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Supporting Information for

Activation of autophagy rescues synaptic and cognitive deficits in Fragile X mice

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Fig. S1. Validation of Raptor and Atg7 shRNA viruses in mouse brain.

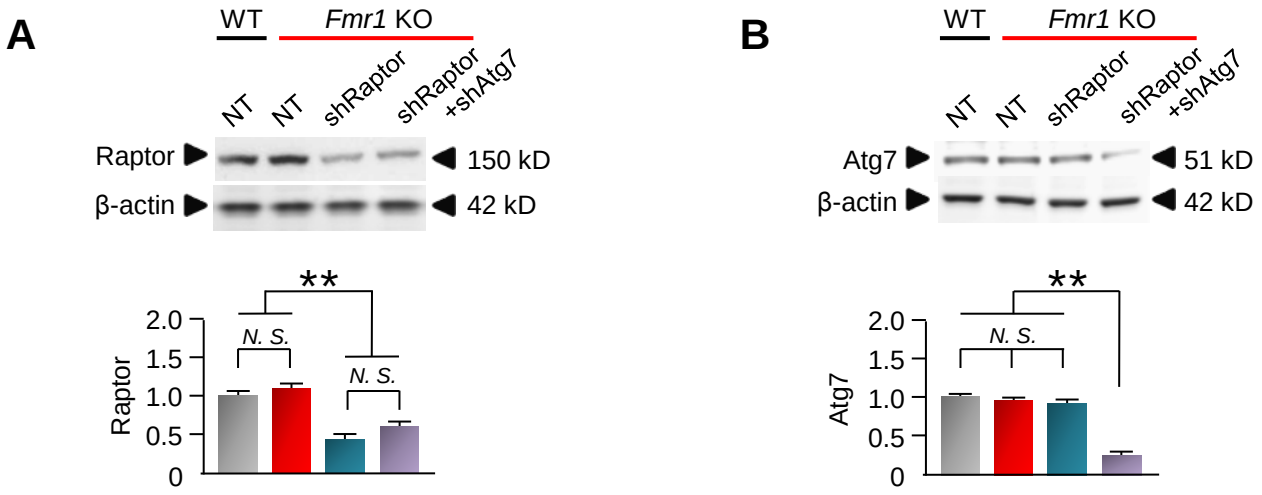

Fig. S1. Validation of Raptor and Atg7 shRNA viruses in WT and *Fmr1* KO mouse brain. *Fmr1* KO mice (male, 5-week-old) were bilaterally injected in the hippocampal CA1 with lentiviruses expressing GFP with **NT** shRNA, **shRaptor** alone, or shRaptor together with shRNA to Atg7 (**shRaptor + shAtg7**). Age-matched WT male mice expressing **NT** shRNA were used as control. One week after injection, we assessed the ability of shRaptor and shAtg7 to suppress Raptor and Atg7 abundance in whole-cell lysates of CA1 by Western blot analysis. Upper, representative Western blot. Lower, summary data show Raptor (**A**) and ATG7 (**B**) abundance in CA1 of mouse brains (normalized to the value for WT mice expressing NT shRNA). ** $p < 0.01$. Statistics were calculated by two-way ANOVA with Tukey's test. n = 4 WT mice in each group.

Fig. S2. Autophagic degradation of PSD-95 in neurons of WT and FXS mice.

