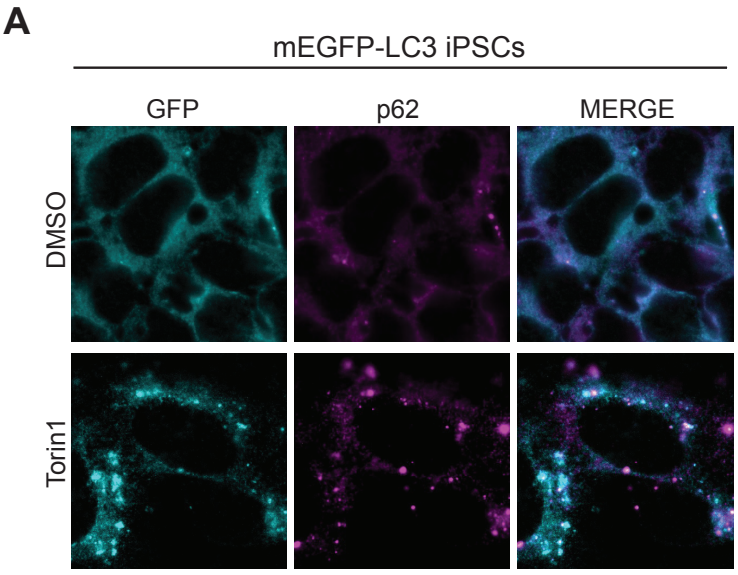
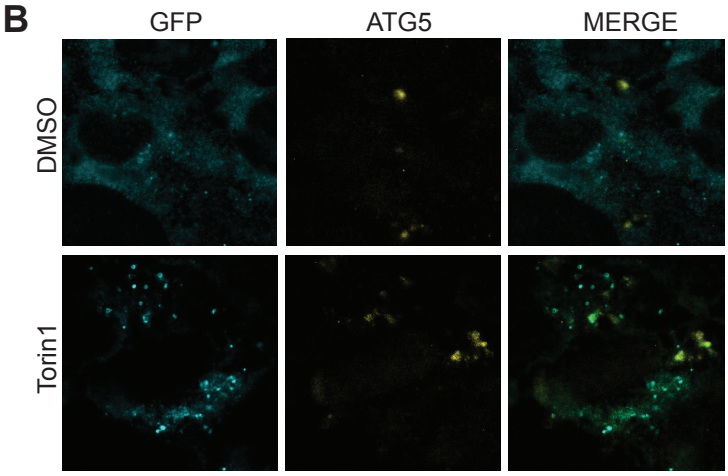

A
mEGFP-LC3 iPSCs
GFP
p62
MERGE
DMSO
Torin1


B
GFP
ATG5
MERGE
DMSO
Torin1

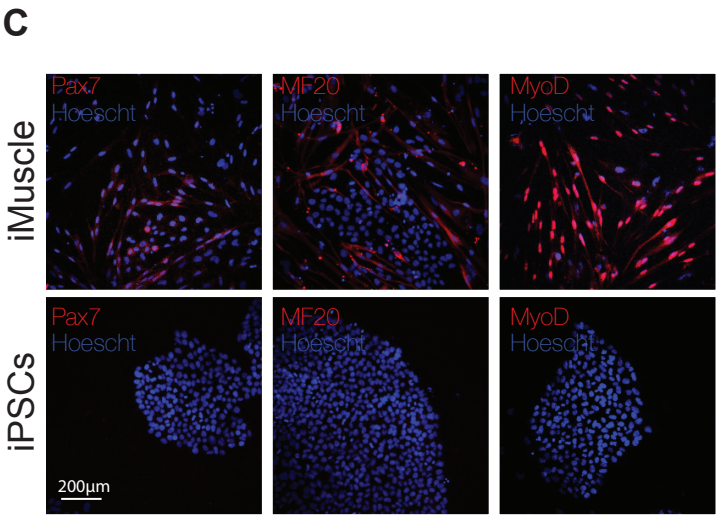

C
iMuscle
iPSCs
Pax7
Hoescht
MF20
Hoescht
MyoD
Hoescht
200µm

Figure S1. Immunocytochemical markers in mEGFP-LC3 cells, related to Figure 1. (**A-C**) Undifferentiated mEGFP-LC3 iPSCs were treated with 250nM Torin1 or DMSO vehicle for 4h, fixed, then immunostained for p62 (A), ATG5 (B), and LC3 (C) to visualize the distribution of these markers in relation to mEGFP. (**D**) After differentiating mEGFP-LC3 iPSCs into iMuscle, cells were fixed and immunostained for Pax7, MF20, and MyoD to confirm skeletal muscle maturity (top panels) and compare against undifferentiated iPSCs (bottom panels).

