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ErbB Receptor stimulation is required for mouse colon adenoma organoids to form crypts

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Abstract

The majority of colon adenomas harbor genetic mutations in the APC gene. APC mutation leads to changes in Wnt signalling and cell-cell adhesion: as a consequence, intestinal crypt budding increases and the excess crypts accumulate to form adenomas that progress to colon cancer. When cultured with Wnt, R-spondin, EGF, Noggin, myofibroblast conditioned medium and Matrigel, crypts from normal mouse colon mucosa form crypt-producing organoids and can be passaged continuously. Under the same culture and passage conditions, crypts isolated from colon adenomas derived from *Apc*^{min/+} mice typically grow as spheroidal cysts and do not produce crypts. The adenoma organoid growth requires EGF, but not Wnt, R-spondin or Noggin. However, when mouse colon adenoma spheroids are grown for more than 10 days in the presence of EGF, crypt formation occurs. EGF, EREG, β -cellulin, Neuregulin-1 or AREG are sufficient for initiating crypt formation, however, neuregulin-1 is more potent than the other EGF-family members. EGFR and ErbB2 inhibitors both prevent crypt formation in adenoma cultures. Either EGFR:ErbB2 or ErbB3:ErbB2 signalling is sufficient to initiate adenoma crypt budding and elongation. ErbB2 inhibitors may provide a therapeutic avenue for controlling and ablating colon adenomas.

Introduction

The intestine is a highly regenerative organ with cells being replaced continuously. The epithelium surface is made up of a sheet of epithelial cells folding into glandular like crypts. Under normal homeostatic conditions, regeneration is maintained by the production of new cells from actively cycling stem cells located at the base of each crypt as well as the production of new crypts. During developmental growth or repair of the intestinal epithelium, new crypts are produced by crypt budding^{1,2}, where the new crypt is initiated at the base of an existing crypt and elongates as the upper rim migrates to the top of the crypt³⁻⁵. Aberrant crypt budding has been implicated as a potential mechanism responsible for colorectal hyperplastic polyps⁶ and linked to adenoma formation⁷. These observations have been corroborated in the intestinal and colonic epithelium of *Apc*^{min/+} mice during polyp formation⁸.

Intestinal epithelial cells can be cultured *in vitro* to produce multicellular three-dimensional structures called organoids⁹⁻¹¹. The development of small intestine¹¹ and colon^{9,10,12} organoid cultures allows the direct study of both intra-crypt cell production and crypt budding in organoids. Organoids can be used to measure the effects of factors regulating crypt initiation and survival or intra-crypt cell production in normal, adenomatous or cancerous colon mucosa^{9,10,12}.

Previous reports show that factors such as epimorphin¹³, bone morphogenetic proteins (BMPs)^{14,15}, Wnt^{16,17}, Epidermal Growth Factor (EGF)¹⁸, TGFβ¹⁹ and Hedgehog¹⁶ can affect the formation of the villus-crypt structure in the small intestine. Whereas mouse small intestinal organoid growth requires R-Spondin, Noggin and EGF, but not Wnt, colon organoids require the addition of Wnt¹². We have previously reported our results with mouse colon organoids looking at the effect of the structural environment and biochemical factors^{9,10} on development of crypts in wildtype mouse colon organoids. Our previous study suggests that crypt formation in mouse colon organoids also requires the conditioned medium derived from mouse myo-fibroblast cells (WEHI-YH2)²⁰. In contrast, mouse colon adenomatous organoids grow and passage weekly as spheres or cysts and have not been previously reported to form crypts *in vitro*^{21,22}. In this study, we report that whilst colon adenoma cells from *Apc*^{min/+} mice initially form spheres, when cultured 10 or more days without splitting the cultures, in the presence of EGF or selected members of the ErbB family of ligands^{23,24}, adenomatous crypts will form. Our results provide insights into the mechanisms initiating crypt production and the aberrant growth of adenomas and colon cancers.

Results

Crypt formation is induced by EGF stimulation in long term colon adenoma cultures

The normal mouse colon is lined with a single layer of polarised epithelial cells that form the regular array of crypts which create the flat luminal surface (Fig. 1a). Colon crypts were labelled with phalloidin to visualise F-actin (luminal-specific) and E-cadherin (baso-lateral specific). The high resolution 3D imaging reveals the highly organised crypt structures and apical/basolateral polarity (Fig. 1a, Sub Fig. 1a,b). In contrast to the normal colonic epithelial mucosa, adenomatous colonic polyps from $Apc^{min/+}$ mice display irregular crypt structure and disorganised packing of the crypts i.e. the crypts no longer stack in perpendicularly to the mucosa surface (Fig. 1b, Sub Fig. 1c,d). The crypts are still tightly packed, but the luminal axes are no longer aligned. Individual adenomatous crypts retain the apical/basolateral polarity of the epithelial cells (Fig. 1b).

