Aberrant claudin-6–adhesion signal promotes endometrial cancer progression via estrogen receptor α

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Abstract

Cell adhesion proteins not only maintain tissue integrity but also possess signaling abilities to organize diverse cellular events in physiological and pathological processes; however, the underlying mechanism remains obscure. Among cell adhesion molecules, the claudin (CLDN) family often possesses aberrant expression in various cancers, but the biological relevance and molecular basis have not yet been established. Here, we show that high CLDN6 expression promotes endometrial cancer progression and represents the poor prognostic marker. The second extracellular domain and Y196/200 of CLDN6 were required to recruit and activate Src-family kinases (SFKs) and to stimulate malignant phenotypes. Importantly, we demonstrate that the CLDN6/SFK/PI3K-dependent AKT and SGK (serum- and glucocorticoid-regulated kinase) signalings target Ser518 in the human estrogen receptor α and ligand-independently activate target genes in endometrial cancer cells, resulting in cancer progression. The identification of this machinery highlights regulation of the transcription factors by cell adhesion to advance cancer progression.

Introduction

Endometrial cancer represents the most common gynecological malignancy in developed countries, with an increased prevalence worldwide (1). Although it has been considered to occur during the postmenopausal period, cases diagnosed in premenopausal women are growing (2). The risk factors for endometrial cancer include an excess of endogenous and exogenous estrogens, older age, obesity, and nulliparity (3, 4). Patients with endometrial cancer are often found at the early stages and possess a relatively favorable prognosis. However, up to 20% of cases recur after primary surgery, and the 5-year overall survival rates for the International Federation of Gynecology and Obstetrics (FIGO) stages III and IV are 57–66% and 20–26%, respectively (5). Therefore, biomarkers that reflect the malignant behavior of endometrial cancer are required to identify patients with poor outcome.

Claudins (CLDNs) are major proteins of tight junctions, the apical-most components of apical junctional complexes (6-9). The CLDN family is composed of 24 members in humans, and displays distinct expression patterns in tissue- and cell-type selective manners. CLDNs also show aberrant expression in a variety of cancer tissues (10). These tetraspanning membrane proteins have a short cytoplasmic N-terminus, two extracellular loops (EC1 and EC2) and a C-terminal cytoplasmic domain. CLDNs act as paracellular barriers or pores via the EC1 to regulate selective transport of ions and substances. On the other hand, CLDN-EC2 participates not only in the binding for *Clostridium Perfringens* enterotoxin (CPE), but also in *trans*-interaction between the plasma membranes of neighboring cells. Furthermore, the C-terminal cytoplasmic domain of CLDNs is thought to propagate intracellular signals, but the underlying molecular basis has not been determined (Cavallalo and Dejana, 2011).

Among the CLDN family, CLDN6 is expressed in several types of embryonic epithelial cells but not largely in normal adult cells (11-15). In addition, CLDN6 is highly expressed in germ cell tumors, including seminomas, embryonal carcinomas and yolk sac tumors, as well as in some cases of gastric adenocarcinomas, lung adenocarcinomas, ovarian adenocarcinomas and endometrial carcinomas (16, 17). However, the biological significance of CLDN6 expression in these cancers remains unclear.

We have recently uncovered that the EC2-dependent engagement of CLDN6 recruits and activates Src-family kinases (SFKs), which in turn phosphorylate CLDN6 at Y196/200 and propagate the PI3K/AKT pathway, and this signaling axis stimulates the retinoic acid receptor γ (RARγ) and estrogen receptor α (ERα) activity (18). Taken together with the notion that ERα acts as a master transcription factor in endometrial cancers (19), we postulated that the CLDN6 signaling modulates the malignant behavior of endometrial cancer cells via ERα. Here, we show that high CLDN6 expression, which expects poor prognosis in endometrial cancer, advances tumor progression. We also demonstrate that the CLDN6/SFK/PI3K axis propagates AKT and SGK (serum- and glucocorticoid-regulated kinase) and targets ERαS518, leading to stimulation of the ERα activity and malignant behaviors in endometrial cancer cells.

Results

Establishment of an anti-human CLDN6 mAb

We first generated a novel monoclonal antibody (mAb) against the C-terminal cytoplasmic region of human CLDN6 (*Supplementary Figure S1A*) using the iliac lymph node method (20). Among 384 hybridomas, 24 clones were selected by enzyme-linked immunosorbent assay (ELISA), 20 of which were able to detect CLDN6 by Western blot in HEK293T cells transfected with the corresponding expression vector (*Supplementary Figure S1B and C*). To check the specificity of an anti-human CLDN6 mAb (clone #15) and the previously established anti-mouse CLDN6 polyclonal antibody (pAb; 21), HEK293T cells were transiently transfected with individual CLDN expression vectors, followed by Western blot and immunohistochemical analyses. Clone #15 selectively recognized CLDN6 but not CLDN1, CLDN4, CLDN5 or CLDN9, which are closely related to CLDN6 within the CLDN family (*Supplementary Figure S1D and E*). On the other hand, the anti-CLDN6 pAb reacted not only with CLDN6 but also with overexpressed CLDN4 and CLDN5 to a lesser extent. We also clarified the complementarity–determining regions of clone #15 (*Supplementary Figure S1F*).

High expression of CLDN6 correlates with poor prognosis in endometrial cancer

Using immunohistochemistry, we next evaluated the expression of CLDN6 in endometrial cancer tissues that resected from 173 patients. Based on semi-quantification using the immunoreactive score, 10 of the 173 cases (5.8%) showed high CLDN6 expression (score 3+). Among the low expression group, 19 (11.0%), 18 (10.4%) and 126 (72.8%) cases had scores 2+, 1+ and 0, respectively. CLDN6 was distributed along the cell membranes of endometrial carcinoma cells (*Figure 1A*). Interestingly, CLDN6 exhibited intratumor heterogeneity, and CLDN6-positive and negative subpopulations were observed in endometrial cancer tissues even in the high CLDN6 expression subjects (*Figure 1B*).

Kaplan-Meier plots revealed significant differences in overall survival and recurrence-free survival between the two groups (*Figure 1C* and *Supplementary Figure S2*). The five-year survival rate in the high CLDN6 expression group remained at approximately 30%, whereas that in the low expression group was 90%. Among the clinicopathological factors, the high CLDN6 expression was significantly associated with surgical stages

III/IV (p<0.001), histological type (p=0.030), histological grade 3 (p=0.004), lymphovascular space involvement (LVSI; p=0.005), lymph node metastasis (p=0.001) and distant metastasis (p<0.001), but not with younger age (*Supplementary Table S1*). In addition, using the Cox multivariable analysis, stages III/IV (hazard ratio [HR] 10.93, p=0.002), distant metastasis (HR 4.68, p=0.006) and high CLDN6 expression (HR 3.50, p=0.014) possessed independent prognostic variables for overall survival of endometrial cancer patients (*Supplementary Table S2*).

CLDN6 promotes malignant phenotypes of endometrial carcinoma cells *in vitro* and *in vivo*

We subsequently generated, using the lentiviral vector system, the human endometrial carcinoma cell line Ishikawa expressing CLDN6 (Ishikawa:*CLDN6*; *Figure 2A*). CLDN6 was detected along the cell borders in Ishikawa:*CLDN6* cells, indicating that CLDN6 acted as a cell adhesion molecule (*Figure 2B*). BrdU assay revealed that cellular proliferation was significantly increased in Ishikawa:*CLDN6* cells compared with parental Ishikawa cells (*Figure 2C and D*). In contrast, on the TUNEL assay, few apoptotic cells were observed in both cell lines (*Supplementary Figure S3*). Moreover, wound healing assay demonstrated that cell migration in Ishikawa:*CLDN6* cells was significantly accelerated compared with that in Ishikawa cells (*Figure 2E and F*).

We then validated whether the high CLDN6 expression also promoted malignant phenotypes of human endometrial carcinoma cells *in vivo*. Four weeks after inoculation in SCID mice, the tumor weight of Ishikawa:*CLDN6* xenografts was significantly increased compared with that of Ishikawa (*Figure 2G and H*). Neither lymph node nor distant metastasis was grossly evident in these xenografts. Microscopically, Ishikawa:*CLDN6* xenografts were equivalent to Grade 3 endometrial carcinomas that were rich in solid components (*Figure 2I*). Furthermore, intratumor heterogeneity of CLDN6 expression was observed in Ishikawa:*CLDN6* xenograft tissues as in the high CLDN6 expression cases of endometrial cancer subjects. It is also noteworthy that invasion into the fibrous capsule around the tumor was prominent in Ishikawa:*CLDN6* xenografts but hardly in Ishikawa ones.

The EC2 and Y196/200 of CLDN6 are required for the signaling to activate SFKs in endometrial carcinoma cells and to promote their progression

We next verified the involvement of CLDN6-EC2 and CLDN6-Y196/200 in activation of SFKs and formation of the CLDN6/pSFK complex in human endometrial carcinoma cells. Double immunofluorescence staining showed that pSFK appeared to be concentrated to cell boundaries together with CLDN6 in Ishikawa:*CLDN6* cells (*Figure 3A*). When Ishikawa:*CLDN6* cells were exposed to C-terminal half of CPE (C-CPE), which binds to the EC2 of CLDN6 and excludes CLDN6 from cell membranes without alteration in its total protein levels (15, 18), the pSFK immunoreactivity was markedly reduced. On Western blot, the levels of pSFK were elevated in Ishikawa:*CLDN6* cells compared with Ishikawa cells, and decreased in both Ishikawa:*CLDN6Y196A* and Ishikawa:*CLDN6Y200A* cells (*Figure 3B*). Immunoprecipitation assay revealed that CLDN6 was associated with pSFK in Ishikawa:*CLDN6* cells, and the CLDN6/pSFK complex was diminished in Ishikawa:*CLDN6* cells on C-CPE treatment as well as in Ishikawa:*CLDN6Y196A* and Ishikawa:*CLDN6Y200A* cells (*Figure 3C and D*).

We also demonstrated that CLDN6 was highly tyrosine-phosphorylated in Ishikawa:*CLDN6* cells, and the phospho-tyrosine levels were suppressed by C-CPE exposure and in both Ishikawa:*CLDN6Y196A* and Ishikawa:*CLDN6Y200A* cells (*Figure 3E and F*). In addition, the promoted cell proliferation and migration in Ishikawa:*CLDN6* cells were reversed by C-CPE treatment (*Figure 2C–F*). Moreover, the CLDN6-enhanced cell proliferation was prevented in Ishikawa:*CLDN6Y196A* or Ishikawa:*CLDN6Y200A* cells (*Figure 3G*). Taken collectively, these results indicated that the CLDN6 signaling activated SFKs and accelerated endometrial cancer progression in the EC2- and Y196/200-dependent manners.

We subsequently validated the involvement of PI3K and the two major downstream cascades AKT and SGK (serum- and glucocorticoid-regulated kinase), which shares the high degree of homology and the same consensus phosphorylation motif (22), in the CLDN6/SFK signaling, using the respective protein kinase inhibitors LY294001, AKT inhibitor VIII and SGK1 inhibitor. The enhanced cell proliferation in Ishikawa:*CLDN6* cells was significantly prevented by these inhibitors and the SFK inhibitor PP2 (*Supplementary Figure S4A*). In addition, the CLDN6-facilitated cell migration in endometrial cancer cells was reversed by these four inhibitors,

though slight but significant difference in migration between Ishikawa and Ishikawa:*CLDN6* cells remained upon the AKT inhibitor VIII treatment (*Supplementary Figure S4B*).

The CLDN6/SFK/PI3K-dependent AKT and SGK signalings target ERα in endometrial carcinoma cells

To evaluate whether the CLDN6-adhesion signaling stimulates the malignant behavior of endometrial carcinoma cells via ERα, we then generated both Ishikawa:*ESR1*$^{-/-}$ and Ishikawa:*CLDN6*:*ESR1*$^{-/-}$ cells, and compared their phenotypes. Knockout of *ESR1* genes in both cell lines was confirmed by DNA sequence, Western blot and immunostaining (*Figure 4A–C*). In the absence of ERα, CLDN6 did not alter cell proliferation or migration capacity in Ishikawa cells (*Figure 4D–G*).

We also used HEC-1A cells, in which neither CLDN6 nor ERα were expressed, and established cell lines expressing either CLDN6, ERα, or both together (*Figure 5A–C*). Cell growth was significantly elevated in HEC-1A:*ESR1:CLDN6* cells but not in HEC-1A:*CLDN6* or HEC-1A:*ESR1* cells compared with parental HEC-1A cells, (*Figure 5D*). Cell migration was also significantly increased in HEC-1A:*ESR1:CLDN6* cells compared with HEC-1A and HEC-1A:*ESR1* cells, and was raised in HEC-1A:*CLDN6* cells less efficiently than in HEC-1A:*ESR1:CLDN6* cells (*Figure 5E*). Taken together, these results strongly suggested that the CLDN6-adhesion signaling links to ERα in endometrial cancer cells. In addition, exposure of HEC-1A:*ESR1:CLDN6* cells to C-CPE reversed the increase in cell proliferation and migration (*Figure 5D and E*), again indicating the critical role of the EC2 in the CLDN6 signaling.

