subjected to western blotting for the indicated proteins.

Figure S9. Neuronal activity and death after KA injection into the hippocampi of WT

and *Atf6b*^{-/-} mice. A, Total RNA was isolated from the CA3 area of the hippocampus from WT and *Atf6b*^{-/-} mice after KA injection. n=3–5 mice. Data are shown as mean ± SEM. *p < 0.01, **p < 0.01 between two genotypes by a two-way ANOVA followed by the Bonferroni test. B, Brain sections including the CA3 area of the hippocampus obtained from WT and *Atf6b*^{-/-} mice at 1 h after KA injection were subjected to immunohistochemical staining for c-Fos. The right graph depicts the number of c-Fos-positive neurons. n=3 mice. Data are shown as mean ± SEM. *p < 0.05 by a two-way ANOVA followed by the Bonferroni test. C, D, Brain sections including the CA3 area of the hippocampus obtained from WT and *Atf6b*^{-/-} mice 3 days after injection with 2-APB or salubrinal alone (n=4 mice) (C) or with KA plus 2-APB and KA plus salubrinal (n=6 mice) (D) were subjected to Nissl staining. The right graph depicts the number of surviving CA3 neurons. Data are shown as mean ± SEM. **p < 0.01, ***p < 0.001 by a two-way ANOVA followed by the Bonferroni test.

Figure S10. Uncropped images of western blots from Figures 1E, 2B, and 3B. Western

blots of ATF6β from Figure 1E (A), Calreticulin, Calnexin, GRP78, and GAPDH from Figure 2B (B), and Calreticulin, Calnexin, GRP94, GRP78, and GAPDH from Figure 3B (C). In all cases bands of interest are marked with pink arrows.

Figure S11. Uncropped images of western blotting from Figures 5A, 6B, and S8 C.

Western blots of Calreticulin, Canexin, and GAPDH from Figure 5A (A), Calreticulin, Calnexin, GRP94, GRP78, and GAPDH from Figure 6B (B), and Calreticulin, Calnexin, GAPDH from Figure S8 C (C). In all cases bands of interest are marked with pink arrows. Pink rectangles indicate the parts used in Figure 5A.