Cells from normal colon mucosa form colonospheres in matrigel cultures (with the full complement of growth factors) and from Day 7 the colonospheres begin to produce crypt buds (Fig. 1c). As previously shown, the addition of myofibroblast conditioned media (WEHI-YH2) is required for optimal crypt production⁹. Most budding crypt organoids produce multiple crypt structures formed from epithelial cells with typical apical/basolateral polarity (Fig. 1c and inset, Fig. 1f). In contrast, cells from $Apc^{min/+}$ colon adenomas require only the addition of EGF in the basic culture media to proliferate and form spheroids, (cyst-like) structures (Fig. 1d). The $Apc^{min/+}$ adenomas organoids grow similarly with or without the addition of Wnt, R-spondin and Noggin²². The spheroids are readily visible by Day 6 and continue to grow but do not form crypt-like structures when in culture and passaged for up to 10 days (Fig. 1d). Adenoma cultures can be passaged and grown in long term culture, but require passaging after 7-10 days and continue to grow as spheroids. Under these conditions, in contrast to the organoids from normal colon, $Apc^{min/+}$ adenoma organoids do not appear to produce crypts. This was surprising given that adenoma tissue contains tightly packed and irregular crypt structures (see Fig. 1b) and suggested that the culture conditions did not provide the appropriate micro-environment to form adenomatous crypts. In order to investigate adenoma organoid growth further, the $Apc^{min/+}$ adenoma cultures were allowed to grow for more than 7 days without mechanical disruption and in the presence of EGF, but in the absence of R-spondin and Wnt3a. Under these conditions, the organoids produced crypt-like structures from Day 12 (Fig. 1d). Budding crypt structures were evident in a significant proportion of the organoids, similar to the normal colon organoids (Fig. 1c, d). To ensure that the crypt formation phenomenon presented by the current colon adenoma line was not an isolated event (i.e. specific to a specific organoid line), adenoma cell lines derived from separate $Apc^{min/+}$ adenomas were tested and similar crypt-like structures were observed when cultured in the presence of EGF up to 20 days (adenoma line #B13 shown in Supplementary Fig. 2). Time-lapse imaging of another crypt forming organoid suggest that the crypt-like structures can occasionally start to appear after 7 days (Supplementary Movies S1 and S2). $Apc^{min/+}$ colon adenoma organoids require EGF for both growth of the colonospheres and crypt-like structure production. The adenoma colonospheres grew

poorly without EGF (Fig. 1e, h). The $Apc^{min/+}$ adenoma organoid crypt structures have a similar morphology to cultures of normal colon organoids with similar dimensions (eg. diameters of $\sim 40 \mu m^5$) but produce many more crypt structures per organoid (Fig. 1c, d, f, g, i). High resolution 3D imaging of an immunostained $Apc^{min/+}$ adenoma organoid with multiple crypts reveals the structural organisation of the crypts with strong peripheral staining of E-cadherin and β -catenin (Fig. 1i).

Effect of EGF on the formation of colon adenoma organoid crypts

The EGF signalling pathway is activated in the intestinal epithelial stem cell niche²⁵. EGF increases the proliferation of mouse intestinal stem cells²⁶, mouse embryonic intestinal tissue²⁷ and is an important component for small intestinal¹¹ and colon organoid cultures^{9,10,12}. To investigate the amount of EGF needed to trigger crypt formation, colon adenoma cultures were treated with different concentrations of EGF and imaged daily over 21 days. Organoids remained as spheroids at 0.005 ng/ml EGF but produced crypts between 0.05 – 5.0 ng/ml EGF (Fig. 2a-b). The proportion of crypt-containing organoids was scored and is presented in Fig. 2b. In the absence or at lower concentrations of EGF the organoids remain as spherical cysts (Fig. 2a, b). The crypt formation colon adenoma cultures increased with increasing EGF concentration, from 0.05 ng/ml to 20 ng/mL (Fig. 2b) and was significantly increased above 0.5 ng/ml (Fig. 2b).

Different morphological shapes of colon adenoma organoids.

The colon $Apc^{min/+}$ adenoma organoids have four three distinctive morphologies: Spheroids (see Fig 3a up to day 8) , Large body organoids with crypt extensions (Fig 3a), Lenticular organoids (Fig 3b) and Hyperbudding organoids (Fig. 3c). The Large body extension organoids had crypts forming after the organoid had grown to a significant size. Crypts extensions then appeared from the main body, leading to the gradual regressing/reduction of the size of the main organoid body. Lenticular organoids started to form crypt extensions as they grew, reshaping the organoid into a series of interconnected tubular crypts. These extensions could be very long ($> 100 \mu m$, twice the maximum mean crypt width of normal murine crypt⁵) with occasional occurrence of secondary branches (Fig. 3b). Finally, Hyper-budding organoids produced large numbers of small crypt extensions on the surface of the organoid formed at the same time (Fig. 3c). The timing of crypt extensions formation is different for each class of morphology, and varied between day 8 and day 14. For example, the Hyper-budding organoids shown in Fig. 3c formed budding structures on day 11 and day 15, respectively.

The elongation rate of crypts in adenoma organoids is independent of EGF concentration.

We analysed the number and length of the adenoma crypt extensions over a range of EGF concentrations (Fig. 4a). There was little crypt production at low concentrations of EGF (5 pg/ml) but the number of crypts increased dramatically at 0.5 ng/ml (Fig. 4a). The number of crypt extensions per organoid increases with EGF concentration, peaking at 0.5 ng/mL. The crypt lengths over the culture period of 10-25 days at three EGF concentrations (50, 5, and 0.5 ng/mL) were measured (Fig. 4b-d). The mean length of crypts at 50 ng/mL EGF appears to be constant ($\sim 50 \mu m$) between day 14

and day 24 (Fig. 4b). In contrast, at lower concentrations of 5 ng/mL and 0.5 ng/mL EGF the crypt lengths continued to increase throughout the time course (Fig. 4c, d, respectively), at a rate of 2.24 $\mu\text{m}/\text{day}$ and 2.03 $\mu\text{m}/\text{day}$ respectively (Fig. 4e).

Collectively, this data shows that a minimal concentration of EGF is required for crypt production, at EGF concentrations (i.e. between 0.5~5ng/mL), the crypt extends at a rate of ~2 $\mu\text{m}/\text{day}$. At higher concentrations of EGF, crypt length rapidly reaches ~50 μm and does not extend any further

Effect of EGF-family ligands and the ErbB signaling pathway in colon adenoma crypt formation.

Members of the epidermal growth factor receptor family include EGFR, ErbB2 (also known as HER2), ErbB3/HER3, and ErbB4/HER4. EGFR and ErbB2 have been associated with the growth of many human cancers, including colorectal cancer^{28,29} (Fig.5a). Ligand binding to the extracellular domain of EGFR, ErbB3 or ErbB4 induces the receptors to form oligomers and consequentially activate the intracellular kinase³⁰. ErbB2 does not bind a ligand but is in a conformation which allows it to bind to other EGFR family members and when the co-receptor is in the ligand-activated conformation, the ErbB2 kinase is activated³¹. Interestingly, the intracellular domain of ErbB3 has no measurable kinase activity of its own, but when the ligand bound form of ErbB3 combines with ErbB2, the heterodimer (or higher order aggregates of ErbB3 and ErbB2) activates the ErbB2 kinase activity.