Notably, AKT and SGK1 were associated with transiently introduced ERα, but not with ERαΔC, in Ishikawa:*ESR1*$^{-/-}$ cells (*Figure 6*A and B), indicating that both kinases target either the LBD/AF2 or F region of ERα. We next determined whether the CLDN6 signaling directed to ERαS518 and ligand (estradiol)-independently stimulated the ERα activity in endometrial cancer cells, as in MCF-7 cells (18). To this end, we generated Ishikawa:*CLDN6*:*ESR1*$^{-/-}$:*ESR1-wt (wild-type)* and Ishikawa:*CLDN6*:*ESR1*$^{-/-}$:*ESR1S518A* cells, in the latter of which ERαS518 was substituted for an alanine residue, and cells were grown in phenol red-free medium with charcoal-treated FBS to exclude fat-soluble ligands. As expected, the transcript levels of the four ER target

genes (*BCL2*, *CCND1*, *MYC*, and *VEGFA*; 23) were significantly higher in Ishikawa:*CLDN6* cells than in Ishikawa cells (*Figure 6C*). More importantly, the expression levels of these target genes were significantly reduced in Ishikawa:*CLDN6*:*ESR1*$^{-/-}$:*ESR1S518A* cells compared with those in Ishikawa:*CLDN6*:*ESR1*$^{-/-}$:*ESR1-wt* cells (*Figure 6C*). Furthermore, cell proliferation was decreased in in Ishikawa:*CLDN6*:*ESR1*$^{-/-}$:*ESR1S518A* and HEC-1A:*CLDN6*:*ESR1S518A* cells compared with those in Ishikawa:*CLDN6*:*ESR1*$^{-/-}$:*ESR1-wt* and HEC-1A:*CLDN6*:*ESR1-wt* cells, respectively (*Figure 6D*). In addition, cell migration was significantly diminished in Ishikawa:*CLDN6*:*ESR1*$^{-/-}$:*ESR1S518* cells compared with those in Ishikawa:*CLDN6*:*ESR1*$^{-/-}$:*ESR1-wt* cells (*Figure 6E*). Hence, the CLDN6-adhesion signaling directed to ERαS518 for promoting the ERα activity and malignant phenotypes in endometrial cancer cells.

The CLDN6 signaling ERα-dependently and independently modulates gene expression in endometrial carcinoma cells

To identify downstream molecules that expression is altered by the CLDN6 signaling, we next compared, using RNA sequencing, the transcriptome in Ishikawa:*CLDN6* cells with that in Ishikawa cells (*Figure 7A*). Among the CLDN6-activated genes, the gene products associated with malignant phenotypes, including *ADAMTS18* (a disintegrin and metalloproteinase with thrombospondin motifs; 24) and the transmembrane receptor-associated tyrosine kinase *AXL* (25), as well as the soluble factors *CTGF* (Connective tissue growth factor; 26), *CXCL1* (C-X-C motif ligand 1; 27), *FGFBP1* (Fibroblast growth factor binding protein 1; 28), *NRG1* (Neuregulin 1; 29), *NTN4* (Netrin 4; 30) and *TGFB2* (Tumor growth factor beta 2; 31), were detected. We then by semi-quantitative RT-PCR clarified the expression of these eight genes in Ishikawa, Ishikawa:*CLDN6*, Ishikawa:*ESR1*$^{-/-}$ and Ishikawa:*CLDN6*:*ESR1*$^{-/-}$ cells (*Figure 7B*). CLDN6 appeared to induce the expression of *ADAMTS18*, *AXL*, *CTGF*, *NRG1*, *NTN4* and *TGFB2* transcripts via ERα. By contrast, CLDN6 activated the mRNA expression of *CXCL1* and *FGFBP1* in an ERα-independent manner. Thus, the CLDN6-activated genes can be classified into at least two groups with distinct ERα-dependence.

Discussion

In the present study, we demonstrated that high CLDN6 expression in endometrial cancer tissues, in which the strong and moderate signal intensity on cell membranes was observed at greater than 30% and 50%, respectively, was significantly related to several clinicopathological features such as surgical stages III/IV, histological type, histological grade 3, LVSI, lymph node metastasis and distant metastasis. Importantly, the high CLDN6 expression represented an independent prognostic factor (HR 3.50), and the five-year survival rate was about 30%, which was one third of that in the low expression group. Thus, the aberrant CLDN6 expression appeared to corelate with poor outcome in patients with endometrial cancer. Taken together with the finding that CLDN6 is barely expressed in normal adult cells as described above, the established anti-human CLDN6 mAbs would provide powerful tools that selectively recognize CLDN6 protein in a range of cancer tissues. Extremely high

CLDNs comprise a gene family as described above, and some anti-CLDN Abs are known to react not only with the corresponding CLDN but also with other CLDN subtypes (32). Therefore, it is of particular importance to verify the specificity of the anti-CLDN Abs used. Along this line, we previously established the anti-CLDN pAbs that selectively recognize CLDN1, CLDN5, CLDN6, CLDN7, CLDN12 or CLDN15 as far as we determined (33-35). The anti-CLDN6 pAb is one of the most reliable anti-CLDN6 Abs, and is used for immunohistochemical staining of formalin-fixed paraffin-embedded human tissues (17, 35, 36). However, we noticed in the present work that it also reacted with highly expressed CLDN4 and CLDN5 less efficiently than CLDN6, reinforcing the importance of validating the selectivity of each anti-CLDN Ab.

We also showed that CLDN6 accelerated endometrial cancer progression *in vitro* and *in vivo*. This was obvious because introduction of the human *CLDN6* gene was enough to promote cell proliferation and migration in two distinct endometrial cancer cell lines Ishikawa and HEC-1A:*ESR1*. In addition, overexpression of CLDN6 in Ishikawa cells led to enhanced tumor growth and invasion into the fibrous capsule in xenografts. Thus, we established the clinicopathological and biological relevance of the high CLDN6 expression in endometrial cancer.

Another finding of the present study is that the EC2 and Y196/200 of CLDN6 are responsible for recruiting and activating SFKs in endometrial cancer cells, as well as promoting the malignant properties. This conclusion was drawn from the following results: 1) the pSFK levels were increased in Ishikawa:*CLDN6* cells but not in Ishikawa:*CLDN6Y196A* or Ishikawa:*CLDN6Y200A* cells; 2) colocalization of CLDN6 and pSFK along cell boundaries was evident in Ishikawa:*CLDN6* cells, and diminished by C-CPE treatment; 3) a CLDN6-pSFK complex was formed in Ishikawa:*CLDN6* cells, and their association was decreased upon C-CPE exposure and in Ishikawa:*CLDN6Y196A* and Ishikawa:*CLDN6Y200A* cells; 4) the increased cell growth and migration in both Ishikawa:*CLDN6* and HEC-1A:*ESR1:CLDN6* cells were abrogated upon C-CPE treatment; 5) the CLDN6-stimulated cell proliferation was not detected in Ishikawa:*CLDN6Y196A* or Ishikawa:*CLDN6Y200A* cells. We also demonstrated that SFKs in turn phosphorylated CLDN6 at both Y196 and Y200, and tyrosine-phosphorylation of CLDN6 was governed by the EC2 domain. We previously reported that similar reciprocal regulation between CLDN6 and SFKs is also observed in mouse F9 stem cells (18), further strengthening our conclusion. Moreover, using the respective protein kinase inhibitors, we revealed that the PI3K-dependent AKT and SGK cascades contributed to the CLDN6/SFK signaling in endometrial cancer progression.

The most important conclusion of the present work is that the CLDN6/SFK/PI3K-dependent AKT and SGK signalings target ERα in endometrial cancer cells. This was apparent because CLDN6-accerelated cell growth and migration were hindered in Ishikawa:*CLDN6*:*ESR1*$^{-/-}$ cells. Using HEC-1A expressing CLDN6 and/or ERα, it was confirmed that the CLDN6 signaling in endometrial cancer advancement was mediated via ERα. Furthermore, AKT and SGK1 formed a complex with ERα in endometrial cancer cells, reinforcing the conclusion. On the other hand, neither kinases were associated with ERαΔC, indicating that they do not directly target the known AKT substrate S167 (37-40) at least in endometrial cancer cells. Instead, our RT-qPCR analysis indicated that the CLDN6 signaling directed to S518 in ERα and ligand-independently activated a range of the oncogenic target genes. We also revealed that ERα-S518 is responsible for the CLDN6-accelerated malignant behaviors in endometrial cancer cells. The pathobiological relevance of the ERαS518 phosphorylation

should be determined not only in endometrial cancer tissues, but also in other hormone-dependent tumors, such as ovarian cancer and breast cancer, in future experiments.

Our RNAseq analysis revealed that a variety of gene expression, including the *SGK1* gene, was altered between Ishikawa and Ishikawa:*CLDN6* cells. Among eight representative gene products associated with tumor progression in various cancers, the *ADAMTS18*, *AXL*, *CTGF*, *NRG1*, *NTN4* and *TGFB2* genes were activated by the ERα-dependent CLDN6 signaling. By contrast, the expression of *CXCL1* and *FGFBP1* transcripts was induced by CLDN6 in an ERα-independent manner. Interestingly, the novel AKT/SGK-consensus phosphorylation motif is conserved in 14 of 48 members of human nuclear receptors (18). Taken together, CLDN6 may also target these nuclear receptors and possibly other transcription factors in order to regulate the expression of certain genes. Importantly, we previously reported that the CLDN6 signal targets RARγ in mouse F9 stem cells to initiate epithelial differentiation (18).

Genomic and non-genomic heterogeneity among distinct cell populations within cancers is known to influence tumour behaviors (41). Our immunohistochemical study revealed intratumor heterogeneity on the CLDN6 expression within human endometrial cancer and Ishikawa:*CLDN6* xenograft tissues. These tumors were composed of CLDN6-positive and negative subpopulations, even in endometrial cancer tissues with high CLDN6 expression. Hence, the expression of CLDN6 should be carefully evaluated when small biopsy specimens and tissue arrays were subjected to immunohistochemistry. Of note, since the gene expression of various diffusive factors was induced in Ishikawa:*CLDN6* as described above, non-cell-autonomous paracrine effects between CLDN6-positive and negative cancer cells may also contribute to the enhancement of tumor progression.

In summary, we here established that high expression of CLDN6 protein in endometrial cancer leads to more aggressive tumors and predicts poor prognosis. We also demonstrated that the CLDN6/SFK/PI3K-dependent AKT and SGK cascades direct to S518 in human ERα and stimulated its activity, resulting in progression of tumor behaviors in endometrial cancer. Therefore, in addition to the PI3K/AKT pathway, which is frequently altered in endometrial cancers (42-45), the CLDN6/SFK, SGK and ERαS518 may be promising therapeutic

targets for endometrial cancer. It would also be interesting to determine whether a similar link between cell adhesion and nuclear receptor signaling regulates tumor progression in various types of cancers.

Materials and Methods

Antibodies

The antibodies used in this study are listed in Supplementary Table S3. A rabbit pAb against CLDN6 was generated in cooperation with Immuno-Biological Laboratories as described previously (21).

Rat mAbs against CLDN6 were established using the iliac lymph node method (20). In brief, a polypeptide, (C)SRGPSEYPTKNYV corresponding to the cytoplasmic domain of CLDN6, was coupled via the cysteine to Imject™ Maleimide-Activated mcKLH (Thermo Fisher SCIENTIFIC). The conjugated peptide was subcutaneously injected with Imject™ Freund's Complete Adjuvant (Themo Fisher SCIENTIFIC) into the footpads of anesthetized eight-week-old female rats. The animals were sacrificed 14 days after immunization, and the median iliac lymph nodes were collected, followed by extraction of lymphocytes by mincing. Extracted lymphocytes were fused to a SP2 mouse myeloma cell line by polyethylene glycol. Hybridoma clones were maintained in GIT medium (Wako) with supplementation of 10% BM-Condimed (Sigma-Aldrich). The supernatants were screened by ELISA.

Tissue collection, immunostaining, and analysis

Paraffin-embedded tissue sections were obtained from 173 patients with uterine endometrial cancer who underwent hysterectomy, bilateral saplingo-oophrectomy, and/or lymphadenectomy between 2003 and 2012 at Fukushima Medical University Hospital (FMUH) and Iwaki City Medical Center (ICMC). Informed consent was obtained from all the patients. The subjects were limited to patients with confirmed 5-year outcomes and who died due to uterine endometrial cancer and metastasis. The clinicopathological characteristics of patients are summarized in Supplementary Table S4. The detailed information, including postoperative pathology diagnosis reports, age, stage (FIGO2008), histological type, histological grade, lymph-vascular space invasion (LVSI), lymph node metastasis, distant metastasis, overall survival (OS), and recurrence-free survival (RFS), were also obtained. The staging of patients between 2003 and 2007 were modified in accordance with the FIGO 2008 systems. Distant metastasis was judged by diagnostic imaging. The study was approved by the ethics committee of FMUH and ICMC.

