Online Supplementary Methods

Organoid cultures

The patient-derived organoid cultures used in the current study (58 gastric tumour organoids derived from 54 patients, and representative paired normal organoids) were either previously described and characterised, or newly established and characterised using the same protocol (See online supplementary table 1).[1] They were established from fresh gastrectomy specimens from gastric cancer patients who underwent surgical resection of gastric cancer at Queen Mary Hospital. Informed consent was obtained from all patients, with the research protocol approved by the Institutional Review Board of the University of Hong Kong and the Hospital Authority Hong Kong West Cluster. The clinicopathological data for the patient cohort, including age, sex, tumour subtype, stage and infiltration patterns, are listed in online supplementary table 1. Key oncogenic driver mutations were derived from exome sequencing data whereas oncogenic fusion transcripts involving ARHGAP genes were derived from transcriptome analysis using bioinformatics pipelines as described in our previous study.[1] Details are provided in online supplementary table 1.

Normal and tumour gastric organoids were established and cultured under standard stomach medium as previously described.[1] Briefly, normal tissues were washed and cut into ~5 mm^2 pieces, followed by incubation in a PBS based chelating buffer (10mM EDTA, 0.5mM DL-dithiothreitol, 1x P/S and 100µg/ml Primocin) at 4˚C for 45 minutes. The tissue-pieces were then transferred to a petri dish and the gastric glands were released by pressing the tissues using a glass-slide. The released gastric glands were washed twice with PBS and resuspended with Matrigel. A drop of 50µl Matrigel-cell mixture was added to each well of a pre-warmed 24-well

plate. For tumour tissues, the specimens were chopped and minced before incubation in a digestion buffer (advanced DMEM/F12 containing 1x P/S, 100µg/ml Primocin, 2.5% FBS, 0.6mg/ml collagenase, 20mg/ml hyaluronidase and 10µM Y-27632) at 37˚C. The incubation times usually last for 1 hour, and was extended to overnight for diffuse-type tumours with very tough stroma. The tumour cell clusters were then collected by passing through a cell strainer, washed twice using AdDMEM medium, and resuspended in Matrigel for plating in a 24-well plate, similar to the procedure for normal glands. 500µl of standard gastric organoid medium (advanced DMEM/F12, 1x GlutaMax, 1x HEPES, 1x P/S, 50% Wnt3a, 10% RSPO-1, 10% Noggin, 1xB27, 50ng/ml EGF, 200ng/ml FGF10, 1mM N-Acetylcystenine, 1nM Gastrin, 2µM A83-01) and 10µM Y-27632 were added to each well. The culture medium was changed every 2-3 days and primocin was added during the first 2 weeks of culture.

Passage of the organoid cultures was performed two weeks after isolation, when the Matrigel droplets containing organoids reached approximately ~80% to 90% confluence, and being split at a 1:3 or 1:4 ratio. Normal gastric organoids were disrupted mechanically using a fire-polished glass Pasteur pipette, while tumour organoids were passaged using a trypLE solution at 37℃ for three to five minutes. For some tumour organoids that were difficult to dissociate, 50mM EDTA was added with the trypLE to improve the dissociation efficiency into single cells. The disrupted organoids were collected by centrifugation, re-embedded in Matrigel and cultured in fresh gastric medium. 10µM Y-27632 was added to the culture medium right after passage and covering up to the first 2-3 days and then omitted in subsequent culture medium change during expansion. All cultures were regularly checked and confirmed to be mycoplasma free.

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DNA and RNA were extracted from the organoids, as well as their paired frozen tissues, if adequate spare adjacent tumour tissues were available. For organoids, including the ARHGAP fusion knock out clones, 5-6 Matrigel droplets at 90% confluent were incubated with Cell Recovery solution (500µl Cell Recovery solution per 50µl Matrigel) at 4℃ for at least thirty minutes. After incubation, the released organoids were washed twice with 1X PBS and centrifuged at 600-800 x g for five minutes. The organoid pellets were lysed with lysis buffer from the QIAGEN All prep DNA/RNA extraction kit, (QIAGEN), and DNA and RNA were extracted following the manufacturer's protocol. To delineate the transcriptomic changes brought about by ARHGAP fusion and corresponding KO clones under ROCK inhibition withdrawal condition, representative fusion knockout organoids spanning the 3 ARHGAP fusion subtypes (GX052-TO g1B, GX052-TO g3B, GX058-TO g2B, GX080-TO g3A), and their corresponding un-induced fusion positive control (GX052-TO g1-Ctrl, GX052-TO g3-Ctrl, GX058-TO g2-Ctrl, GX080-TO g3-Ctrl) were trypsinized into single cells, re-embedded into Matrigel and overlaid with standard human gastric organoid medium without Y-27632. After 24 hours, the cells were released by cell recovery solution, washed and lysed for DNA/RNA extraction as described above.