To investigate which ErbB receptors might be driving colon adenoma crypt formation, we used recombinant preparations of EGF-like ligands: Amphiregulin (AREG), Epiregulin (EREG), β -cellulin (BTC) and Neuregulin (NRG- β 1, Heregulin- β 1) to stimulate the colon adenoma cells in the 3D-Matrigel cultures. Notably, AREG has been implicated in intestinal epithelial regeneration³² and has been implicated in a pro-neoplastic role in colorectal carcinogenesis³³. Mouse colon adenoma cells were cultured in the presence of different concentrations of each of the EGF-like ligands and the crypts per organoids scored (Fig.5b). All the EGF-like ligands triggered crypt formation with Heregulin /NRG- β 1 being the most potent (80-fold more active than EGF) (Fig. 5b). BTC and EGF showed a similar dosage range for stimulating the formation of organoids with crypts; AREG and EREG both required higher concentrations to stimulate crypt formation (Fig. 5b). Given that AREG binds to EGFR but with a significantly lower affinity than that of EGF^{34,35}, it is not surprising that a higher concentration of AREG is required to stimulate the same level of crypt formation in colon adenoma organoid assay (Fig.5b). Similarly, EREG binds to both the EGFR (~2.8 μM) and ErbB4 (>5 μM) but with a significantly lower affinity than EGF binding to these same receptors (1.9 nM and 49 nM, respectively)³⁶, so higher concentrations of EREG were required to stimulate crypt formation (Fig. 5b).

Although NRG- β 1 (Heregulin- β 1) has a high affinity for the ErbB3 and ErbB4 homodimers (IC₅₀ ~5 nM), it has an even higher affinity for the ErbB3/ErbB2 Erb4/ErbB2 heterodimers (IC₅₀ 0.1-0.2 nM)³⁶. EGF's affinity for EGFR homodimer and the EGFR/ErbB2 heterodimer is between 1.2 to 1.8 nM. Heregulin- β 1 stimulates crypt formation more potently than EGF (Fig.5b, Supplementary Fig. 2). This result indicates that a pathway stimulated by ErbB3/ErbB1, ErbB4/ErbB1, ErbB3/ErbB2 or

ErbB4/ErbB2 heterodimer could initiate and maintain adenoma crypt formation. BTC binds to the EGFR homodimer, the EGFR/ErbB2 heterodimer, the ErbB4 homodimer and the ErbB2/ErbB4 heterodimer with relatively high affinities (1.4 nM, 1.7nM, 3.6 nM and 0.2 nM respectively)³⁶. The affinities are very similar to the binding of EGF to the EGFR homodimer and ErbB1/ErbB2 heterodimer and would account for the similarity of their potencies for inducing colon adenoma crypt formation (Fig.5b).

The mean number of spheroids and organoids with crypts were also scored for each of the EGF-like ligands (Fig.5c). NRG- β 1 and BTC appeared to be the most potent in terms of organoid formation with a consistently higher mean organoids formed, particularly at the lower ligand concentrations. Both EGF and AREG, which stimulate the slowest organoid formation rate, signal through the EGFR homodimer. EREG, which has a faster rate of organoid formation compared to EGF and AREG, can signal through EGFR/ErbB3, E/ErbB2, ErbB1/ErbB4 and ErbB4/ErbB2 heterodimers. With BTC and NRG- β 1 predominant signaling through ErbB4/ErbB3, ErbB2/ErbB4 heterodimers.

It is clear that ErbB signalling plays an essential role in facilitating both colon adenoma organoid formation and crypt formation with combinations of EGFR, ErbB2, ErbB3 and ErbB4 heterodimers identified as key regulators with varying crypt stimulating capacities. ErbB receptor inhibition studies were conducted to further detail the roles each ErbB family member in crypt formation.

Inhibition of ErbB Receptor signaling reduces colon adenoma crypt formation.

The colon adenoma cultures were treated with different ErbB receptor inhibitors (see Supplementary Table 1 for specificity and IC50s) in the presence of either 0.5 ng/mL EGF (Fig. 2b) or 0.005 ng/mL Heregulin/NGR- β 1 (Fig.5b) and the proportion of crypts per organoid was assessed (Fig. 6). Upon EGF stimulation, the EGFR/ErbB2 compound PCI-32765 inhibited crypt formation efficiently at concentrations above 0.5 pM (Fig. 6a). Both the ErbB2 antagonist CP724714 and the EGFR inhibitor AG1478 also inhibited crypt formation but less efficiently, requiring concentrations greater than 10 nM (Fig. 6a). These results suggest that EGF-stimulated crypt formation requires both EGFR and ErbB2 signalling.

The colon adenoma organoid cultures were stimulated with NRG- β 1 (Heregulin- β 1) and treated with ErbB inhibitors (Fig. 6b). Crypt formation was reduced with inhibition of ErbB2 (CP724714), suggesting that NRG- β 1 (Heregulin- β 1) stimulates crypt formation via the ErbB3/ErbB2 heterodimers. EGFR (AG1478) inhibition, which signals through ErbB3/EGFR heterodimers, did not inhibit crypt formation significantly (Fig. 6b). However, the pan-ErbB family inhibitor (HM781-36B) demonstrated the most potent inhibition and was more than 10-fold more effective at inhibiting crypt formation than ErbB2-specific inhibitors (Fig. 6b). This suggests that NRG-b1-stimulated crypt formation can act through both ErbB3 and ErbB2 receptors. ErbB2 signalling is important for crypt formation in the colonadenoma organoids via either EGFR/ErbB2 heterodimers upon EGF stimulation, or via ErbB3/ErbB2 heterodimers when stimulated with NRG- β 1.

