The LC3B FRET biosensor monitors the modes of action of ATG4B during autophagy in living cells

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Supplementary information

Figure S1

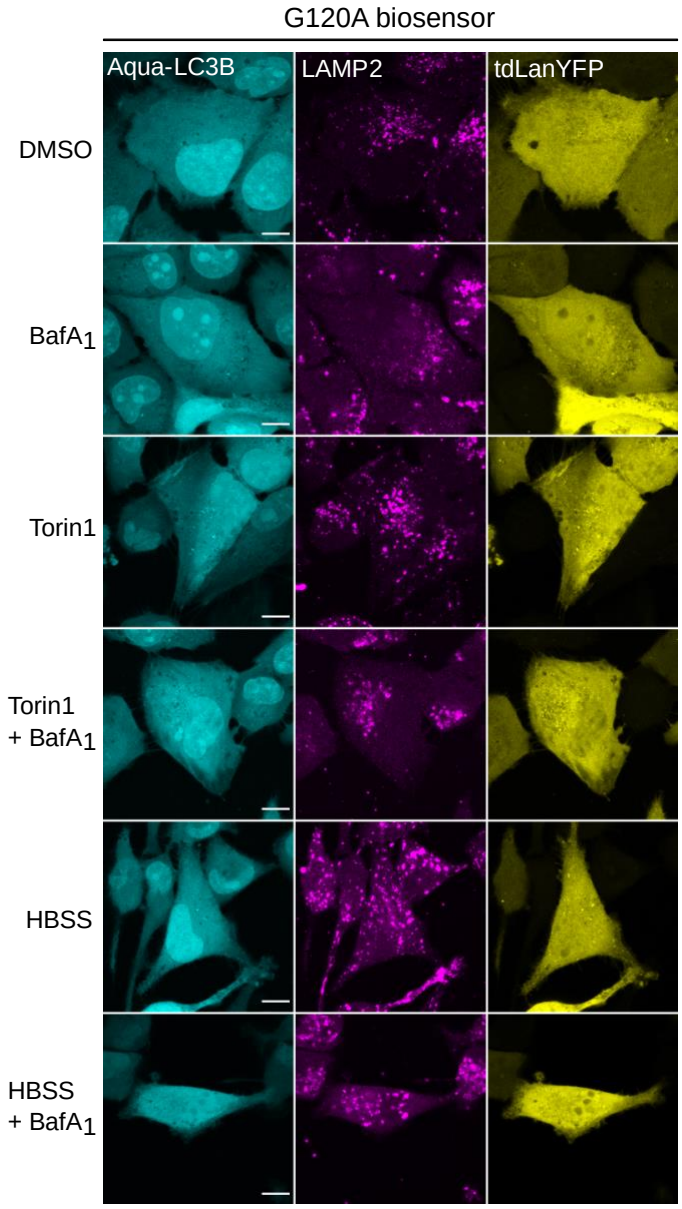

Figure S1. The G120A LC3B biosensor is not sensitive to autophagy induction and/or lysosomal inhibition, and it does not colocalize with LAMP2. Representative fluorescence images of U2OS expressing the G120A biosensor and stained for endogenous LAMP2. To investigate the changes in Aqua-LC3B puncta numbers and their colocalization with LAMP2, cells were treated with: DMSO (6h), BafA1 (6h, 100 nM), Torin1 (3h, 250 nM), Torin1 (3h, 250 nM) + BafA1 (6h, 100 nM), HBSS (1h), HBSS (1h) + BafA1 (6h, 100 nM). Scale bar: 9 µm.