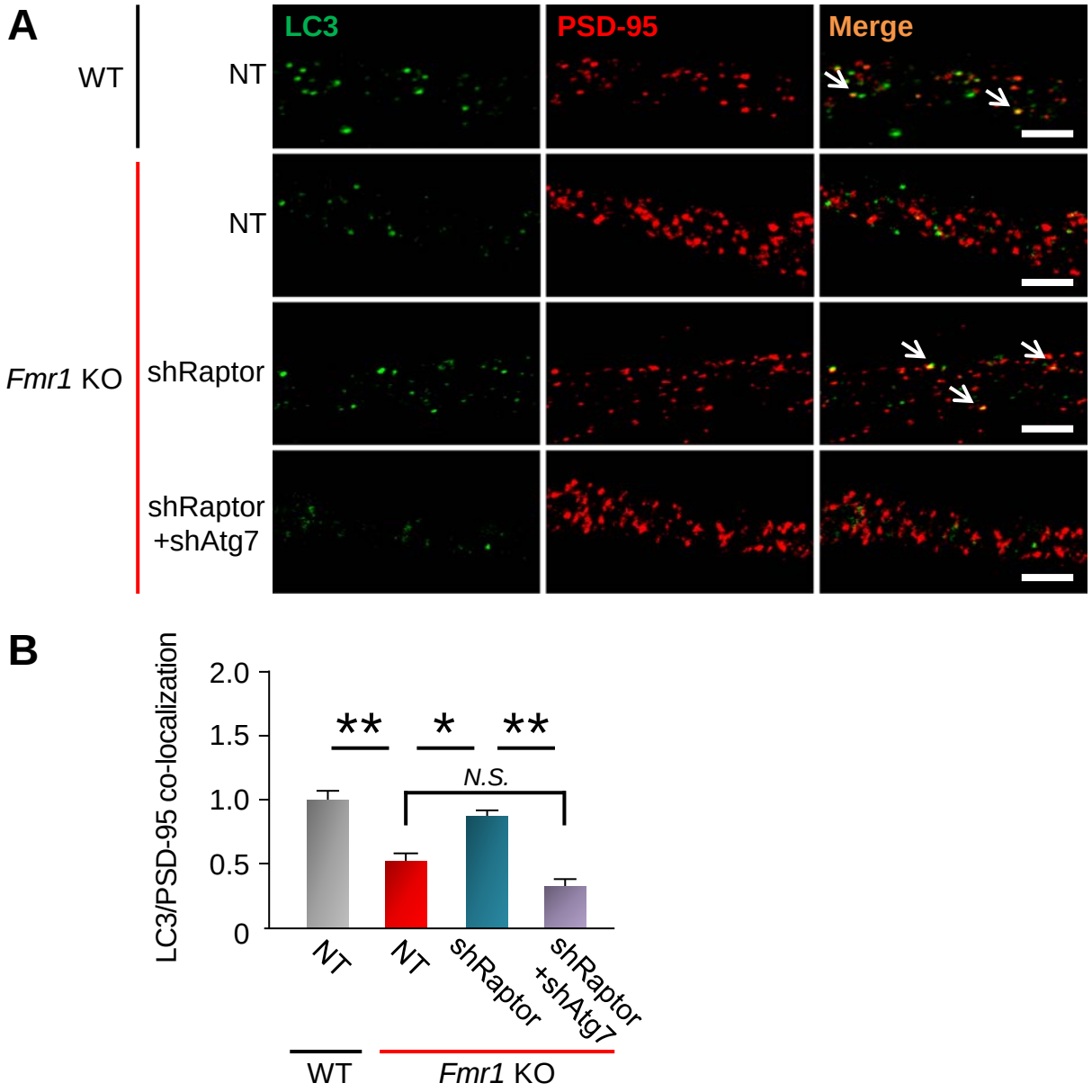

Fig. S2. Autophagy promotes degradation of PSD-95 in neurons of Fragile X mice. Primary cultures of hippocampal neurons from *Fmr1* KO mice were transfected with lentivirus expressing NT shRNA (negative control), shRaptor, or shRaptor + shAtg7. Values for KO neurons were normalized to the corresponding values for WT neurons expressing NT shRNA. 72 h after transfection, localization of PSD-95 to LC3(+) autophagosomes was determined by immunolabeling. (***A***) Representative images of PSD-95 and LC3 immunolabeling. Scale bar, 5 µm; (***B***) Summary data showing co-localization of PSD-95 puncta with LC3(+) puncta.). * $p < 0.05$, ** $p < 0.01$, *N.S.*: no significant difference. Statistics were calculated by two-way ANOVA with Tukey's test; $n = 4$ independent experiments with separate batches of neurons cultured from different litters and a minimum of 50 cells per group were analyzed. Values reflect mean ± s.e.m.

Fig. S3. Autophagic degradation of Arc in WT and KO neurons.

