

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

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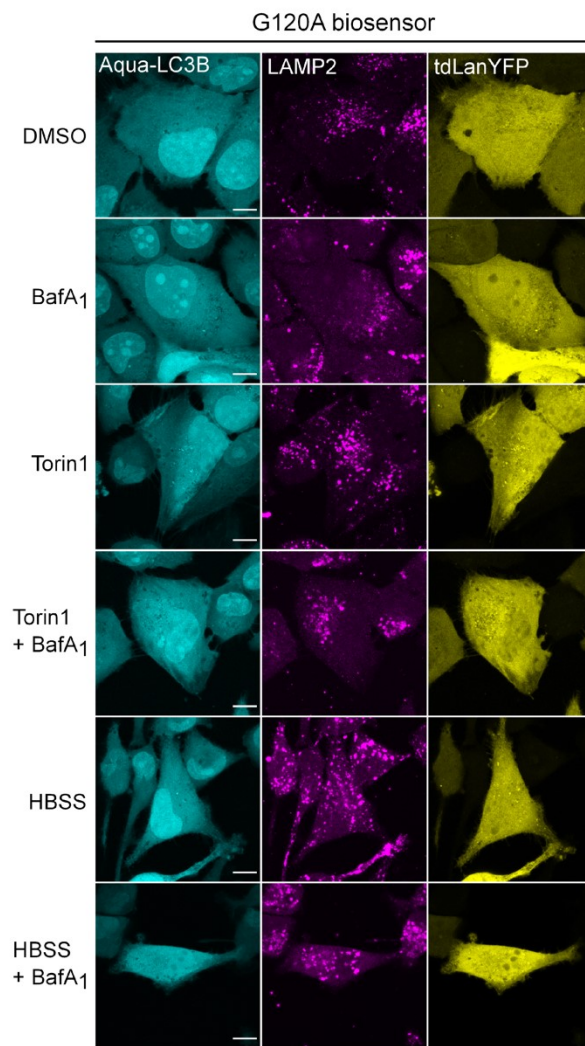
4 **Elif Begüm Gökerküçük et al.**

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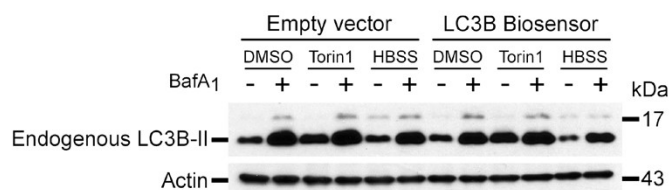
6 **Supplementary figures**

Figure S1

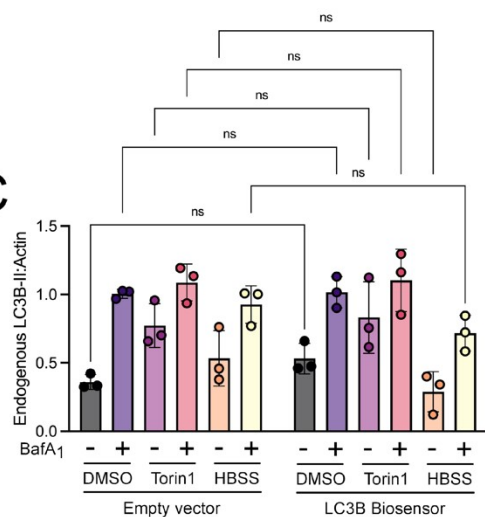
A



B



C



7

8 **Figure S1.** The G120A LC3B biosensor is not sensitive to autophagy induction and/or

