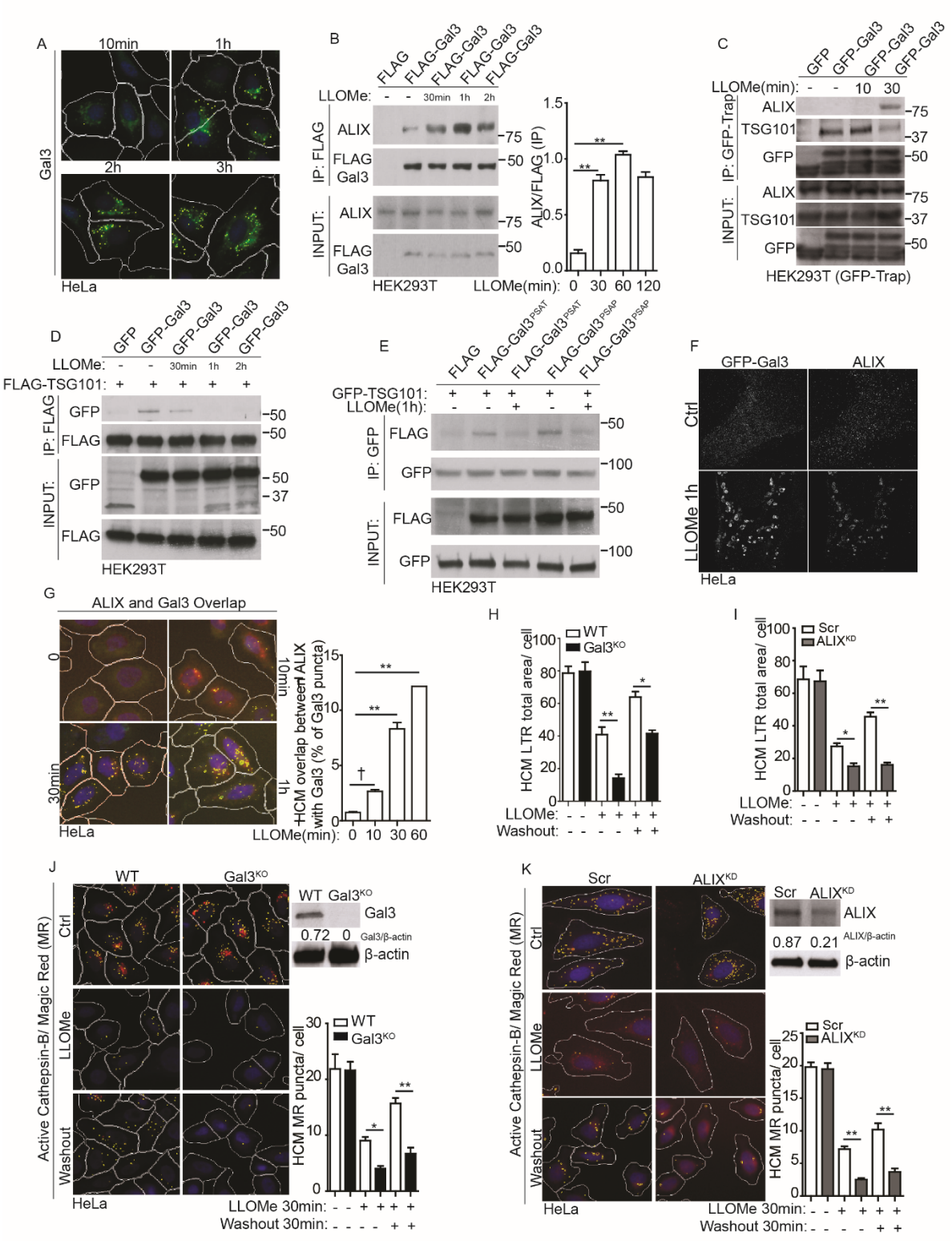


Figure S1, related to Figure 1. Gal3 and ALIX interact and counteract damage to lysosomes.

(A) Examples of HCM images used for time-response HCM quantification of Gal3 during lysosomal damage in Fig. 1A. **(B)** Co-immunoprecipitation analysis and