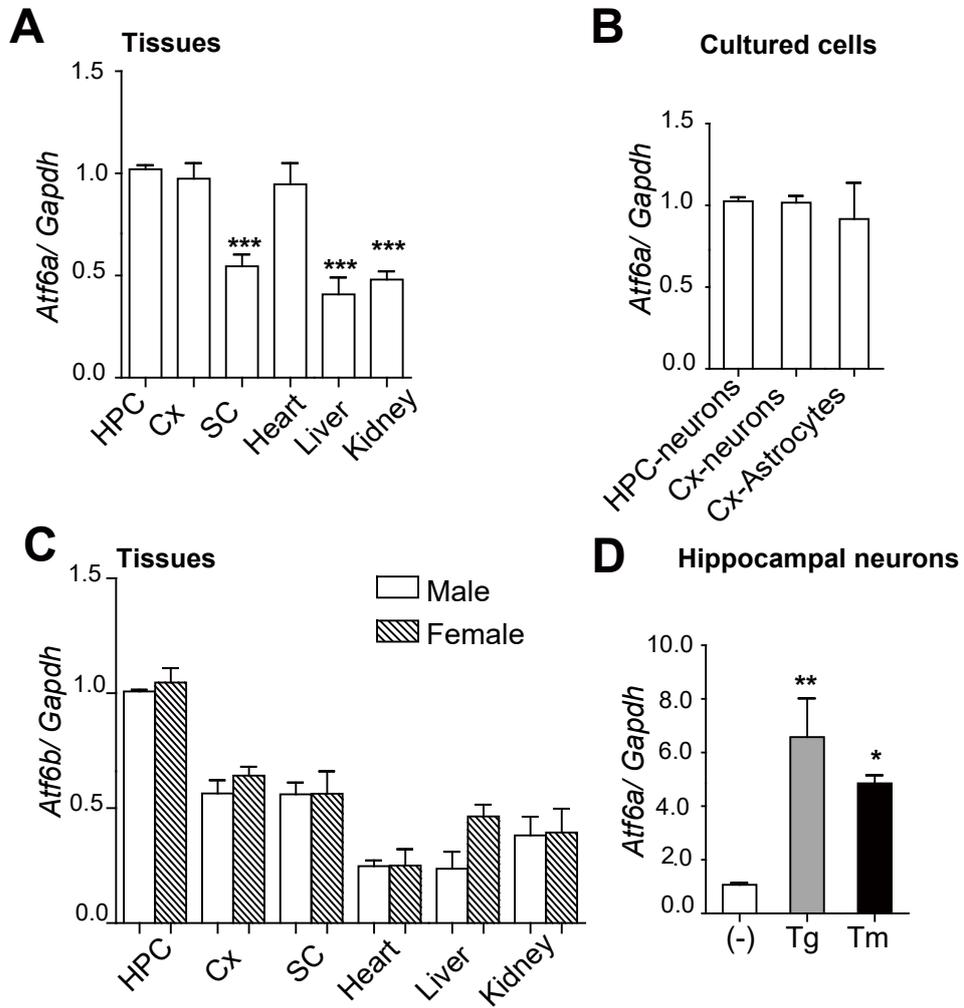


Figure S1

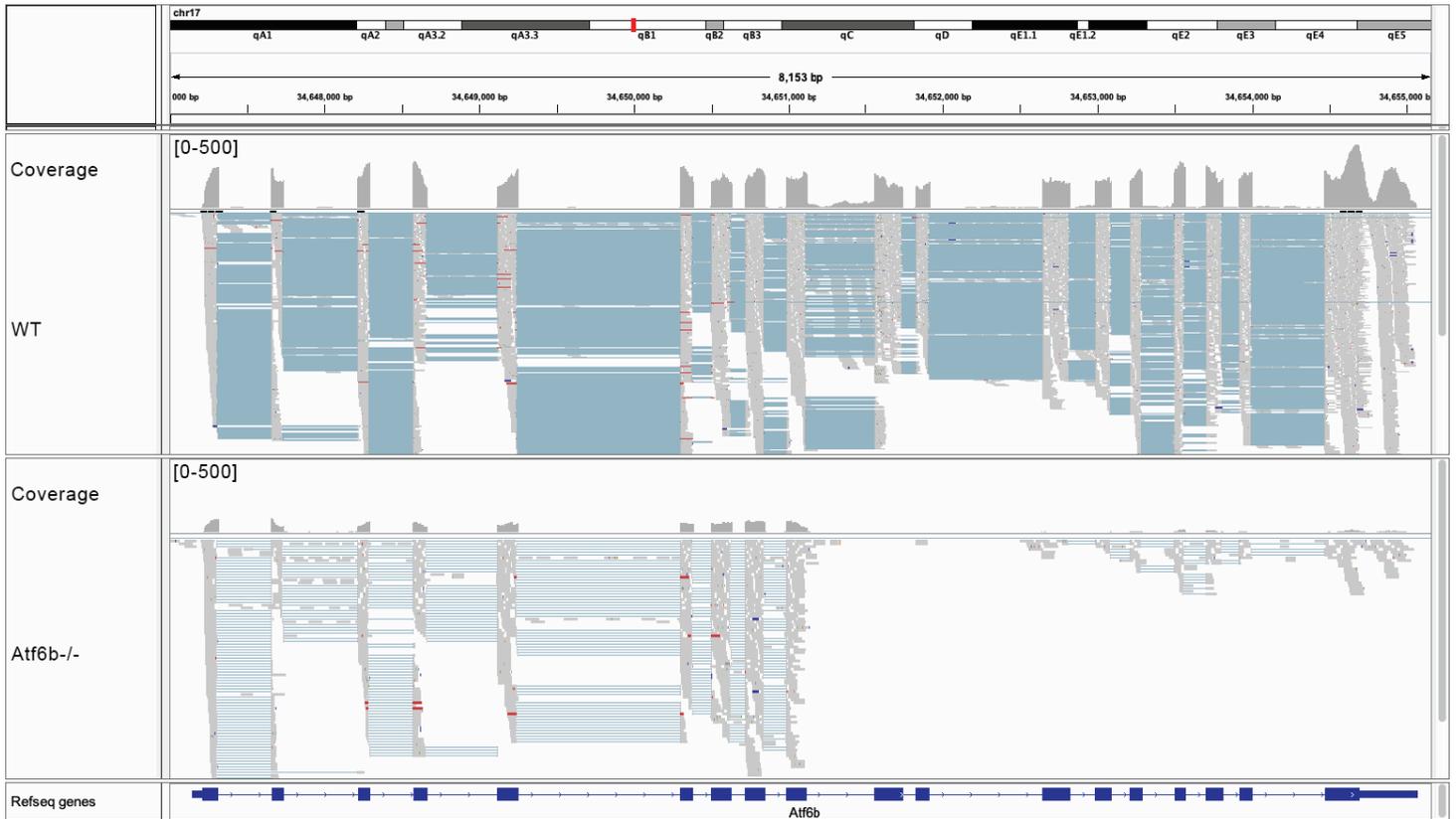
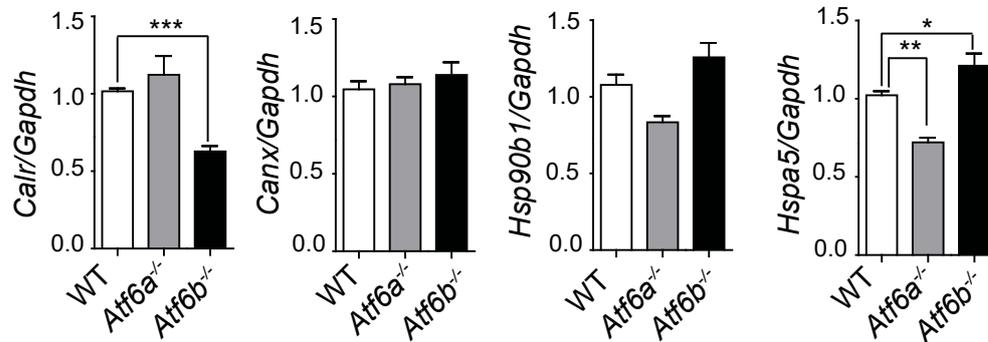
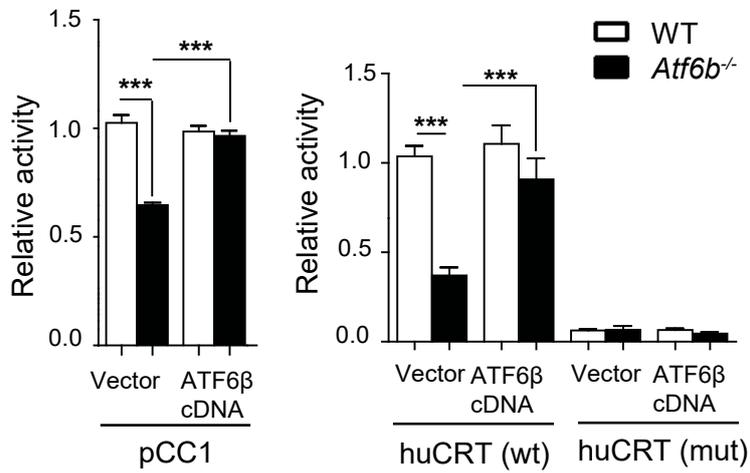
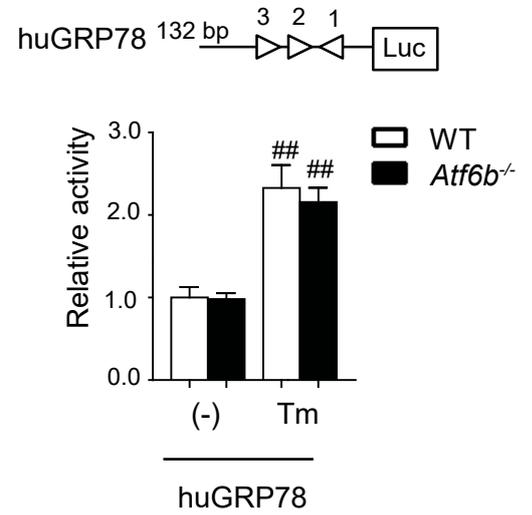