For immunostaining, uterine endometrial cancer tissues were obtained, and the 10% formalin-fixed and paraffin-embedded tissue blocks were sliced into 5-μm-thick sections, then deparaffinized with xylene and rehydrated using a graduated series of ethanol. The sections were immersed in 0.3% hydrogen peroxide in methanol for 20 min at room temperature to block endogenous peroxidase activity. Antigen retrieval was performed by incubating the sections in boiling citric acid buffer (pH 6.0) for antigen retrieval in a microwave. After blocking with 5% skimmed milk at room temperature for 30 min, the sections were incubated overnight at

4°C with the primary antibodies. Histofine SAB-PO kit for rabbit (Nichirei) or VECTASTAIN Elite ABC HRP Kit for rat (VECTOR LABORATORIES) was used for 3',3'-diaminobenzidine (DAB) staining.

Immunostaining results were interpreted by three independent pathologists and one gynecologist using a semi-quantitative scoring system, immunoreactivity score (IRS; 46). The immunostaining reactions were evaluated according to signal intensity (SI: 0, no stain; 1, weak; 2, moderate; 3, strong) and percentage of positive cells (PP: 0, <1%; 1, 1 to 10%; 2, 11 to 30%; 3, 31 to 50%; and 4, >50%). The SI and PP were then multiplied to generate the IRS for each case. We divided the samples into two groups based on the results of the immunostaining in the tissues: low expression (IRS<8) and high expression (IRS≥8) (Supplementary Table S5).

Cell lines and cell culture

The Ishikawa cell line was obtained from Kasumigaura Medical Center and from Dr.Yamada (Wakayama Medical University). The HEC-1A cell line (47) were obtained from National Institute of Biomedical Innovation, Health and Nutrition (Japan). F9:*Cldn6* was previously established (15). Cells were grown in Roswell Park Memorial Institute (RPMI) 1640 (Ishikawa), McCoy's 5A (HEC-1A), or Dulbecco's Modified Eagle Medium (DMEM; HEK293T and F9:*Cldn6*), with 10% Fetal bovine serum (FBS; Sigma-Aldrich) and 1% Penicillin-streptomycin mixture (Gibco, Waltham, MA). Ishikawa and HEC-1A cells were treated with 1 µM of C-CPE, 10 µM of PP2 (Calbiochem), 10 µM of LY294002 (Cell Signaling TECHNOLOGY), 10 µM of AKT inhibitor VIII (funakoshi), or 0.1 nM of SGK-1 inhibitor (Santa Cruz Technology) one or two days after plating. For preparation of charcoal-treated FBS, 500 ml of FBS was treated with 0.5 g of Charcoal, dextran coated (Sigma) overnight at 4ºC followed by filtration using 0.22 µm cellulose acetate filter membranes. Establishment of stable cell lines, transient overexpression of target genes, and C-CPE production and purification were performed as described previously (18).

Expression vectors and transfection

The protein coding regions of human *CLDN1*, *CLDN4*, *CLDN5*, *CLDN6*, *CLDN9*, and *ESR1* were cloned into the *BamHI*/*Not*I site of the CSII-EF-MCS-IRES2-Venus (RIKEN, RDB04384) plasmid. Hemagglutinin (HA) tag was added by PCR with tailed primer. Expression vectors of mutant genes (*CLDN6Y196A*, *CLDN6Y200A*, *ESR1ΔC*, and *ESR1S518A*) were established by a standard site-directed mutagenesis protocol using KOD -Plus-Mutagenesis Kit (TOYOBO) following the providers protocol.

For transient expression of the target genes (*CLDN1*, *CLDN4*, *CLDN5*, *CLDN6*, *CLDN9*; *SI Appendix*, Fig. S1), 5×10^6 cells were transfected with 10 µg of the indicated vectors using 30 µg of Polyethylenimine Max (PEI Max, Cosmo Bio) 8 h after passage. Lentiviral vectors (*CLDN6*, *CLDN6Y196A*, *CLDN6Y200A*, *ESR1*, *ESR1ΔC*, and *ESR1S518A*) were generated by transfecting HEK293T cells with 10 µg of the CSII plasmids containing the target genes, 5 µg of packaging plasmids psPAX2 (Addgene, #12260) and pCMV-VSV-G-RSV-Rev (RIKEN,

RDB04393) using PEI Max. Culture media containing recombinant lentiviruses were collected 72 h after transfection. The lentiviral vectors were added to cell culture medium of Ishikawa or HEC-1A cell lines after filtration. More than 48 h after transfection, the cells were used for further analysis.

Genome editing

To establish the *ESR1*$^{-/-}$ cell lines, transcription activator-like effector nucleases (TALENs) were designed by TALEN Targeter 2.0 software (https://tale-nt.cac.cornell.edu/node/add/talen; 48). The expression vector of the TALENs were cloned by using Platinum TALEN Kit (49). The plasmids were transiently transfected by Polyethylenimine Max (PEI Max, Cosmo Bio). Next, 24–48 h after transfection, the cells were exposed to 100 µg/ml of hygromycin for positive selection, followed by limiting dilution and genotyping with PCR-based restriction fragment length polymorphism (RFLP; 50).

Immunoprecipitation and immunoblot

Immunoprecipitation was performed using an Immunoprecipitation kit (Protein G, Sigma), following the manufacturer's protocol. Immunoblot analysis was performed as previously described (51). Each blot was stripped with Restore Western blot stripping buffer (Pierce Chemical) and immunoprobed with anti-actin antibody. Signals in the immunoblots were quantified using ImageJ software (Wayne Rasband National Institutes of Health). The protein levels were normalized to the corresponding actin levels, and their relative levels were then presented.

RNA extraction, RT-PCR, and RNA sequencing

RNA extraction and RT-PCR were performed as described previously (18). The primers for RT-PCR are listed in Supplementary Table S6. RNA sequencing and mapping were performed by TaKaRa. The mapped bam files were imported into SeqMonk software (Babraham Bioinformatics; https://www.bioinformatics.babraham.ac.uk/projects/seqmonk/) and were quantitated by the default RNA-Seq quantitation pipline.

Fluorescence Immunohistochemistry

Cells were grown on coverslips coated by Cellmatrix Type I-A (Nitta gelatin). The samples were fixed in 1% paraformaldehyde and 0.1% Triton-X for 10 min at room temperature. After washing with PBS, they were preincubated in PBS containing 5% skimmed milk. They were subsequently incubated overnight at 4ºC with primary antibodies in PBS, then rinsed again with PBS, followed by a reaction for 1 h at room temperature with appropriate secondary antibodies. All samples were examined using a laser-scanning confocal microscope

(FV1000, Olympus). Photographs were processed with Photoshop CC (Adobe) and ImageJ software (Wayne Rasband National Institutes of Health).

Cell proliferation, migration, and apoptosis assays

Cell proliferation index was evaluated by incorporation of bromodeoxy uridine (5-Bromo-2-DeoxyUridine, BrdU, sigma). Cells were exposed to BrdU for 5 min after passage. The specimens were fixed with 4% paraformaldehyde and 0.1% Triton-X, followed by immunostaining with anti-BrdU antibody (BD) and its standard protocol.

For evaluating cell migration, wound areas were generated by scratching with disposable 1,000 μl pippette tips 24–48 h after passage. Culture media were changed daily. Photographs of the wound areas were taken at the same locations, using a phase-contrast microscope. Wound healing was calculated as the percentage of the remaining cell-free area compared with the initial wound area using ImageJ software.

in situ Cell Death Detection Kit (Roche) was used for evaluation of cell apoptosis.

Xenograft model

Xenograft studies were performed in 8-week-old NOD/ShiJic-scid mice (CLEA-Japan). 1×10^7 cells were subcutaneously injected into the back of anesthetized mice. Then, 28 d after injection, the mice were ethically sacrificed. All animal experiments conformed to the National Health Guide for the Care and Use of Laboratory Animals, and were approved by the Animal Committee at Fukushima Medical University.

Statistical analysis

We used the chi-squared test to evaluate the relationship between CLDN6 expression and various clinicopathological parameters (age, stage, histological type, histological grade, LVSI, lymph node metastasis, distant metastasis, 5-year OS, and 5-year RFS). Survival analysis was performed using the Kaplan-Meier method, and differences between the groups were analyzed using the log-rank test. The Cox regression multivariate model was used to detect the independent predictors of survival. Two-tailed P-values less than 0.05 were considered to indicate a statistically significant result. All statistical analyses were performed using SPSS software version 23.0 (IBM).

The PCR values are presented as the mean ± SD from three samples. Original values were quantified by ImageJ software (Wayne Rasband National Institutes of Health). The expression levels of the target genes in RT-PCR were divided by the corresponding *GAPDH* signal intensity. Their relative levels were analyzed by paired sample two-tailed t-test to evaluate statistical significance.

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Author contributions: K.S. and H.C. designed research. M.K., K.S., M.T., N. I.-T., K.K, H.K. performed research; M.K., Y.E., T.H, S.F., H.N., T.W., S.S., and K.F. acquired and managed patients; M.K., K.S., M.T., Y.E., N. I.-T., K.K, H.K., T.H., S.F., H.N., T.W., S.S., K.F., and H.C. analyzed data; and M.K., K.S., and H.C. wrote the paper.

Competing financial interests

The authors declare no competing financial interests.

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Figure legends

Figure 1. Overexpression of CLDN6 is associated with poor outcome in endometrial cancer patients. (*A*) Representative immunohistological images of high and low CLDN6 expression in endometrial cancer tissues. HE, hematoxylin-eosin. Scale bar, 50 μm. (*B*) Intratumor heterogeneity of CLDN6 protein in the high CLDN6 expression subjects of endometrial cancer. The blue and yellow squares indicate CLDN6-positive and negative subpopulations, respectively. Scale bar, 200 μm. (*C*) Kaplan-Meier plots for high and low CLDN6 expression groups in endometrial cancer subjects. *$p<0.001$.

Figure 2. CLDN6 enhances malignant behavior of endometrial carcinoma cells *in vitro* and *in vivo*. (*A* and *B*) Western blot (*A*) and confocal images (*B*) for the indicated proteins in Ishikawa and Ishikawa:*CLDN6* cells. N.S., nonspecific signals. (*C* and *D*) Representative (*C*) and quantitative (*D*) BrdU assay for the indicated cells grown in the presence of absence of 1.0 μg/ml C-CPE. The BrdU/DAPI levels are shown in histograms (mean ± SD; $n = 6$). (*E* and *F*) Typical (*E*) and quantitative (*F*) wound healing assay for the indicated cells grown in the presence of absence of 1.0 μg/ml C-CPE. The values represent wound closure rates (mean ± SD; $n = 12$). (*G–I*) Gross and microscopic appearances (*G* and *I*) and weight (*H*) of the indicated xenografts at 28 d after the inoculation. The tumour weight is shown in histograms (mean ± SD; $n = 4$). The regions corresponding the squares include the fibrous capsule around the xenograft tumors, and are enlarged. The boundaries between cancer tissues and the fibrous capsule around the tumor are shown in dashed green lines. *$p<0.05$. Scale bars, 20 μm (*B* and *C*); 50 μm (*E*); 1 cm (*G*); 200 μm (*I*)

Figure 3. CLDN6 activates SFKs in endometrial carcinoma cells via the EC2 and Y196/200. (*A*) Confocal images for the indicated proteins in Ishikawa and Ishikawa:*CLDN6* cells. Ishikawa:*CLDN6* cells were grown in the presence or absence of 1.0 μg/ml C-CPE. Arrowheads indicate the remaining CLDN6/pSFK signals. Scale bar, 20 μm. (*B*) Western blot for the indicated proteins in the revealed Ishikawa cells. (*C* and *D*) Association between CLDN6 and pSFK in the indicated Ishikawa cell lines. Ishikawa:*CLDN6* cells were exposed to the

vehicle or 1.0 μg/ml C-CPE. (*E* and *F*) Tyrosine-phosphorylation of CLDN6 in Ishikawa:*CLDN6* (*E*) and the indicated Ishikawa mutant cells (*F*). Ishikawa:*CLDN6* cells were cultured in the presence or absence of 1.0 μg/ml C-CPE. Quantification of the protein levels is shown in the histograms. (*G*) BrdU assay for the indicated Ishikawa cells. The BrdU/DAPI levels are shown in histograms (mean ± SD; $n = 6$). *$p<0.05$.

Figure 4. ERα is required for CLDN6-stimulated malignant phenotypes of endometrial carcinoma cells. (*A*) Knockout (KO) of the *ESR1* gene encoding human ERα in Ishikawa cells using the TALEN method. The KO in Ishikawa:*ESR1*$^{-/-}$ cells is confirmed by sequencing. (*B* and *C*) Absence of ERα protein in Ishikawa:*ESR1*$^{-/-}$ and Ishikawa:*CLDN6*:*ESR1*$^{-/-}$ cells on Western blot (*B*) and immunofluorescence (*C*) analyses. (*D* and *E*) Representative (*D*) and quantitative (*E*) BrdU assay for the indicated cells. The BrdU/DAPI levels are shown in histograms (mean ± SD; $n = 6$). (*F* and *G*) Typical (*F*) and quantitative (*G*) wound healing assay for the indicated cells. The values represent wound closure rates (mean ± SD; $n = 12$). Scale bars, 50 μm (*F*); 20 μm (*C* and *D*). N.S., not significant.

