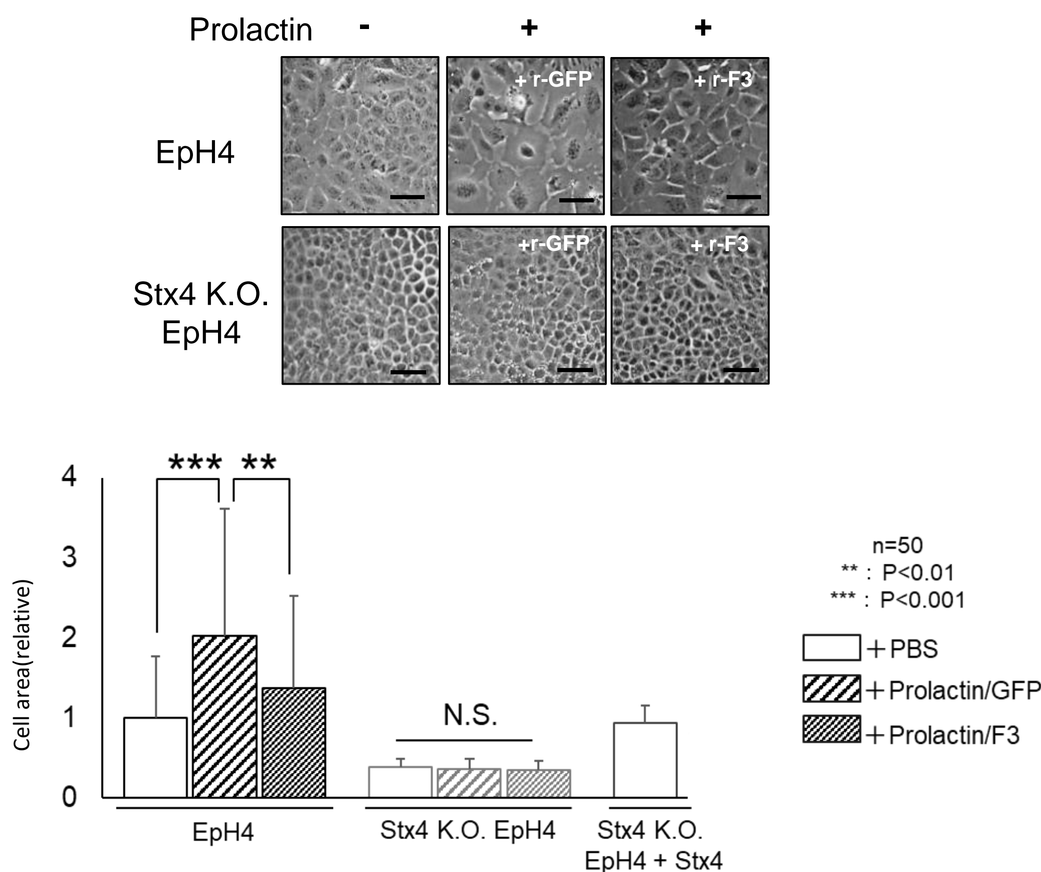

Fig. S1. Prolactin induced flattened morphology in parent EpH4 cells, but not in Stx4 K.O. EpH4 cells, which was blocked by the addition of membrane-impermeable syntaxin4 antagonist r-F3 (Hirose et al., 2017), but not of r-GFP control (50 µg/ml). As Stx4 K.O. clones generated by different gRNAs behaved similarly and exogenous syntaxin4 could be expressed in clone2, the cell size was quantitated only for clone2. Compared to parent EpH4 cells, the size of Stx4 K.O. cells was apparently small, however, re-expression of syntaxin4 recovered the cell size. Bars, 50 µm. Area occupied by a cell is shown. n= 40, ***; p< 0.001, **; p< 0.01.