Transwell migration assay

Two to three Matrigel droplets containing organoids at ~80% confluence were trypsinized into single cells. $5x10^5$ cells were seeded into a 6.5mm insert with an 8µm pore polycarbonate membrane, in triplicate. The cells were cultured in plain Ad/F12 DMEM medium with Y-27632 and 10% Noggin. 500µl of complete gastric medium containing Y-27632 and 20% FBS as a chemoattractant was added to the lower chamber. After 48 hours, cells in the inserts were

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carefully removed using moistened cotton buds. The cells that migrated across the transwell inserts were fixed by immersing the inserts into methanol for 10min, and then stained with 0.5% crystal violet for visualization after being air-dried. The migrated cells were imaged under a microscope, and quantitated using Image J.

ROCK inhibitor (Y-27632) withdrawal assay in Matrigel (Cell-cell adhesion dependence)

Two to three Matrigel droplets containing organoids at ~80% confluence were trypsinized into single cells. 5,000 cells were seeded in Matrigel in triplicate and cultured in standard gastric organoid culture medium with or without Y-27632. The culture medium, with or without Y-27632, was changed every 2-3 days, and bright field images were taken on day 14 to document organoid survival in different conditions. Fast growing organoids, cultured with or without Y-27632, were passaged once at day 7. At day 14, the organoids were collected and cell viability was analysed by CellTiter-Glo luminescent cell viability assay (Promega, Fitchburg, WI, USA), according to the manufacturer's protocol. Luminescence was recorded using a multi-functional plate reader (SpectraMaxL, Molecular Devices), with one second exposure time. Organoid viability upon Y-27632 withdrawal was normalized against the measured luminescence of lysate from cells cultured with Y-27632. A viability ratio of 70% or less is considered cell-cell adhesion dependent (CCd) whereas >70% is considered cell-cell adhesion independent (CCi). The assay was repeated once using a different cell passage to ensure consistent results.

Spheroid formation (cell-matrix adhesion dependence) and double challenge assays

Two to three Matrigel droplets containing organoids at ~80% confluence were trypsinized into a single cell suspension. 5,000 cells in 500μL of standard gastric organoid medium, with or

without Y-27632, were seeded in ultra-low binding plates, without Matrigel, in triplicate. 100μL of fresh gastric organoid medium, with or without Y-27632, was added to each well every 2-3 days. At day 14, bright field images were taken to document spheroid formation and survival in medium with or without Y-27632, and the number of viable cells in each treatment group was determined by an automated cell counter (TC20™ cell counter, Bio-Rad or LUNA™ Automated Cell Counter, Logos Biosystems) after incubation with trypan blue (Bio-rad), following the manufacturer's recommendation. Spheroid viability in the presence of Y-27632 was calculated by dividing the final viable cell count with the initial number of seeded cells (i.e. 5,000 cells) to derive the cell-matrix adhesion dependence. Fold change great than 1 is considered cell-matrix independent (CMi) state, meaning the organoids can expand in number without attachment to matrix. For spheroid viability assay performed without Y-27632 (double challenge), the ratio of final viable cell counts without Y-27632 normalized to the final viable cell counts with Y-27632 was calculated and taken as the CC/CM dependence index. Tumour organoids that were CCi and had a viability ratio >70% under double challenge was considered to be CCi/CMi.