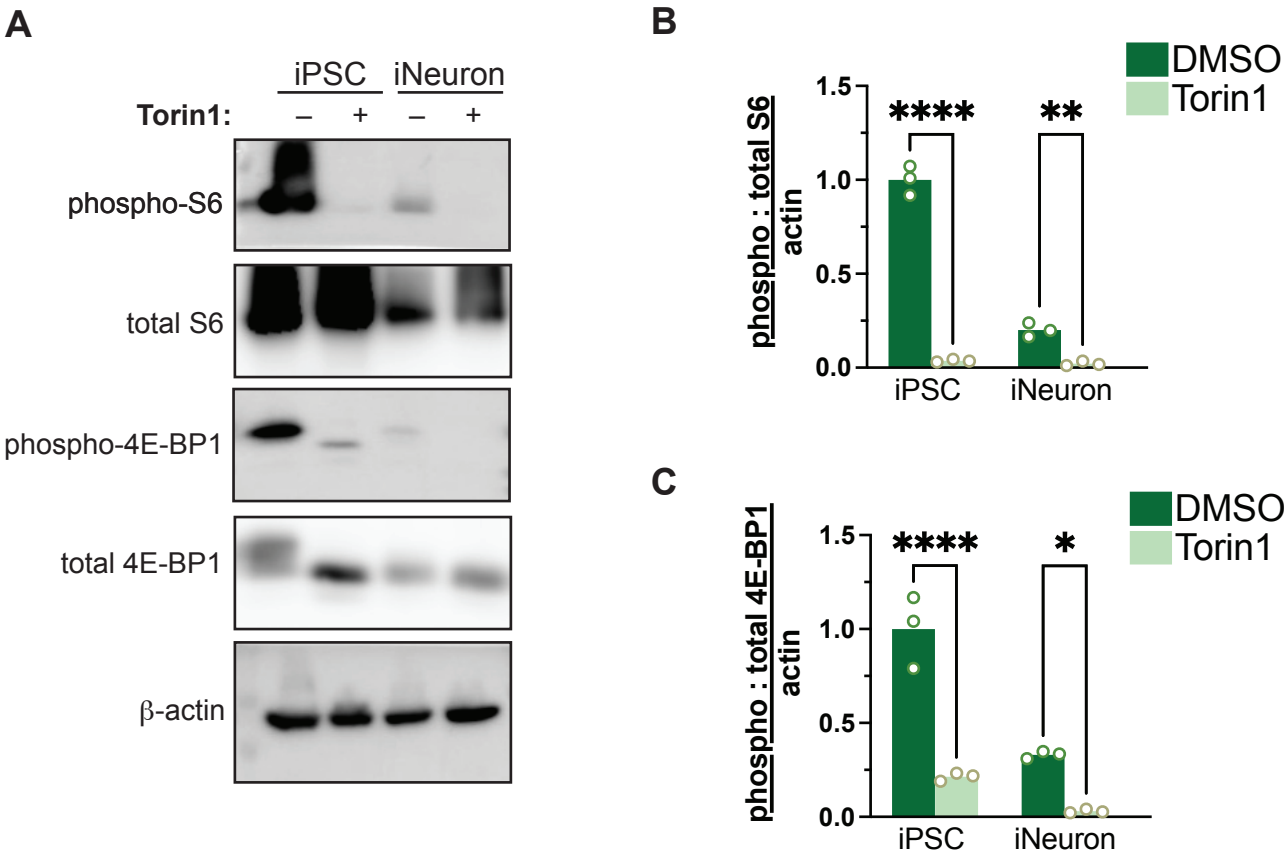
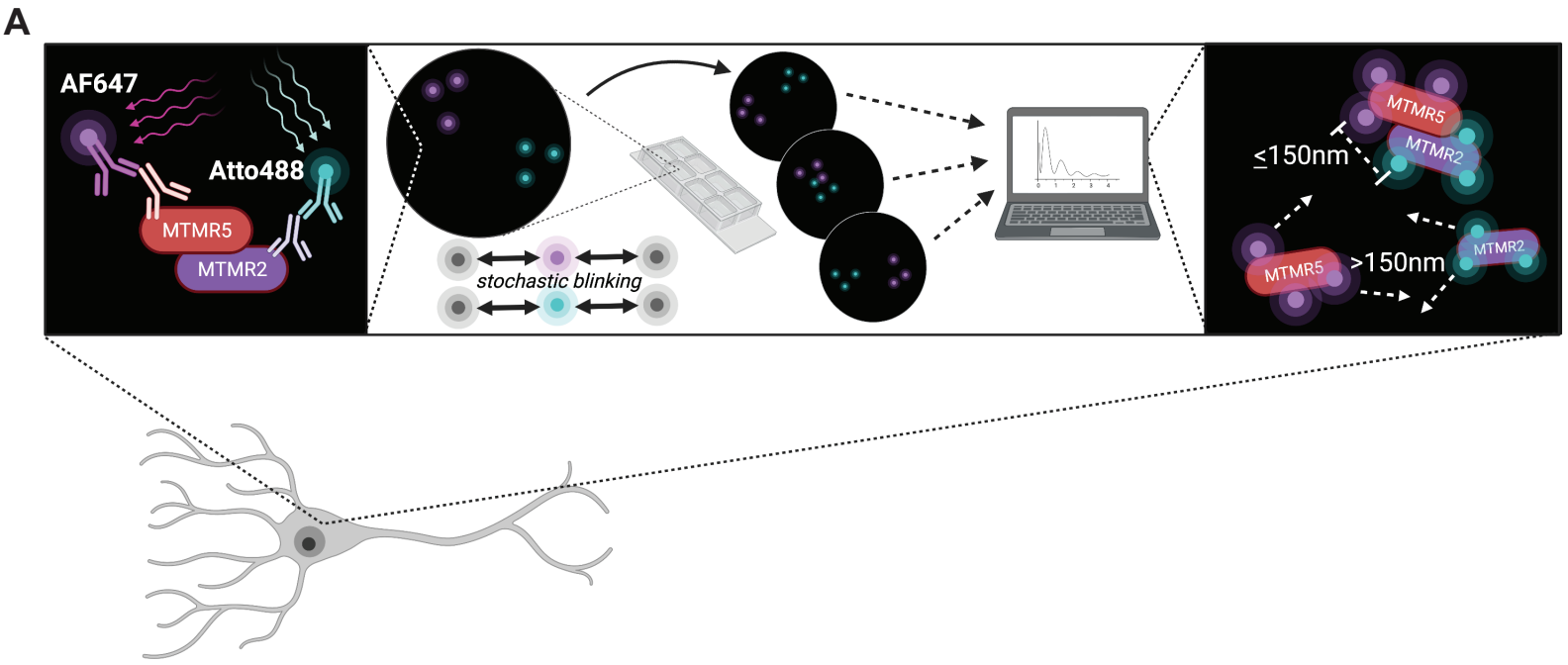