	EpH4 +prolactin	T7-Stx4 EpH4 (ON) +prolactin	Sig-T7-Stx4 EpH4(ON)
Cells with extracellular syntaxin-4	partial	partial	total
phenotype	multiple lumen	multiple lumen	single lumen

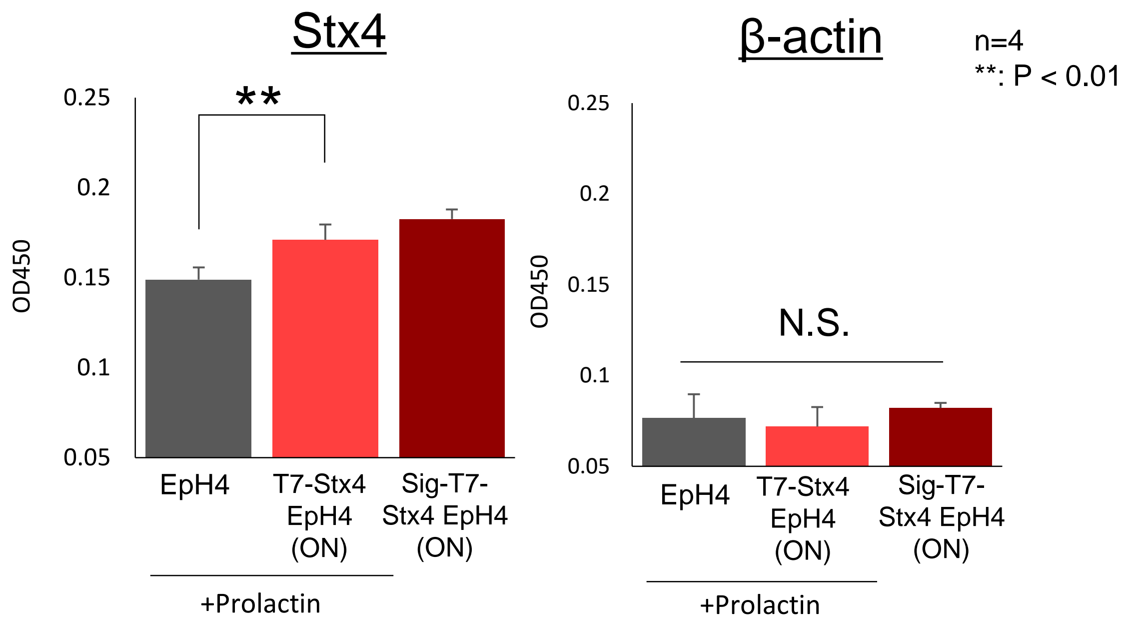

Fig. S2. Upper, the relationship between the mode of expression of extracellular syntaxin-4 and the phenotype. Lower, amount of extracellular syntaxin4 (Stx4) in parental EpH4 cells with prolactin, T7-Stx4 EpH4 cells (ON) with prolactin, and Sig-T7 Stx4 EpH4 cells (ON) without prolactin. Non-permeabilized cells in 96-well plate were incubated with primary antibody against syntaxin4 or β-actin, and with HRP-conjugated secondary antibodies. After excessive washing with TBS, amount of syntaxin4 or β-actin on the cell surface was quantified with TMB solution (Scy Tek, UT, USA) using the plate leader (Thermo Scientific, Finland) . n = 4. **: $P < 0.01$. As the signal intensity of syntaxin4 in the permeabilized cells is weaker than or similar to that of β-actin (Fig 1B), the lower left graph reflects the expression amount of extracellular syntaxin4.

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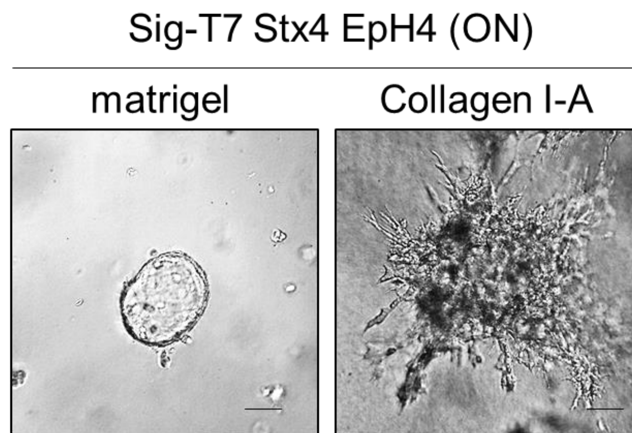