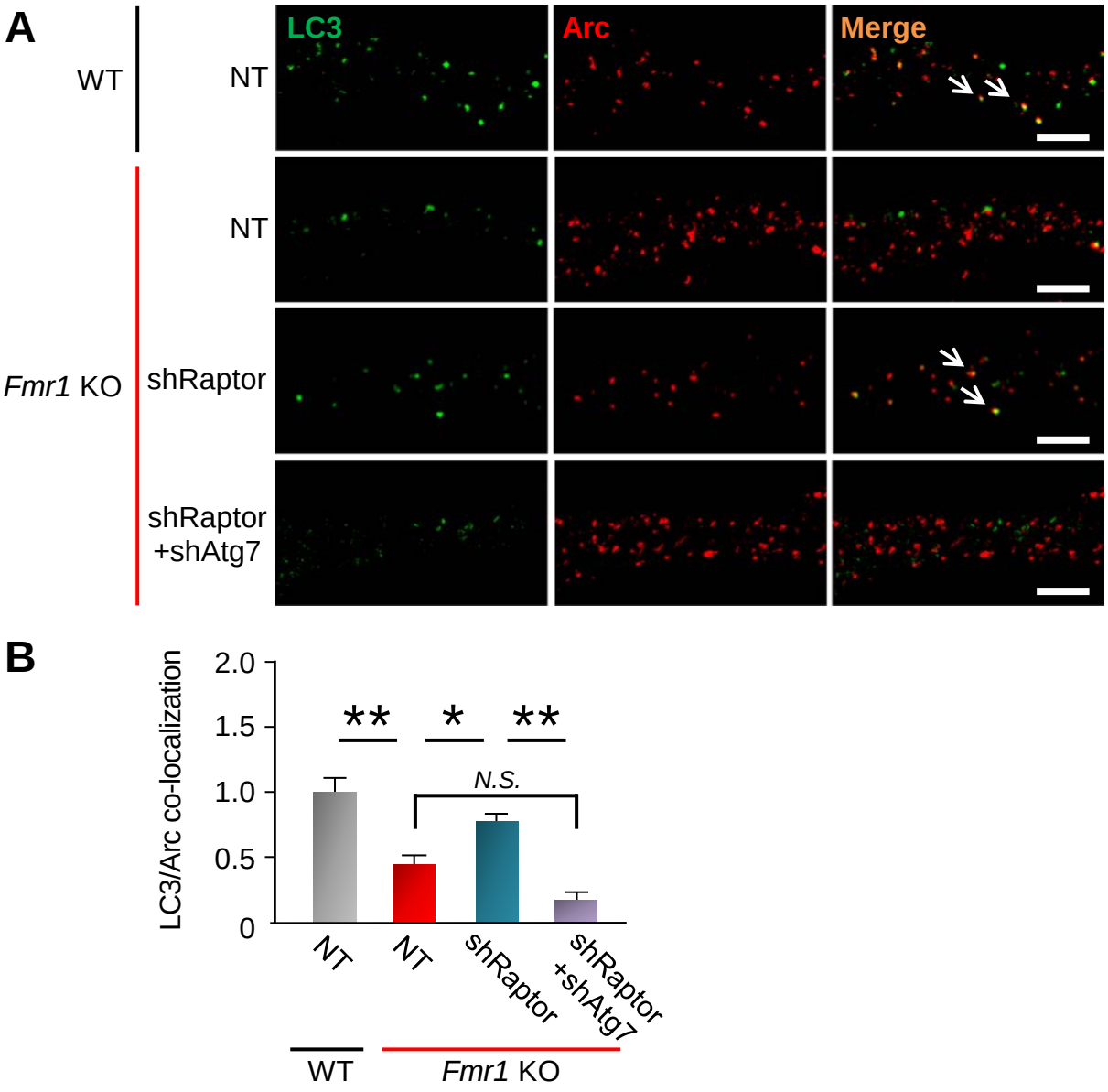

Fig. S3. Autophagy promotes degradation of Arc in neurons of Fragile X mice. Primary cultures of hippocampal neurons from *Fmr1* KO mice were transfected with lentivirus expressing NT shRNA (negative control), shRaptor, or shRaptor + shAtg7. 72 h after transfection, localization of Arc to LC3(+)-autophagosomes was examined by immunolabeling. (***A***) Representative images of Arc and LC3 immunolabeling. Scale bar, 5 µm; (***B***) Summary data showing co-localization of Arc puncta with LC3(+) puncta. Values for KO neurons were normalized to the corresponding values for WT neurons expressing NT shRNA. * $p < 0.05$, ** $p < 0.01$, *N.S.*: no significant difference. Statistics were calculated by two-way ANOVA with Tukey's test; $n = 4$ independent experiments with separate batches of neurons cultured from different litters and a minimum of 50 cells per group were analyzed. Values reflect mean ± s.e.m.