quantification of changes in interactions between Gal3 and ALIX during lysosomal damage. HEK293T cells transiently expressing FLAG-Gal3 were treated with 1mM LLOMe for the indicated time. Cell lysates were immunoprecipitated (IP) with anti-FLAG antibody, immunoblotted for endogenous ALIX, intensities quantified and normalized. **(C)** Co-immunoprecipitation analysis of changes in interactions between Gal3 and ALIX or TSG101 during lysosomal damage. HEK293T cells transiently expressing GFP-Gal3 were treated with 1mM LLOMe for the indicated time. Cell lysates were immunoprecipitated (IP) with GFP-Trap beads and immunoblotted for endogenous ALIX or TSG101. **(D)** Co-immunoprecipitation analysis of changes in interactions between Gal3 and TSG101 during lysosomal damage. HEK293T cells transiently expressing GFP-Gal3 and FLAG-TSG101 were treated with 1mM LLOMe for the indicated time. Cell lysates were immunoprecipitated (IP) with anti-FLAG antibody and immunoblotted for GFP-Gal3. **(E)** Co-immunoprecipitation analysis of interactions between Gal3 variants and TSG101 during lysosomal damage. HEK293T cells transiently expressing FLAG tagged variants of Gal3 and GFP-TSG101 were treated with 1mM LLOMe for 1h. Cell lysates were immunoprecipitated (IP) with anti-GFP antibody and immunoblotted for FLAG tagged variants of Gal3. **(F)** Unmerged (separate channels) images of super-resolution analysis in Fig. 1D. **(G)** Quantification by HCM of overlaps between Gal3 and ALIX. HeLa cells were treated with 1mM LLOMe for the indicated time and subjected to HCM analysis of overlaps between Gal3 and ALIX. White masks, computer algorithm defined cell boundaries (primary objects); Yellow masks, computer-identified overlap of ALIX and Gal3. **(H, I)** HCM analysis for the total area of LTR puncta as shown in Fig. 1F and Fig. 1G. **(J)** Magic Red (MR) analysis of the status of acidified organelles in parental HeLa (WT) and Gal3-knockout HeLa cells ($Gal3^{KO}$) during lysosomal damage. Cells were treated with 1mM LLOMe for 30 min followed by 30 min washout, and MR^+ puncta quantified by HCM. Untreated cells were used as control (Ctrl). White masks, algorithm defined cell boundaries; Yellow masks, computer-identified MR^+ puncta. **(K)** Analysis of the status of acidified organelles in ALIX knockdown ($ALIX^{KD}$) cells by MR during lysosomal damage. Cells transfected with scrambled siRNA as control (Scr) or ALIX siRNA were treated with 1mM LLOMe for 30 min followed by 30 min washout, and then quantified for MR puncta using HCM. White masks, algorithm-defined cell boundaries; Yellow masks, computer-identified MR^+ puncta.

