Supporting Information

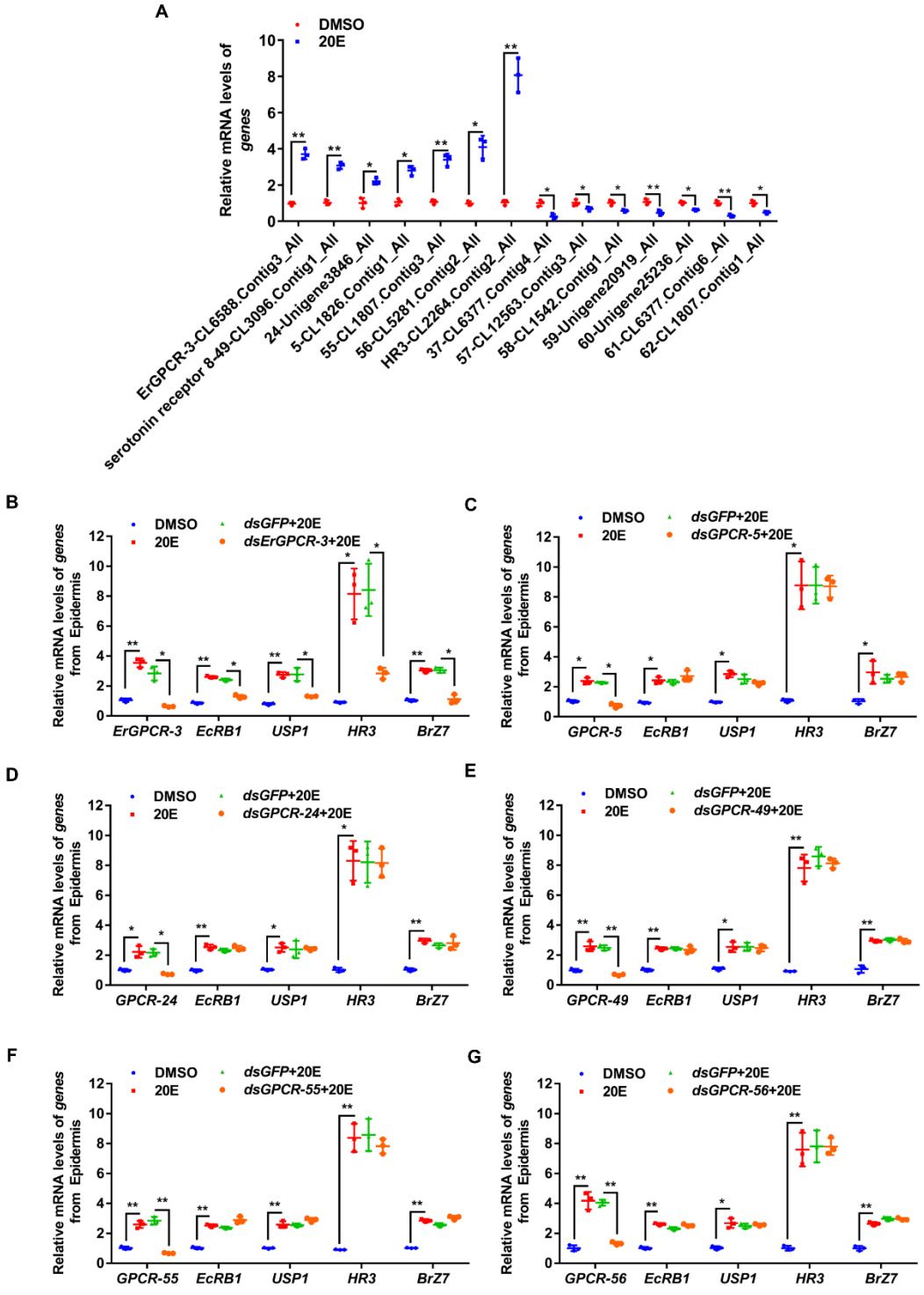

Figure S1. qRT-PCR screening of *ErGPCR-3*. A. mRNA levels of thirteen *GPCRs* from the larval epidermis treated with 20E (500 ng/larva) for 20 h, with *HR3* as a positive control, compared with DMSO. **B.** to **G**. qRT-PCR showing the mRNA levels of 20E-response genes after the six upregulated *GPCR* knockdown in larvae at 6th 72 h. 500 ng/larva at sixth instar 6 h (thrice at an 18 h interval) and 20E treatment (500 ng/larva) at the third injection. Error bars represent the mean ±SD of three replicates. Asterisks indicate significant differences according to Student's *t*-tests (*$p < 0.05$ and **$p < 0.01$).