Figure S2

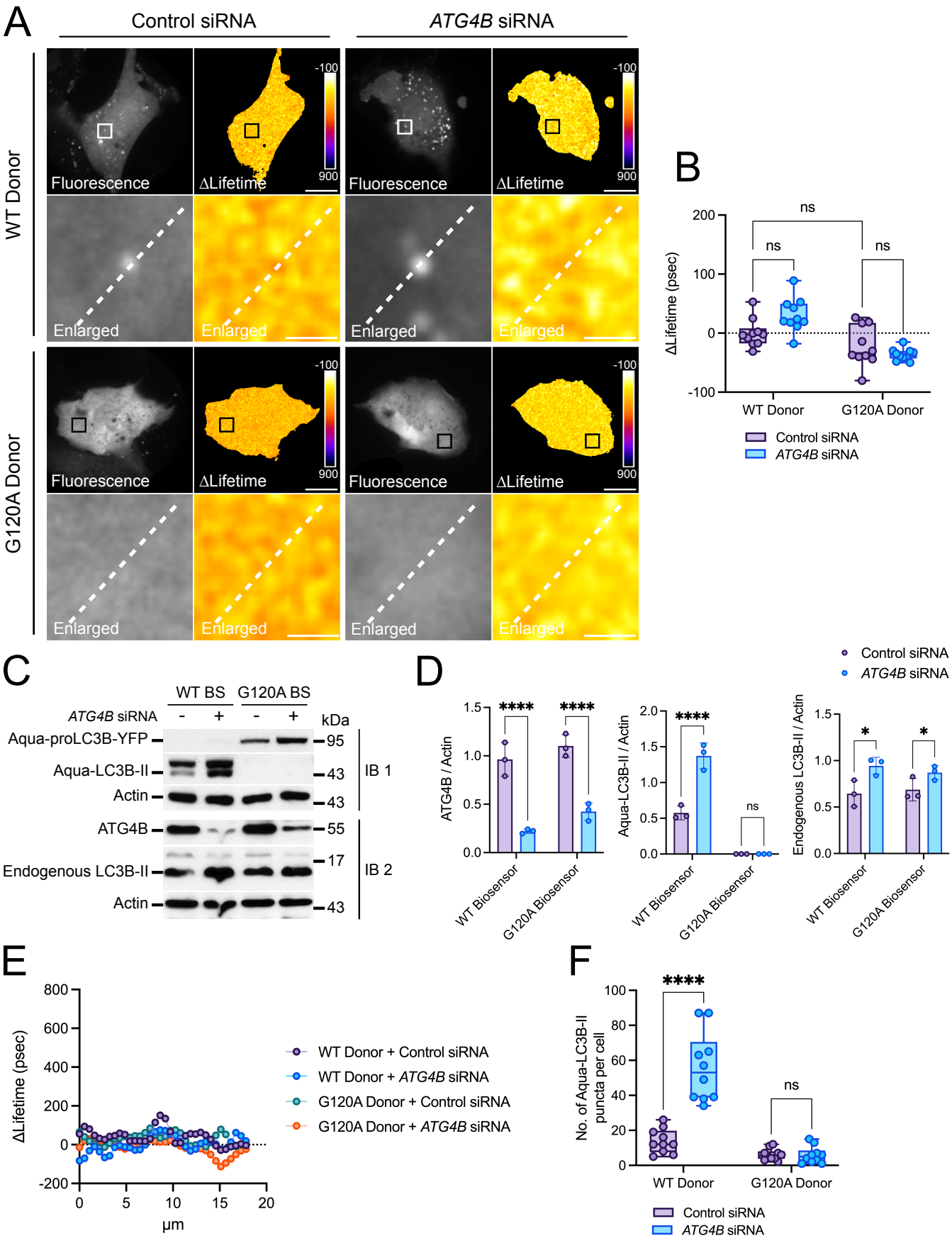
A
Control siRNA
ATG4B siRNA
WT Donor
G120A Donor
Fluorescence
ΔLifetime
Enlarged
-100
900
B
ΔLifetime (psec)
200
100
0
-100
ns
WT Donor
G120A Donor
Control siRNA
ATG4B siRNA
C
WT BS
G120A BS
ATG4B siRNA
kDa
Aqua-proLC3B-YFP
Aqua-LC3B-II
Actin
ATG4B
Endogenous LC3B-II
95
43
55
17
IB 1
IB 2
D
ATG4B / Actin
Aqua-LC3B-II / Actin
Endogenous LC3B-II / Actin
****
*
ns
WT Biosensor
G120A Biosensor
E
ΔLifetime (psec)
μm
WT Donor + Control siRNA
WT Donor + ATG4B siRNA
G120A Donor + Control siRNA
G120A Donor + ATG4B siRNA
F
No. of Aqua-LC3B-II puncta per cell
WT Donor
G120A Donor
Control siRNA
ATG4B siRNA