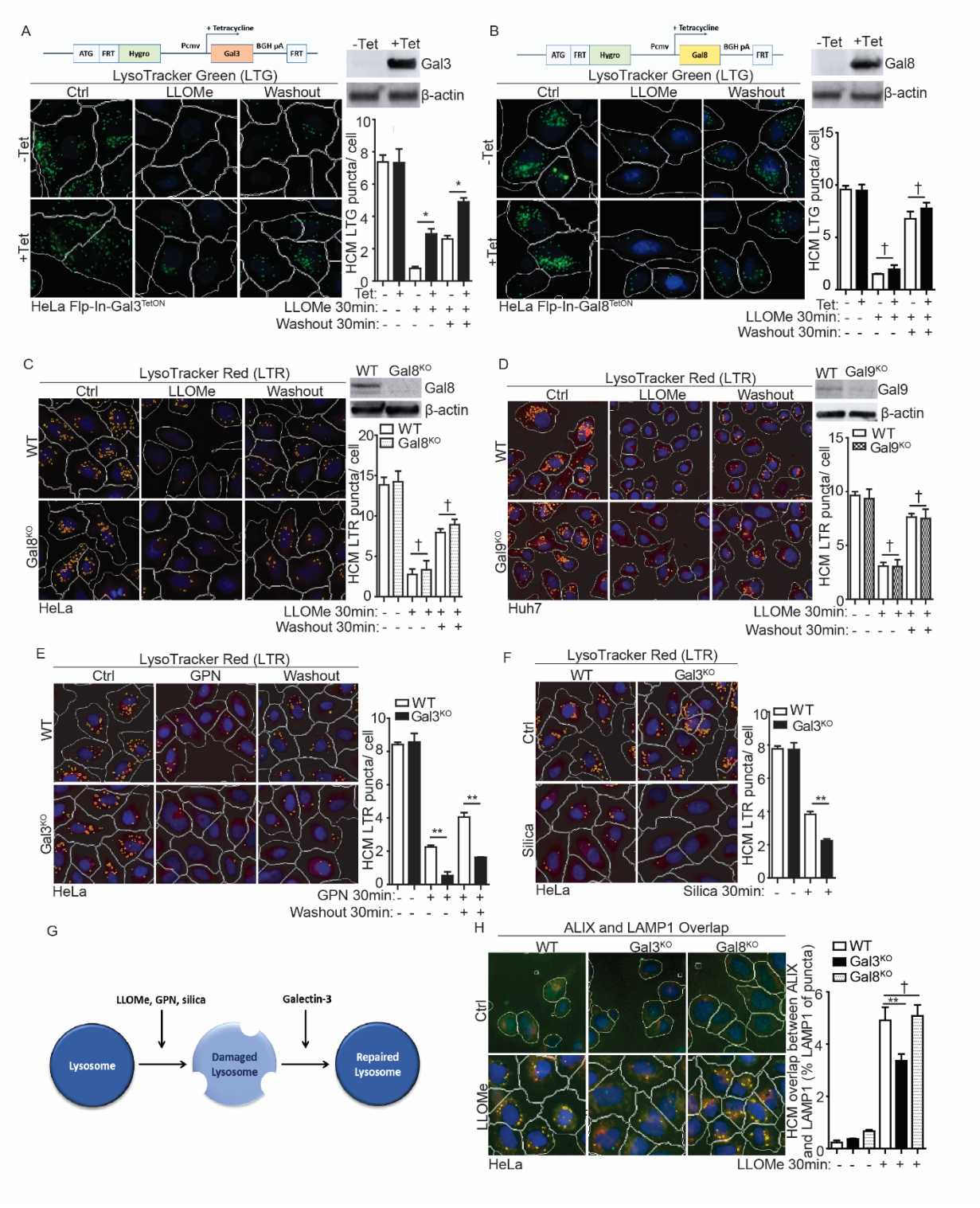
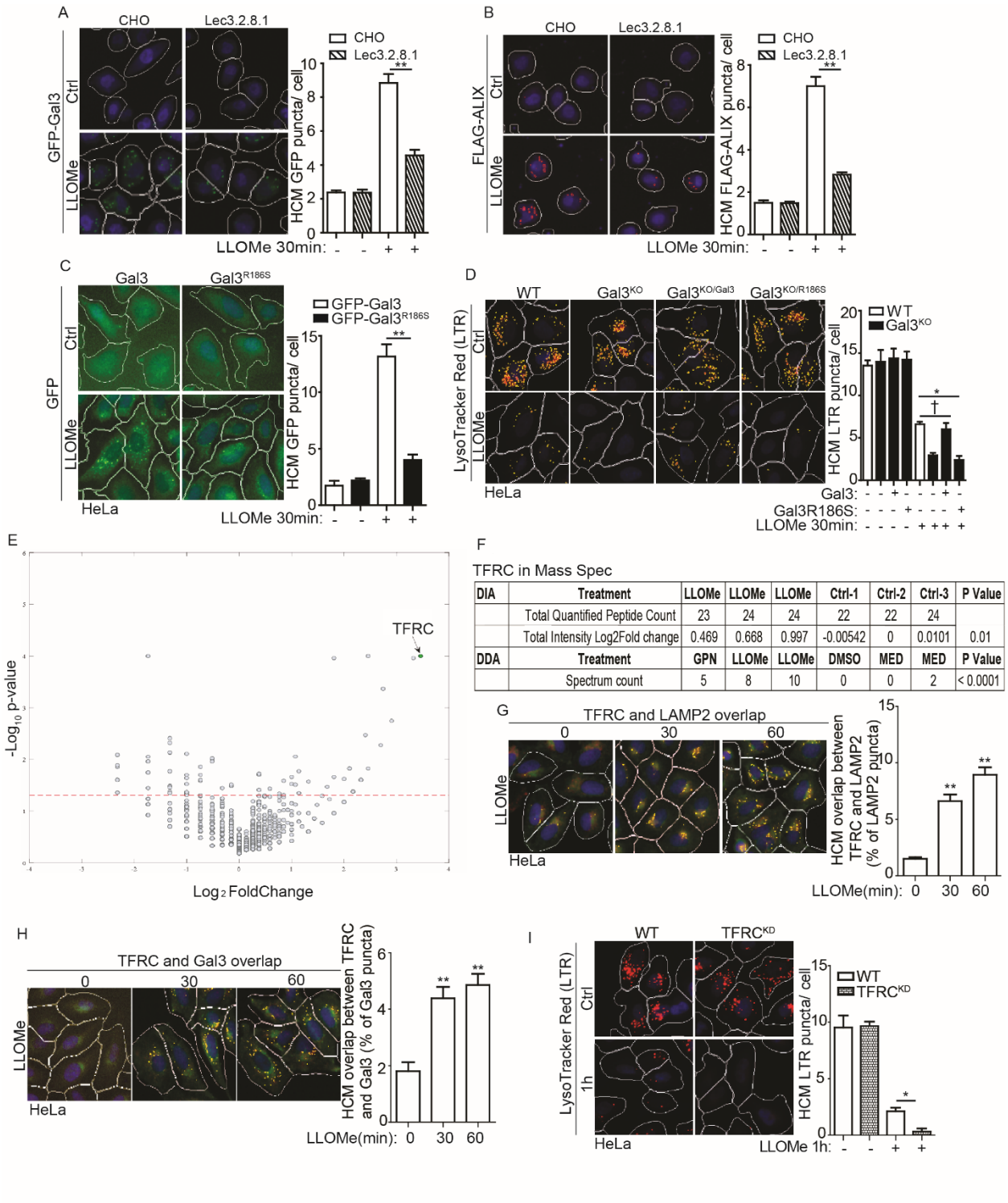


Figure S2, related to Figures 1 and 2. Gal3 protects against lysosomal damage and recruits ALIX to damaged lysosomes.