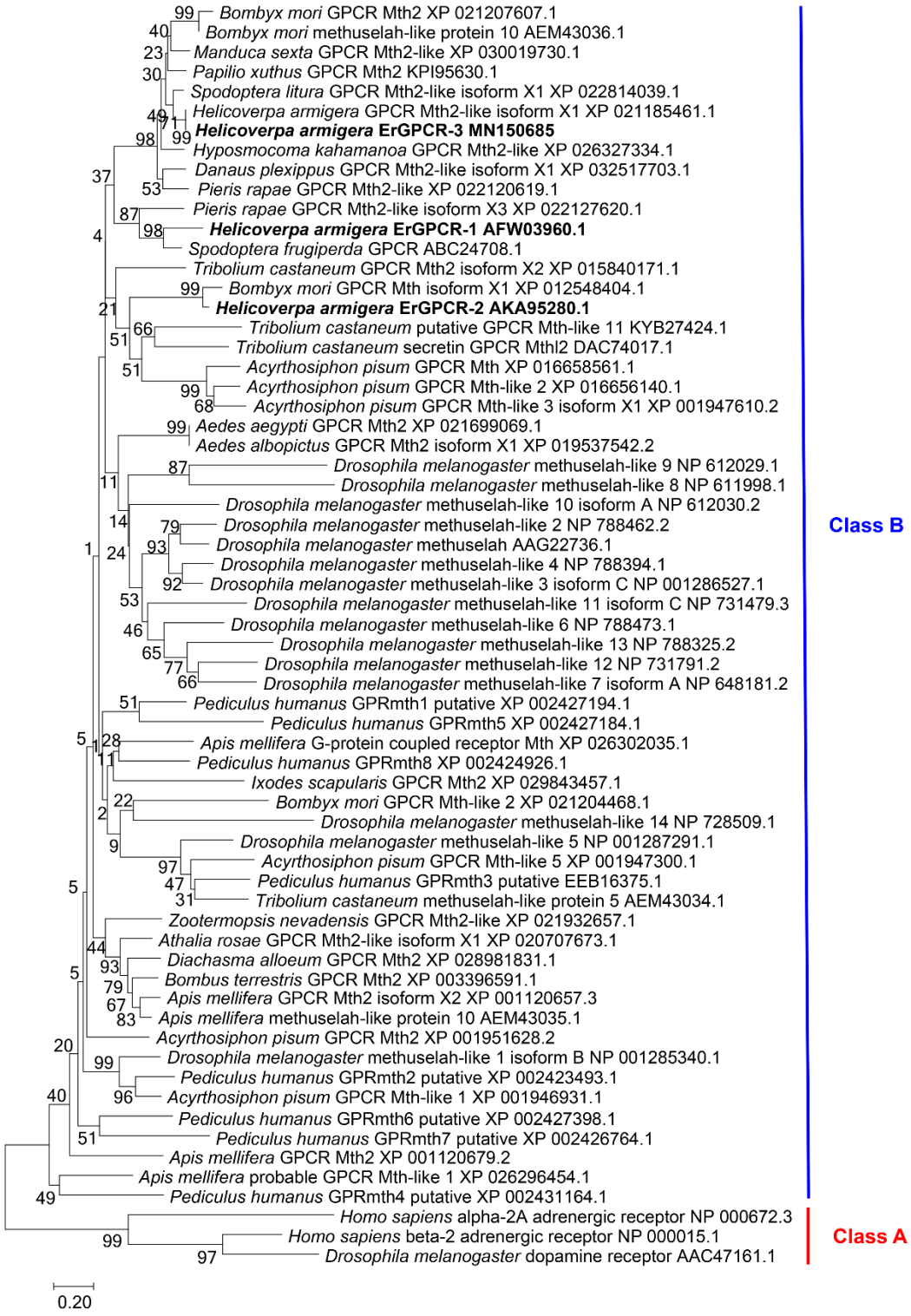

Figure S2. Phylogenetic tree of ErGPCR-3 and GPCRs based on amino acid sequences (NJ method). Numbers above branches support values (%) based on 1,000 replicates are indicated, the scale bar represents 0.2% amino acid substitutions per site, and the GenBank accession numbers are behind the Latin names.

