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Supplementary Information

Title:

Environmental pH stress influences cellular secretion and uptake of extracellular vesicles

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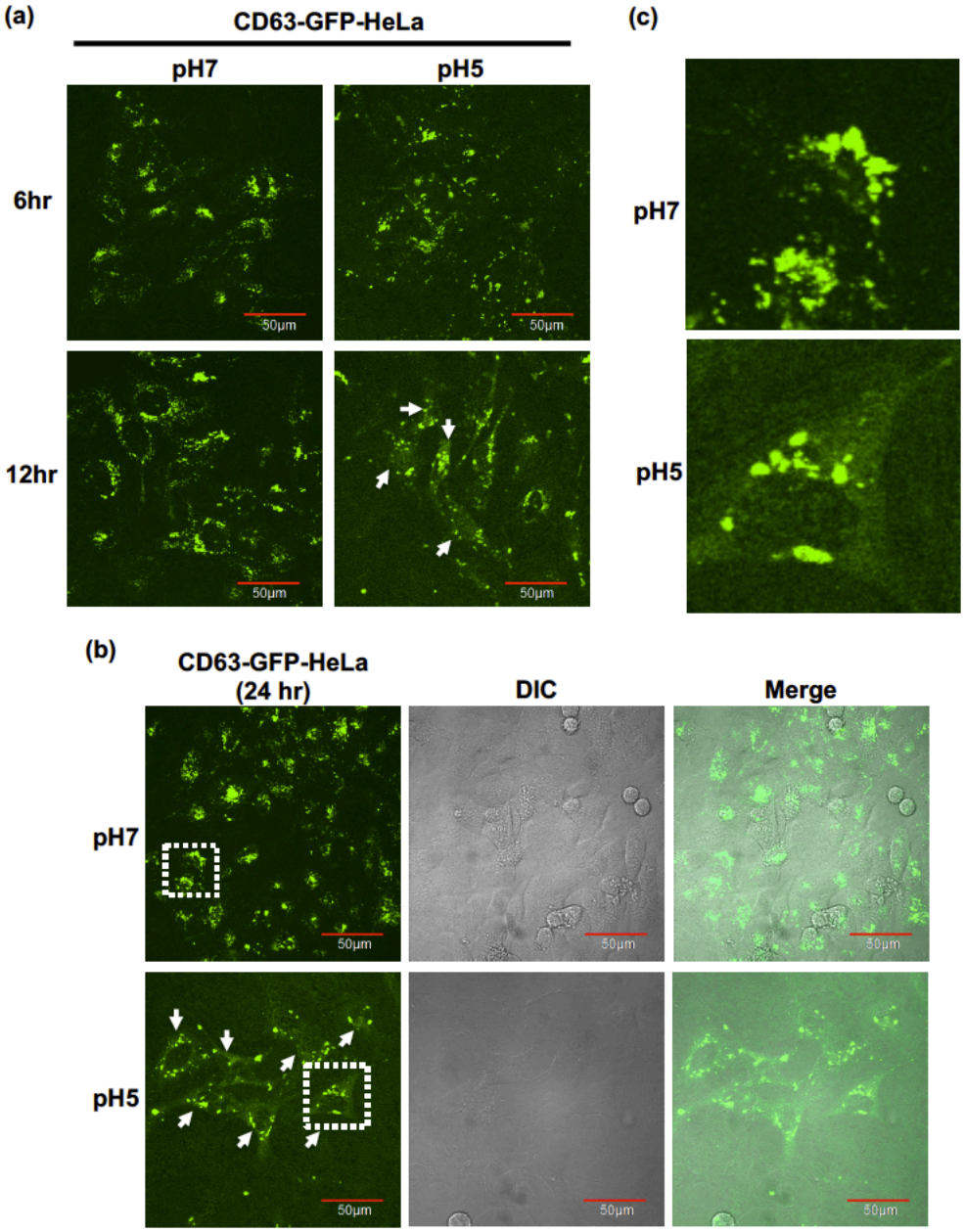
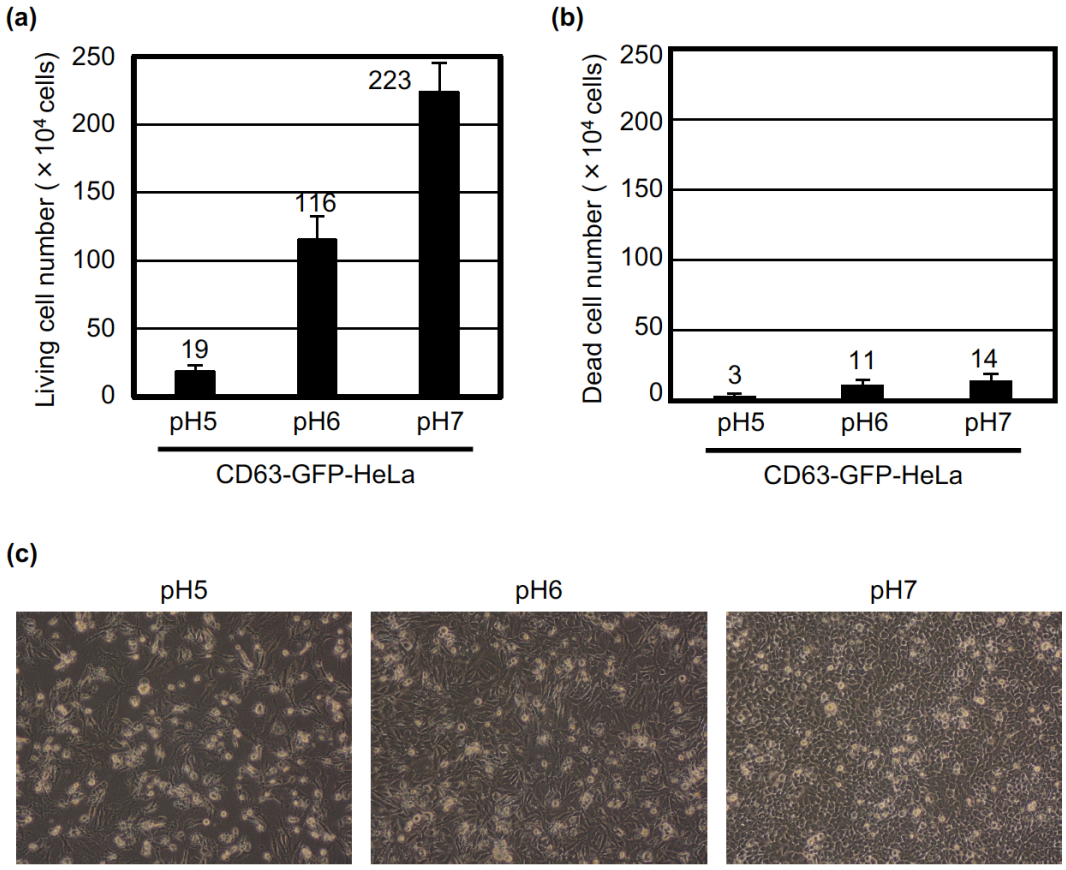
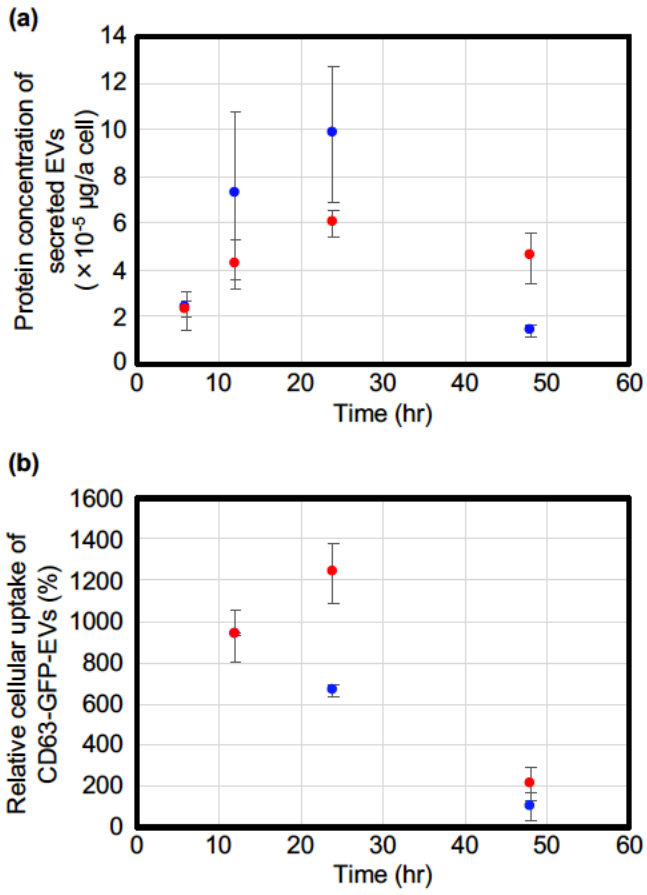


