CALCIUM SIGNALLING IN HUMAN COLONIC EPITHELIUM

By

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A thesis submitted for the degree of Master of Science by Research (MSc(R))

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March 2019

Abstract

Intracellular calcium signals within intestinal epithelial cells are a critical integrator of a multitude of extracellular stimuli that regulate intestinal stem cell (ISC) driven tissue homeostasis and almost every other cellular activity. Calcium is an important second messenger that has an emerging role in maintaining intestinal tissue homeostasis and in the development of malignant colorectal cancer (CRC). Understanding the regulation of intestinal tissue homeostasis through intracellular calcium signals is essential for the development of novel therapies. Cholinergic signals have been shown to stimulate calcium signals, but the mechanism and impact on the intestinal epithelium is not fully understood. The aims of this study were to firstly, investigate the spatiotemporal characteristics of intracellular calcium signals, using an *ex vivo* 3D native human colonic crypt model system. Secondly, investigate whether these calcium signals are remodelled during colon carcinogenesis, using an *in vitro* 3D model of organoids (health) and tumouroids (CRC).

Fura-2 calcium imaging revealed the spatiotemporal characteristics of muscarinic-induced intracellular calcium signals, through addition of Carbachol (Cch). Calcium signals evoked by Cch in human colonic crypts initiate at the apical pole in the base of crypts. Spreading to neighbouring cells as the calcium wave propagates across all cell types along the crypt axis. This study has found the extracellular environment is critical in the mobilisation of intracellular calcium signalling e.g. store operated calcium entry (SOCE). The higher the extracellular calcium concentration the greater the intracellular calcium response. Additionally, there appears to be an important role of non-neuronal ACh synthesis by Tuft cells in mobilising intracellular calcium at the base of crypts via muscarinic receptor activation. Even though pharmacological inhibition of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump substantially reduces Cch-induced calcium mobilisation there was still a significant calcium response after Thapsigargin treatment. The acidic lysosome was confirmed as the initiation site of intracellular calcium signals via 100 µM GPN stimulation with a lack of subsequent Cch-induced calcium response. Confirmed through coupling of fluorescence imaging and previous immunocytochemistry data.

In full culture media, there were intracellular calcium oscillations from a single 10 µM Cch stimulation, intensity of which was amplified using 100 µM Cch. In a perfusion chamber, oscillations are also induced through the constant addition of fresh 10 µM Cch solution. Inhibition of mechanistic target of Rapamycin (mTOR), important for TPC-mediated calcium mobilisation from lysosomes, remarkably had no effect on the generation of a calcium signal. Organoid formation assays investigated the effect of muscarinic receptor activation on the proliferative activity of ISCs , measured by organoid bud formation. Inhibition of receptors that facilitate mobilisation of calcium from lysosomal and ER stores, along with Cch and UTP, were used to determine if there was remodelling of the calcium signalling pathway in colon carcinogenesis. There appears to be some differentiation in relative store size in colon cancer.

In conclusion, this study identifies lysosomes at the apical of cells within the ISC niche as the site of intracellular calcium initiation in human colonic crypts. These propagate to the basal pole within cells as the signal travels up the crypt axis. Calcium signals are induced through muscarinic activation and the calcium concentration has an important role in this response, to be explored further. TPC-mediated calcium release from lysosomes is under the regulation of mTOR which itself is regulated by numerous different factors. Inhibition of mTOR did not significantly impact calcium mobilisation. Finally, this study potentially indicates a difference in ER and lysosomal calcium store sizes between health and disease. Therefore, the remodelling of these stores could serve as potential targets for novel treatments of CRC.

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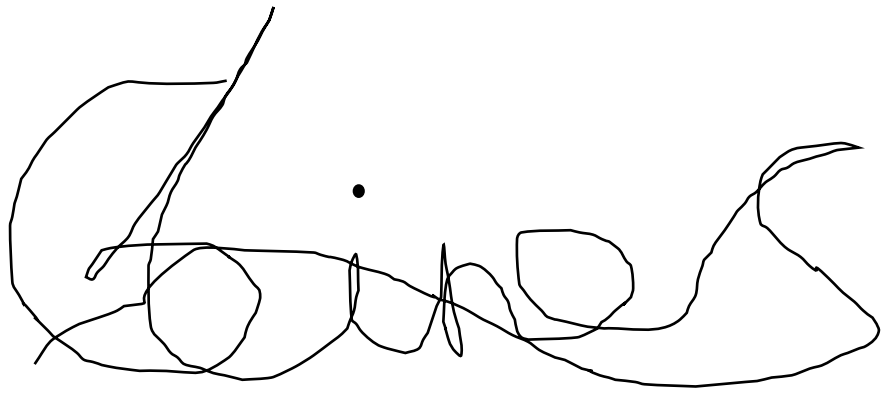
Declaration

I declare that thesis represents my own work, except where due acknowledgement is made, and that it has not been previously included in a thesis, dissertation or report submitted to this University or to any other institution for a degree, diploma or other qualifications



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Masters by Research candidate.

Acknowledgments

This project would not have been possible without the unconditional support of many people.

First of all, I would like to say a huge thank you to my primary supervisor Dr. Mark Williams for this opportunity. Also, for his continued support and guidance throughout the year and having patience with me as I developed as a researcher. I am privileged to have gained so much from his wealth of knowledge and to have spent time with someone who is so passionate about his work that it becomes infectious. So much so, that my passion for the study of human colonic crypts and stem cells began from the first seminar Mark gave, 5 years ago. Additionally, thank you for your support and encouragement in helping me attain a PhD at Cambridge University, I am looking forward to the collaboration between the two labs and four more years of working, in part, with the Williams laboratory.

A big thank you to former master student and now PhD candidate Alvin Lee for his time and patience in teaching me the methods for Fura-2 calcium imaging and since becoming a good friend of mine. Furthermore, I would like to say a massive thank you to Dr. Victoria Jones and Nicolas-Palaez Llaneza, PhD candidate for their continuous support and guidance throughout this year both in terms of science but also in my personal life. Both of which have become very good friends of mine now and for the foreseeable future.

I am grateful for the support and beneficial discussions had with Dr. Alyson Parris, unfortunately only for a short time during my project before Alyson retired. I wish her well in her retirement.

For scientific contribution to the current work, thanks go to former PhD student Christy Kam, present post-doc, Dr. Victoria Jones and to previous master student Alvin Lee.

Finally, I would like to take this opportunity to thank all the new friendships made at the UEA, mainly through football and the emotional and work-related support that they have given me. Without them, this year would have been far more difficult. My family, I do not know what I would do without their unconditional love and support in everything I do. I hope that thus far I am repaying that immense love and support that has been there for the past 23 years. Also, I hope that I have and will continue making them proud. My incredibly supportive and pulchritudinous partner Hannah, who for the past year, especially, has been my rock and has given me support, advice, care and love regardless throughout the good, and the bad times.

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1. Introduction

This project has focused on the functioning of the human colonic epithelium under homeostasis and colon cancer. The intestinal epithelium is one of the most rapidly renewing tissues within the human body, thanks to the small population of ISCs located at the base of crypts. Crypts are small invaginations of the epithelial layer that are present along the length of the colon. A key component regulating homeostasis of this epithelial monolayer are intracellular calcium signals. There is also an emerging role for the calcium signalling pathway in colon carcinogenesis. Using agonists and antagonists of the calcium pathway, this project has investigated the mechanics of calcium signalling and whether this pathway is remodelled in colon cancer.

1.1 Human Intestinal Physiology

The intestinal epithelium is the most rapidly renewing tissue in the human body with the entire epithelium replaced every 3-5 days in human (2-3 days in mice) (Wath, et al., 2013). The intestines have several layers with the outermost layer, a single layer of columnar epithelial cells. Along the length of the large intestine this layer forms millions of invaginations into the underlying mucosa, known as the crypts of Lieberkühn, (Humphries & Wright, 2008).

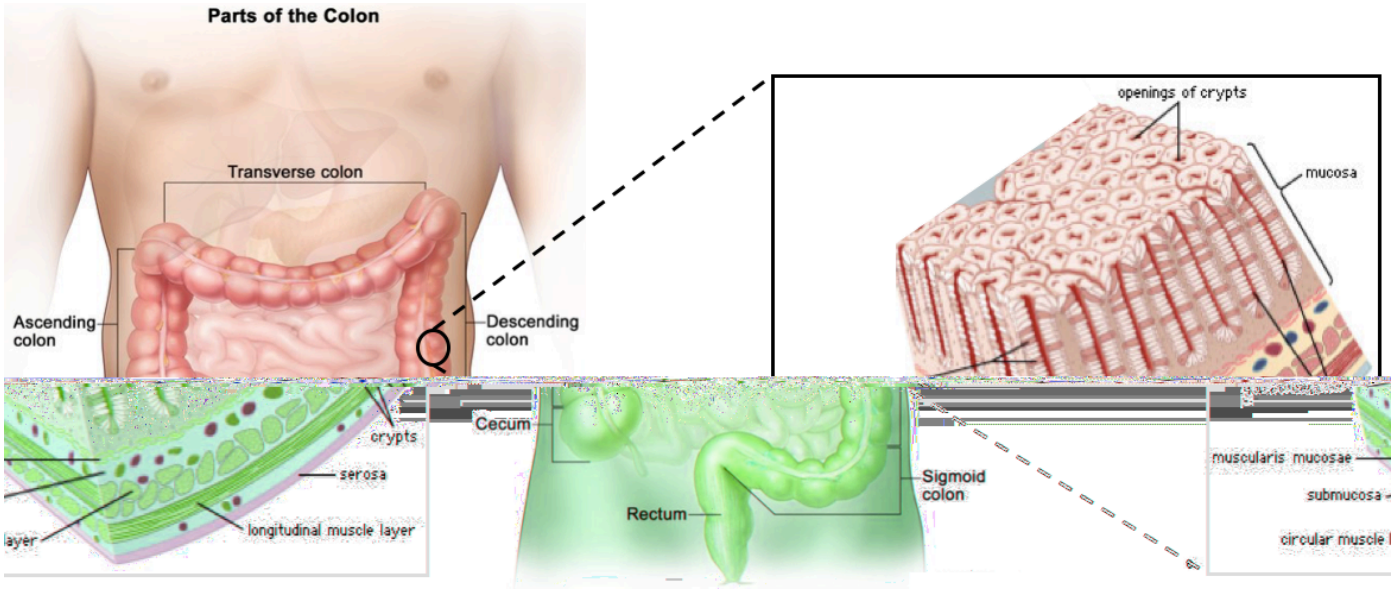


Figure 1.1.1- (left) An overview of the intestinal anatomy and the areas of the colon and rectum that can be affected by colon cancer (NIH, 2011). **(right)** A schematic diagram of the native arrangement of the colonic epithelium, with colonic crypts the site of intestinal self-renewal (Vector-Illustration, 2018).

The intestinal epithelium is a selectively permeable barrier that facilitates the absorption of nutrients and waste secretion whilst also providing a physical barrier between the internal milieu and the harsh external environment (Anderson, et al., 2011). This selective permeability is mediated by trans-epithelial and paracellular pathways (Tsukita, et al., 2001). The topographical organisation of the colonic crypt epithelium is one of three intersecting zones (Gibson, et al., 1996), **(Figure 1.1.2)**. At the base resides a concentrated population of Leucine-rich repeat- containing G-protein coupled receptor 5-positive ($Lgr5^{+}$) ISCs also known as crypt base columnar (CBC) ISCs (Li & Jasper, 2016) (Beumer & Clevers, 2016). These undergo symmetrical division whereby stochastically one daughter cell remains a SC and one becomes a transit amplifying cell entering the 'proliferation zone' undergoing multiple rounds of rapid cell division and maturation into the differentiated cell types **(Figure 1.1.2)**. The upper region of the crypt, 'maturation zone', is where differentiated cell types are expressing a full array of functional activities and at the top of the crypt axis are shed into the lumen as there is a constant movement of cells from the crypt base to the top of the crypt axis **(Figure 1.1.2)**.

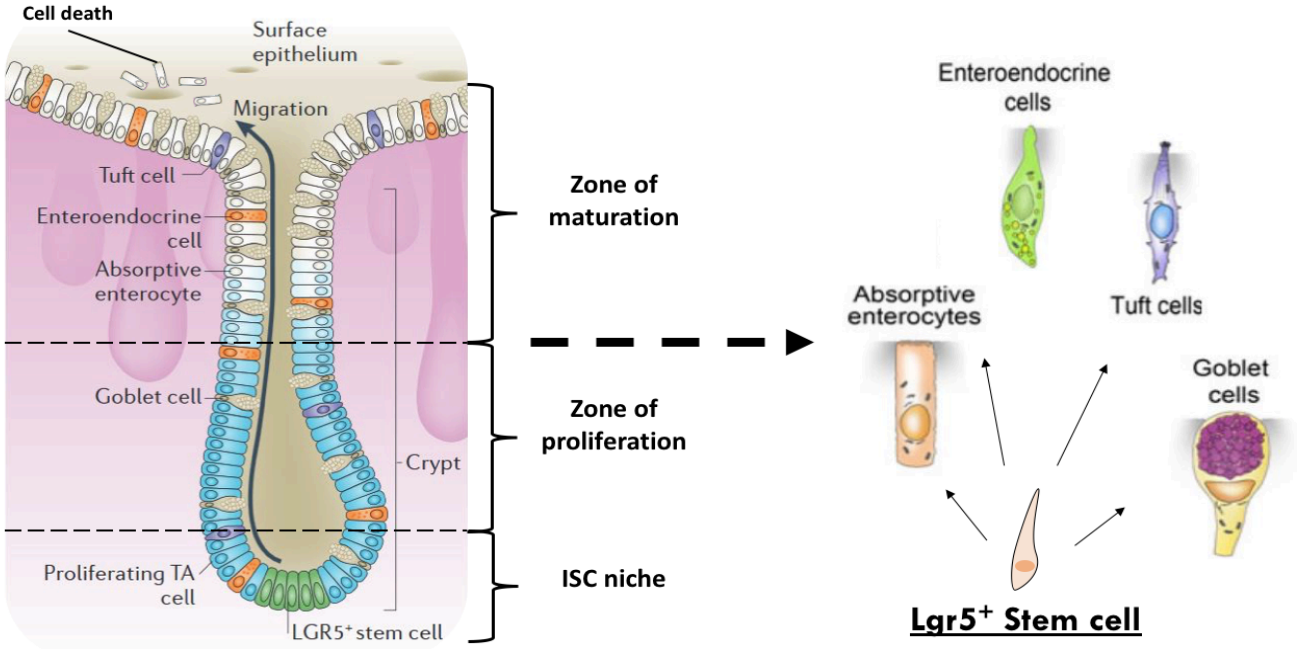


Figure 1.1.2- **(left)** Schematic diagram of a typical human colonic crypt, $Lgr5^{+}$ ISCs located at the base within the ISC niche. Cells migrate up the crypt axis passing through several zones until they reach the top of the crypt axis and undergo cell shedding. **(right)** The differentiated cell types of colonic crypts that $Lgr5^{+}$ stem cells produce. (Adapted from: (Barker, 2014)).

These differentiated cell types of the colonic crypt include are absorptive enterocytes, goblet cells, enteroendocrine and newly discovered tuft cells. Enterocytes aid absorption of nutrients from the gut lumen and are located at the crypt surface, goblet cells in the mid-crypt maintain a thick double mucus lining to protect the underlying epithelium. Enteroendocrine cells are concentrated at the crypt base and produce hormones controlling many processes such as glucose levels and food intake, finally tuft cells can 'taste' luminal contents and secrete signals on the serosal side impacting the cellular process of other epithelial and immune cells **(Figure 1.1.2)**.

In addition to the Lgr5⁺ 'workhorse' ISCs there has been a recently discovered subpopulation of quiescent +4 Bmi1⁺ ISCs (Li & Jasper, 2016) (Beumer & Clevers, 2016), **(Figure 1.1.4)**. Bmi1⁺ ISCs are labelled +4, because they are located four-cell diameters apical of the crypt base (Li & Jasper, 2016), **(Figure 1.1.4)**. The activity of these two stem cell compartments in maintaining homeostasis is controlled through a number of different signalling pathways and ligands secreted by supporting epithelial cells, mesenchymal cells and myenteric neurons (Powell, et al., 2011), **(Figure 1.1.5)**.

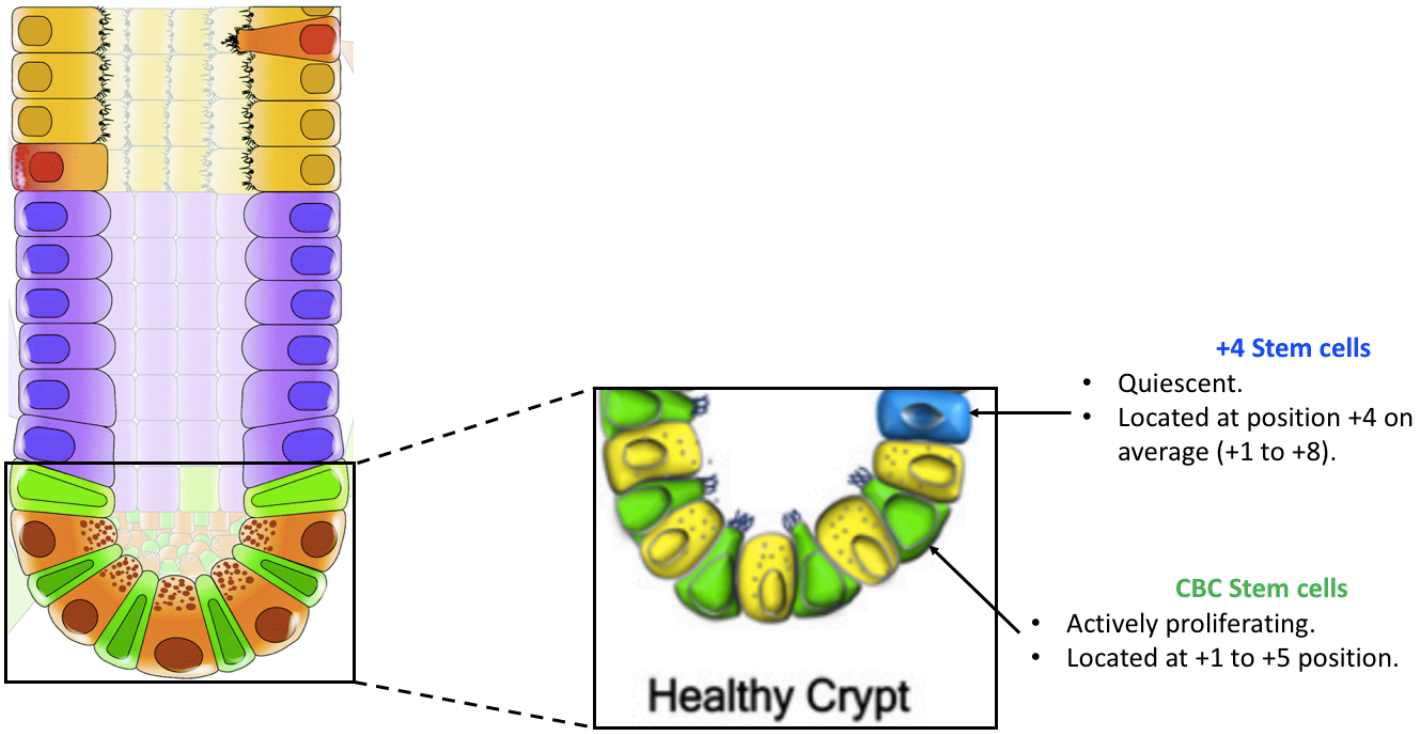


Figure 1.1.4- **(left)** A schematic diagram of a typical colonic crypt, with the ISC niche located at the base, (Adapted from: (Carulli, et al., 2014)). **(right)** Recent discovery of a new second stem cell niche, located on average at four-cell diameters apical of the crypt base, in **blue**. CBC stem cells that are the typical Lgr5⁺ 'workhorse' stem cells are shown in **green** (Adapted from: (Barker, et al., 2012)).

Several key signalling pathways such as Wnt, epidermal growth factor (EGF) and bone morphogenic protein (BMP) regulate ISC driven homeostasis. The Wnt signalling pathway regulates proliferation, through Wnt ligands e.g. Wnt3a secreted by neighbouring differentiated epithelial cells and cells of the underlying submucosa **(Figure 1.1.5)**. ISCs express the frizzled-LRP5/6 receptor on their cell surface upon ligand binding the APC destruction complex is inhibited, resulting in increased cytosolic β-catenin concentration, β-catenin can now freely translocate into the nucleus and is a co-factor for T-cell factor (TCF) transcription factors that results in downstream transcription of Wnt target genes (Beumer & Clevers, 2016).

EGF regulates proliferation rates of CBC stem cells (Sato, et al., 2011), similarly to Wnt, EGF ligands are expressed by neighbouring cells that interact with the EGF receptor (EGFR) on the surface of ISCs. Resulting in upregulation of the downstream expression of ErbB that increases ISC proliferation. EGF induced proliferation is regulated by the ErbB inhibitor Lrig1, loss of which results in rapid expansion of stem cells and subsequent dysregulation of the intestinal epithelium (Beumer & Clevers, 2016).