Figure 5. Expression of both CLDN6 and ERα accelerates malignant behavior of HEC-1A cells. (*A*) The construct of *ESR1* and/or *CLDN6* expression vector. EF-1α, elongation factor-1α; IRES, internal ribosome entry site; 2A, self-cleaving peptide. (*B* and *C*) Western blot (*B*) and confocal images (*C*) for the indicated proteins in the revealed cell lines. (*D*) BrdU assay for the indicated cells. The BrdU/DAPI levels are shown in histograms (mean ± SD; $n = 6$). (*E*) Wound healing assay for the indicated cells. The values represent wound closure rates (mean ± SD; $n = 12$). HEC-1A: *ESR1*$^{-/-}$:*CLDN6* cells were grown in the presence of absence of 1.0 μg/ml C-CPE (*D* and *E*). *$p<0.05$; **$p<0.01$; ***$p<0.001$. Scale bars, 20 μm.

Figure 6. The CLDN6 signaling targets ERαS518 in endometrial carcinoma cells. (*A*) The construct of wild-type and mutant HA-ESR1 expression vectors. (*B*) Association of between either pAKT or SGK1 and ERα in Ishikawa:*ESR1*$^{-/-}$ cells transiently transfected with the HA-ESR1 expression vector. In the input lanes, 10% for

HA, 1% for SGK1, or 0.1% for AKT of the input protein samples were loaded. (*C*) RT-qPCR for the indicated molecules in the revealed Ishikawa cells. The relative expression levels are shown in the histograms (mean ± SD; $n = 3$). (*D*) BrdU assay for the indicated Ishikawa and HEC-1A cells. The BrdU/DAPI levels are shown in histograms (mean ± SD; $n = 6$). (*E*) Wound healing assay for the revealed Ishikawa cells. The values represent wound closure rates (mean ± SD; $n = 16$). *$p<0.01$; **$p<0.01$; ***$p<0.001$.

Figure 7. The CLDN6 signaling ERα-dependently and independently activates genes in endometrial carcinoma cells. (*A*) Heatmap of RNA sequencing comparing Ishikawa:*CLDN6* to Ishikawa. RNAseq was performed in two biological replicates, and genes for which expression was significantly altered are indicated. (*B*) RT-PCR analysis for the indicated genes in the revealed cell lines. The expression levels relative to *GAPDH* are shown in the histograms (mean ± SD; $n = 3$). N.S., not significant; *$p<0.05$; **$p<0.01$; ***$p<0.001$.

Figure. 1

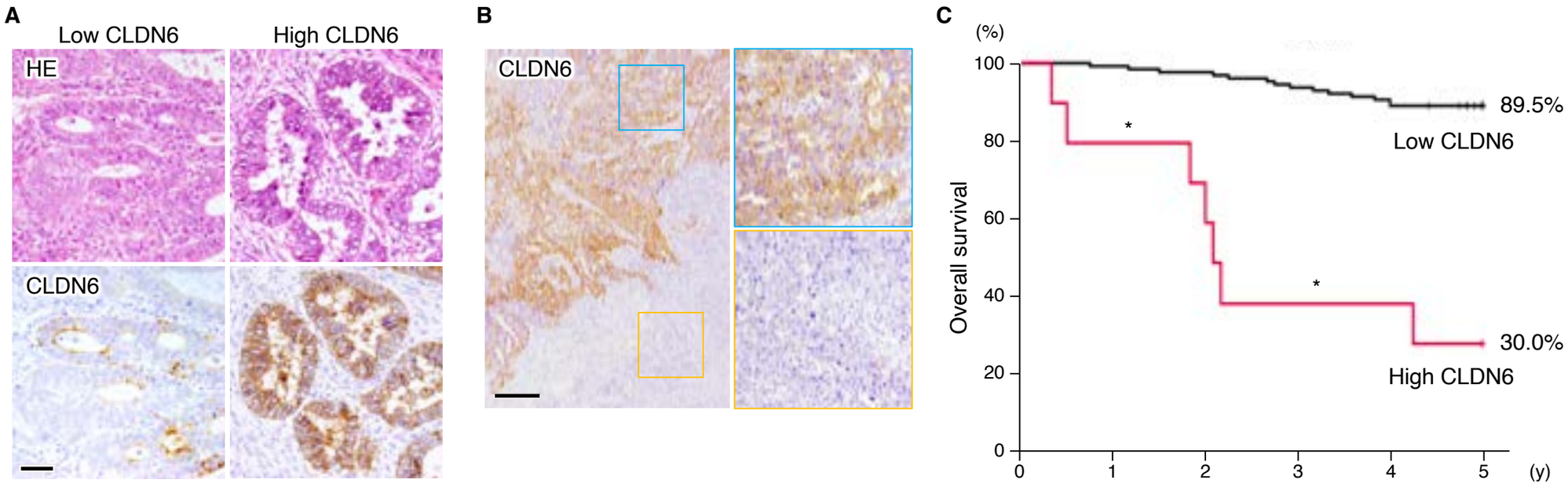

A
Low CLDN6
High CLDN6
HE
CLDN6
B
CLDN6
C
(%)
100
80
60
40
20
0
Overall survival
89.5%
Low CLDN6
30.0%
High CLDN6
*
*
0
1
2
3
4
5
(y)

Figure. 2

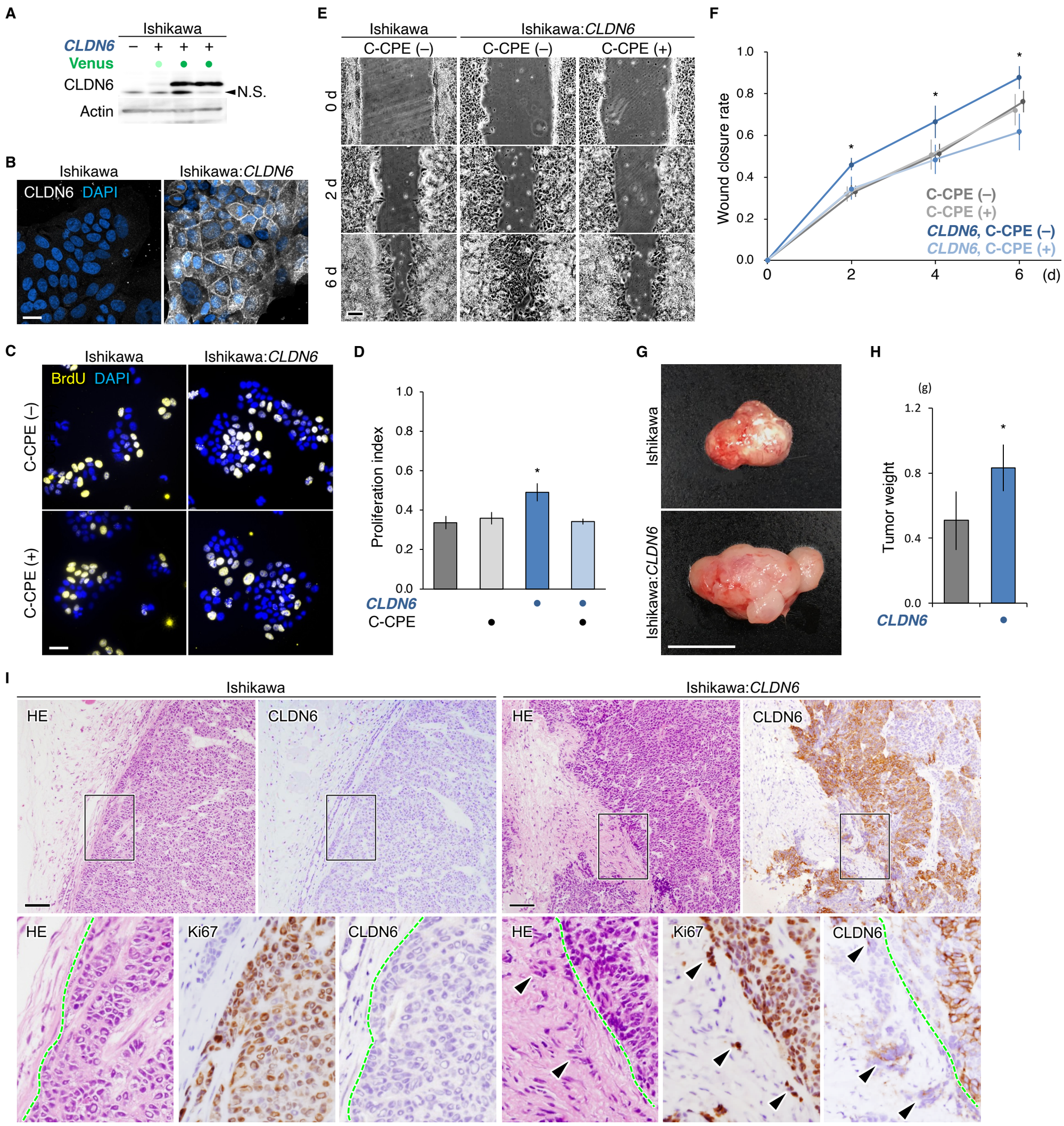

A
Ishikawa
CLDN6 – + + +
Venus
CLDN6
N.S.
Actin
B
Ishikawa
Ishikawa:CLDN6
CLDN6 DAPI
C
Ishikawa
Ishikawa:CLDN6
BrdU DAPI
C-CPE (–)
C-CPE (+)
D
Proliferation index
1.0
0.8
0.6
0.4
0.2
0.0
*
CLDN6
C-CPE
E
Ishikawa
Ishikawa:CLDN6
C-CPE (–)
C-CPE (–)
C-CPE (+)
0 d
2 d
6 d
F
Wound closure rate
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
(d)
C-CPE (–)
C-CPE (+)
CLDN6, C-CPE (–)
CLDN6, C-CPE (+)
G
Ishikawa
Ishikawa:CLDN6
H
(g)
Tumor weight
1.2
0.8
0.4
0.0
CLDN6
I
Ishikawa
Ishikawa:CLDN6
HE
CLDN6
Ki67

Figure. 3

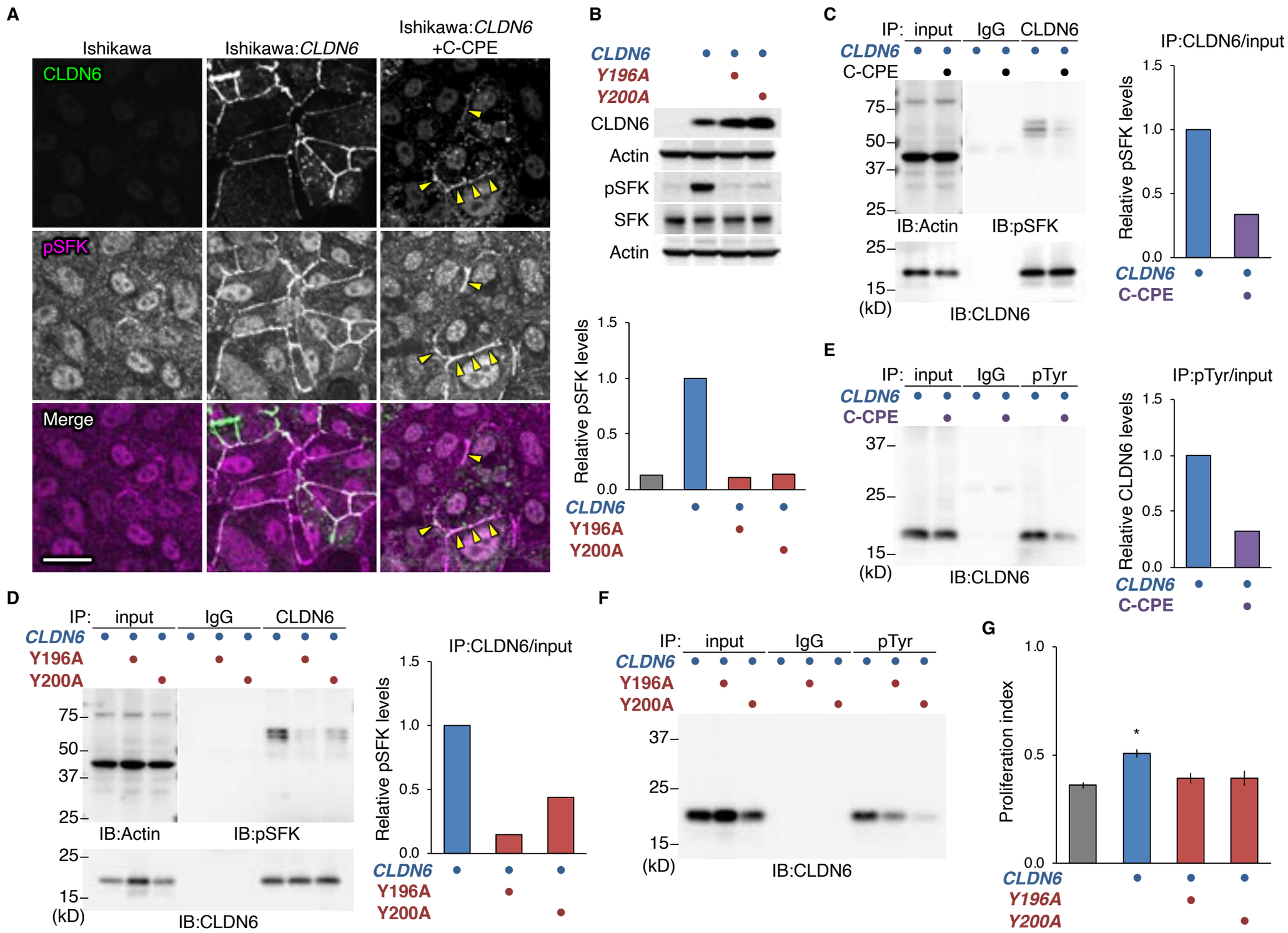

A
Ishikawa
Ishikawa:CLDN6
Ishikawa:CLDN6 +C-CPE
CLDN6
pSFK
Merge
B
CLDN6
Y196A
Y200A
CLDN6
Actin
pSFK
SFK
Actin
Relative pSFK levels
C
IP: input IgG CLDN6
CLDN6
C-CPE
IB:Actin
IB:pSFK
IB:CLDN6
(kD)
IP:CLDN6/input
Relative pSFK levels
E
IP: input IgG pTyr
IP:pTyr/input
Relative CLDN6 levels
D
IP:CLDN6/input
F
G
Proliferation index