Supplemental Table 1. Summary of statistics for **Figure 2**

Figure	N	Mean ± SEM	p value	Statistical Test
B	WT = 5 cultures KO = 5 cultures	1.00 ± 0.08 1.18 ± 0.12	*p*=0.24214	Two-tailed unpaired *t* test for WT vs KO
C	WT = 5 cultures KO = 5 cultures	1.00 ± 0.08 0.43 ± 0.12	**, 0.004	
D	WT = 5 cultures KO = 5 cultures	1.00 ± 0.08 0.36 ± 0.05	**, 0.00145	
F	WT = 5 cultures (54 neurons) KO = 5 cultures (60 neurons)	1.00 ± 0.09 0.46 ± 0.10	**, 0.007	
H	WT = 5 cultures (53 neurons) KO = 5 cultures (50neurons)	1.00 ± 0.13 1.51 ± 0.15	*, 0.03409	
J	WT = 4 mice KO =4 mice	1.00 ± 0.17 2.65 ± 0.35	**, 0.00578	

Supplemental Table 2. Summary of statistics for **Figure 3**

Figure	N	Mean ± SEM	p value	Statistical Test
A (p-mTOR)	WT = 4 cultures KO = 4 cultures	1.00 ± 0.17 2.46 ± 0.15	**, 0.0007	Two-tailed unpaired *t* test for WT vs KO
A (Total mTOR)	WT = 4 cultures KO = 4 cultures	1.00 ± 0.05 1.09 ± 0.05	*N.S.* 0.25281	
B (p-ULK-1)	WT = 4 cultures KO = 4 cultures	1.00 ± 0.14 2.16 ± 0.18	**, 0.0026	
B (Total ULK-1)	WT = 4 cultures KO = 4 cultures	1.00 ± 0.09 1.10 ± 0.06	*N.S.* 0.43538	
C (p-ULK-1)	WT = 4 cultures KO = 4 cultures	1.00 ± 0.11 0.51 ± 0.09	*, 0.01491	
C (Total ULK-1)	WT = 4 cultures KO = 4 cultures	1.00 ± 0.05 1.18 ± 0.06	*N.S.* 0.0765	
D (p-Beclin-1)	WT = 4 cultures KO = 4 cultures	1.00 ± 0.10 0.39 ± 0.11	*, 0.02987	
D (Total Beclin-1)	WT = 4 cultures KO = 4 cultures	1.00 ± 0.05 1.07 ± 0.07	*N.S.* 0.44426	
E (Total)	WT = 5 mice KO = 5 mice	1.00 ± 0.08 0.96 ± 0.06	*N. S.*,0.71384	
E (Lysosomal)	WT = 5 mice KO = 5 mice	1.00 ± 0.16 2.37 ± 0.33	**, 0.00612	
F (Lysosomal)	WT = 4 cultures (32 neurons) KO = 4 cultures (30 neurons)	1.00 ± 0.14 2.10 ± 0.32	*, 0.02122	
F(Total)	WT = 4 cultures KO = 4 cultures	1.00 ± 0.07 0.97 ± 0.11	*N. S.*,0.81132	

Supplemental Table 3. Summary of statistics for **Figure 4**

Figure	N	Mean ± SEM	p value	Statistical Test
A	NT shRNA = 4 WT mice shRatpor = 5 WT mice	1.00 ± 0.08 0.43 ± 0.05	**, 0.0004	Two-way ANOVA with Tukey's post-test
B	WT + NT shRNA = 4 cultures KO + NT shRNA = 4 cultures KO + shRaptor = 4 cultures	1.00 ± 0.07 1.78 ± 0.16 1.15 ± 0.12	WT + NT *vs.* KO + NT **, 0.00372 KO + NT *vs.* KO + shRaptor *, 0.01355 WT + NT *vs*. KO + shRaptor *N. S.*, 0.66532	
C	WT + NT shRNA = 4 cultures KO + NT shRNA = 4 cultures KO + shRaptor = 4 cultures	1.00 ± 0.08 1.95 ± 0.18 1.36 ± 0.09	WT + NT *vs.* KO + NT **, 0.0006 KO + NT *vs*. KO + shRaptor *, 0.01366 WT + NT *vs*. KO + shRaptor *N. S.*, 0.12451	
D (LC3-II)	WT + NT shRNA = 4 cultures KO + NT shRNA = 4 cultures KO + shRaptor = 4 cultures	1.00 ± 0.05 0.52 ± 0.06 0.88 ± 0.06	WT + NT *vs.* KO + NT **, 0.0008 KO + NT *vs*. KO + shRaptor **, 0.0055 WT + NT *vs*. KO + shRaptor *N. S.*, 0.37854	
D (LC3-II/I ratio)	WT + NT shRNA = 4 cultures KO + NT shRNA = 4 cultures KO + shRaptor = 4 cultures	1.00 ± 0.08 0.52 ± 0.05 0.99 ± 0.10	WT + NT *vs.* KO + NT **, 0.00417 KO + NT *vs*. KO + shRaptor **, 0.00499 WT + NT *vs*. KO + shRaptor *N. S.*, 0.99142	
F	KO + NT shRNA = 4 cultures (60 neurons) KO + shRaptor = 4 cultures (50 neurons)	1.00 ± 0.16 2.14 ± 0.27	*, 0.01113	