(A, B) Analysis of the status of acidified organelles by LysoTracker Green (LTG) in Gal3 or Gal8 FLIP-IN stable cell lines during lysosomal damage. HeLa FLIP-IN cells stably

expressing Gal3 or Gal8 induced by tetracycline (Tet), were treated with 1mM LLOMe for 30 min followed by 30 min washout, and LTG^+ puncta quantified by HCM. Ctrl, control untreated (no LLOMe) cells. White masks, algorithm-defined cell boundaries (primary objects); Green masks, computer-identified LTG^+ puncta (target objects). **(C)** Analysis of the status of acidified organelles in parental HeLa (WT) and Gal8-knockout HeLa cells ($Gal8^{KO}$) by LysoTracker (LTR) staining during lysosomal damage. Untreated cells were used as control (Ctrl). White masks, algorithm defined cell boundaries; Yellow masks, computer-identified LTR puncta. **(D)** Analysis of the status of acidified organelles in parental Huh7 (WT) and Gal9-knockout Huh7 cells ($Gal9^{KO}$) by LTR staining and HCM during lysosomal damage. Ctrl, control untreated (no LLOMe) cells. White masks, algorithm-defined cell boundaries; Yellow masks, computer-identified LTR^+ puncta. **(E)** Analysis of the status of acidified organelles in parental HeLa (WT) and Gal3-knockout HeLa cells ($Gal3^{KO}$) by LTR during glycyl-L-phenylalanine 2-naphthylamide (GPN) treatment. Cells were treated with 200 µM GPN for 30 min followed by 30 min washout, and LTR^+ puncta quantified by HCM. White masks, algorithm defined cell boundaries; Yellow masks, computer-identified LysoTracker Red puncta (target objects). **(F)** Analysis of the status of acidified organelles in parental HeLa (WT) and Gal3-knockout HeLa cells ($Gal3^{KO}$) by LTR during silica treatment. Cells were treated with 400 µg/mL silica for 30 min, and LTR^+ puncta quantified by HCM. White masks, algorithm-defined cell boundaries; Yellow masks, computer-identified LysoTracker Red puncta. **(G)** Schematic summary of the findings in Figure S2A-F. **(H)** Quantification by HCM of overlaps between ALIX and LAMP1 in parental HeLa (WT), Gal3-knockout HeLa cells ($Gal3^{KO}$) and Gal8-knockout HeLa cells ($Gal8^{KO}$) during lysosomal damage. Cells were treated with 1mM LLOMe for 30 min, and HCM analysis of overlaps between ALIX and LAMP1 carried out. White masks, algorithm-defined cell boundaries; Yellow masks, computer-identified overlap of ALIX and LAMP1. Data, means ± SEM; HCM: $n \geq 3$ (each experiment: 500 valid primary objects/cells per well, ≥ 5 wells/sample). † $p \geq 0.05$ (not significant), *$p < 0.05$, **$p < 0.01$, ANOVA.



DIA	Treatment	LLOMe	LLOMe	LLOMe	Ctrl-1	Ctrl-2	Ctrl-3	P Value
	Total Quantified Peptide Count	23	24	24	22	22	24	
	Total Intensity Log2Fold change	0.469	0.668	0.997	-0.00542	0	0.0101	0.01
DDA	**Treatment**	**GPN**	**LLOMe**	**LLOMe**	**DMSO**	**MED**	**MED**	**P Value**
	Spectrum count	5	8	10	0	0	2	< 0.0001

Figure S3, related to Figure 3. Glycosylation is important for Gal3 recognition of lysosomal damage.

(A, B) Response of Gal3 or ALIX in wild-type CHO cells and mutant Lec3.2.8.1 during lysosomal damage. Cells transfected with GFP-Gal3 (A) and FLAG-ALIX (B) were treated with 1mM LLOMe for 30min, and responses of Gal3(A) and ALIX (B) by their

puncta formation were quantified by HCM. Ctrl, untreated control cells. White masks, algorithm-defined cell boundaries; Green or red masks, computer-identified Gal3 or ALIX puncta, respectively. **(C)** Response of Gal3 and its mutant $Gal3^{R186S}$ during lysosomal damage. HeLa cells transfected with GFP-tagged WT Gal3 or $Gal3^{R186S}$ were treated with 1 mM LLOMe for 30min. White masks, algorithm-defined cell boundaries; Green masks, computer-identified GFP puncta. **(D)** Analysis of the status of acidified organelles by Gal3 and its mutant $Gal3^{R186S}$. Gal3-knockout HeLa cells ($Gal3^{KO}$) transfected with Gal3 or $Gal3^{R186S}$ were treated with 1 mM LLOMe for 30min. LTR (LysoTracker Red) puncta were quantified by HCM. White masks, algorithm-defined cell boundaries; Yellow masks, computer-identified LTR^{+} puncta. **(E)** Proteomic analysis of Gal3 binding partners by proximity-biotinylation using APEX2-Gal3 during lysosomal damage with 200 μM glycyl-L-phenylalanine 2-naphthylamide (GPN) for 1h or with 1mM LLOMe for 1h. Scatter (volcano) plot shows fold change (log2 FoldChange) and –log10 p-value for the proteins quantified (spectral counts; LC/MS/MS) in three independently replicated experiments with lysosomal damage (see Table S1, Tab 4). **(F)** Summary of APEX2-Gal3 LC/MS/MS proteomic DIA or DDA analysis (see STAR methods, and Table S1, Tabs 1, 2 and 4). **(G, H)** Quantification by HCM of overlaps between TFRC and LAMP2 or Gal3. HeLa cells were treated with 1 mM LLOMe for the indicated time, and HCM analysis carried out for overlaps between TFRC and LAMP2 or Gal3. White masks, algorithm-defined cell boundaries; Yellow masks, computer-identified overlap of TFRC with LAMP2 or Gal3 puncta. **(I)** Analysis of the status of acidified organelles in parental HeLa (WT) and TFRC-knockdown HeLa cells ($TFRC^{KD}$) by LTR staining during lysosomal damage. Cells were treated with 1mM LLOMe for 1h and LTR^{+} puncta quantified by HCM. White masks, algorithm defined cell boundaries; Yellow masks, computer-identified LTR puncta. Data, means ± SEM; HCM: $n \geq 3$ (each experiment: 500 valid primary objects/cells per well, ≥ 5 wells/sample). † $p \geq 0.05$ (not significant), *$p < 0.05$, **$p < 0.01$, ANOVA.