Figure. 4

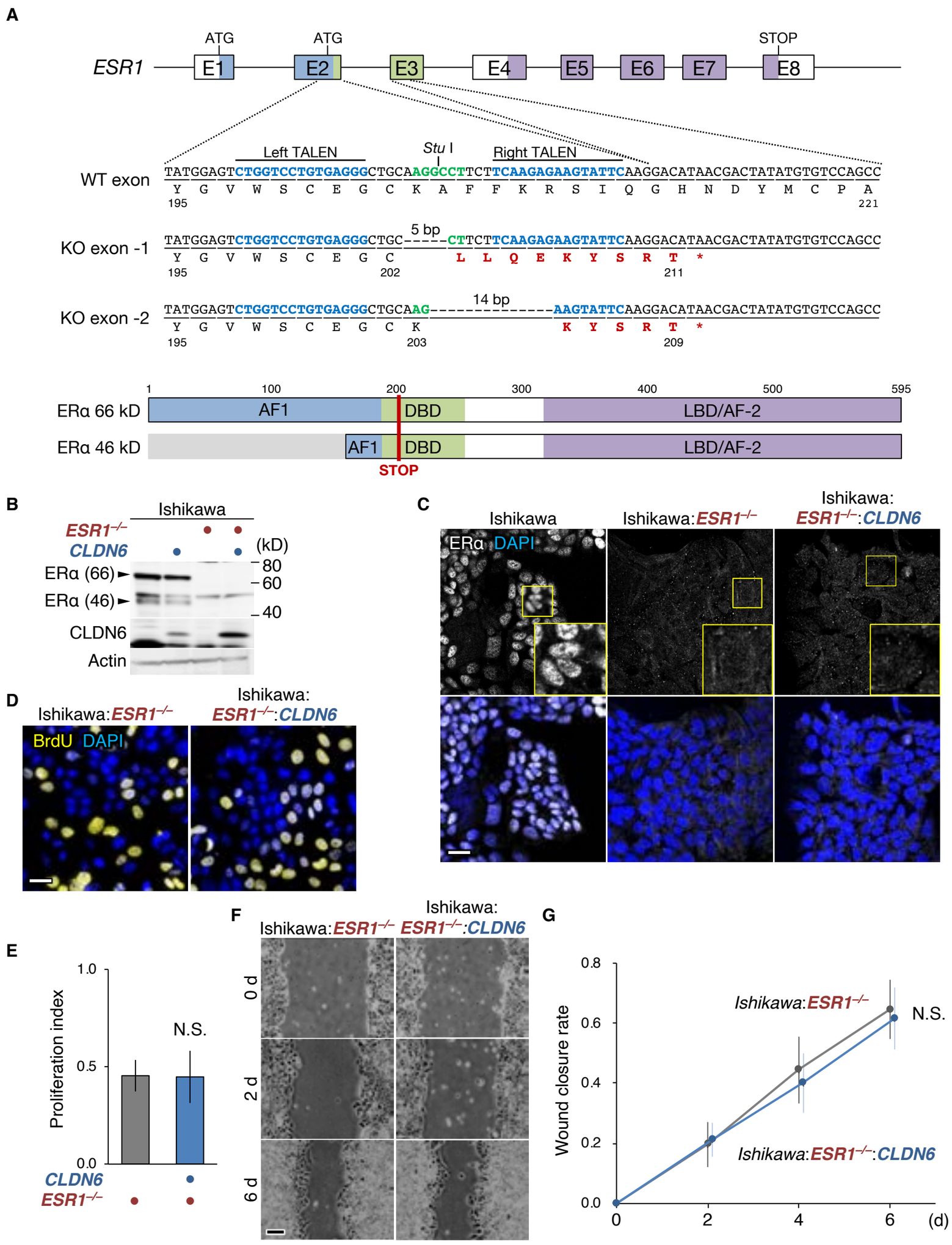

A
ESR1
ATG
ATG
STOP
E1
E2
E3
E4
E5
E6
E7
E8
Left TALEN
Stu I
Right TALEN
WT exon
TATGGAGTCTGGTCCTGTGAGGGCTGCAAGGCCTTCTTCAAGAGAAGTATTCAAGGACATAACGACTATATGTGTCCAGCC
Y G V W S C E G C K A F F K R S I Q G H N D Y M C P A
195
221
KO exon -1
5 bp
TATGGAGTCTGGTCCTGTGAGGGCTGC-----CTTCTTCAAGAGAAGTATTCAAGGACATAACGACTATATGTGTCCAGCC
Y G V W S C E G C L L Q E K Y S R T *
195
202
211
KO exon -2
14 bp
TATGGAGTCTGGTCCTGTGAGGGCTGCAAG----------AAGTATTCAAGGACATAACGACTATATGTGTCCAGCC
Y G V W S C E G C K K Y S R T *
195
203
209
1
100
200
300
400
500
595
ERα 66 kD
AF1
DBD
LBD/AF-2
ERα 46 kD
AF1
DBD
LBD/AF-2
STOP
B
Ishikawa
ESR1−/−
CLDN6
(kD)
ERα (66)
ERα (46)
CLDN6
Actin
80
60
40
C
Ishikawa
Ishikawa:ESR1−/−
Ishikawa:
ESR1−/−:CLDN6
ERα DAPI
D
Ishikawa:ESR1−/−
Ishikawa:
ESR1−/−:CLDN6
BrdU DAPI
E
Proliferation index
1.0
0.5
0.0
N.S.
CLDN6
ESR1−/−
F
Ishikawa:ESR1−/−
Ishikawa:
ESR1−/−:CLDN6
0 d
2 d
6 d
G
Wound closure rate
0.8
0.6
0.4
0.2
0.0
Ishikawa:ESR1−/−
N.S.
Ishikawa:ESR1−/−:CLDN6
0
2
4
6
(d)

Figure. 5

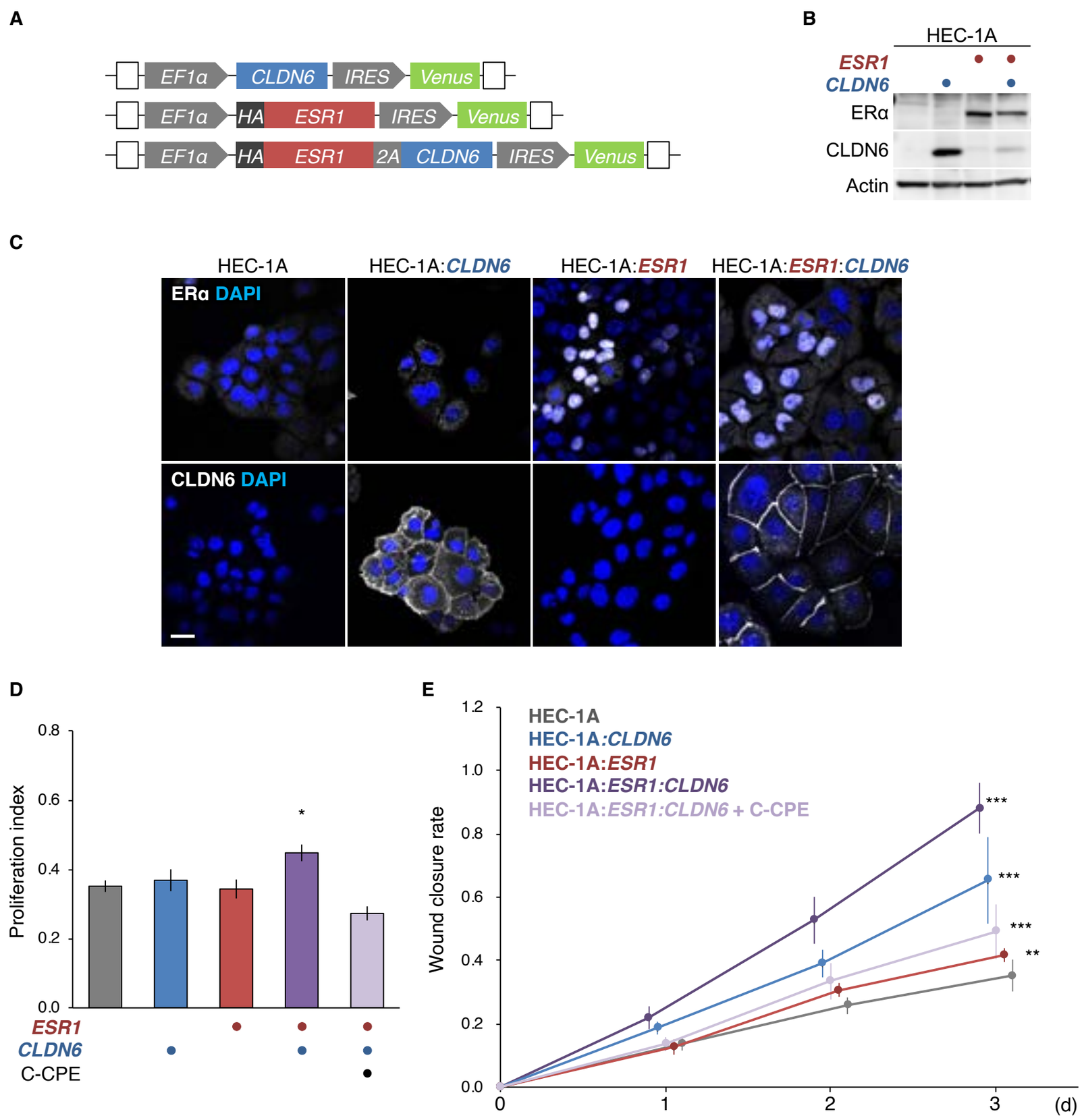

A
EF1α
CLDN6
IRES
Venus
HA
ESR1
2A
B
HEC-1A
ESR1
CLDN6
ERα
CLDN6
Actin
C
HEC-1A
HEC-1A:CLDN6
HEC-1A:ESR1
HEC-1A:ESR1:CLDN6
ERα DAPI
CLDN6 DAPI
D
Proliferation index
0.8
0.6
0.4
0.2
0.0
*
ESR1
CLDN6
C-CPE
E
Wound closure rate
1.2
1.0
0.8
0.6
0.4
0.2
0.0
HEC-1A
HEC-1A:CLDN6
HEC-1A:ESR1
HEC-1A:ESR1:CLDN6
HEC-1A:ESR1:CLDN6 + C-CPE
***
***
***
**
0
1
2
3
(d)