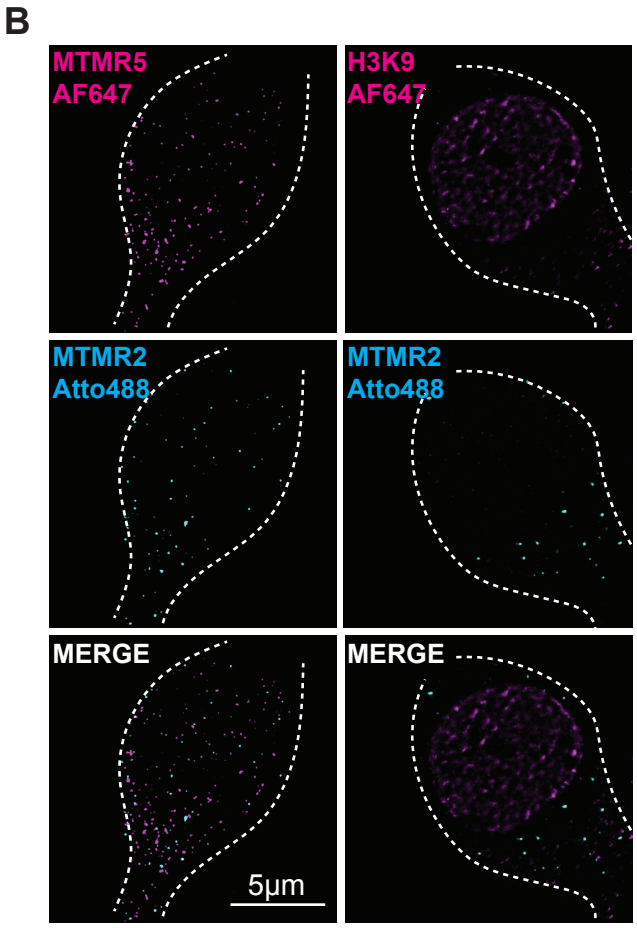
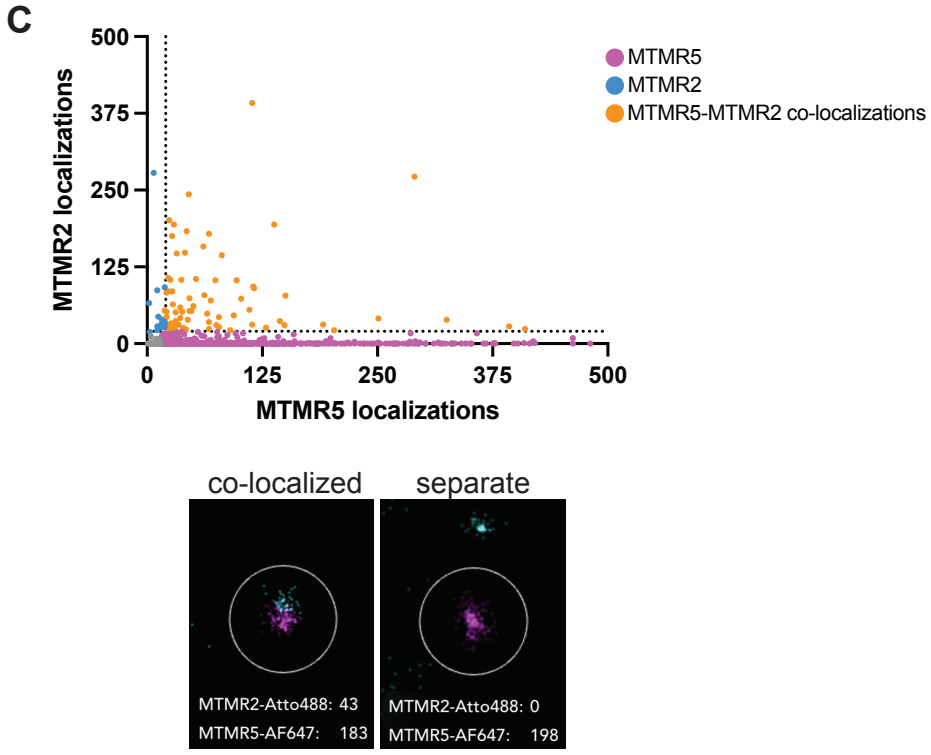