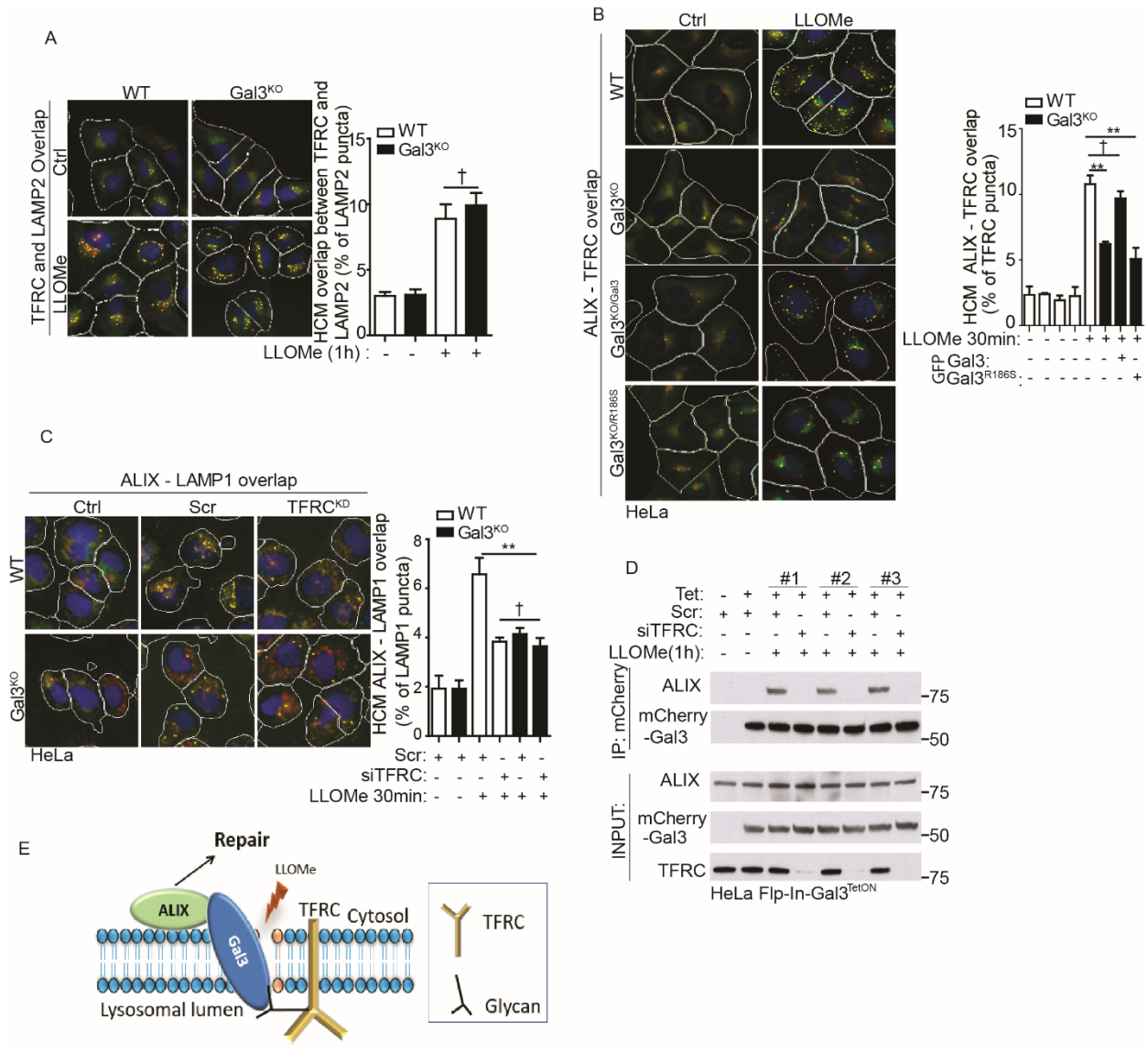


Figure S4, related to Figure 3. TFRC plays a role in Gal3 recognition of lysosomal damage.