Figure. 6

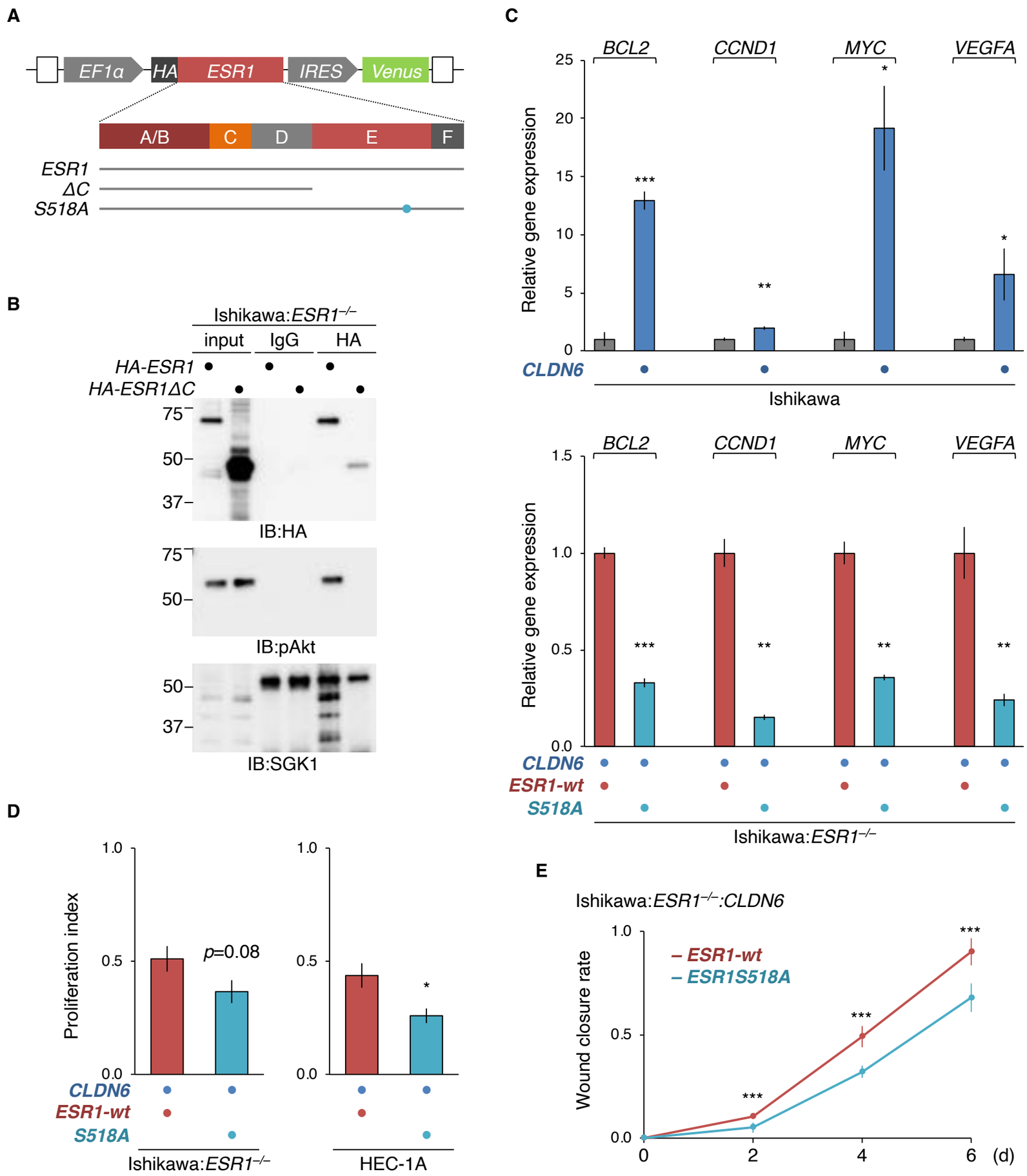
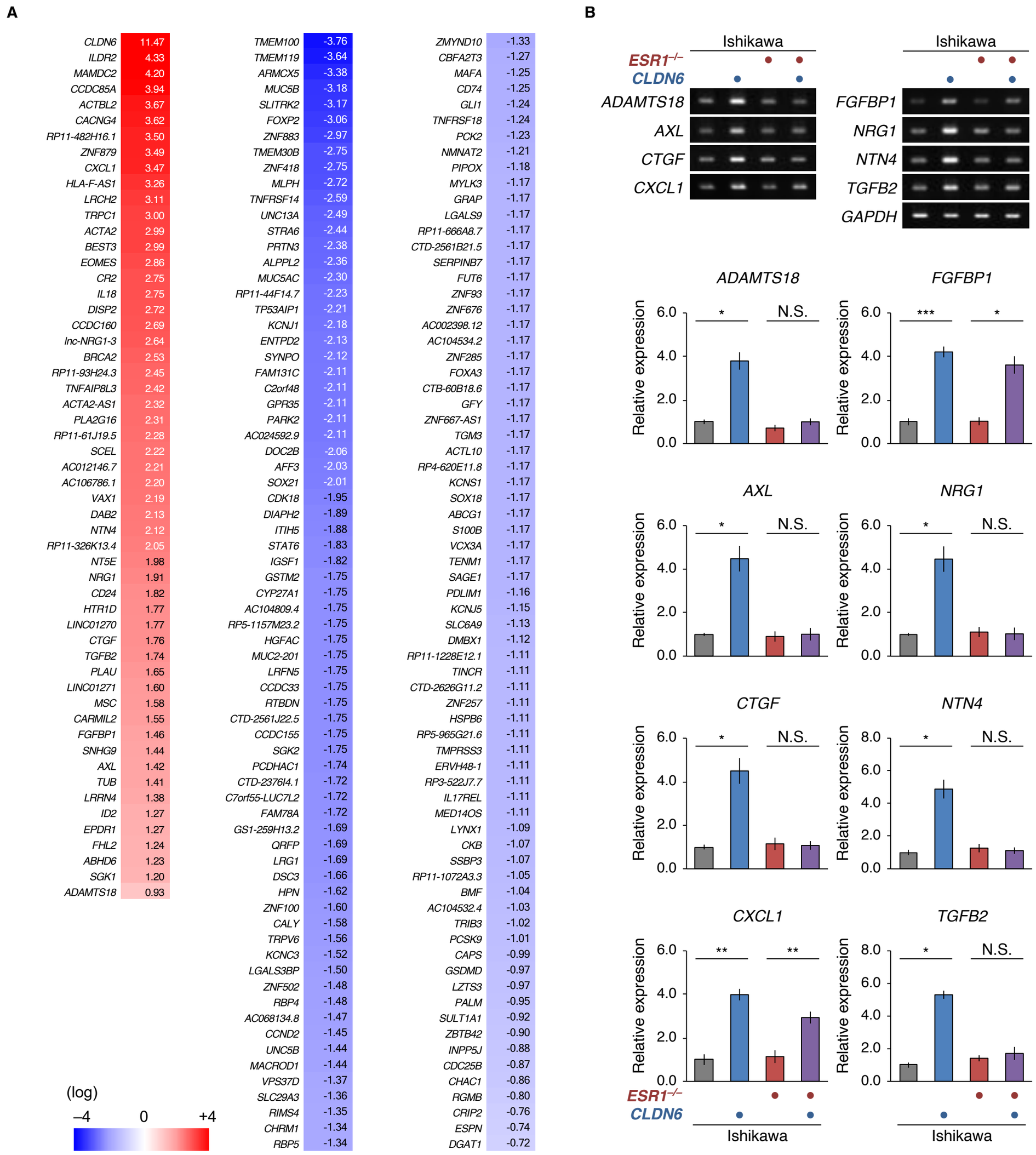

A
EF1α
HA
ESR1
IRES
Venus
A/B
C
D
E
F
ESR1
ΔC
S518A
B
Ishikawa:ESR1−/−
input
IgG
HA
HA-ESR1
HA-ESR1ΔC
75
50
37
IB:HA
IB:pAkt
IB:SGK1
C
BCL2
CCND1
MYC
VEGFA
Relative gene expression
CLDN6
Ishikawa
ESR1-wt
S518A
Ishikawa:ESR1−/−
D
Proliferation index
p=0.08
Ishikawa:ESR1−/−
HEC-1A
E
Ishikawa:ESR1−/−:CLDN6
Wound closure rate
ESR1-wt
ESR1S518A
(d)

Figure. 7


A
CLDN6 11.47
ILDR2 4.33
MAMDC2 4.20
CCDC85A 3.94
ACTBL2 3.67
CACNG4 3.62
RP11-482H16.1 3.50
ZNF879 3.49
CXCL1 3.47
HLA-F-AS1 3.26
LRCH2 3.11
TRPC1 3.00
ACTA2 2.99
BEST3 2.99
EOMES 2.86
CR2 2.75
IL18 2.75
DISP2 2.72
CCDC160 2.69
lnc-NRG1-3 2.64
BRCA2 2.53
RP11-93H24.3 2.45
TNFAIP8L3 2.42
ACTA2-AS1 2.32
PLA2G16 2.31
RP11-61J19.5 2.28
SCEL 2.22
AC012146.7 2.21
AC106786.1 2.20
VAX1 2.19
DAB2 2.13
NTN4 2.12
RP11-326K13.4 2.05
NT5E 1.98
NRG1 1.91
CD24 1.82
HTR1D 1.77
LINC01270 1.77
CTGF 1.76
TGFB2 1.74
PLAU 1.65
LINC01271 1.60
MSC 1.58
CARMIL2 1.55
FGFBP1 1.46
SNHG9 1.44
AXL 1.42
TUB 1.41
LRRN4 1.38
ID2 1.27
EPDR1 1.27
FHL2 1.24
ABHD6 1.23
SGK1 1.20
ADAMTS18 0.93
TMEM100 -3.76
TMEM119 -3.64
ARMCX5 -3.38
MUC5B -3.18
SLITRK2 -3.17
FOXP2 -3.06
ZNF883 -2.97
TMEM30B -2.75
ZNF418 -2.75
MLPH -2.72
TNFRSF14 -2.59
UNC13A -2.49
STRA6 -2.44
PRTN3 -2.38
ALPPL2 -2.36
MUC5AC -2.30
RP11-44F14.7 -2.23
TP53AIP1 -2.21
KCNJ1 -2.18
ENTPD2 -2.13
SYNPO -2.12
FAM131C -2.11
C2orf48 -2.11
GPR35 -2.11
PARK2 -2.11
AC024592.9 -2.11
DOC2B -2.06
AFF3 -2.03
SOX21 -2.01
CDK18 -1.95
DIAPH2 -1.89
ITIH5 -1.88
STAT6 -1.83
IGSF1 -1.82
GSTM2 -1.75
CYP27A1 -1.75
AC104809.4 -1.75
RP5-1157M23.2 -1.75
HGFAC -1.75
MUC2-201 -1.75
LRFN5 -1.75
CCDC33 -1.75
RTBDN -1.75
CTD-2561J22.5 -1.75
CCDC155 -1.75
SGK2 -1.75
PCDHAC1 -1.74
CTD-2376I4.1 -1.72
C7orf55-LUC7L2 -1.72
FAM78A -1.72
GS1-259H13.2 -1.69
QRFP -1.69
LRG1 -1.69
DSC3 -1.66
HPN -1.62
ZNF100 -1.60
CALY -1.58
TRPV6 -1.56
KCNC3 -1.52
LGALS3BP -1.50
ZNF502 -1.48
RBP4 -1.48
AC068134.8 -1.47
CCND2 -1.45
UNC5B -1.44
MACROD1 -1.44
VPS37D -1.37
SLC29A3 -1.36
RIMS4 -1.35
CHRM1 -1.34
RBP5 -1.34
ZMYND10 -1.33
CBFA2T3 -1.27
MAFA -1.25
CD74 -1.25
GLI1 -1.24
TNFRSF18 -1.24
PCK2 -1.23
NMNAT2 -1.21
PIPOX -1.18
MYLK3 -1.17
GRAP -1.17
LGALS9 -1.17
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SERPINB7 -1.17
FUT6 -1.17
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AC104534.2 -1.17
ZNF285 -1.17
FOXA3 -1.17
CTB-60B18.6 -1.17
GFY -1.17
ZNF667-AS1 -1.17
TGM3 -1.17
ACTL10 -1.17
RP4-620E11.8 -1.17
KCNS1 -1.17
SOX18 -1.17
ABCG1 -1.17
S100B -1.17
VCX3A -1.17
TENM1 -1.17
SAGE1 -1.17
PDLIM1 -1.16
KCNJ5 -1.15
SLC6A9 -1.13
DMBX1 -1.12
RP11-1228E12.1 -1.11
TINCR -1.11
CTD-2626G11.2 -1.11
ZNF257 -1.11
HSPB6 -1.11
RP5-965G21.6 -1.11
TMPRSS3 -1.11
ERVH48-1 -1.11
RP3-522J7.7 -1.11
IL17REL -1.11
MED14OS -1.11
LYNX1 -1.09
CKB -1.07
SSBP3 -1.07
RP11-1072A3.3 -1.05
BMF -1.04
AC104532.4 -1.03
TRIB3 -1.02
PCSK9 -1.01
CAPS -0.99
GSDMD -0.97
LZTS3 -0.97
PALM -0.95
SULT1A1 -0.92
ZBTB42 -0.90
INPP5J -0.88
CDC25B -0.87
CHAC1 -0.86
RGMB -0.80
CRIP2 -0.76
ESPN -0.74
DGAT1 -0.72
(log)
–4 0 +4
B
Ishikawa
ESR1–/–
CLDN6
ADAMTS18
AXL
CTGF
CXCL1
FGFBP1
NRG1
NTN4
TGFB2
GAPDH
Relative expression
6.0
4.0
2.0
0.0
8.0
*
**
***
N.S.

