RESEARCH ARTICLE

Figures and figure supplements

A novel gene *REPTOR2* activates the autophagic degradation of wing disc in pea aphid

Erliang Yuan *et al*.

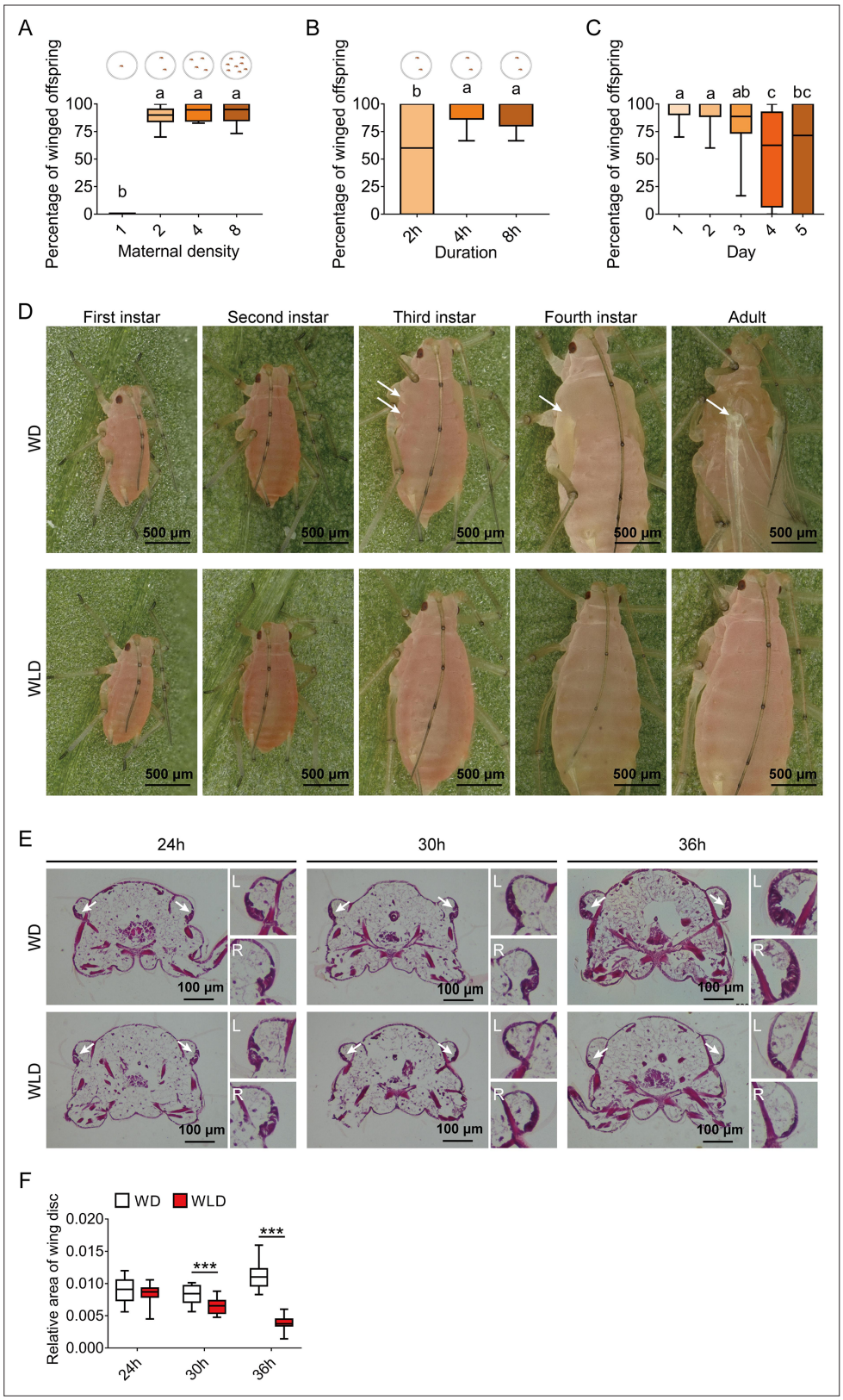

Figure 1. Wing dimorphism in pea aphids is transgenerational with high sensitivity to maternal density and duration, and first instar nymphs at 30-hr postbirth is a critical stage for developmental plasticity of the wing disc. (**A**) Two female adults in a petri dish can efficiently induce a high proportion of winged offspring. (**B**) Percentage of winged offspring produced by the two-adult contacting treatment for different durations. Circles in the

Figure 1 continued on next page

Figure 1 continued

diagrams above the figure panels represent petri dishes. (**C**) Daily percentage of winged offspring for the two-adult contacting treatment for 4-hr. (**D**) Developmental morphology of aphid wings from the first instar nymphal to adult stages in winged and wingless aphids. White arrows: locations of the wing bud or wing in the winged morph. (**E**) Histological comparisons of wing disc in the first instar nymphs at 24-, 30-, and 36-hr postbirth, and wing discs existed in both winged- and wingless-destined aphids. White arrows indicate the wing disc. L, left wing disc; R, right wing disc. (**F**) The relative area of the wing disc measured by ImageJ. WD, winged-destined; WLD, wingless-destined. Boxes show the interquartile range, and the line is the median value of each group (n>16). Tukey's multiple range tests at $p<0.05$ were used to compare means, and different lowercase letters indicate significant differences. Independent sample t test was used to compare means of the relative area of the wing disc, andsignificant difference at $p<0.001$ is indicated by asterisks (***).