Discussion

This study demonstrates that EGFR/ErbB family signalling is required for crypt production in organoids produced by mouse colon adenoma stem cells. Although excess crypt formation appears to be the defining feature of adenomas, the current paradigms for intestinal repair³⁷, colon adenoma and colon cancer focuses on excess intra-crypt cell proliferation³⁸. Cell production within normal colon crypts occurs at a rapid rate, indeed the rate of cell production in the intestines³⁹ (including the colon) exceeds all other tissues. The renewal of the colon epithelial cell occurs rapidly and continuously within the crypt, however under normal homeostatic conditions, crypt production is a rare event: less than 1 in 200 crypts in the mouse colon are in the process of budding^{2,40} and in the normal human colon less than 1 in 2000 crypts are producing crypt buds⁴¹. During development⁴², intestinal renewal after physical damage, radiation, infection and adenoma formation, crypt budding occurs frequently⁴³. In normal tissue, once homeostasis is achieved, crypt budding ceases. However, in adenoma tissue, crypt formation is uncontrolled and leads to the formation of adenomatous polyps.

It was surprising that our initial cultures of mouse colon adenomas only yielded spheroids – there were no crypts²². The spheroids grow rapidly, so the cultures were split for passaging after 7 days. Normal mouse colon organoids started to form crypt-like structures within 5 days. It was not until we allowed the adenoma organoids to grow for 10 days or more that the spheroids initiated crypt buds. Although normal colon stem cells require Wnt, R-spondin and EGF to proliferate *in vitro*, Apc^{+/min} mouse colon adenoma cells only require an EGF ligand family member to proliferate and for the spheroids to produce new crypts. The five EGF family members EGF, NRG-1, AREG, EREG and β -cellulin) all stimulated crypt formation; however, NRG-1 (EC50 2 pg/mL) was almost 100-fold more active than the other ligands. EGF and β -cellulin were active at 200 pg/mL, but AREG and EREG required almost 1ng/mL to stimulate crypt formation.

NRG- β 1 stimulates the aggregation of ErbB3 (HER3) homodimers and heterodimers, included within these aggregates are the ligand-bound ErbB3 in complex with ErbB2 (HER2), where the ErbB2 kinase is activated⁴⁴. In the presence of sufficient concentration of an ErbB2 inhibitor, NRG- β 1 stimulated crypt formation is blocked. This inhibition is amplified if both EGFR and ErbB2 are inhibited. A similar situation occurs when EGF is used to induce crypt formation: the EGF activates both the EGFR kinase and the ErbB2 kinases through ligand stimulated EGFR:ErbB2 hetero-oligomers^{45,46}. EGF stimulated crypt formation can be blocked by either an ErbB2 inhibitor (CP724714) or an EGFR inhibitor (AG1478), however, inhibiting both receptors with PCI-32765 blocks crypt formation profoundly. When crypt formation is stimulated by NRG- β 1 (i.e. via ErbB3), the EGFR inhibitor AG1478 does not block crypt formation, indicating that NRG- β 1 stimulates crypt formation through activation of ErbB3:ErbB2 hetero-oligomers rather than EGFR:ErbB3 oligomers. The EGFR:ErbB2 oligomers require the ligand-activated EGFR kinase to stimulate the formation of oligomers of active ErbB2 kinase. Thus, it appears that the activation of the ErbB2 kinase, via ligand activated EGFR:ErbB2 or ErbB2:ErbB3 oligomers, is required to initiate crypt formation.

EGF family members stimulate crypt formation in the context of a mature colon spheroid. It is not clear what changes occur in the adenoma spheroids after 7-10 days in culture, but it is likely that ErbB2 activation leads to changes in key components of cell-cell adhesion⁴⁷ and/or cytoskeletal proteins at the apical surface⁴⁸ which induce the cell to form a rhomboidal shape– thus inducing the tissue curvature seen at the base of intestinal crypts. This curvature, together with the proliferation of the crypt stem cells leads to crypt bud formation. Consequential to bud formation, crypt elongation occurs via ligand (EGF-family) stimulated proliferation of progenitor (transit amplifying) cells.

Intriguingly, ErbB2 signalling has also been implicated in both mammary duct formation^{49,50} and alveolar formation in the developing lung^{49,51} and may be a key regulator of budding morphogenesis in a number of tissues.

Crypt cell proliferation and crypt bud formation are different processes. Adenomas are grow by ligand dependent, ErbB2 kinase dependent crypt budding processes, so it is likely that both colon adenoma and colon cancers will be susceptible to drugs or combinations of drugs which inhibit both the EGFR kinase and ErbB2 kinase or to combinations of antagonists such as anti-EGFR, anti-ErbB2 or anti-ErbB3 antibodies) which inhibit the activation of the ErbB3:ErbB2 hetero-oligomers. Once the EGFR family kinase activity is blocked the colon adenoma cancer cancer cells will be more susceptible to pro-apoptotic drugs.

Methods

Ligands and inhibitors of the ErbB family. The following EGF-like ligands and ErbB kinase inhibitors were used in this study: Recombinant mouse EGF (Peprotech #315-09), recombinant human Epiregulin (Peprotech # 100-04), recombinant human β -cellulin (Peprotech # 100-50), recombinant human Amphiregulin (Peprotech # 100-55B) and recombinant human NRG- β 1 (Peprotech # 100-03). EGFR tyrosine kinase inhibitor Tyrphostin AG-1478 mesylate (Institute of Drug Technology, IDT Australia, provided by Prof Andrew Scott #T4182), selective inhibitor of ErbB2 CP-724714 (Selleck Chemicals #S1167), irreversible pan-HER inhibitor for EGFR/ErbB2/ErbB4 Poziotinib (HM781-36B, Selleck Chemicals, Houston, TX, USA. Cat No. S7358) and Btk and EGFR/ ErbB2 inhibitor Ibrutinib (PCI-32765, Selleck Chemicals, Houston, TX, USA. Cat No. S2680). Affinities for the respective ErbB receptors in cell-free assays are as shown in Supplementary Table 1. The ligands were reconstituted in 0.1%(w/v) Bovine Serum Albumin (BSA) in distilled filtered water while the inhibitors were reconstituted in dry dimethyl sulfoxide (DMSO).