Figure S2. The WT or G120A donors do not respond to *ATG4B* silencing. (**A**) Representative fluorescence and ∆Lifetime images of U2OS cells co-expressing the WT or G120A donor with control or *ATG4B*-specific siRNAs, and analyzed by FRET/FLIM. Squares on the top images of WT or G120A donor panels illustrate the location of the enlarged images. Dotted lines on the enlarged images illustrate where the line analysis was performed. Pseudocolor scale: pixel-by-pixel ∆Lifetime. Scale bars: overviews, 40 µm; enlarged, 6 µm. (**B**) Mean ∆Lifetime analysis of U2OS cells co-expressing the WT or G120A donor with control or *ATG4B* siRNA. Representative western blotting images (**C**) and corresponding quantifications (**D**) of total lysates from U2OS cells co-expressing the WT or G120A biosensor with control or *ATG4B*-specific siRNAs. IB1 and IB2 correspond to the same lysates blotted for overexpressed (IB1) or endogenous (IB2) LC3B forms. Loading control: Actin. n = 3 independent experiments. (**E**) Line and (**F**) number of Aqua-LC3B-II puncta analyses of U2OS cells co-expressing the WT or G120A donor with control or *ATG4B* siRNA. n = 10 cells per condition from one representative experiment (of three) in (**B**) and (**F**). *P < 0.05, ****P < 0.0001, ns (not significant) as determined by two-way ANOVA with Tukey's multiple comparison test in (**B**) and (**F**), and with two-stage step-up method of Benjamini, Krieger and Yekutieli's multiple comparison test to control the false discovery rate in (**D**).

Figure S3

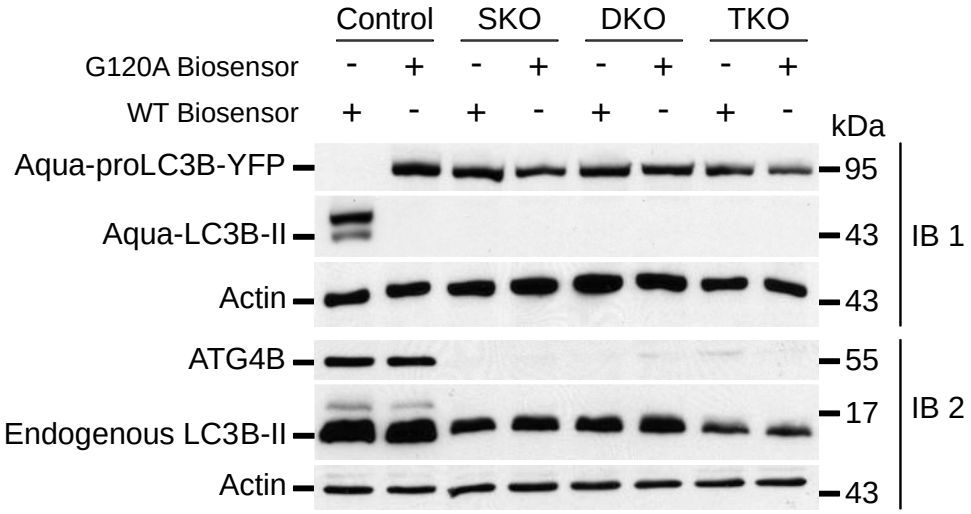

Figure S3. The LC3B biosensor and endogenous LC3B proteins cannot be primed in cells lacking ATG4B. Representative western blotting images of total lysates from HeLa control, *ATG4B* SKO, *ATG4A/B* DKO, *ATG4A/B/C* TKO cells expressing the WT or G120A biosensor. IB1 and IB2 correspond to the same lysates blotted for overexpressed (IB1) or endogenous (IB2) LC3B forms. Loading control: Actin. *n* = 3 independent experiments.