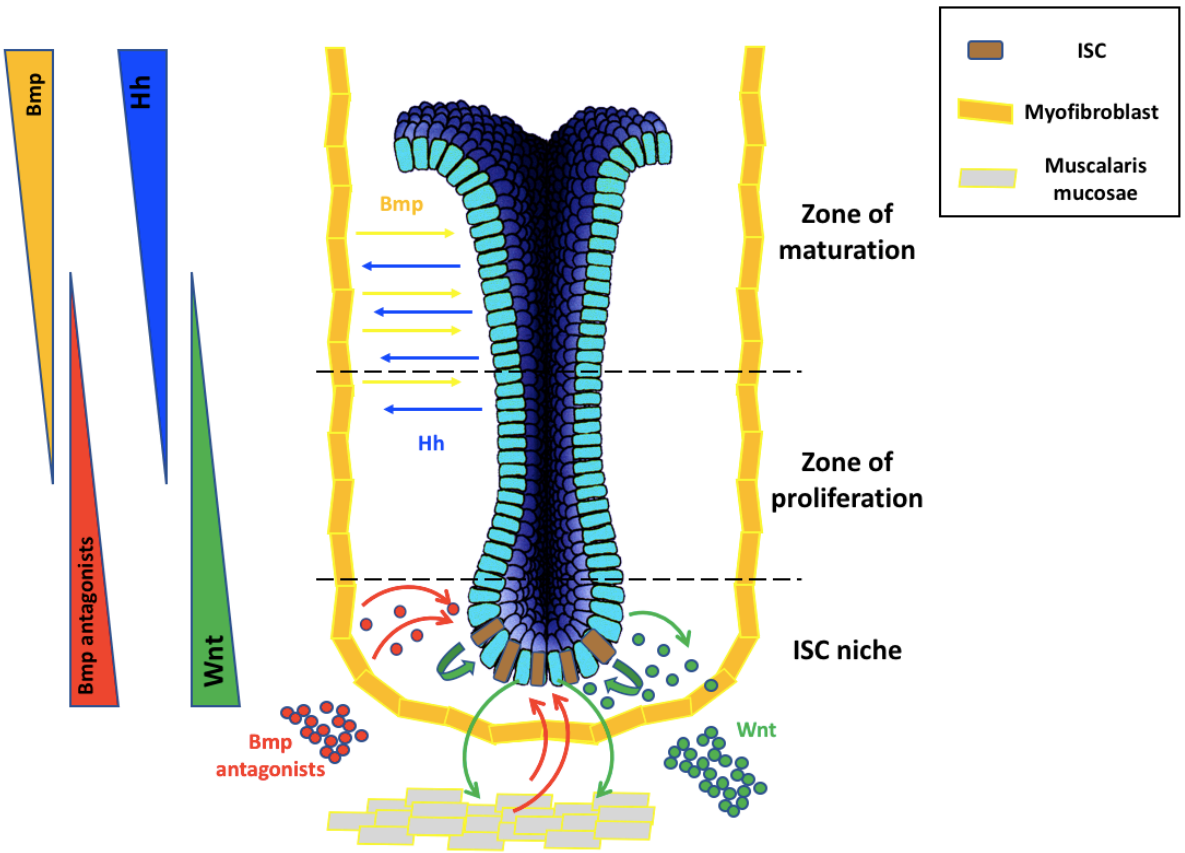


Figure 1.1.5- Schematic diagram of the numerous signalling factors that result in signalling gradients that regulate ISC-driven homeostasis. Signals emanate from cells within the underlying lamina propria, such as myofibroblasts and the muscularis mucosae. (Adapted from: (Powell, et al., 2011)).

Finally, BMP ligands induce differentiation and thus a BMP gradient is maintained that follows the reverse order to Wnt, **(Figure 1.1.5)**. There is a high concentration of BMP inhibitors, e.g. noggin, at the crypt base preventing early differentiation, secreted by mesenchymal cells surrounding the ISC niche (Kosinski, et al., 2007) **(Figure 1.1.5)**. Together these are just some examples of how different factors and pathways interact to maintain cell hierarchy and regulate ISC driven renewal and regulation of the intestinal epithelium. How all these different components are interpreted by ISCs and epithelial cells has been investigated and there is an emerging role of calcium as a key regulator in integrating these different signalling pathways. However, dysregulation of these processes through mutations, poor lifestyle choices, chronic inflammation will result in disease such as colorectal cancer (CRC).

1.2 Colorectal cancer

In the Western world, CRC is the third most common cancer in men and second in women (Boyle & Ferlay, 2005). Worldwide it is the third most common and the fourth largest cause of death (Haggar & Boushey, 2009). It is estimated globally there are 1.4 million new CRC cases with 693,900 each year (Xie & Raufman, 2016). The majority of CRC cancers are a result of sporadic mutations whilst the remaining fraction is hereditary. Benign dysplastic adenomatous polyps have an increased susceptibility to subsequent cancer formation, **(Figure 1.2.1)**. Further mutations within these benign polyps, such as *p53*, turn these polyps malignant (Boland, et al., 1995).

The rate of polyp formation is heavily influenced by lifestyle choices such as diet. In the USA, it is predicted that over 50% of the population will develop a minimum of one adenomatous polyp by 70 years of age, of which one-tenth will progress into cancer (Arnold, et al., 2005). Polyps form within the epithelial cell layer and is subsequently the site of CRC initiation and development into a malignant tumour. As this tumour develops it begins to protrude into the lumen and the underlying lamina propria **(Figure 1.2.1)**. In latter stages metastasis can occur, this is often to the liver (Huang, et al., 2018).

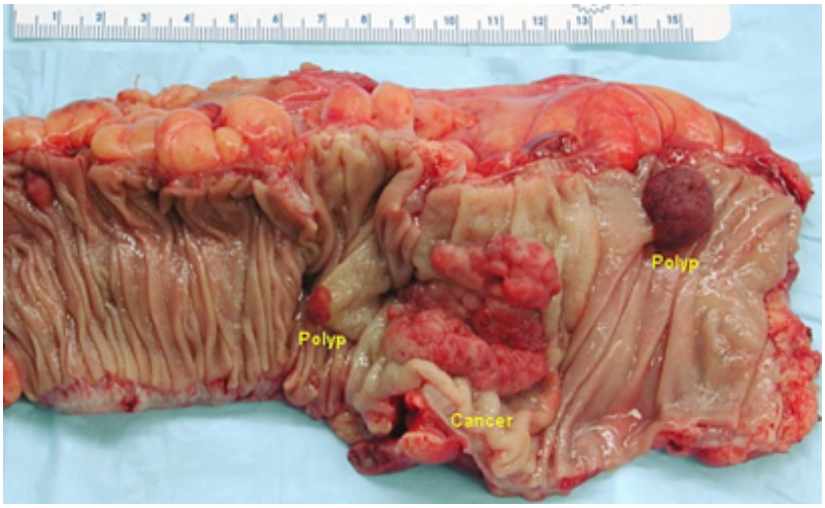


Figure 1.2.1- Longitudinal cross-section of a human colon, (left) is the normal mucosa of the colonic epithelium. (label 'Polyp') Polyps themselves are benign, however, they are common precursors for colorectal cancer. (centre label 'cancer') CRC is characterised by uncontrolled cell growth, the tumour protrudes into the lumen and also into the lamina propria that lies below the monolayer of epithelial cells.

As polyps are often a precursor to CRC formation, those with the hereditary single gene disorder familial adenomatous polyposis (FAP), have a high predisposition to CRC, developing hundreds to thousands of polyps. FAP is an autosomal dominant disease resulting from germline mutations of the tumour suppressor gene, *APC* (Arnold, et al., 2005) (Barker, et al., 2009). The outcome of loss of function mutations in *APC* is increased cell proliferation **(Figure 1.2.1)**. The location of mutations within the *APC* gene impacts on the severity of the FAP phenotype (Brensinger, et al., 1998). Severe forms of FAP are due to deletions or premature truncations between codons 1250 and 1464 in the *APC* gene. On the contrary, mutations

occurring within the first 4 exons of *APC* display an attenuated phenotype which results in later manifestation with far fewer polyps (Spirio, et al., 1993).

CRC metastasis is promoted by mutations in the key driver genes, KRAS and p53 (Huang, et al., 2018). For early-stage CRC, surgical and endoscopic treatment is highly effective, those with late-stage CRC are largely resistant to both radiotherapy and chemotherapy (Xie & Raufman, 2016). There are a number of different symptoms of CRC, the most common are; rectal bleeding, changes in bowel habit and abdominal pain (Ballinger & Anggiansah, 2007). These symptoms are problematic as they often present during late stages of CRC development, greatly impairing treatment efficacy (Ballinger & Anggiansah, 2007). The effects of genetic factors in CRC formation and progression can be confounded by environmental factors, such as; age, obesity, inflammatory bowel disease, mutational site, etc (Huang, et al., 2018). These data indicate the growing need for more research into the molecular and cellular causes of colon cancer, from which novel therapeutic methods can be developed.

1.3 Intracellular calcium signalling

Within the Williams laboratory there is a particular focus on the calcium signalling pathway and its role in homeostasis within the healthy mucosa and its role in CRC initiation and development. Calcium is a vital second messenger in almost all cell types, originally, investigations into calcium were with excitable cell types, those of the heart and neurons (Carafoli, 2002). However, subsequent research identified calcium as an important second messenger in non-excitable cell types such as the epithelia (Berridge, et al., 2000).

Calcium is thought to be a key integrator of signals regulating the cellular functions of crypt epithelial cells, such as ISC proliferation, fluid and mucus secretion, cell migration and shedding. These data show that intracellular calcium is ubiquitous and context dependent, and the role in which the calcium signalling toolkit has in CRC development and progression has been underestimated (Monteith, et al., 2017). More recently studies show modulation of calcium pumps and channels result affect the rate of cancer progression (Monteith, et al., 2017). These data identify the importance of intracellular calcium in maintaining homeostasis but also its role in CRC.

Calcium ions have a rapid local action, with an estimated migration distance of 0.1-0.5 µm in ~50 µs before interacting with calcium binding protein e.g. calcineurin (Clapham, 1995). These rapid signals are regulated by calcium ion channels, pumps and exchangers that are collectively termed the calcium signalling toolkit (Montieth, et al., 2017). Calcium homeostasis is regulated by sustaining a high extracellular calcium concentration (1mM) and a much lower cytosolic calcium concentration (~100 nM), 10,000-fold difference **(Figure 1.3.1)**. This steep concentration gradient facilitates the rapid influx of calcium into cells upon membrane depolarisation via voltage operated calcium channels (VOCCs) (Carafoli, 2002). Furthermore, this steep concentration gradient is maintained by divalent calcium ions being too large to cross the plasma membrane. However, some leakage does occur due to the steepness of the gradient but is countered by the plasma membrane Ca^{2+}-ATPase (PMCA) pump that actively removes excess calcium from the cytosol (Montieth, et al., 2017).
Intracellular organelles sequester excess cytosolic calcium through calcium binding proteins, for example calreticulin. Within the ER, calreticulin binds up to 40 calcium ions that results in

an osmotic value of just one, making the ER the largest intracellular calcium store in cells (Mekahli, et al., 2011). Lysosomes are another intracellular calcium store that sequester excess cytosolic calcium. Therefore, these two intracellular stores have a greater calcium concentration than the cytosol setting up yet another steep concentration gradient. The ER-cytosol gradient is maintained through Sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pumps actively sequestering calcium (Kania, et al., 2017) and IP_3Rs releasing calcium upon IP3 binding from upstream G-protein coupled receptor (GPCR) activation **(Figure 1.3.1)**. The endolysosome-cytosol gradient is maintained through calcium-proton exchange pumps that actively sequester calcium against a concentration gradient driven by the energy released from protons down a concentration gradient. Calcium is released from endolysosomes via two-pore channels (TPCs) facilitated by Nicotinic acid adenine dinucleotide phosphate (NAADP) binding (Pitt, et al., 2016). After a stimulus-induced intracellular calcium signalling, the intracellular stores must be replenished while the high cytosolic calcium concentration is returned back to resting ~100 nM. ER stores are replenished through store operated calcium entry (SOCE). This process involves the interaction of calcium release-activated calcium channel protein 1 (ORAI1) and the ER calcium sensor, stromal interaction molecule 1 (STIM1) at ER-plasma membrane junctional sites, (Feske, 2007) (Lynes & Simmen, 2011) **(Figure 1.3.1)**.

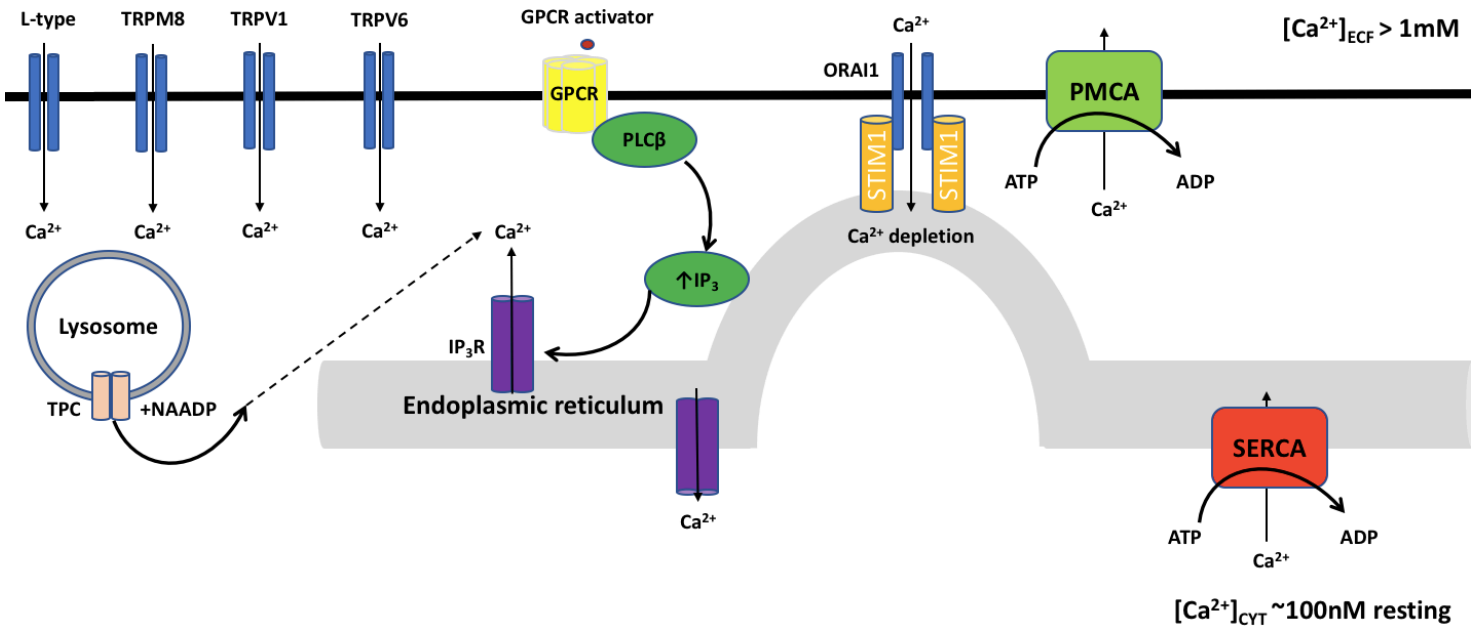


Figure 1.3.1- A schematic diagram representing examples of the major Ca^{2+}-permeable channels, pumps and exchangers located at the plasma membrane and intracellular organelles, lysosomes and ER. This diagram shows how calcium homeostasis is maintained within a normal colonic epithelial cell. (Adapted from: (Montieth, et al., 2017).

The spatial and temporal characteristics of calcium signals from a stimulus dictate the overall cellular response to that specific stimulus (Berridge, et al., 2003). A large, prolonged signal often results in cell death whilst localised alterations in cytosolic calcium concentrations regulate directional migration (Montieth, et al., 2017). In *Drosophila*, calcium is a central regulator of ISCs, integrating a number of metabolic and proliferative signals, for example; inflammatory signals, growth factors, nutrients and cancer driver mutations (Deng, et al., 2015), **(Figure 1.3.2)**. Deng, *et al*., 2015 assessed the role diet-derived amino acids played in intestinal homeostasis, through supplementing *Drosophila* food with L-Glutamate (L-Glu). Discovering the sole addition of L-Glu was sufficient to stimulate ISC proliferation and gut growth (Naszai & Cordero, 2016), **(Figure 1.3.2)**. L-Glu was found to be absorbed by enterocytes, activating a signalling cascade, resulting in an increase in IP_3, substrate for IP_3Rs (Deng, et al., 2015). During *Drosophila* homeostasis, there are frequent low amplitude calcium oscillations in quiescent ISCs, but, upon introduction of L-Glu become less frequent with a higher peak amplitude. This alteration increases the rate of ISC proliferation (Deng, et al., 2015), **(Figure 1.3.2)**. This increase in proliferation was an outcome of elevated cytosolic calcium activating calcium-dependent phosphatase calcineurin. In turn stimulating CREB regulated transcription co-activator (CRTC), promoting a switch from quiescence to actively dividing (Deng, et al., 2015).

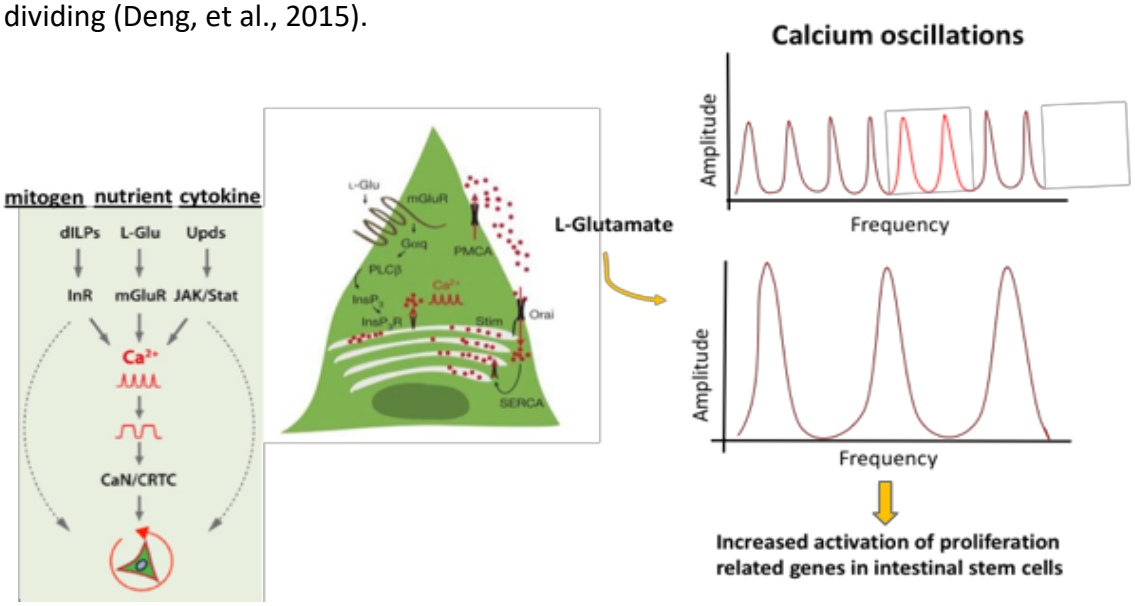


Figure 1.3.2- Adaptation of Deng, *et al*., 2015 findings of calcium having a key role in integrating several metabolic and proliferative signals. One of which highlighted here is L-Glu, which induces increased ISC proliferation in *Drosophila* (Deng, et al., 2015).

These previous data indicate the role of the calcium signalling pathway in health and disease. In the Williams laboratory the focus is to develop on previous knowledge to understand the role of calcium in gut health and disease using novel 3D *ex vivo* human culture models; crypts, organoids and tumouroids. Here we investigate the role of endolysosomes and ER in health and disease, following previous work in the Williams laboratory. Previous immunohistochemistry data identified the location of endolysosomes to the apical pole and ER to the basal pole of colonic crypts, using specific markers for each, Lamp-1 and KDel respectively **(Figure 1.3.3 a-b)**. Furthermore, expression patterns are conserved in the other *ex vivo* model, organoids **(Figure 1.3.3 c-d)**. These two markers were used as Lamp-1 is

expressed in the endolysosomal cell wall (Eskelinen, 2006) whilst KDel is a receptor expressed on the ER membrane surface (Capitani & Sallese, 2009).

In vivo endolysosomes and ER have specific calcium release channels regulating calcium activity to a plethora of external and internal stimuli. Previous work by Christy Kam confirmed expression of these receptors was conserved within our *ex vivo* culture models. TPCs facilitate endolysosomal calcium release, whilst IP_3R and Ryanodine receptor (RyR) subtypes facilitate ER calcium release, immunohistochemistry confirmed the expression of all three receptors and their subtypes **(Figure 1.3.4)**.

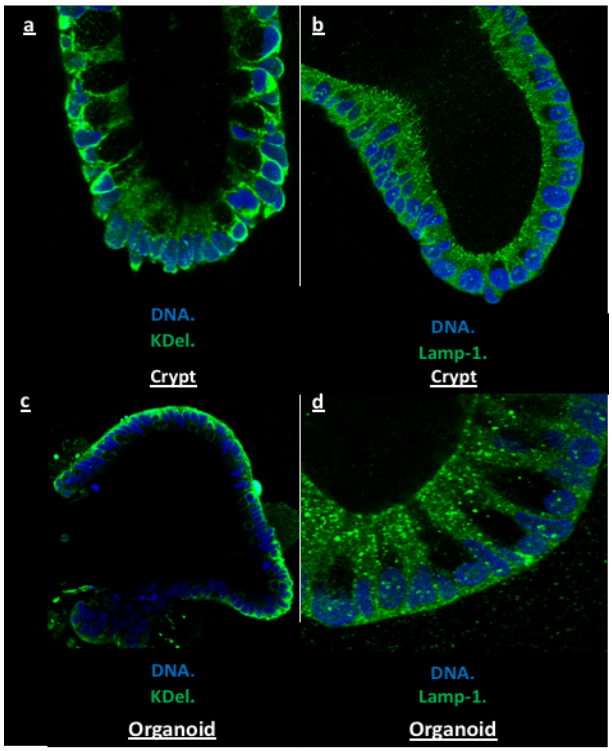


Figure 1.3.3 (a-d)- **(a)** Immunohistochemistry of a human colonic crypt using an ER antibody marker, KDel. Highly expressed at the basal pole. **(b)** Immunohistochemistry of a human colonic crypt using the endolysosomal antibody marker, Lamp-1, highly expressed at the apical pole. **(c)** Expression of KDel at the basal pole is conserved in organoids, KDel only expressed at the basal pole. **(d)** Expression of Lamp-1 at the apical pole is conserved in organoids, greater number of puncta at the apical pole. (Work by Victoria Jones).

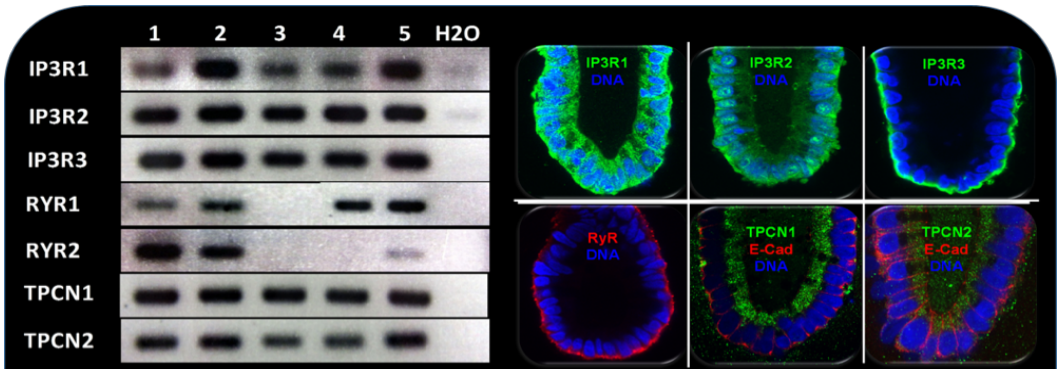


Figure 1.3.4- (**Left)** RT-PCR of intracellular calcium receptors, IP_3R 1-3 and RyR 1-2 **(ER)** including TPC 1-2 **(endolysosomes)**. **(Right)** immunohistochemistry of intracellular calcium receptors, IP_3R 1-3 and RyR 1-2 expressed on the basal pole while TPC 1-2 expressed on the apical pole of human colonic crypts. (Work by Christy Kam).