(A) Analysis of overlaps between TFRC and LAMP2 in parental HeLa (WT) and Gal3-knockout HeLa cells (Gal3^{KO}). Cells were treated with 1 mM LLOMe for 1h and overlaps between endogenous TFRC and LAMP2 quantified by HCM. Untreated cells (no LLOMe) were used as control (Ctrl). White masks, algorithm-defined cell boundaries (primary objects); Yellow masks, computer-identified overlap of TFRC and LAMP2 puncta. **(B)** Quantification by HCM of overlaps between ALIX and TFRC affected by Gal3 and its mutant Gal3^{R186S}. Gal3-knockout HeLa cells (Gal3^{KO}) transfected with GFP-tagged Gal3 or Gal3^{R186S} were treated with 1 mM LLOMe for 30 min. HCM analysis of overlaps between ALIX and TFRC. White masks, algorithm-defined cell boundaries; Yellow masks, computer-identified overlap of ALIX and TFRC. **(C)** Quantification by HCM of overlaps between ALIX and LAMP1 in parental HeLa (WT), Gal3-knockout HeLa cells (Gal3^{KO}) and TFRC-knockdown HeLa cells (TFRC^{KD}) during

LLOMe treatment. Cells were treated with 1 mM LLOMe for 30 min, and HCM analysis of overlaps between ALIX and LAMP1 carried out. Scr, cells transfected with scrambled siRNA as control. White masks, algorithm-defined cell boundaries; Yellow masks, computer-identified overlap of ALIX and LAMP1. **(D)** Co-immunoprecipitation analysis of changes in interactions between Gal3 and ALIX in TFRC-knockdown HeLa cells ($TFRC^{KD}$) during LLOMe treatment. HeLa Flp-In-$Gal3^{TetON}$ cells stably expressing Gal3 (induced when necessary by tetracycline; Tet) were subject to TFRC knockdown and then treated with 1mM LLOMe for 1 h. Cell lysates were immunoprecipitated (IP) with anti-mCherry antibody and immunoblotted for endogenous ALIX. **(E)** Schematic summary of the findings in Figures 3, S3 and S4.

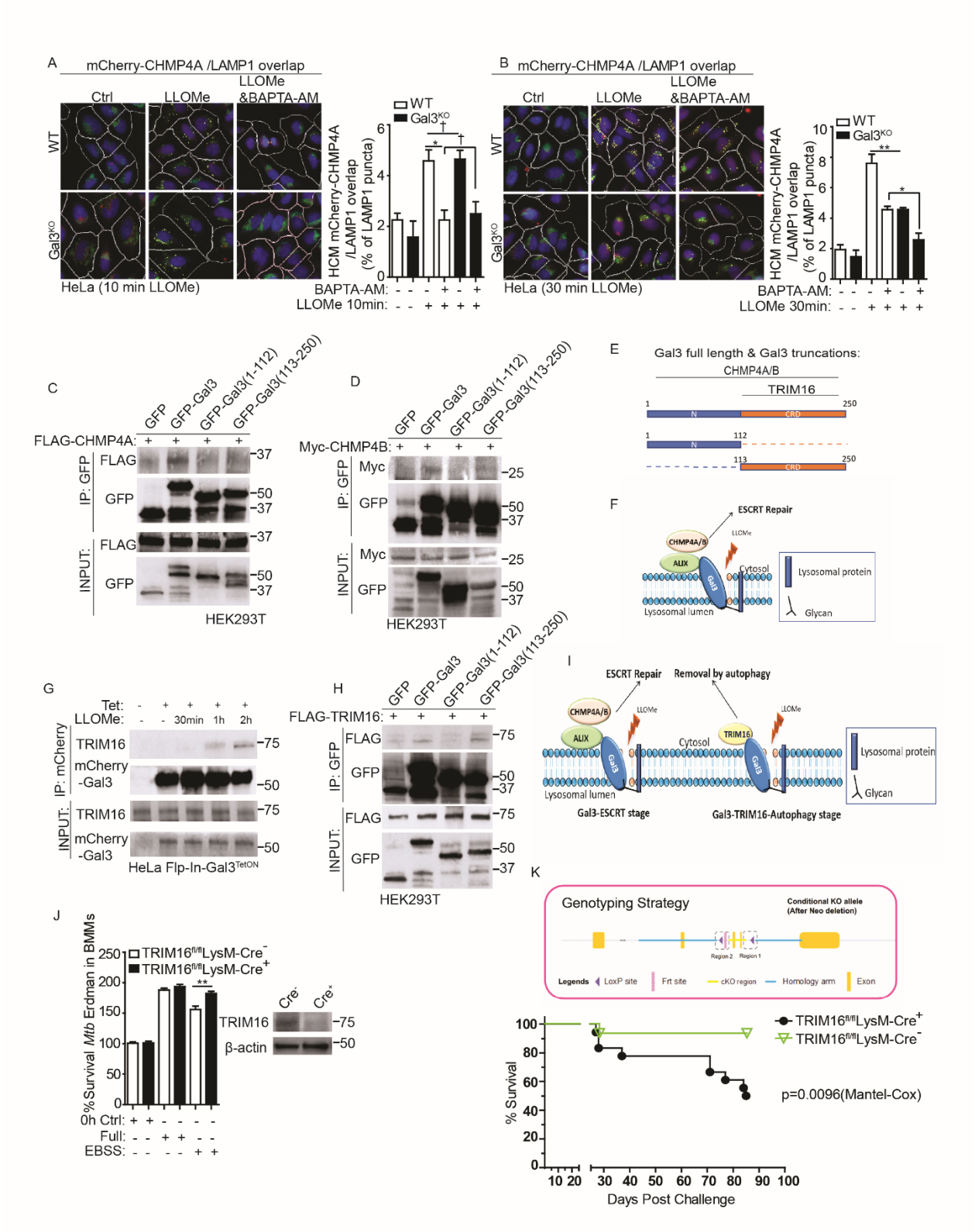


Figure S5, related to Figures 5 and 6. Gal3 is important for autophagy response during lysosomal damage.