Normal mouse colon cultures. All animal studies were carried out in accordance with Australian NHMRC Animal Ethics requirements and experimental protocols approved by the Animal Ethics Committee of the Walter and Eliza Hall Institute of Medical Research. C57Bl6 mouse colon tissue was isolated by resection and the tissue opened longitudinally from the anus to the caecum. The tissue was washed with PBS and the distal two-thirds of the colon retained. After 5 minute incubation in PBS containing 0.04% hyperchlorite, the tissue was washed and incubated in PBS containing 3 mM EDTA. Crypts were released by vigorous shaking in PBS (20 seconds) and pelleted at 100 xg. Crypts were digested with Dispase (0.1 mg/ml Dispase (Life technologies #17105-041) in DMEM-F12 and then by incubation in TrypLE. Single cell suspensions were prepared by passing the digested crypts through a 26G needle and plated at 500 cells/well in 384 well plates coated with 8 μ l Matrigel (BD Biosciences #356231). Growth media (Intesticult (Stem Cell Technologies #06005) supplemented with penicillin/streptomycin (Gibco), 10x concentrated WEHI-YH2 conditioned medium²⁰ and 10 μ M Y27632 was added within 30 minutes of plating and cultures were maintained in the same media but without Y27632.

Colon adenoma organoid cultures. The colon adenoma crypts were prepared from a polyp taken from an *Apc*^{+/min} mouse⁵² colon (kindly provided by Dr Anuratha Sakthianandeswaren, The Walter and Eliza Hall Institute of Medical Research, Victoria, Australia), preparation and culturing of the WEHI-Ad67 colon adenoma organoid line was described previously⁹. Using the same technique, we prepared another colon adenoma organoid line: WEHI-AdB13 using a polyp from a separate *Apc*^{+/min} mouse. The colon adenoma organoid lines were passaged weekly (details as described in ref ⁹) in 96 well plates precoated with 15 μ L of Matrigel (BD Biosciences #356231) and basic growth medium with recombinant mouse EGF (50 ng/mL, PeproTech, #315-09). The basic colon adenoma organoid growth medium consisted of DMEM-F12 (Gibco/Life Technologies, #10565018) supplemented with additives: 1x penicillin-streptomycin (Gibco/Life Technologies, #15140-122), 1x GlutaMAX (Gibco/Life Technologies, #35050-061), 1x N2 (Gibco/Life Technologies, #17502), 1x B27 (Gibco/Life Technologies, #17504) and HEPES (10 mM).

372

373 **Colon adenoma organoid proliferation and crypt formation assays.** Organoid
374 harvesting and preparation: The colon adenoma organoids were harvested from the
375 96 well plates, collected and pipetted with a 26G needle for 5-10 times, visually
376 checking the size of the resulting fragments after each round of pipetting (~10 cell
377 fragments are ideal to work with). The fragments were resuspended with DMEM/F12
378 and centrifuged at $8500 \times g$ for 5 minutes. The supernatant fluid was discarded, and
379 the pellet resuspended with 1mL DMEM/F12. The suspension was centrifuged at
380 $10000 \times g$ for 3 minutes and supernatant fluid discarded. After resuspension of the
381 pellet with 150 μ L basic growth medium, an aliquot of the fragment suspension was
382 removed for counting using a haemocytometer (Hausser Scientific).

383 Based on the number of wells to be plated and required seeding density per well, the
384 volume and fragment concentrations of the seeding mixture were determined as
385 described in Sub Fig. 3a. The seeding mixture is divided equally between two columns
386 of a skirted twin.tec 96-well PCR plate (Eppendorf cat#951020401) sitting on a pre-
387 cooled thermo-block (total 14 out of 16 wells, leaving out the first and last well to be
388 filled with buffer solution or water, Sub Fig. 3a). The PCR plate was placed onto the
389 working stage of an Eppendorf epMotion® P5073c liquid handling machine (fitted with
390 a Clean Cap filter to keep the samples sterile) for automatic plating.

391 Automated seeding: The colon adenoma fragments suspended in the 50% Matrigel
392 were pipetted (8 μ L) into a 384-well optical plate (Corning, #3985) epMotion®. An 8-
393 channel dispensing tool (TM 50, Eppendorf cat# 5280000215) was used to extract the
394 adenoma mixture from the preloaded twin.tec 96-well PCR plate (Eppendorf
395 cat#951020401), kept cool by sitting on a thermo-block (precooled at -20°C). The
396 robotic arm was programmed and customized to transfer viscous fluid and to dispense
397 near the bottom of the well (Sub Fig. 3c). After dispensing the adenoma cells, the plate
398 was centrifuged for 1 minute at 450 RCF (Eppendorf 5810 R centrifuge) and incubated
399 at 37°C for 1 hour (Sub Fig. 3d).

400 Reagent preparation and application: The respective EGF-like ligand stocks were
401 prepared in basic growth medium while the ErbB receptor inhibitors were made up in
402 basic growth medium containing recombinant mouse EGF (0.5 ng/mL, PeproTech,
403 #315-09). The study used a 5-point titration of the ligands using 1 in 10 dilutions. The
404 reagents were pipetted into the designated wells of a 96-well deep well plate
405 (Eppendorf cat#951033529) with layout as per described in Sub Fig. 3. The plate was
406 placed on the stage of the Eppendorf epMotion® P5073c along with the previously
407 incubated optical 384-well plate with the plated fragment mixture. The liquid handling
408 machine was programmed and customized to transfer liquid and to dispense from the
409 top of the well. The TM-50 was used to dispense reagents from the 96-well deep well
410 plate to the 384well plate in an alternating manner (Sub Fig. 3f). The 96-well deep well
411 ligand stock plate was covered, sealed and stored at 4°C for use when the medium
412 was to be replaced. In the case of significant bubble formation, the culture plate was
413 centrifuged for 2 minutes at 450 RCF (Eppendorf 5810 R centrifuge) and before being
414 placed in the at 37°C incubator.