The mitochondria are another important organelle in calcium signalling, although not investigated in this project. Mitochondrial calcium levels are maintained through actively up-taking calcium through the mitochondrial calcium uniporter (MCU) and the Na^+/Ca^{2+} exchanger releases calcium (Stefani, et al., 2016). The low affinity of MCUs coupled to these efflux pumps prevents massive energy dissipation from calcium cycling across the inner mitochondrial membrane (Stefani, et al., 2016). Crosstalk between the ER and mitochondria help regulate cellular processes through dynamic contacts known as mitochondria-associated membranes (MAMs) (Giorgi, et al., 2015) Rapid IP_3R-mediated calcium release from the ER to the mitochondria promote pro-survival effects but prolonged gross signals induce apoptotic cell death (Pedriali, et al., 2017). IP_3R-mediated calcium release is controlled by luminal calcium levels, which in turn balances the shift between pro-apoptotic and pro-survival signalling through dampening or augmenting calcium transfer between the ER and mitochondria (Pedriali, et al., 2017).

ER-mediated calcium release is activated by upstream purinergic receptor GPCR coupled activation (Montieth, et al., 2017). Upon purinergic receptor activation, phospholipase C (PLC) is upregulated subsequently cleaving phosphatidylinositol 4,5-bisphosphate (PIP_2) producing IP_3. IP_3 is a substrate for IP_3R mediated ER calcium release (Montieth, et al., 2017), **(Figure 1.3.5a)**. As the ER is the largest organelle store of calcium we aimed to elucidate the function and size of this store in intracellular calcium signalling in health and disease using the SERCA inhibitor Thapsigargin, blocking calcium sequestering resulting in calcium leak from the ER (Lytton, et al., 1991), **(Figure 1.3.5a)**. This calcium leak can be visualised by the ratiometric calcium fluorescent dye Fura-2 (Tsien, et al., 1982).

As mentioned earlier the acidic endolysosomal organelle is a recently discovered intracellular store at the apical pole that is proposed to be the initiation site of cholinergic-induced intracellular calcium signals. Muscarinic receptors are GPCRs that are activated by ACh and its analogues, for example Cch. Muscarinic receptor activation results in a downstream signalling cascade that activates the enzyme CD38 which catalyses the production of NAADP (Pitt, et al., 2016) (Lin, et al., 2017), **(Figure 1.3.5b)**. NAADP is a substrate for TPC1-2, activating TPC-mediated calcium release from endolysosomes **(Figure 1.3.5b)**. Calcium released from these stores upon muscarinic receptor activation can be augmented by calcium-induced-calcium release (CICR) from the ER (Galione, 2014), (Rios, 2018). Calcium from acidic stores travels along calcium microdomains coupling these two calcium storage organelles (Penny, et al., 2015). This phenomenon has been termed the “trigger hypothesis” (Kinnear, et al., 2004) (Boittin, et al., 2002) in an attempt to unify the differing aspects and findings surrounding lysosomal calcium signalling (Galione, 2014), (Lin, et al., 2017). In addition to NAADP, a further TPC1 substrate is cytosolic calcium and TPC2 can interact with PIP_2 (Pitt, et al., 2016). We aim to investigate the endolysosomal function and size in health and disease using the lysosomotropic agent Gly-Phe β-naphthylamide (GPN) (Kilpatrick, et al., 2014).

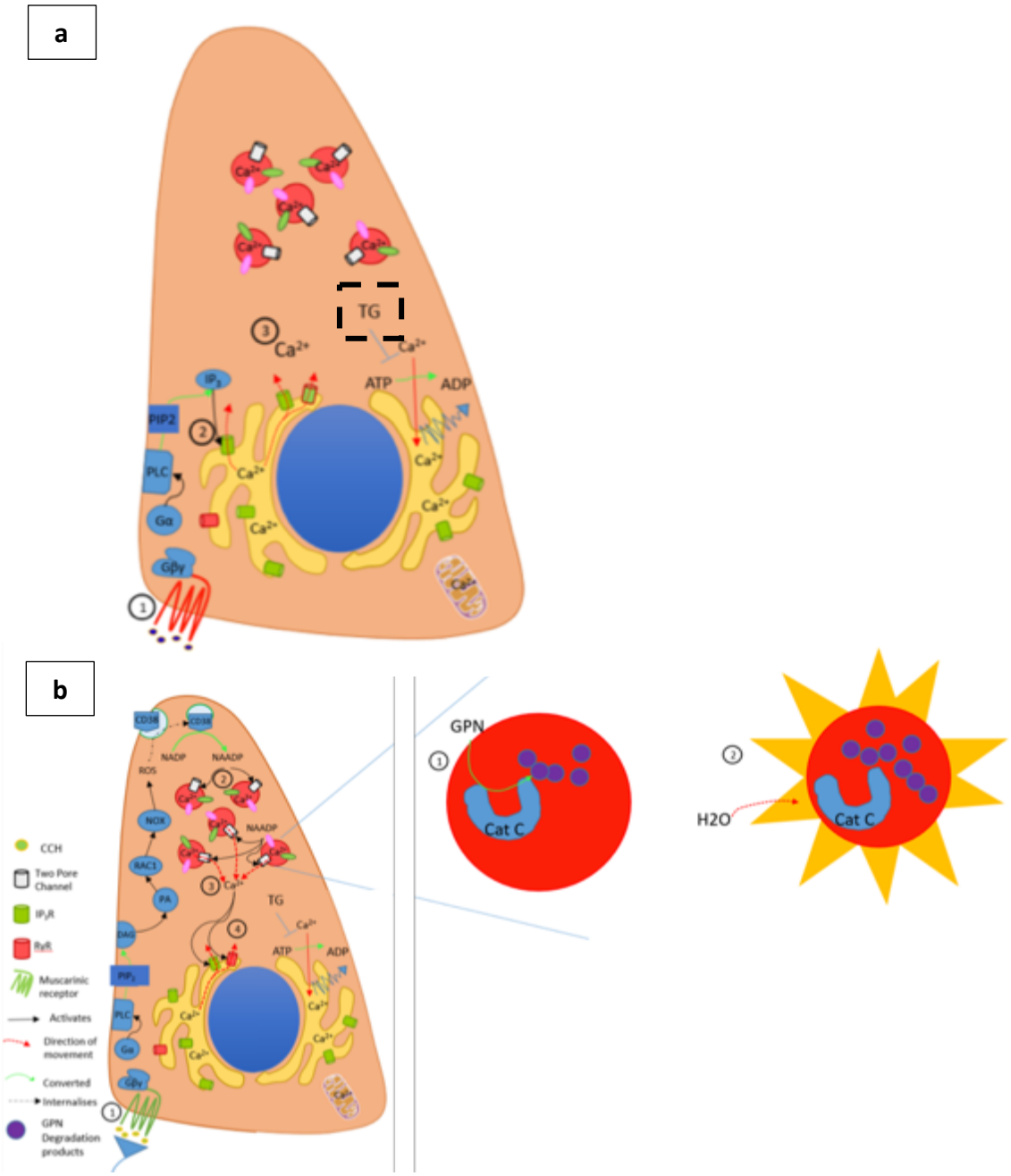


Figure 1.3.5 (a-b)- **(a)** Schematic diagram of purinergic receptor activation and the downstream signalling pathway, resulting in ER mediated calcium release at the basal pole. Additionally, Thapsigargin (Tg) is highlighted, which inhibits SERCA pump activity. **(b)** Schematic diagram of muscarinic receptor activation, the downstream signalling pathway and TPC mediated calcium release from endolysosomes at the apical pole. Subsequently resulting in calcium-induced calcium release from the ER at the basal pole. Also, the mode of action for GPN is shown, hydrolysed by Cathepsin C, producing amino acids, resulting in lysis via water entering along an osmotic gradient.

With increasing data on endolysosomes as an important calcium storage organelle, recent research has uncovered an emerging role of mammalian target of rapamycin (mTOR) in regulating TPC-mediated calcium mobilisation. mTOR is a serine/threonine protein kinase, that regulates a number of different cellular processes, such as; growth, proliferation and survival, and as is often the case, upregulated in cancer. Therefore, mTOR is of particular importance for developing therapeutic cancer drugs (Ballou & Lin, 2008). Ogunbayo, *et al*., 2018 identified mTORC1 controls TPC-mediated calcium release from lysosomes, **(Figure 1.3.6)**. When mTOR is active, it has an inhibitory effect on TPC-mediated calcium release (Ogunbayo, et al., 2018).

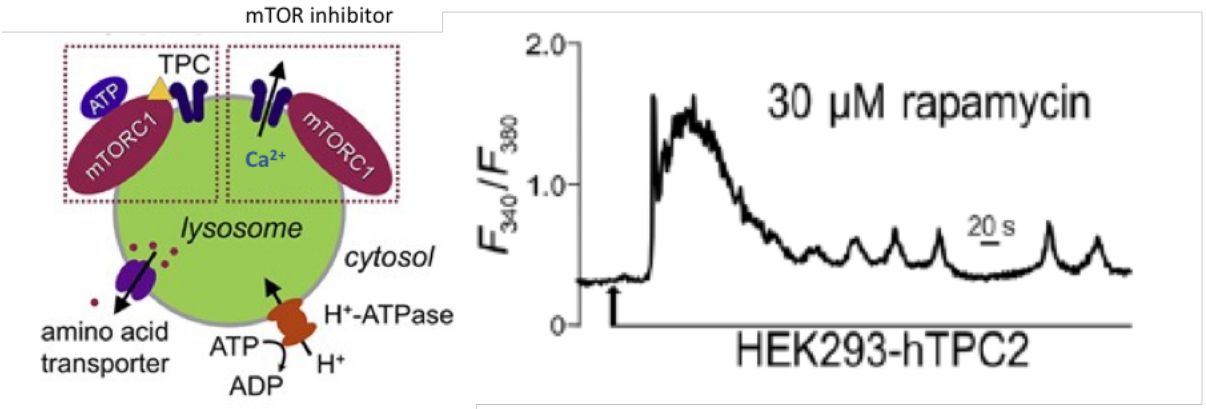


Figure 1.3.6- mTOR under normal conditions inhibits TPC activity, using an mTOR inhibitor such as Rapamycin induces an almost immediate calcium response in HEK cells (Ogunbayo, et al., 2018)

mTOR has two complexes, mTORC 1&2 that regulate different cellular processes, and collectively are controlled by a number of different signalling pathways (Ogunbayo, et al., 2018). Taking inspiration from Ogunbayo *et al*., 2018 we also use the mTOR inhibitor rapamycin to investigate the role of mTOR in health and disease **(Figure 1.3.6)**. Determining the effects of mTOR on calcium signalling is difficult due to its numerous stimuli and the number of complexes it can form that have opposing rapamycin sensitivity (Ballou & Lin, 2008), regulating these variables *ex vivo* is a challenge.

Previous work by Alvin Lee (Williams laboratory), has inhibited muscarinic receptor induced calcium release from endolysosomes using the NAADP antagonist NED-19. Furthermore, NED-19 reduces calcium release from the ER however does not affect purinergic receptor induced calcium release from the ER. Confirming that the two pathways are separate and the specificity of NED-19. Other data supports these results, at high concentrations (1 µM) NED-19 inhibits NAADP but at lower concentrations appears to enhance TPC-mediated calcium mobilisation (Pitt, et al., 2010). Within this project GPN is used to investigate the endolysosomal store, GPN is a cathepsin C substrate that is hydrolysed to amino acids. Thus, increasing the osmolarity inside endolysosomes, water enters along a concentration gradient causing lysis (Berg, et al., 1994), **(Figure 1.3.5b)**.

As cholinergic calcium signalling is important in regulating ISC driven regulation of the intestinal epithelium we investigated the emerging role of non-neuronal sources of ACh in *ex vivo* crypts. The Williams laboratory has previously demonstrated the neurotransmitter ACh mobilises intracellular calcium in $Lgr5^+$ stem cells at the base of human colonic crypts (Reynolds, et al., 2007). The initiation site is found to be at the apical pole, potentially from endolysosomes (Williams, unpublished). Hayakawa *et al*., (2017), discovered non-neuronal sources of ACh in mouse intestinal epithelium, from by Choline acetyl transferase positive ($ChAT^+$) tuft cells after sensing changes in the gut lumen. Furthermore, Yajima, *et al.,* (2011) demonstrated ACh is synthesised in rat colonic epithelial cells and secreted on the serosal side of the colonic epithelium after the addition of, short-chain fatty acid, propionate into the lumen (Yajima, et al., 2011). Ablation of tuft cells in mouse epithelium resulted in reduced proliferation and tumourigenesis in an ACh-muscarinic dependent manner (Hayakawa, et al., 2017). Here we aim to recapitulate these results in our human *ex vivo* crypt cultures. From these data we used the potent muscarinic receptor agonist Oxotremorine to investigate the growth rate of organoids and ISC proliferative activity. Comparing organoids in 10% organoid culture media with 1 µM Oxotremorine against control over the course of a week. These data would provide insights on the effects of cholinergic-calcium signals on ISC activity.

Finally, data indicates mutations and alterations in the calcium signalling toolkit can lead to cancer. In mice, microbial ligands promoting calcineurin-dependent activation of nuclear factor of activated T cells) NFAT can lead to the development of intestinal tumours (Peuker, et al., 2016). In humans, increased nuclear labelling of NFATc3 is associated with increased risk of CRC death (Peuker, et al., 2016). Overexpression of STIM 1 and SERCA 2 (Wang, et al., 2016) (Lyu, et al., 2017) or reduced SERCA3 expression (Gelebart, et al., 2002) can lead to tumour formation. However, it is not known whether changes in the calcium signalling toolkit expression impact signalling status in colorectal carcinogenesis.

From these data we hypothesised that calcium signalling is remodelled during colon carcinogenesis. The aims of this project were:

I. Characterise the cholinergic calcium signalling pathway, using an *ex vivo* 3D native human colonic crypt model system.

II. Compare ER storage capacities between *in vitro* 3D models of organoids and tumouroids via Thapsigargin stimulation.

III. Compare endolysosomal storage capacities between *in vitro* 3D models of organoids and tumouroids via GPN.

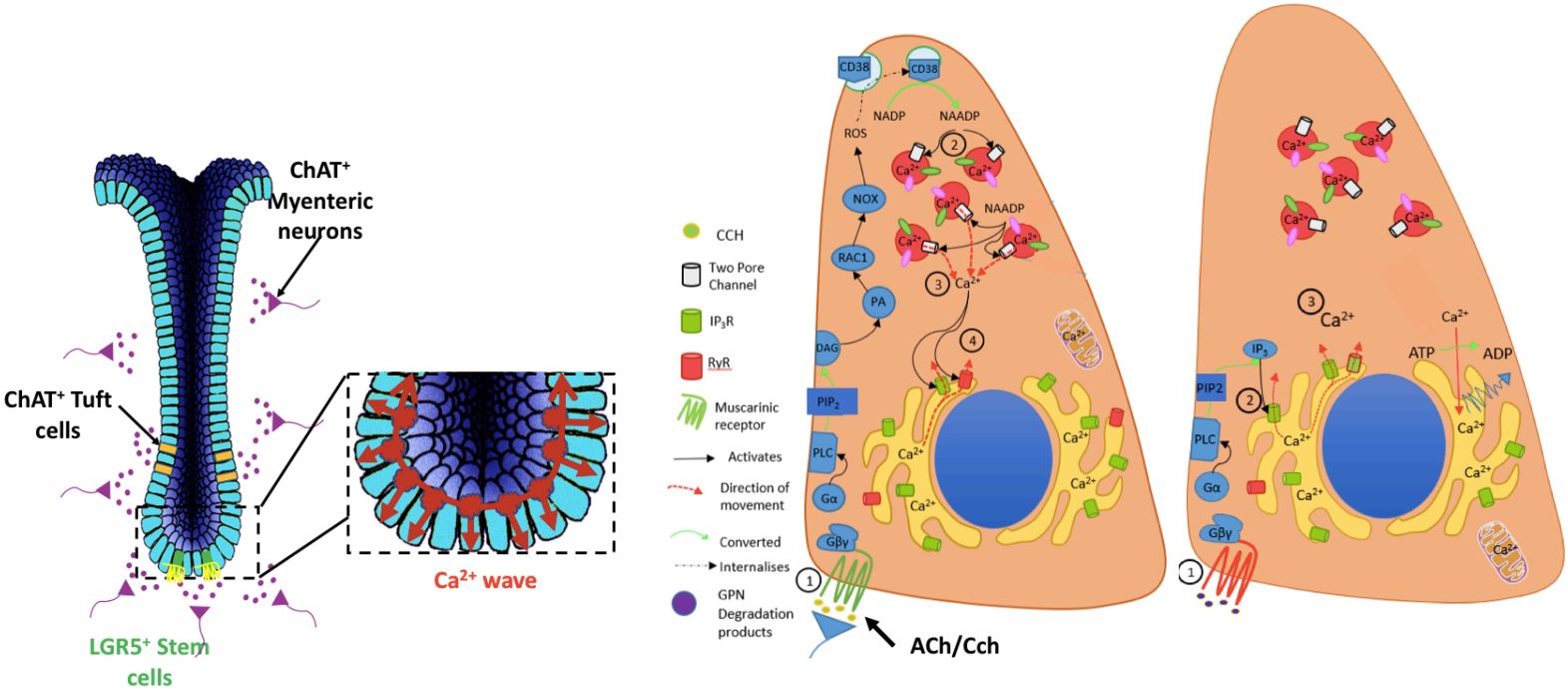


Figure 1.3.7- (left to right) Schematic diagram of the neuronal and non-neuronal sources of cholinergic signals that activate muscarinic receptor mediated intracellular calcium signals. Intracellular calcium signals are suggested to initiate at the apical pole and propagate to the basal pole. The intercellular calcium wave propagates from the crypt base up the crypt axis via gap junctions. On the right, the downstream signalling pathways of muscarinic or receptor activation and the downstream signalling that activates TPC-mediated calcium release from the lysosomes. Additionally, purinergic receptor activation downstream signalling pathway induces calcium release directly from the ER.

2. Materials & Methods

2.1 Tissue sample collection

Biopsy samples of healthy colonic epithelial tissue were collected from patients who underwent exploratory colonoscopy at the Norfolk & Norwich University Hospital (NNUH) by members of the Williams laboratory. These samples were collected from patients who had given their consent for a biopsy to be taken, following strict ethical procedures and with NHS research & development approval. Samples collected were kept cool within a sealed container whilst being transported by members of the Williams laboratory to the laboratory.

2.2 Human colonic crypt isolation

Human colonic crypts collected from the NNUH were isolated following previously described protocol (Lindqvist, et al., 1998) and (Parris & Williams, 2015). Human colonic crypts are isolated using dissecting scissors, forceps and knives. Human colonic crypts are isolated from the lamina propria using universal bottles containing 25mls crypt isolation buffer, that contains a number of different factors; described in (Parris & Williams, 2015). Afterwards, universal bottles are filled with 25mls DMEM culture medium (Parris & Williams, 2015). Human colonic crypts are then seeded onto coverslips containing Matrigel (mouse) of a 12-well plate **(Figure 2.2.1)**. Then, around 300ul of culture media is added to each cover slip and the 12-well plates are kept in an incubator replicating *in vivo* CO_2 levels and temperature, 5% and 37^{O}C respectively. The culture media contains several important growth factors required for maintenance of crypts in culture, namely Wnt and Noggin (Parris & Williams, 2015). Isolated colonic crypts can be further dissociated and grown into organoids within a 3D Matrigel contained on a glass slide within a 12-well plate **(Figure 2.2.1)**. Again, this 3D culture system replicates the *in vivo* environment of the colonic epithelium *in vitro*. With plates being stored in an incubator at 37^{O}C, 5% CO_2, ready for later use in organoid formation assays.

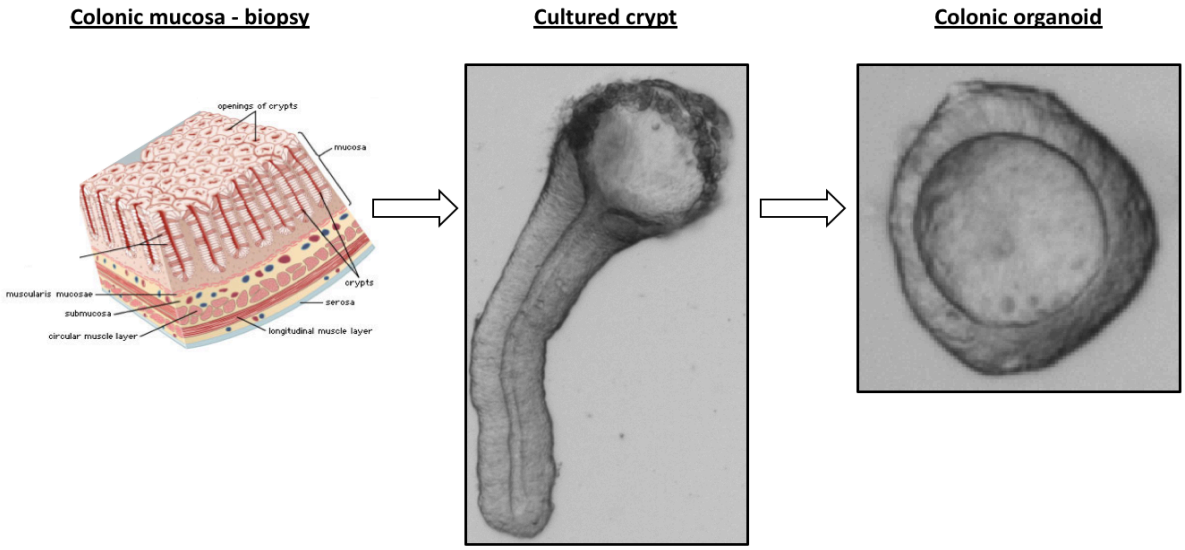


Figure 2.2.1- (left) Schematic diagram of the intact mucosa of patient biopsy before the start of colonic crypt isolation (Vector-Illustration, 2018). **(middle)** Brightfield image of a typical human colonic crypt in culture. **(right)** Brightfield image of a typical organoid in culture at day 1.

2.3 Organoid formation assay

Intestinal crypt cultures were fragmented via several rounds of centrifugation, subsequent small crypt fragments were then plated onto Matrigel dishes in a twelve-well plate. There were four different culture mediums used for the formation assay, these were; Full (WREN) media, (control), Full (WREN) media, (1µM Oxotremorine), 10% (WREN) media, (control) and 10% (WREN) media, (1µM Oxotremorine). These cultures were then analysed over the period of around a week to observe organoid forming and organoid budding capabilities using FIJI ImageJ **(Figure 2.3.1)**. A Nikon TMS light microscope was used to image organoid cultures (X40 magnification), **(Figure 2.3.1),** and in-between imaging, cultures were kept in a 37^0C incubator, 5% CO_2.