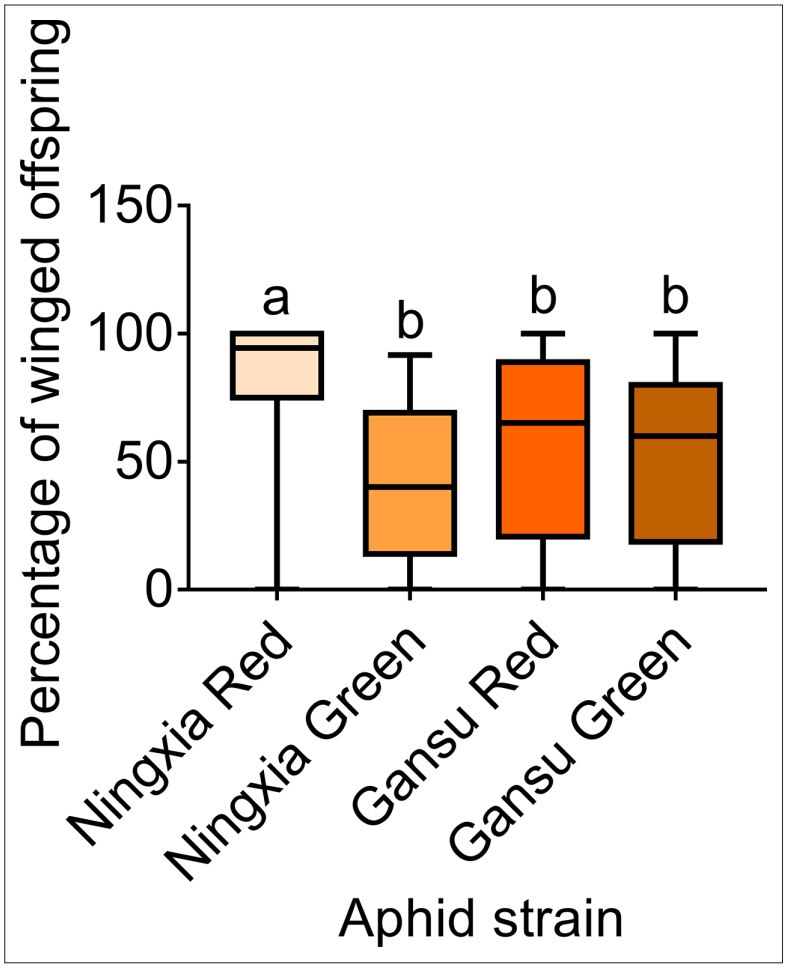

Figure 1—figure supplement 1. The percentage of winged offspring produced by two-adult contacting treatment for 4-hr of different pea aphid strains. Boxes show the interquartile range, and the line is the median value of each group (n=40). Tukey's multiple range tests at $p<0.05$ were used to compare means, and different lowercase letters indicate significant differences.

