PCDH17 increases the sensitivity of colorectal cancer to 5-fluorouracil treatment by inducing apoptosis and autophagic cell death

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5-Fluorouracil (S-FU) is known as a first-line chemotherapeutic agent against colorectal cancer (CRC), but drug resistance occurs frequently and significantly limits its clinical success. Our previous study showed that the protocadherin 17 (PCDH17) gene was frequently methylated and functioned as a tumor suppressor in CRC. However, the relationship between PCDH17 and S-FU resistance in CRC remains unclear. Here, we revealed that PCDH17 was more highly expressed in S-FU-sensitive CRC tissues than in S-FU-resistant CRC tissues, and high expression of PCDH17 was correlated with high BECN1 expression. Moreover, this expression profile contributed to superior prognostic and increased survival in CRC patients. Restoring PCDH17 expression augmented the S-FU sensitivity of CRC in vitro and in vivo by promoting apoptosis and autophagic cell death. Furthermore, autophagy played a dominant role in PCDH17-induced cell death, as an autophagy inhibitor blocked cell death to a greater extent than the pancaspase inhibitor Z-VAD-FMK. PCDH17 inhibition by siRNA decreased the autophagy response and 5-FU sensitivity. Mechanistically, we showed that c-Jun NH2-terminal kinase (JNK) activation was a key determinant in PCDH17-induced autophagy. The compound SP600125, an inhibitor of JNK, suppressed autophagy and S-FU-induced cell death in PCDH17-reexpressing CRC cells. Taken together, our findings suggest for the first time that PCDH17 increases the sensitivity of CRC to S-FU treatment by inducing apoptosis and JNK-dependent autophagic cell death. PCDH17 may be a potential prognostic marker for predicting 5-FU sensitivity in CRC patients.

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INTRODUCTION

Colorectal cancer (CRC) ranks third in morbidity and second in mortality among various malignancies, although much progress has been achieved in CRC therapy in past years.1 Currently, the antimetabolite 5-fluorouracil (S-FU) is widely used as chemotherapeutic drug therapy in various solid tumors such as colorectal cancer and gastric cancer. Although S-FU in combination with other chemotherapeutic agents improves the prognosis of CRC patients, S-FU resistance occurs frequently and significantly limits its clinical success.2-3 Epigenetic and genetic disruptions of tumor suppressor genes (TSGs) are considered to be partly attributed to S-FU resistance.4,5 Therefore, the identification of novel genes that have therapeutic potential as predictive biomarkers of 5-FU chemosensitivity is urgently needed.

The protocadherin 17 (PCDH17) gene, a member of the protocadherin family, is frequently methylated and associated with poor prognosis in various cancer types, including esophageal squamous cell carcinoma (ESCC),6 urological cancer,7 gastric cancer,6 and nasopharyngeal cancers.9 In a previous study, we demonstrated that PCDH17 was silenced in most CRC cell lines and that restoring PCDH17 expression reduced tumor cell growth.10 Therefore, PCDH17 is a potential tumor suppressor in CRC. However, the relationship between PCDH17 and S-FU resistance in CRC remains unclear.

Autophagy, an important homeostatic cell recycling system, plays an important role in cellular component degradation and recycling.11,12 Chemotherapy agents such as S-FU may give rise to autophagic responses. This autophagic response can have a prodeath or a prosurvival role and thus contribute to anticancer
efficacy or drug resistance, respectively. Therefore, targeting autophagy will provide a potential therapeutic strategy to overcome drug resistance and augment the clinical outcomes of anticancer therapies for patients with cancer. However, to our knowledge, there are still no reports about the role of PCDH17 in regulating autophagy and 5-FU sensitivity in CRC.

In this study, we first demonstrated that PCDH17 and BECN1 (Beclin 1, which is autophagy-related) were more highly expressed in 5-FU-sensitive CRC tissues than in 5-FU-resistant CRC tissues and showed a significant positive relationship between high expression of these genes and superior prognosis. Next, we found that PCDH17 reexpression augmented the 5-FU sensitivity of CRC cells by promoting apoptosis and autophagic cell death. Furthermore, JNK activation was proven to confer 5-FU sensitivity in PCDH17-expressing cells by inducing death-promoting autophagy. Thus, our findings indicate that PCDH17 plays a critical role in augmenting 5-FU sensitivity by promoting autophagy. PCDH17 will hopefully become a potential predictive biomarker for CRC patients treated with 5-FU.