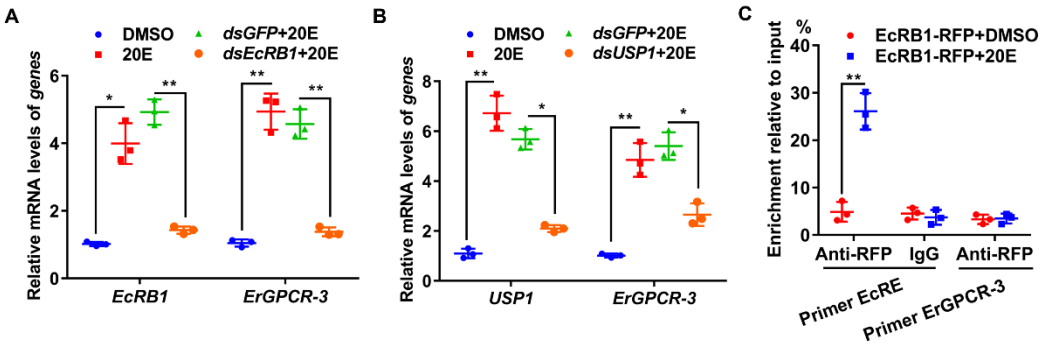

Figure S3. 20E upregulates ErGPCR-3 A. and **B.** The mRNA levels of *ErGPCR-3* after knockdown of *EcRB1* and *USP1* by *dsEcRB1* and *dsUSP1* (2 μg/mL for 48 h) followed 20E (2 μM for 6 h) induction in HaEpi cell. *dsGFP* (2 μg/mL for 48 h) was the negative control. DMSO was the solvent control for 20E. **C.** ChIP assay of EcRB1 binding to the upstream region of *ErGPCR-3* using primers (Table S1). EcRB1-RFP-His was overexpressed in HaEpi cells for 72 h. The cells were treated with 2 μM 20E for 3 h. DMSO treatment was used as a control. Error bars represent the mean ± SD of three replicates. Asterisks indicate significant differences according to Student's *t*-tests (*$p < 0.05$ and **$p < 0.01$).