Fig. S3. Sig-T7 Stx4 EpH4 cell aggregates with extracellular syntaxin-4 (ON) were embedded in Matrigel or collagen I and compared the morphological appearance on day 5. In collagen gel Sig-T7 Stx4 EpH4 cells scattered/disseminated and never underwent luminal morphogenesis.

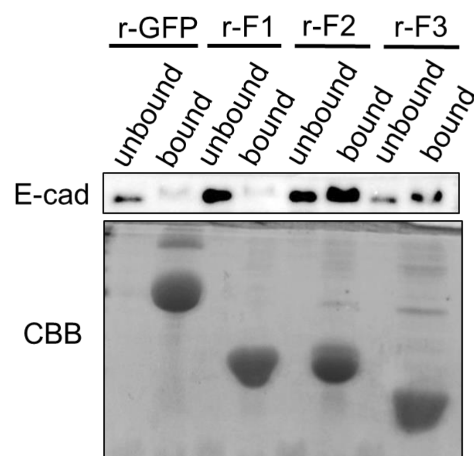

Fig. S4. Syntaxin4 binds to E-cadherin in EpH4 cells via its central and membrane proximal domain. Syntaxin4 fragments (r-F1:Met1-Glu110, r-F2:Ala111-Arg197, r-F3:Glu198-Lys272) tagged with 6X histidine residues were prepared, trapped to Ni-NTA agarose beads, and incubated with EpH4 cell lysate. Unbound and bound materials to the beads were collected and analyzed for E-cadherin by immunoblotting (upper). Equivalent amount of each fragment on the beads was apparent, as judged by Coomassie Brilliant Blue (CBB) staining (lower). The central domain (F2) and membrane proximal domain (F3) bound to E-cadherin expressed in EpH4 cells.