A
iPSC
iNeuron
Torin1:
– + – +
phospho-S6
total S6
phospho-4E-BP1
total 4E-BP1
β-actin
B
DMSO
Torin1
phospho : total S6
actin
1.5
1.0
0.5
0.0
****
**
iPSC
iNeuron
C
DMSO
Torin1
phospho : total 4E-BP1
actin
1.5
1.0
0.5
0.0
****
*
iPSC
iNeuron

Figure S2. Torin1 inhibits the mTOR pathway in neurons, related to Figure 1. (**A**) Representative Western analysis of undifferentiated iPSCs (lanes 1-2) and iNeurons (lanes 3-4) after treatment with 250nM Torin1 or DMSO vehicle for 4h. (**B**) Band intensity quantifications of the mTOR pathway substrate, phospho-S6, normalized to total S6 and actin loading control. Data are from three independent experiments. ns, not significant; **p<0.01; ****p<0.0001, one-way ANOVA. (**C**) Band intensity quantifications of the mTOR pathway substrate, phospho-4E-BP1, normalized to total 4E-BP1 and actin loading control. Data are from three independent experiments. ns, not significant; *p<0.05; ****p<0.0001, one-way ANOVA.


A
AF647
Atto488
MTMR5
MTMR2
stochastic blinking
≤150nm
>150nm
MTMR5
MTMR2
MTMR5
MTMR2


B
MTMR5
AF647
H3K9
AF647
MTMR2
Atto488
MTMR2
Atto488
MERGE
MERGE
5µm


C
MTMR5
MTMR2
MTMR5-MTMR2 co-localizations
MTMR2 localizations
MTMR5 localizations
0
125
250
375
500
co-localized
separate
MTMR2-Atto488: 43
MTMR5-AF647: 183
MTMR2-Atto488: 0
MTMR5-AF647: 198

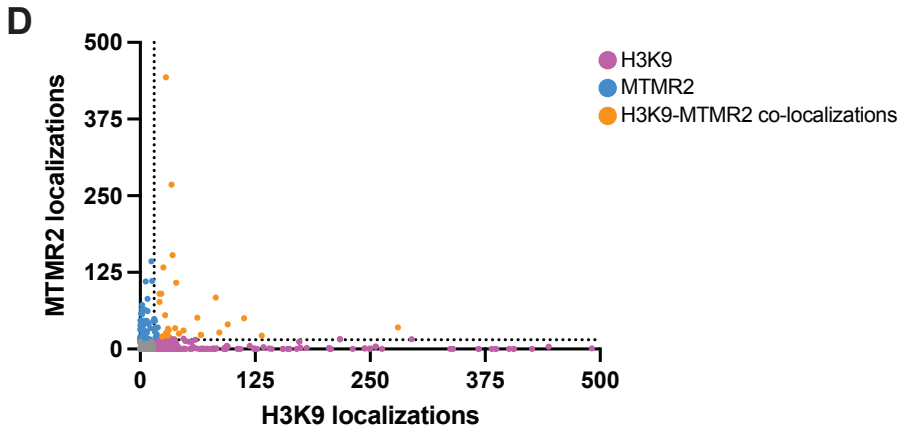

D
H3K9
MTMR2
H3K9-MTMR2 co-localizations
MTMR2 localizations
H3K9 localizations
0
125
250
375
500