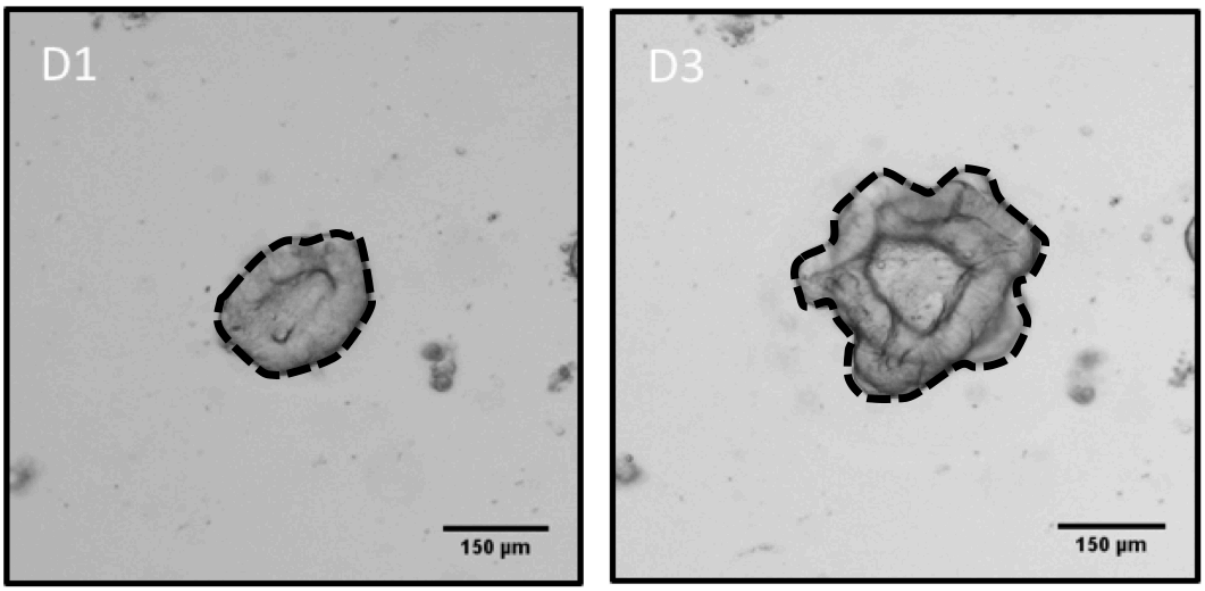


Figure 2.3.1- Average cross-sectional area (CSA) of organoids, cultured in 10% media with or without 1µM Oxotremorine over the time course of nine-days, was measured through drawing around the circumference of individual organoids, using FIJI ImageJ (With Nico Pelaez-Llaneza).

2.4 Fluorescent imaging of intracellular Ca^{2+}

Isolated colonic crypts were loaded with either Fura-2-acetoxymethyl ester(AM) (10µM, 2 h) to monitor Ca^{+2} as previously described. Fura-2AM is the ratiometric calcium fluorescent dye Fura-2 conjugated to an ester, the ester facilitates Fura-2 crossing the cell membrane into the cell cytoplasm. Cellular esterases break down the AM ester, subsequently Fura-2 remains within the cell and is bound to free intracellular calcium. As a ratiometric fluorescent dye, Fura-2 is excited by two wavelengths, 340nm and 380nm, and emits at a single wavelength 515nm **(Figure 2.4.1)** , regardless of the presence of calcium. Excitation at two wavelengths gives a ratio which is directly correlated to the concentration of intracellular calcium, also using a ratio abolishes variability in dye concentration and cell thickness thus removing any artefacts (Paredes, et al., 2008).

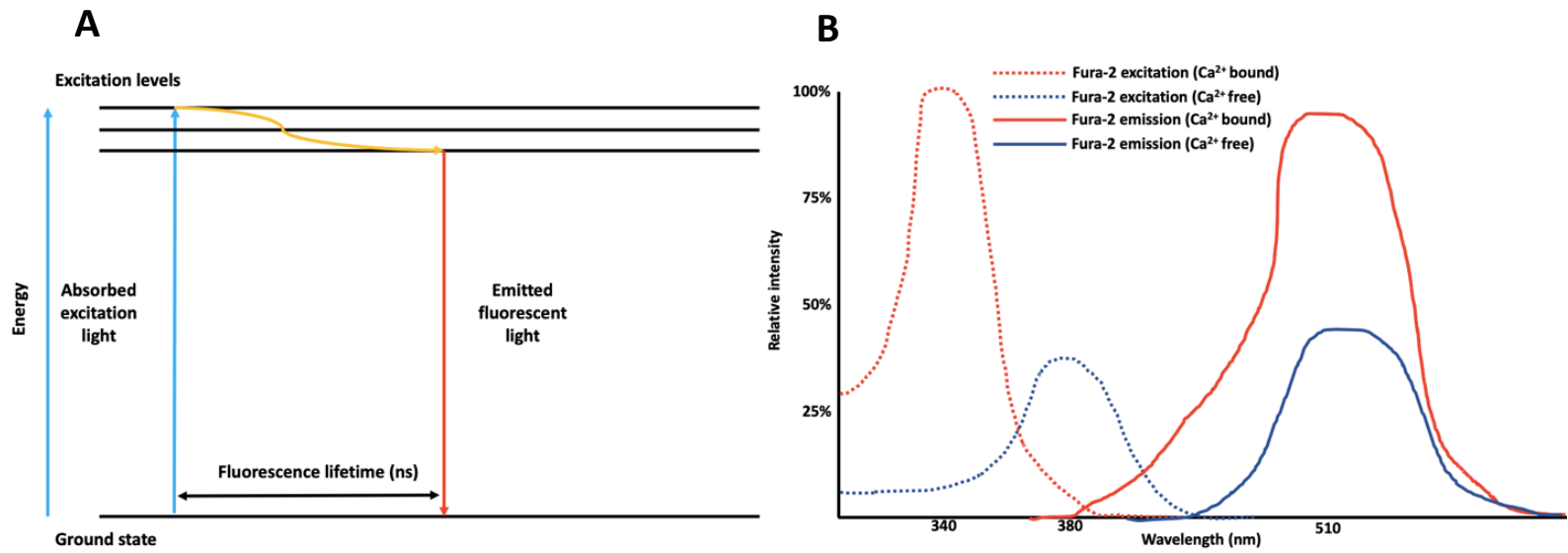


Figure 2.4.1- Excitation and emission of the ratiometric fluorescent dye Fura-2 **(A)** The Jablonski diagram illustrating the excitation of electrons from ground state and the emission at a longer wavelength as the electron returns to ground state. (Lleres, et al., 2007) **(B)** Fura-2 ratiometric dye is excited at 340nm (calcium bound) and 380nm (calcium free), **dotted lines.** Regardless of the presence or absence of calcium, Fura-2 emits energy in the form of light at a single 510nm wavelength, **solid lines** (Mudraboyina, et al., 2014).

The Fura-2 loading phase and subsequent washing of crypts was carried out using HBS made in house following the recipe in **section 2.5**. The Fura-2 loaded specimen was placed in a POC chamber located on the stage of an inverted epifluorescence microscope (×40 Nikon magnification objective, oil immersion, N.A.=1.30) at RT **(Figure 2.4.3)**. Samples visualised and imaged using a compact digital camera (CDC) with a sampling frequency of one event per second **(Figure 2.4.3)**.

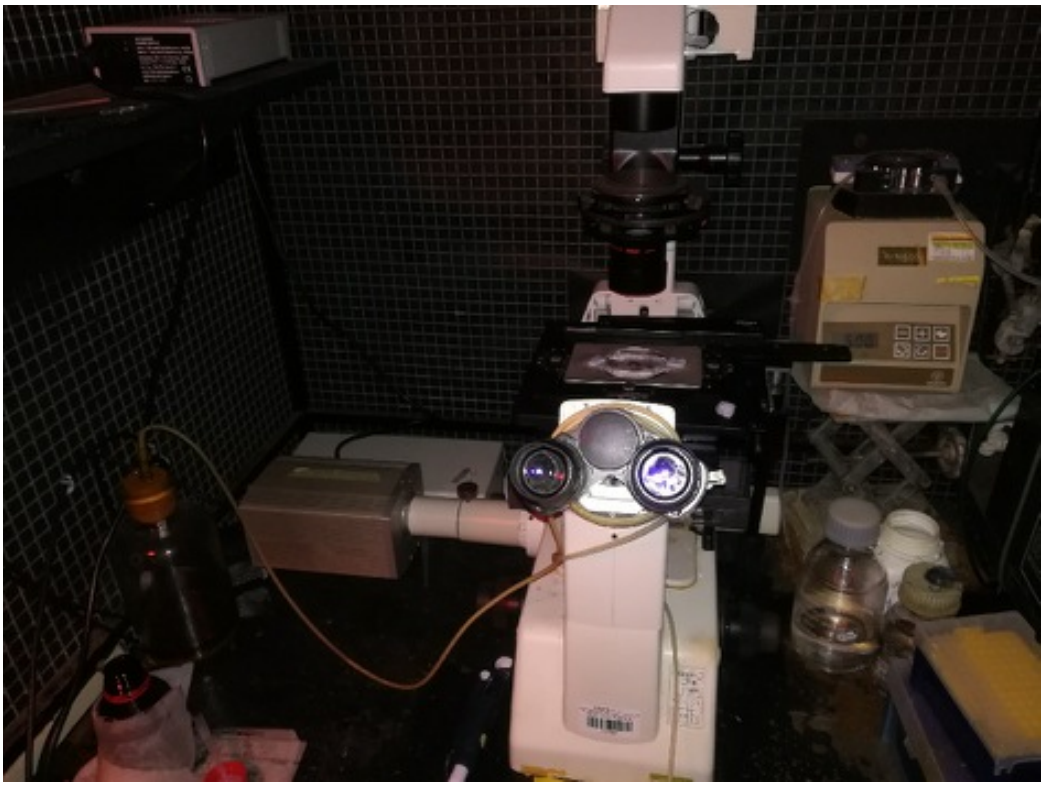


Figure 2.4.2- Inverted epifluorescence microscope with a compact digital camera
Inverted epifluorescence microscope used for imaging Fura-2 loaded crypts, organoids and tumouroids using a compact digital camera to capture culture models at rest, during and after cholinergic-calcium signals.

Crypts were placed in a volume of 200µl of supplemented HBS medium, ACh analogues were administered to the chamber and carefully removed using an aspirator. Calcium signalling was measured using EasyRatio Pro software (Photon Technology International). Fluorescence ratio data are presented in the form of pseudo colour images or as traces, where the average ratio values within regions of interest (ROIs) located along the crypt axis are plotted with respect to time **(Figure 2.4.3)**.

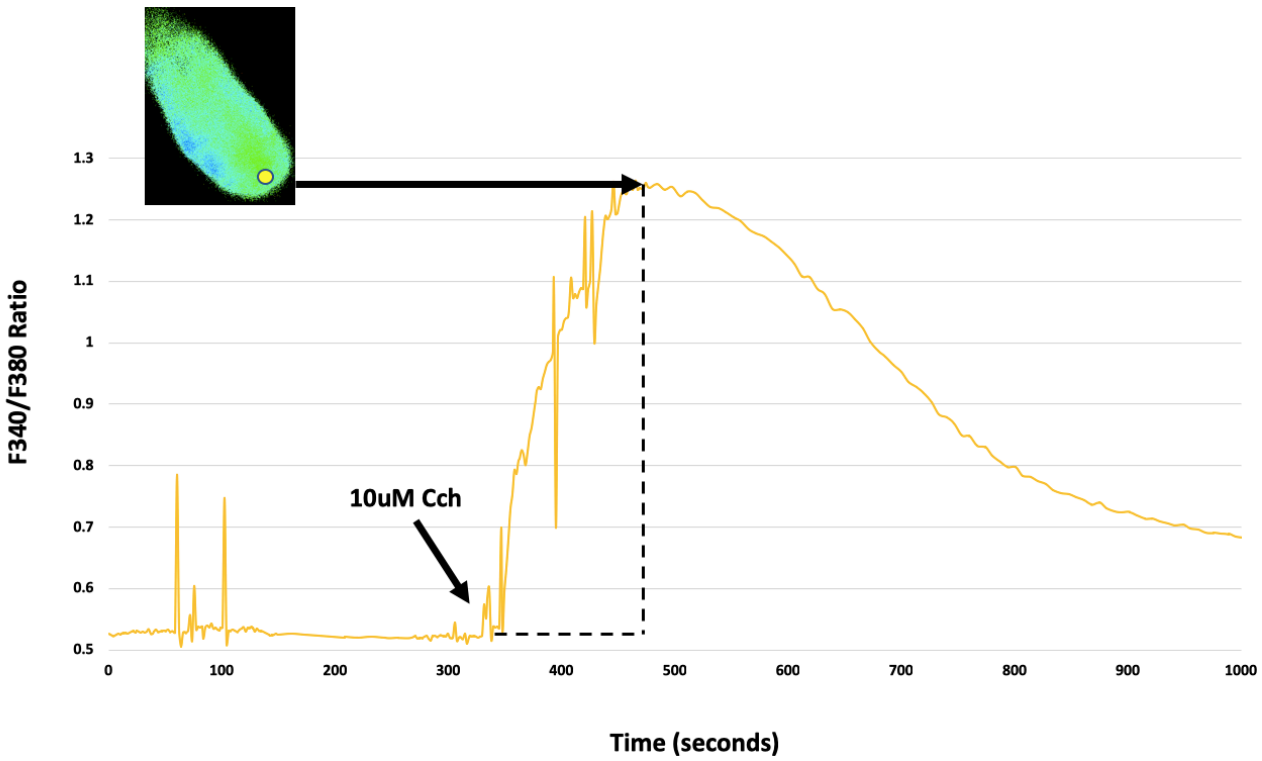


Figure 2.4.3- A typical human colonic crypt calcium response to a single 10uM Cch stimulation. Visualised via the calcium sensitive fluorescent dye Fura-2. (Dashed line) represents how the amplitude of response was measured in each case taking the difference between the base ratio and peak ratio.

2.5 Chemicals and reagents

Chemicals, reagents, buffers and medium	Supplier
Bovine serum albumin (BSA)	Sigma-Aldrich
(500ml) Hepes Buffered Saline (HBS)	
Disodium hydrogen orthophosphate (Na_2HPO_4); 0.07g, **(5.5mM)**	Fisons scientific apparatus
Potassium chloride (KCl); 0.185g, **(5mM)**	Fisher scientific
Sodium bicarbonate ($NaHCO_3$); 0.21g **(5mM)**	Fisher scientific
D-Glucose; 1.57g, **(17.5mM)**	Fisher scientific
HEPES; 1.19g, **(10mM)**	Fisher scientific
Sodium Chloride (NaCl); 4.091g, **(140mM)**	Fisher scientific
Calcium Chloride (CaCl); 0.5ml, **(1M)**	BDH Prolabo (VWR international)
Magnesium Chloride (MgCl); 0.5ml, **(0.5M)**	Fluka (Sigma-Aldrich)
L-Glutamine; 7.5ml, **(15ul/ml)**	Gibco
Non-essential amino acids; 5ml, **(10ul/ml)**	Gibco
Penicillin Streptomycin; 5ml, **(10ul/ml)**	Fisher scientific
ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetra acetic acid (EGTA)	Sigma-Aldrich
Fura-2 AM	Invitrogen (Molecular probe)
Matrigel	BD Biosciences
Oxotremorine (Oxo)	Sigma-Aldrich
DMEM	Invitrogen
Thapsigargin	Tocris Bioscience
Gly-Phe β-naphthylamide (GPN)	Cayman chemical company
Rapamycin	Tocris Bioscience
Rivastigmine	Tocris Bioscience
Essential amino acids	Gibco
Ethanol	Sigma
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich

3 Results

3.1 Spatio-temporal characteristics of cholinergic calcium signals

The spatio-temporal characteristics of cholinergic calcium signalling was investigated using 3D *ex vivo* human colonic crypt cultures and 10 µM Cch. Fresh crypt cultures were prepared for calcium imaging by 'loading' with the calcium fluorescent dye Fura-2 for two hours, protocol section 2.4. Using strategically placed ROIs post-hoc, using the EasyRatio Pro software, at the apical and basal pole along the length of the crypt axis we investigated the intracellular characteristics of calcium **(Figure 3.1.1-3.1.2)**. Under organoid culture conditions we observed cholinergic induced calcium signals originate at the apical pole of cells located at the base of the crypts and propagate to the basal pole. In addition, these signals repeat in the same fashion up the crypt axis, **(Figure 3.1.1-3.1.2)**. Coupling these data to previous immunohistochemistry in the Williams laboratory strongly suggest endolysosomes are the organelle responsible for the initiation of intracellular calcium signals upon muscarinic receptor activation. After the initial aim of confirming these acute spatio-temporal characteristics and the effects of chronic stimulation of muscarinic receptors, investigations were aimed at identifying conditions permissive for calcium oscillations.

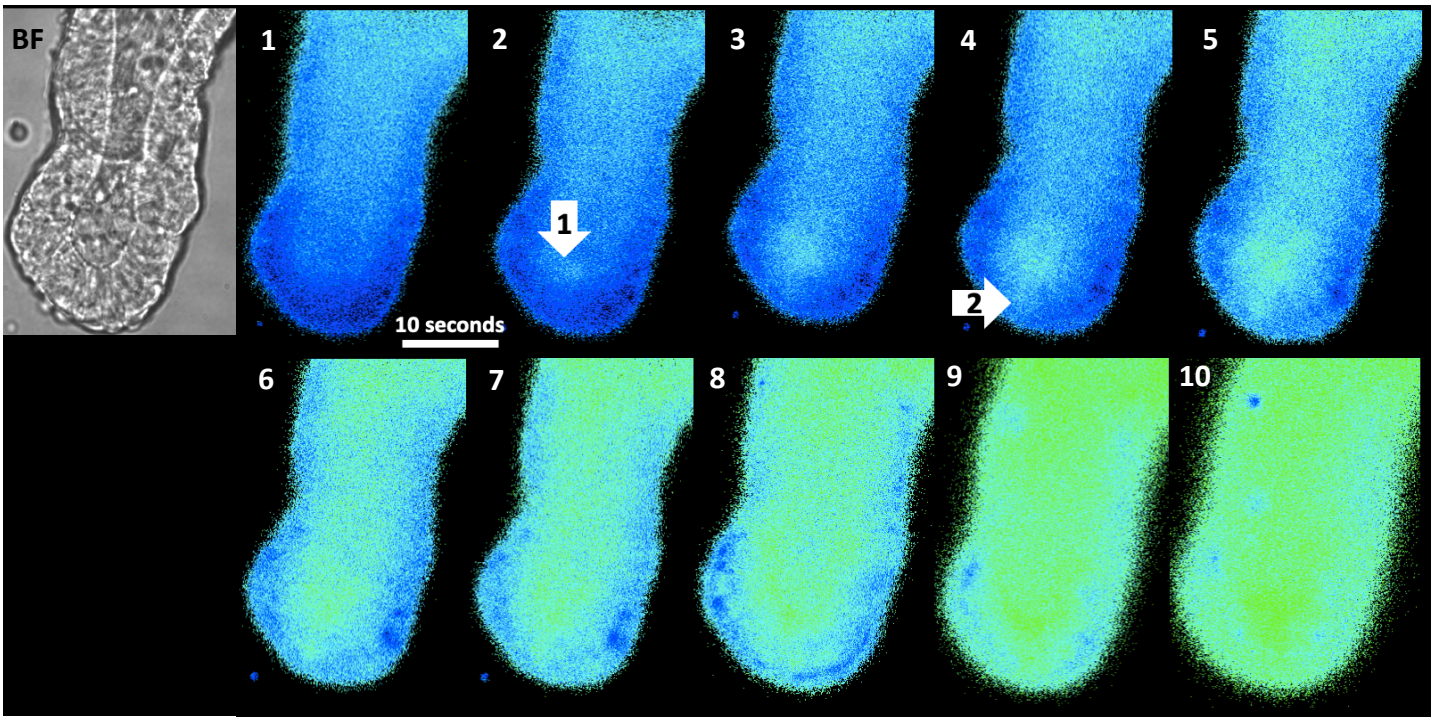


Figure 3.1.1 (A)- Spatio-temporal characteristics of cholinergic calcium signalling.
Brightfield image (BF) of human colonic crypt in 3D culture followed by immunofluorescence of the ratiometric calcium sensitive dye Fura-2 in an unstimulated crypt (**frame 1)**. Subsequent freeze frames depict changes in the ratio of the Fura-2 dye in the cytoplasm of colonic crypt epithelial cells in 10 second periods, indicating an intracellular increase in calcium concentration. **(white arrows)** Indicate the initiation site of intracellular calcium signals at the apical pole and the propagation of the intracellular calcium wave to the basal pole and subsequent spreading of the calcium wave up the crypt axis.