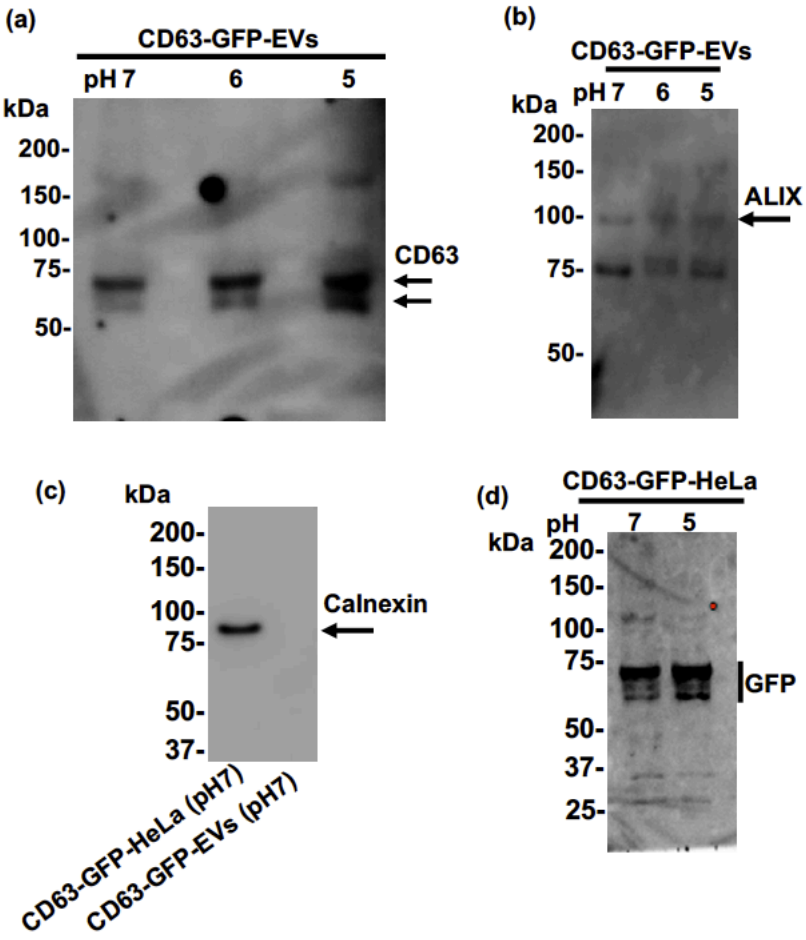
Supplementary Figure S1. Effects of low pH cell culture condition on expression of CD63-GFP fusion proteins. (**a**, **b**) Confocal microscopic observation of CD63-GFP fusion proteins stably expressing HeLa cells cultured for 6, 12 (**a**), and 24 h (**b**) at 37°C in α-MEM (pH 7 or 5) with 10% FBS. Scale bar, 50 μm. Arrows show typical cells with diffused GFP signals in cytosol. (**c**) Enlarged pictures of (**b**) (areas within the white dot squares).