Figure S3. MTMR5 associates with MTMR2 in neurons, related to Figure 5 and Figure 6. (**A**) Schematic workflow of dSTORM microscopy. Single-molecule fluorophores of differing emission wavelengths and bound to MTMR5 and MTMR2 are stochastically activated in clusters by high intensity lasers in a selected ROI, followed by photobleaching ("blinking"). Analytic software detects localization clusters of each fluorophore wavelength with 20-nm resolution, then determines the proximity of clusters within a 150-nm radius. (**B**) Representative localization clusters for MTMR5, MTMR2, and H3K9 proteins in iNeurons. Scale bar, 5μm. Knockdown of MTMR5 or MTMR2 enhances autophagy (see Figure 5 and Figure 6), which is dependent on PtdIns3P. (**C**) Scatter plot of MTMR5 and MTMR2 localizations within 150nm of each other, with co-localizations scored for clusters containing $\geq$15 localizations for both proteins (top panel). Example clusters (bottom panel) scored as co-localized (left) and separate (right). (**D**) Scatter plot of H3K9 and MTMR2 localizations within 150nm of each other, with co-localizations scored for clusters containing $\geq$15 localizations for both proteins.

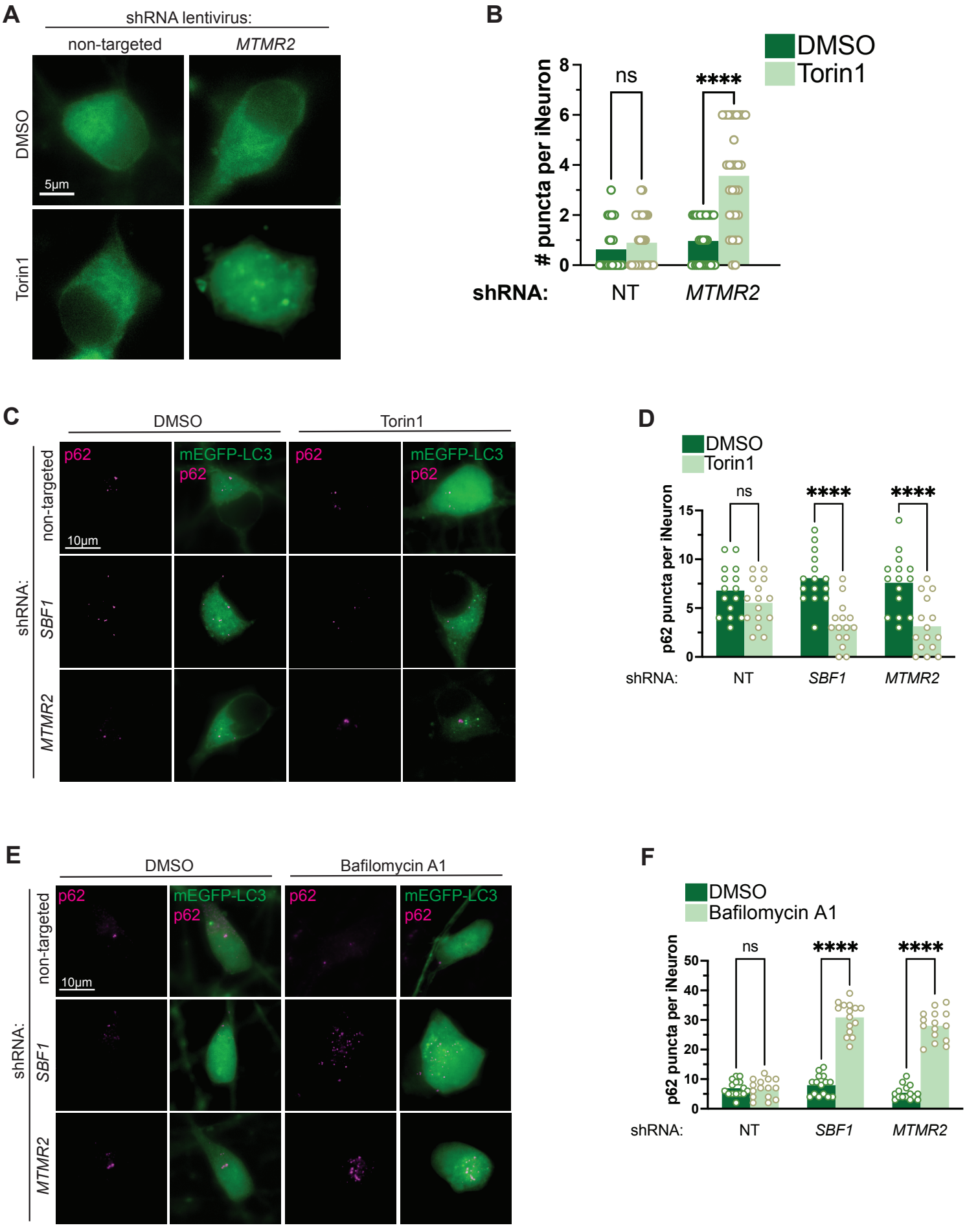

A
shRNA lentivirus:
non-targeted
MTMR2
DMSO
Torin1
5μm
B
DMSO
Torin1
puncta per iNeuron
ns
****
shRNA:
NT
MTMR2
C
DMSO
Torin1
p62
mEGFP-LC3
p62
non-targeted
SBF1
MTMR2
shRNA:
10μm
