Extracellularly Extruded Syntaxin-4 Is a Potent Cornification Regulator of Epidermal Keratinocytes

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Supplementary Figure S1. Effect of a recombinant form of syntaxin-4 (r-stx4) and rabbit antiserum against syntaxin-4 (anti-stx4 pAbs) on the epidermal histogenesis. Skin rudiments prepared from 13-g.d. mouse embryos were cultured for seven days with medium that contained r-Stx4 or r-GFP (a dorsal skin culture), and antibodies against r-stx4 or against AHF (an upper lip skin culture). Pictures represent hematoxylin-stained transverse sections of the rudiments before (Day 0, lower left) and after (Day 7, right) the cultivation. Upper left, a schematic image of the organ culture. Yellow bar, epidermis. Red bar, horny layer. *, epidermal basal layer. Scale bar, 10 μm. Antistx4 pAbs attenuated and r-stx4 promoted formation of the denucleated horny layer.

Experimental Methods: Small pieces of skin rudiments were microsurgically collected from 13-g.d embryos of ICR mice (Japan SLC) and placed on a transparent porous membrane floating on the medium and cultured for one week. Upper lip or dorsal skin rudiments were used to test the inhibitory or inductive effect of extracellular syntaxin-4, respectively, since the epidermis of the upper lip is supported by the thick mesenchyme and undergoes vigorous stratification and differentiation in this culture. Rabbit antibodies against syntaxin-4 or those against cytoplasmic protein AHF, both of which were similarly purified by an ammonium sulfate precipitation method from the antiserum, were added to the upper lip skin culture at a final concentration of 1%. A recombinant form of syntaxin-4 or GFP was added to the dorsal skin culture at 50 μg/ml.

Supplementary Figure S2. Number of cells bound to the surface coated with syntaxin-4-mutant proteins. The recombinant forms of soluble syntaxin-4 mutants were prepared as described for r-stx4—see ref. (11) in the paper. HaCaT cells suspended in DH medium supplemented with 5% BSA were seeded onto wells of 96-well plate (40,000 cells/well), each of which has been coated with one of these recombinant proteins (~15 μg/well). After 2.5 hours, non-adherent cells were washed away with medium and the relative number of cells bound to each syntaxin-4 mutant was measured using Alamar Blue reagent (Bio-rad). As a control, well coated with r-GFP (15 μg/well) was used. HaCaT cells appeared to bind to StxΔ4 and Stx(2)4, but not to StxG4. ***, P <0.01, N=4.
Supplementary Figure S3. Effect of ST4n1 in epidermal hyperkeratosis generated with low-Mg diet in HR-1 mice. (a), Schematic diagram of the experimental procedure. (b), Cryosections of dorsal skin of HR-1 mice treated with 50% ethanol (placebo) or ST4n1 in 50% ethanol (ST4n1) were stained with hematoxylin (H) or DAPI. Arrows, cells with DAPI-positive but hematoxylin-negative nuclei. Scale bar, 10 μm. (c), Analyses of the epidermis from three independent animals (A–C) for each category. Three different sections for each animal were analyzed. The DAPI-positive and hematoxylin-negative property may be attributable to the excess formation of CCE in the as-yet-immature keratinocyte. ST4n1 reduced the frequency of the appearance of this abnormal keratinocytes (upper), even when the thickness of the epidermis was not significantly altered (lower). (d) HaCaT keratinocytes with calcium influx-triggered CCE often demonstrated similar staining properties.

Experimental Method: Six male Hr-1 mice (4-weeks of age) were fed a low-Magnesium diet (Hoshino). After 40 days, these mice were divided into two groups and the shriveled dorsal skins were treated daily with the placebo or peptide sample every week day for 4 weeks.