SUPPLEMENTARY INFORMATION

Autophagy induction enhances TDP43 turnover and survival in neuronal ALS models

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SUPPLEMENTARY RESULTS

Supplementary Figure 1: Automated microscopy of primary neurons expressing TDP43-EGFP. Most neurons displayed nuclear localization of TDP43-EGFP (a–d), but a significant fraction exhibited cytoplasmic mislocalization (e–h) and/or cytoplasmic aggregates of TDP43-EGFP (i–l). (a, e, i) mApple; (b, f, j) TDP43-EGFP; (c, g, k) Hoechst nuclear stain; (d, h, l) merged images. Scale bar = 25 μM.

Supplementary Figure 2: The survival of control neurons is unaffected by the amount of transfected EGFP or autophagy inducers at low doses. Primary rat cortical neurons were dissected, cultured, and transfected with mApple and EGFP, then imaged using automated fluorescence microscopy. (a) The amount of EGFP DNA did not affect the survival of transfected neurons (0.13 ng/μl, n=317, reference group; 0.27 ng/μl, n=330; 0.54 ng/μl, n=327; 0.67 ng/μl, n=325). Data were pooled from 8 wells per condition, performed in duplicate. (b) Neurons expressing EGFP were unaffected by treatment with 0.5 μM of NCP, MTM, or FPZ (DMSO, n=480, reference group; NCP, n=211; MTM, n=262; FPZ, n=267). Data were combined from eight wells per condition in each of five experiments. (c) At higher doses, NCP causes significant toxicity in control primary neurons expressing only EGFP. (DMSO, n=126, reference group; 0.5 μM NCP, n=113 neurons; 1.5 μM NCP, n=112 neurons, HR=1.61; 2.5 μM NCP, n=64 neurons, HR=1.81). In contrast, neither MTM (d) nor FPZ (e) had significant effects upon survival at doses up to 2.5 μM. (For panel d, DMSO, n=96, reference group; 0.5 μM MTM, n=82 neurons; 1.5 μM MTM, n=109 neurons; 2.5 μM MTM, n=78 neurons. For panel e, DMSO, n=245, reference group; 0.5 μM FPZ, n=256 neurons; 1.5 μM FPZ, n=236 neurons; 2.5 μM FPZ, n=225 neurons.) Data combined from eight wells per condition, in duplicate. *$p < 0.01$; ns, not significant ($p > 0.05$) by Cox proportional hazards analysis.

Supplementary Figure 3: Determining the "dose" of exogenous TDP43 in transfected neurons. Primary neurons were isolated, cultured, and transfected with TDP43-EGFP and mApple. At 24 h after transfection, immunocytochemistry was used to determine the total amount of TDP43 within transfected neurons. (a-d) Micrographs of neurons transfected with TDP43-EGFP (a) and mApple (c), stained with anti-TDP43 antibodies (b) and Hoechst dye (d). The intensity of TDP43 in untransfected neurons (arrowheads, b) was used as a reference to calculate the amount of total TDP43 in transfected neurons (arrows, b). Scale bar = 10 μm. (e) Plot depicting the relation between the concentration of TDP43(WT)-EGFP (blue) or TDP43(A315T)-EGFP (red) DNA and total normalized TDP43. By linear regression, the total normalized TDP43 is $5.4x_{WT} + 1.1 \pm 0.5$ for TDP43(WT)-EGFP ($R^2 = 0.9105$), and $4.1x_{A315T} + 1.4 \pm 0.3$ for TDP43(A315T)-EGFP ($R^2 = 0.9450$), where x_{WT} is the concentration of TDP43(WT)-EGFP DNA (ng/μl), and x_{A315T} is the concentration of TDP43(A315T)-EGFP DNA (ng/μl). Error bars represent ± SEM. (f) Downregulation of endogenous TDP43 by exogenous TDP43-EGFP. Primary neurons were transfected with vector DNA encoding TDP43(WT)-EGFP or TDP43(A315T)-EGFP at the indicated concentrations, and the

amount of TDP43 determined by quantitative immunocytochemistry. Downregulation of endogenous TDP43 is apparent in cells with a fold endogenous TDP43 < 1. Horizontal bars indicate mean fold endogenous TDP43 levels for each condition. Values were pooled from 17–30 neurons per condition, performed in duplicate.