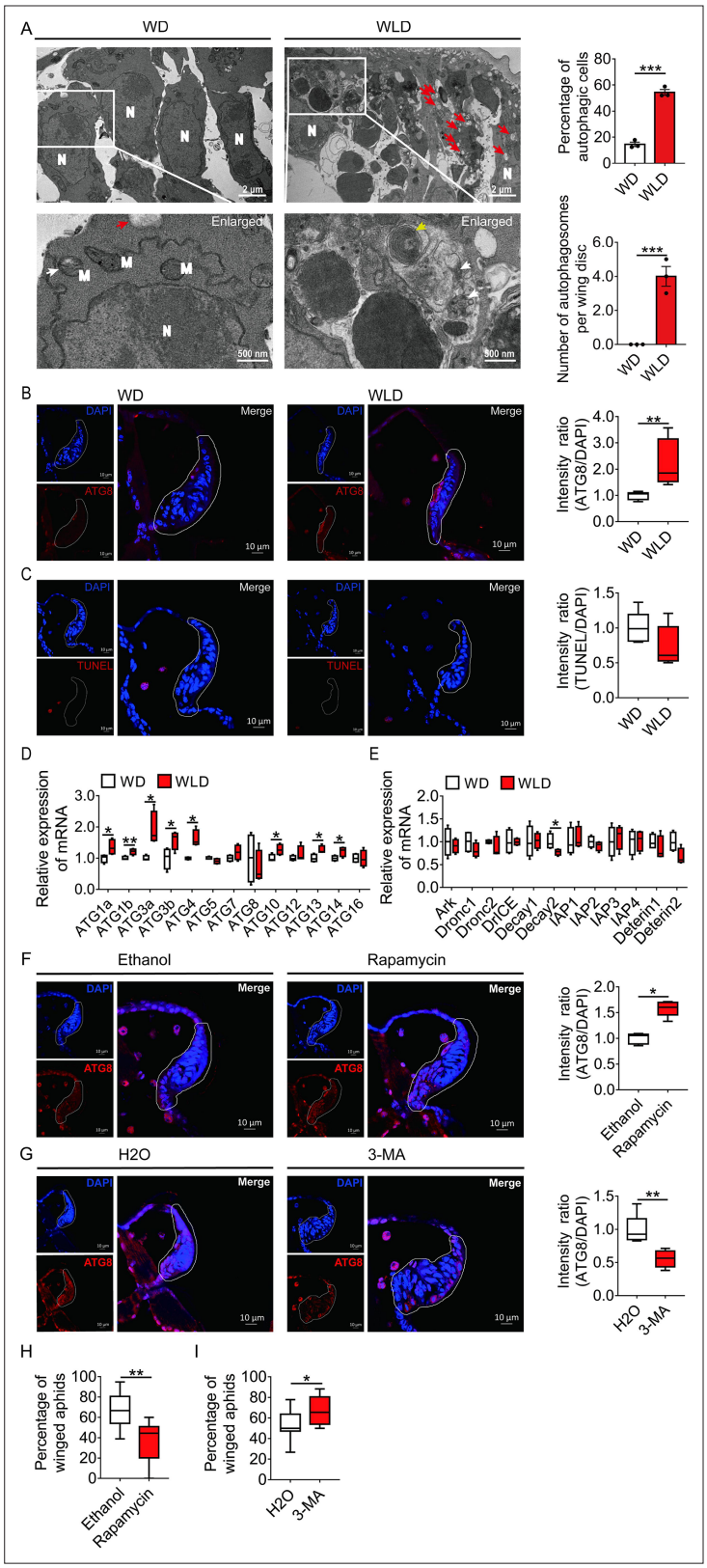

Figure 2. Autophagy, and not apoptosis, is responsible for degeneration of the wing disc in wingless-destined aphids. (**A**) TEM images of wing discs of winged- and wingless-destined aphids at 30-hr postbirth. N, nucleus; M, mitochondrion. Yellow and white arrows indicate autophagosomes containing membranous whorls and remnants of cellular organelles, respectively. Red arrows indicate the vacuoles (n=3). Values in bar plots are means (± SEM).

Figure 2 continued on next page

Figure 2 continued

(**B**) Autophagy in the wing disc was indicated by the hallmark ATG8 (red) in immunofluorescence. The nuclei (blue) were stained by DAPI (n=7). (**C**) Apoptosis was determined by TUNEL assays (red) (n=5). (**D**) Autophagy-related genes (*ATG1a*, *ATG1b*, *ATG3a*, *ATG3b*, *ATG4*, *ATG5*, *ATG7*, *ATG8*, *ATG10*, *ATG12*, *ATG13*, *ATG14*, and *ATG16*) were determined by qPCR (n=4). (**E**) Pro-apoptotic genes (*Ark*, *Dronc1*, *Dronc2*, *DrICE*, *Deterin1*, and *Deterin2*), anti-apoptotic genes (*IAP1*, *IAP2*, *IAP3*, *IAP4*, *Decay1*, and *Decay2*) were determined by qPCR (n=4). (**F**) The effect of autophagy agonist rapamycin, and (**G**) autophagy inhibitor 3-MA on autophagy in the wing disc. Autophagy was noted by ATG8 (red) in immunofluorescence, and nuclei were stained by DAPI (blue). All relative intensity was quantified by ImageJ (n>5). (**H**) The effect of autophagy agonist rapamycin, and (**I**) autophagy inhibitor 3-MA on the proportion of winged aphids. Ethanol and H_2O were used as control, respectively (n>9). Wing disc shown in confocal was highlighted by white circle. WD, winged-destined; WLD, wingless-destined. Boxes show the interquartile range, and the line is the median value of each group. Independent sample t test was used to compare means, and significant differences between treatments are indicated by asterisks, *p<0.05, **p<0.01, ***p<0.001. qPCR, quantitative polymerase chain reaction; TEM, transmission electron microscopy.