D
DMSO
Torin1
p62 puncta per iNeuron
ns
****
****
shRNA:
NT
SBF1
MTMR2
E
DMSO
Bafilomycin A1
p62
mEGFP-LC3
p62
non-targeted
SBF1
MTMR2
shRNA:
10μm
F
DMSO
Bafilomycin A1
p62 puncta per iNeuron
ns
****
****
shRNA:
NT
SBF1
MTMR2

Figure S4. Knockdown of MTMR2 sensitizes neurons to Torin1, related to Figure 5. (**A**) Representative images of mEGFP-LC3 iNeurons transduced with non-targeted or *MTMR2* shRNA and treated with DMSO vehicle or 250nM Torin1 for 4h. Scale bar, 5μm. (**B**) Scatterplots of blinded manual quantifications of mEGFP-LC3-positive puncta imaged in iNeurons as treated in (A). Data are from three independent experiments; ns, not significant; ****p<0.0001, one-way ANOVA. (**C**) Representative images of mEGFP-LC3 iNeurons transduced with non-targeted, *SBF1*, or *MTMR2* shRNA, treated with DMSO vehicle or 250nM Torin1 for 4h, fixed, and immunostained for p62. Scale bar, 10μm. (**D**) Quantifications of p62 puncta from (C). Data are from three independent experiments; ns, not significant; ****p<0.0001, one-way ANOVA. (**E**) Representative images of mEGFP-LC3 iNeurons transduced with non-targeted, *SBF1*, or *MTMR2* shRNA, treated with DMSO vehicle or 100nM bafilomycin A1 for 4h, fixed, and immunostained for p62. Scale bar, 10μm. (**F**) Quantifications of p62 puncta from (C). Data are from three independent experiments; ns, not significant; ****p<0.0001, one-way ANOVA.