Supplementary Figure 4: Complementary methods for measuring neuronal TDP43 turnover. (a) Representative, full-length autoradiographic exposures of immunoprecipitated material from metabolically labeled rodent primary cortical neurons. TDP43 was immunoprecipitated at 0, 5, 15, or 25 h after pulse labeling by (1) anti-TDP43 antibodies from ThermoScientific (PA5-17011), (2) anti-TDP43 antibodies from G. Yu and J. Herz (University of Texas, Southwestern), or control anti-IgG antibodies. * denotes full-length TDP43. Molecular weight in kDa is shown to the left of each gel. (b) Schematic diagram comparing metabolic pulse chase (MPC, top) with optical pulse labeling (OPL, bottom). (c-e) Half-life values for individual cells expressing TDP43(WT)-Dendra2 (c), TDP43(A315T)-Dendra2 (d), or Dendra2 (e) were plotted against the normalized RFP intensity immediately after photoconversion. By linear regression, there was no significant relationship between half-life and expression level for any of the constructs. TDP43(WT)-Dendra2, n=207, R^2 = 0.0039. TDP43(A315T)-Dendra2, n=251, R^2 = 0.0108; Dendra2, n=546, R^2 = 0.0052. Values were pooled from 8–12 wells per construct, and five independent experiments. Dotted lines represent 95% confidence intervals.

Supplementary Figure 5: ALS-associated TDP43(M337V). (a) Probability density plot of single-cell half-life measurements from neurons expressing TDP43(WT)-Dendra2 (cyan, n=128) and TDP43(M337V)-Dendra2 (green, n=111), demonstrating a higher probability of observing a low TDP43-Dendra2 half-life in neurons expressing TDP43(M337V)-Dendra2. * $p=1.6\times10^{-4}$, two-sided Kolmogorov-Smirnov test). Values were obtained from eight wells per condition, performed in duplicate. (b) FPZ lowered the risk of death in neurons expressing TDP43(M337V)-EGFP. By Cox proportional hazards analysis, for TDP43(M337V)-EGFP + DMSO vs EGFP, HR=1.49, ** $p=6.2\times10^{13}$; for TDP43(M337V)-EGFP + FPZ vs EGFP, HR=1.17, * p=0.006; and for TDP43(M337V)-EGFP + DMSO vs TDP43(M337V)-EGFP + FPZ , HR=0.78, $p=1.7\times10^{6}$. EGFP, n=851; TDP43(M337V)-EGFP + DMSO, n=914; TDP43(M337V)-EGFP + FPZ, n=931. Data were pooled from 8–12 wells per condition, performed in duplicate.

Supplementary Figure 6: Small-molecule autophagy inducers effectively stimulate autophagic flux in primary neurons. (a) Primary neurons were treated for 24 h with the indicated autophagy inducer at 0.5 or 5.0 μM, then analyzed by SDS-PAGE and western blotting using anti-GAPDH antibodies (left) or anti-LC3 antibodies (right). ♦, GAPDH; *, LC3-I; **, LC3-II. (b) Autophagic induction by small molecules in the presence of NH_4Cl. Primary neurons were incubated with vehicle or drug (5 μM) for 24 h, and NH_4Cl (50 mM) for 6 h before lysis, SDS-PAGE, and western blotting for LC3. * $p < 0.05$; ns, $p > 0.05$, by one-way ANOVA with Dunnett's test. Values were pooled from 9 independent experiments. (c-i) LC3 immunocytochemistry, demonstrating the formation of large LC3-positive puncta in primary neurons treated with 5 μM but not 0.5 μM of each compound. Scale bar = 10 μM. (j) FPZ, MTM and NCP fail to increase LC3-

Dendra2 turnover in the absence of ATG7, a critical component of autophagy. Primary neurons were co-transfected with LC3-Dendra2 and shRNA directed against *ATG7* (KD-ATG7) or scrambled shRNA (Sc), photoactivated, and half-life calculated from cell intensities measured by automated microscopy. Half-life was then plotted using a probability density function. Sc + DMSO, n=917 neurons; Sc + FPZ, n=262 neurons; Sc + MTM, n=665 neurons; Sc + NCP, n=666 neurons; KD-ATG7 + DMSO, n=917 neurons; KD-ATG7 + FPZ, n=273 neurons; KD-ATG7 + FPZ, n=695 neurons; KD-ATG7 + NCP, n=698 neurons. Sc + DMSO *vs.* KD-ATG7 + DMSO, * $p < 0.05$ for right-shift in curve; KD-ATG7 + DMSO *vs.* KD-ATG7 + FPZ, ns ($p > 0.05$); KD-ATG7 + DMSO *vs.* KD-ATG7 + MTM, ‡ $p < 0.05$ for right-shift in curve; KD-ATG7 + DMSO *vs.* KD-ATG7 + NCP, ns ($p > 0.05$). All comparisons by two-sided Kolmogorov-Smirnov test. Data were pooled from 8-12 wells per condition, performed in duplicate.