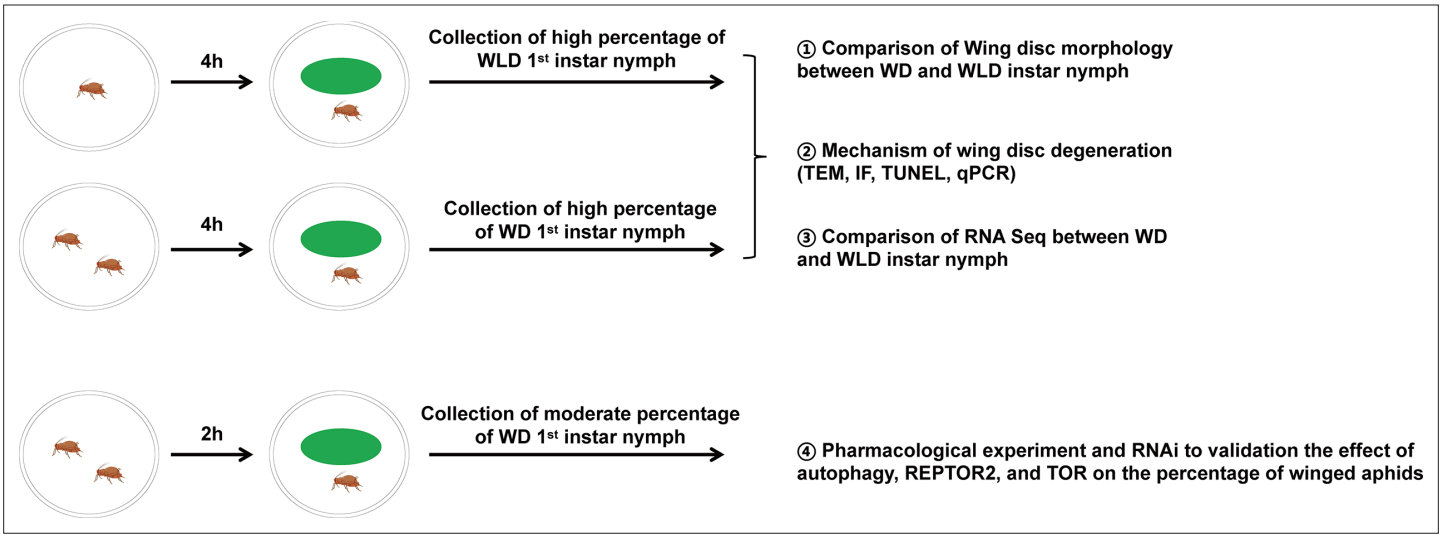

Figure 2—figure supplement 1. Experimental design. A flow diagram of the sampling procedure for maternal treatment and offspring collection. Female adults of pea aphids were subjected to single adult or two adult individual treatement in the petri dish for 4 hr. Since the solitary mother produced high proportion of wingless offspring (nearly 100%), the collected newborn nymphs during the first 2 days named wingless-destined aphids. Likewise, the two-adult individuals contacting for 4 hr resulted in high proportion of winged offspring (>90%), so newborn nymphs at first 2 days named winged-destined aphids. Alternatively, two adult individuals contacting for 2 hr could cause a moderate proportion of winged offspring (~50%). WD, winged-destined; WLD, wingless-destined.