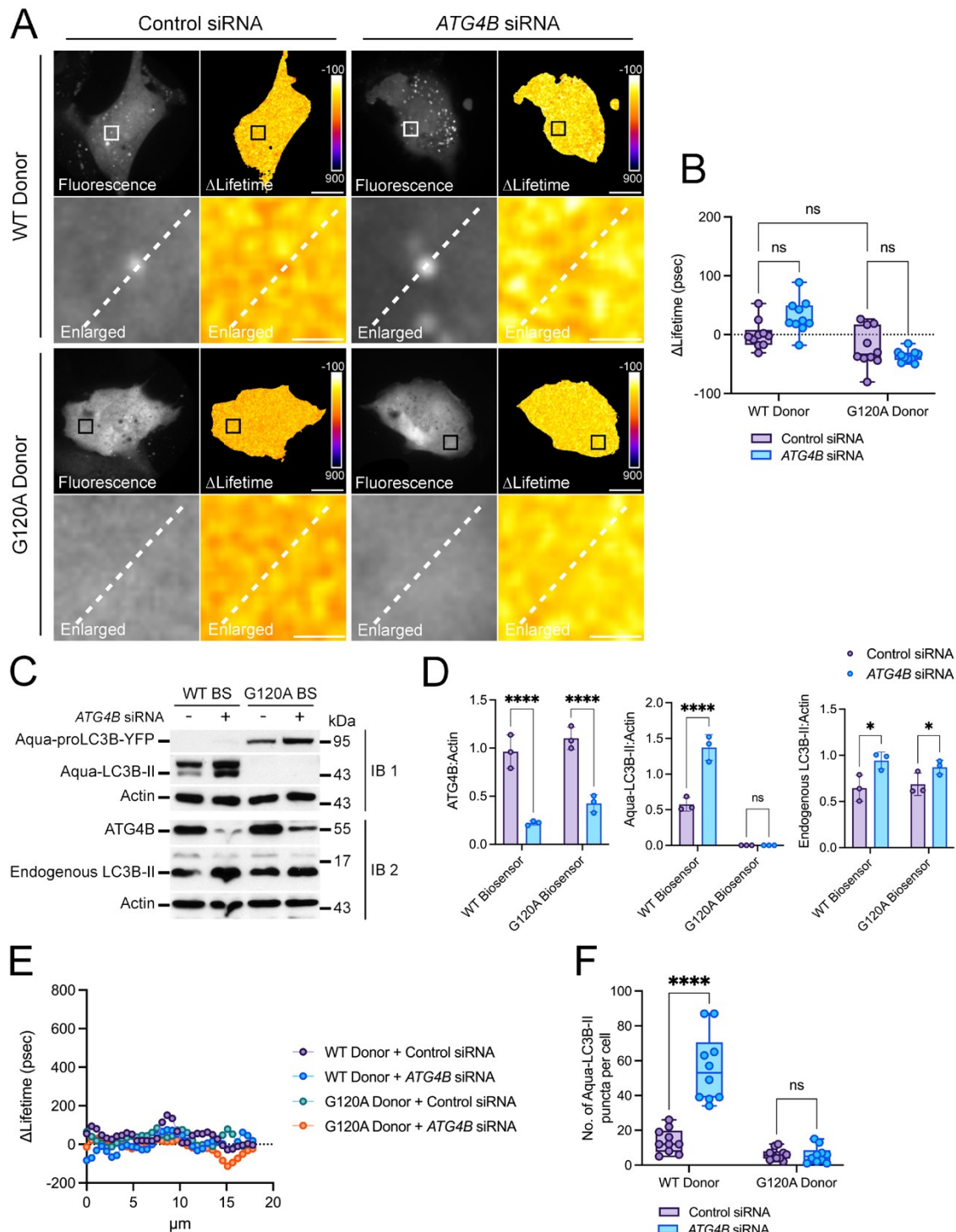
9 lysosomal inhibition, it does not colocalize with LAMP2, and the expression of the WT

10 biosensor does not perturb the abundance of the endogenous LC3B protein. (A) Representative

11 fluorescence images of U2OS expressing the G120A biosensor and stained for endogenous

12 LAMP2. To investigate the changes in Aqua-LC3B puncta numbers and their colocalization
13 with LAMP2, cells were treated with: DMSO (6 h), BafA₁ (6 h, 100 nM), torin1 (3 h, 250 nM),
14 torin1 (3 h, 250 nM) + BafA₁ (6 h, 100 nM), HBSS (1 h), HBSS (1 h) + BafA₁ (6 h, 100 nM).
15 Scale bar: 9 μm. **(B)** Representative western blotting images and corresponding quantifications
16 **(C)** of total lysates from U2OS cells expressing the empty vector or the WT LC3B biosensor
17 and treated as indicated. Loading control: Actin. *n* = 3 independent experiments. ns (not
18 significant) as determined by two-way ANOVA with two-stage step-up method of Benjamini,
19 Krieger and Yekutieli's multiple comparison test to control the false discovery rate in **(C)**.