RESULTS
Loss of the PCDH17 and BECN1 proteins is associated with 5-FU resistance and poor prognosis in CRC patients
In a previous study, we demonstrated that lower protein expression of PCDH17 was significantly correlated with low T stage, decreased lymph node metastasis, and low tumor stage in CRC patients compared with paired surgical margin tissues. However, the relationship between PCDH17 expression and the 5-FU sensitivity of CRC patients is still unclear. Therefore, we examined PCDH17 expression in 21 chemosensitive and 39 chemoresistant CRC tissues by immunohistochemistry (IHC) staining. To explore the potential mechanism of PCDH17 in cell autophagy, the protein expression of BECN1 was simultaneously detected in the same paraffin-embedded block. The results showed that PCDH17 and BECN1 were simultaneously expressed in the cytoplasm of cancer cells (Fig. 1a), and their expression was significantly correlated (Table 1, p < 0.05). In addition, high expression of PCDH17 and BECN1 was significantly correlated with low levels of lymph node metastasis in both chemosensitive and chemoresistant CRC tissues (Tables 1 and 2, p < 0.05). Furthermore, the percentages of cases with high expression of PCDH17 and BECN1 were 52.4% (11/21 cases) and 81% (17/21 cases), respectively, in chemosensitive CRC cases but only 7.7% (2/22 cases) and 30.8% (1272 cases), respectively, in chemoresistant tissues. Kaplan–Meier survival curve analysis indicated that CRC patients with high PCDH17 expression had significantly better overall survival (OS, p = 0.0173) than patients with low PCDH17 expression. The relationship between BECN1 protein expression and OS was consistent with that of PCDH17 (Fig. 1b). Moreover, patients with PCDH17high/BECN1high and PCDH17low/BECN1low disease had better OS than those with PCDH17low/BECN1high and PCDH17low/BECN1low disease, respectively (Fig. 1b). All these data showed that PCDH17 expression was correlated with sensitivity to 5-FU and autophagy in colorectal cancer patients.

Ectopic expression of PCDH17 induces caspase-dependent apoptosis and autophagy in CRC cells after 5-FU treatment
Our previous study showed that methylation mediated the transcriptional silencing of PCDH17 in CRC cell lines, including HCT116 and SW480 cells. We also established 5-FU-resistant HCT116 cells. However, in agreement with the results in 5-FU-sensitive HCT116 cells, we did not observe PCDH17 expression in HCT116/5-FU-resistant cells (Supplemental Fig. 1). In this study, we detected the effects of ectopic PCDH17 expression on CRC cell growth. The forced expression of PCDH17 in HCT116 and SW480 cells was confirmed by RT-PCR and western blot (Fig. 2a). We next investigated whether tumor cell growth was inhibited by ectopic PCDH17 expression. The CCK-8 assay results showed that PCDH17-transfected cells exhibited significantly lower viability than controls after treatment with various concentrations of 5-FU for 24 h (Fig. 2b, c). We further studied the effect of PCDH17 expression on apoptosis and autophagy with 5-FU treatment. As shown in Fig. 2d, an accumulation of PCDH17 protein was detected in CRC cells treated with different concentrations of 5-FU. The accumulated PCDH17 caused apoptotic cell death and triggered autophagy in a 5-FU dose-dependent manner, indicating that apoptosis and autophagy were involved in the effect of PCDH17 on CRC cell viability. Therefore, we used 20 μM 5-FU in PCDH17-transfected HCT116 and SW480 cells in subsequent experiments.