Supplemental Table 4. Summary of statistics for **Figure 5**

Figure	N	Mean ± SEM	p value	Statistical Test
B (Spine density)	WT + NT shRNA = 6 mice (30 neurons) KO + NT shRNA = 6 mice (30 neurons) KO + shRaptor = 6 mice (30 neurons) KO+ shRaptor +shAtg7 = 6 mice (30 neurons)	9.50 ± 0.61 14.90 ± 0.33 10.57 ± 0.73 13.77 ± 0.31	WT + NT *vs.* KO + NT **, p<0.0001 KO + NT *vs*. KO + shRaptor **, 0.0006 KO + shRaptor *vs*. KO + shRaptor + shAtg7 **, 0.00191	Two-way ANOVA with Tukey's post-test
C (%Stubby/ Mushroom)	WT + NT shRNA = 6 mice (30 neurons) KO + NT shRNA = 6 mice (30 neurons) KO + shRaptor = 6 mice (30 neurons) KO+ shRaptor +shAtg7 = 6 mice (30 neurons)	33.91 ± 3.76 19.50 ± 2.06 34.52 ± 3.15 21.75 ± 2.49	WT + NT *vs.* KO + NT **, 0.00723 KO + NT *vs.* KO + shRaptor **, 0.00257 KO + shRaptor *vs.* KO + shRaptor + shAtg7 **, 0.00992	
C (%Thin/ Filopodia)	WT + NT shRNA = 6 mice (30 neurons) KO + NT shRNA = 6 mice (30 neurons) KO + shRaptor = 6 mice (30 neurons) KO+ shRaptor +shAtg7 = 6 mice (30 neurons)	8.67 ± 0.91 18.54 ± 2.48 10.84 ± 1.01 16.14 ± 1.80	WT + NT *vs.* KO + NT **, 0.0039 KO + NT *vs.* KO + shRaptor: *, 0.01661 KO + shRaptor *vs.* KO + shRaptor + shAtg7: *, 0.02784	
D (head width, µm)	WT + NT shRNA = 6 mice (27 neurons) KO + NT shRNA = 6 mice (32 neurons) KO + shRaptor = 6 mice (27 neurons) KO+ shRaptor +shAtg7 = 6 mice (28 neurons)	0.51 ± 0.04 0.32 ± 0.03 0.45 ± 0.04 0.33 ± 0.03	WT + NT *vs.* KO + NT: **, 0.00022 KO + NT *vs.* KO + shRaptor **, 0.00655 KO + shRaptor *vs.* KO + shRaptor + shAtg7 **, 0.00868	