Figure S4

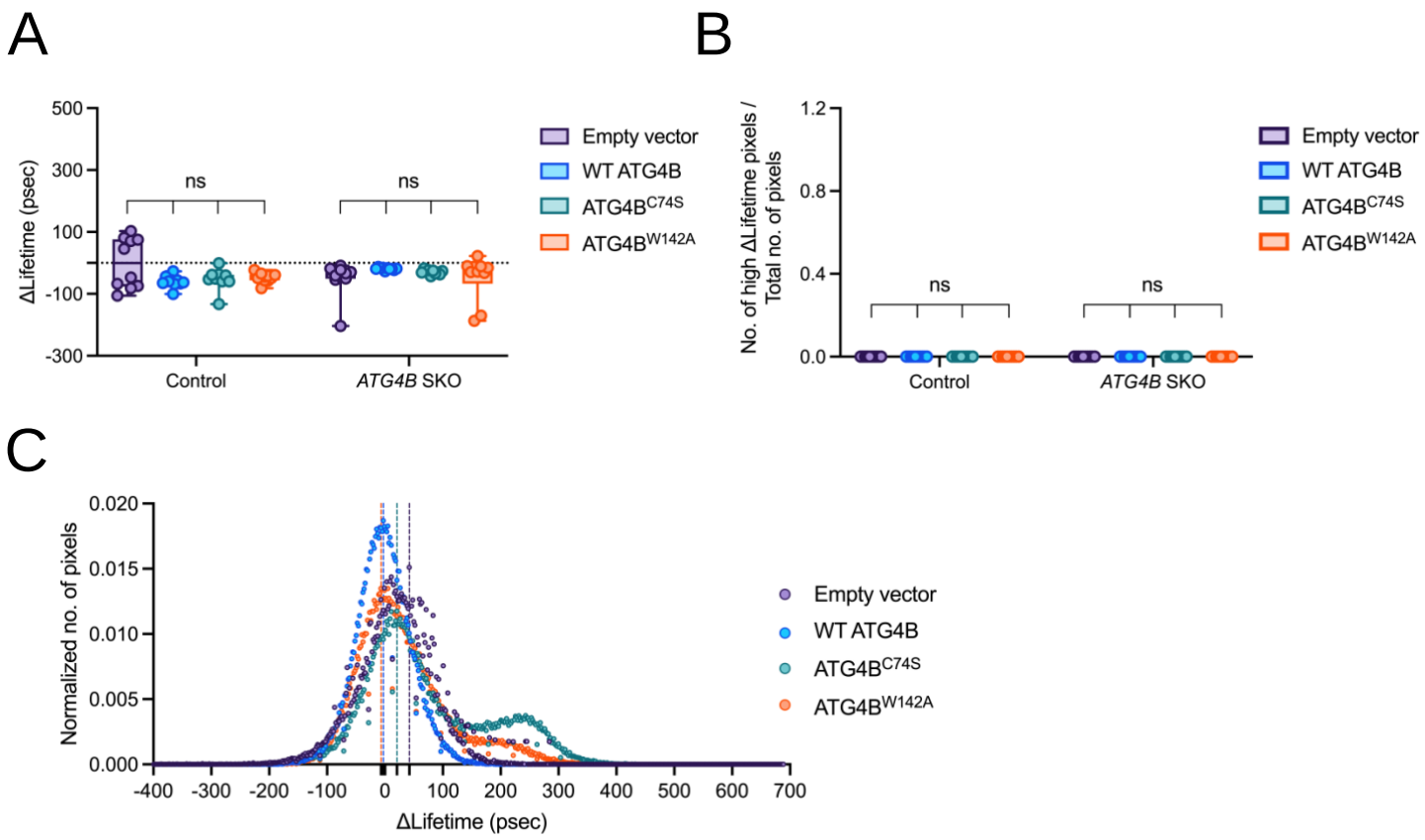

Figure S4. The ectopic expression of active ATG4B does not alter LC3B donor-only ΔlifeTime variations in *ATG4B* SKO cells. Mean ΔLifetime (**A**) and number of high ΔLifetime pixels (**B**) analyses of control and *ATG4B* SKO HeLa cells co-expressing the WT donor with an empty vector, or with vectors expressing WT ATG4B, ATG4B^{C74S} or ATG4B^{W142A}. (**C**) Histogram analysis of HeLa control cells co-expressing the WT biosensor with an empty vector, or with vectors expressing WT ATG4B, ATG4B^{C74S} or ATG4B^{W142A}. Vertical dotted lines on each histogram depict the mode value. *n* = 10 cells per condition from one representative experiment (of three) in (**A**), (**B**) and (**C**). ns (not significant) as determined by two-way ANOVA with Tukey's multiple comparison test in (**A**) and (**B**).