Restoration of PCDH17 expression activates autophagic cell death in CRC cells
It is known that autophagy is one of the conserved protein degradation processes, and it can lead to resistance to chemotherapy treatment through a cell survival or cell death mechanism, depending on the cellular status. To determine the effect of PCDH17 on the autophagy induced by 5-FU, we investigated the level of LC3-II, the number of autophagic puncta and the formation of autophagosomes in HCT116 and SW480 cells. Western blotting results revealed that PCDH17 increased the turnover of the autophagic marker LC3-II and SQSTM1 (sequestosome 1) degradation (Fig. 3a). Moreover, LC3-II levels were further increased and SQSTM1 was increased after BafA1 (bafilomycin A1) treatment, indicating that 5-FU induced the autophagic flux of PCDH17-transfected CRC cells. In accordance with these results, the number of GFP-RFP-LC3-II-positive puncta in PCDH17-transfected CRC cells was significantly higher than that in the control cells after 24 h exposure to 20 μM 5-FU, whereas little signal was observed after treatment with the autophagy inhibitor 3-MA (3-methyladenine) (Fig. 3b, c). The TEM results also indicated that there were more autophagosomes and autolysosomes in the cytoplasm of PCDH17-transfected cells than in that of control cells (Fig. 3d).

To investigate whether PCDH17-triggered autophagy plays a prosurvival or prodeath role, PCDH17-transfected CRC cells were exposed to 5-FU with or without different inhibitors. The 5-FU treatment combined with Z-VAD-FMK (a pancaspase inhibitor) or the autophagy inhibitor chloroquine (CQ), BafA1 and 3-MA, but not with the necroptosis inhibitor necrostatin-1, prevented the PCDH17-induced cell growth inhibition (Fig. 4a–e). Furthermore, autophagy played a dominant role in PCDH17-induced cell death, since the autophagy inhibitors blocked cell death to a greater extent than the pancaspase inhibitor Z-VAD-FMK (Fig. 4a, c–e). These results strongly indicate that PCDH17-induced apoptosis and autophagy collectively trigger cell death. Additionally, autophagy serves as the predominant method by which PCDH17 induces cell death.

PCDH17 regulates autophagy and apoptosis and augments 5-FU sensitivity
Next, we tested the effect of PCDH17 knockdown on autophagy and 5-FU sensitivity by using short hairpin RNA (shRNA) to knockdown PCDH17 expression in PCDH17-transfected CRC cells. Both HCT116/PCDH17 and SW480/PCDH17 cells were transfected with PCDH17-specific shRNA. Decreased expression of PCDH17 was confirmed by western blotting (Fig. 4f). We found that PCDH17 knockdown by specific shRNA significantly inhibited LC3-II turnover and the expression of BECN1 and active caspase-3 (Fig. 4f); however, BECN1 overexpression did not affect PCDH17 expression (Fig. 4g), indicating that PCDH17 can modulate BECN1 and apoptosis.

We then assessed PCDH17 regulation of 5-FU sensitivity in HCT116 and SW480 cells. The CCK-8 assay showed that the cytotoxic effect of 5-FU was significantly inhibited in a dose-
dependent manner when PCDH17 was knocked down compared with the controls (Fig. 4h, i). To quantify the cell viability, we performed an Annexin V-FITC dual staining assay and then flow cytometry. In line with the above findings, flow cytometry results also showed that PCDH17 knockdown significantly decreased 5-FU sensitivity in HCT116/PCDH17 and SW480/PCDH17 cells (Fig. 4j, k). Taken together, these data suggested that PCDH17 could modulate autophagy and augment the 5-FU sensitivity of CRC cells.

Restoration of PCDH17 expression activates autophagic cell death to augment the 5-FU sensitivity of CRC cells. To investigate whether PCDH17 can sensitize cells to the anticancer effects of 5-FU in vivo, xenograft tumor models of HCT116/PCDH17 cells were generated. When the size of tumor xenografts reached 100 mm³, the mice were randomly divided into 4 groups: the control group, 5-FU group (40 mg/kg intraperitoneal injection every 2 days for 28 days), 3-MA group (20 mg/kg intraperitoneal injection every 2 days for 28 days), and 5-FU plus 3-MA group. As shown in Fig. 5a, b, not 3-MA but 5-FU alone (p < 0.01) exhibited a significant effect on the growth of HCT116/PCDH17 CRC cells. In accordance with the in vitro results, the tumor size of the 5-FU plus 3-MA group was significantly larger than that of the 5-FU group (p < 0.01), indicating that PCDH17 can induce prodeath autophagy in vivo when CRC cells are treated with 5-FU. Furthermore, no significant hepatic toxicity or weight loss was observed in the 5-FU group or the 5-FU plus 3-MA group (data not shown).