Supplementary Figure legends

Figure S1. Generation of rat anti-human CLDN6 mAbs. (*A*) Amino acid sequences of the antigenic peptide of the C-terminal cytoplasmic domains of human CLDN6 and the corresponding regions of the closely related CLDNs. Conserved amino acids are shown in red. (*B*) The construct of CLDN1/4/5/6/9 expression vectors and the representative fluorescence images of the transfected HEK293T cells. EF-1α, elongation factor-1α; IRES, internal ribosome entry site. (*C*) Twenty-four hybridoma clones were screened by Western blot for CLDN6 in HEK293T cells that transiently transfected with the CLDN6 or empty expression vector. (*D* and *E*) HEK293T cells were transfected with individual CLDN expression vectors, and subjected to Western blot and imnunohistochemical analyses using the indicated anti-CLDN6 Abs. (*F*) The complementary determining-regions (CDRs) of an anti-human CLDN6 mAb (clone #15). Scale bars, 100 μm.

Figure S2. The 5-year recurrence-free survival for high and low CLDN6 expression groups in endometrial cancer subjects. *$p<0.001$.

Figure S3. Apoptosis are not detected in Ishikawa and Ishikawa:*CLDN6* cells. Cells are subjected to TUNEL assay together with DAPI staining. As a positive control, cells were treated with DNase. Scale bars, 20 μm.

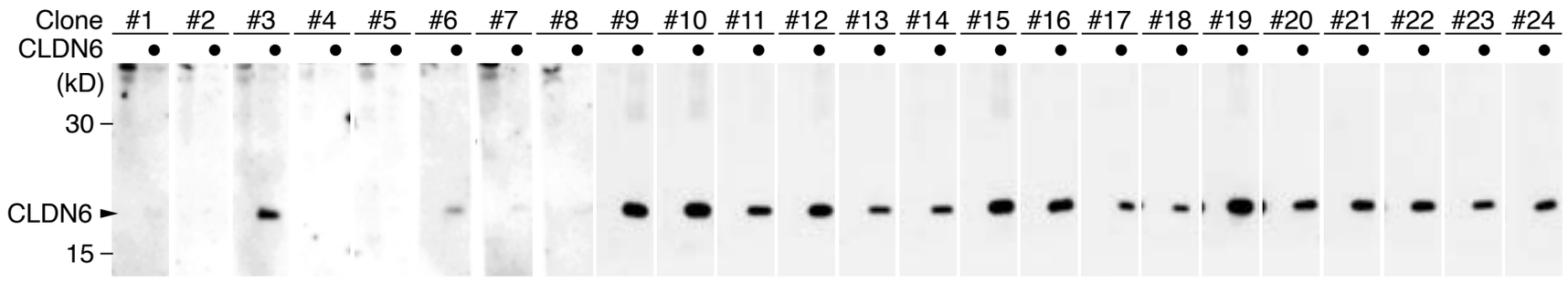
Figure S4. The SFK/PI3K-dependent AKT and SGK pathways are involved in the CLDN6-accererated endometrial cancer progression. (*A* and *B*) Effects of SFK, PI3K, AKT and SGK1 inhibitors (PP2, LY294002 and AKT inh VIII, 10 μM; SGK1 inh, 1 nM) on cell proliferation (*A*) and migration (*B*). Ishikawa and Ishikawa:*CLDN6* cells were grown as in the indicated culture condition. The BrdU/DAPI levels are shown in histograms (mean ± SD; $n = 6$). The values for wound healing assay represent wound closure rates (mean ± SD; $n = 12$ to 16). *$p<0.05$; ***$p<0.001$.

Figure. S1

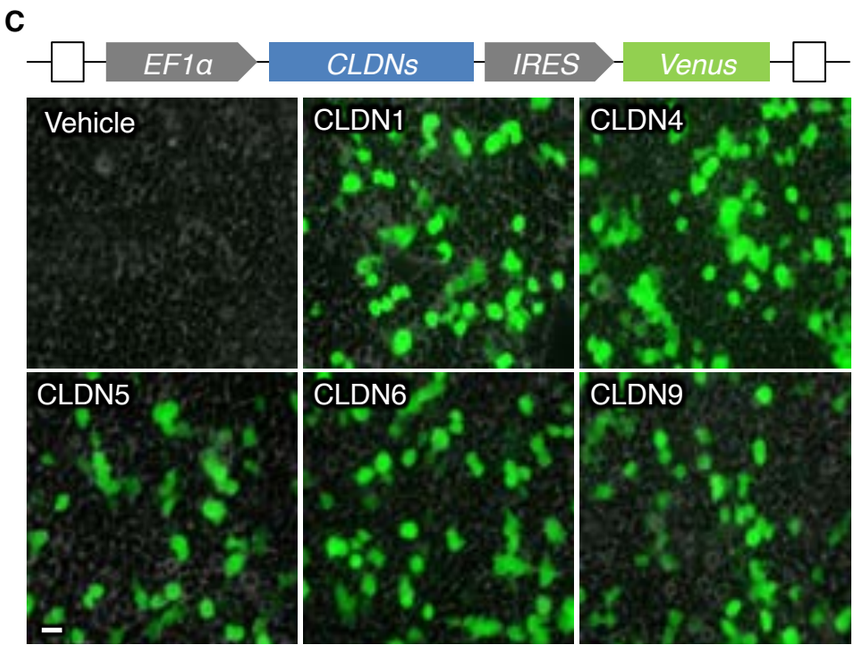
A

```
          208                                   220
CLDN6     CSRGPSEYPT--------------------KNYV
CLDN1     PPRTDKPYSAKYSAARSA----AAS------NYV
CLDN4     TGRPDLSFPVKYSAPRRP----TATGDYDKKNYV
CLDN5     QVERPRGPRLGYSIPSRS----GASG-LDKRDYV
CLDN9     CPRKTTSYPTPRPYPKPAPSSG--------KDYV
```

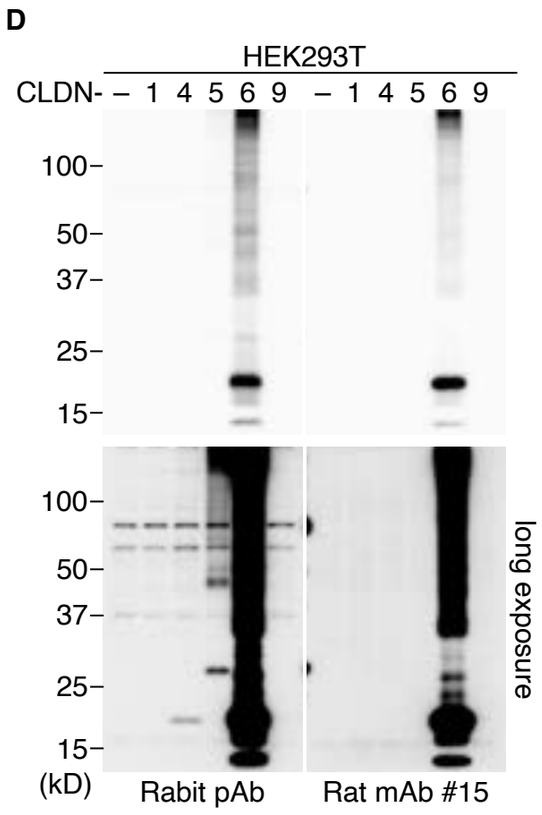
B



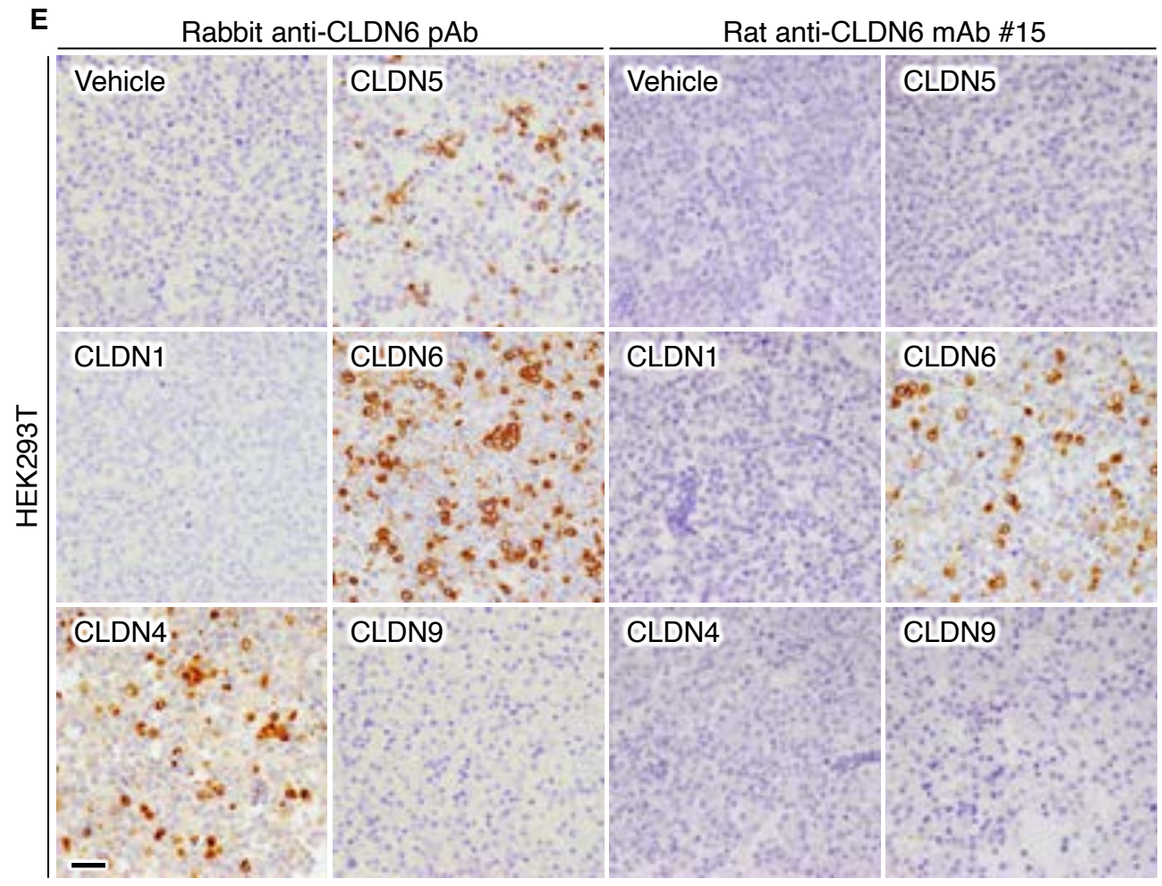
C



D



E



F

Heavy chain
```
EVKLVETGGGLVKPGGSLRLSCATSGFNFNDYFMNWVRQAPGKGLEWVAQIRNKNYNYATYYAESLEGRVTISRDDSKSSVYLQVS
                         CDR1                    CDR2
SLRAEDTAIYYCTRGAYWGQGVMVTVSS
              CDR3
```

Light chain
```
DIVMTQSPSFLSASVGERVTLSCRASQNINKYLDWYQQKLGEAPKLLIYDTNNLHAGIPSRFSGSGSGTDYTLTISSLQPEDVATY
                       CDR1                    CDR2
FCLQRNSWPYTFGAGTKLELK
   CDR3
```

Figure. S2

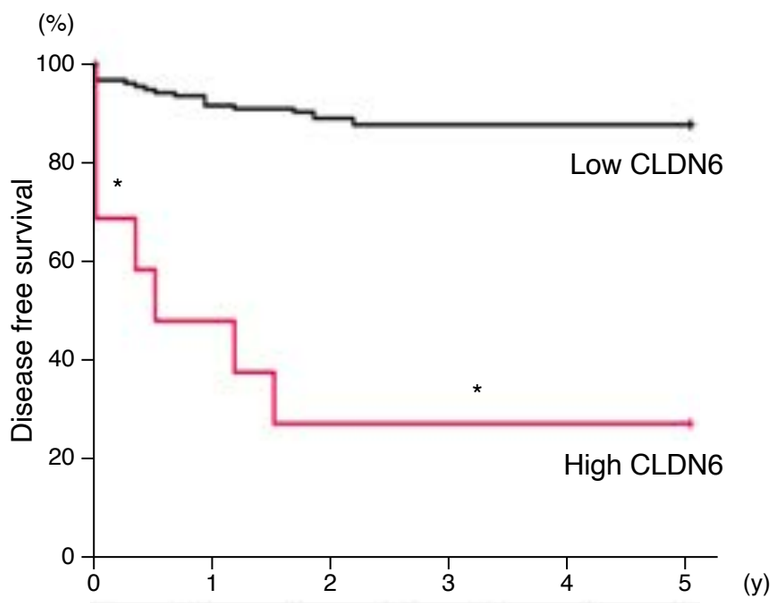

(%)
100
80
60
40
20
0
Disease free survival
Low CLDN6
High CLDN6
*
*
0
1
2
3
4
5
(y)