Figure S2

A**Mouse hippocampi****B****Mouse embryonic fibroblasts****C****Mouse embryonic fibroblasts****Figure S3**

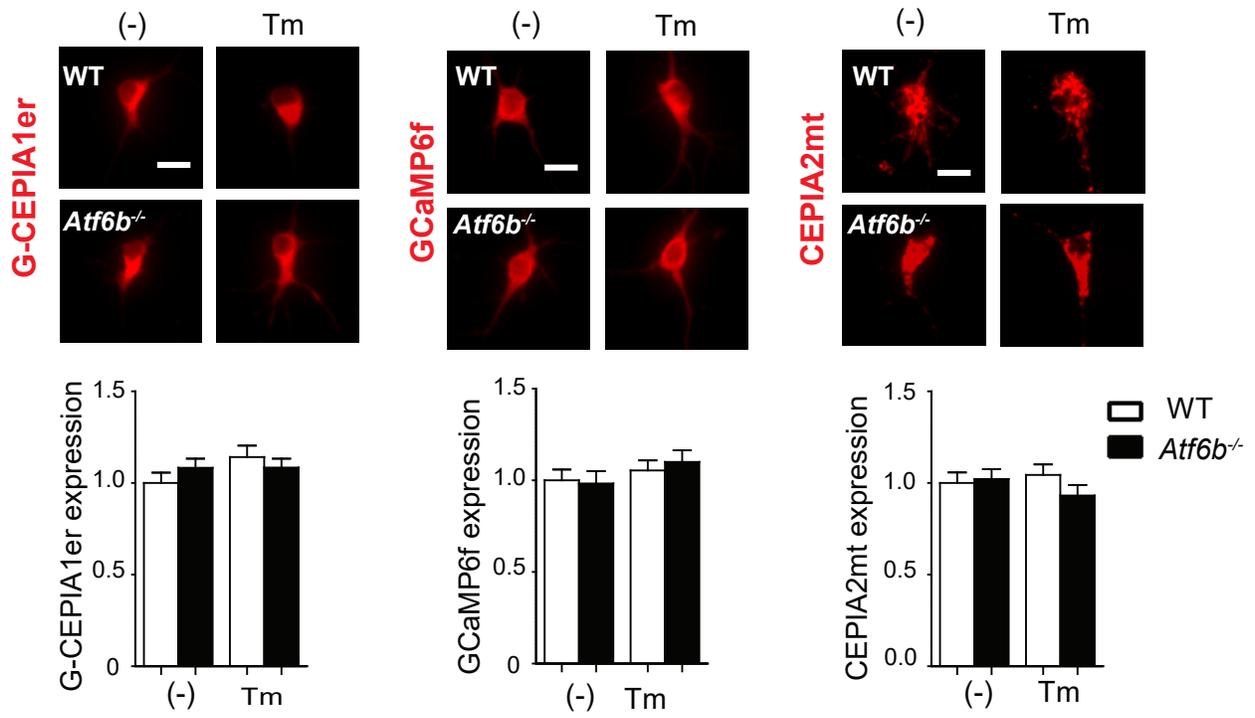


Figure S4

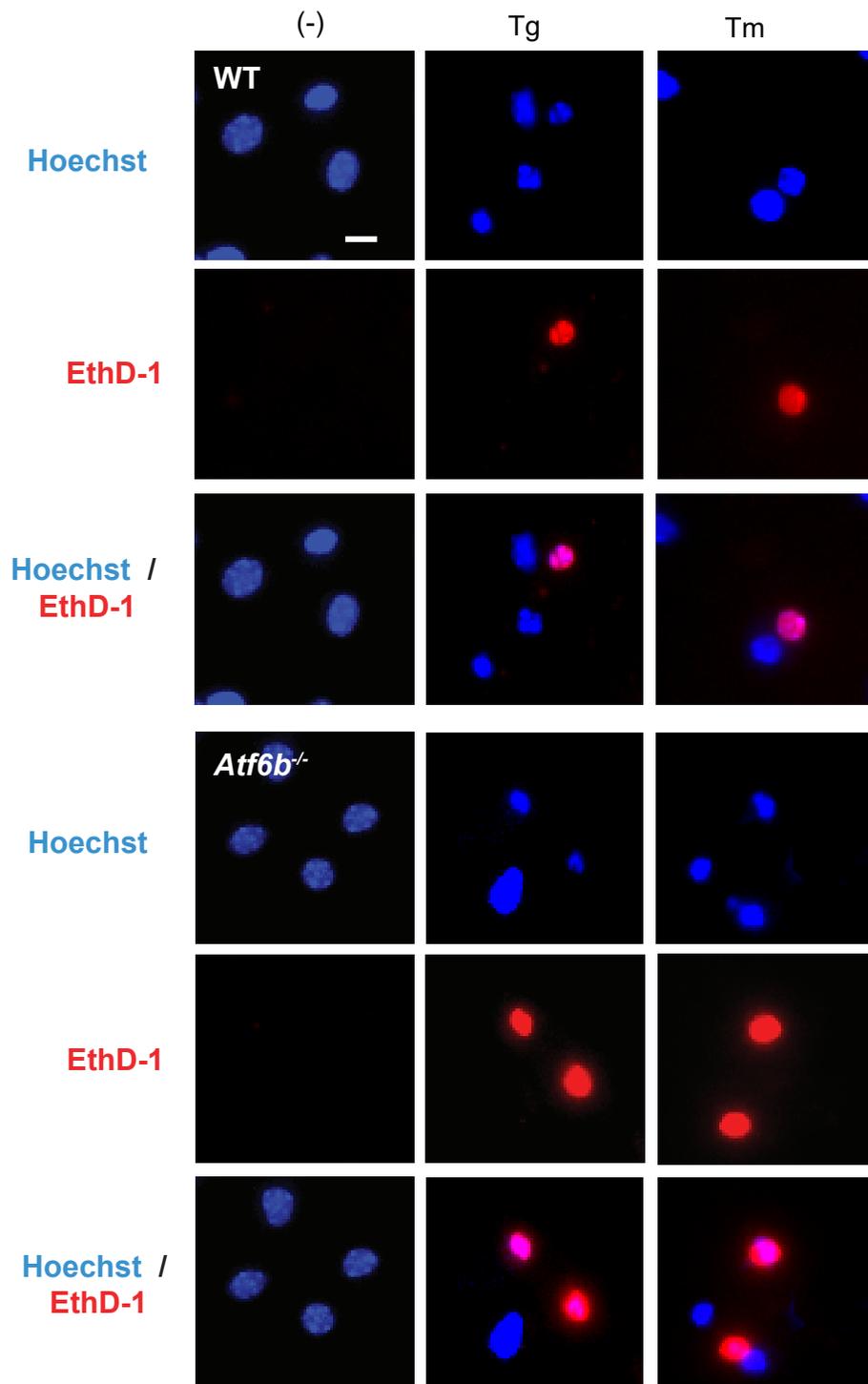


Figure S5

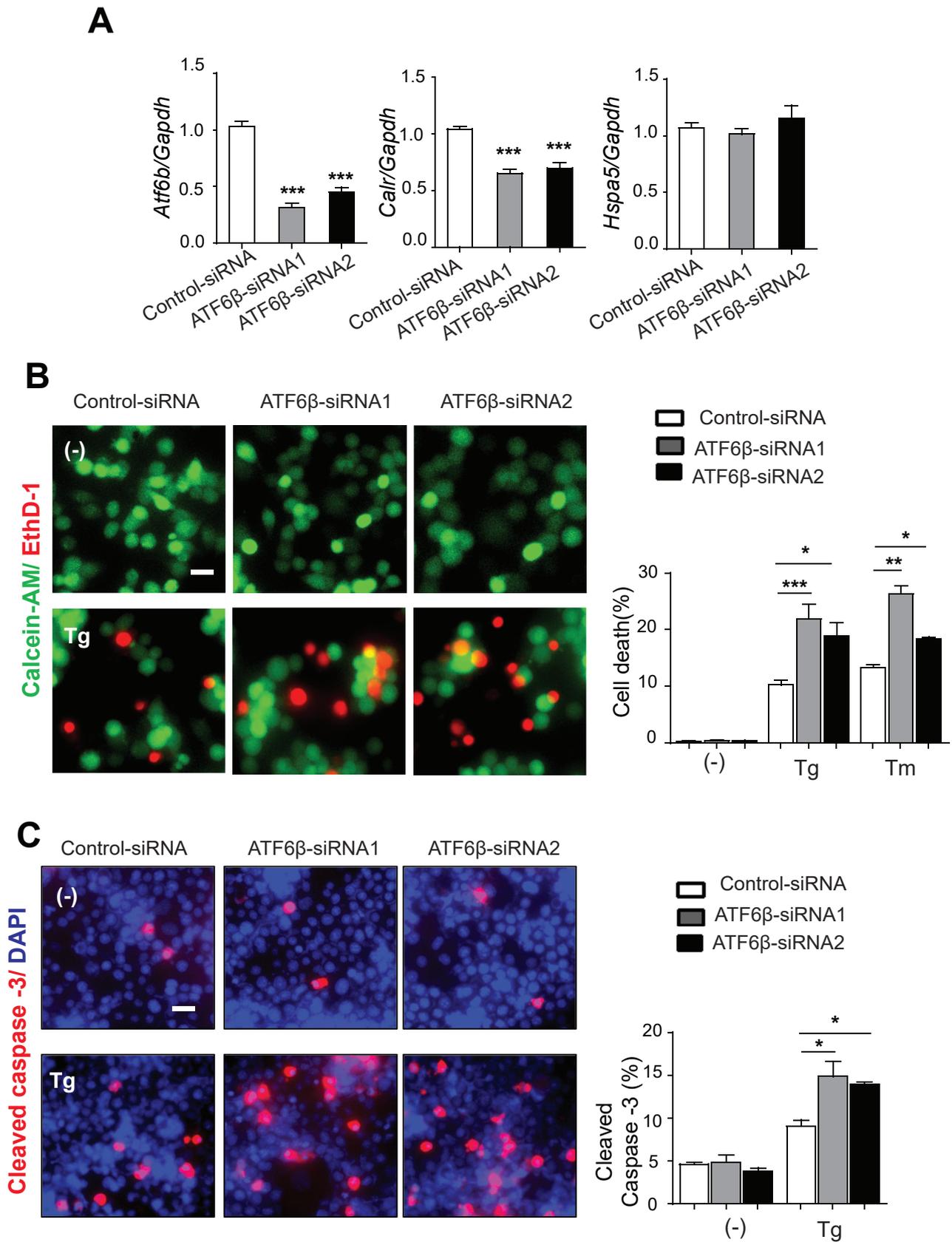