Figure S2

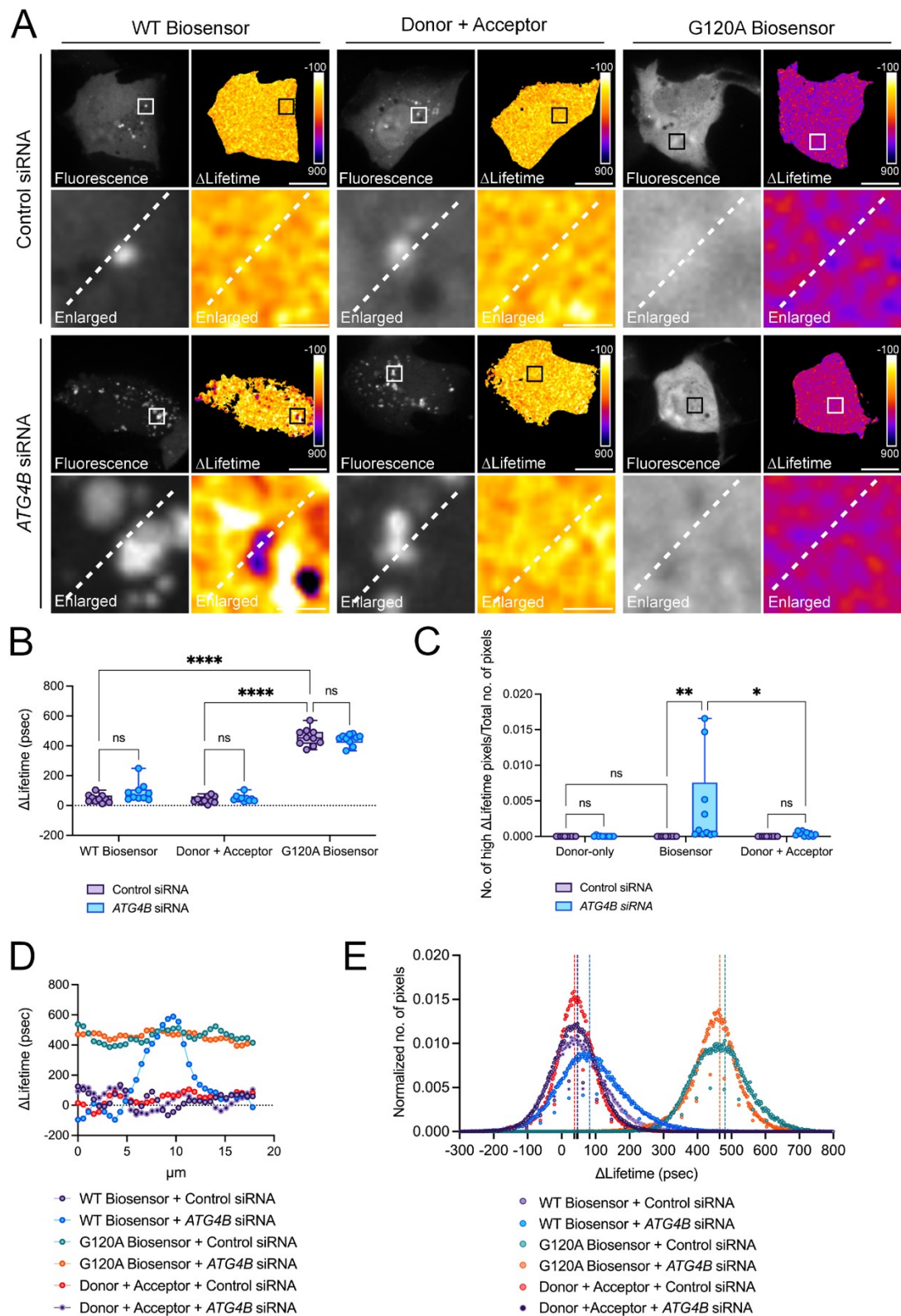


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21 **Figure S2.** The WT or G120A donors do not respond to *ATG4B* silencing. (A) Representative
 22 fluorescence and Δ Lifetime images of U2OS cells co-expressing the WT or G120A donor with
 23 control or *ATG4B*-specific siRNAs, and analyzed by FRET-FLIM. Squares on the top images
 24 of WT or G120A donor panels illustrate the location of the enlarged images. Dotted lines on

25 the enlarged images illustrate where the line analysis was performed. Pseudocolor scale: pixel-
26 by-pixel Δ Lifetime. Scale bars: overviews, 40 μ m; enlarged, 6 μ m. **(B)** Mean Δ Lifetime
27 analysis of U2OS cells co-expressing the WT or G120A donor with control or *ATG4B* siRNA.
28 Representative western blotting images **(C)** and corresponding quantifications **(D)** of total
29 lysates from U2OS cells co-expressing the WT or G120A biosensor with control or *ATG4B*-
30 specific siRNAs. IB1 and IB2 correspond to the same lysates blotted for overexpressed (IB1)
31 or endogenous (IB2) LC3B forms. Loading control: Actin. $n = 3$ independent experiments. **(E)**
32 Line and **(F)** number of Aqua-LC3B-II puncta analyses of U2OS cells co-expressing the WT
33 or G120A donor with control or *ATG4B* siRNA. $n = 10$ cells per condition from one
34 representative experiment (of three) in **(B)** and **(F)**. $*P < 0.05$, $****P < 0.0001$, ns (not
35 significant) as determined by two-way ANOVA with Tukey's multiple comparison test in **(B)**
36 and **(F)**, and with two-stage step-up method of Benjamini, Krieger and Yekutieli's multiple
37 comparison test to control the false discovery rate in **(D)**.

Figure S3



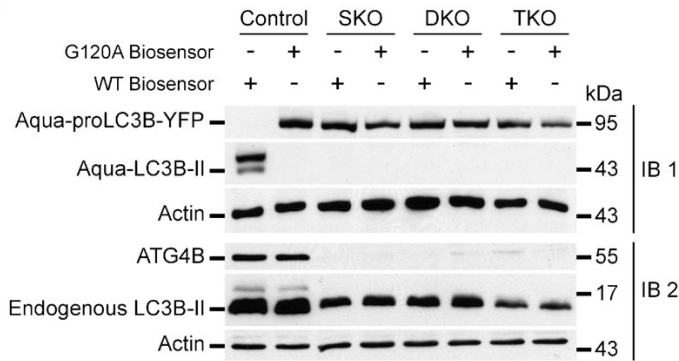
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39 **Figure S3.** No FRET is detected when donor-bound LC3B and acceptor-bound LC3B are co-
 40 expressed. (A) Representative fluorescence and Δ Lifetime images of U2OS cells co-expressing
 41 the WT or G120A biosensor, or donor (Aquamarine-proLC3B) + acceptor (proLC3B-