415

Medium replacement: The crypt forming assays usually ran for three weeks with weekly replacement of medium. The Eppendorf epMotion® P5073c was used for this replacement. Using the 8-channel dispensing tool (TM-50), 40µL of medium was removed and replaced with 40µL of fresh reagent.

Colon adenoma organoid imaging assay. The functional imaging assay was conducted with two systems both using an optical 384-well plate. The first system initial imaging data were acquired using a Nikon Eclipse Ti-U microscope with a 4x objective lens and motorised stage (Prior Scientific, H117). Bright field image stacks of each well were captured on a Nikon DS-Ri2 camera (Nikon Inc, MQA17000) using the NIS-Elements software (Nikon, Basic Research version 4.40). The depth images of each FOV (well) were then stacked using the EDF algorithm in Fiji for image processing and analysis. The whole plate assay data were acquired using a customized Nikon High Content Analysis platform consisting of a Nikon C2+ confocal microscope with a LIS/PRIOR automated stage, plate loader and cell culture incubation throughout the experiment (including during the imaging of the plate). Brightfield image stacks of individual 384 well were acquired on a Nikon Qi2 grayscale camera and NIS-Elements High Content Analysis software module (Nikon, High Content Analysis version 5.11). For both systems, each experiment was followed and imaged daily for up to 3 weeks. Each dataset which represents all the wells of a specific timepoint was processed using a customized Fiji script⁵³ to extract each individual well's image stack, before digitizing, saving as tiff images and organizing into folders for analysis.

Computational image analysis for organoid imaging assay. For analysis, a customised Fiji script was used to batch process and organise all the images in each experiment, this includes steps to focus the multiple images at different focal planes for each well, background and debris removal, as well as image segmentation to extract organoids and for feature selection. Specific details of each processing steps are briefly described below.

The individual well's Z-stack bright-field images were flattened using ImageJ/Fiji software⁵³. The projection of z-stack images onto a single in-focus image was carried out using the Stack Focuser plugin (<https://imagej.nih.gov/ij/plugins/stack-focuser.html>) (parameters: *select=10 variance=0.000 edge select_only*) and exported as high-quality tiff files.

Well exterior, background and debris removal as well as organoid selection and feature extraction were performed were achieved using sequential steps of the following operations: conversion to 8-bit, "Auto Local Threshold" (method=Phansalkar, radius=150, parameter_1=0 parameter_2=0 white stack), set Black Background, "Convert to Mask" (method=Default background=Default calculate black list), "Find Edges" as stack, "Invert" stack, set Foreground Color as white, clear boundary (in steps), "Dilate" as stack, "Fill Holes" as stack and "Remove Outliers" (radius=7 threshold=50 which=Dark stack). Analyses of object sizes performed using "Analyze Particles" function (size=1000-Infinity circularity=0.30-1.00).

The script was used to processes all well images and used to threshold, segment and count the individual organoids. The physical parameters of each organoid, including

shape, size, intensity and circularity were recorded. These features for all organoids identified per well/image were exported as text files (.csv) for analysis and tabulation of the parameters of interest (e.g. mean organoid size). Overlaid images with selection masks were exported for visual curation and validation. The results were curated to remove false positives and to add missing organoids.

Whole mount staining and imaging. Colon tissue (normal and adenoma) was cleared and stained as described in ref ⁵⁴. Briefly, the tissues were fixed, cleared and stained with rat monoclonal anti-E-cadherin (Clone ECCD-2, Thermo Fisher Scientific Cat #13-1900 (1:250)), goat anti-Rat IgG (H+L) Cross-Absorbed Secondary Antibody, Alexa Fluor 488 (Thermo Fisher Scientific Cat #A-11006 (1:500)), Rhodamine Phalloidin (Molecular Probes/Invitrogen(1:200)) and DAPI (Thermo Fisher Scientific Cat# 62248 (1:1000)). For the adenoma tissues, 3D image stacks were acquired on a Leica SP8 Resonance Scanning Confocal microscope using a 20x objective for 100µm depth at 0.5 µm per section. For normal tissues, 3D image stacks were acquired on an Olympus IX-81 microscope with an Olympus FV1000 Spectral Confocal attachment and a 20x objective for ~55 µm depth at 1 µm per section. All acquired image data were processed and rendered using Imaris software package (Bitplane, Zürich, Switzerland).

Immunofluorescent staining of colon adenoma organoid. Organoid cultures were harvested from selected wells of 384 well plates and transferred into a 1.5 mL Eppendorf microtube. The organoids were either allowed to settle to the bottom of the tube. The supernatant fluid was discarded carefully and in the remaining fluid (~20 µL) the pellet was mixed with an equal volume of Matrigel. The mixture was plated as domes into the wells of an 8-well chamber slide (Ibidi cat#80826) and incubated at 37°C for 1 hour. 300 µL of MT-PBS was added to wash the organoid-Matrigel domes twice. The MT-PBS was removed and 150 µL of 0.1% Paraformaldehyde (PFA) in PBS was added to each well and incubated at 4 °C for 48 hours. The paraformaldehyde was removed from the wells and individual wells were washed with 150 µL of PBS twice. The MT-PBS was removed, 300 µL of 0.2% (v/v) TritonX-100 in MT-PBS added and the dome incubated for 5 minutes at room temperature. The liquid was removed, and the dome washed twice with 300 µL of blocking buffer [0.2% (w/v) Bovine serum albumin (BSA) in MT-PBS] before leaving in the blocking buffer for 24 hr. After removing the blocking solution and 200 µL of the primary antibody solution containing rabbit anti-β-catenin (Sigma-Aldrich #C2206, 1:400) and rat anti-E-cadherin (Invitrogen #13-1900, 1:200, monoclonal ECCD-2) was added and the incubation continued at 4°C for 24 hr. The antibody-stained domes were washed with 300 µL per well of blocking buffer and followed by 300 µL per well of 0.1% (v/v) TritonX-100 in MT-PBS for 5 min at room temperature. After removing the Triton X100 buffer, wash twice with 300 µL of blocking buffer before adding 200 µL of secondary antibody solution (0.2% BSA (w/v) in MT-PBS) containing Alexa Fluor® 546 goat anti-rat IgG (1:400) and Alexa Fluor® 488 goat anti- rabbit IgG (1:400). The domes were incubated with the secondary antibody solution at 4°C for 18 hours. After removing the secondary antibody solution, the domes were washed twice with the Triton X100 buffer before counter staining the nuclei with 1 nM DAPI (4, 6-diamidino-2-phenyl indole Nucleic Acid Stain, cat# D1306, Molecular Probes Inc, Eugene, OR, Invitrogen) in MT-PBS

(200 μ L) for 5 minutes at room temperature in the dark. The organoids were imaged in the 8-well Ibidi slides on the confocal microscope.