Figure. S3

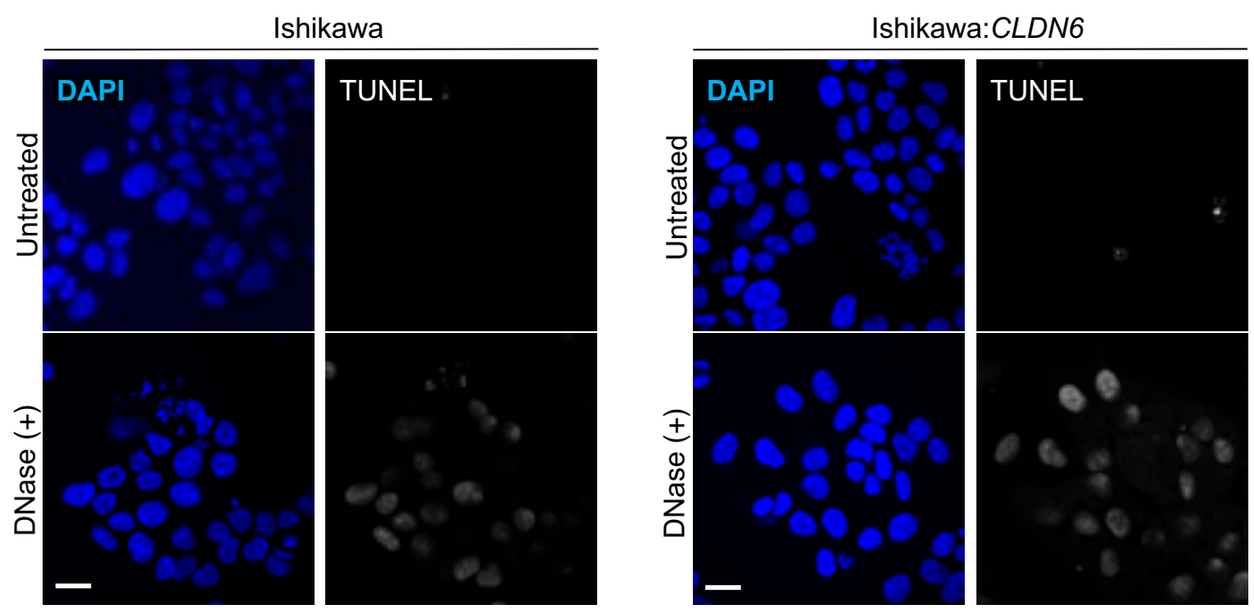

Ishikawa
Ishikawa:*CLDN6*
DAPI
TUNEL
DAPI
TUNEL
Untreated
DNase (+)
Untreated
DNase (+)

Figure. S4

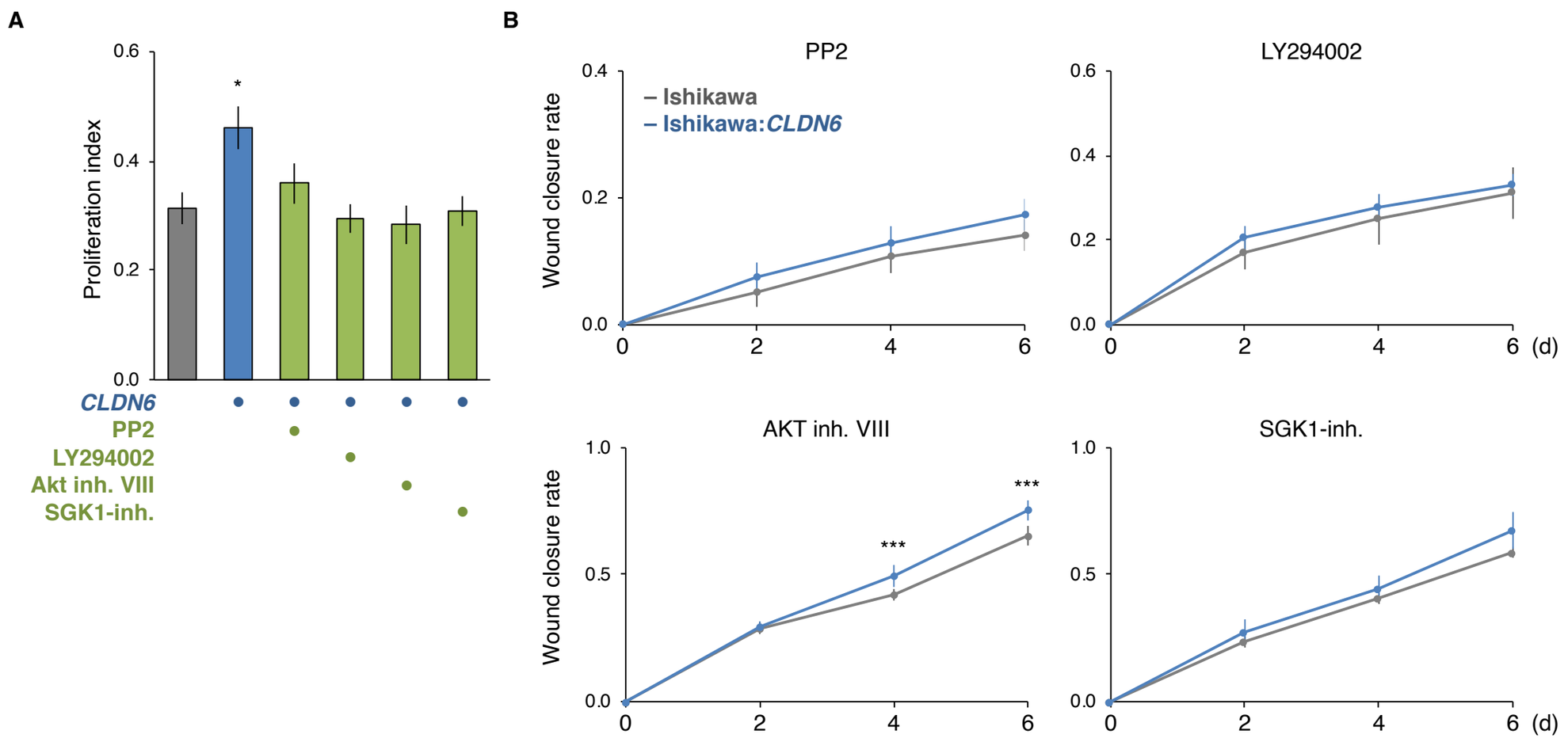

A
Proliferation index
0.6
0.4
0.2
0.0
*
CLDN6
PP2
LY294002
Akt inh. VIII
SGK1-inh.
B
PP2
LY294002
AKT inh. VIII
SGK1-inh.
Wound closure rate
– Ishikawa
– Ishikawa:CLDN6
1.0
0.5
0.0
***
0
2
4
6
(d)

Table. S1

Relevance between CLDN6-expression and clinicopathological factors

parameter	total (n=173)	CLDN6-low (n=163)	CLDN6-high (n=10)	*p*-value
Age <50	32 (18%)	32 (20%)	0 (0%)	0.122
≥50	141 (32%)	131 (80%)	10 (100%)	
Stage I-II	139 (80%)	136 (83%)	3 (30%)	0.001
III-IV	34 (20%)	27 (17%)	7 (70%)	
Endometrioid	164 (95%)	156 (90%)	8 (80%)	0.087
Non-endometrioid	9 (5%)	7 (10%)	2 (20%)	
Histological Grade 1-2	140 (85%)	136 (87%)	4 (50%)	0.004
3	24 (15%)	20 (13%)	4 (50%)	
LVSI (–)	120 (69%)	117 (72%)	3 (30%)	0.001
LVSI (+)	53 (31%)	46 (28%)	7 (70%)	
N0	145 (85%)	140 (88%)	5 (50%)	0.012
N1	25 (15%)	20 (12%)	5 (50%)	
M0	163 (94%)	156 (96%)	7 (70%)	0.014
M1	10 (6%)	7 (4%)	3 (30%)	

LVSI, lymphovascular space involvement; N0/1, negative/positive for lymphnode metastasis; M0/1, negative/positive for distant metastasis.

Table. S2

Cox multivariable analysis

variable	HR	95%CI	*p*-value
Age ≥50	1.61	0.39 – 6.66	0.513
Stage III or IV	10.93	2.48 – 48.04	0.002
Histological Grade 3	2.18	0.24 – 3.60	0.091
LVSI (+)	1.91	0.51 – 7.18	0.340
N1	0.45	0.13 – 1.61	0.220
M1	4.68	1.57 – 14.01	0.006
CLDN6-high	3.50	2.42 – 9.43	0.014

HR, hazard ratio; CI, confidence interval; LVSI, lymphovascular space involvement; N1, positive for lymphnode metastasis; M1, positive for distant metastasis.

Table. S3

Antibodies

antibodies	host	source	identifier	IP	IB	IHC
AKT	rabbit	Cell Signaling Technology	4691S		1:1,000	
βActin	mouse	Thermo Fisher Scientific	A5441		1:10,000	
BrdU	rat	Creative Diagnostics	DPATB-H81231			1:1,000
Claudin-6	rabbit	Immuno-Biological Laboratories	18865	2 μg	1:2,000	1:200
ERα(HC-20)	rabbit	Santa Cruz Technology	sc-543		1:1,000	1:200
HA	rat	Roche	11867423001	1 μg	1:1,000	
Ki67	mouse	Dako	M7240			1:200
PI3K (55)	rabbit	Cell Signaling Technology	11889S		1:1,000	
Phospho-AKT	rabbit	Cell Signaling Technology	4060S		1:1,000	
Phospho-PI3K (85/55)	rabbit	Bioss	bs-3332R		1:1,000	
Phospho-Tyrosine	mouse	Santa Cruz Technology	Sc-508	2 μg	1:1,000	1:100
Phospho-SFK (Tyr416)	rabbit	Cell Signaling Technology	2101		1:1,000	1:100
Phospho-SGK1		MERK				
SFK	mouse	Cell Signaling Technology	2102S		1:1,000	
SGK1	rabbit	Cell Signaling Technology	12103S		1:1,000	
mouse IgG (HRP)	sheep	GE Health Care	NA931V		1:10,000	
rabbit IgG (HRP)	donkey	GE Health Care	NA934V		1:2,000	
rat IgG (HRP)	goat	GE Health Care	NA935V		1:2,000	
rabbit IgG (Cy3)	donkey	Jackson ImmunoResearch	711-165-152			1:300
rat IgG (Cy3)	donkey	Jackson ImmunoResearch	712-165-150			1:300
rat IgG(Alexa Fluor 647)	donkey	Jackson ImmunoResearch	712-605-153			1:300

IP, Immunoprecipitation; IB, Immunoblotting; IHC, Immunohistochemistry

Table. S4

Clinicopathological characteristics of patients with uterine endometrial carcinoma

All patients	173
Age (years)	33-83 (59±11)
Stage I	138
II	1
II	24
IV	10
Endometrioid	164
Grade 1	110
Grade 2	30
Grade 3	24
Serous	3
Mucinous	2
Clear	4
Relapse (+)	20
Relapse (-)	145
Non-CR	8

Table. S5

Immunoreactivity score (IRS)

Score	Signal intensity (SI)	Percentage of positive cells (PP)
0	negative	<1%
1	weak	1-10%
2	moderate	11-30%
3	strong	31-50%
4		>50%

SI × PP	IRS	
0	Score 0	CLDN6 low
1–2	Score 1+	CLDN6 low
3–6	Score 2+	CLDN6 low
8–12	Score 3+	CLDN6 high

Table. S6

Primers

Gene	Forward primer	Reverse primer	Product size (bp)
ADAMTS18	CGAGGTGCAGCAATGCTTCT	ACAGTACGTGAGGATGGTGA	194
AXL	TGAGGATGAACAGGATGACT	GCTTGGCAGCTCAGGTTGAA	219
CTGF	GCCTATTCTGTCACTTCGGCT	ACGAACGTCCATGCTGCACA	191
BCL2	GACAACATCGCCCTGTGGATG	AGAAATCAAACAGAGGCCGCA	127
CCND1	TCTACACCGACAACTCCATCCG	TCTGGCATTTTGGAGAGGAAGTG	133
CXCL1	CCTGCAGGGAATTCACCCCAA	CCTCCCTTCTGGTCAGTTGGA	193
FGFBP1	GGACTTCACAGCAAAGTGGTCT	ATTGCCAGCAAAGACACAGGA	210
GAPDH	TTGTTGCCATCAATGACCCC	TGACAAGCTTCCCGTTCTCA	117
MYC	CCTGGTGCTCCATGAGGAGAC	CAGACTCTGACCTTTTGCCAGG	128
NRG1	TGCCAGAGAAACCCCTGATT	CGCCATGGAAGGCATGGACA	181
NTN4	TGGGAGGCAGCTGATGGCAA	TAGAAGCCTGGCTTGCACCT	191
TGFB2	GAGTACTACGCCAAGGAGGT	CCAAATTGGAAGCATTCTTCTCCA	145
VEGFA	TTGCCTTGCTGCTCTACCTCCA	GATGGCAGTAGCTGCGCTGATA	126