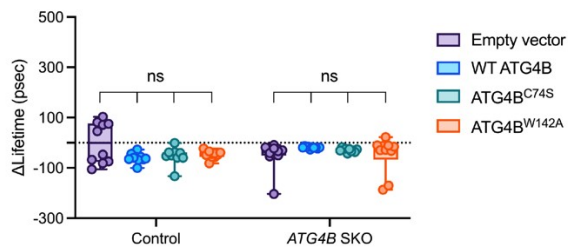
42 tdLanYFP) constructs with control or *ATG4B*-specific siRNAs, and analyzed by FRET-FLIM.
43 Squares on the top images of WT or G120A biosensor, or donor + acceptor panels illustrate
44 the location of the enlarged images. Dotted lines on the enlarged images illustrate where the
45 line analysis was performed. Pseudocolor scale: pixel-by-pixel Δ Lifetime. Scale bars:
46 overviews, 40 μ m; enlarged, 6 μ m. Mean Δ Lifetime (**B**), number of high Δ Lifetime pixels (**C**),
47 line (**D**), and histogram (**E**) analyses of U2OS cells co-expressing the WT or G120A biosensor,
48 or donor + acceptor constructs with control or *ATG4B*-specific siRNAs in (**B**), (**D**), and (**E**),
49 and the WT donor, biosensor or donor + acceptor constructs with control or *ATG4B* siRNA in
50 (**C**). Vertical dotted lines on each histogram depict the mode value in (**E**). $n = 10$ cells per
51 condition from one representative experiment (of three) in (**B**), (**C**), and (**E**). $*P < 0.05$, $**P <$
52 0.01 , $****P < 0.0001$, ns (not significant) as determined by two-way ANOVA with Tukey's
53 multiple comparison test in (**B**) and (**C**).

Figure S4

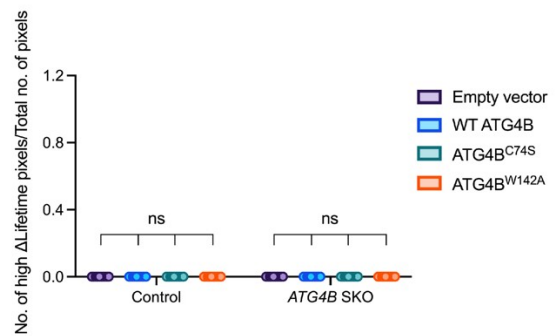
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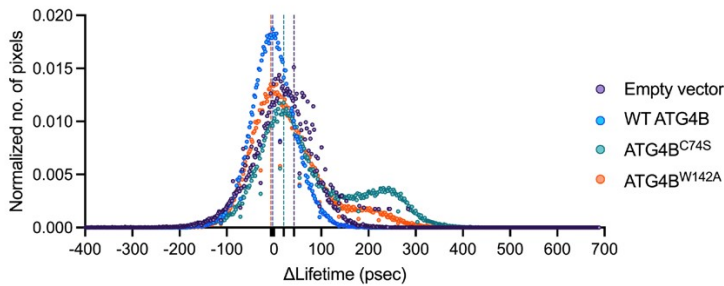
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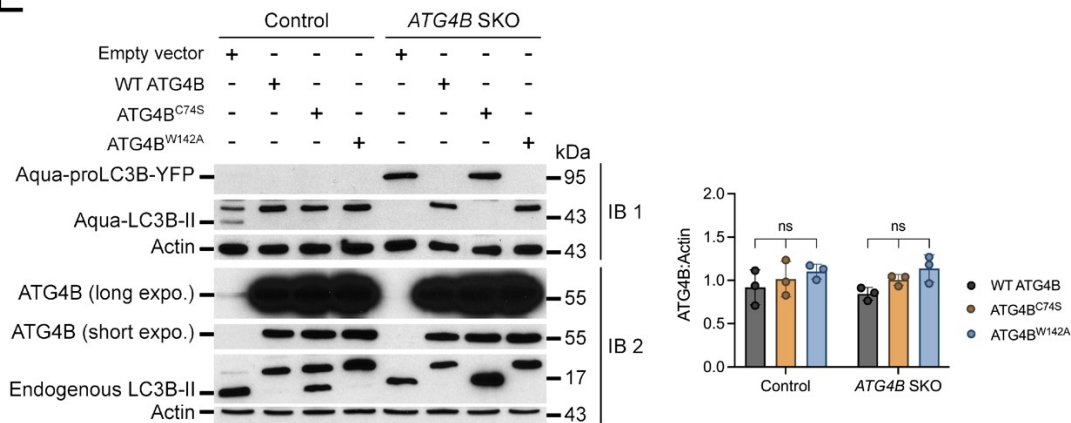
C