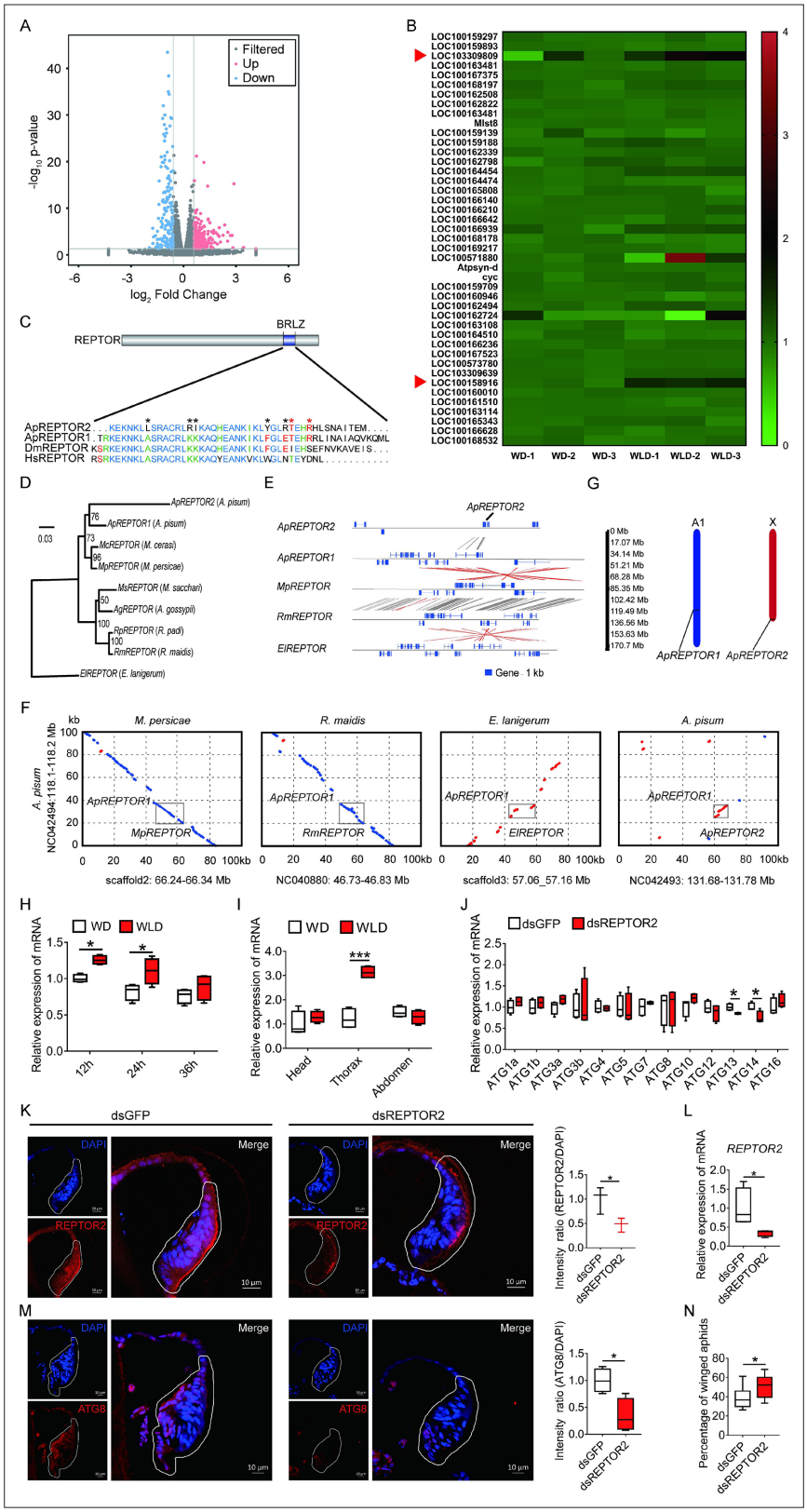

Figure 3. Pea aphid-specific *REPTOR2* is highly expressed in wingless-destined morph that activates autophagy in the wing disc. (**A**) The volcano plot of log-transformed FPKM showing the number of differentially expressed genes (DEGs) in wingless- versus winged-destined aphids at 24-h postbirth. Three biological replications were conducted in the RNA-seq analysis. (**B**) Transcript mapping to TOR signaling pathway of pea aphids and DEGs

Figure 3 continued on next page

Figure 3 continued

in two wing morphs. Red arrows indicated two DEGs, *REPTOR2* (LOC103309809) and *sodium-coupled neutral amino acid transporter 9* (LOC100158916), in wingless- versus winged-destined aphids. (**C**) Homology analysis of pea aphid REPTOR2. The amino acid sequence of conserved basic region leucine zippers (BRLZ) domain of pea aphid REPTOR2 was aligned with pea aphid REPTOR1 (ApREPTOR1), human REPTOR (HsREPTOR) and *Drosophila* REPTOR (DmREPTOR). Red and black asterisks indicated pea aphid-specific variation and ApREPTOR2-specific variation in amino acid, respectively. (**D**) An unrooted phylogenetic tree was constructed by the IQ-TREE method with coding sequence alignments for aphid REPTOR. The sequences of REPTOR identified from eight aphid species as well as the candidate pea aphid *REPTOR2* were compared. (**E**) Sequence comparisons of the LOC103309809 (*ApREPTOR2*) locus and its homologous regions in aphids. Homologous sequences were linked by gray dashes corresponding to the positive strand and by red dashes corresponding to the negative strand. (**F**) Gray boxes highlight the similarity of aphid REPTOR across 100 kb covering the REPTOR loci. (**G**) Chromosomal distribution of two REPTOR genes in pea aphids. (**H**) Relative expression levels of *REPTOR2* in first instar nymph at 12-, 24-, and 36-h postbirth in winged- and wingless-destined aphids (n=4). (**I**) Expression levels of *REPTOR2* in head, thorax and abdomen of winged- and wingless-destined aphids at 24-h postbirth (n=4). (**J**) Effects of ds*REPTOR2* on the expression of genes related to autophagy in whole body of the first instar nymphs (n=4). (**K**) Knockdown of *REPTOR2* reduced its expression in wing disc at 24-h postbirth. Fluorescence in situ hybridization (FISH) was used to detect the *REPTOR2* expression level in aphid wing discs. *REPTOR2* was hybridized with 5-CY3 in red, and nuclei were stained with DAPI in blue (n=3). (**L**) *dsREPTOR2* feeding decreased the gene expression of *REPTOR2*, as determined by qPCR (n=4). (**M**) Knockdown of *REPTOR2* resulted in the decline of autophagy in the wing disc at 30-h postbirth. Autophagy was noted by ATG8 (red) in immunofluorescence, and nuclei were stained by DAPI (blue) (n=4). (**N**) *dsREPTOR2* treatment increased the proportion of winged aphids (n=10). Wing discs shown in confocal were highlighted by white circles. WD, winged-destined; WLD, wingless-destined. Boxes show the interquartile range, and the line is the median value of each group. Independent sample t test was used to compare mean, and significant differences between treatments are indicated by asterisks: *p<0.05, **p<0.01, ***p<0.001.