Supplemental Table 5. Summary of statistics for **Figure 6**

Figure	N	Mean ± SEM	p value	Statistical Test
B (%, fESP in last 10 min)	WT + NT shRNA = 6 mice KO + NT shRNA = 6 mice KO + shRaptor = 6 mice KO+ shRaptor +shAtg7 = 6 mice (2-3 slices for each mouse)	87.19 ± 3.34 75.05 ± 3.24 93.17 ± 3.04 83.05 ± 2.53	WT + NT *vs*. KO + NT *, 0.0401 KO + NT *vs*. KO + shRaptor **, 0.00286 KO + shRaptor *vs*. KO + shRaptor + shAtg7 **, 0.00681	Two-way ANOVA with Tukey's post-test
C (Interaction time with novel)	WT + NT shRNA = 10 mice KO + NT shRNA = 9 mice KO + shRaptor = 8 mice KO+ shRaptor +shAtg7 = 8 mice	73.50 ± 3.21 76.11 ± 3.34 85.63 ± 4.13 72.50 ± 5.59	WT + NT novel *vs.* WT + NT familiar **, 0.0005 KO + shRaptor novel *vs*. KO + shRaptor familiar *, 0.04339	
C (Interaction time with familiar)	WT + NT shRNA = 10 mice KO + NT shRNA = 9 mice KO + shRaptor = 8 mice KO+ shRaptor +shAtg7 = 8 mice	47.93± 4.31 77.78 ± 2.48 66.00 ± 5.59 74.52 ± 5.61	WT + NT novel *vs*. WT + NT familiar **, 0.0005 KO + shRaptor novel *vs.* KO + shRaptor familiar *, 0.04339	
D (Preference to Novel)	WT + NT shRNA = 10 mice KO + NT shRNA = 9 mice KO + shRaptor = 8 mice KO+ shRaptor +shAtg7 = 8 mice	0.621 ± 0.019 0.495 ± 0.006 0.568 ± 0.015 0.493 ± 0.014	WT + NT *vs.* KO + NT: **, p<0.0001 KO + NT *vs*. KO + shRaptor *, 0.02261 KO + shRaptor *vs.* KO + shRaptor + shAtg7 *, 0.02808	

Supplemental Table 6. Summary of statistics for **Figure 7**

Figure	N	Mean ± SEM	p value	Statistical Test
B (Ubiquitinated)	WT = 4 cultures (50 neurons) KO = 4 cultures (50 neurons)	1.00 ± 0.15 1.92 ± 0.22	*, 0.01429	Two-tailed unpaired *t* test for WT vs KO
C (Co-localized with p62)	WT = 4 cultures (53 neurons) KO = 4 cultures (57 neurons)	1.00 ± 0.17 3.52 ± 0.33	**, 0.0005	
D (PSD-95, Input)	WT = 4 cultures KO = 4 cultures	1.00 ± 0.04 2.75 ± 0.12	**, $p<0.0001$	
D (PSD-95, Co-Ip)	WT = 4 cultures KO = 4 cultures	1.00 ± 0.08 2.02 ± 0.12	**, 0.0004	
E (Arc, Input)	WT = 4 cultures KO = 4 cultures	1.00 ± 0.06 2.03 ± 0.11	**, 0.0005	
E (Arc, Co-Ip)	WT = 4 cultures KO = 4 cultures	1.00 ± 0.07 2.34 ± 0.08	**, 0.0001	

Supplemental Table 7. Summary of statistics for **Figure 8**

Figure	N	Mean ± SEM	p value	Statistical Test
A (PSD-95, Input)	WT + Veh = 4 cultures WT + Lac= 4 cultures WT + 3-MA= 4 cultures WT + lyso inhibitor = 4 cultures	1.00 ± 0.05 2.26 ± 0.19 1.74 ± 0.11 2.45 ± 0.16	Veh *vs*. Lac: **, 0.0001 Veh *vs*. 3-MA: **, 0.00942 Veh *vs*. lyso inhibitor: **, 0.00003	Two-way ANOVA with Tukey's post-test
A (PSD-95, Co-Ip)	WT + Veh = 4 cultures WT + Lac= 4 cultures WT + 3-MA= 4 cultures WT + lyso inhibitor = 4 cultures	1.00 ± 0.08 2.69 ± 0.14 1.71 ± 0.07 2.16 ± 0.09	Veh *vs*. Lac: **, 0.0003 Veh *vs*. 3-MA: **, 0.00142 Veh vs. lyso inhibitor: **, 0.00002	
B (Arc, Input)	WT + Veh = 4 cultures WT + Lac= 4 cultures WT + 3-MA= 4 cultures WT + lyso inhibitor = 4 cultures	1.00 ± 0.07 2.06 ± 0.11 1.94 ± 0.08 2.73 ± 0.12	Veh *vs.* Lac: **, p<0.0001 Veh *vs*. 3-MA: **, 0.0001 Veh *vs*. lyso inhibitor: **, p<0.00001	
B (Arc, Co-Ip)	WT + Veh = 4 cultures WT + Lac= 4 cultures WT + 3-MA= 4 cultures WT + lyso inhibitor = 4 cultures	1.00 ± 0.06 1.52 ± 0.08 1.72 ± 0.10 1.61 ± 0.08	Veh *vs*. Lac: **, 0.00383 Veh *vs*. 3-MA: **, 0.0003 Veh *vs*. lyso inhibitor: **, 0.0011	
C	WT + NT shRNA = 4 cultures KO + NT shRNA =4 cultures KO + shRaptor = 4 cultures KO+ shRaptor +shAtg7 =4 cultures	1.00 ± 0.12 3.27 ± 0.22 1.57 ± 0.14 2.92 ± 0.16	WT + NT *vs*. KO + NT **, p<0.00001 KO + NT *vs*. KO + shRaptor **, 0.00004 KO + shRaptor *vs*. KO + shRaptor + shAtg7 **, 0.0004 WT + NT *vs*. KO + shRaptor *N. S.* , 0.15983	
D	WT + NT shRNA = 4 cultures KO + NT shRNA = 4 cultures KO + shRaptor = 4 cultures KO+ shRaptor +shAtg7 = 4 cultures	1.00 ± 0.06 1.96 ± 0.20 0.85 ± 0.08 1.43 ± 0.09	WT + NT *vs.* KO + NT **, 0.0006 KO + NT *vs.* KO + shRaptor **, 0.0002 KO + shRaptor vs. KO + shRaptor + shAtg7 *, 0.02925 WT + NT *vs*. KO + shRaptor *N. S.* , 0.84261	