Figure S5

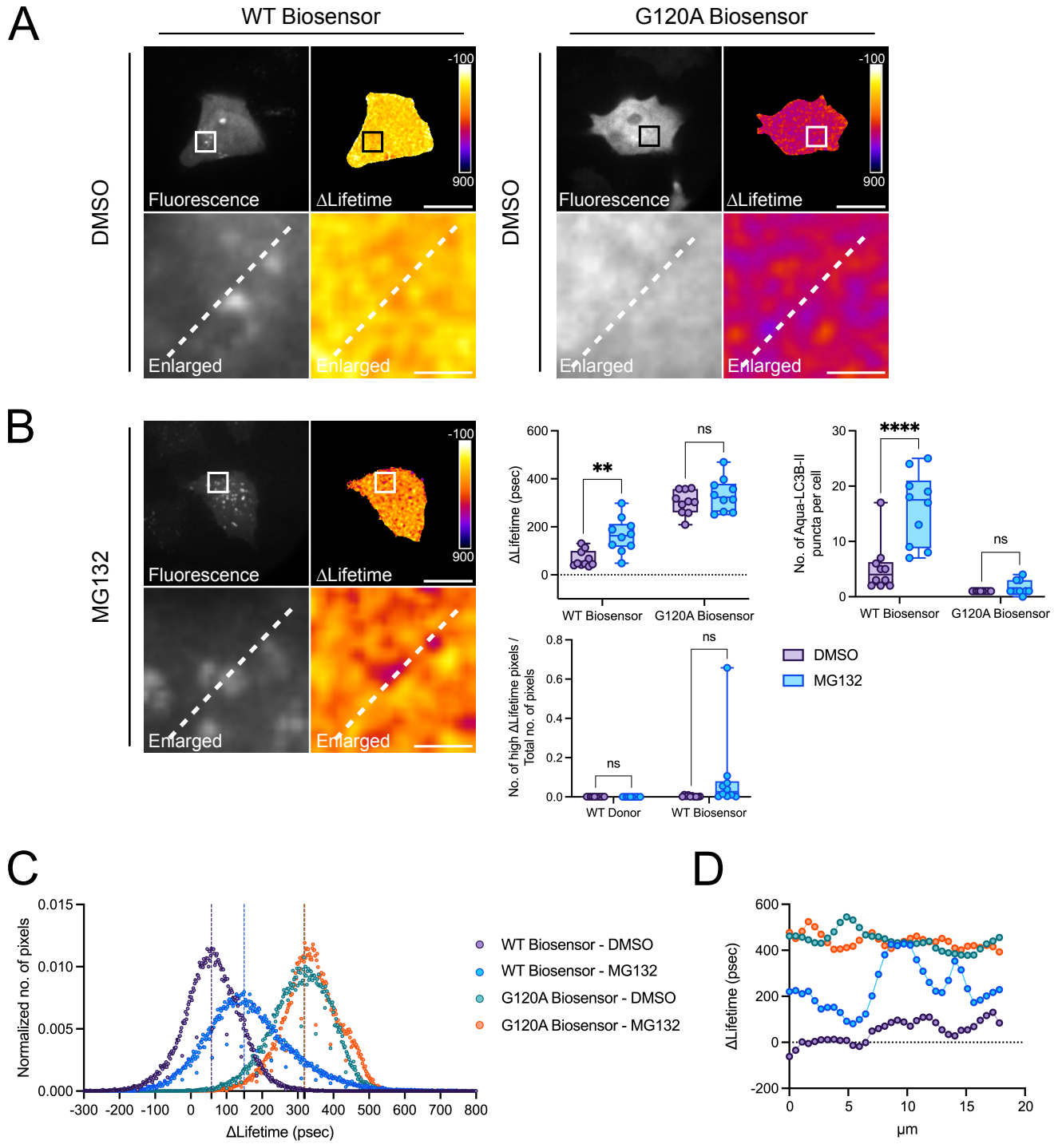
A
WT Biosensor
G120A Biosensor
DMSO
DMSO
Fluorescence
ΔLifetime
-100
900
Enlarged
B
MG132
ΔLifetime (psec)
WT Biosensor
G120A Biosensor
**
ns
No. of Aqua-LC3B-II puncta per cell
****
No. of high ΔLifetime pixels / Total no. of pixels
WT Donor
DMSO
MG132
C
Normalized no. of pixels
ΔLifetime (psec)
WT Biosensor - DMSO
WT Biosensor - MG132
G120A Biosensor - DMSO
G120A Biosensor - MG132
D
ΔLifetime (psec)
μm

Figure S5. The LC3B biosensor reveals the mode of action of MG132 in cells. (**A and B**) Representative fluorescence and ∆Lifetime images of HeLa cells expressing the WT or G120A biosensor, treated with DMSO (6h) or MG132 (6h, 1 µM), and analyzed by FRET/FLIM. Pseudocolor scale: pixel-by-pixel ∆Lifetime. Scale bars: overviews, 40 µm; enlarged, 6 µm. Mean ∆Lifetime (**B**), number of Aqua-LC3B-II puncta (**B**), histogram (**C**), and line (**D**) analyses of HeLa cells expressing the WT or G120A biosensor and treated with DMSO (6h) or MG132 (6h, 1 µM). Number of high ∆Lifetime pixels analysis (**B**) of HeLa cells expressing the WT donor or biosensor and treated with DMSO (6h) or MG132 (6h, 1 µM). Vertical dotted lines on each histogram depict the mode value in (**C**). *n* = 10 cells per condition from one representative experiment (of three) in (**B**) and (**C**). ***P* < 0.01, *****P* < 0.0001, ns (not significant) as determined by two-way ANOVA with Tukey's multiple comparison test in (**B**).