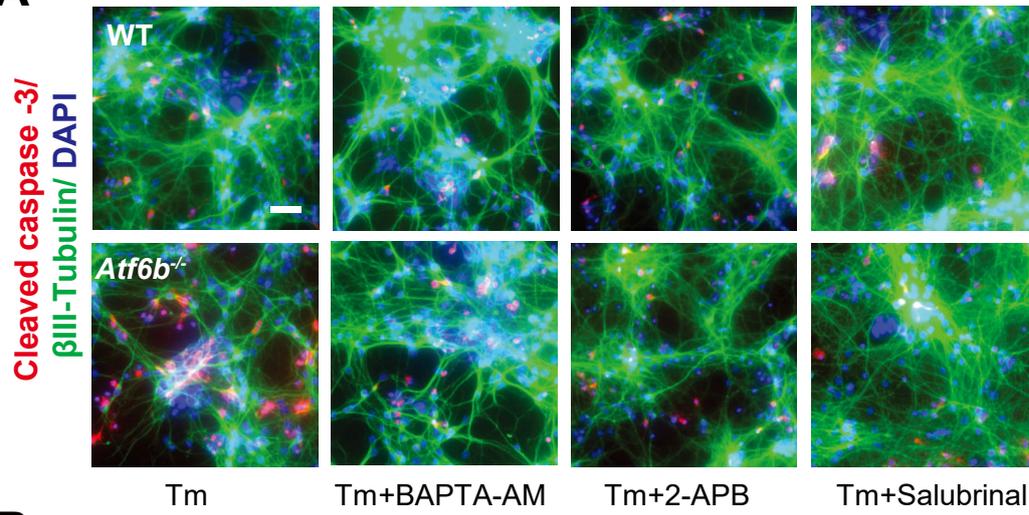
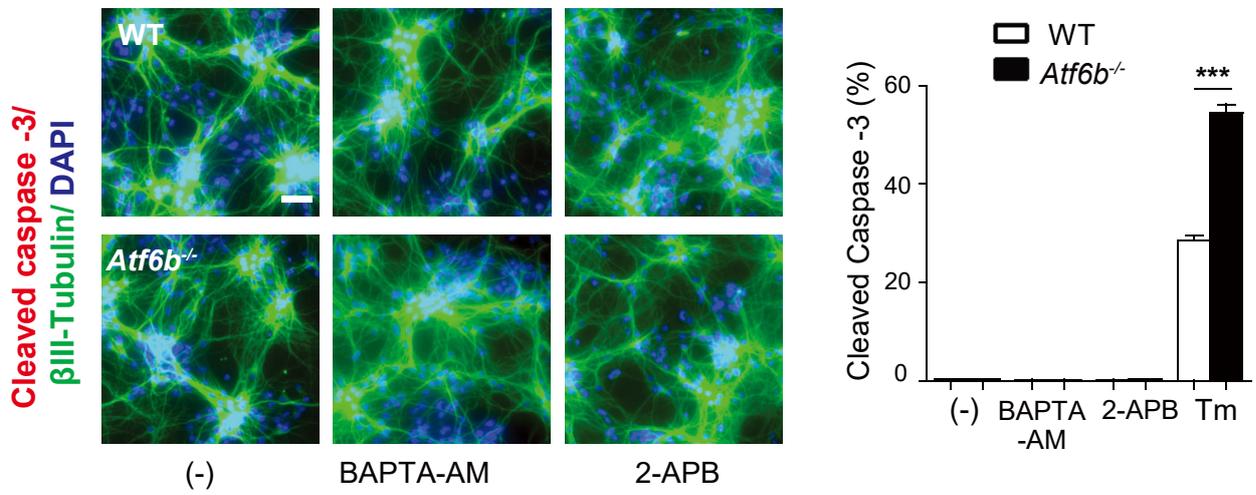
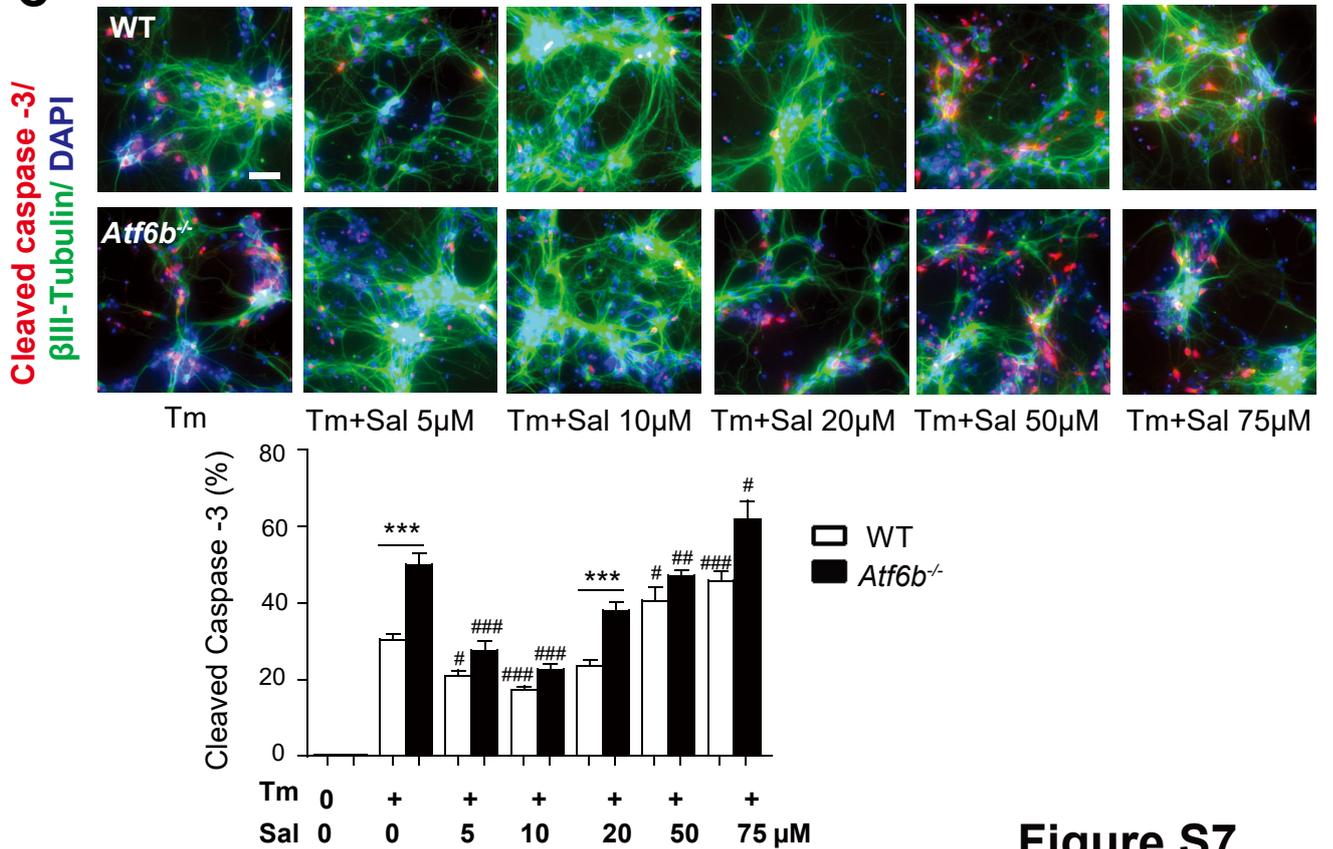