Proliferation Assay

The doubling time of the organoids was measured as previously described.[2] Briefly, the organoids were trypsinized into a single cell suspension. 10,000 cells were seeded in Matrigel in triplicate and cultured in standard stomach organoid culture medium with Y-27632 for 7 days. Afterward, the organoids were dissociated into single cells again and the number of viable cells was counted by an automated cell counter as described above. The growth rate was calculated from the mean of 3 replicates using the equation y(t) = y0 x e (growth rate x t) (y = number of cells at final time point, y 0 = number of cells at initial time point, t = time). The mean

doubling time (in hours) from 3 replicates was calculated as Doubling time = In(2)/Growth rate.

Generation of the ARHGAP fusion knockout tumour organoid lines

An all-in-one doxycycline inducible CRISPR/Cas9 system was used to create *ARHGAP* fusion knockout (KO) tumour organoids. The TLCV2 vector (Addgene plasmid #87360) was used for the cloning of single guide RNA (sgRNA). Two sgRNAs targeting *CLDN18* exon 5 and two sgRNAs targeting *CTNND1* exon 16 were designed (but only one sgRNA successfully generated clones for *CTNND1*) (Online supplementary table 4), with the aim of create INDELs near the fusion junction. 10μM sense and antisense paired oligos for each sgRNA were cloned into a digested TLCV2 vector following a previously described protocol.[3] Clones with the correct insertion were confirmed by Sanger sequencing using a U6 promoter forward primer (5' > 3' sequence: GGACTATCATATGCTTACCG). The desired sgRNA plasmids and viral packaging vectors were transfected into 293T/17 cells for lentiviral production. In brief, $3x10^7$ 293T/17 cells were seeded in a 150mm tissue culture dish in 25ml of medium (DMEM with 1x P/S, 10% FBS). One day after, 15μg of each viral packaging plasmid (pVSVG, pMDL and pRSV) and 45μg of recombinant plasmid were transfected into the 293T/17 cells using polyethylenimine (PEI). At 16 hours post transfection, the medium was changed. After 3 days, the viral particles were collected by centrifugation at 25,000rpm for 2.5 hours at 4°C, and transduced to ARHGAP fusion positive tumour organoids using a previously described protocol.[4] Briefly, the viruses were resuspended in gastric organoid medium supplemented with 10μM Y-27632 and 8mg/ml polybrene. 400μl of virus was mixed with 100μl of trypsinized organoids from 4 droplets of Matrigel at 85% confluence, in gastric medium with Y-27632. The cells were spinoculated at 300x g for 1 hour at room temperature, followed by incubation at 37°C for 4 hours. After that,

the cell-virus suspension was collected and the cells were centrifuged, re-embedded in Matrigel and cultured in gastric medium with 10μM Y-27632. The lentiviral transduced *ARHGAP* fusion knockout organoids underwent puromycin selection (1μg/ml) for 7 days and were then stocked as an un-induced fusion-positive control. In parallel, the transduced organoids were trypsinized into single cells and serially diluted in a 24-well plate. Cas9 expression was induced by 1μg/ml doxycycline for at least 2 weeks, followed by picking individual clones for clonal expansion. Mutations at the DNA level for each of the clonal organoid lines were validated by PCR and Sanger sequencing using primers flanking the genomic DNA sequence around the sgRNA cut sites (primer sequences were listed in online supplementary table 5). Fusion transcript expression levels in the parental tumour organoids and some of the knockout clones were quantitated by qRT-PCR using QuantiFast SYBR Green PCR kit using primers specifically amplifying the fusion transcripts (the primer sequences were listed in online supplementary table 6). Protein lysates were also obtained from representative ARHGAP fusion organoids and corresponding KO clones, using a protein lysis buffer (150mM NaCl, 50mM Tris pH7.4, 1% NP40 & 1mM EDTA), supplemented with protease and phosphatase inhibitors (Roche) for western blotting. 50-80μg of protein was resolved on SDS-polyacrylamide gels (7.5% home-made gel or 4-15% precast gradient gel) in a running buffer (25mM Tris base, 192mM glycine, 0.1% SDS) and transferred to a nitrocellulose membrane at 110V for 2 hours in a cold transfer buffer (25mM Tris base, 192mM glycine, 20% methanol). The membrane was blocked with 5% non-fat milk in Tris-buffered saline (TBS) for 2 hours and incubated with the respective primary antibodies (Anti-ARHGAP6 at a 1:400 dilution; Anti-ARHGAP26 at a 1:1,000 dilution) overnight at 4 °C, followed by incubation with a secondary antibody (Anti-rabbit at a 1:5,000 dilution) for 1 hour at room temperature. Protein expression was detected using either ECL Prime, ECL, or

WESTSAVE Up. The membrane was re-probed with an anti-β-actin antibody (1:5,000) as a loading control. Chemiluminescence was visualized by X-ray film or by digital imaging using Alliance Q9 Advanced.