Figure S6

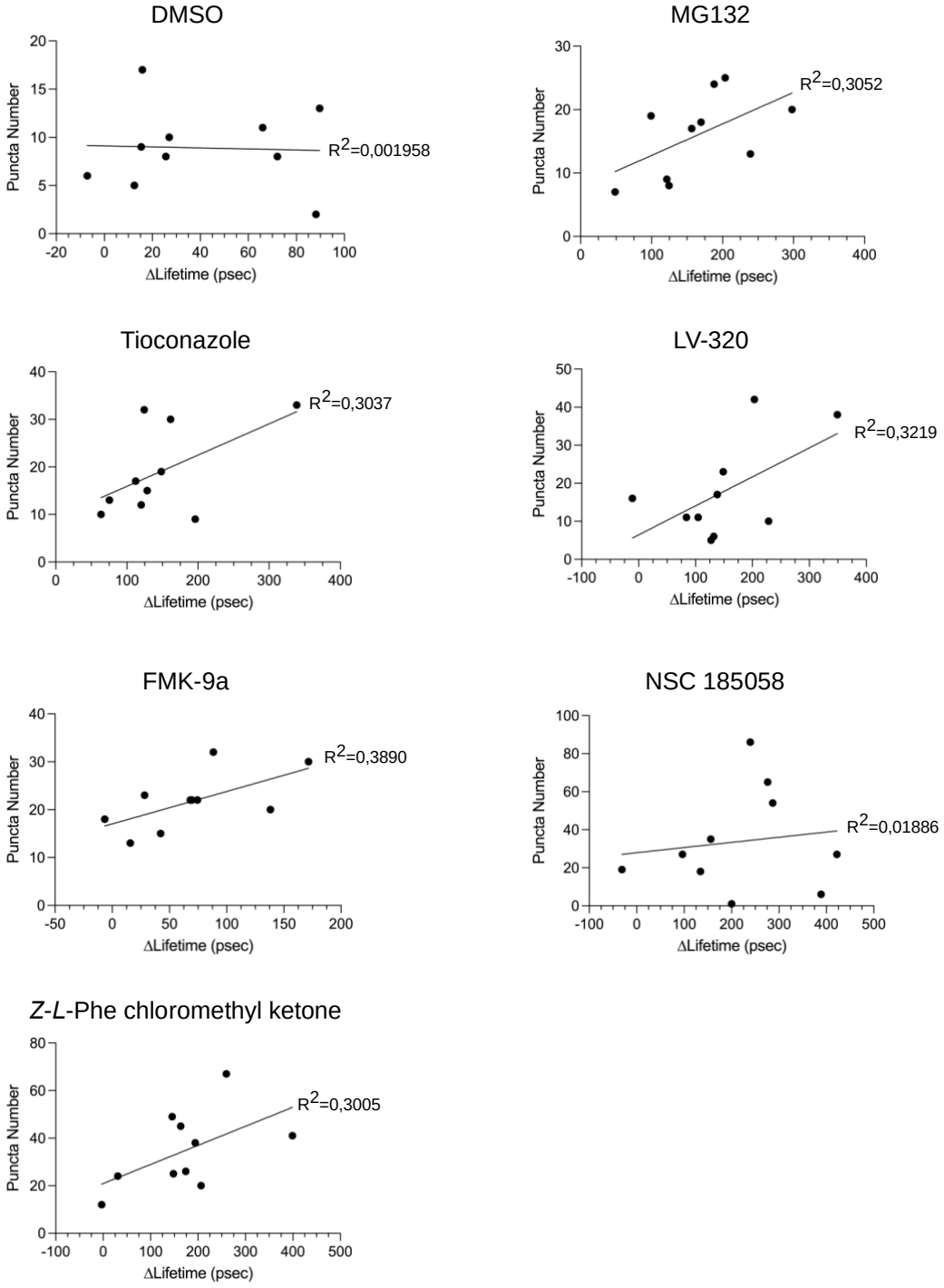

Figure S6. The number of Aqua-LC3B-II puncta and the mean ΔLifetime values do not correlate. Correlation analyses of Aqua-LC3B-II puncta and the mean ΔLifetime values of HeLa cells expressing the WT biosensor and treated with DMSO (6h), MG132 (6h, 1 µM), Tioconazole (6h, 4 µM), LV-320 (6h, 120 µM), FMK-9a (6h, 10 µM), NSC 185058 (6h, 100 µM), or *Z-L*-Phe chloromethyl ketone (6h, 3 µM). *n* = 10 cells per condition from one representative experiment (of three).