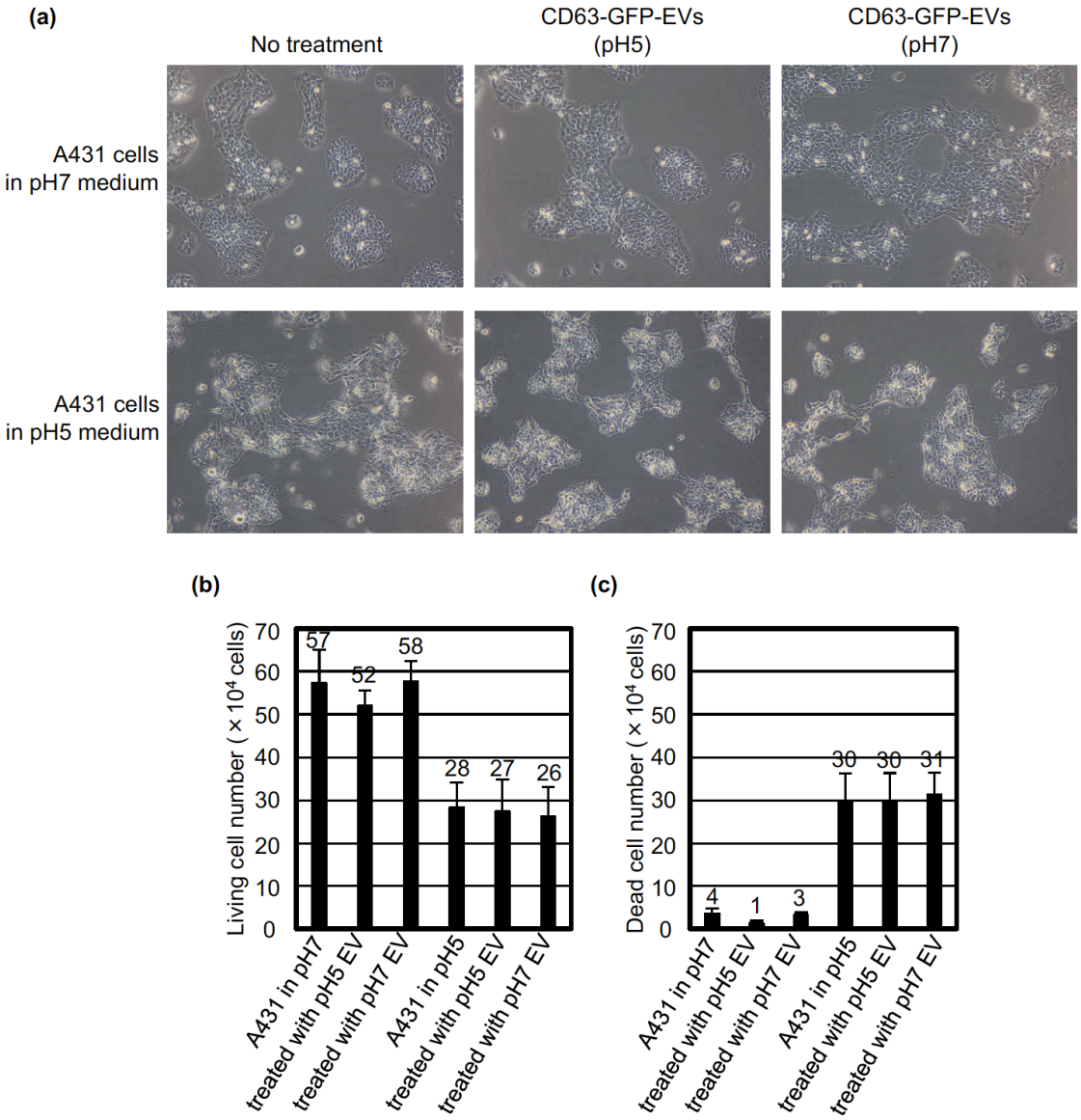
Supplementary Figure S2. Viability of cells stably expressing the CD63-GFP fusion protein. (**a**, **b**) Living (**a**) or dead (**b**) cell counting assay after culture of HeLa cells stably expressing the CD63-GFP fusion protein for 24 h at 37°C in α-MEM (pH 5, 6, or 7) with 10% FBS in trypan blue stain methods. The data are expressed as the mean (± SD) of three experiments. (**c**) Microscopic observation of CD63-GFP fusion proteins stably expressing HeLa cells in α-MEM (pH 5, 6, or 7) with 10% FBS for 48 h at 37°C.



