Molecular Biology of the Cell (re-revised manuscript: E11-08-0683)

Cell surface-annexins regulate ADAM-mediated ectodomain shedding of pro-amphiregulin

Hironao Nakayama, Shinji Fukuda, Hirofumi Inoue, Hisayo Nishida-Fukuda, Yuji Shirakata, Koji Hashimoto and Shigeki Higashiyama

Figure S1_Nakayama

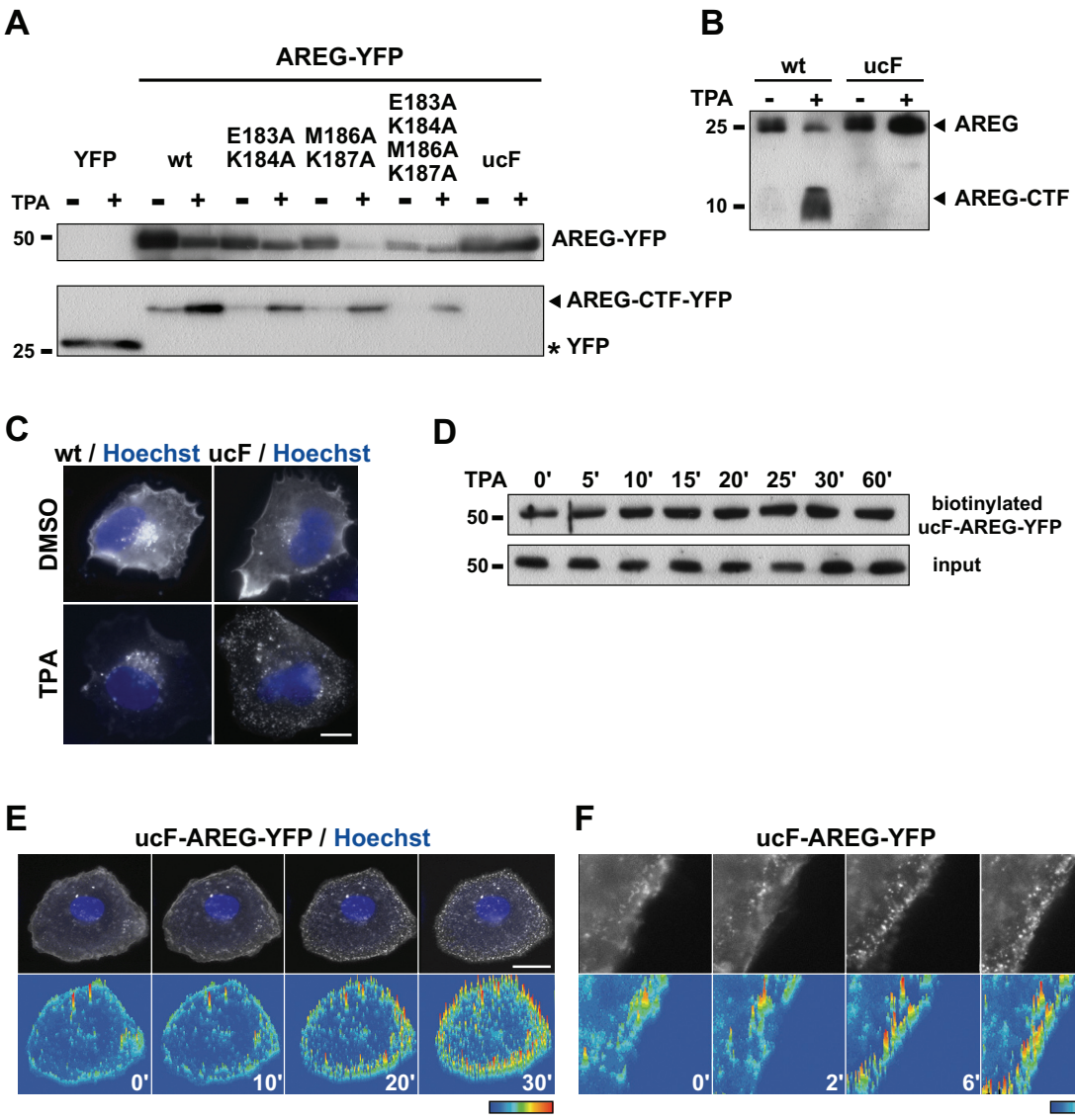

Fig. S1. Uncleavable mutant proAREG is efficiently assembled after TPA stimulation. (A) HT1080 cells were transiently transfected with YFP-fused wt- or mutant proAREG expression vectors. Cells were incubated with 100 nM TPA for 60 min. Western blotting was performed with an anti-GFP antibody. (B) HT1080 cells were transiently transfected with wt- or ucF-proAREG expression vectors. Cells were incubated with 100 nM TPA for 60 min. Western blotting was performed with an anti-AREG-CTF antibody. (C) HT1080 cells stably expressing untagged wt- or ucF-proAREG were incubated with 20 nM TPA for 30 min. Cells were immunostained with an anti-AREG-N antibody. (D) The ucF-proAREG-YFP cells were incubated with 100 nM TPA for the indicated time intervals, and biotinylated with a membrane-impermeable biotinylation reagent. The biotinylated proteins were immunoprecipitated with an anti-GFP antibody, and analyzed by western blotting using HRP-conjugated streptavidin. (E, F) Cells were treated with 10 μg/ml cycloheximide for 4 h

to block *de novo* protein synthesis. The time-lapse images of ucF-proAREG-YFP were obtained at the indicated time points after 20 nM TPA stimulation. The fluorescent signal was visualized in color images with LuminaVision. The scale bars indicate 20 μm.