Figure S7

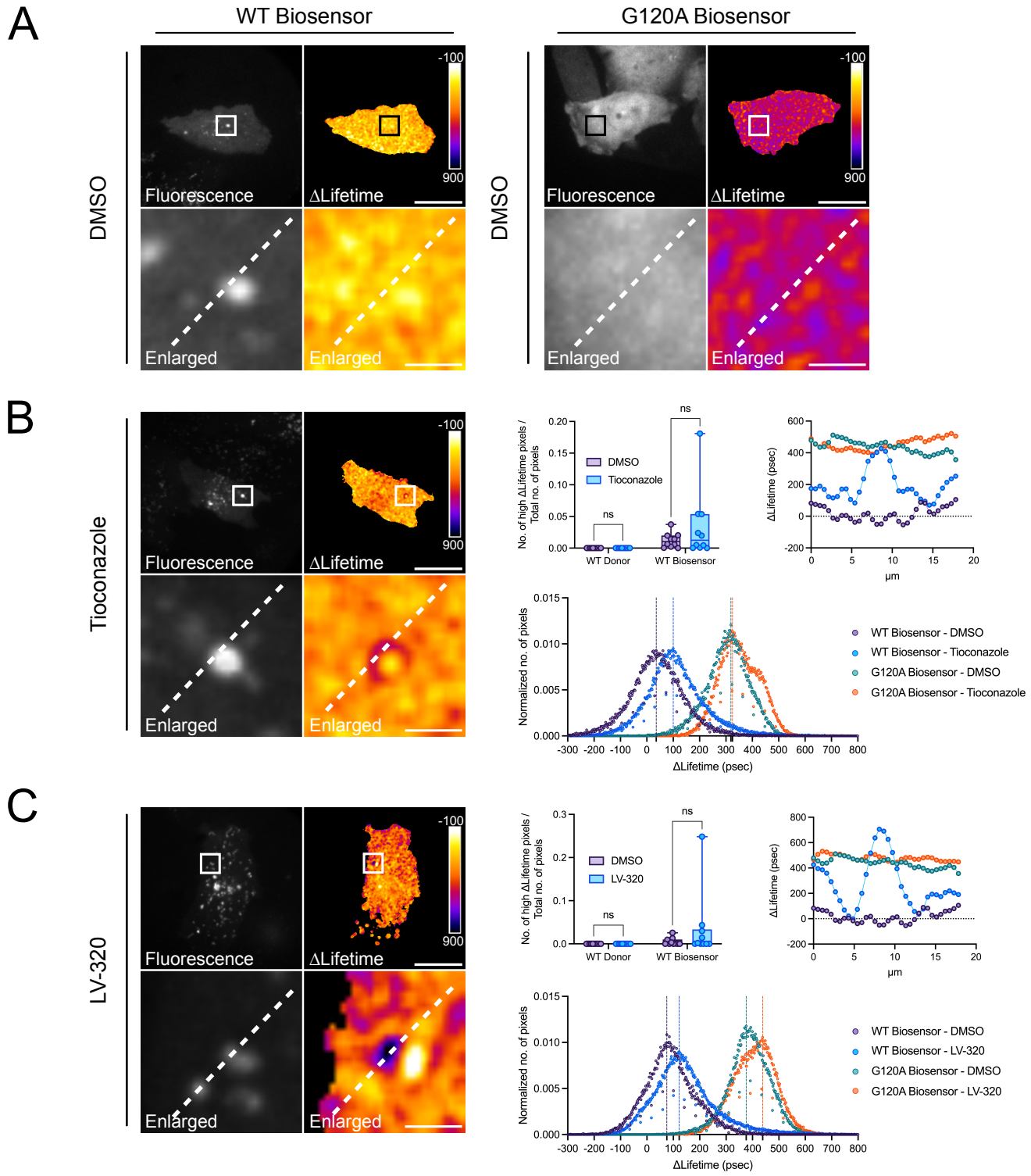
A
WT Biosensor
G120A Biosensor
DMSO
DMSO
Fluorescence
ΔLifetime
-100
900
Enlarged
B
Tioconazole
No. of high ΔLifetime pixels / Total no. of pixels
DMSO
Tioconazole
ns
WT Donor
WT Biosensor
ΔLifetime (psec)
μm
Normalized no. of pixels
ΔLifetime (psec)
WT Biosensor - DMSO
WT Biosensor - Tioconazole
G120A Biosensor - DMSO
G120A Biosensor - Tioconazole
C
LV-320
DMSO
LV-320
WT Biosensor - DMSO
WT Biosensor - LV-320
G120A Biosensor - DMSO
G120A Biosensor - LV-320