(A) Quantification by HCM of overlaps between mCherry-CHMP4A and endogenous LAMP1 in parental HeLa (WT) and Gal3-knockout HeLa cells ($Gal3^{KO}$) treated with BAPTA-AM during lysosomal damage. Cells treated with 15 μM BAPTA-AM for 1h were subject to 1 mM LLOMe treatment for 10 min and overlaps between mCherry-CHMP4A and LAMP1 quantified by HCM analysis of. Ctrl, untreated (no LLOMe) cells. White masks, algorithm-defined cell boundaries; Yellow masks, computer-identified overlap of mCherry-CHMP4A and LAMP1. **(B)** Quantification by HCM of overlaps between mCherry-CHMP4A and endogenous LAMP1 in parental HeLa (WT) and Gal3-knockout HeLa cells ($Gal3^{KO}$) treated with BAPTA-AM during lysosomal damage. Cells treated with 15 μM BAPTA-AM for 1 h, were subject to 1 mM LLOMe treatment for 30 min and overlaps between mCherry-CHMP4A and LAMP1 quantified by HCM analysis. White masks, algorithm-defined cell boundaries; Yellow masks, computer-identified overlap of mCherry-CHMP4A and LAMP1. **(C)** Mapping the interaction between Gal3 and CHMP4A. HEK293T cells overexpressing GFP-tagged full-length or truncated Gal3 and FLAG-CHMP4A were subjected to anti-GFP immunoprecipitation, followed by immunoblotting for FLAG-CHMP4A. **(D)** Mapping the interaction between Gal3 and CHMP4B. HEK293T cells overexpressing GFP-tagged full-length or truncated Gal3 and Myc-CHMP4B were subjected to anti-GFP immunoprecipitation, followed by immunoblotting for Myc-CHMP4B. **(E)** Schematic diagram of Gal3 domains, its deletion constructs and of mapping the interaction between Gal3 and CHMP4A/B or TRIM16. **(F)** Schematic summary of the findings in Figures 5 and S5. **(G)** Co-immunoprecipitation analysis of changes in interactions between Gal3 and TRIM16 during the process of lysosomal damage. HeLa Flp-In-$Gal3^{TetON}$ cells stably expressing Gal3 induced by tetracycline (Tet) were treated with 1 mM LLOMe for the indicated time. Cell lysates were immunoprecipitated (IP) with anti-mCherry antibody and immunoblotted for endogenous TRIM16. **(H)** Mapping the interaction between Gal3 and TRIM16. HEK293T cells overexpressing GFP-tagged full-length or truncated Gal3 and FLAG-TRIM16 were subjected to anti-GFP immunoprecipitation, followed by immunoblotting for FLAG-TRIM16. **(I)** Schematic summary of the findings in Figures 6 and S6. **(J)** *M. tuberculosis* survival assay in murine bone marrow-derived macrophages (BMMs) from $TRIM16^{fl/fl}$LysM-Cre^{-} and $TRIM16^{fl/fl}$LysM-Cre^{+} mice. BMMs were infected with *M. tuberculosis* strain Erdman at MOI 10 and incubated with full medium for total 18 h (-) or for 16 h in full medium plus last 2 h in EBSS (+; starvation). CFU were enumerated by plating and counting after 3 weeks of incubation. **(K)** Survival curves of mice infected by aerosol with virulent *M. tuberculosis* Erdman. Mice with a TRIM16 knockout specific for myeloid cells ($TRIM16^{Fl/Fl}$ LysM-Cre^{+}) and their $TRIM16^{Fl/Fl}$ LysM-Cre^{-} littermates (negative for LysM-Cre) were subjected to a model of respiratory infection with *M. tuberculosis*. Initial lung deposition, 1,372 CFU/mouse of *M. tuberculosis* Erdman.