Figure S2_Nakayama

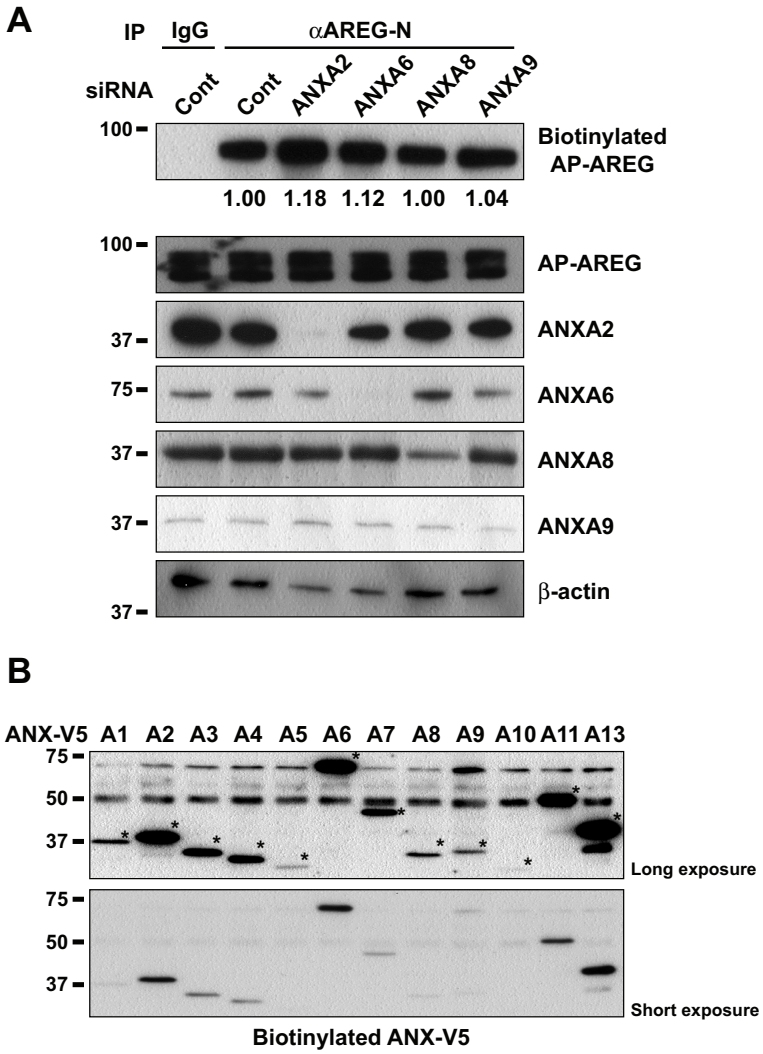

Fig. S2. Annexins are expressed on the cell surface. (A) AP-AREG cells were transfected with siRNA, and biotinylated with a membrane-impermeable biotinylation reagent. Biotinylated proteins were immunoprecipitated with normal goat IgG or an anti-AREG-N antibody, and analyzed by western blotting using HRP-conjugated streptavidin. The intensity of biotinylated AP-AREG bands is represented as fold change relative to control siRNA. (B) HT1080 cells were transiently transfected with ANX-V5, and biotinylated with a membrane-impermeable biotinylation reagent. Biotinylated proteins were immunoprecipitated with an anti-V5 antibody, and analyzed by western blotting using HRP-conjugated streptavidin. The asterisks indicate biotinylated annexins tagged with V5-epitope.