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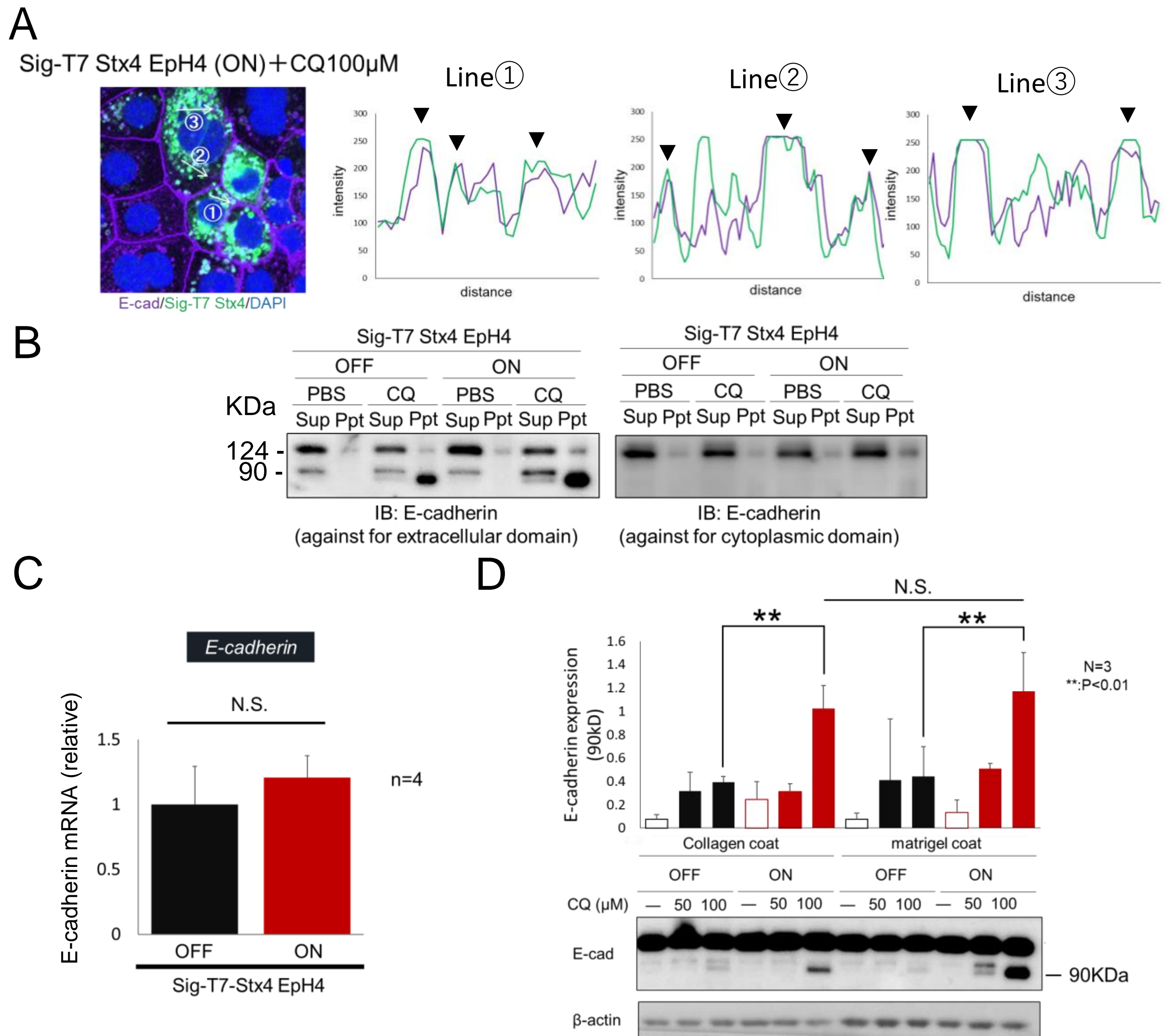

Fig. S5. A, Line scan analyses of E-cadherin (magenta) and sig-T7-Stx4 (green) in sig-T7-Stx4 EpH4 (ON) treated with CQ (same as a lower panel in Fig. 6E). Signal intensities of E-cadherin and sig-T7-Stx4 were analyzed along lines ①, ②, ③ using imageJ. Overlap of each maximum signal intensity shows co-localization of E-cadherin and sig-T7-Stx4 (triangles). B, The 90 kDa form of E-cadherin lacks cytoplasmic tail for cytoskeletal linkage. A monoclonal antibody against extracellular domain of E-cadherin (ECCD2) bound both the full length and the 90 kDa form of E-cadherin, whereas polyclonal antibodies against the cytoplasmic tail (Takara, Japan) failed to recognize the latter. **C**, Expression of extracellular syntaxin4 for 3 days did not affect the expression of E-cadherin mRNA in EpH4 cells. n= 4. D, Signals from Matrigel did not affect the generation of 90 kDa E-cadherin. Sig-T7 Stx4 EpH4 cells on Matrigel or collagen I were treated with various amount of CQ, then the expression of E-cadherin was analyzed. These cells produced 90kDa E-cadherin in response to extracellular syntaxin4 even on Matrigel. N= 3, **; p< 0.01.