Supplementary Figure 7: Autophagic induction does not affect the survival of WT human iPSC-derived neurons and astrocytes. Human iPSCs carrying WT TDP43 were differentiated into motor neurons or astrocytes, treated with 0.5 μM MTM, FPZ, or vehicle control, then followed by longitudinal fluorescence microscopy. (a) Autophagy inducers did not significantly affect the survival of WT human iPSC-derived motor neurons expressing HB9::GFP (ns, $p > 0.05$ by Cox proportional hazards analysis. DMSO, n=11; FPZ, n=40; MTM, n=40). Data were pooled from 8-12 wells per condition, performed in triplicate. (b) There was no significant effect of autophagy inducers on the survival of WT human iPSC–derived neurons expressing MAP2::mApple (ns, $p > 0.05$ by Cox proportional hazards analysis. DMSO, n=112; FPZ, n=131; MTM, n=138). Data were pooled from 8–12 wells per condition, performed in triplicate. (c) Autophagy inducers also failed to significantly affect survival in WT human iPSC-derived astrocytes (ns, $p > 0.05$ by Cox proportional hazards analysis. DMSO, n=127; FPZ, n=208; MTM, n=131). Results were combined from 8–12 wells per condition, performed in duplicate.

Supplementary Table 1: Cox proportional hazards analysis of TDP43-EGFP in primary rodent cortical neurons.

	DNA (ng/μl)	N	HR	95% CI	p†
TDP43(WT)-EGFP	0.13	290	1.56	1.31–1.86	6.2×10^{-7}
	0.27	334	1.69	1.43–2.01	2.2×10^{-9}
	0.54	321	2.56	2.16–3.04	$<2\times10^{-16}$
	0.67	342	2.98	2.51–3.53	$<2\times10^{-16}$
TDP43(A315T)-EGFP	0.13	315	1.28	1.08–1.54	5.6×10^{-3}
	0.27	332	1.89	1.60–2.25	2.9×10^{-13}
	0.54	319	2.63	2.22–3.13	$<2\times10^{-16}$
	0.67	324	2.99	2.52–3.55	$<2\times10^{-16}$

N, number of neurons per group. HR, hazard ratio, with EGFP (0.13 ng/μl) as the reference group. CI, confidence interval. † The minimum p value for this assay is 2×10^{-16}.

Supplementary Table 2: Effect of small-molecule autophagy inducers on the risk of death in primary neurons expressing TDP43-EGFP.

	Drug (0.5 μM)	N	HR	95% CI	p[†]
EGFP	DMSO	480	Ref	–	–
TDP43(WT)-EGFP	DMSO	347	1.90	1.56–2.25	1.1×10^{-11}
	FPZ	143	1.22	0.93–1.60	0.15
	MTM	132	1.40	1.07–1.83	0.01
	NCP	160	1.70	1.33–2.16	1.8×10^{-5}
TDP43(A315T)-EGFP	DMSO	331	1.77	1.47–2.14	3.4×10^{-9}
	FPZ	200	1.07	0.84–1.35	0.60
	MTM	248	1.28	1.03–1.59	0.02
	NCP	193	1.51	1.19–1.90	5.6×10^{4}