Figure S3_Nakayama

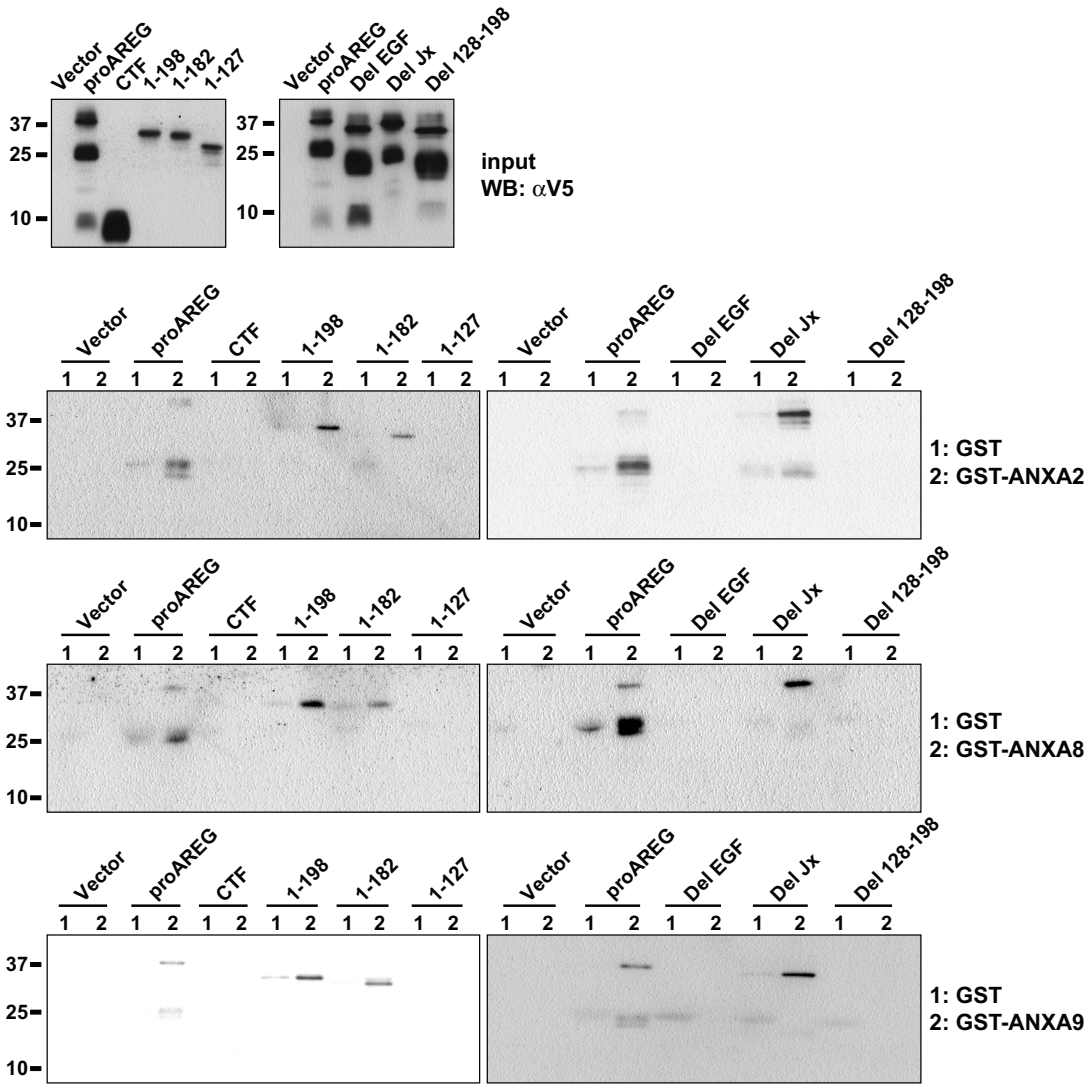

Fig. S3. Annexins interact with EGF-like domain of proAREG. A GST pull-down assay was performed using annexins produced in BL21 as GST fusion proteins. Cell lysates containing the V5-tagged AREG derivatives were incubated with glutathione-sepharose beads immobilized to GST or GST-annexin, and bound proteins were detected by western blotting with an anti-V5 antibody. Expression of the derivatives was also confirmed by western blotting using the same antibody.