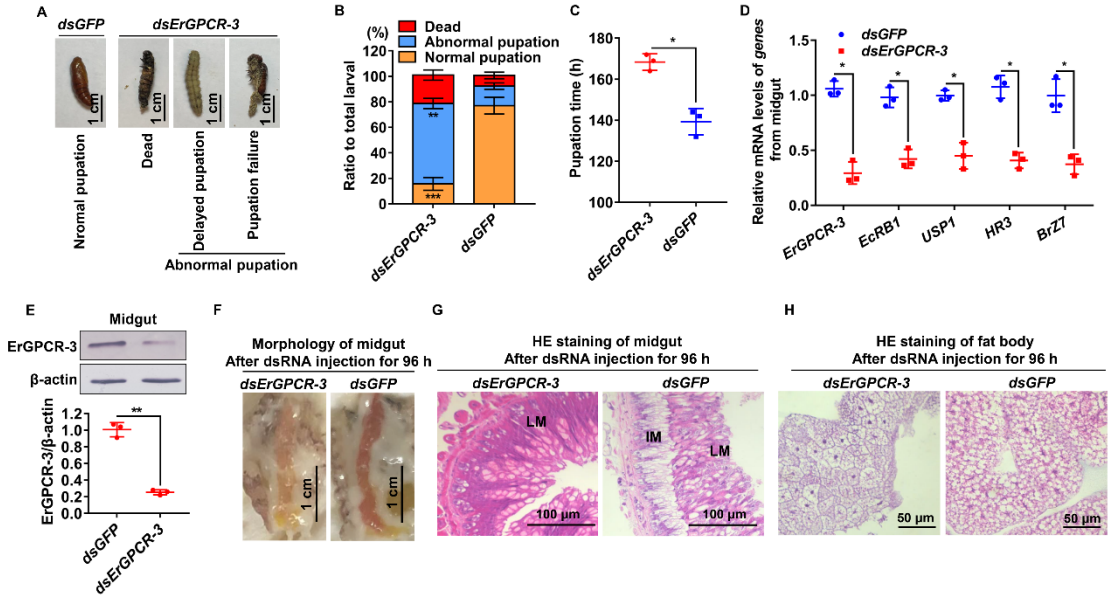

Figure S4. *ErGPCR-3* knockdown via dsRNA injection in larvae causes abnormal pupation. A. Phenotypes after *ErGPCR-3* knockdown (500 ng/larva at sixth instar 6 h, thrice at an 18 h interval). Images were obtained at six instar larvae 140 h according to the *dsGFP* injection control group. Scale bar = 1 cm. **B.** Percentages of the phenotype in A. **C.** Statistical analysis of pupation time from 6th instar 0 h larvae to pupae. The data were calculated from 30 larvae × 3 experiments. **D.** qRT-PCR showing the mRNA levels of 20E-response genes after *ErGPCR-3* knockdown in larvae at 6th 72 h. **E.** Efficiency analysis of *ErGPCR-3* knockdown using western blotting at the protein levels. **F.** Morphology of the midgut 96 h after the first *dsRNA* injection. Scale bar = 1 cm. **G** and **H.** HE-stained midgut and fat body after knockdown of *ErGPCR-3*. *dsGFP* was used as a control. LM: larval midgut; IM: imaginal midgut. Scale bar indicates 100 μm and 50 μm, respectively. The bars indicate the mean ± SD, and asterisks indicate significant differences using Student's *t*-test based on three replicates (*$p < 0.05$, ** $p < 0.01$, and ***$p < 0.001$) in B, C, D, and E.

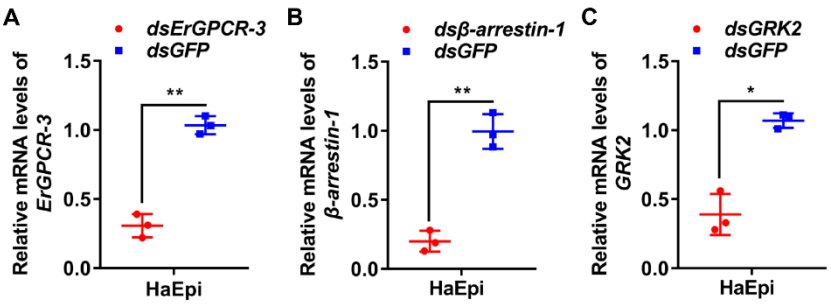

Figure S5. Efficiency of gene knockdown in HaEpi cells assessed using qRT-PCR. HaEpi cells were treated with 2 μg/mL *dsErGPCR-3*, *dsβ-arrestin-1*, and *dsGRK2* for 48 h, respectively, with *dsGFP* as a control. The bars indicate the mean ± SD, and asterisks indicate significant differences using Student's *t*-test based on three replicates (*$p < 0.05$ and **$p < 0.01$).

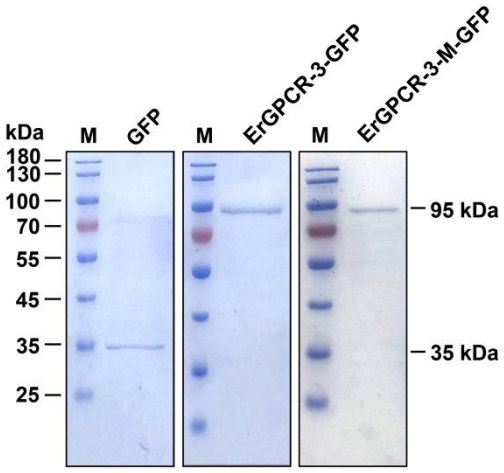

Figure S6. SDS-PAGE (12.5%) with Coomassie brilliant blue staining showing the purity of the isolated GFP, ErGPCR-3-GFP, and ErGPCR-3-M-GFP.