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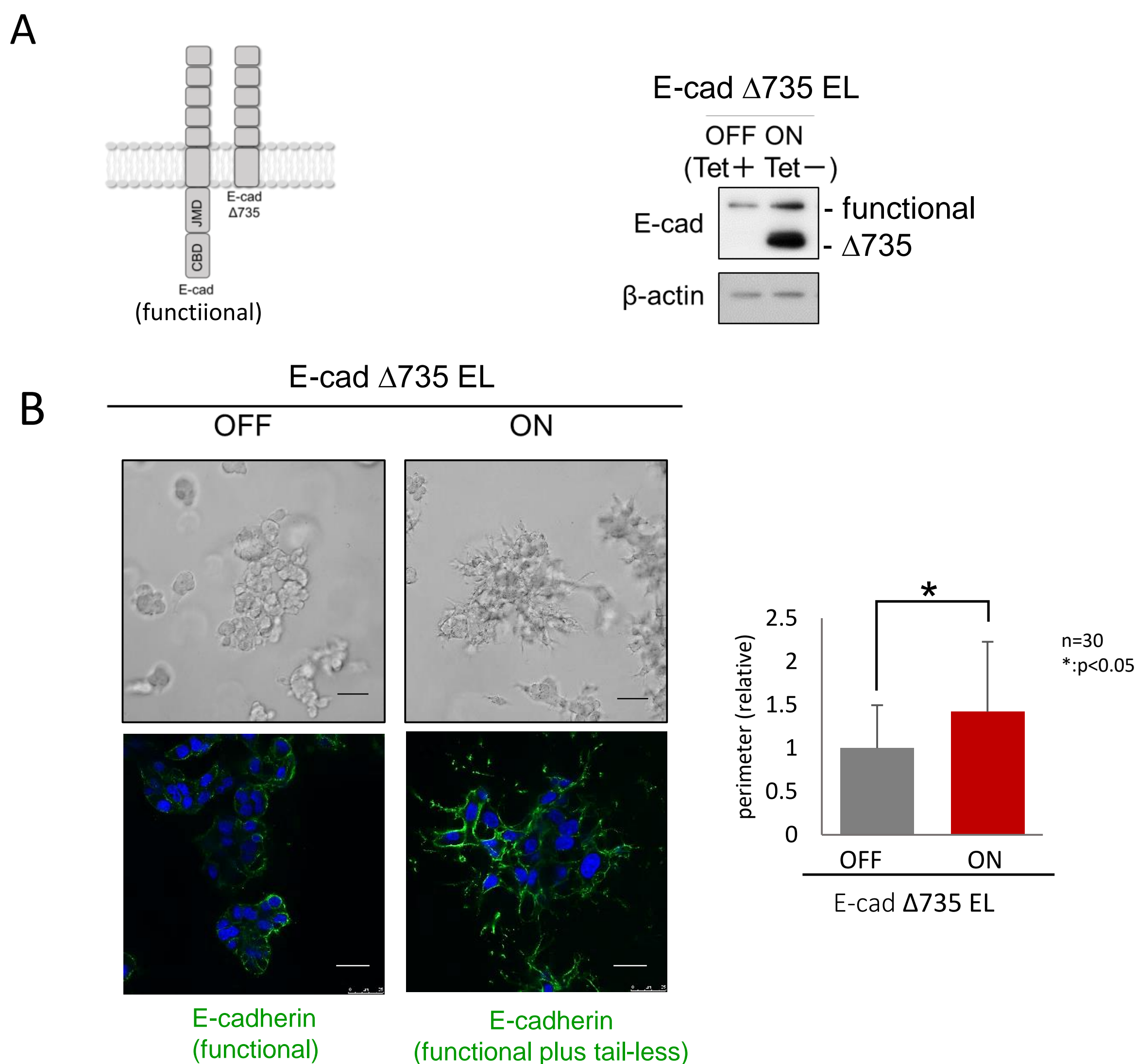


Fig. S6. A, Left, a schematic image of full length (functional) and "tailless" E-cadherin-mutant (E-cad Δ735: Met1~Arg735). Right, EL cells with inducible expression of "tailless" E-cadherin-mutant (E-cadΔ735 EL). EL cells were stably transfected with the PiggyBac-based tet-regulatable expression plasmid containing cDNA for Met1-Arg735 in *E-cadherin* (NCBI 12550). Upon removal of tetracycline (ON), these cells expressed "tailless" E-cadherin-mutant without affecting the amount of functional E-cadherin. B, Left, microscopy images and distribution of E-cadherin in E-cadΔ735 EL cell aggregates embedded in Matrigel for three days. Bars, 50 µm (upper) and 25 µm (lower). Right, quantification of the perimeter of the cell aggregates. n= 30, *; $p < 0.05$. Cell aggregates with only functional E-cadherin remained as cell clumps, whereas those additionally with E-cadΔ735 were dissociated and scattered.