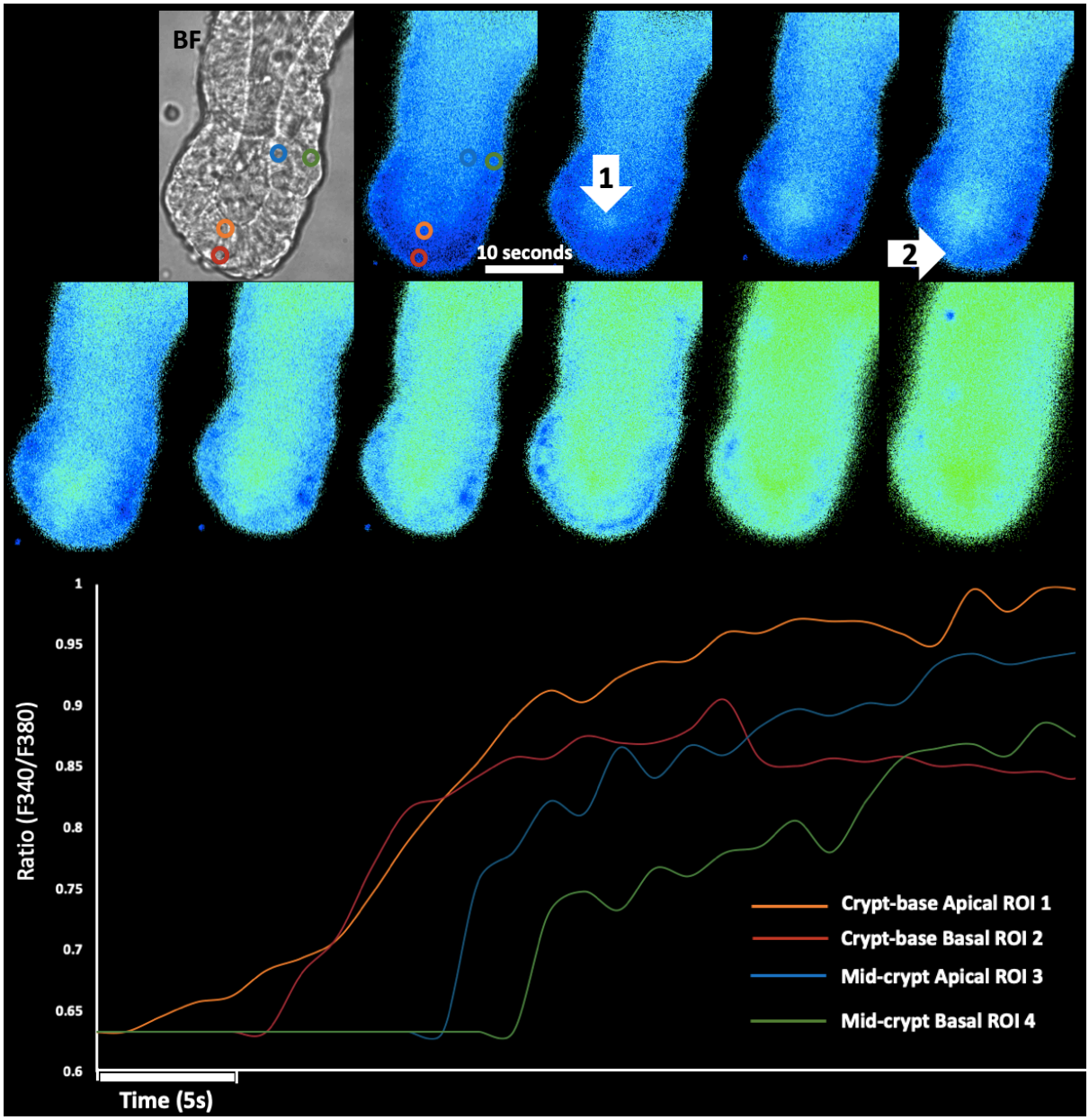


Figure 3.1.2- **Strategically positioned ROIs determined the initiation site and dynamics of the cholinergic calcium wave in organoid culture media**

ROIs are placed at the apical and basal pole opposite each other along the length of the crypt axis. From which we observe the initiation of the intracellular calcium at the apical pole in the base of the crypt. This is observed through the apical ROI at the crypt base **(orange)** displaying a calcium response initially. Followed by a calcium response in the basal ROI **(red)** opposite its corresponding apical ROI **(orange**. The intracellular calcium wave therefore begins at the apical pole and propagates up the crypt axis. Further up the crypt axis the same apical-basal pole dynamics are observed with a calcium response at the apical ROI **(blue)** before its corresponding basal ROI **(green)**.

3.2 Biological activity of non-neuronal acetylcholine secreted by colonic crypt cells

Tuft cells sense changes in luminal content, triggering synthesis of ACh via the ChAT enzyme within these cells. Newly synthesised ACh is secreted to the serosal side to the underlying lamina propria (Hayakawa, et al., 2017). We theorise this non-neuronal ACh can activate muscarinic receptors on the basal membrane of ISC, to regulate their activity. Investigation of this endogenous ACh synthesis used freshly isolated human colonic crypts cultured overnight with Rivastigmine, an ACh esterase inhibitor within the culture media. After 24 hours, conditioned culture media was removed and added to naïve crypts that had been loaded with Fura2 and kept in standard HBS for some time. Before the addition of this crypt 'conditioned' media, naïve media was first added as a vehicle control, no response. Addition of crypt 'conditioned' media to naïve crypts resulted in a rapid and sizeable intracellular response, inhibited by the muscarinic receptor antagonist atropine at 10uM, **(Figure 3.2.1 a-c)**. In parallel experiments the concentration of ACh within the crypt conditioned media was quantified using liquid chromatography tandem mass spectroscopy at ~90nM. Satisfyingly, addition of exogenous ACh at a concentration of 100 nM elicited a calcium response of a similar amplitude **(Figure 3.2.1 c)**.

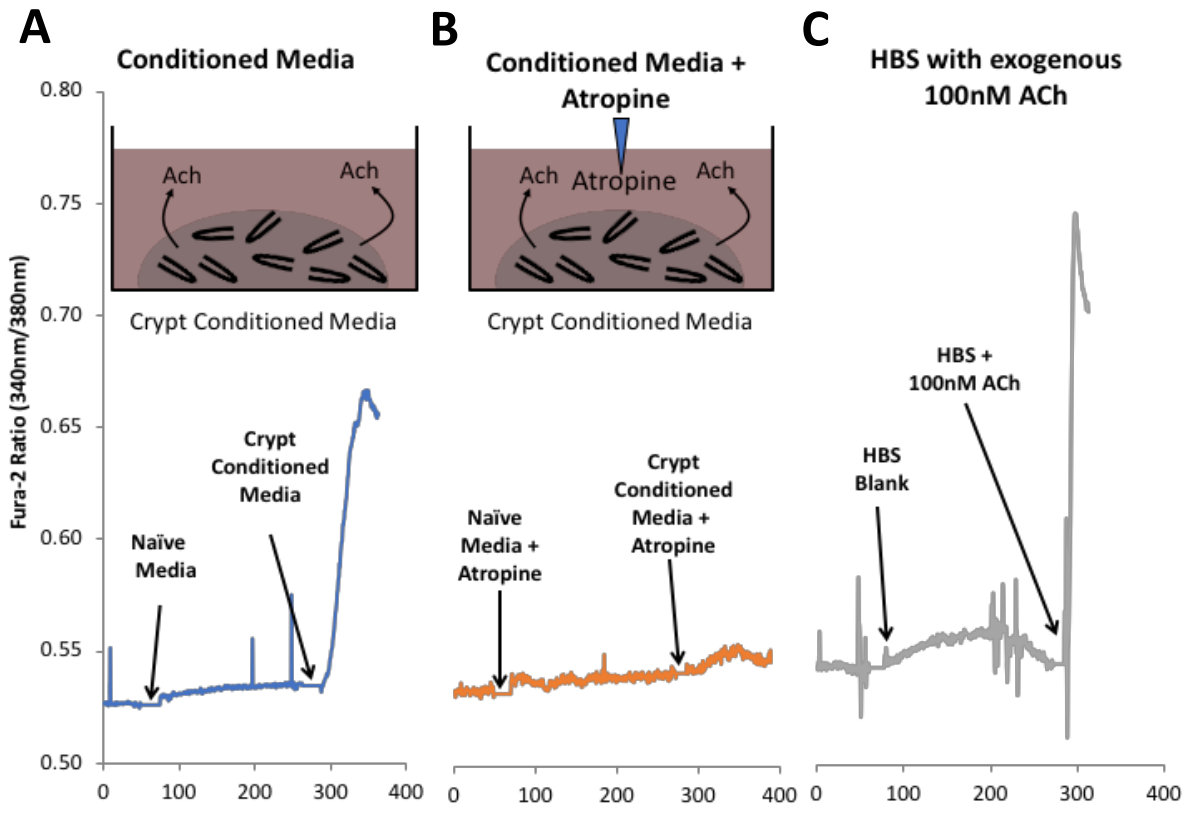


Figure 3.2.1 (a-c)- **(a)** There are no calcium responses in cultured crypts to naïve media, however there is a considerable calcium response to conditioned media containing ACh **(b)** To confirm that these calcium responses were cholinergic muscarinic receptor activation we used the muscarinic receptor inhibitor Atropine (10µM) **(c)** Mass spectrometry quantified the concentration of ACh within conditioned media at 90nM. Qualitatively we used 100nM ACh and observed a similar rate and amplitude of response to that of conditioned media **(a)** (With Alvin Lee).

3.3 Insights into the source of intracellular calcium and the influence of extracellular calcium

The spatiotemporal characteristics of calcium signals are highly consistent; apical initiation within the ISC niche, propagating to the basal pole as the wave extends along the crypt axis. However, we had yet to identify the lysosomes as the specific store in which calcium was originally mobilised from. Previous data strongly indicates acidic stores as the intracellular calcium initiation site **(Figure 3.1.1, 1.3.3 & 1.3.4)**. To confirm this theory, we used the lysosomotropic agent GPN, 100 µM. A substrate for lysosomal Cathepsin C, producing amino acids resulting in lysosomal lysis via osmosis. Addition of GPN caused an initial train of oscillations followed by a sustain increase in intracellular calcium. Subsequent addition of 10 µM Cch resulted in reduced calcium signals, suggesting lysosomes are the initiation site of cholinergic-calcium signals **(Figure 3.3.1)**.

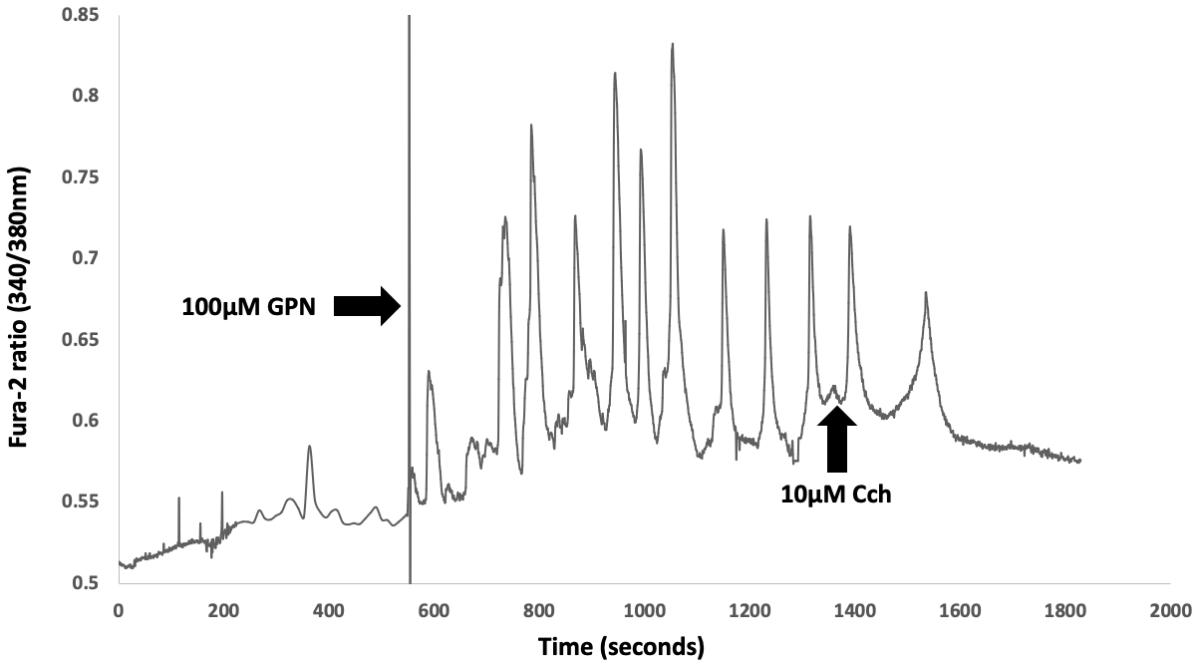


Figure 3.3.1- Determining the intracellular calcium initiation store downstream of cholinergic activation.
Intracellular calcium is mobilised upon addition of the lysosomotropic agent GPN, 100 µM to cultured crypts, oscillatory waves are observed. Subsequent 10 µM Cch stimulation results in a very small calcium response. Indicating muscarinic receptor activation coupled to endolysosomal calcium stores.

Calcium signalling is a fast-acting local response, requiring a large concentration gradient between the cytoplasm and the extracellular matrix. A low cytosolic concentration is maintained through PMCAs actively removing any calcium leakage across the cell membrane. Additionally, intracellular calcium stores, for example endolysosomes and ER, sequestering excess cytosolic calcium. Upon stimulation, VOCCs open and there is a fast response due to the high concentration gradient, highlighting why calcium is key for fast muscle contractions, e.g. in the heart. In organoid culture media and HBS solution there is 1mM calcium to replicate that of *in vivo* extracellular calcium. After stimulation, resting cytosolic calcium is restored whilst depleted intracellular calcium storage organelles are refilled (Manjarres, et al., 2011). Calcium refilling is mediated by a number of different calcium sensitive channels on the plasma membrane, for example TRPM sub-types and ORAI1 (Manjarres, et al., 2011). In regard to the oscillations observed, calcium was mobilised from the apical pole of cells within the ISC niche, similar to that of the initial Cch-induced calcium response **(figure 3.3.4 a)**. Also, oscillations occurred at uniform time points, for example in **(Figure 3.3.4 b)** from peak to peak, oscillations were 14 minutes apart. For some responses at 2mM calcium the Fura-2 ratio never returned to baseline and remained elevated, signifying that increased extracellular calcium could overwhelm the calcium transporters (e.g. PMCA1). As a result, elevated intracellular calcium levels impart negative feedback on the oscillatory machinery of the cell.

From these data, we aimed to investigate the effects of decreasing and increasing extracellular calcium concentrations on cholinergic induced calcium signals **(Figure 3.3.2 & 3.3.3)**. Oscillations were observed from altering the extracellular calcium **(Figure 3.3.5 a)** as well as a number of other differing responses **(Table 3.3.1)**. Different culture media were used: organoid culture media (i.e. nutrient replete, 5% CO_2/ 37 degree Celsius) and physiological HBS conditions **(Figure 3.3.5 b)**.

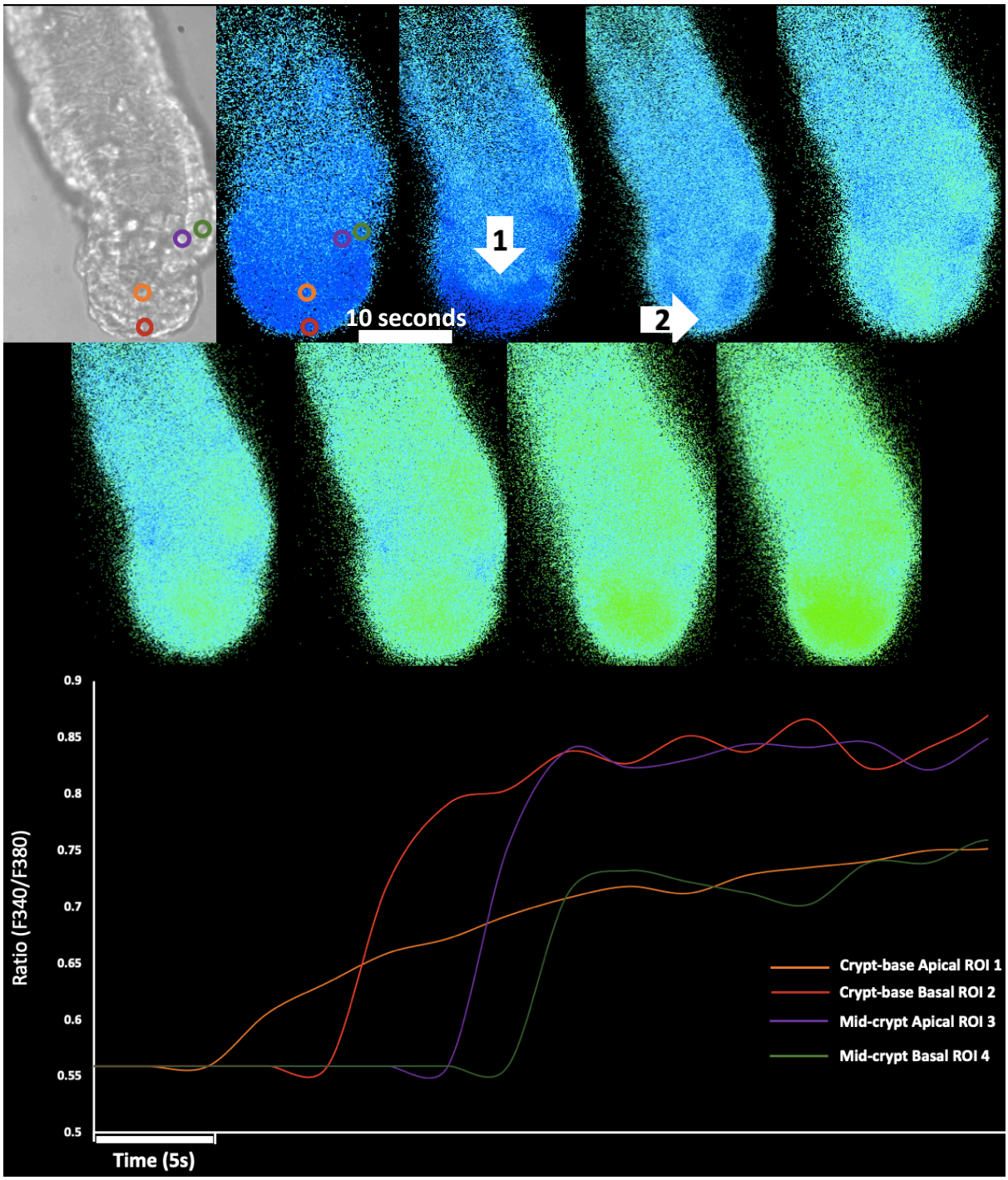


Figure 3.3.2- Strategically positioned ROIs determined the initiation site and dynamics of the cholinergic calcium wave in high calcium (2mM) organoid culture media

Intracellular calcium signals originate at the base of colonic crypts, in the apical pole **(orange)** and propagate to the basal pole **(red)** as is the case in standard organoid culture media calcium signals propagate up the crypt axis and for each cell calcium initiates in the apical pole **(purple)** to the corresponding basal pole **(green)**.

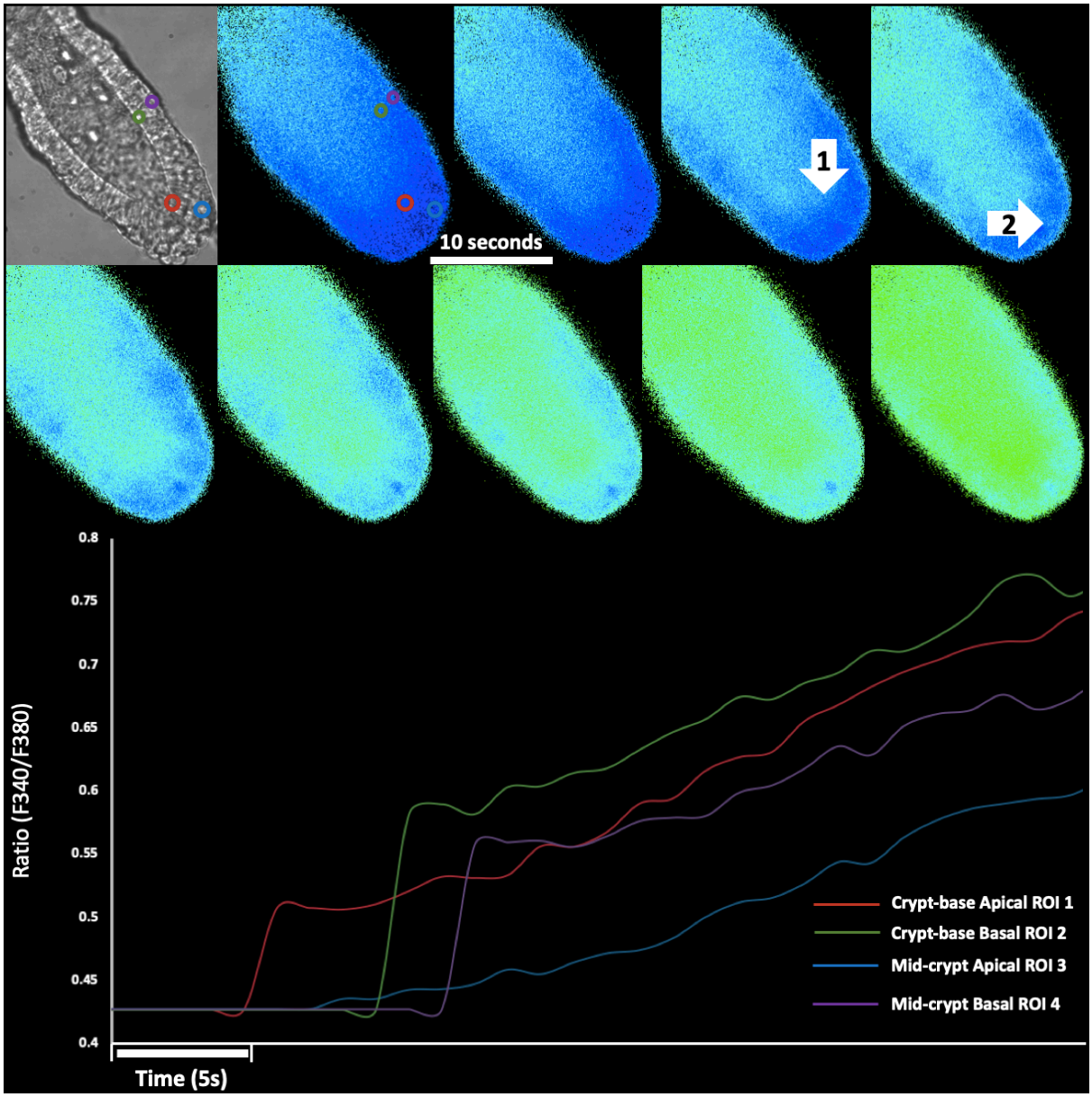


Figure 3.3.3- Strategically positioned ROIs determined the initiation site and dynamics of the cholinergic calcium wave in reduced calcium (0.5mM) Hepes Buffered Saline (HBS) solution

Strategically placed ROIs determined that under reduced extracellular calcium and in non-culture media, cholinergic calcium signals still originate in the apical pole of cells at the crypt base **(red)**. The intra- and intercellular dynamics remain the same with an apical-basal propagation as the wave extends up the crypt axis. This is observed through paired ROIs along the crypt axis with apical ROIs at the base highlighting the initiation of calcium signals **(red)** spreading to its neighbouring basal ROI **(blue)** and later a reciprocal pattern is observed higher up the crypt axis **(green and purple)**.

Calcium oscillations have been formerly described within the *Drosophila* mid-gut (Deng, et al., 2015). In the *Drosophila* midgut calcium is suggested to be a key central regulator of ISC activity (Deng, et al., 2015). Furthermore, Deng, *et al.,* 2015 discovered the frequency of calcium oscillations and the average cytosolic calcium concentration resulted in differing outcomes of ISC activity. Increased cytoplasmic calcium concentration but decreased oscillation frequency, ISC proliferation is favoured. However, decrease in cytoplasmic calcium concentration and increased oscillation frequency is found within quiescent stem cells (Deng, et al., 2015). Calcium oscillations were not reproducible in any circumstances, in addition to extracellular calcium levels, the concentration of stimulation was altered by using lower and higher concentrations of Cch, yielding similar results. Oscillatory waves occur at a higher concentration of Cch, 100 µM but are absent at a lower 1 µM Cch concentration **(Figure 3.3.6)**.