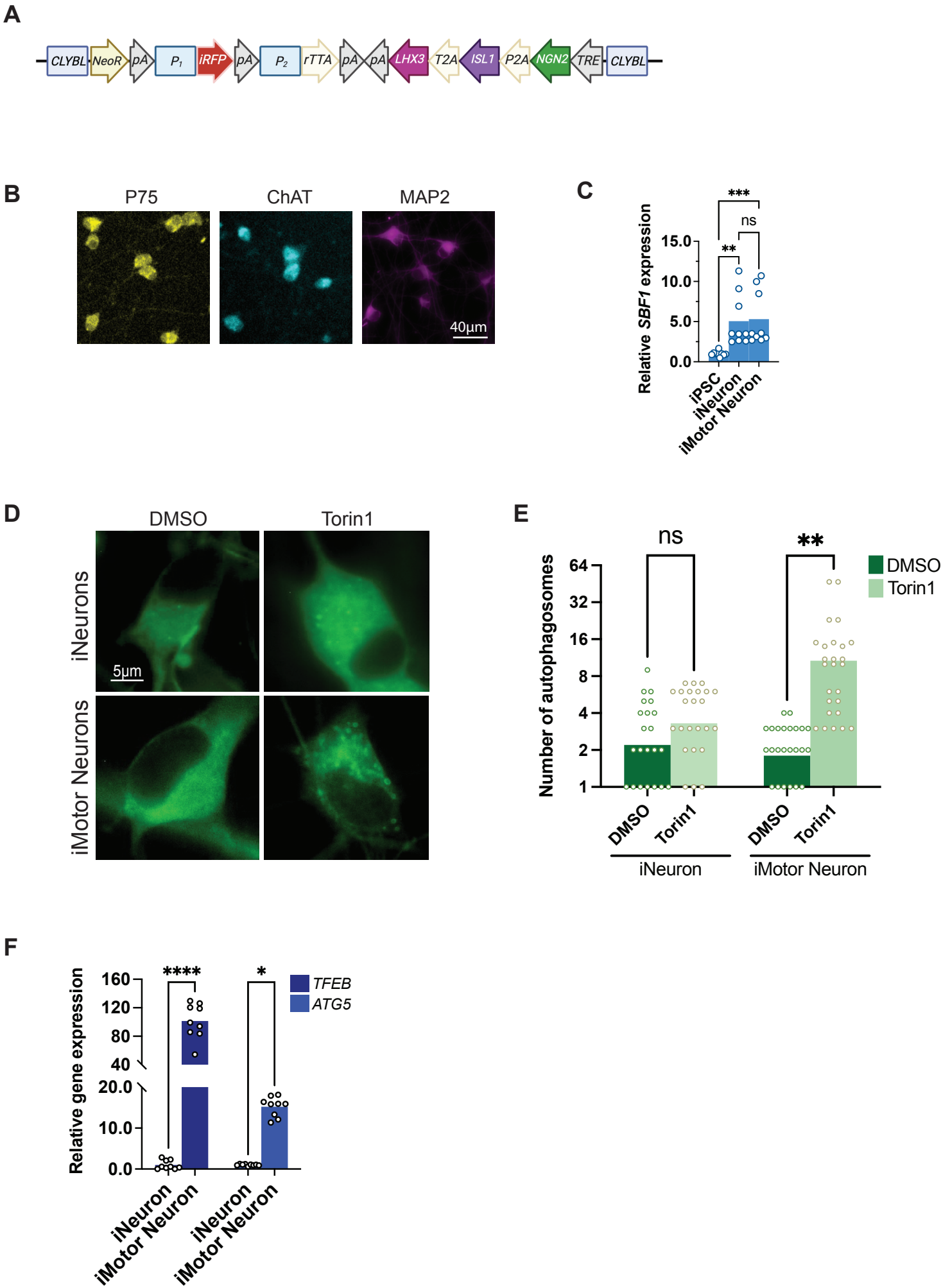

A
CLYBL
NeoR
pA
P1
iRFP
pA
P2
rTTA
pA
pA
LHX3
T2A
ISL1
P2A
NGN2
TRE
CLYBL
B
P75
ChAT
MAP2
40μm
C
Relative SBF1 expression
15.0
10.0
5.0
0.0
***
ns
**
iPSC
iNeuron
iMotor Neuron
D
DMSO
Torin1
iNeurons
iMotor Neurons
5μm
E
Number of autophagosomes
64
32
16
8
4
2
1
ns
**
DMSO
Torin1
DMSO
Torin1
DMSO
Torin1
iNeuron
iMotor Neuron
F
Relative gene expression
160
120
80
40
20.0
10.0
0.0
****
*
TFEB
ATG5
iNeuron
iMotor Neuron
iNeuron
iMotor Neuron

Figure S5. iMotor Neurons have unique profiles of autophagy factor expression and sensitivity to Torin1, related to Figure 1 and Figure 2. (**A**) Schematic of the cassette used to integrate *LHX3*, *ISL1*, and *NGN2* at the *CLYBL* safe harbor locus under the control of a Tet-ON system. *Neo*, neomycin-resistance gene; *pA*, poly-A tail; P_1, P_2, promotors; *iRFP*, near-infrared fluorescent protein; *rTTA*, reverse tetracycline-controlled transactivator; *NGN1* and *NGN2*, neurogenin-1 and -2; *T2A*, self-cleaving peptide; *TRE*, tetracycline response element. (**B**) Immunocytochemical staining of DIV14 iMotor Neurons for motor neuron markers P75, choline acetyltransferase (ChAT), and MAP2. (**C**) RT-PCR measurements of human *SBF1* RNA in iPSCs and iNeurons expanded to include relative expression in isogenic iMotor Neurons; ns, not significant; **p<0.01; ***p<0.001, one-way ANOVA. (**D**) Representative images of mEGFP-LC3-positive vesicles in iNeurons and iMotor Neurons after treatment with DMSO vehicle or 250nM Torin1 for 4 hours. (**E**) Scatterplots of blinded manual quantifications of mEGFP-LC3-positive vesicles imaged as in (D). Data are from three independent experiments; ns, not significant; **p<0.01, one-way ANOVA. (**F**) RT-PCR measurements of human *TFEB* and *ATG5* RNA in iMotor Neurons compared to iNeurons; *p<0.05; ****p<0.0001, one-way ANOVA.