Figure S6

A**B****C****Figure S7**

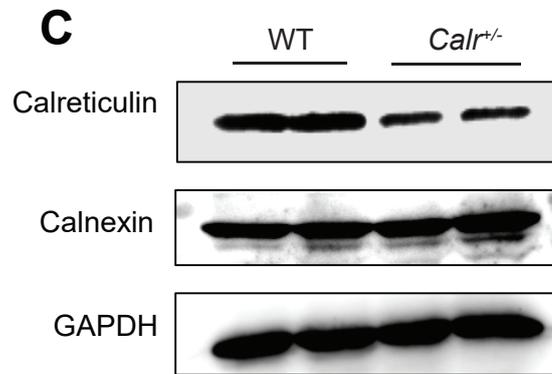
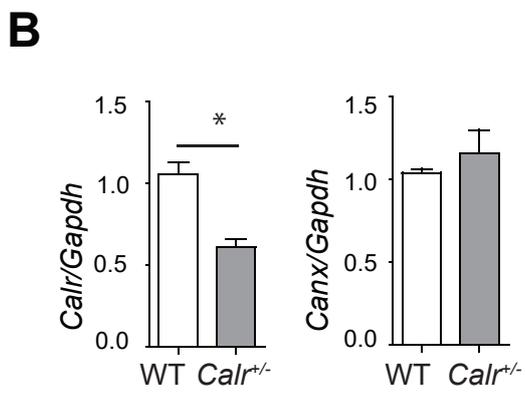
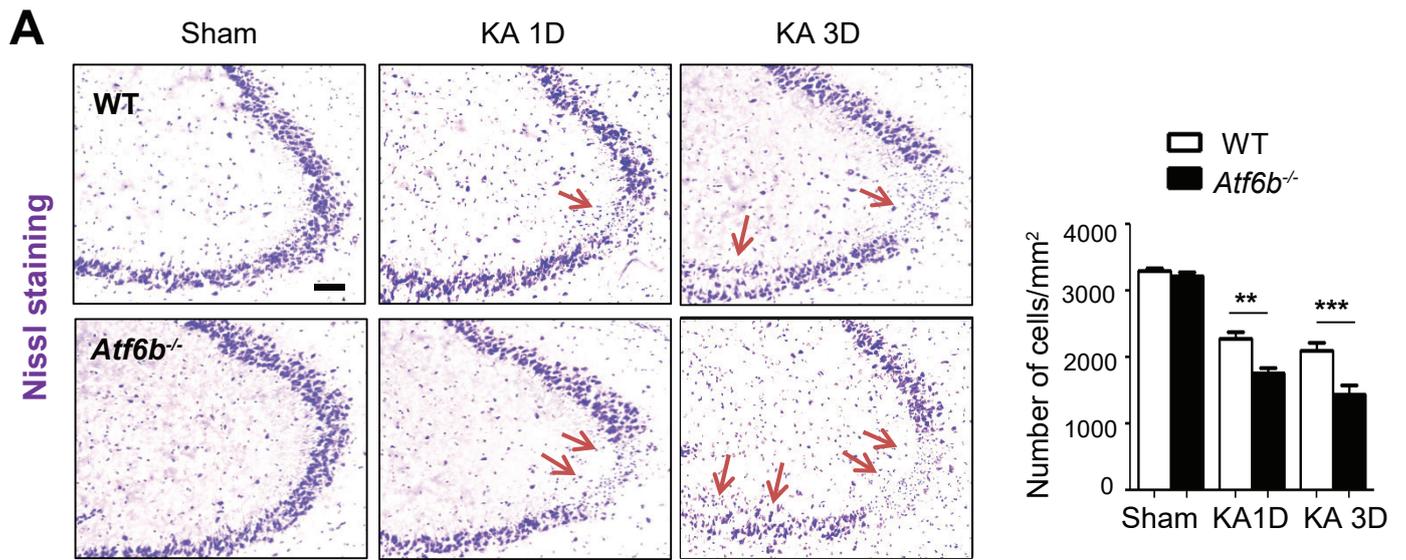