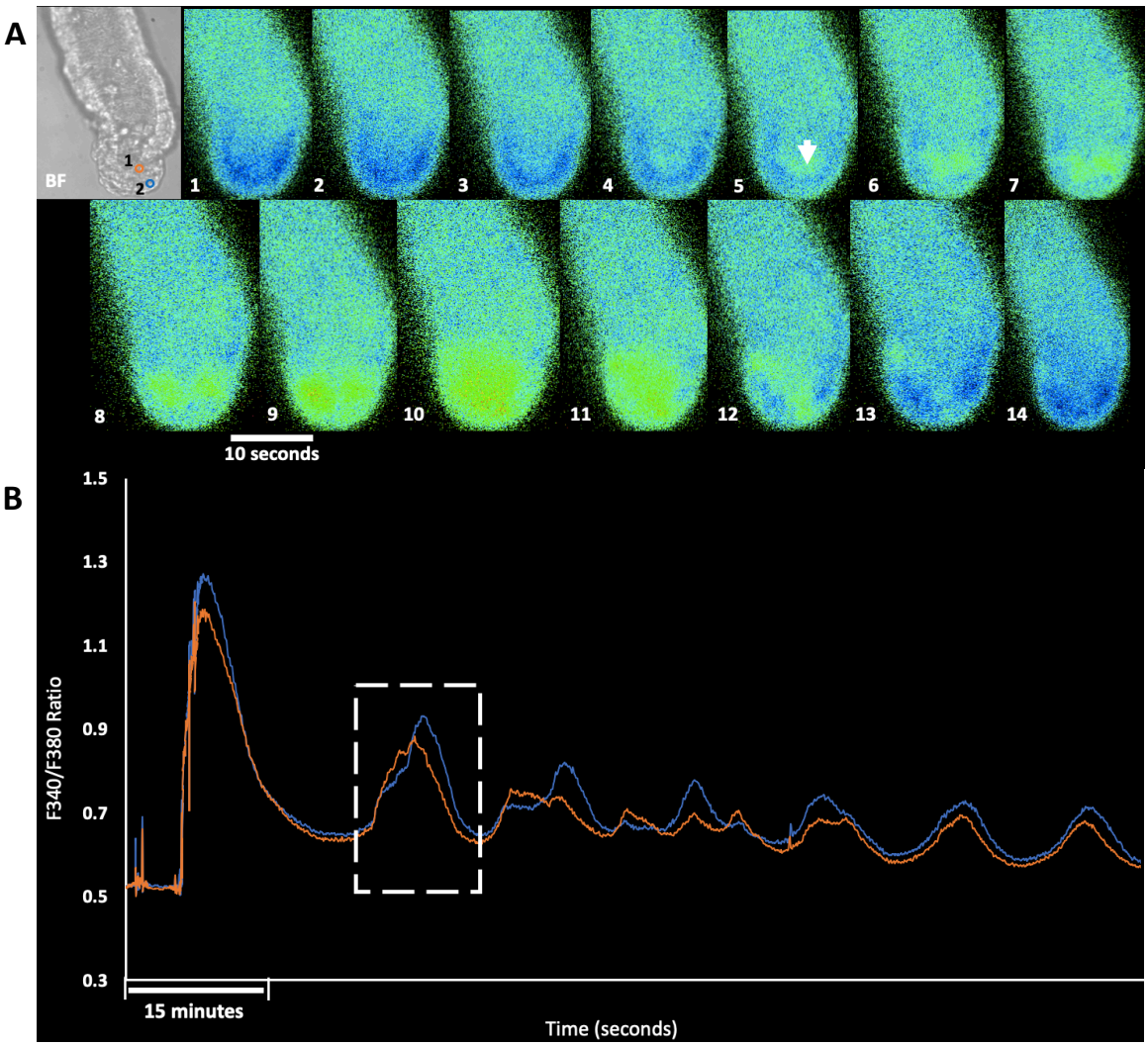


Figure 3.3.4- Cholinergic calcium oscillations occur in *ex vivo* human colonic crypts cultured in organoid culture media, with high extracellular calcium concentration. (A) Brightfield image of an *ex vivo* cultured crypt with ROIs strategically placed at the Apical and basal pole of regions of activity and interest using the EasyRatio Pro software **(ROIs labelled 1 & 2)**. Furs-2 labelled crypts allow us to observe the rise and fall of a calcium oscillatory wave as shown by images **1-14**, initiation of this wave at the apical pole within the ISC niche. **(B)** Crypts are incubated in Fura-2 for 2 hours, washed with HBS three times and then incubated for 30 minutes in organoid culture media with high calcium (2mM) before imaging. A single addition of 10 µM Cch is followed by a rapid and large intracellular calcium response, after which oscillations occur at an equal frequency of 14 minutes.

Table 3.3.1- Different culture mediums used for analysing calcium signals in Fura-2 labelled human colonic crypts. Crypts in each culture medium were stimulated by the muscarinic agonist Cch at a working concentration of 10uM. Number of crypts in which oscillations or two calcium waves (bi-phasic) occurred and the number of times after the calcium response did the Fura-2 ratio return to resting potential.

	Oscillations	Bi-phasic	Returned to Baseline (No oscillations)	Elevated Baseline (No oscillations)
HBS			2	1
HBS (1/2 CaCl)			3	
HBS (X2 CaCl)	1		2	1
Full Media	2		2	6
Full Media (X2 CaCl)	3		3	14
*Full Media (100uM CCh)	1	1		
*Full Media (1uM CCh)				1

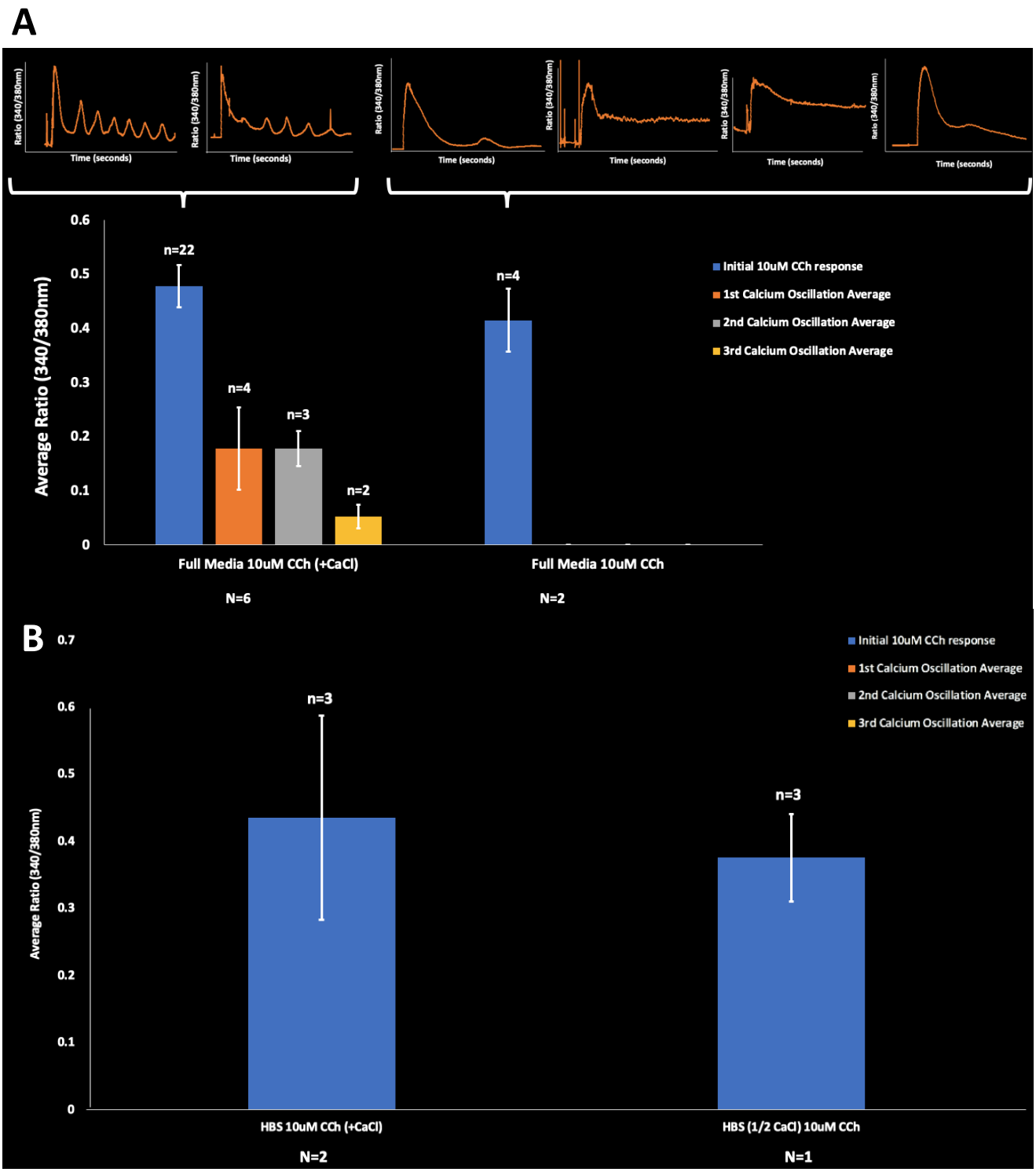


Figure 3.3.5- Identifying the conditions permissive for cholinergic induced intracellular calcium oscillations. N= number of patients, n= number of crypts imaged. **(A)** Under full organoid culture media and high calcium (2mM) calcium oscillations are observed. However, oscillations were absent in full organoid culture media with *in vivo* concentration of calcium (1mM). **(B)** Calcium oscillations are entirely absent from crypts image in HBS buffer solution, regardless of calcium concentrations. Potentially indicating extracellular calcium and components within full organoid culture media are permissive for intracellular calcium oscillations.

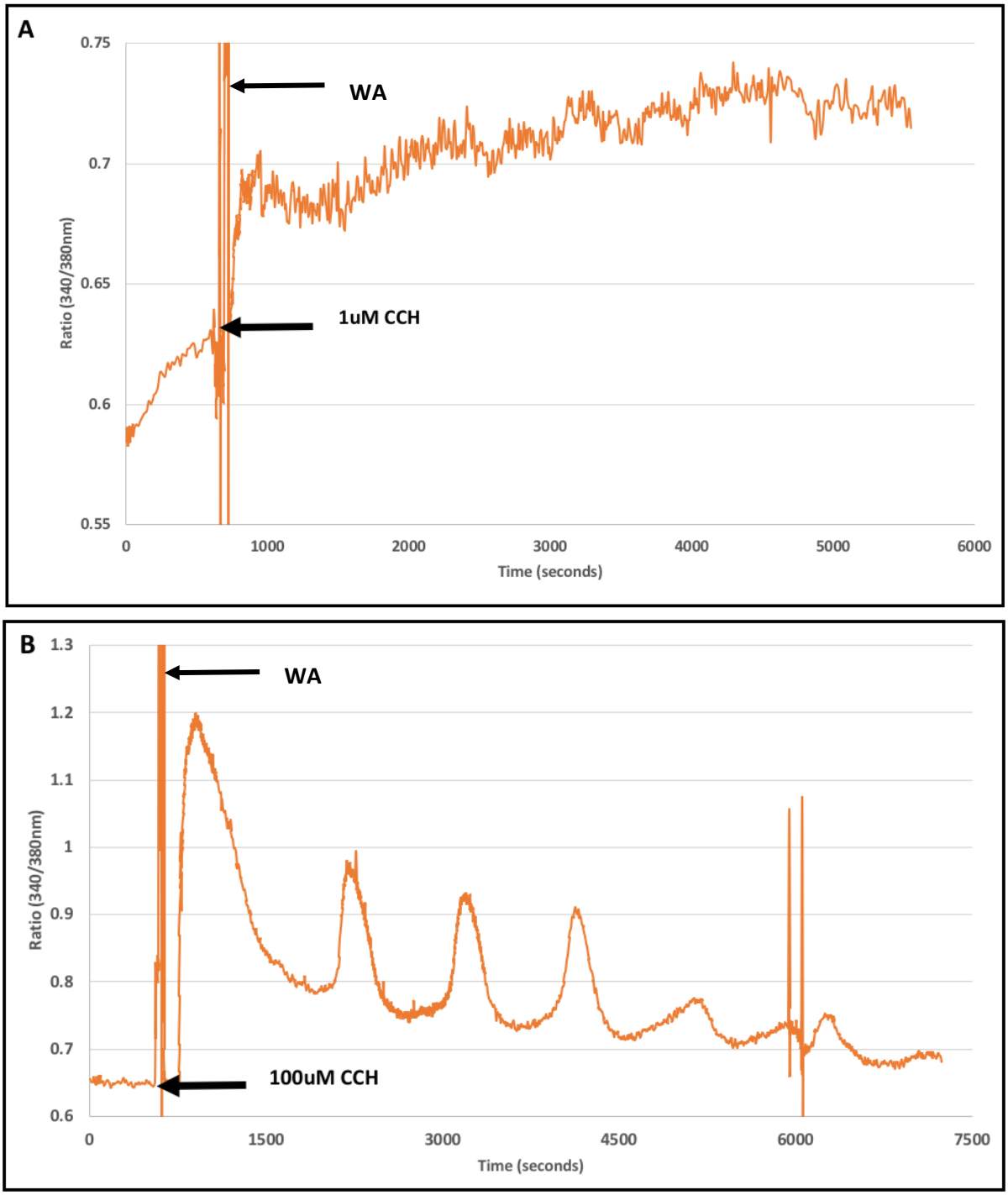


Figure 3.3.6 a-b- Concentration of Cch permissive for cholinergic-induced intracellular calcium oscillations
(A) Fura-2 Ca^{2+} imaging trace of a calcium response to 1uM Cch. **(B)** Fura-2 Ca^{2+} imaging trace to the addition of 100uM Cch, single addition yields oscillations. **WA**= wash artefact, this is disturbance caused by removing bathing solution and replacing with the initial muscarinic receptor agonist solution.

Additionally, by decreasing or increasing the concentration of Cch to 1 µM (N=1, n=1) or 100 µM (N=1, n=2) respectively characteristics of calcium signals change **(Figure 3.3.6 a-b)**. At 1 µM Cch no oscillations observed **(Figure 3.3.6 a)**. Administration of 100 µM, induced calcium oscillations, the rate of which were faster than those observed at 10 µM Cch and the total number of oscillations were reduced **(Figure 3.3.6 b)**. Potentially representing over stimulation of the cholinergic calcium signalling pathway by an excessively high concentration of agonist.

So far, these data have been collected from a single administration of Cch, therefore these crypts have been stimulated under static conditions. We theorised that the heterogeneity of calcium responses **(Table 3.3.1)** could be due to the static environment. This theory was challenged by removing the Cch solution from colonic crypts after the Fura-2 ratio returned to baseline after stimulation and replaced with fresh Cch solution. **(Figure 3.3.7)**. Calcium responses were observed after each re-administration of 10 µM Cch **(Figure 3.3.7)**. Reason being, under static conditions there is a possible accumulation of negative factors that prevent Cch binding to muscarinic receptors and therefore further calcium responses are prohibited, potentially via a negative feedback loop.

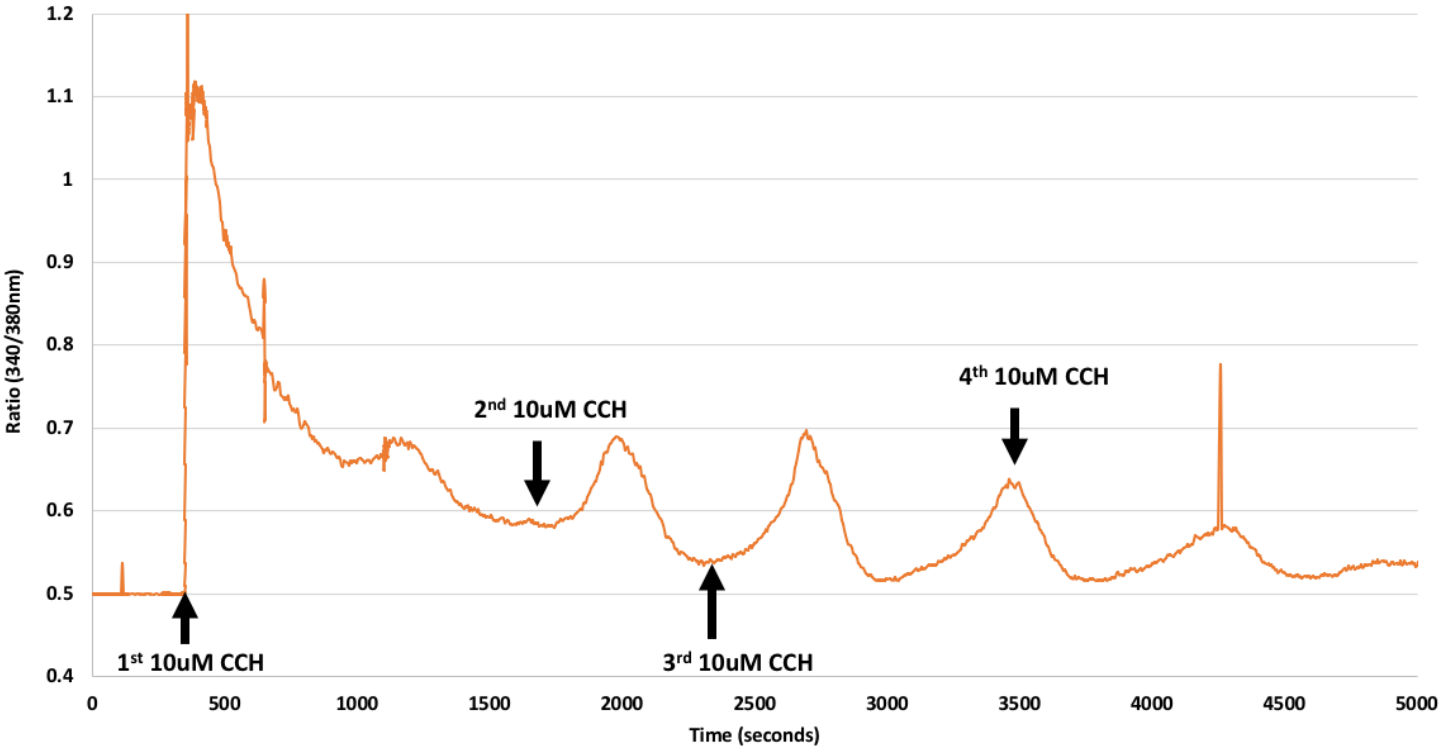


Figure 3.3.7- Dynamic cholinergic calcium stimulation with Cch results in repeated intracellular calcium signals Once a calcium response has returned to baseline and plateaued the old 10 µM Cch solution is replaced with fresh 10 µM Cch solution. Each re-addition of Cch induces a calcium response although at a reduced amplitude compared to the initial response, eventually the re-addition of Cch induces no further calcium response. Potentially, each re-addition removes negative factors that may accumulate within a static environment.

3.4 The role of the mTOR pathway on intracellular calcium signalling

As mTOR has been identified as a vital regulator of TPC-mediated calcium release, the effect of inhibiting mTOR activity was explored using well known mTOR inhibitors, Rapamycin and Torin 2 (Ogunbayo, et al., 2018). Spatiotemporal characteristics of calcium signals in the presence of these inhibitors showed calcium mobilisation from the apical pole, where endolysosomes seem to be concentrated, **(Figure 3.4.1)**. Previous research from Ogunbayo, *et al.,* 2018 determined calcium transients are initiated in pulmonary arterial myocytes from 100 nM Rapamycin. At higher concentrations, such as 30 µM, calcium oscillations result.

Following on from these data we aimed to use Rapamycin and Torin 2 to inhibit mTOR to facilitate TPC-mediated calcium release. Although there was an increase in the calcium response in the presence of either 3 µM or 30 µM Rapamycin and 100 µM Torin2 **(Figure 3.4.2 a-b)**, these differences were not significant. Additionally, with more time the chronic calcium signals from Rapamycin stimulation would have been investigated.

So far, investigations have demonstrated the spatiotemporal characteristics of cholinergic signalling and strongly infer cholinergic calcium mobilisation is via TPCs on the endolysosomal membrane. Furthermore, altering extracellular calcium conditions and Cch concentration resulted in no reproducible chronic calcium signals. Furthermore, mTOR inhibitors Rapamycin and Torin2 had minimal effect upon intracellular calcium mobilisation.

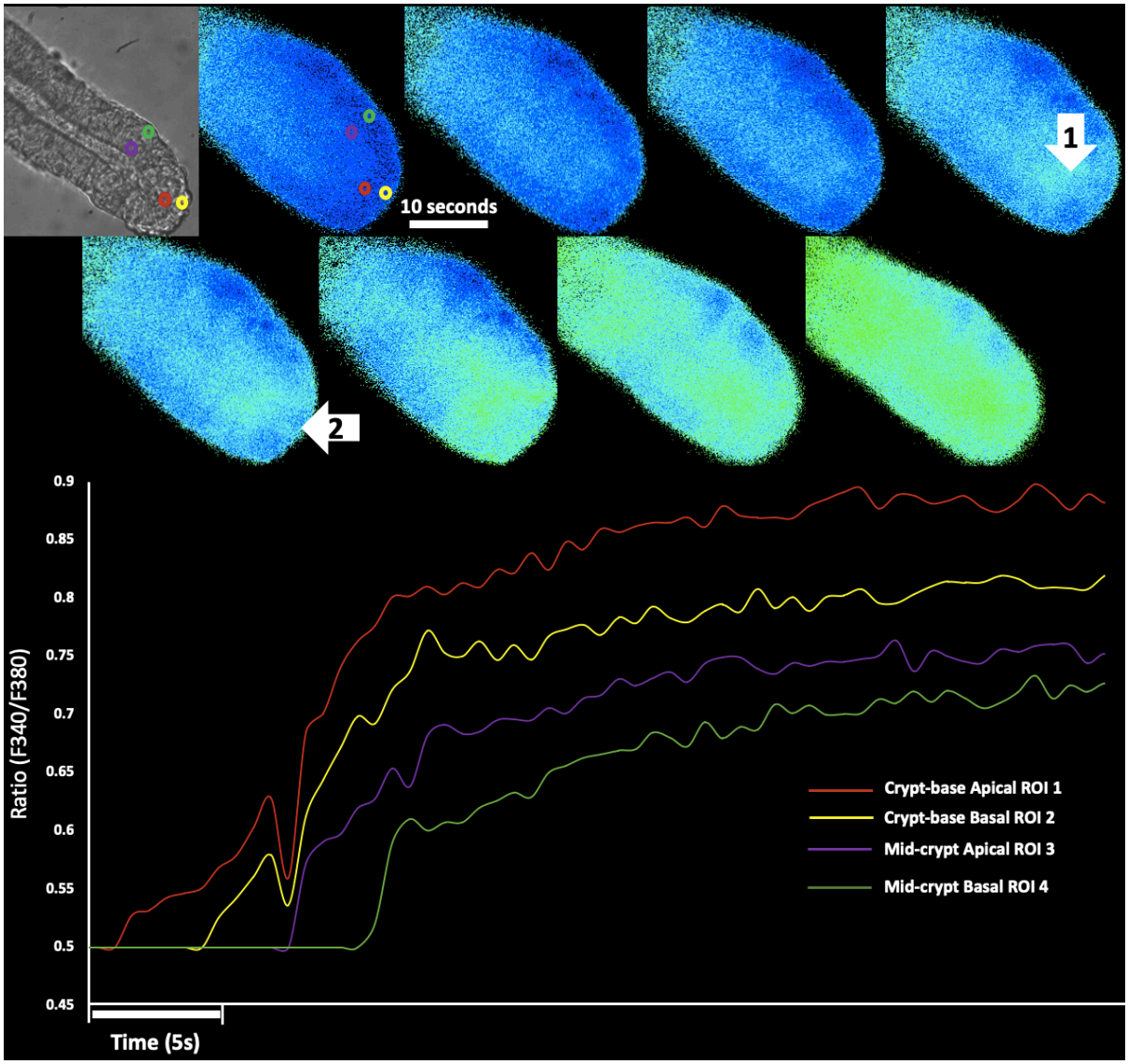


Figure 3.4.1- The effect of mTOR inhibitor Rapamycin on the size and dynamics of human crypt cholinergic calcium signalling.