D



E



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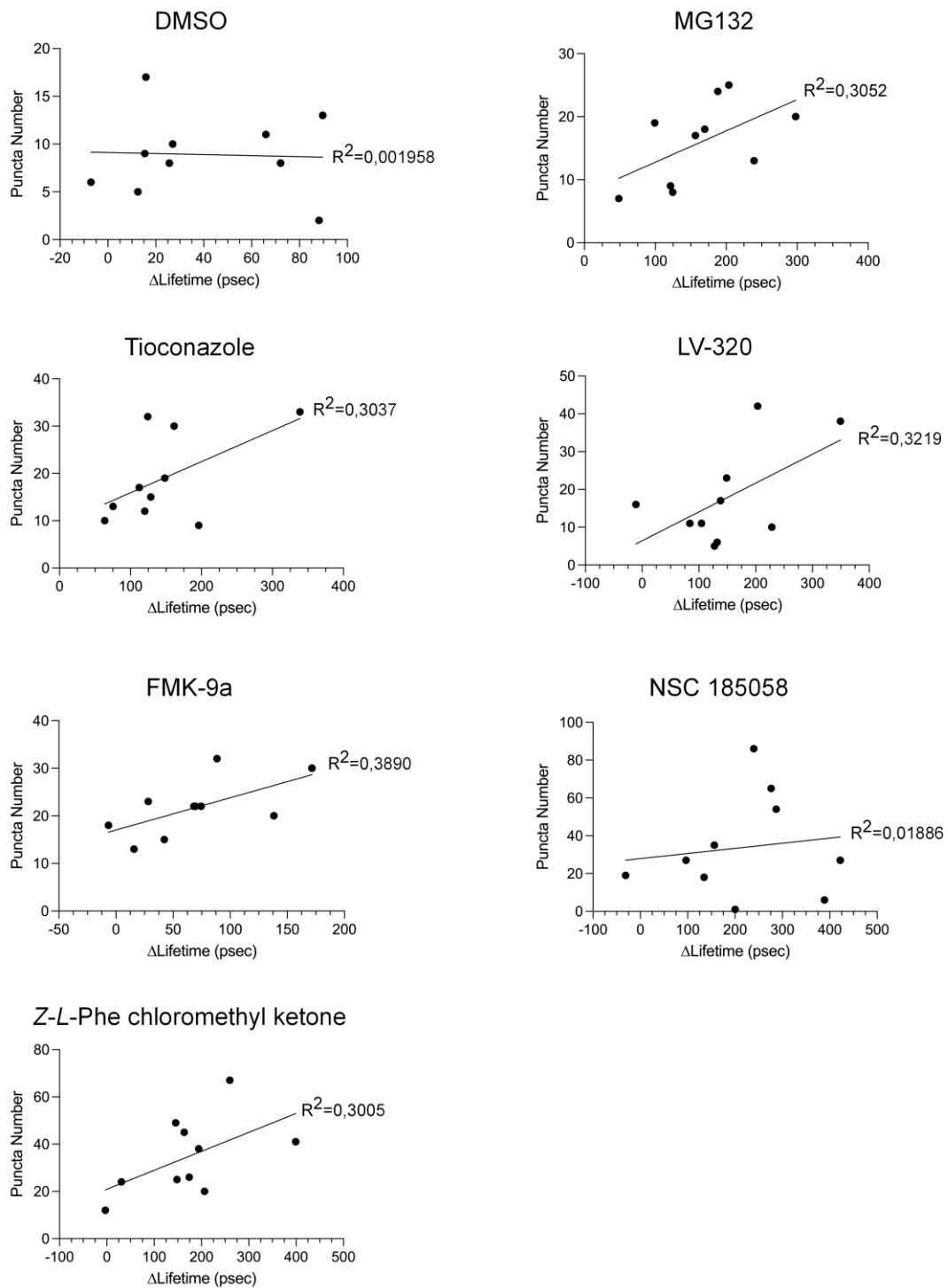
55 **Figure S4.** The LC3B biosensor and endogenous LC3B proteins cannot be primed in cells

56 lacking ATG4B and the ectopic expression of active ATG4B does not alter LC3B donor-only

57 Δlifetime variations in *ATG4B* SKO cells. (A) Representative western blotting images of total

58 lysates from HeLa control, *ATG4B* SKO, *ATG4A ATG4B* DKO, *ATG4A ATG4B ATG4C* TKO
59 cells expressing the WT or G120A biosensor. IB1 and IB2 correspond to the same lysates
60 blotted for overexpressed (IB1) or endogenous (IB2) LC3B forms. Loading control: Actin. $n =$
61 3 independent experiments. Mean Δ Lifetime (**B**) and number of high Δ Lifetime pixels (**C**)
62 analyses of control and *ATG4B* SKO HeLa cells co-expressing the WT donor with an empty
63 vector, or with vectors expressing WT ATG4B, ATG4B^{C74S} or ATG4B^{W142A}. (**D**) Histogram
64 analysis of HeLa control cells co-expressing the WT biosensor with an empty vector, or with
65 vectors expressing WT ATG4B, ATG4B^{C74S} or ATG4B^{W142A}. Vertical dotted lines on each
66 histogram depict the mode value. $n = 10$ cells per condition from one representative experiment
67 (of three) in (**B**), (**C**) and (**D**). (**E**) Representative western blotting images and corresponding
68 ATG4B:Actin quantification of total lysates from HeLa control and *ATG4B* SKO cells co-
69 expressing the WT biosensor with an empty vector, or with vectors expressing WT ATG4B,
70 ATG4B^{C74S} or ATG4B^{W142A}. IB1 and IB2 correspond to the same lysates blotted for
71 overexpressed (IB1) or endogenous (IB2) LC3B forms. Short and long ATG4B exposures are
72 shown. Loading control: Actin. $n = 3$ independent experiments. ns (not significant) as
73 determined by two-way ANOVA with Tukey's multiple comparison test in (**B**) and (**C**), and
74 with two-stage step-up method of Benjamini, Krieger and Yekutieli's multiple comparison test
75 to control the false discovery rate in (**E**).