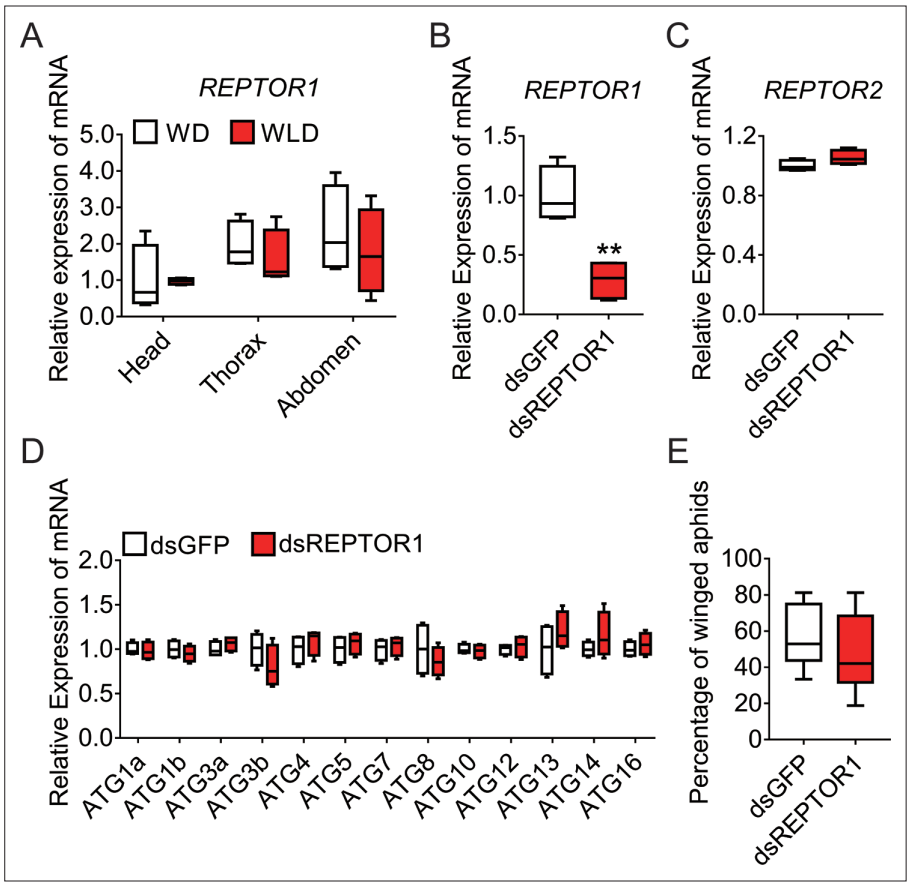

Figure 3—figure supplement 1. *REPTOR1* was not involved in activation of autophagic degradation in the wing disc of the winged-destined morph. (**A**) Expression levels of *REPTOR1* in head, thorax, and abdomen of winged- and wingless-destined aphids at 24-h postbirth (n=4). (**B–D**) Knockdown of *REPTOR1* decreased the gene expression of *REPTOR1*, but did not affect the gene expression of *REPTOR2* and *ATG*s in whole body of the first instar nymphs, as determined by qPCR (n=4). (**E**) *dsREPTOR1* did not affect the proportion of winged aphids (n=11). Boxes show the interquartile range, and the line is the median value of each group. Independent sample t test was used to compare means. Significant differences between treatments are represented by asterisks: **p<0.01.