Crypts were incubated with Fura-2, washed with HBS and then incubated in organoid culture media containing 30 µM Rapamycin. Following pre-treatment with 30 µM Rapamycin for 30 minutes, crypts were stimulated with 10 µM Cch. From which we observed a typical intracellular calcium wave response using strategically positioned ROIs. The calcium signal starts at the crypt-base apical pole **(red)** and propagates to the crypt-base basal pole **(yellow)** and then migrates up the crypt axis following the same apical-basal propagation **(purple to green)**. This apical-basal wave from crypt-base to crypt-opening is confirmed through plotting changes in Fura-2 ratio from which we analysed the calcium signalling wave dynamics.

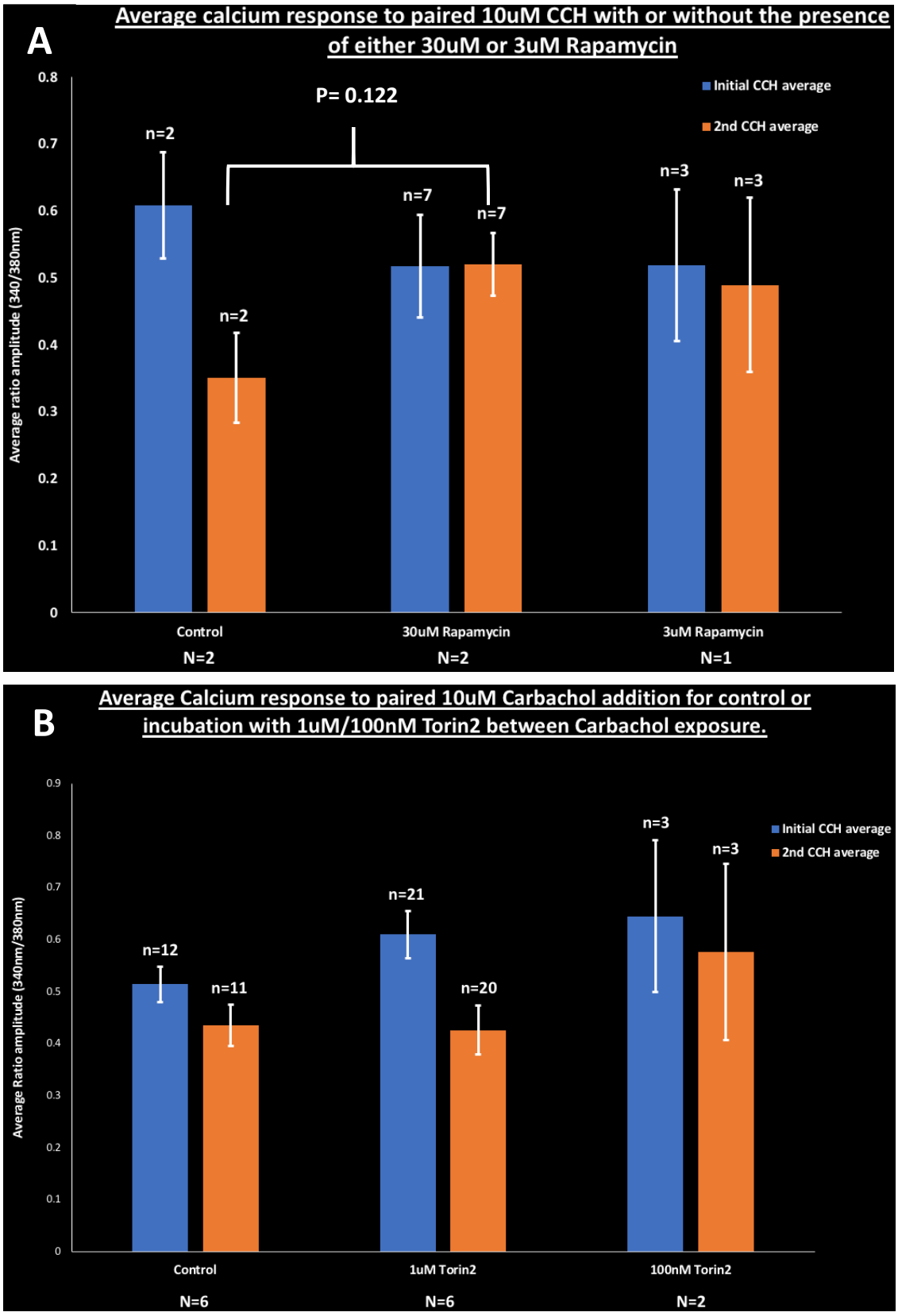


Figure 3.4.2- The effect of mTOR inhibitors Rapamycin and Torin 2 on the size and dynamics of human crypt cholinergic calcium signalling.

(A) In standard physiological conditions the mTOR inhibitor Rapamycin at 3 µm or 30 µm has no significant effect on cholinergic-induced intracellular calcium signalling. **(B)** Similarly, under standard physiological conditions Torin 2 at high or low concentrations had no significant impact on cholinergic-induced calcium signals.

3.5 Organoid growth rate enhanced through muscarinic receptor activation and potentially downstream intracellular calcium signals

Given that calcium signals have been demonstrated to regulate ISC biology in *drosophila*, the effects of muscarinic receptor activation on ISC activity in human organoids was investigated **(Figure 3.5.1)**. Muscarinic receptor agonist Oxotremorine, 1 μM, stimulated muscarinic receptor activation on organoid membrane surface. To analyse the effects of this agonist on organoid growth, the cross-sectional area (CSA) of a sample of twenty organoids for each culture medium was measured. Also counted the number of organoid buds formed for each individual organoid, as an indicator of ISC activity. Increase in budding number suggests increased mAChR activation enhances ISC proliferation. Firstly, culture crypts derived from a biopsy taken from the normal mucosa of a CRC patient where fragmented and plated. These small crypt fragments were plated into culture media with or without the presence of 1 μM Oxotremorine and were analysed over the period of around a week. The CSA raw data was averaged, and the results plotted, the data shows that organoids cultured in Oxotremorine have a greater increase and final organoid CSA compared to control, **(Figure 3.5.1 a)**.

From **(Figure 3.5.1 a)**, it can be seen on day-nine the greatest difference in CSA, therefore, further analysed the difference in CSA of organoids culture with or without Oxotremorine, **(Figure 3.5.1 b-c)**. For each culture condition, a sample of twenty-organoids were measured, in **(Figure 3.5.1 c)**, organoids sorted evenly into the smallest (ten) and largest (ten) based on their initial CSA size. For both small and large organoids, Oxotremorine cultured organoids had a larger average CSA, however, the difference was more profound between the initial smaller organoids, **(Figure 3.5.1 c)**. To measure whether mAChR activation increased ISC activity, the number of buds per organoid were measured, **(Figure 3.5.1 d)**. These data show a significant difference of Oxotremorine on ISC proliferation compared to control **(Figure 3.5.1 d)**.

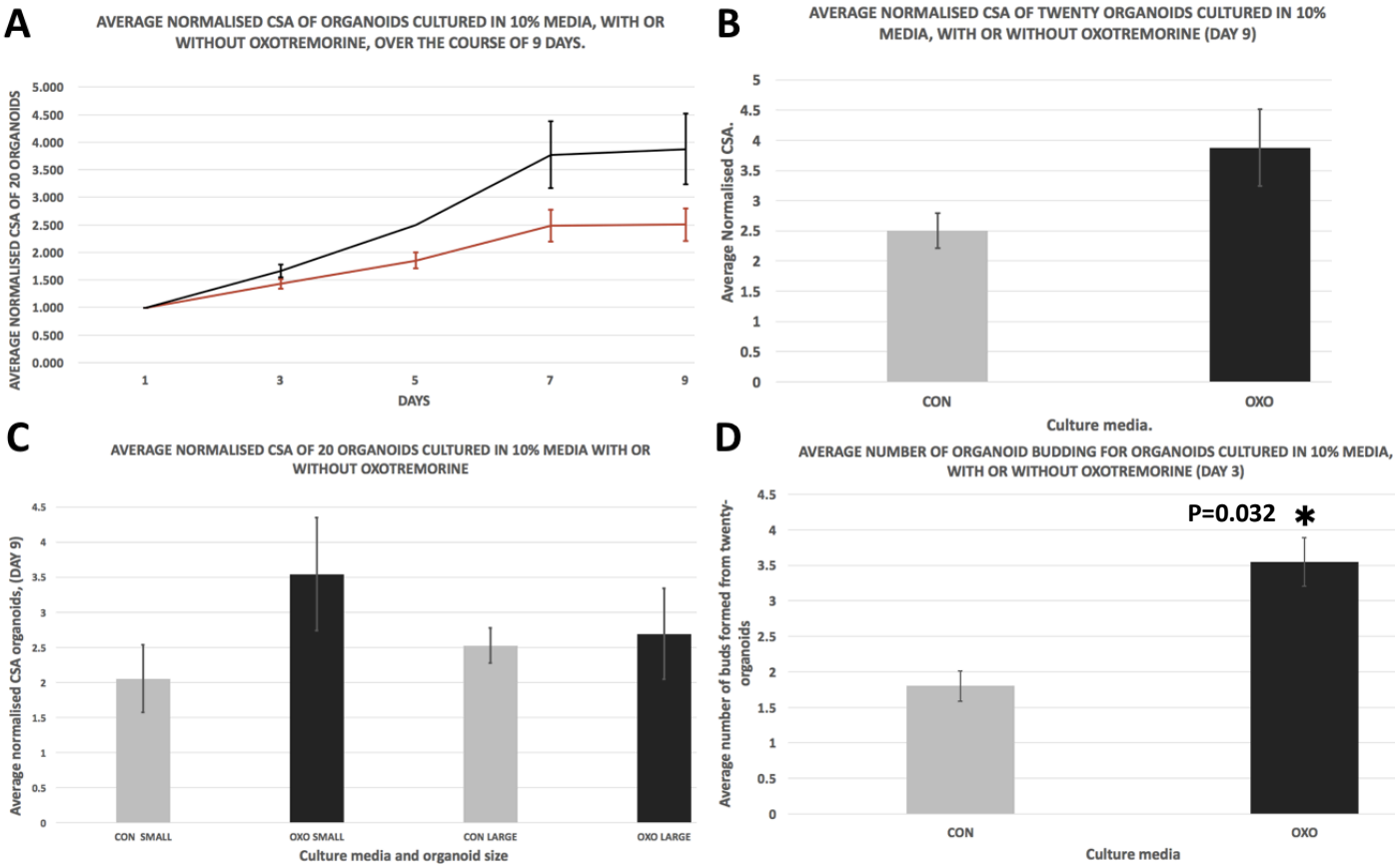


Figure 3.5.1- **(A)** Average CSA of organoids cultured in 10% media with or without 1µM Oxotremorine over the time course of nine-days. The CSA of Oxotremorine cultured organoids is greater than control. **(B)** The average CSA of organoids at the final day of culturing (day-nine) found that Oxotremorine cultured organoids had a larger final average CSA than control. **(C)** The twenty organoid samples were sorted based on their initial size (day-one) and compared, from which there was a more profound difference between organoids that were smaller in initial size. **(D)** Average number of buds formed from twenty organoids cultured in full media, with or without Oxotremorine, budding number used as an indicator of ISC activity. Significant difference observed when, p=0.05.

3.6 Cholinergic calcium signalling status in colorectal cancer

In the Williams laboratory a biobank of several patient matched organoid and tumouroid samples have been created and stored. In addition to understanding the mode and effects of cholinergic calcium signals in health it is important to comprehend changes or remodelling of calcium within tumourigenesis. We aimed to unravel differences between health and disease using the 3D experimental models, organoids and tumouroids.

Firstly, to gain insight into the relative sizes of endolysosomal and ER calcium stores in health and disease the cholinergic and purinergic calcium pathways were stimulated with 10 µM Cch and 50 µM UTP respectively **(Figure 3.6.1)**. From which we observed a significant difference in cholinergic calcium signalling but not with purinergic calcium signalling **(Figure 3.6.1)**.

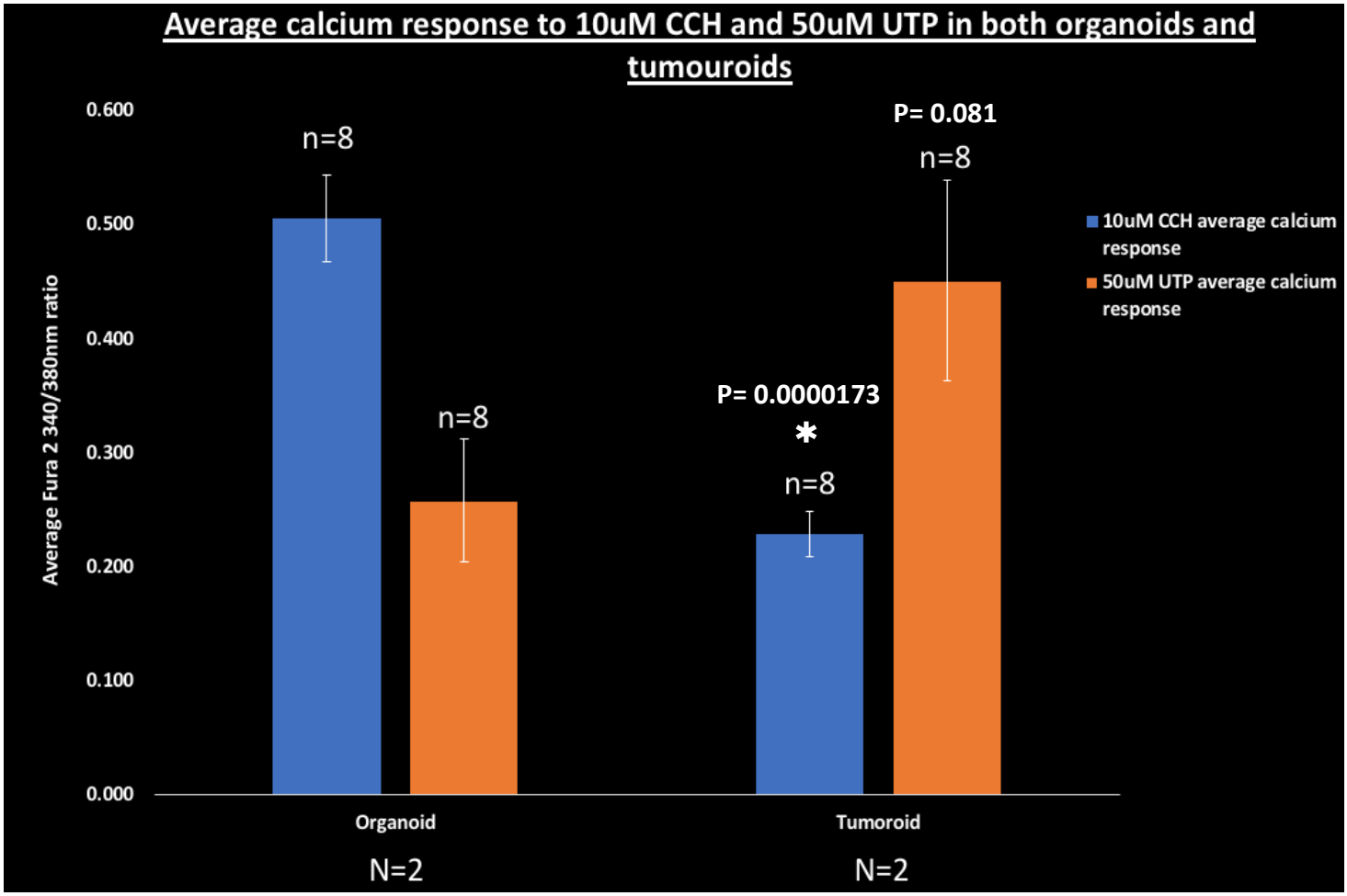
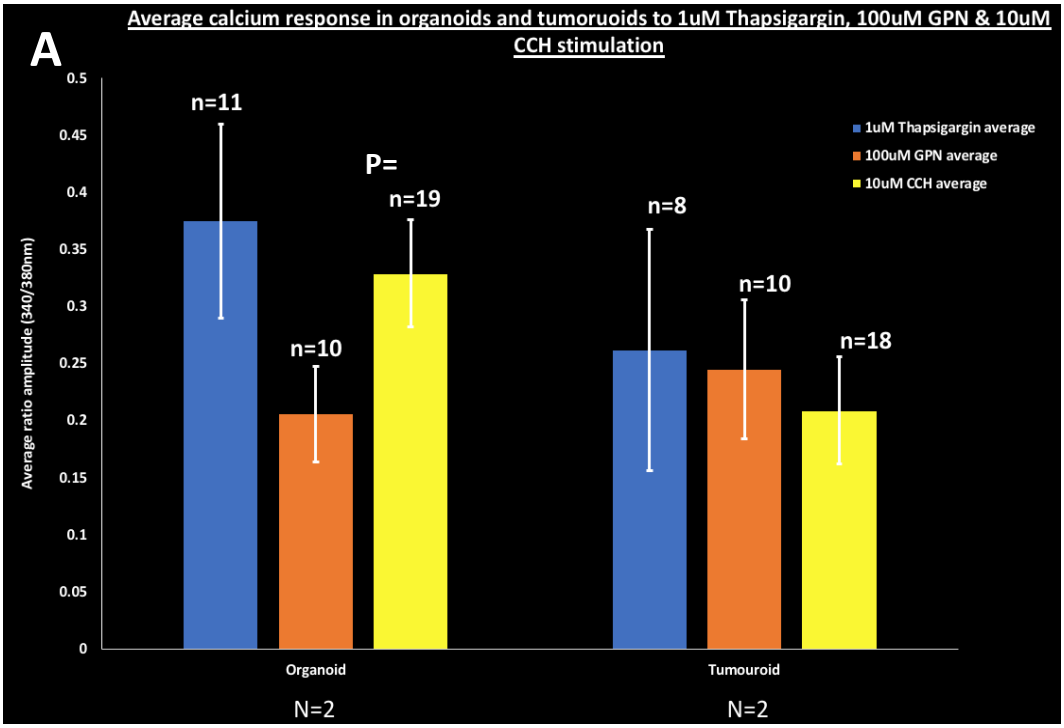


Figure 3.6.1- Insights into the potential size of intracellular calcium stores in health vs disease
10 µM Cch stimulates cholinergic-induced calcium release from the apically concentrated endolysosomal calcium stores, there is a significant difference in these calcium responses between health and disease. The ER calcium store is targeted using the purinergic agonist UTP, 50 µM. There is no significant difference between Purinergic-induced calcium release from the ER between health and disease.

Follow up experiments using Thapsigargin and GPN further investigated the differences between these intracellular calcium stores in health and disease **(Figure 3.6.2)**. Thapsigargin is a Ca^{2+}-ATPase inhibitor, and is therefore an effective SERCA pump inhibitor, resulting in ER calcium leak. This calcium leakage can be observed via Fura-2 and provide data on the relative size of the ER in health and disease. The lysosomotropic agent GPN lyses endolysosomes, releasing their calcium contents again providing data on potential changes in tumourigenesis **(Figure 3.6.2)**.

Organoid and tumouroids are patient matched, derived from the same patient, for these data two patient samples have been used 24th October 2017 & 13th November 2017, the data for which has been collated in **(Figure 3.6.2 a)**. Experiments were carried out in HBS conditions with typical extracellular calcium levels, 1 mM.

A more accurate measure of the respective calcium stores was the removal of extracellular calcium followed by the brief (5 minutes) addition of the calcium chelator, ethylene glycol-bis (β-aminoethyl ether)-*N*, *N*, *N*, *N*-tetra-acetic acid), (EGTA). This aimed to give a more well-defined view of the lysosomal and ER calcium stores in organoids and tumouroids and found no significant difference in the size of the calcium store, further research is required **(Figure 3.6.2 b)**. Previous literature has found a decrease in calcium of the ER in certain cancer, e.g. bladder cancer cells, this also attenuates apoptosis through prevention of mitochondrial calcium overload, this cross-talk is mediated via mitochondria-associated ER membranes (MAMs) (Kerkhofs, et al., 2018).



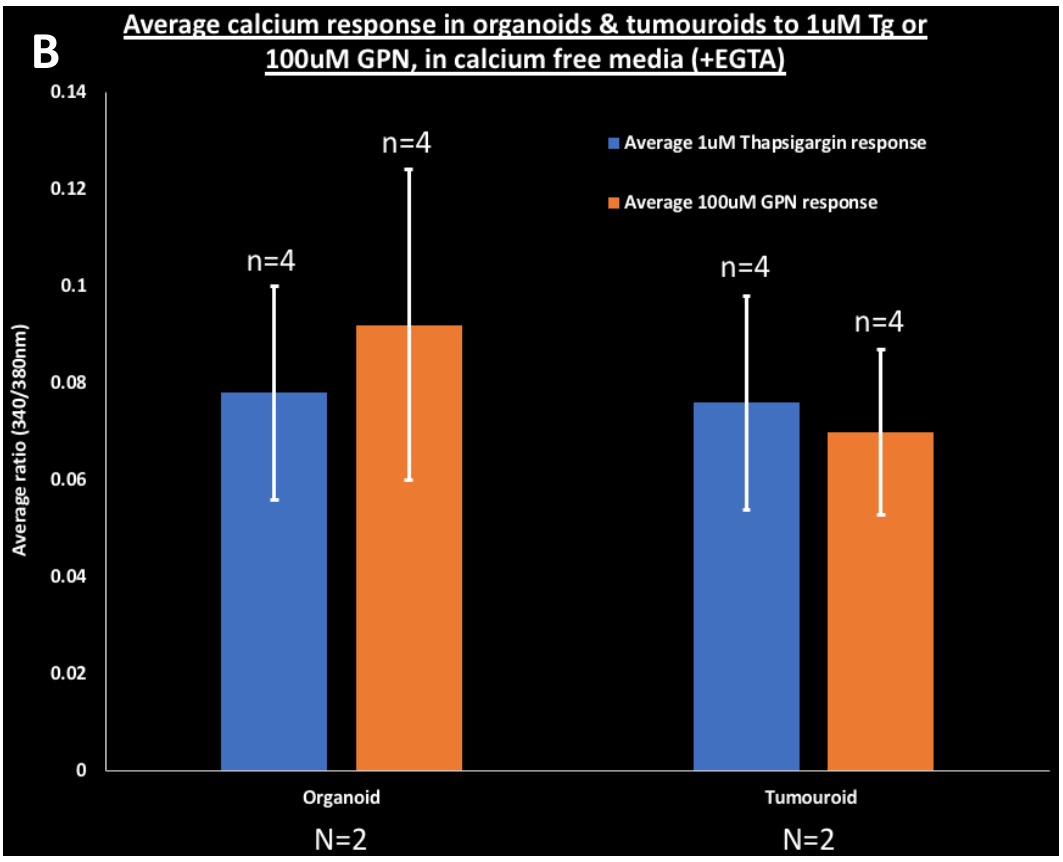


Figure 3.6.2- Potential impact of extracellular factors on the size of calcium response in endolysosomal and ER intracellular calcium stores in health vs disease
(A) Intracellular calcium store size in organoids and tumouroids measured through changes in ratio amplitude using the SERCA pump inhibitor Thapsigargin (1uM) or the lysosomotropic agent GPN (100uM). **(B)** Ratio amplitude change from using the SERCA pump inhibitor Thapsigargin (1uM) or the lysosomotropic agent GPN (100uM), in calcium free media, +EGTA.