Next, we examined the protein expression of PCDH17, BECN1 and LC3B by immunohistochemical staining. As shown in Fig. 5c, 5-FU treatment resulted in an upregulation of PCDH17 and autophagy in HCT116/PCDH17 CRC cells. Taken together, our data indicated that PCDH17 sensitized CRC cells to the anticancer effects of 5-FU in vivo by inducing...
autophagic cell death. By inhibiting autophagy, 3-MA potently attenuated the antitumor activity of 5-FU in vivo.

**PCDH17** activates autophagy via the c-Jun N-terminal kinase (JNK) pathway.

c-Jun N-terminal kinase (JNK) plays a key role in the sensitivity to and outcome of anticancer therapies. JNK activation can mediate BECN1 expression to induce autophagic cell death in response to chemotherapeutic agents.15 Because we observed that autophagic cell death was involved in the effect of **PCDH17** on CRC cell viability, we next detected the protein levels of phosphorylated-JNK (p-JNK) to determine whether the JNK pathway is activated in these cells exposed to 5-FU (Fig. 6a).

To determine whether JNK activation may be correlated with autophagy induction in HCT116/PCDH17 and SW480/PCDH17 cells in response to 5-FU, we specifically inhibited JNK activation with the JNK inhibitor SP600125 (10 µM). As shown in Fig. 6c, inhibiting JNK in **PCDH17**-transfected CRC cells significantly decreased the level of phosphorylated c-Jun and the ratio of LC3-II/I but had no effect on LC3-II/I turnover (Fig. 6b), indicating that **PCDH17** can modulate JNK activation and the induction of autophagy.

**DISCUSSION**

**CRC** is the third most common malignancy worldwide and associated with high-mortality. To date, 5-FU-based chemotherapy remains widely used in the clinical treatment of CRC patients, but resistance to 5-FU is a major drawback in its clinical use.16 Thus, overcoming 5-FU resistance or increasing 5-FU treatment efficacy is urgently needed.

**PCDH17**, a member of the cadherin superfamily, functions as a potential tumor suppressor and is found to be transcriptionally silenced in various human cancer types due to molecular mechanisms such as deletion, mutation, and promoter methylation.17,18 Our previous study reported that **PCDH17** is frequently methylated and inactivated in gastric and colorectal cancers, in

| Table 1. The association between **PCDH17** expression with clinicopathological background and BECN1 expression. |
|---------------------------------------------------------------|
| **PCDH17** expression | N | High (%) | Low (%) | **p** value |
| Total               | 60 | 13 (21.7) | 47 (78.3) | |
| Gender              |   |   |   |   |
| Male                | 34 | 8 (23.5) | 26 (76.5) | 0.7603 |
| Female              | 26 | 5 (19.2) | 21 (80.8) | |
| Age                 |   |   |   |   |
| Median              | 61 |   |   |   |
| ≥61                 | 28 | 5 (15.2) | 23 (84.8) | 0.9999 |
| <61                 | 32 | 8 (29.6) | 24 (70.4) | |
| Histopathological grading |   |   |   |   |
| Well/moderately     | 48 | 10 (20.8) | 38 (79.2) | 0.7114 |
| Poorly              | 12 | 3 (27.2) | 9 (81.8) | |
| pT categories       |   |   |   |   |
| pT2                 | 9  | 0 (0) | 9 (100) | 0.1839 |
| pT3                 | 51 | 13 (25.5) | 38 (74.5) | |
| pN categories       |   |   |   |   |
| pN0/1               | 31 | 11 (35.5) | 20 (64.5) | 0.0109 |
| pN2                 | 29 | 2 (6.9) | 27 (93.1) | |
| Stage               |   |   |   |   |
| II                  | 19 | 6 (31.6) | 13 (68.4) | 0.3119 |
| III                 | 41 | 7 (17.1) | 34 (82.9) | |
| Chemosensitivity    |   |   |   |   |
| Sensitive           | 21 | 11 (52.4) | 10 (47.6) | <0.0001 |
| Resistant           | 39 | 2 (7.7) | 37 (92.3) | |
| BECN1 expression    |   |   |   |   |
| High                | 29 | 10 (34.5) | 19 (65.5) | 0.0283 |
| Low                 | 31 | 3 (9.7) | 28 (90.3) | |