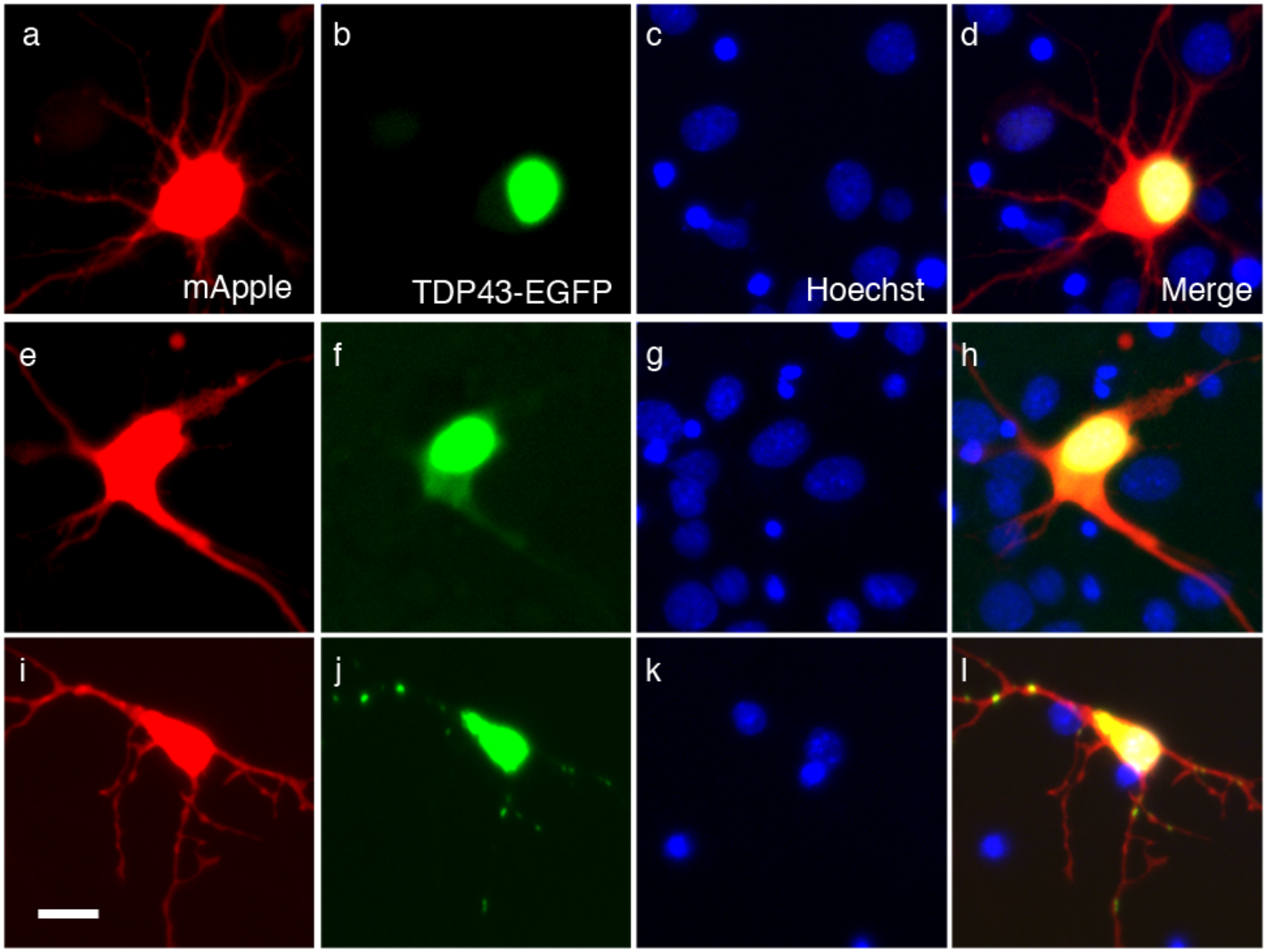
N, number of neurons per group. HR, hazard ratio, with EGFP + vehicle (DMSO) as the reference group (Ref). CI, confidence interval. In Fig. 6a, ns, $p > 0.05$; *** $p=1\times10^{-11}$; ** $p=2\times10^{-5}$; * $p=0.01$. In Fig. 6b, ns $p > 0.05$; *** $p=3\times10^{-9}$; ** $p=6\times10^{-4}$; * $p=0.02$. All p values determined by Cox proportional hazards analysis. [†] The minimum p value for this assay is 2×10^{-16}.

Supplementary Table 3: Effect of small molecule autophagy inducers on the risk of death in iPSC-derived neurons and astrocytes.