Figure S5



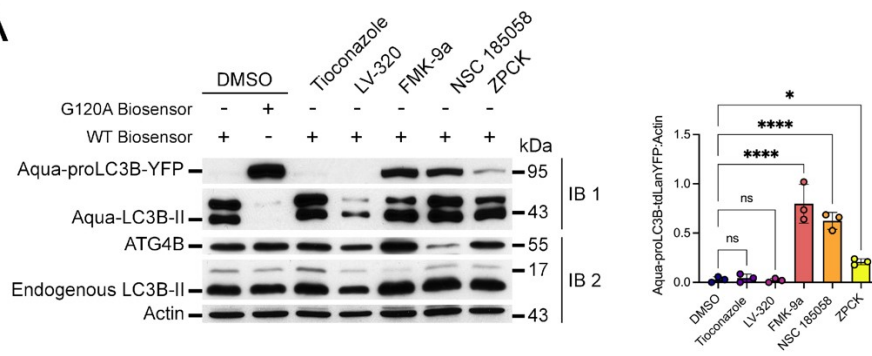
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77 **Figure S5.** The number of Aqua-LC3B-II puncta and the mean Δ Lifetime values do not
78 correlate. Correlation analyses of Aqua-LC3B-II puncta and the mean Δ Lifetime values of
79 HeLa cells expressing the WT biosensor and treated with DMSO (6 h), MG132 (6 h, 1 μ M),
80 tioconazole (6 h, 4 μ M), LV-320 (6 h, 120 μ M), FMK-9a (6 h, 10 μ M), NSC 185058 (6 h, 100

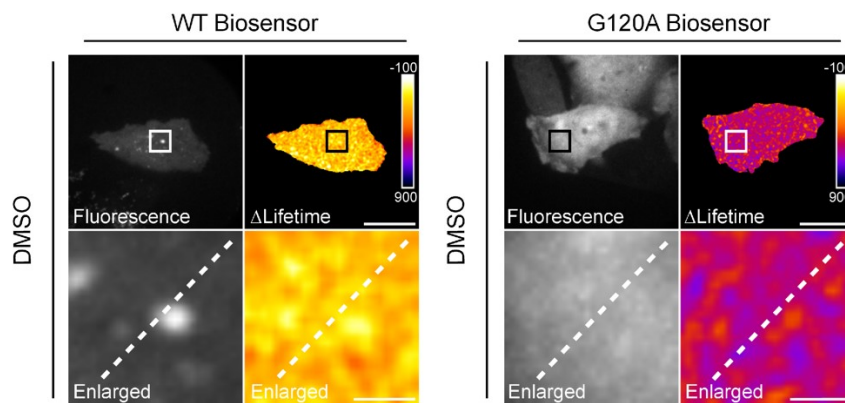
81 μM), or *Z-L*-Phe chloromethyl ketone (6 h, 3 μM). $n = 10$ cells per condition from one
82 representative experiment (of three).