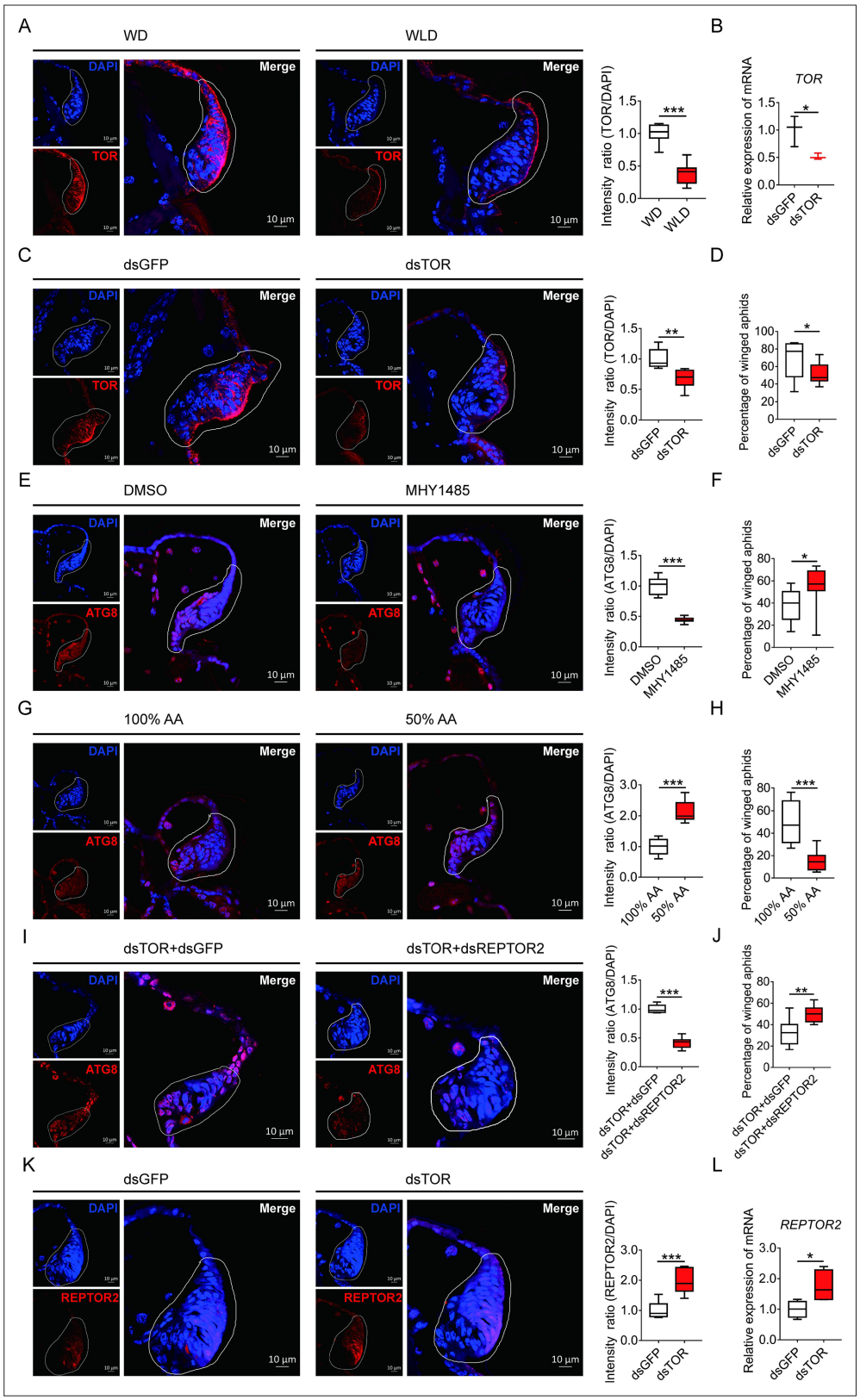

Figure 4. TOR negatively regulated autophagy in the wing disc and positively affected the proportion of winged aphids. (**A**) *TOR* expression in the wing disc of first instar nymph of winged- and wingless-destined aphids at 24-h postbirth, as determined by mRNA-FISH. *TOR* was hybridized with 5-CY3 in red, and nuclei were stained with DAPI in blue (n=9). (**B**) Newborn nymphs fed with dsRNA reduced *TOR* expression in whole body 24-h postbirth,

Figure 4 continued on next page

Figure 4 continued

as determined by qPCR (n=3). (**C**) Knockdown of *TOR* reduced its expression in the wing disc 24-h postbirth, shown in mRNA-FISH (n>7), and (**D**) increased the proportion of winged aphids (n=10). (**E**) Application of TOR agonist MHY1485 attenuated autophagy in the wing disc 30-h postbirth (n=9). (**F**) Activation of TOR increased the proportion of winged aphids (n>8). (**G**) Amino acids supplied at 50% of the standard diet (50% AA) activated autophagy in the wing disc 30-h postbirth (n=8). (**H**) 50% AA reduced the proportion of winged aphids (n=8). Autophagy in the wing disc was indicated by the hallmark protein ATG8 (red) in immunofluorescence. The nuclei (blue) were stained by DAPI. (**I**) Knockdown of both *TOR* and *REPTOR2* attenuated autophagy in the wing disc 30-h postbirth, relative to the control, ds*TOR*+ds*GFP* (n=7), and (**J**) increased the proportion of winged aphids (n=8). (**K**) Knockdown of *TOR* increased *REPTOR2* expression in the wing disc 24-h postbirth, shown in mRNA-FISH (n=7). *REPTOR2* was hybridized with 5-CY3 in red, and nuclei were stained with DAPI in blue. (**L**) Newborn nymphs fed with ds*TOR*-RNA increased *REPTOR2* expression in whole body 24-h post-birth, as determined by qPCR (n=4). WD, winged-destined; WLD, wingless-destined. Relative intensity was quantified by ImageJ. Wing discs shown in confocal was highlighted by white circles. Boxes show the interquartile range, and the line is the median value of each group. Independent sample t test was used to compare mean, and significant differences between treatments are indicated by asterisks: *p<0.05, **p<0.01, ***p<0.001.