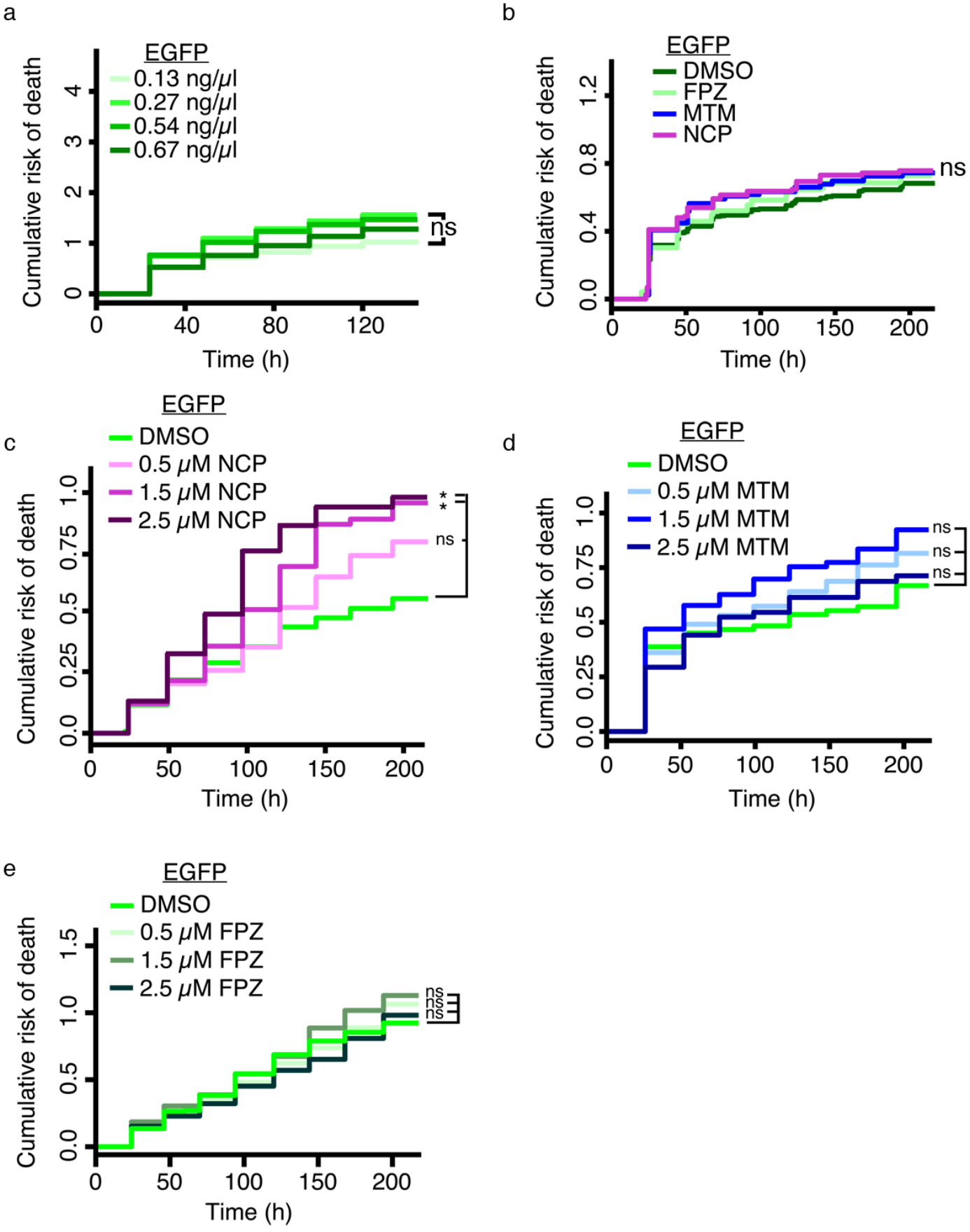
		Drug (0.5 μM)	N	HR	95% CI	p[†]
HB9+ neurons	**WT**	DMSO	11	Ref	–	–
	M337V	DMSO	33	2.64	1.02–6.82	0.04
		FPZ	86	1.45	0.57–3.68	0.43
		MTM	64	1.70	0.66–4.34	0.27
MAP2+ neurons	**WT**	DMSO	112	Ref	–	–
	M337V	DMSO	133	1.44	1.05–1.97	0.02
		FPZ	116	1.12	0.79–1.59	0.51
		MTM	150	0.91	0.66–1.27	0.59
Astrocytes	**WT**	DMSO	127	Ref	–	–
	M337V	DMSO	147	2.53	1.93-3.33	2.5×10^{-11}
		FPZ	139	1.64	1.24-2.18	5.2×10^{-4}
		MTM	125	1.51	1.12-2.01	6.1×10^{-3}

N, number of neurons per group. HR, hazard ratio, with WT + vehicle as the reference group (Ref). CI, confidence interval. In Figure 6c and d, ns $p > 0.05$; * $p=0.02$. In Fig. 6e, *** $p=2\times10^{-11}$; ** $p=5.2\times10^{4}$; * $p=6.1\times10^{-3}$. All p values determined by Cox proportional hazards analysis. [†] The minimum p value for this assay is 2×10^{-16}.