Figure S8

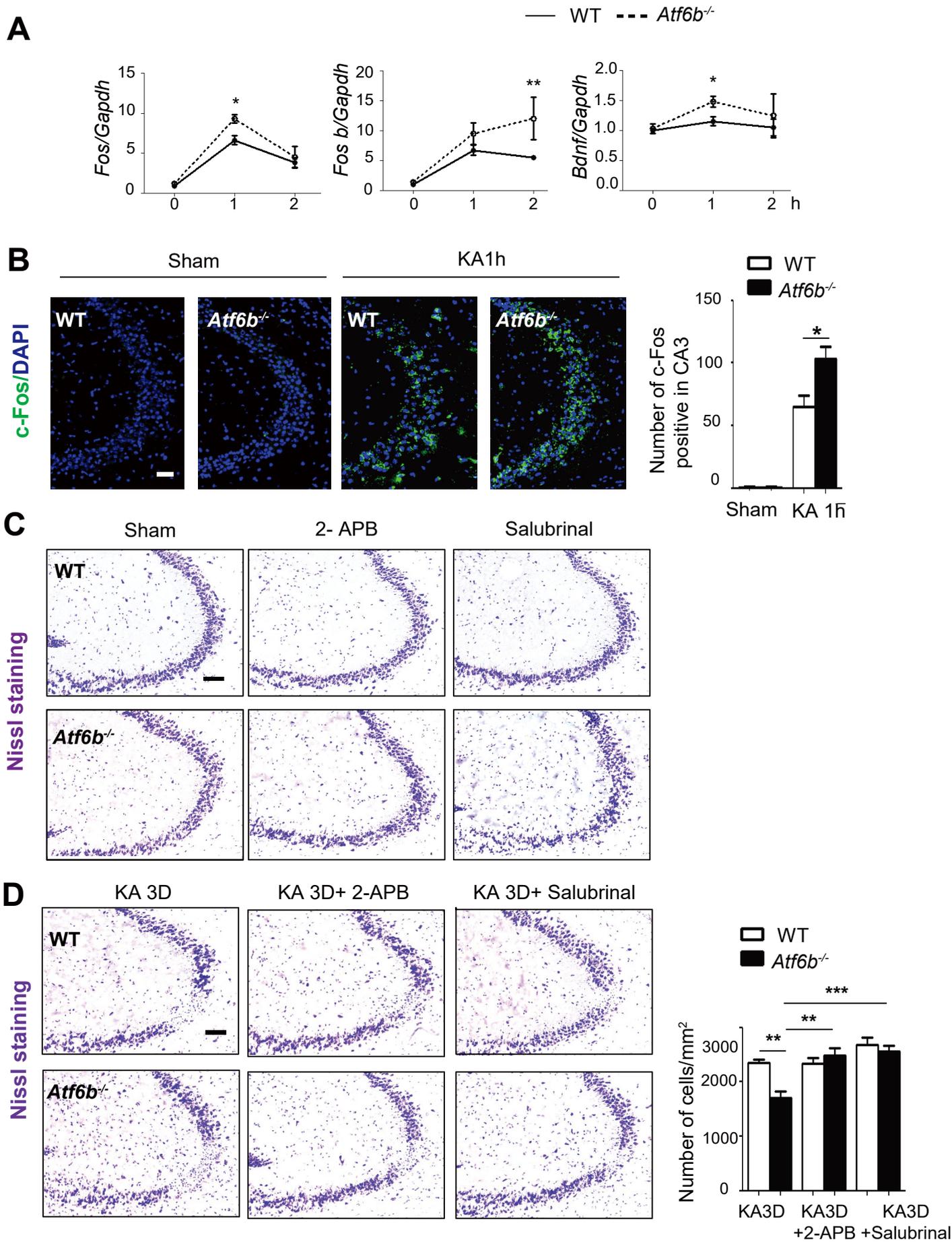


Figure S9

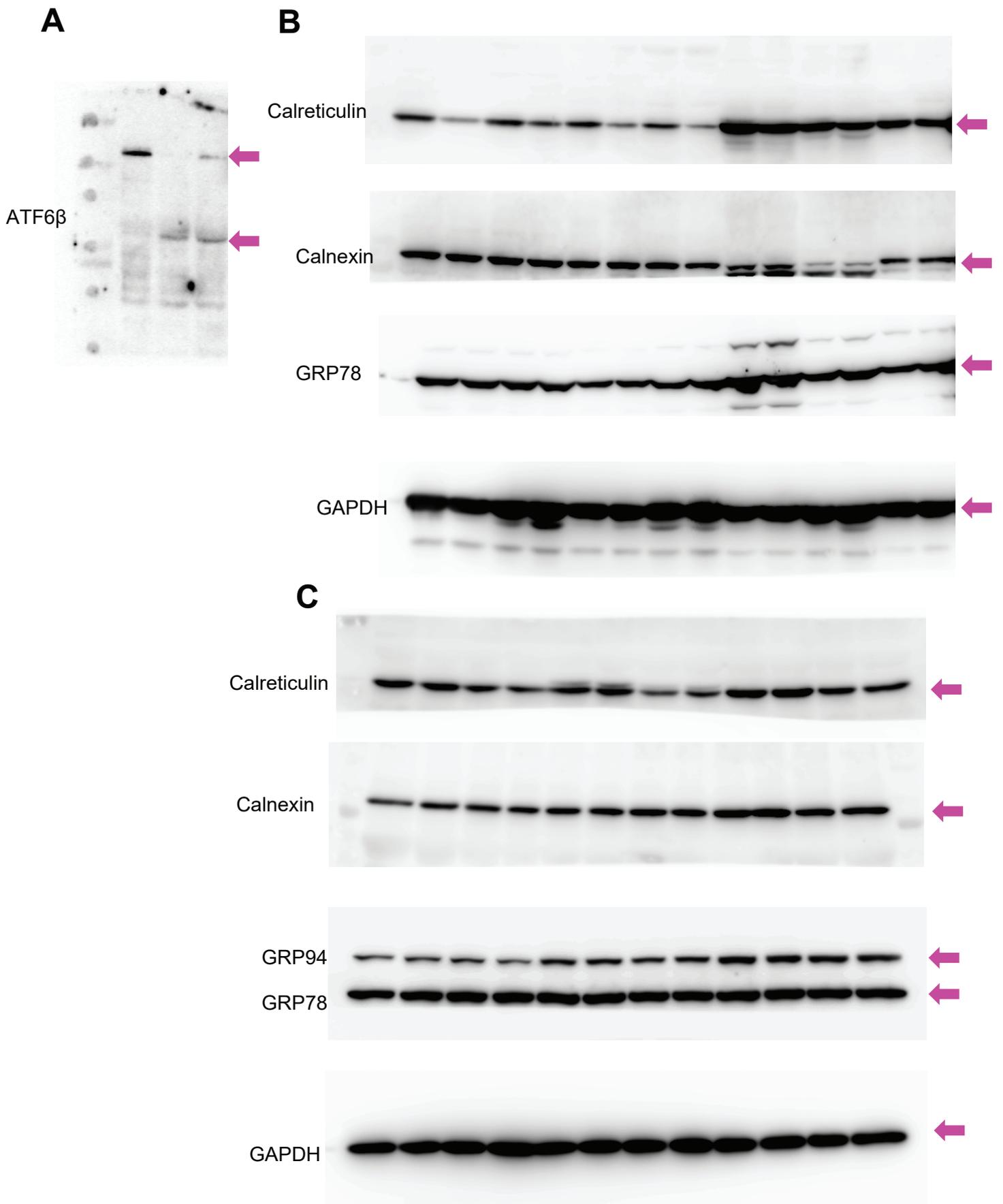