Western blot was also performed to study the change in protein expression of adhesion molecules in the organoids upon introducing different driver mutations, using the following primary antibodies: Anti-E-cadherin (1:2,000 dilution), anti-catenin alpha-1 (1:2,500 dilution) and anti-β-catenin (1:1,000 dilution) antibodies.

Rhotekin-RBD pulldown assays and immunoblotting

The effect of CLDN18-ARHGAP26 fusion on RHO GTPase was quantified using an Active Rho Pull-down and Detection Kit (Thermo Scientific), following the manufacturer's protocol. Briefly, the expression vector encoding the *CLDN18-ARHGAP26* fusion transcripts were transiently transfected into 293T/17 cells alone or in combination with a previously cloned wild-type RHOA expression vector using PEI. At 48 hours post transfection, the cells were serum starved overnight and stimulated with 10% FBS for 30 min to activate RHOA prior to collecting the protein lysate. 800μg of cell lysate was used per active Rho pulldown reaction. The eluted Rhotekin-Rho bound proteins were resolved by 12% SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane, blocked with 3% BSA and incubated for 1 hour with primary mouse anti-Flag antibody (Sigma-Aldrich 3165, clone M2; 1:2,000 dilution). Total cell lysates (10μg) were blotted with anti-Flag antibody and anti-Myc-antibody (R951-25, Novex by Invitrogen, Life Technologies) to quantitate the total Flag-tagged RHOA and Myc-tagged fusion

proteins in the 293T/17 cells, respectively. The membrane was re-probed with an anti-GAPDH antibody (1:1,000) as a loading control.

Overexpression of CLDN18-ARHGAP26 cDNA and immunofluorescence staining of ARHGAP fusion proteins in 293T/17 cells

CLDN18-ARHGAP26 transcripts were amplified from gastric cancer samples and cloned into the pcDNA3.1+Myc-His version C vector (Thermo Fisher Scientific) using the primers listed in online supplementary table 7. The nucleotide sequence of all constructs was verified by Sanger sequencing and the plasmid DNA was purified using the Plasmid Maxi Kit (Qiagen) for transfection into 293T/17 cell lines, using PEI as previously described.[5] After 72 hours, the transfected cells were fixed in ice-cold methanol for 10 minutes and washed with PBS, before blocking with 10% normal goat serum in PBS for 30 minutes. The blocking solution was then drained off and the cells were incubated with 1:500 anti-Myc antibody (R950-25, Novex by Invitrogen, Life Technologies) in 1% normal goat serum in PBS, in a moist chamber at 4°C overnight. The cells were washed 3 times with PBS before incubating in 1:100 goat anti-mouse Alexa Fluor 594 secondary IgG (A-11032, Invitrogen) in 10% normal goat serum for 30 minutes, at room temperature. Finally, the cells were rinsed 3 times with PBS then mounted using Vectashield Mounting Medium for Fluorescence with DAPI (H-1200 Vector Laboratories). The fluorescence was visualized under a confocal microscope (Carl Zeiss LSM780) within one week. The confocal images were captured and analysed using the ZEN 2012 SP1 (black edition, 64 bit) software by Carl Zeiss Microscopy.