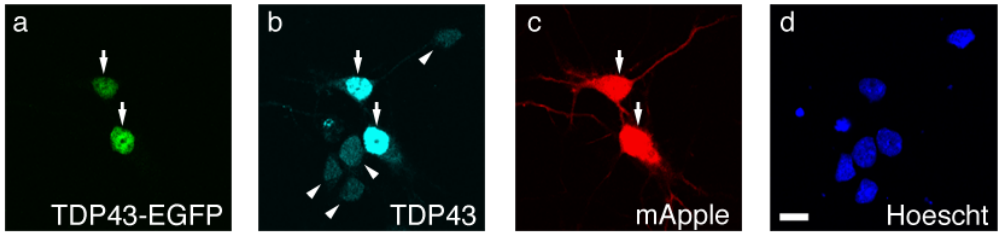
Supplementary Figure 1

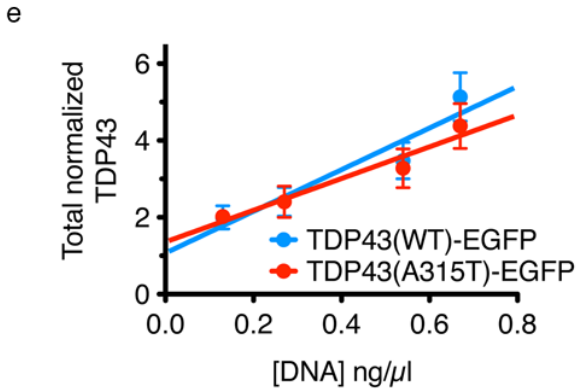


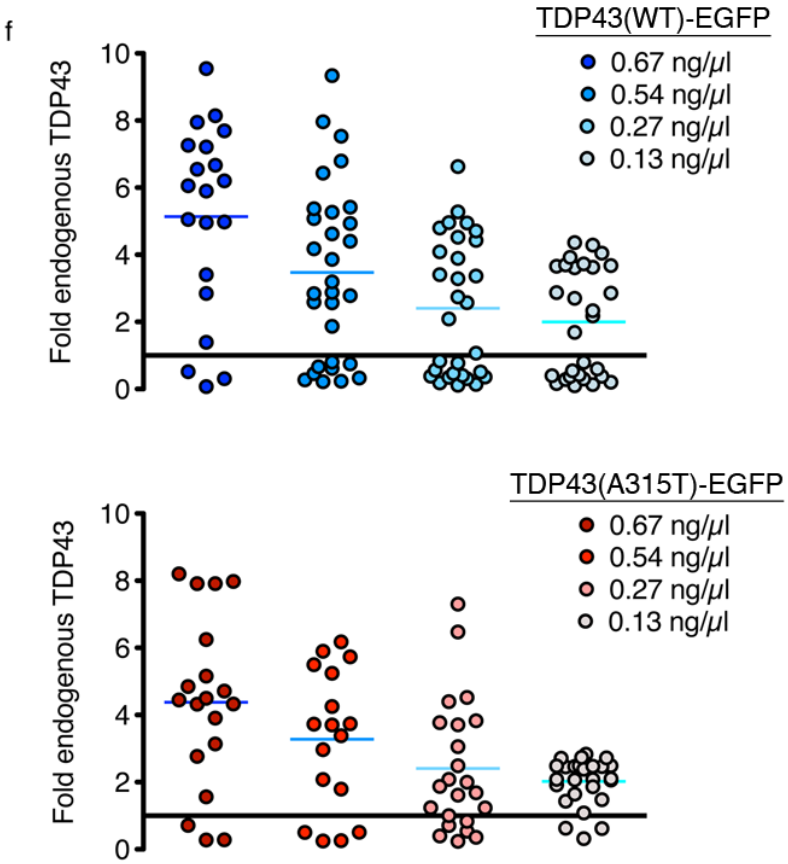