| Table 2. The association between BECN1 expression with clinicopathological background. |
|---------------------------------------------------------------|
| **BECN1** expression | N | High (%) | Low (%) | **p** value |
| Total               | 60 | 29 (48.3) | 31 (51.7) | |
| Gender              |   |   |   |   |
| Male                | 34 | 17 (50.0) | 17 (50.0) | 0.7997 |
| Female              | 26 | 12 (46.2) | 14 (53.8) | |
| Age                 |   |   |   |   |
| Median              | 61 |   |   |   |
| ≥61                 | 28 | 9 (15.2) | 19 (84.8) | 0.0227 |
| <61                 | 32 | 20 (62.5) | 12 (70.4) | |
| Histopathological grading |   |   |   |   |
| Well/moderately     | 48 | 23 (20.8) | 25 (79.2) | >0.05 |
| Poorly              | 12 | 6 (27.2) | 6 (81.8) | |
| pT categories       |   |   |   |   |
| pT2                 | 9  | 3 (33.3) | 6 (66.7) | 0.4743 |
| pT3                 | 51 | 26 (51)  | 25 (49)  | |
| pN categories       |   |   |   |   |
| pN0/1               | 31 | 25 (80.6) | 6 (19.4)  | <0.001 |
| pN2                 | 29 | 4 (13.8) | 25 (86.2) | |
| Stage               |   |   |   |   |
| II                  | 19 | 8 (42.1) | 11 (57.9) | 0.5853 |
| III                 | 41 | 21 (51.2) | 20 (48.8) | |
| Chemosensitivity    |   |   |   |   |
| Sensitive           | 21 | 17 (81)  | 4 (19)   | 0.0003 |
| Resistant           | 39 | 12 (30.8) | 27 (69.2) | |

blotting was used to confirm the reduced expression of **PCDH17** (Fig. 6b). **PCDH17** knockdown by specific shRNA significantly inhibited JNK activation and LC3B-II turnover (Fig. 6b), indicating that **PCDH17** can modulate JNK activation and the induction of autophagy.
which PCDH17 functions as a tumor suppressor by inducing apoptosis and autophagy. However, no further detailed analysis of the biological roles of PCDH17 in autophagy and the sensitivity of CRC cells to 5-FU has been conducted to date.

In this study, the protein expression of PCDH17 and BECN1 was detected in CRC tissues by IHC. We first revealed that the expression of PCDH17 and BECN1 was significantly upregulated in chemosensitive tissues compared with chemoresistant tissues. Moreover, the clinical analysis results indicated that high expression of PCDH17 augments the 5-FU sensitivity of CRC cells by promoting JNK-dependent autophagic cell death, which might support the clinical potential of PCDH17 for predicting 5-FU sensitivity in CRC patients.

**METHODS**

Cell lines and reagents

The human colorectal cancer cell lines HCT116 and SW480 were obtained from the ATCC (LGC Standards SLU, Barcelona, Spain). They were cultured in McCoy’s 5A or Dulbecco’s modified Eagle’s medium (DMEM; Gibco BRL, Rockville, MD, USA) containing 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 µg/mL streptomycin (Invitrogen), and 2 mmol/L L-glutamine in a humidified atmosphere of 5% carbon dioxide in air at 37 °C. The fetal bovine serum was obtained from Corilis (184590, Australia). 5-FU was obtained from Jinyao Amino Acid Co., Ltd. (Tianjin, China).