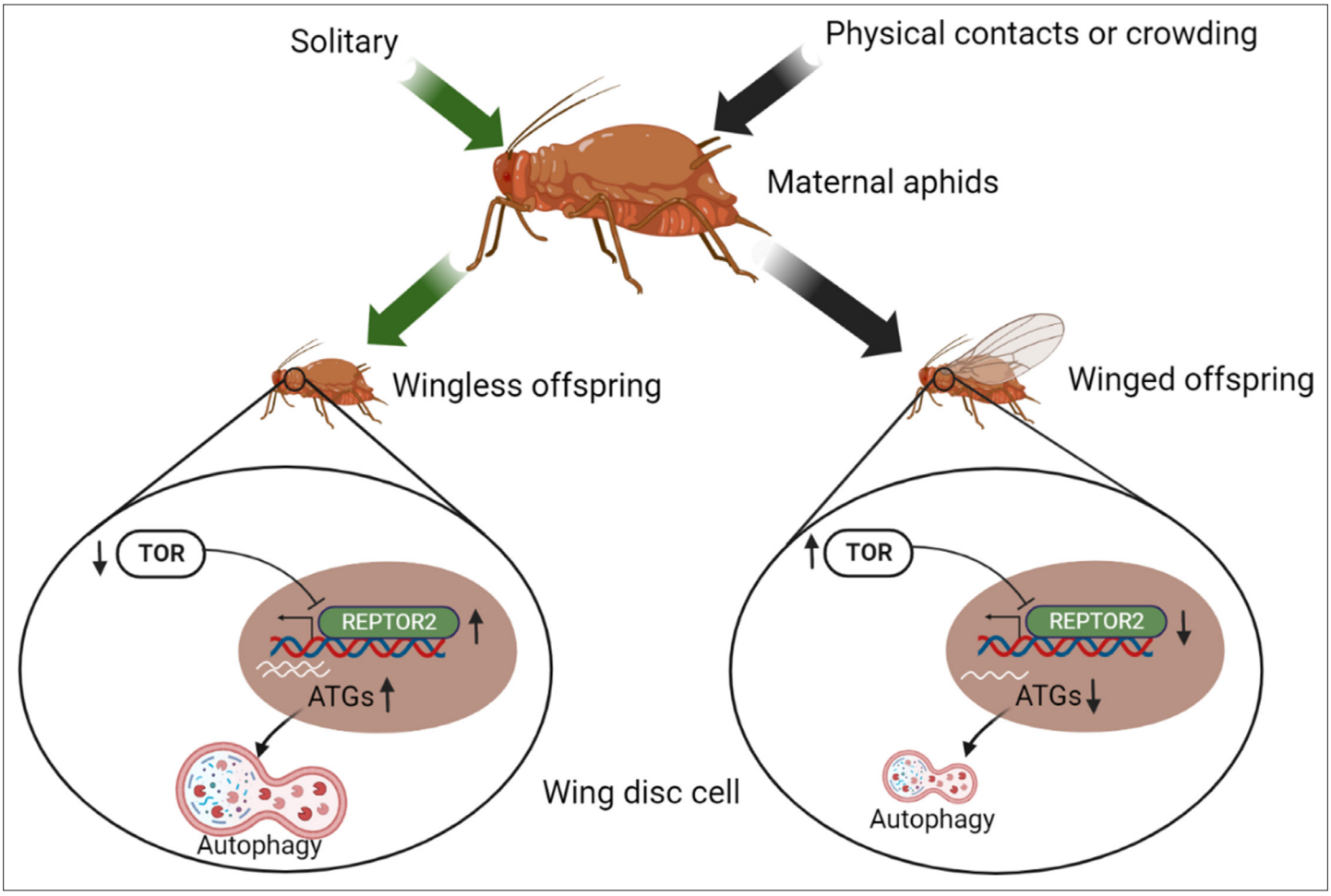

Figure 5. Illustration of the TOR signaling pathway-controlled wing disc degeneration of the first instar pea aphid nymphs. All treatments, indicated along the top, were applied to wingless maternal aphids. Arrows correspond to the treatments, coded by different colors. ATG, autophagy related gene; REPTOR2, repress by TOR 2; TOR, target of rapamycin. This graph was created with BioRender.com.