Supplementary Figure 2



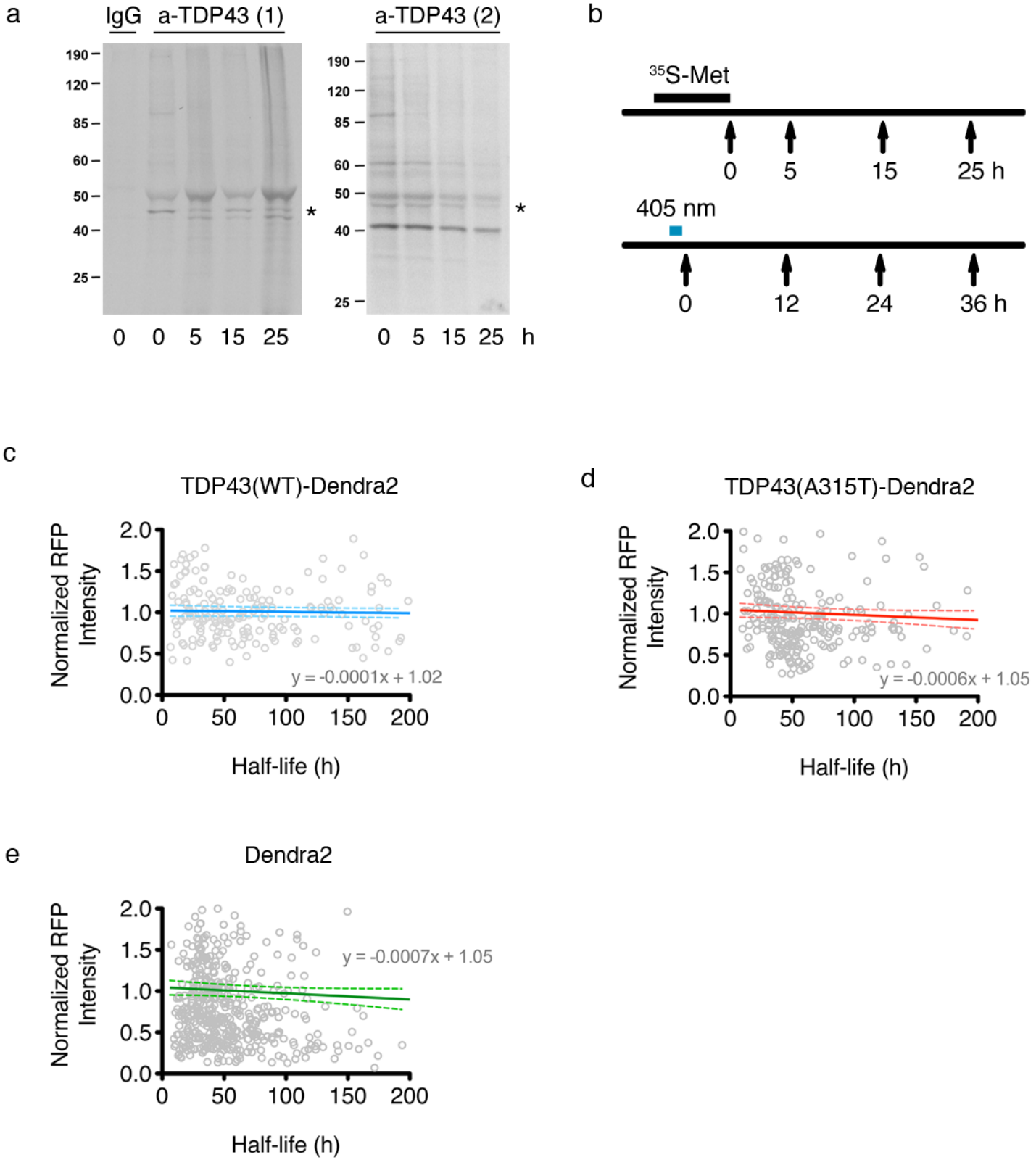
Supplementary Figure 3



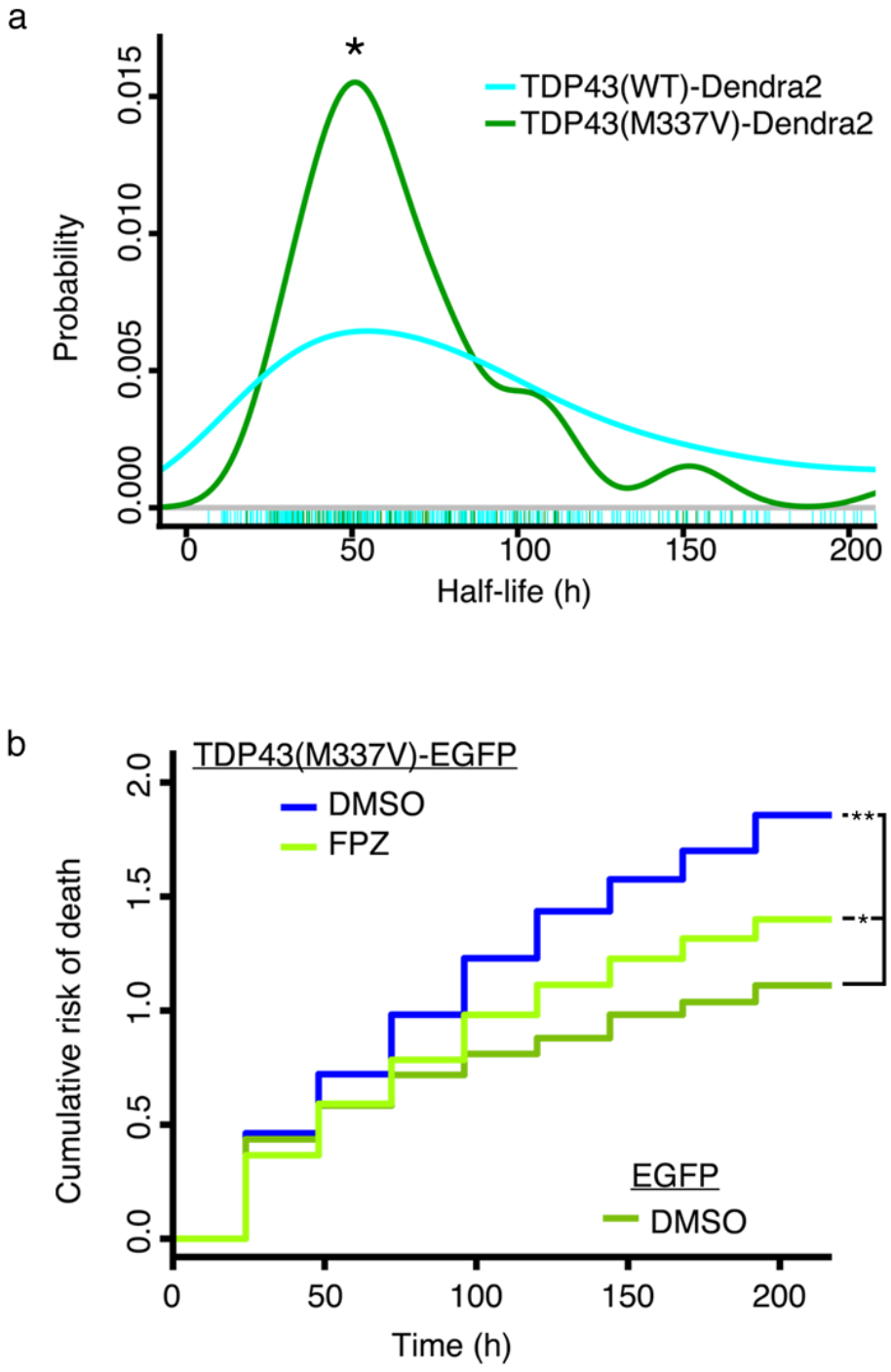