Figure S6

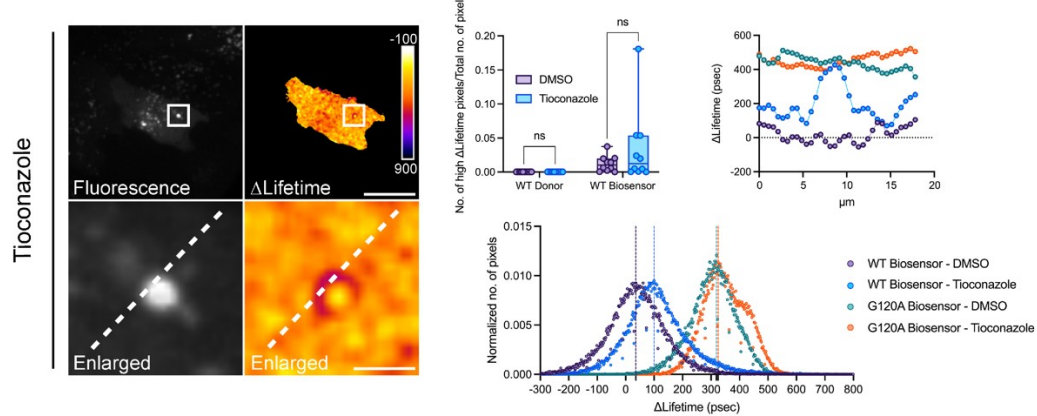
A



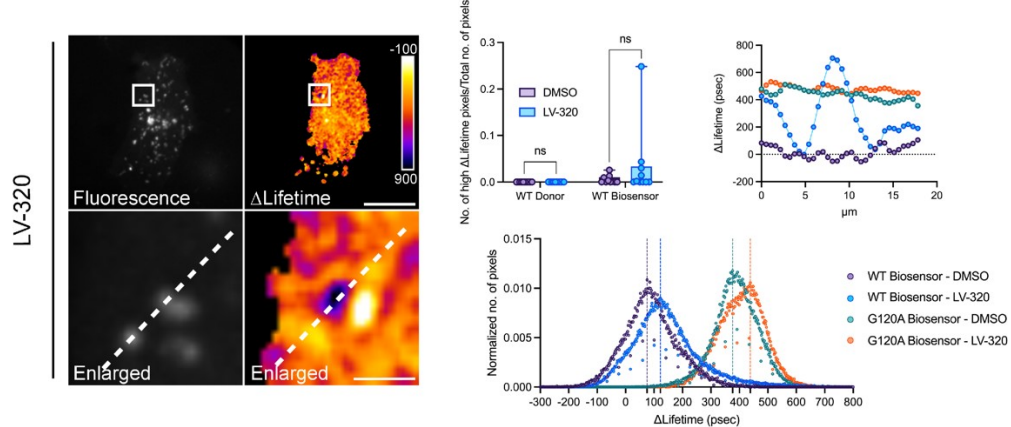
B



C



D

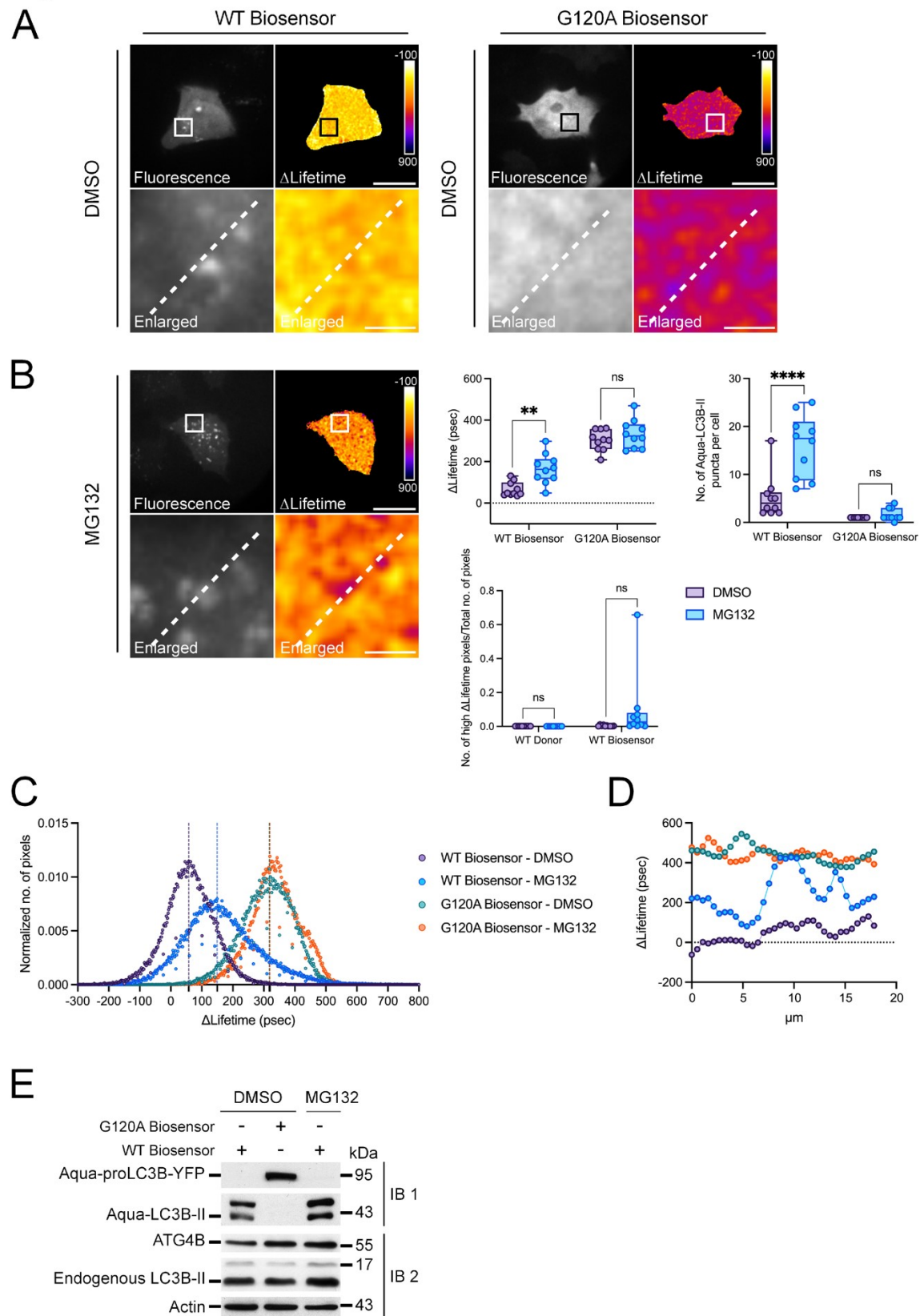


83

84 **Figure S6.** Treatment with FMK-9a, NSC and ZPCk results in the accumulation of the
 85 unprimed biosensor and FRET-FLIM data analyzed by using sensitive methods reveal the