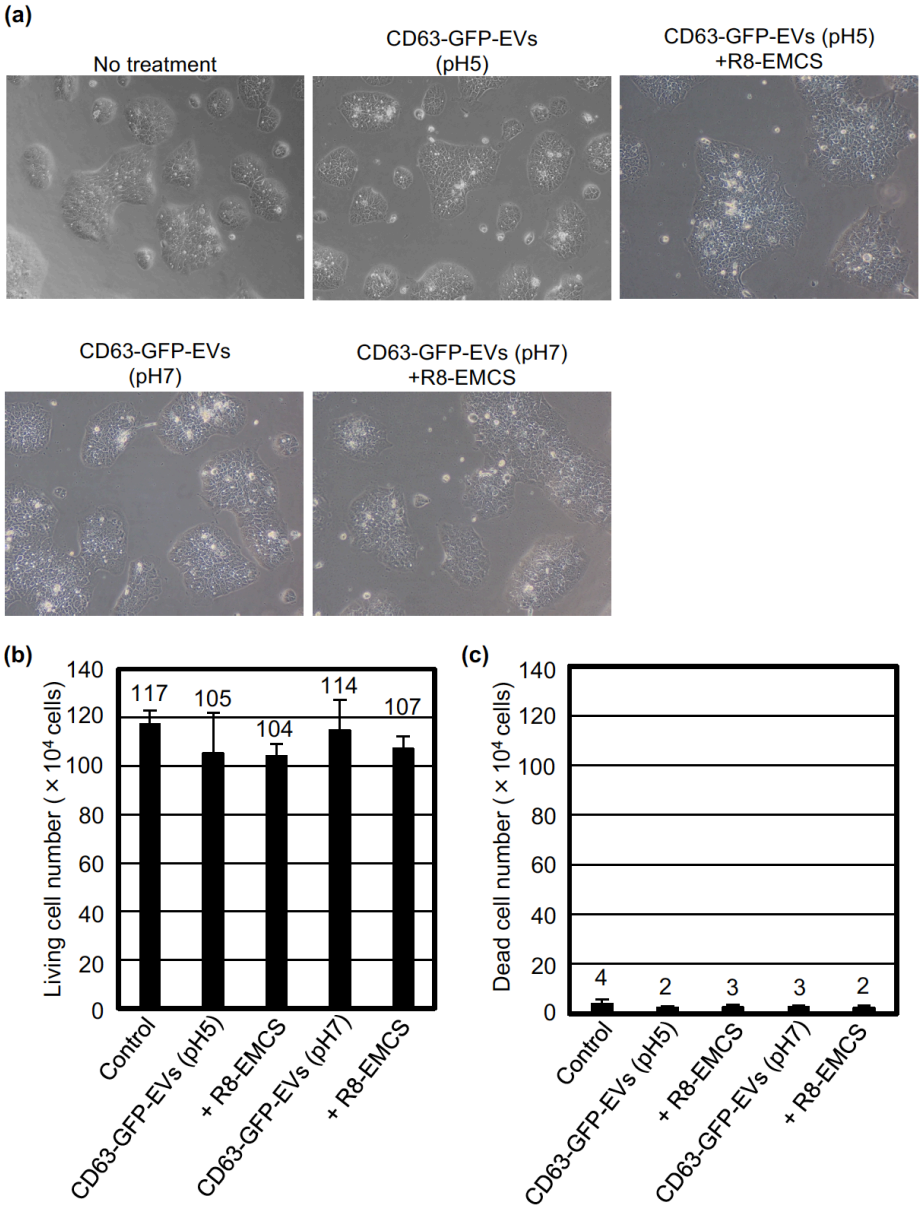
Supplementary Figure S3. Protein content of secreted EVs under low pH cell culture condition and their cellular uptake. (**a**) Relative protein concentration of EVs/cell number under different pH (pH 5 (red) or 7 (blue)) cell culture condition. EVs secreted from CD63-GFP-HeLa cells (6, 12, 24, or 48 h, 37°C) were analyzed by BCA protein assay, and each protein concentration was divided by the cell number. The data are expressed as the mean (± SD) of three experiments. (**b**) Cellular uptake (serum-starved condition) of isolated EVs. The relative cellular uptake of isolated CD63-GFP-EVs (20 μg/mL for 12, 24, or 48 h at 37°C in MEM (pH 7) without FBS) secreted under pH 7 (blue) or pH 5 (red) cell culture condition, analyzed using a flow cytometer (detection for 10,000 live cells). The data are expressed as the mean (± SD) of three experiments.



Supplementary Figure S4. Western blot analyses of CD63-GFP-EVs and CD63-GFP-HeLa cells. Western blot analyses (anti-CD63 (**a**), anti-ALIX (**b**), anti-calnexin (**c**), and anti-GFP (**d**)) showing the EVs secreted from CD63-GFP-HeLa under pH5, 6, or 7 condition (48 hr) (**a**, **b**), lysate of CD63-GFP-HeLa (pH7, 48 hr) and CD63-GFP-EVs (pH7, 48 hr) (**c**), and lysate of CD63-GFP-HeLa (pH7 or pH5, 48 hr) (**d**).



Supplementary Figure S5. Cell viability under cellular EV uptake experiments in serum-starved condition. (**a**) Microscopic observation of A431 cells treated with isolated CD63-GFP-EVs (20 μg/mL for 48 h at 37°C in MEM (pH 5 or 7) without FBS) secreted under pH 7 or pH 5 cell culture condition. (**b**, **c**) Living (**b**) or dead (**c**) cell counting assay after cellular EV uptake experiments of (**a**) in trypan blue staining method. The data are expressed as the mean (± SD) of three experiments.



Supplementary Figure S6. Cell viability under cellular EV uptake experiments in 10% FBS containing condition. (**a**) Microscopic observation of A431 cells treated with isolated CD63-GFP-EVs (20 μg/mL for 24 h at 37°C in MEM (pH 7) with 10% FBS), secreted under pH 7 or pH 5 cell culture condition. (**b**, **c**) Living (**b**) or dead (**c**) cell counting assay after cellular EV uptake experiments of (**a**) by trypan blue staining. The data are expressed as the mean (± SD) of three experiments.