BafA1 (B1793), 3-methyladenine (M9281), chloroquine (C6628), the pancaspase inhibitor Z-VAD-FMK (C2105), necrostatin-1 (N9037) and SP600125 (S5567) were purchased from Sigma Aldrich. Anti-LC-3B (#3868), anti-p62 (#8025), anti-cleaved caspase-3 (#9661), anti-SQSTM1/p62 (#5114), anti-BECN1 (#3495), anti-SAPK/JNK (#9252), anti-phospho-SAPK/JNK (Thr183/Tyr185)
anti-phospho-c-Jun (Ser63), anti-GAPDH, and anti-PCDH17 antibodies were purchased from Cell Signaling Technology (CST). Anti-PCDH17 (HPA026817) for IHC was obtained from Sigma Aldrich. Anti-PCDH17 (ab128815) for WB was obtained from Abcam. The PCDH17 plasmid (NM_001040429) was purchased from OriGene.

**Patient-specimen selection and immunohistochemistry**

IHC was performed with 60 formalin-fixed and paraffin-embedded tissue samples obtained from CRC patients diagnosed from January 2008 to October 2010. All patients volunteered to participate in the initial surgery and then received chemotherapy based on 5-FU. The follow-up deadline was June 30, 2018. Patients who developed disease during the initial treatment or relapsed within 6 months after completing the initial treatment were termed 5-FU-resistant. Patients who relapsed after 6 months or had no recurrence were termed 5-FU-sensitive.

Briefly, according to the instructions of the ChemMate EnVision test kit (Dako, Carpinteria, California, USA), the sections were incubated with the respective primary antibody overnight at 4 °C. The ChemMate EnVision/HRP, Rabbit/Mouse (ENV) reagent was applied to these sections, and then the ChemMate DAB chromogen was used. The slides were then slightly counterstained with hematoxylin.

The expression of PCDH17 and BECN1 proteins was scored according to the intensity of membrane and cytoplasmic staining using a four-point system: 0, negative; 1, weak; 2, moderate; and 3, strong. To investigate the correlation between PCDH17 and BECN1 expression and clinicopathological features, CRC patients were divided into two groups: low expression (0 and 1, -) or high expression (2 and 3, +). Immunostaining was independently scored by two investigators who did not know the patient’s clinical information in separate cases.

**PCDH17-expressing plasmids and transfection**

The pCMV6 Entry-PCDH17 plasmid or empty vector (pCMV6 Entry-mock; OriGene, MD, USA) was transfected into the colorectal cell lines HCT116 and SW480 with the MegaTran 1.0 transfection reagent (OriGene), followed by selection of stable clones expressing PCDH17 or the empty vector for further study.

**Cell Viability and Apoptosis Measurement**

Cell viability was assessed using the Cell Counting Kit-8 (CCK-8) (LJ621, DOJINDO, JAPAN) following the product’s introduction.
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**Fig. 4** Autophagy contributes to PCDH17-induced growth inhibition in CRC cells, and PCDH17 regulates autophagy and 5-FU sensitivity. a HCT116/PCDH17 and SW480/PCDH17 cells were treated with 5-FU with or without the pancaspase inhibitor Z-VAD-FMK for 24 h, and cell viability was assayed. The experiments were performed in triplicate. *p < 0.05. b HCT116/PCDH17 and SW480/PCDH17 cells were treated with 5-FU with or without the necroptosis inhibitor necrostatin-1 for 24 h, and cell viability was assayed. The experiments were performed in triplicate. **p < 0.01. c HCT116/PCDH17 and SW480/PCDH17 cells were treated with 5-FU with or without the autophagy inhibitor Baf-A1 (0.05 nM) for 24 h, and cell viability was assayed. The experiments were performed in triplicate. **p < 0.01. d HCT116/PCDH17 and SW480/PCDH17 cells were treated with 5-FU with or without the autophagy inhibitor 3-MA (5 μM) for 24 h, and cell viability was assayed. The experiments were performed in triplicate. **p < 0.01. e HCT116/PCDH17 and SW480/PCDH17 were transfected with PCDH17-specific shRNA, and then PCDH17, caspase-3, BECN1 and LC3B levels were assessed by western blotting. g HCT116/PCDH17 and SW480/PCDH17 cells were transfected with BECN1 plasmid, and then BECN1 and PCDH17 levels were assessed by western blotting. h i HCT116/PCDH17 and SW480/PCDH17 cells were transfected with PCDH17-specific shRNA, and then the cells were exposed to 5-FU at various final concentrations for 24 h. Cell viability was measured by CCK-8 assay after 48 h. Data are presented as the means ± standard deviations (SDs). The experiments were performed in triplicate.