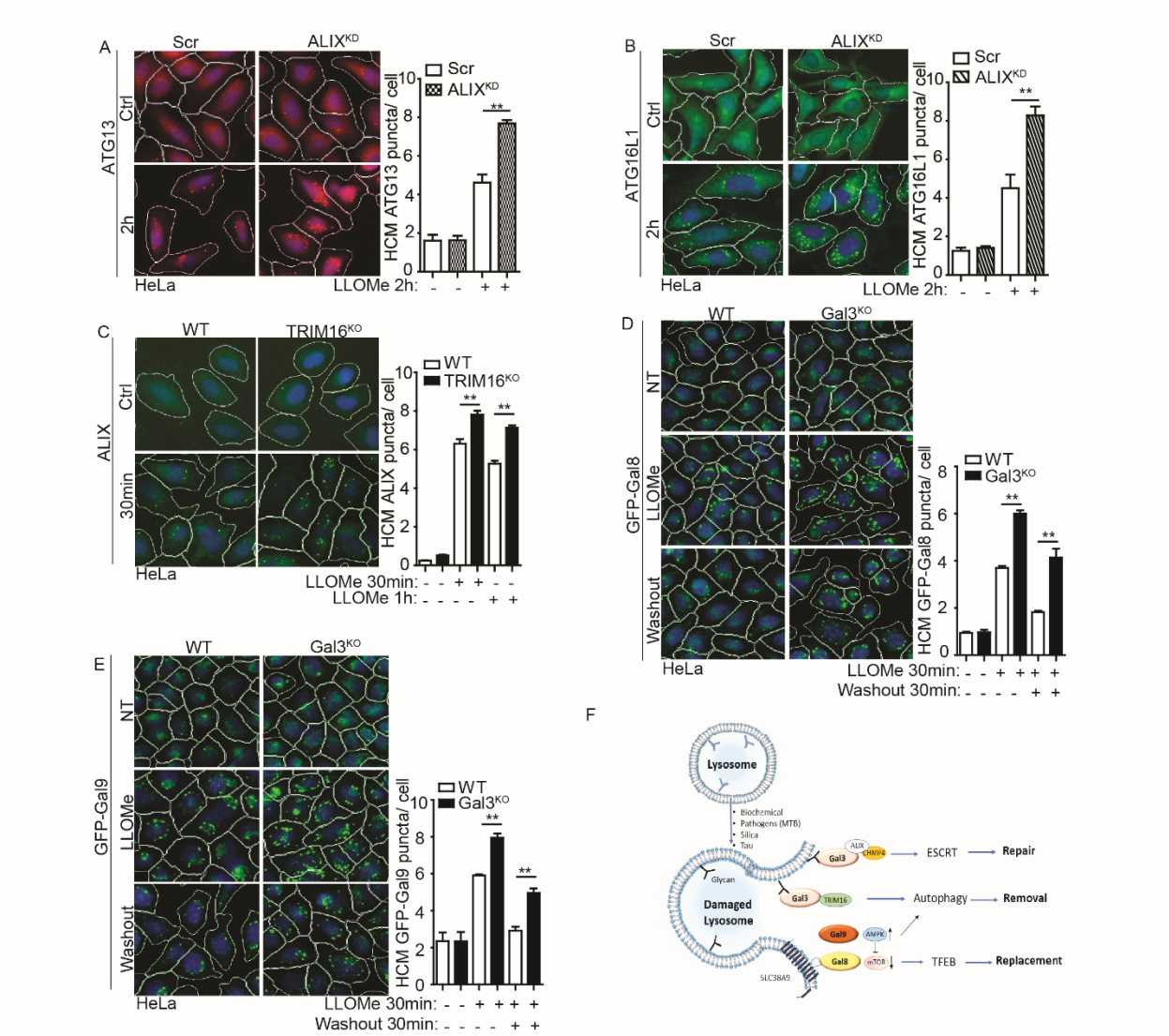


Figure S6, related to Figure 7. Gal3 acts as a switch between repair of damaged membranes by ESCRT and their removal by autophagy.

(A) ATG13 response in ALIX knockdown (ALIX^{KD}) cells during lysosomal damage. Cells transfected with scrambled siRNA as control (Scr) or ALIX siRNA were treated with 1 mM LLOMe for 2 h, and ATG13 puncta quantified by HCM. Untreated (no LLOMe) cells were used as control (Ctrl). White masks, algorithm-defined cell boundaries (primary objects); Red masks, computer-identified ATG13 puncta (target objects). **(B)** ATG16L1 response in ALIX knockdown (ALIX^{KD}) cells during lysosomal damage. Cells transfected with scrambled siRNA as control (Scr) or ALIX siRNA were treated with 1 mM LLOMe for 2 h, and ATG16L1 puncta were quantified using HCM. White masks, algorithm-defined cell boundaries; Green masks, computer-identified ATG16L1 puncta. **(C)** ALIX response in parental HeLa (WT) and TRIM16-knockout HeLa cells (TRIM16^{KO}) during lysosomal damage. HeLa cells were treated with 1 mM LLOMe for the indicated time, and ALIX puncta quantified by HCM. White masks, algorithm-defined cell boundaries; Green masks, computer-identified ALIX puncta. **(D, E)** Analysis of galectins' response (Gal8(D) or Gal9(E)) in parental HeLa (WT) and Gal3-knockout HeLa cells (Gal3^{KO})

during lysosomal damage. Cells transiently transfected with GFP-Gal8or GFP-Gal9 were treated with 1 mM LLOMe for 30 min followed by 30 min washout, and GFP-Gal8 or GFP-Gal9 puncta quantified by HCM. White masks, algorithm-defined cell boundaries; Green masks, computer-identified GFP-Gal8/9 puncta. **(F)** Schematic summary of Figures 7 and S6.