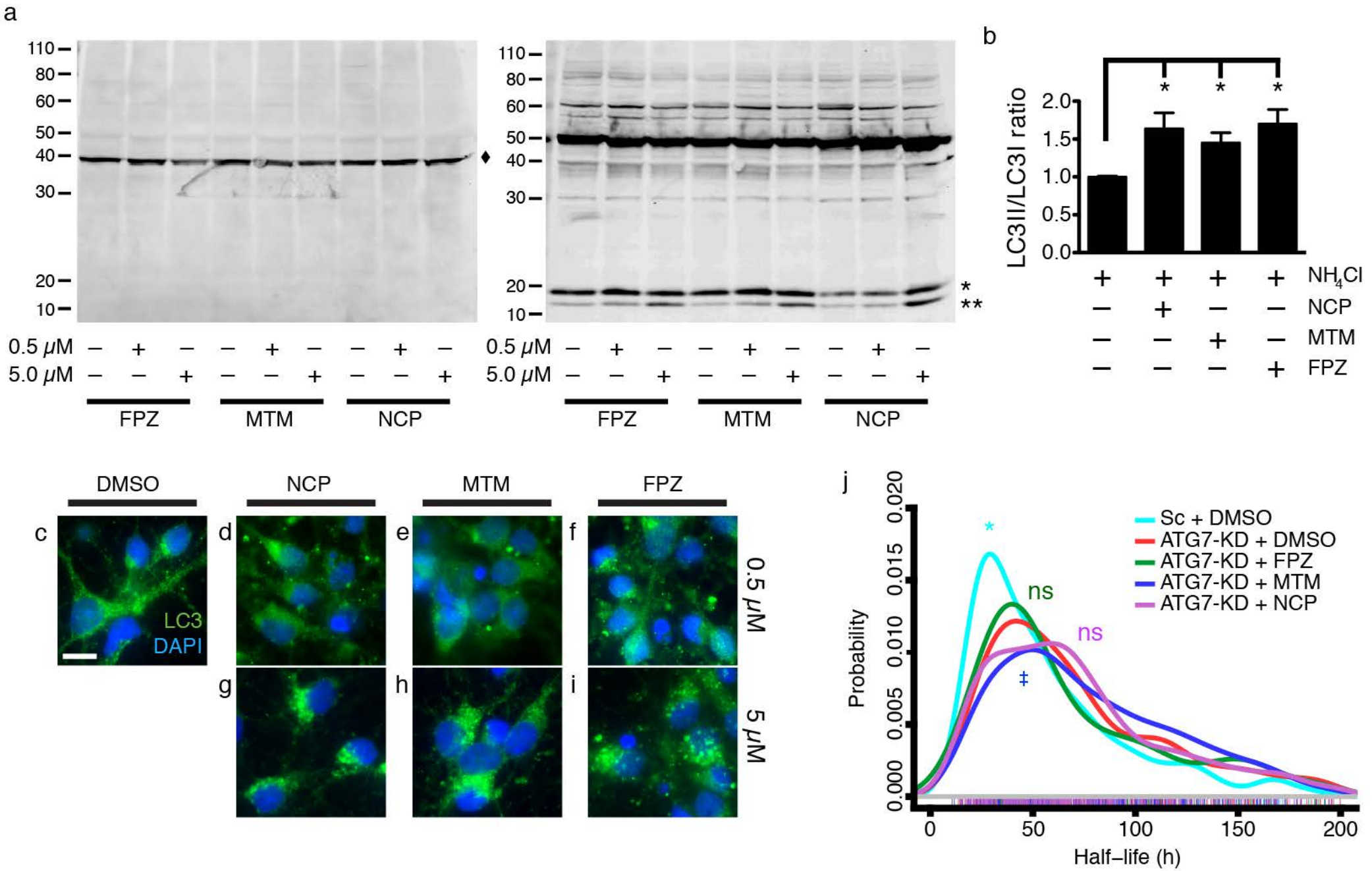
Supplementary Figure 4



Supplementary Figure 5



Supplementary Figure 6



Supplementary Figure 7