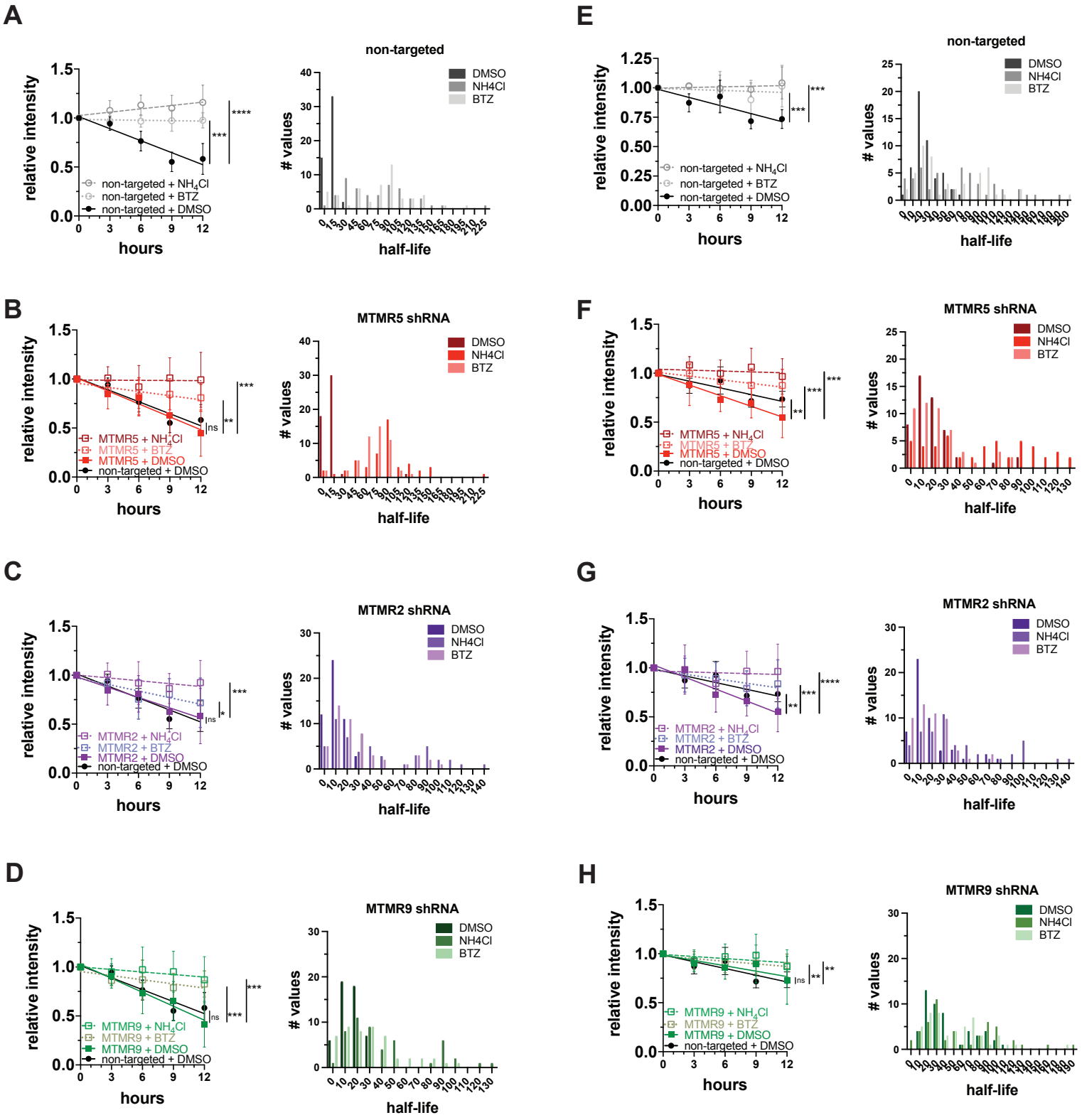

A
relative intensity
hours
non-targeted + NH4Cl
non-targeted + BTZ
non-targeted + DMSO
****
***
non-targeted
values
half-life
DMSO
NH4Cl
BTZ
B
MTMR5 shRNA
MTMR5 + NH4Cl
MTMR5 + BTZ
MTMR5 + DMSO
non-targeted + DMSO
ns
**
***
C
MTMR2 shRNA
MTMR2 + NH4Cl
MTMR2 + BTZ
MTMR2 + DMSO
non-targeted + DMSO
*
D
MTMR9 shRNA
MTMR9 + NH4Cl
MTMR9 + BTZ
MTMR9 + DMSO
non-targeted + DMSO
E
F
G
H

Figure S6. Degradation of autophagy substrates is not limited by MTMR5-MTMR2 in iMotor Neurons, related to Figure 6 and Figure 7. (**A**-**D**) Fluorescence of TDP-43-Dendra2 (A-D) or Dendra2 (E-H) measured in iMotor Neurons transduced with lentivirus expressing non-targeted shRNA (A-B, top panels) *SBF1* shRNA (A-B, bottom panels), *MTMR2* shRNA (C), or *MTMR9* shRNA (D) and after the indicated drug treatments (left panels), and histogram plots of the half-lives of each measured iMotor Neuron (right panels). Data are represented at each time point as mean ± SD; ns, not significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001, one-way ANCOVA.