3D Confocal Fluorescence Imaging. Immunofluorescent images of the organoids were recorded using an Olympus IX-81 microscope with an Olympus FV1000 Spectral Confocal attachment and a 60x water immersion lens. DAPI, β -catenin and E-cadherin fluorescence were excited with the 405 nm, 488 nm and 546 nm laser lines, respectively and the emissions were measured at 405 nm, 473 nm and 559 nm, respectively. 3D image stacks were captured encompassing the entire depth of the organoid in the field of view using Olympus FluoroView software (Version 1.7c). For larger organoids, tiled image stacks were acquired and merged. For quantitative measurements and 3D reconstruction, cubic voxels were acquired (typically up to 0.33 μ m) for each image stack with the output analogue signal, representing the fluorescence intensities digitized to 16 bits resolution at 65536 levels of grey and saved as Olympus Image Binary (OIB) files. The Bioformats plugin was used to import the images into the ImageJ/Fiji software processing and visualization.

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Author contributions

R.Z., A.W.B. and C.T. conceived and designed the experiments. R.Z., S.R.K., X.Z., M.C.F and C.T. performed the experiments. R.Z., M.A., M.C.F, A.W.B. and C.T. performed the data analysis and interpretation. R.Z., A.W.B., M.C.F and C.T. wrote the manuscript. All authors critically reviewed and approved the manuscript prior to submission.

Competing financial interests

The authors declare no competing financial interests.

714 **Figure Legends**

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Fig. 1 Crypt formation in colon $Apc^{min/+}$ adenoma organoids requires EGF.

Whole-mount mouse colon crypts from **(a)** an C57Bl6 adult mouse and **(b)** $Apc^{min/+}$ adenoma tissue were immunolabelled for E-cadherin (E-cad) (green) and labelled with phalloidin (magenta) and DAPI (blue) to visualise F-actin and nuclei, respectively. Shown are single section images. **(c)** Normal mouse colon organoids form crypts in culture. Bright-field extended depth of field (EDF) time-lapse images of wildtype mouse colon organoids grown over 10 days. Insets at Day 9 are shown as enlarged images in panel f. **(d)** Mouse colon adenoma organoids derived from $Apc^{min/+}$ mice form crypts when the cells are cultured in the presence of EGF. Brightfield EDF time-lapse images of $Apc^{min/+}$ adenoma organoids grown over 21 days with EGF. Organoids cultured with EGF remain spherical and cyst-like in the first 7-9 days. Crypt extensions start to appear in some organoids after 10 days, some with multiple extensions projecting from the main organoid body. **(e)** $Apc^{min/+}$ adenoma spheroids grown without EGF. Brightfield EDF time-lapse images of $Apc^{min/+}$ adenoma organoids grown over 19 days without EGF. Enlarged images of budding organoids from **(f)** normal mouse colon (Day9) and **(g)** colon adenoma (Day21) with EGF and **(h)** colon adenoma (Day19) without EGF. **(i)** Colon adenoma organoids were immunolabelled with β -catenin (green) and E-cadherin (red) and co-stained with DAPI (blue). Single section images acquired on an Olympus FV100 confocal microscope shown (merged/overlayed image shown in the top left panel). The crypt extensions formed are physiologically similar in shape to crypts isolated from mice colons, with a crypt width of $\sim 40 \mu\text{m}$, as indicated in the β -catenin image. Merged 3D reconstruction of the stained crypt forming organoid shown in the right panel. Scale bars: (a-b) $50 \mu\text{m}$, (f-h) $100 \mu\text{m}$.

Fig. 2 Effect of EGF on crypt formation in colon adenoma organoids.

Colon adenoma organoids formed crypts in an EGF dependent manner. Colon organoids were cultured in different amounts of recombinant mouse EGF, monitored and imaged for up to 24 days. The numbers of spheroidal and crypt-forming organoids were quantified and the % of organoids with crypts were tabulated. **(a)** EDF images of representative wells at different concentrations of EGF (day indicated below each image). **(b)** The mean proportion of organoids with crypts for different EGF concentrations. Scoring conducted using a customized Fiji script (see Methods section) for first occurrence of organoids with crypts during the duration of the culture. Error bars and analysis: Mean $\pm$ SEM, $n \geq 5$ experiments, *** $p < 0.001$, ordinary 1-way ANOVA, Holm-Sidak's multiple comparisons test with respect to no added EGF.

Fig. 3 Colon adenoma crypt-forming organoids can have different morphologies.

The mouse $Apc^{min/+}$ organoids with crypts can be categorized into **(a)** large body extension organoids, **(b)** lenticular organoids and **(c)** hyper-budding organoids. Large body extensions occur from different sized starting points from a large cyst like body. Lenticular organoids have crypt buds extending in length with reshaping of the organoid. Hyper-budding organoids have large number of crypt buds forming on the organoid. Concentration of EGF used in the respective cultures are as per indicated.

Fig. 4 The initial growth rate of the crypt extensions is stimulated by EGF.