Mouse gastric xenograft model to characterise tumorigenicity of ARHGAP fusion

The tumorigenicity of *ARHGAP* fusion was studied using a mouse gastric xenograft model, which was approved by the Committee on the Use of Live Animals in Teaching and Research (CULATR no. 5084-19). A representative *ARHGAP* fusion KO tumour organoid line (GX052-TO KO-g1B) and its corresponding un-induced fusion-positive tumour organoid line (GX052-TO g1-Ctrl) was first transduced with virus containing pHIV-Luc-ZsGreen (Addgene plasmid # 39196), a pHIV-ZsGreen plasmid with firefly luciferase (Luc2P). Transduced organoids were enriched by FACS before expanding for mouse injection. Briefly, the organoids were trypsinized into single cells and resuspended in a cell sorting medium (1x PBS supplemented with 1x B27, 2% FBS, 10μM Y-27632) to achieve a concentration of ~$1x10^6$ live cells/ml. The cells were filtered through a 40μm cell strainer into a round-bottom tube before loading to a BD Influx™ cell sorter (BD Biosciences). For each organoid line, GFP-positive singlets with FITC signal between 1-3 log intensity were sorted into a collection medium (2:1 AdvDMEM/F12:standard gastric medium, 10μM Y-27632). The sorted cells were collected by centrifugation at 1,000x g for 5 minutes, embedded in Matrigel, and cultured in the presence of Y-27632 and Primocin for 1 week before orthotopic injection into the mouse stomach submucosa. 6-8-week-old NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG, NOD scid gamma) mice were fasted overnight before surgery. On the day of surgery, luciferase-tagged organoids with or without *ARHGAP* fusion were trypsinized and resuspended in a 1:1 mixture of 2x gastric medium:Matrigel (2x gastric medium contained double the concentration of growth factors compared to the standard gastric medium). The mice were anaesthetized and the stomach was exteriorized with forceps. A final volume of 50μl of cell suspension, with $2.5x10^6$ cells, was injected into the stomach submucosa. The mice were monitored by bi-weekly bioluminescence imaging, using the IVIS® Spectrum *in vivo*

imaging system, starting from week 3 post surgery. Tumour volume was represented as bioluminescence intensity (photons/second) and bioluminescence intensity normalized to luciferase activity of corresponding organoid line. Luciferase activity of GX052-TO g1-Ctrl and GX052-TO KO-g1B lines at passage prior to orthotopic injection was measured using a Luciferase assay kit (Promega). Briefly, 20,000 cells in triplicate were lysed, followed by adding in Luciferase assay buffer II to generate a luminescent signal. Luminescence was recorded using a multi-functional plate reader (SpectraMaxL, Molecular Devices). At week 15 post surgery, the mice were euthanized by cervical dislocation under anaesthesia. The stomach was isolated and imaged with the IVIS® Spectrum by directly applying luciferin solution to the stomach surface. The tumour region was evenly divided into two tissue slices. One was fixed in 10% neutral buffered formalin (NBF) for paraffin sectioning, while the other was snap frozen. The other internal organs were also checked for any abnormalities or possible metastasis. The stomach tumour region frozen and paraffin blocks were serially sectioned and examined for presence of tumour by a pathologist. The slides with the maximum amount of tumour were scanned by nanozoomer and the maximum tumour area (mm^2) were traced and measured by the computer software.

Immunohistochemistry

Immunostaining for Ki-67 protein was performed on 6mm paraffin sections of xenografts from the mouse stomach block. Briefly, staining was performed using the Roche Ventana Discovery Ultra automatic slide stainer with a monoclonal mouse anti-human Ki-67 antibody (Dako, M7240) at 1:250 dilution, after heat-mediated antigen retrieval at 100ºC for 20 min and detected using DAB as the chromogen from Bond Polymer Refine Detection (Leica Biosystems, Cat No: DS9800). Negative controls were performed by omitting the primary antibody.

Genome editing to create CDH1 or RHOA mutation in normal gastric organoids via CRISPR/Cas9