Figure S10

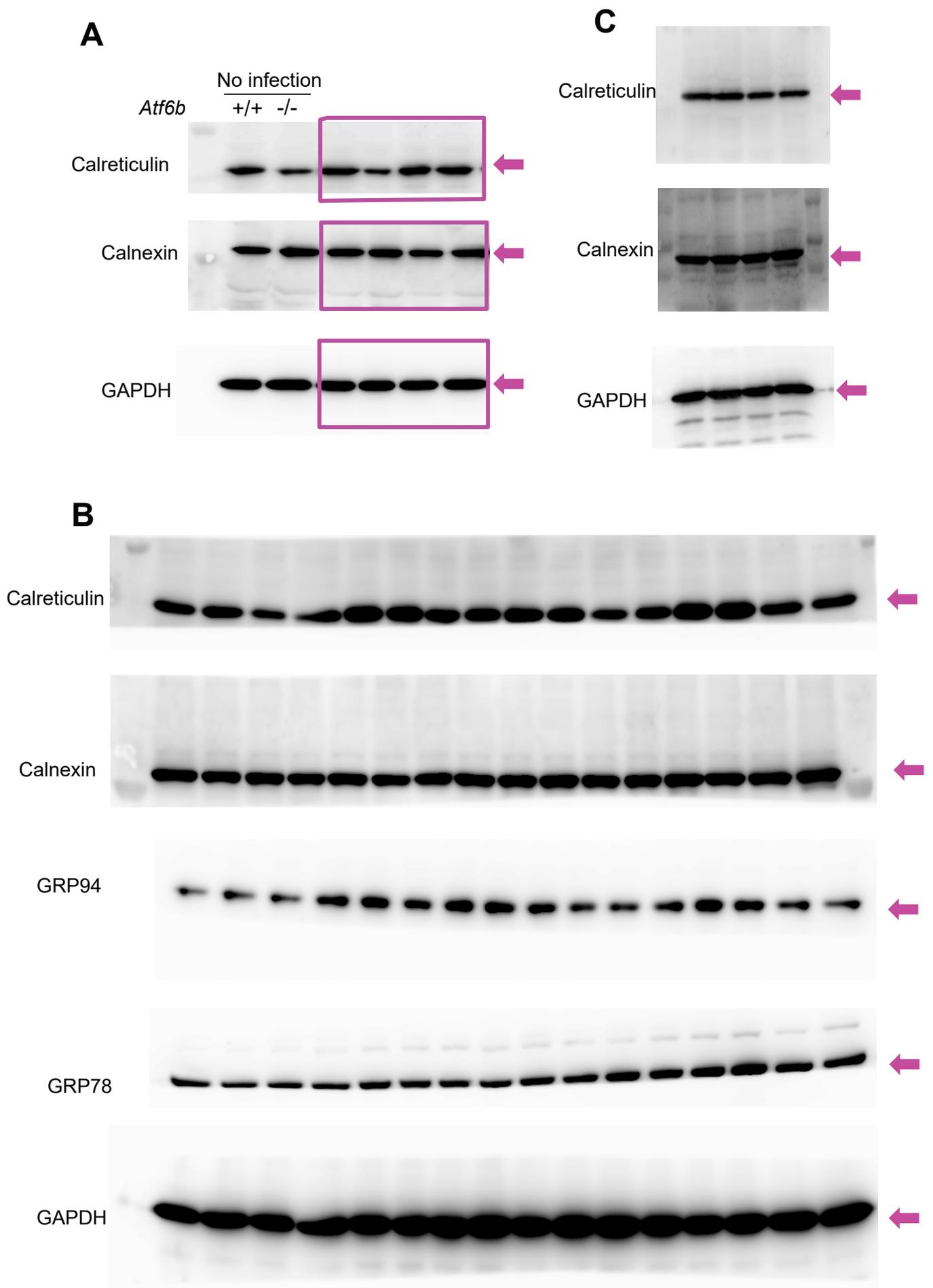


Figure S11