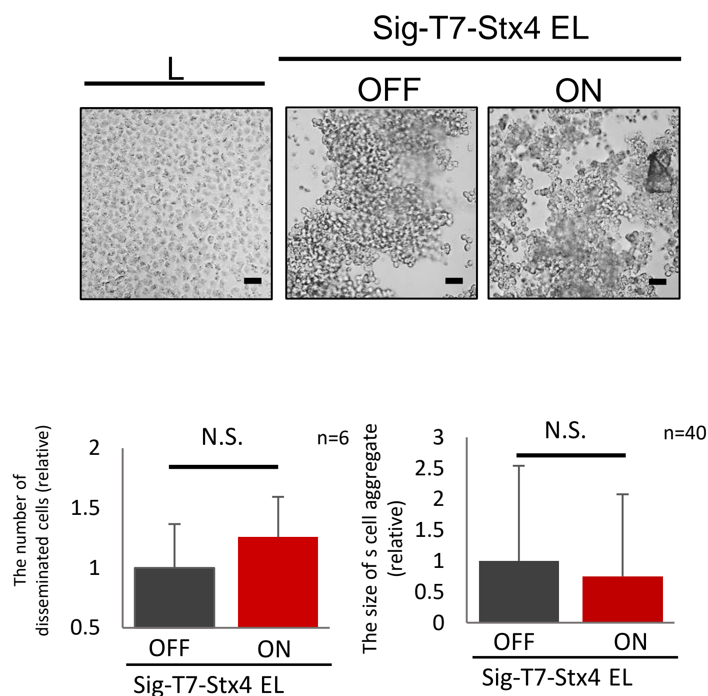
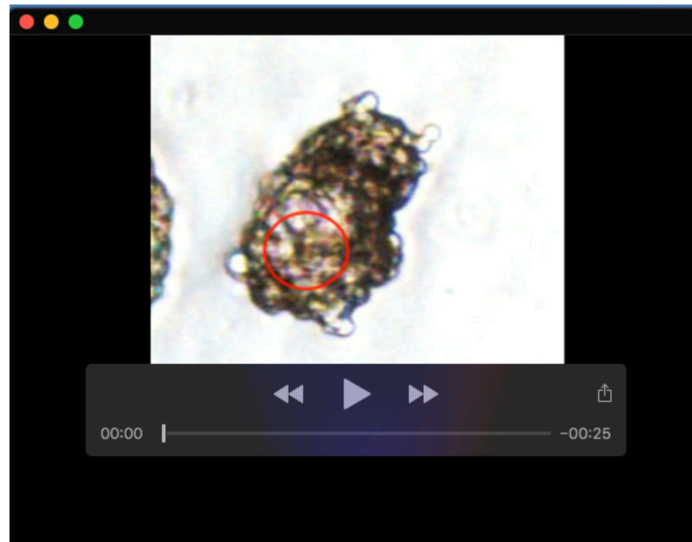


Fig. S7. Upper, light microscopy images of sig-T7-Stx4-EL cells with (ON) and without (OFF) extracellular expression of syntaxin4 in the cell dissociation assay. L cells that have no cadherins were completely dissociated by this treatment. Bars, 50 µm. Lower, relative number of dissociated single cells (left, n= 6), and the size of the undissociated cell aggregates (right, n= 40) after treatment with trypsin in the presence of Ca^{2+}.



Movie 1. Sig-T7-Stx4 EpH4 aggregates were embedded in Matrigel and time-lapse images was acquired from 48 h of culture and ran for 120 h. Movie was constructed from expanded images from 99 h to 164 h when active cell movement was observed. Red circle indicates the inner cell populations in the aggregate.

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