Supplemental Table 8. Summary of statistics for **Fig. S1.**

Figure	N	Mean ± SEM	p value	Statistical Test
A (Raptor)	WT + NT shRNA = 4 cultures KO + NT shRNA =4 cultures KO + shRaptor = 4 cultures KO+ shRaptor +shAtg7 =4 cultures	1.00 ± 0.06 1.10 ± 0.06 0.42 ± 0.08 0.50 ± 0.06	WT + NT *vs*. KO + NT *N. S*., 0.70715 KO + NT *vs*. KO + shRaptor **, 0.00004 KO + shRaptor *vs*. KO + shRaptor + shAtg7 *N. S*., 0.80533 WT + NT *vs*. KO + shRaptor **, 0.0002 WT + NT *vs*. KO + shRaptor + shAtg7 **, 0.00008	Two-way ANOVA with Tukey's post-test
B (Atg7)	WT + NT shRNA = 4 cultures KO + NT shRNA = 4 cultures KO + shRaptor = 4 cultures KO+ shRaptor +shAtg7 = 4 cultures	1.00 ± 0.04 0.95 ± 0.05 0.92 ± 0.05 0.24 ± 0.05	WT + NT *vs*. KO + NT *N. S*., 0.90262 KO + NT *vs*. KO + shRaptor *N. S*., 0.9699 KO + shRaptor *vs*. KO + shRaptor + shAtg7 **, $p<0.00001$ WT + NT *vs*. KO + shRaptor *N. S*., 0.9699 WT + NT *vs*. KO + shRaptor + shAtg7 **, $p<0.00001$	

Supplemental Table 9. Summary of statistics for **Fig. S2.**

Figure	N	Mean ± SEM	p value	Statistical Test
B	WT + NT shRNA = 4 cultures (63 neurons) KO + NT shRNA = 4 cultures (52 neurons) KO + shRaptor = 4 cultures (54 neurons) KO+ shRaptor +shAtg7 = 4 cultures (50 neurons)	1.00 ± 0.08 0.52 ± 0.08 0.86 ± 0.05 0.32 ± 0.07	WT + NT *vs.* KO + NT **, 0.00281 KO + NT *vs.* KO + shRaptor *, 0.02874 KO + shRaptor *vs.* KO + shRaptor + shAtg7 **, 0.00109 KO + NT *vs.* KO + shRaptor + shAtg7 *N. S.*, 0.27085	Two-way ANOVA with Tukey's post-test

Supplemental Table 10. Summary of statistics for **Fig. S3.**

Figure	N	Mean ± SEM	p value	Statistical Test
B	WT + NT shRNA = 4 cultures (54 neurons) KO + NT shRNA = 4 cultures (50 neurons) KO + shRaptor = 4 cultures (50 neurons) KO+ shRaptor +shAtg7 = 4 cultures (52 neurons)	1.00 ± 0.12 0.44 ± 0.08 0.78 ± 0.05 0.18 ± 0.05	WT + NT *vs.* KO + NT **, 0.00162 KO + NT *vs.* KO + shRaptor *, 0.04791 KO + shRaptor *vs.* KO + shRaptor + shAtg7 **, 0.0009 KO + NT *vs.* KO + shRaptor + shAtg7 *N. S.*, 0.16783	Two-way ANOVA with Tukey's post-test