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Cells were seeded at a density of 5,000 cells per well in 96-well bottom microtiter plates. After 24 h, 5-FU was added at different times at the indicated concentrations. The absorbance was measured at 450 nm on a microplate reader (Synergy HT, Bio-Tek, USA). Apoptosis was detected using a Pharmingen Annexin V-FITC Apoptosis Detection Kit I (BD, USA) following the manufacturer’s instructions. Briefly, cells were seeded at a density of 2 × 10^6 cells per 6 cm culture dish. After attachment overnight, cells were washed twice with PBS and cultured in medium with 20 µM 5-FU for 24 h. All cells were collected and resuspended in ice-cold 1 x binding buffer. Approximately 1 × 10^5 cells were then stained with 5 μL of Annexin V-FITC and 5 μL of PI for 15 min and analyzed using a FACS Calibur flow cytometer (Beckman Coulter, CytoFLEX S, USA).

**Western Blot Analysis**

Cells were collected from cultured dishes and lysed in lysis buffer. The main components of the lysis buffer were 20 mM Tris-HCl pH 7.6, 1 mM EDTA, 140 mM NaCl, 1% NP-40, 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM sodium vanadate. Protein concentration was determined using a BCA Protein Assay Kit (Pierce). Protein samples (40 μg of protein per cell line) were separated on a 5 to 20% Tris-Tricine Ready Gel (Bio-Rad) by SDS-PAGE for nitrocellulose membrane blotting. The blotted membrane was blocked with 5% skim milk for 1 h at room temperature and incubated overnight with primary antibody. The immunoreactive band was observed by enhanced chemiluminescence using horseradish peroxidase-conjugated IgG secondary antibodies. Band density was measured by densitometry, quantified using NIH image 1.62 gel map macros, and normalized to the specified sample in the same membrane.

**RNA interference**

PCDH17 human shRNA (OriGene, TR310584) was transfected with Lipofectamine RNAi Max (Invitrogen, 13778150) following the manufacturer’s instructions.

**Transmission electron microscopy**

The treated cells were washed and fixed in 2.5% glutaraldehyde (Sigma-Aldrich, GS882) for 30 min. The samples were treated with 1.5% osmium tetroxide, dehydrated with acetone and embedded in Durcupan resin. Thin sections were poststained with lead citrate and examined with a 60 kV TECNAI 10 electron microscope (Philips, Holland).
In vivo subcutaneous tumor model
All in vivo experimental protocols were approved by the Animal Protection Committee of Hangzhou Normal University. Viable HCT116/PCDH17 cells (1 × 10^7) were subcutaneously injected into the right dorsal flank of 6-week-old female BALB/c nude mice (five mice per group). Tumor volume was measured every 2 days for 4 weeks and calculated by the following formula: (short diameter)^2 × (long diameter)/2.

Statistical analyses
Data are expressed as the means ± standard deviations (SDs). The experiments were performed in triplicate. *p < 0.05.

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Statistical analyses
Data are expressed as the means ± SD of three independent experiments. Prism 7.0c GraphPad software was used for statistical analysis. Significant differences between groups were analyzed by Student’s t test, and a p value < 0.05 was considered statistically significant.

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AUTHOR CONTRIBUTIONS
X.S. and T.X. designed the research; S.L., H.L. and D.W. performed the immunohistochemical staining, autophagy analyses and cell viability assays. Q.L., H.L., G.L., X.C. and Y.L. provided technical support. P.C., B.Z., W.W., R.Z., B.C., M.Z. and X.H. performed the animal experiments. Q.L., L.C. and Y.L. performed the Kaplan–Meier survival analysis. X.C., G.L., Y.X., T.D., J.F. and J.L. contributed materials and performed data analysis. X.H., Q.Z., T.P., L.Y., T.J., W.Z., L.Z. and Y.S. collected data. X.S. wrote the manuscript with contributions from the other authors.

ADDITIONAL INFORMATION
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