Figure S4_Nakayama

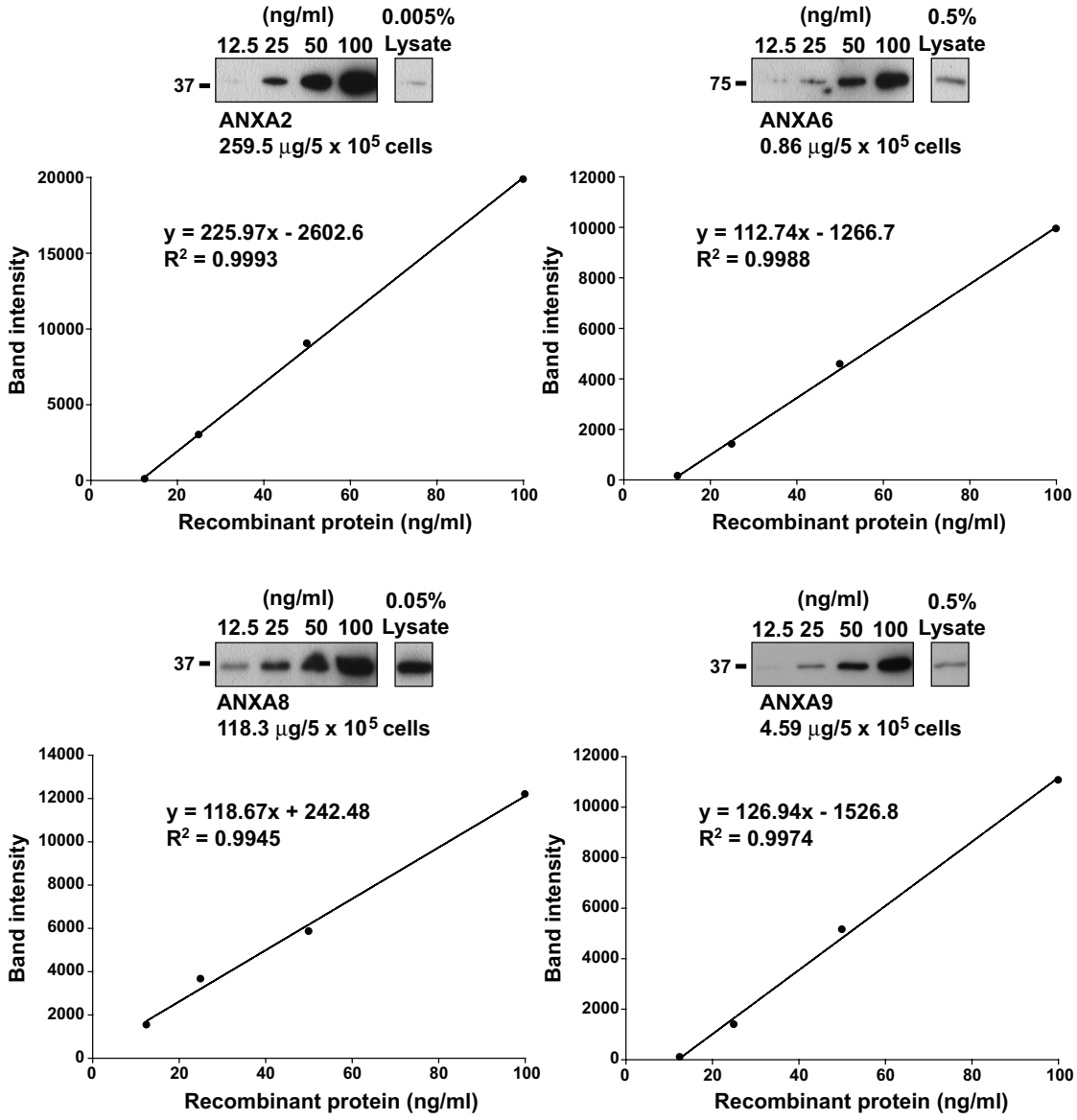

Fig. S4. Protein levels of annexins in human primary keratinocytes. Recombinant annexins were produced in BL21 as GST fusion proteins. Purified proteins were treated with PreScission Protease to remove GST moieties, then used as protein standards. The amount of endogenously expressed annexins in keratinocytes was calculated from the standard curve. Data represent the content of annexin proteins (μg) per 5×10^5 keratinocytes.