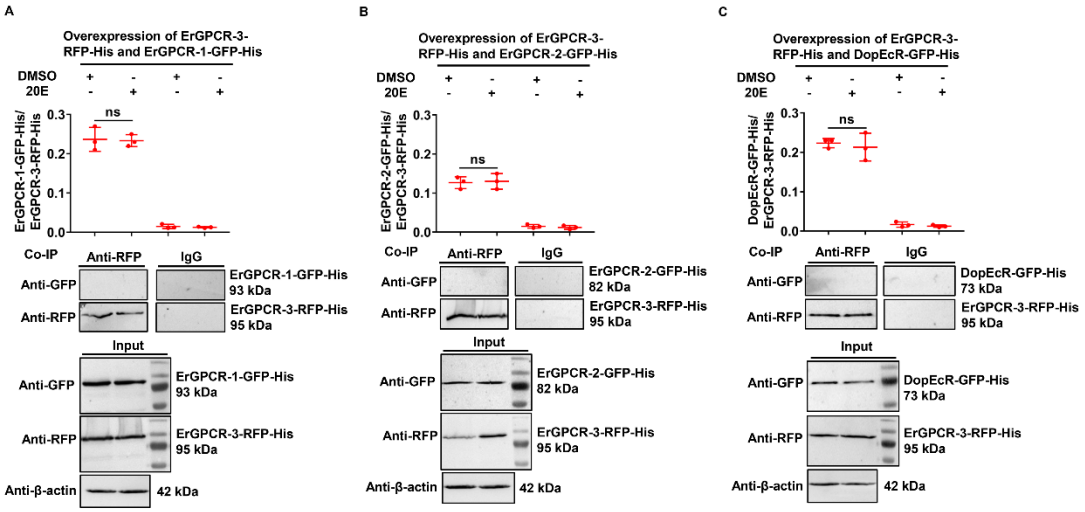

Figure S7. ErGPCR-3 does not interact with other GPCRs after 20E induction. A, B and **C.** Co-IP to detect ErGPCR-3 coupling with other GPCRs under 20E (2 μM for 30 min) induction. DMSO was solvent control. Input: the levels of ErGPCR-3-RFP-His, ErGPCR-3-GFP-His, ErGPCR-2-RFP-His, ErGPCR-1-RFP-His, and DopEcR-RFP-His in the cells detected by an antibody against RFP or GFP. β-actin was a loading control. Co-IP: Anti-RFP antibody co-immunoprecipitated ErGPCR-3-RFP-His and GPCRs-GFP-His. Nonspecific mouse IgG was a negative control. SDS-PAGE gel was 12.5%. Statistical analysis according to three independent replicate experiments by ImageJ software. The bars indicate the mean ± SD. Asterisks indicate significant differences using Student's *t*-test based on three replicates (*$p <$ 0.05).

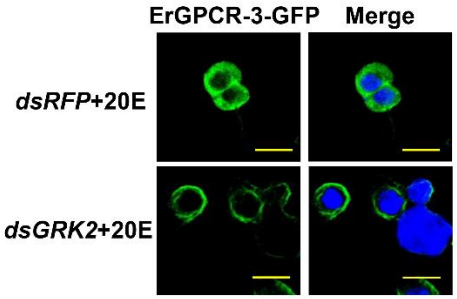

Figure S8. ErGPCR-3-GFP was retained in the cell membrane after knockdown of *GRK2*. HaEpi cells were treated with *dsRFP*, *dsGRK2* for 48 h, followed 1 μM 20E for 30 min, respectively. Green: ErGPCR-3 protein stained with an anti-ErGPCR-3 and secondary antibody labeled with Alexa Fluor 488. Blue: nuclei stained with DAPI. Scale bar = 25 μm.