86 mode of action of tioconazole and LV-320. **(A)** Representative western blotting and
87 corresponding Aqua-proLC3B-tdLanYFP:Actin quantification of total lysates of HeLa cells
88 expressing the WT or G120A biosensor and treated with DMSO (6 h), or expressing the WT
89 biosensor and treated with tioconazole (6 h, 4 μ M), LV-320 (6 h, 120 μ M), FMK-9a (6 h, 10
90 μ M), NSC 185058 (6 h, 100 μ M), or *Z-L-Phe* chloromethyl ketone (6 h, 3 μ M). IB1 and IB2
91 correspond to the same lysates blotted for overexpressed (IB1) or endogenous (IB2) LC3B
92 forms. Aqua-proLC3B-tdLanYFP:Actin quantification is performed by normalizing the
93 density of Aqua-proLC3B-tdLanYFP band in IB1 to that of the respective loading control in
94 IB2. Loading control: Actin. $n = 3$ independent experiments. $*P < 0.05$, $****P < 0.0001$, ns
95 (not significant) as determined by one-way ANOVA with two-stage step-up method of
96 Benjamini, Krieger and Yekutieli's multiple comparison test to control the false discovery rate.
97 **(B)** Representative fluorescence and Δ Lifetime images of HeLa cells expressing the WT or
98 G120A biosensor, treated with DMSO (6 h), and analyzed by FRET-FLIM. Representative
99 fluorescence and Δ Lifetime images of HeLa cells expressing the WT biosensor and treated
100 with the following compounds: tioconazole (6 h, 4 μ M) **(C)** and LV-320 (6 h, 120 μ M) **(D)**.
101 Squares on the top images of WT or G120A biosensor panels illustrate the location of the
102 enlarged images. Dotted lines on the enlarged images illustrate where the line analysis was
103 performed. Pseudocolor scale: pixel-by-pixel Δ Lifetime. Scale bars: overviews, 40 μ m;
104 enlarged, 6 μ m. Number of high Δ Lifetime pixels analysis of HeLa cells expressing the WT
105 donor or biosensor and treated with tioconazole (6 h, 4 μ M) **(C)** or LV-320 (6 h, 120 μ M) **(D)**.
106 Line, and histogram analyses of HeLa cells expressing the WT or G120A biosensor and treated
107 with tioconazole (6 h, 4 μ M) **(C)** or LV-320 (6 h, 120 μ M) **(D)**. $n = 10$ cells per condition from
108 one representative experiment (of three) in **(C, D)**. ns (not significant) as determined by two-
109 way ANOVA with Tukey's multiple comparison test in **(C, D)**.

Figure S7



110

111 **Figure S7.** The LC3B biosensor reveals the mode of action of MG132 in cells. (**A and B**)

112 Representative fluorescence and Δ Lifetime images of HeLa cells expressing the WT or G120A

113 biosensor, treated with DMSO (6 h) or MG132 (6 h, 1 μ M), and analyzed by FRET-FLIM.
114 Pseudocolor scale: pixel-by-pixel Δ Lifetime. Scale bars: overviews, 40 μ m; enlarged, 6 μ m.
115 Mean Δ Lifetime (**B**), number of Aqua-LC3B-II puncta (**B**), histogram (**C**), and line (**D**)
116 analyses of HeLa cells expressing the WT or G120A biosensor and treated with DMSO (6 h)
117 or MG132 (6 h, 1 μ M). Number of high Δ Lifetime pixels analysis (**B**) of HeLa cells expressing
118 the WT donor or biosensor and treated with DMSO (6 h) or MG132 (6 h, 1 μ M). Vertical
119 dotted lines on each histogram depict the mode value in (**C**). $n = 10$ cells per condition from
120 one representative experiment (of three) in (**B**) and (**C**). (**E**) Representative western blotting of
121 total lysates of HeLa cells expressing the WT or G120A biosensor and treated with DMSO (6
122 h), or expressing the WT biosensor and treated with MG132 (6 h, 1 μ M). IB1 and IB2
123 correspond to the same lysates blotted for overexpressed (IB1) or endogenous (IB2) LC3B
124 forms. Loading control: Actin. $n = 3$ independent experiments. $**P < 0.01$, $****P < 0.0001$,
125 ns (not significant) as determined by two-way ANOVA with Tukey's multiple comparison test
126 in (**B**).
127