Figure S5_Nakayama

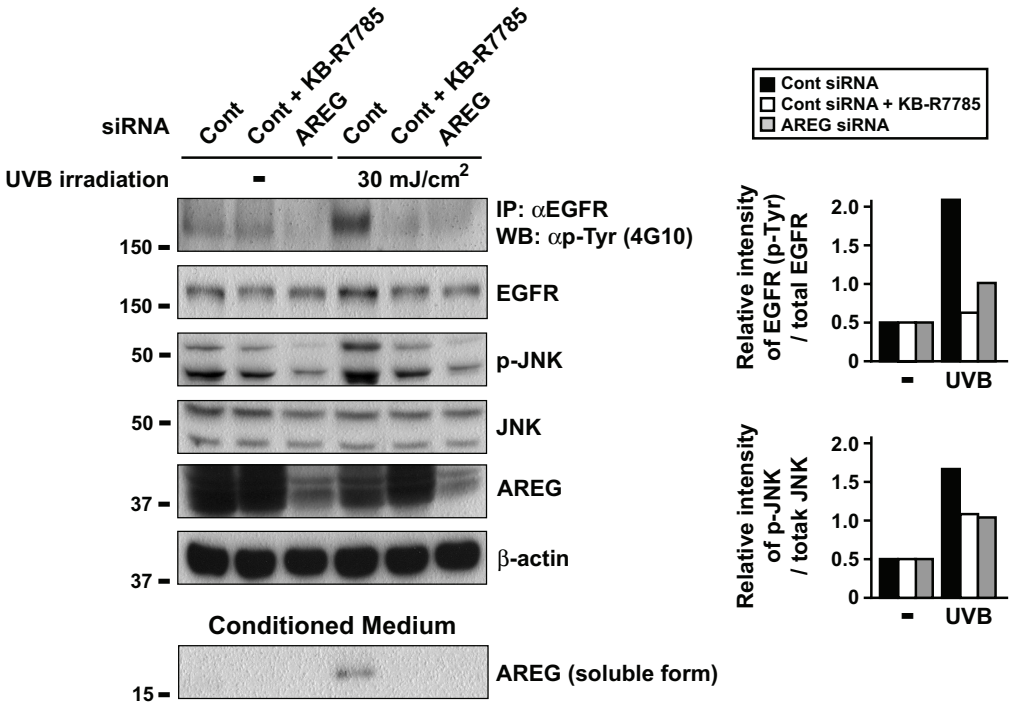

Fig. S5. UV-induced EGFR signal transactivation is dependent on ectodomain shedding in keratinocytes. Keratinocytes were transfected with siRNA. The day before the UVB experiment, cells were incubated with BHE-free medium. Thirty minutes before UVB exposure, cells were re-fed with BHE-free medium with or without KB-R7785 (10 μM). Cells were irradiated with UVB light (30 mJ/cm^2) and incubated for 30 min. Cells were immunoprecipitated with an anti-EGFR antibody. Cell lysates and precipitates were analyzed by western blotting. The intensity of UVB-induced phosphorylated EGFR and JNK was normalized to the respective total protein content, and represented as fold change relative to UVB-untreated cells. Soluble peptides present in the conditioned medium were extracted with a 20% TCA solution.

Figure S6_Nakayama

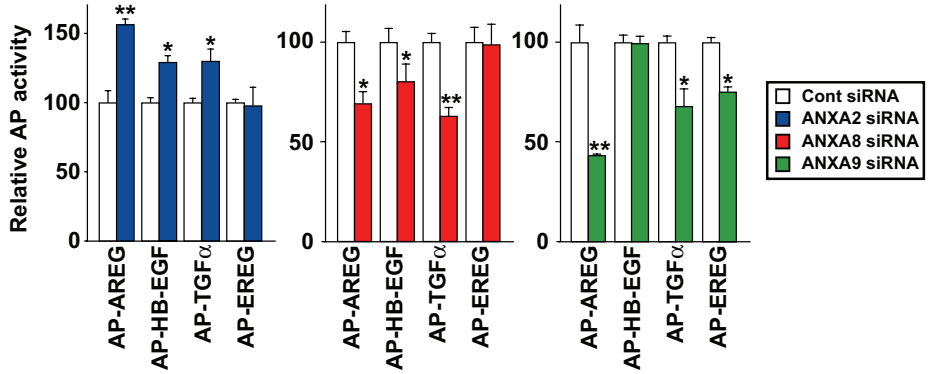

Fig. S6. Annexins work as a shedding platform for EGF family ligands. AP activity was measured in the conditioned medium from HT1080 cells stably expressing each AP-tagged ligand. Cells were transfected with siRNA, and stimulated with 20 nM TPA for 30 min. Data represent the mean ± s.e.m., *$p < 0.05$, **$p < 0.01$.

Table S1_Nakayama

Band NO.	Protein	gi
1	MCF.2 cell line derived transforming sequence-like 2, isoform CRA_c	gi|119598737
2	GRP78 precursor	gi|386758
	albumin, isoform CRA_t	gi|119626083
3	amphiregulin preproprotein	gi|4502199
4	amphiregulin preproprotein	gi|4502199
	actin prepeptide	gi|178067
	annexin VIII	gi|178701
5	anti-colorectal carcinoma heavy chain	gi|425518
6	Csa-19	gi|531171

Table S1. Putative AREG-interacting proteins identified in this study. Band numbers indicate results of the mass spectrometric analysis shown in Fig. 2A.