Analysis of the number and length of crypt extensions at different times in culture **(a)**
The mean number of crypt extensions scored per well for the different concentrations
of EGF. **(b-d)** Mean lengths of the crypts at each timepoint **(b)** 50 ng/mL, **(c)** 5 ng/mL
and **(d)** 0.5 ng/mL EGF. **(e)** Linear Regression Analysis between 5 ng/mL and 0.5
ng/mL. Error bars and analysis: **(a-d)** Mean $\pm$ SEM, $n=6$ experiments, 37 wells, 86
organoids, 661 crypt extensions.

Fig.5 ErbB ligands stimulate crypt formation in colon adenoma organoids.

Colon adenoma organoids were stimulated with different concentrations of EGF-like ligands for up to 21 days and the numbers of spheroids and crypt producing organoids were counted. **(a)** Schematic diagram of the EGF-like ligands and their corresponding ErbB receptor interactions. AREG: Amphiregulin, EREG: Epiregulin, BTC; β -cellulin, NRG- β 1: Neuregulin/Heregulin- β 1. **(b)** The proportion of organoids with crypts and **(c)** the mean numbers of spheroid and crypt-forming organoids scored were plotted. Data shown as Mean $\pm$ SEM for $n \geq 3$ experiments, **(b)** Dose-Response – Stimulation Non-linear Analysis: Best-fit values EC50 for EGF: 0.1430 ng/mL, EREG: ~0.1416 ng/mL, Neuregulin/Heregulin- β 1: 0.001814 ng/mL

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Fig. 6 Inhibition of ErbB receptors prevents crypt formation in EGF or Neuregulin/Heregulin β 1 stimulated colon adenoma organoids.

Production of colon adenoma organoids was assessed with **(a)** 0.5 ng/mL EGF or **(b)** 5 ng/mL Neuregulin/Heregulin β 1 in the presences of different concentration of ErbB pathway inhibitors. The cultures were imaged daily for 21 days, and the numbers of crypt-forming organoids scored. The EGF-stimulated cultures **(a)** were treated with EGFR tyrosine kinase inhibitor AG1478, a selective HER2/ErbB2 inhibitor CP724714 or a potent but less selective Brutons tyrosine kinase (Btk), EGFR and ErbB2 inhibitor, Ibrutinib (PCI-32765). The NRG- β 1-stimulated cultures **(b)** were treated with AG1478, CP724714 or an irreversible pan-HER inhibitor (HER1/2/4) Pozotinib (HM781-36B). Data shown as mean $\pm$ SEM number of organoids with crypts, n=3 experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ordinary 1-way ANOVA. Statistical tests: **(a)** Dunnett's multiple comparisons test with reference to 0.5ng/ml EGF, **(b)** Dunnett's multiple comparisons test with reference to 5 ng/mL NRG- β 1. Dose-Responses – Inhibition Non-linear Analysis, Best-fit values IC50 for **(b)** HM781-36B: $2.89 \pm 2.58 \times 10^{-5}$ nM

Supplementary Information Legends

Supplemental Figure 1 Differences in morphology between normal and adenomatous mouse colon.

3D confocal imaging of tissues from normal mouse colon and Apc^{min/+} mouse colon adenoma showing DAPI in blue, Phalloidin (F-actin) in magenta, E-cadherin in green. Confocal images of a section of the (a) normal epithelium and the (c) adenoma epithelium indicates the differences in crypt morphology. (b) shows the 3D reconstruction of (a) while panel d is a zoomed image of the boxed region in c. Colon tissue was cleared and stained as described in ref ⁵⁴. Briefly, the tissues were fixed, cleared and stained with rat monoclonal anti-E-cadherin (Clone ECCD-2, Thermo Fisher Scientific Cat #13-1900 (1:250)), goat anti-Rat IgG (H+L) Cross-Absorbed Secondary Antibody, Alexa Fluor 488 (Thermo Fisher Scientific Cat #A-11006 (1:500)), Rhodamine Phalloidin (Molecular Probes/Invitrogen (1:200)) and DAPI (Thermo Fisher Scientific Cat# 62248 (1:1000)). Images were acquired on a Leica SP8 Resonant Scanning Confocal microscope with the data processed/rendered using Imaris software package (Bitplane, Zürich, Switzerland). Scale bar: (b) 100µm (c) 50µm.

Supplemental Figure 2 Crypt formation occurs in another colon adenoma organoid line

Colon adenoma organoids derived from other Apc^{min/+} mice were tested for its crypt formation capability using EGF and other ErbB ligands. Adenoma line #B13 organoids were cultured, cultured with EGF or other ErbB ligands for up to 21 days. Representative EDF images of crypt forming organoids from these #B13 cultures stimulated with (a) 5 ng/mL EGF and (b) 0.5 µg/mL Neuregulin/Heregulin-β1. Inset presents zoomed images of selected regions of the respective well on the left. Scale bars: left panel 500 µm, right panels/inset 100 µm.

Supplemental Figure 3 Workflow for colon adenoma organoid imaging analyses

The workflow had been optimised to maximise the use of a 384well optical plate for analysing the growth and morphology of the organoids. Colon adenoma fragments were mixed with Matrigel 50% (v/v). This mixture was then equally divided into two columns of a precooled 96well PCR plate sitting on a cold thermoblock. An automatic liquid handling machine (Eppendorf epMotion® P5073c) was then used to transfer 8 µL per well, 8 wells at a time with an 8-channel pipetting tool. The plate was then centrifuged to move all the cultures to the bottom of the wells before incubating at room temperature for 1 hour. Similarly, media was prepared with the different concentrations of the stock solutions of ligands and inhibitors were prepared in a 96 deep well plate and transferred (80 µL) to the adenoma cultures using the epMotion® liquid handling machine.

844 **Supplementary Table 1**

845 Summary of the IC50 values for the ErbB receptor kinase inhibitors.

846

847 **Supplemental Movie S1**

848 Time-lapse of crypt forming organoid followed for 10 days.

849

850 **Supplemental Movie S2**

851 Time-lapse of crypt forming organoid followed for 24 days.

Figures

Figure 1

please see the manuscript file for the full caption

Figure 2

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Figure 3

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Figure 4

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Figure 5

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Figure 6

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Supplementary Files

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