To create *CDH1* or *RHOA* mutations in normal gastric organoids and evaluate their effects on cell-cell and cell-matrix independence, 2sgRNAs were designed to target exon 3 and 6 of *CDH1*, and 1 sgRNA was designed to target *RHOA* (Online supplementary table 4). The sgRNA oligos were cloned into the pSpCas9(BB)-2A-Puro (PX459) V2.0 vector (Addgene plasmid # 62988), following a previously described protocol.[6] Clones with the correct insertion were confirmed by Sanger sequencing. Normal gastric organoids (GX059-BO) were co-transfected with sgRNA targeting *TP53* in addition to either *CDH1* or *RHOA* sgRNA to increase the success rate of obtaining the desired mutations through Nutlin-3a selection. Transfection was performed by electroporation using a Amaxa^{TM} Mouse/Rat-Hepatocyte-Nucleofector^{TM} kit, as previously described.[7] Briefly, two to three Matrigel droplets containing organoids at ~80% confluence were pooled and trypsinized into a single cell suspension. Then, $1x10^6$ cells were resuspended in 100µl of supplemented nucleofector solution containing 6µg of the desired plasmids. The cell suspension was transfected by electroporation, after which they were re-embedded in Matrigel, with 10µM Nutlin3a added to the culture medium at 48 hours post electroporation for the selection of *TP53* mutant organoids. The surviving clonal organoids were manually picked and expanded for downstream functional assays. Mutations at the DNA level for each of the clonal organoid lines were verified by PCR and Sanger sequencing using the primers listed in online supplementary table 5.

Transcriptomic profiling of organoids, Gene set enrichment analysis (GSEA) and gene signature scores using bulk RNAseq data

Newly established tumour organoids, Crispr engineered organoids and GC frozen tissues were subjected to RNA sequencing (RNAseq) as previously described.[1] Briefly, 4μg of total RNA per sample was subjected to cDNA library construction using the KAPA Stranded mRNA-Seq Kit (KR0960-v3.15) and sequenced using the PE101 HiSeq1500 or PE151 NovaSeq6000. The raw sequence reads were aligned against the reference genome (human genome build 37, hg19) by standard STAR pipeline. The gene expression was imputed by RSEM with default parameters to generate the log 2 TPM value for each gene. Differential gene expression analysis was conducted by the limma empirical Bayes moderation for each gene and each contrast.[8] The gene set enrichment analysis (GSEA) was performed on a collection of functional gene sets (Hallmark gene sets, KEGG pathways, Gene Ontology Biological Process, Molecular Function, Cellular Component). Then the gene ranks by t-statistics between test condition and control were projected into the functional gene set to get the enrichment score and adjusted P-value.[9] Finally, enriched gene sets were ranked based on the adjusted p-value and the top-ranked gene sets were listed according to a normalized enrichment score (NES), from positive enrichment to negative enrichment. Gene set signature scores for individual samples were derived by calculating single sample gene set enrichment scores (ssGSEAs)[9, 10] on a gene expression matrix.

Transcriptome signature of CCi/CMi state and prognostic analysis on GC patient cohorts

The public available gene expression of gastric tumours was collected from three independent GC cohorts: TCGA Gastric Adenocarcinoma (STAD, n=415), Asian Cancer Research Group

(ACRG, n=300, from GSE62254), and the Korean cohort (GSE26253, n=432). Patients without recorded follow-up and vital status were excluded. In each cohort, a cell-cell/cell-matrix adhesion independent (CCi/CMi) score for each patient was calculated by the CCi/CMi gene signatures in two steps: 1. Differential gene expression analysis was conducted as described above.[8] We compared the RNAseq data of CCi/CMi tumour tissues versus non-CCi/CMi tumour tissues after exclusion of MSI samples, using a cut-off of $p<0.01$, log2 fold change >1 or <-1, and obtained 500 up- and 85 down-regulated genes, respectively. We used this gene list to compile a CCi/CMi-up and CCi/CMi-down signature score for every sample in different patient cohorts using the ssGSEA method.[9, 10] 2. the CCi/CMi score was calculated from CCi/CMi-up score – CCi/CMi-down score. In each cohort, patients were grouped by the quantiles ranks of the CCi/CMi scores, that patients in the top quantile were named as "CCi/CMi-high" and patients in bottom quantile were named as "CCi/CMi-low". Survival analysis of both OS (over-all survival) and DFS (disease-free survival) were determined by Kaplan-Meier analysis and the cox regression HR with two-sided 90% CI.

Patient and Public Involvement

As stated above, informed consent was obtained from all patients. However, due to the complexity of our study, it was not appropriate or possible to involve patients or the public in the design, or conduct, or reporting, or dissemination plans of our research.

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