Figure S7. FRET/FLIM data analyzed by using sensitive methods reveal the mode of action of Tioconazole and LV-320 in cells. (**A**) Representative fluorescence and ∆Lifetime images of HeLa cells expressing the WT or G120A biosensor, treated with DMSO (6h), and analyzed by FRET/FLIM. Representative fluorescence and ∆Lifetime images of HeLa cells expressing the WT biosensor and treated with the following compounds: Tioconazole (6h, 4 µM) (**B**), LV-320 (6h, 120 µM) (**C**). Squares on the top images of WT or G120A biosensor panels illustrate the location of the enlarged images. Dotted lines on the enlarged images illustrate where the line analysis was performed. Pseudocolor scale: pixel-by-pixel ∆Lifetime. Scale bars: overviews, 40 µm; enlarged, 6 µm. Number of high ∆Lifetime pixels analysis of HeLa cells expressing the WT donor or biosensor and treated with Tioconazole (6h, 4 µM) (**B**) or LV-320 (6h, 120 µM) (**C**). Line, and histogram analyses of HeLa cells expressing the WT or G120A biosensor and treated with Tioconazole (6h, 4 µM) (**B**) or LV-320 (6h, 120 µM) (**C**). *n* = 10 cells per condition from one representative experiment (of three) in (**B, C**). ns (not significant) as determined by two-way ANOVA with Tukey's multiple comparison test in (**B, C**).

Table S1: List of plasmids used in this study. Table listing the source of each plasmid with the cloning sites and the primers for mutagenesis, if applicable.

Plasmid	Cloning sites	Remarks	Primers for mutagenesis (5' to 3'): sense	Primers for mutagenes (5' to 3'): anti-sense
pcDNA 3.1		Purchased from Thermo Fisher Scientific.		
pCMV Aquamarine C1		Kind gift from F. Merola, Université Paris-Saclay, France		
pCMV Aquamarine-tdLanYFP tandem		Kind gift from F. Merola, Université Paris-Saclay, France		
pcDNA 3.1 mCherry-hLC3B		Addgene plasmid #40827.		
pCMV Aquamarine-proLC3B	BglII/EcoRI	pCMV Aquamarine C1 was used as a backbone vector. Insert proLC3B was subcloned from pcDNA 3.1 mCherry-hLC3B.		
pCMV Aquamarine-proLC3B-tdLanYFP	BglII/EcoRI	pCMV Aquamarine-tdLanYFP tandem was used as a backbone vector. proLC3B was inserted by subcloning it from pcDNA 3.1 mCherry-hLC3B.		
pCMV Aquamarine-proLC3B G120A		Obtained from pCMV Aquamarine-proLC3B	cactgacaatttcatcgcgaacgtctcctggga	tcccaggagacgttcgcgatgaaattgtcagtg
pCMV Aquamarine-proLC3B G120A-tdLanYFP		Obtained from pCMV Aquamarine-proLC3B-tdLanYFP	cactgacaatttcatcgcgaacgtctcctggga	tcccaggagacgttcgcgatgaaattgtcagtg
pGEX GST-ATG4B		Kind gift from R. Ketteler, LMCB, UCL, United Kingdom.		
pCMV WT ATG4B	NheI/EcoRI	pcDNA 3.1 was used as a backbone vector. Insert WT ATG4B was subcloned from pGEX GST-ATG4B.		
pCMV ATG4B C74S		Obtained from pCMV WT ATG4B	ccgcagcatgctgccccagcctg	caggctggggcagcatgctgcgg
pCMV ATG4B W142A		Obtained from pCMV WT ATG4B	gtgttgggcccgtacgcctggcctatggactt	aagtccataggccaggcgtacgggcccaacac