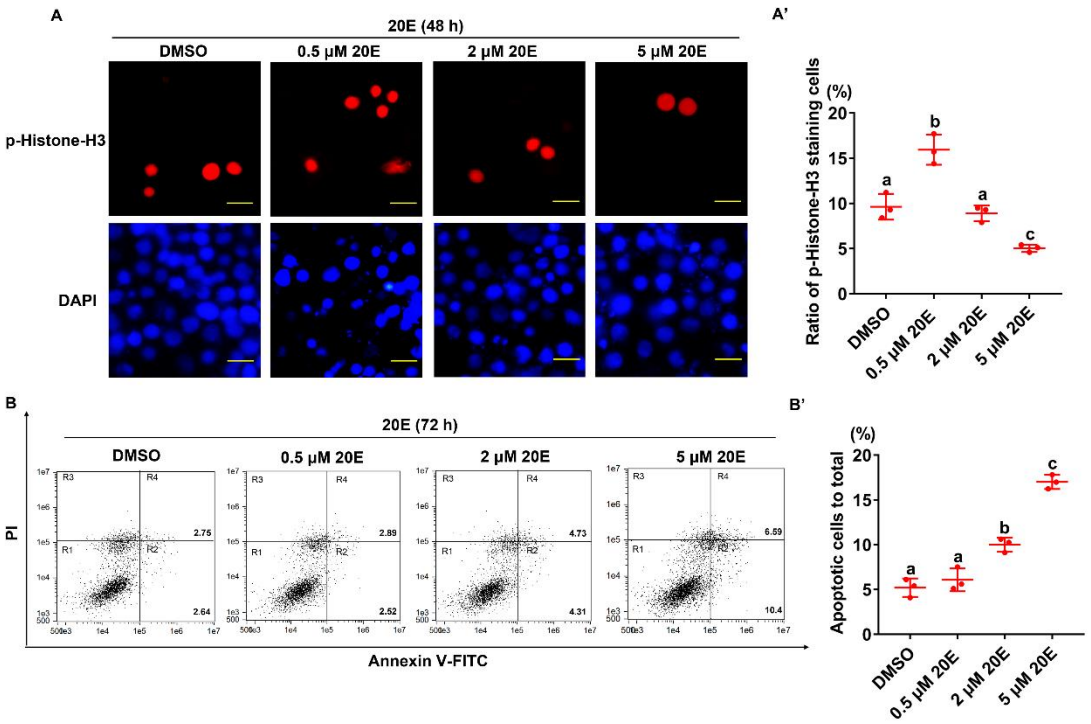

Figure S9. Low concentrations of 20E promoted HaEpi cell proliferation and high concentrations of 20E promoted HaEpi cell apoptosis. A. Detection of cell proliferation. Red fluorescence indicates the p-Histone H3 detected by the phospho-histone H3 antibody and goat anti-mouse IgG Alexa-Fluor 568 (red). Blue indicates nucleus stained by DAPI. Bar indicates 20 μm. **A'.** Ratio of p-Histone H3 staining cells to the total cells (blue) in A. **B.** Flow cytometry analysis by Annexin-V and propidium iodide (PI) staining. R1, normal cells; R2, early apoptotic cells; R3, dead cells; R4, late apoptotic cells. **B'.** The statistical analysis of B. %: the percentage of apoptotic cells (R2 + R4) to total cells. Each bar represents the mean ± SD of three replicates. Significant differences were calculated using ANOVA (different letters represent significant differences, $p < 0.05$).

Supplementary Tables

Table S1 The PCR primer sequences used in this paper

Click here to Download Table S1

Table S2. Predicted binding residues and point mutations.

Proteins	C-score	Ligands	Binding sites*	Mutation sites
ErGPCR-3	0.04	CLR	240M, 241A, 244G, 248V, 276I, 279Y, 331L, 334W	M240A, G244A, L331A, W334A
	0.02	CLR	237M, 240M, 241A, 244G, 327L, 330G, 334W	
	0.02	CLR	222F, 240M, 244G, 247I, 248V, 252F	

*The predicted binding residues of ErGPCR-3 are predicted online at http://zhanglab.ccmb.med.umich.edu/I-TASSER/. C-score is the confidence score of the predicted binding site. **CLR,** cholesterol, cholest-5-en.