4 Discussion

Following previous data within the Williams laboratory and previous literature, it was hypothesised the spatiotemporal characteristics of cholinergic induced calcium signals are remodelled during colon carcinogenesis. The following objectives were designed to test this hypothesis;

1. Characterise the cholinergic calcium signalling pathway in human colonic crypts.

2. Characterise the capacity of intracellular calcium stores, ER and endolysosomes, in health and disease through the use of experimental models, organoids and tumouroids.

4.1 Spatio-temporal characteristics under basic physiological and organoid culture conditions

Initial experiments followed on from recent data within the Williams laboratory indicating the differential location of two main intracellular calcium stores, endolysosomes and ER. Immunohistochemistry analysis showed endolysosomes concentrated at the apical pole and the ER at the basal pole of human colonic crypt epithelial cells **(Figure 1.3.3)**. Additionally, immunohistochemistry analysis of receptors of these intracellular calcium stores complemented the apical-basal expression of the two stores **(Figure 1.3.1)**. PCR analysis, of the TPC and IP_3R Ryanodine receptor subtypes, confirmed conserved expression of all receptor subtypes within *ex vivo* organoid culture model **(Figure 1.3.4)**. These data were the foundation for studying the role and spatiotemporal characteristics of these two organelles and their receptors during intracellular calcium signalling.

In vivo, cholinergic signals involve the multifaceted ACh binding to G-protein coupled muscarinic receptors expressed upon the basolateral surface of epithelial cells. Activation of these receptors invokes an intracellular calcium response. *Ex vivo* experiments recapitulated these signals by using the ACh mimetic Carbachol to avoid the hydrolysis of ACh by endogenous cholinesterases. Initial experiments used organoid culture media to recapitulate the abundance of growth factors and ligands found within an *in vivo* environment. These intracellular calcium dynamics were visualised using the calcium sensitive fluorescent dye, Fura-2, and the propagation of a calcium wave was observed using strategically placed ROIs at hotspot regions **(Figure 3.1.1 & 3.1.2)**. It is known that as a function of time, calcium signals can occur as either oscillations or global transients (Berridge, 2008). Plus, as a function of space, calcium signals can occur within localised domains, such as the ER or lysosomes (Berridge, et al., 2003).

Isolated human colonic crypts were incubated for two hours with Fura-2 and subsequently subjected to a single addition of 10 µM Cch, **(Figure 3.1.1 & 3.1.2)**. Addition of 10 µM Cch induces a calcium response akin to ACh induced intracellular calcium signals previously observed in rat and human crypts (Lindqvist, et al., 1998) (Lindqvist, et al., 2002). From these data it is clear that calcium signals initiate at the apical pole of initiator cells within the ISC niche. However, using an epifluorescence microscope it is impossible to know which cell type these calcium signals originate from. Unpublished data from Fluo-4 calcium confocal imaging

on live human crypts and immunohistochemical labelling indicates that these initiator cells are $Lgr5^+$ stem cells (Nico Pelaez-Llaneza, Williams laboratory). Coupling functional data to previous immunohistochemistry data, strongly suggests endolysosomes are the source of apically localised calcium and the endolysosomal membrane bound TPC 1-2 are involved in calcium mobilisation from these acidic stores. Furthermore, we theorise the progression of an intracellular calcium wave from one cell to its neighbour up the crypt axis via gap junctions or paracrine signalling.

4.2 Biological activity of non-neuronal acetylcholine secreted by colonic crypt cells

Fundamental to understanding cholinergic calcium signalling is knowing the source and type of ligand responsible for intracellular calcium signals. It is well known myenteric neurons within the lamina propria are $ChAT^+$ and a major source of ACh for basolateral membrane bound muscarinic receptors. Additionally, myenteric neurons were thought of as the only source of ACh for the colonic epithelium, however recent research in mouse and rat colonic crypts identified a non-neuronal source of ACh (Hayakawa, et al., 2017) (Yajima, et al., 2011). Therefore, in conjunction with work carried out by Alvin Lee (Williams laboratory), non-neuronal sources of ACh were investigated within *ex vivo* human colonic crypts. Using conditioned media from cultured crypts we observed a calcium response upon addition to naïve crypts **(Figure 3.2.1 a-c)**. ACh can accumulate within the culture media due to the presence of the cholinesterase inhibitor Rivastigmine. Furthermore, any ACh within this media derives from non-neuronal sources as the underlying lamina propria had been stripped away during isolation. Confirmation of muscarinic receptors mediating these calcium signals and ruling out other possible factors inducing calcium signals was achieved using the competitive muscarinic receptor inhibitor 10 μM Atropine, abolishing any calcium signals (Lochner & Thompson, 2016). The amount of endogenous ACh produced was quantified through liquid chromatography tandem mass spectrometry, Alvin Lee (Williams laboratory), at ~90 nM. Subsequently, 100 nM of exogenous ACh produced a similar calcium response to that of the conditioned media **(Figure 3.2.1 c)**.

In mouse, researchers determined non-neuronal ACh, is synthesised and liberated by $ChAT^+$ tuft cells (Hayakawa, et al., 2017) (Takahashi, et al., 2014). Tuft cells make up a small percentage of gut epithelial cells, expressing chemosensory signalling machinery and can therefore 'taste' the contents of the gut lumen (Howitt, et al., 2016). Tuft cells can detect parasites that infect or colonise the gut epithelium, resulting in the rapid expansion of their numbers and secretion of interleukin-25 to clear the parasite and increased production of ACh (Howitt, et al., 2016). It is thought that this non-neuronal source of ACh acts as a 'first responder' to any infection from a parasite or otherwise to prevent the foreign body from entering the underlying mucosa. Furthermore, non-neuronal ACh synthesis and secretion onto the serosal side of rat colonic crypts upon the addition of the short-chain fatty acid propionate into the lumen (Yajima, et al., 2011). The induction of a calcium response through crypt conditioned media may well provide evidence for a role of a non-neuronal source of ACh induced ISC calcium signals that regulate their proliferative activity *in vivo*. As previous data demonstrates the ablation of tuft cells in mouse inhibits epithelial proliferation and tumourigenesis in an ACh-muscarinic receptor dependent manner (Hayakawa, et al., 2017).

4.3 Insights into the source of intracellular calcium and the influence of extracellular calcium

As these data strongly indicate endolysosomes as the site of calcium initiation we aimed to confirm this through the use of the lysosomotropic inhibitor GPN, 100 µM. GPN is a cathepsin C substrate, upon entering endolysosomes it is catalysed into amino acids, increasing the molarity within endolysosomes. Therefore, water enters through osmosis causing endolysosomes to swell and burst. This process can be visualised using the ratiometric calcium dye Fura-2 **(3.3.1)**. To determine whether endolysosomes were the site of cholinergic induced calcium initiation, following GPN we replaced this solution with 10 µM Cch solution, observing a substantial reduction in any subsequent calcium responses. Thus, further justifying the theory of endolysosomes as the intracellular calcium store responsible for initiating cholinergic induced calcium mobilisation.

Previous work in the Williams laboratory and in the wider field of research, identified downstream of muscarinic receptor activation is the mobilisation of the enzyme CD38. In mouse hearts CD38 is critical in the generation of calcium mobilising messengers NAADP and cADPR, substrates for TPC, therefore stimulating calcium release via these channels (Lin, et al., 2017) (Pitt, et al., 2010). TPC2 activation can result in four different characteristics; 1. In the absence of NAADP, there is constitutive, infrequent brief openings of TPC2, not abolished by cytosolic or luminal calcium reduction 2. Transitioning to a sub-conductance state 3. Episodic gating where multiple TPC channels are synchronised to open and close simultaneously 4. Slow-onset in NAADP induced activation (Pitt, et al., 2010). To this day there is still scepticism surrounding endolysosomal calcium signalling and the intracellular synthesis of NAADP, there are conflicting reports with some stating CD38 and ADP-ribosyl cyclases catalyse NAADP (Pitt, et al., 2010) whilst other report in CD38 knockouts, NAADP production is maintained (Soares, et al., 2006).

Further experiments to elucidate the effects of GPN on endolysosomes would confirm beyond any reasonable doubt, endolysosomes as the site of cholinergic calcium mobilisation. Possibly using Lysotracker in live human colonic crypts would facilitate the visualisation and tracking of endolysosomes at rest, during and after GPN incubation and during subsequent replacement with 10 µM Cch. Furthermore, using mathematical equations such as Fura-2 ratio calibration would provide the calcium concentration converted directly from the change in Fura-2 ratio by GPN and Cch induced calcium signals. These experiments would provide insights into the characteristics of endolysosomes and quantify the concentration of calcium within these stores. A potential caveat to these experiments is the high sensitivity of many calcium fluorescent dyes to the extremely low pH within endolysosomes (Lattanzio & Bartschat, 1991).

After an initial apical calcium response, the calcium wave propagates to the basal pole. There appears to be an intimate association of endolysosomes with the ER cisternae through endolysosomal-ER membrane contact sites (MCSs) (Lam & Galione, 2013) (Morgan, et al., 2013). In pulmonary artery smooth muscle cells (PASMCs) NAADP generated calcium release from endolysosomes recruits ryanodine and IP_3Rs to the ER membrane resulting in CICR. These data indicate this phenomenon is achieved through cytoplasmic nanojunctions separated from one another by less than 400 nm (Fameli, et al., 2014). Furthermore, 3D

electron tomography has identified the formation of microdomains between acidic stores and the sarcoplasmic reticulum in cardiac ventricular myocytes upon NAADP stimulated calcium release from acidic stores, this interaction is termed the trigger hypothesis (Aston, et al., 2017). In rat PASMCs a trigger zone for calcium signalling by NAADP is formed through the clustering of a subpopulation of lysosomes within the perinuclear region of PASMCs forming junctions with the sarcoplasmic reticulum which contains a high density of RyR3 (Kinnear, et al., 2008). In addition, previous data suggests bi-directional calcium signalling between acidic calcium stores and the ER. Calcium released from the ER can induce calcium release from acidic stores by either stimulating calcium-dependent synthesis of NAADP or through TPC1 sensitisation to cytosolic calcium (Morgan, et al., 2013) (Faris, et al., 2019).

Future experiments will need to determine the exact dynamics and role endolysosomes have in CICR. Using Lysotracker, endolysosomes can be tracked at rest, during and after addition of 10 µM Cch. Along with Fura-2 there is the potential to observe whether endolysosomes migrate towards the basal pole to form close contacts with the ER. On the other hand, they might remain at their separate poles and a chain reaction of localised CICR in microdomains across the cell links the two stores together. After a global calcium response intracellular calcium stores are emptied, e.g. the ER. For further calcium responses these stores must refill, the ER refills via SOCE. Transient receptor potential canonical (TRPC) and ORAI are two major SOCE channels, that along with STIM1 all rely on each other for regulating calcium influx (Manjarres, et al., 2011) (Cheng, et al., 2013).

Whilst the apical-basal dynamics of the calcium wave remained constant, the same could not be said for the amplitude and frequency of responses to a single addition of 10 µM Cch in full organoid culture or HBS media **(Table 3.3.1) (Figure 3.3.2, 3.3.3 & 3.3.5)**. On occasions we observed oscillatory calcium waves that occurred at regular time intervals, such as 14 minutes apart **(Figure 3.3.4)**. The greatest oscillatory intensity was observed under organoid culture media kept at 37^0C, 5% CO_2 during calcium imaging. However, Fura-2 ratio under these conditions was very poor, potentially due to the increased activity of multi-drug resistant (MDR) channels at 37^0C (human body temperature), resulting in the efflux of Fura-2 from crypt epithelial cells. Calcium oscillations have also been observed in pancreatic acinar cells from 5 or 50 pM Cholecystokinin. Cholecystokinin is a hormone that stimulates NAADP production via CD38, evoking calcium release from acidic stores (Cosker, et al., 2010) **(Figure 4.3.1)**.

Additionally, Deng, *et al*., 2015 provided evidence of calcium oscillations within *Drosophila* mid-gut stimulated by different factors, be it inflammatory cytokines or dietary nutrients (Deng, et al., 2015). An increased average concentration of calcium with decreased oscillation frequency promotes ISC proliferation, whilst reduced calcium concentration with increased oscillation frequency promotes ISC quiescence (Deng, et al., 2015). These data support our evidence of calcium oscillations and suggest a potential role of cholinergic calcium signals in regulating ISC activity within the human intestinal epithelium. This could also be a promising target for future novel chemotherapeutics for CRC. Furthermore, calcium oscillations can be induced by a variety of different substrates. Through the calcium-sensing receptor (CaR), extracellular calcium can induce sinusoidal calcium oscillations while aromatic amino acids trigger transient calcium oscillatory patterns involving the ion channel TRPC1 (Rey, et al., 2010). Transient calcium signals are repetitive low frequency oscillations while sinusoidal

patterns are high frequency oscillations with an elevated plateau (Rey, et al., 2010). Sinusoidal oscillations occur via the negative feedback loop of CaR activation of PKC which in turn phosphorylates CaR, extracellular calcium induced CaR activation can inhibit cell proliferation via sinusoidal, not transient oscillations (Rey, et al., 2010). These were the type of calcium oscillations observed in this project **(Figure 3.3.4)**. As cytosolic calcium increases and ER calcium decreases there is a negative feedback loop allowing the ER to refill and cytosolic calcium to be sequestered, facilitating a further calcium oscillation. Together, these data describe calcium as a key integrator as is the case in *Drosophila* for a number of external stimuli (Deng, et al., 2015). Potentially explaining the heterogeneity observed in calcium responses **(Figure 3.3.5)**.

Due to the lack of reproducibility with calcium oscillations we investigated the effects of extracellular factors on cholinergic calcium signals. Initially the concentration of extracellular calcium was altered as it is known there is a large calcium concentration gradient maintained across the cell plasma membrane. Also, after a global calcium response there is rapid refilling of the ER store via SOCE channels, facilitated by the large concentration of calcium outside of a cell. Therefore, we halved (0.5 mM) and doubled (2 mM) calcium in organoid culture media. At half the concentration there were no calcium oscillations, theoretically indicating a reduction in SOCE, this could prohibit further ER calcium release as this calcium store takes longer to refill or remains empty. On the other hand, doubling the extracellular calcium could increase SOCE to a point where there are no oscillations instead the initial calcium response remains elevated **(Figure 3.3.5)**. Also, these varying calcium concentration experiments were repeated using HBS bathed crypts instead of organoid culture media **(Figure 3.3.3)**. Thus, removing any potential impact of growth factors or ligands within culture media on cholinergic calcium signals, such as Wnt. A number of Wnt ligands induce calcium signals in a multitude of cancer cell lines, for example prostate cancer (PC3) (Thrasivoulou, et al., 2013). Therefore, high Wnt concentration within organoid culture media could have potentially augmented the cholinergic calcium responses observed.

Reducing extracellular calcium reduced cholinergic calcium signals **(Figure 3.3.5 & 4.3.1)**, data not significant. But previous data suggests extracellular calcium as an important factor in facilitating cholinergic calcium signalling **(Figure 4.3.1)**. Muscarinic receptor activation through Cch in mouse duodenum induces a global calcium response, after which SOCE refills depleted intracellular calcium stores of mouse duodenal cells (Yang, et al., 2018). Here SOCE blockers such as 2-Aminoethoxydiphenylborate (2-APB) inhibited short-circuit current (I_{SC}) (Yang, et al., 2018). In addition to inhibiting TRP channels for SOCE, 2-APB inhibits the production of IP_3 and has been used in the Williams lab to inhibit purinergic calcium signals from the ER (Alvin Lee, unpublished).

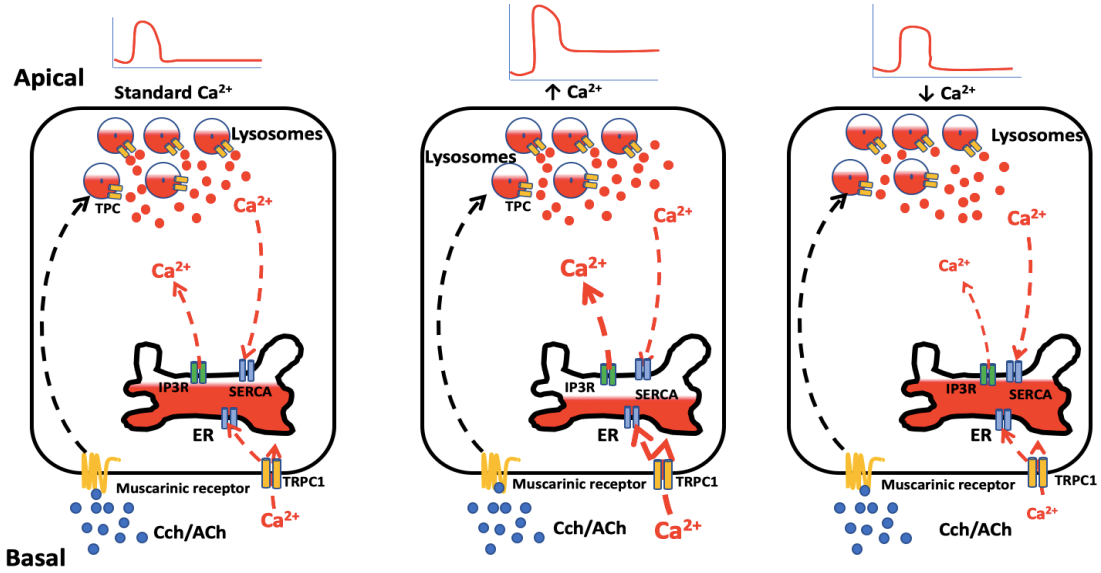


Figure 4.3.1- An overview of the difference in calcium mobilisation in standard, high and low extracellular calcium solution. Increase in SOCE within a high calcium environment mediates an increase and prolonged calcium response within epithelial cells, this is vastly reduced within a calcium low/free environment. In theory a low calcium environment provides a more accurate measure of intracellular calcium stores.

The effect of different concentrations of Cch was also investigated, using 1 µM and 100 µM respectively **(Figure 3.3.6 a-b)**. Decreasing the agonist concentration produced no oscillations and a far higher concentration resulted in more intense oscillations but with a reduced frequency. These data highlight the importance of agonist concentration on stimulating intracellular calcium signals. These data suggest a possible muscarinic receptor threshold to ACh *in vivo* from neuronal and non-neuronal sources which could have important consequences on ISC activity. The variability in calcium responses under the same culture conditions may be due to the accumulation of negative factors around cultured crypts after a single addition of Cch. These negative factors potentially inhibit agonist-receptor interaction under static conditions; therefore, calcium responses were investigated under dynamic conditions **(Figure 3.3.7)**. After an initial 10 µM Cch response returns to baseline the solution was replaced with fresh 10 µM Cch producing another calcium response **(Figure 3.3.7)**. Perfusion removes accumulation of any negative factors and will need further investigating. Furthermore, the impact this has on intracellular calcium stores and the possible effects of perfusing inhibitors remains a possibility but is slightly hindered by cost.

4.4 The role of the mTOR pathway on intracellular calcium signalling

With increasing evidence suggesting endolysosomes initiate calcium signals upon muscarinic receptor activation, we further investigated this acidic calcium store. There is an emerging role of the nutrient sensor mTOR as a key regulator of TPC activity, of which there are two complexes mTORC1 and mTORC2. mTOR interacts with TPC sub-types regulating their function in response to alterations in cytosolic ATP levels (Lin, et al., 2015). In the presence of nutrients, lysosomes are situated beneath the plasma membrane and mTORC1 is recruited to these lysosomes through interaction with their transmembrane proteins such as Rag GTPases. Through this interaction mTORC1 is activated by the GTPase Reb, mTORC1 proceeds to phosphorylate its downstream targets, activating biosynthetic pathways and inhibiting catabolic pathways, thus promoting cell growth and metabolism (Faris, et al., 2019). While mTOR is active, TPCs are inhibited therefore during nutrient starvation or modelling nutrient starvation using mTOR inhibitors, TPCs are active and facilitate calcium release from endolysosomes (Ogunbayo, et al., 2018). Nutrient starvation increases intracellular pH of lysosomes causing perinuclear clustering, inactivating mTORC1 via a number of different mechanisms such as the AMPK phosphorylation cascade (Faris, et al., 2019). Overall inhibition of mTORC1 through nutrient starvation promotes autophagy instead of cell growth and metabolism (Faris, et al., 2019) **(Figure 4.4.1)**. However, prolonged starvation can lead to mTORC1 activity being restored through TRPML1-mediated calcium release, this is alleged to be an adaptive mechanism to maintain protein synthesis (Sun, et al., 2018).

The involvement of mTOR in *ex vivo* human colonic crypt calcium signalling was investigated using mTOR inhibitors Torin2 (1 & 10 µM) and Rapamycin (3 & 30 µM) **(Figure 3.4.1 & 3.4.2)**. The role of mTOR in acidic store mediated calcium release is surrounded by conflicting data. As a nutrient sensor, mTOR activity is augmented and dampened by a multitude of different signalling pathways and growth factors **(Figure 4.4**.1**)**. Additionally, mTOR has a broad physiological role which suggests there are potential downstream pathways that remain undiscovered (Francipane & Lagasse, 2013) **(Figure 4.4.1)**. mTOR inhibits TPC2-mediated calcium release by blocking NAADP binding. Some research suggests TPC2 has dual substrate activation in the form of PI(3,5)P2 as well as NAADP (Dove, et al., 2009). On the contrary, in pulmonary arterial myocytes TPC2 depletion abolishes NAADP induced global calcium signals but has no effect on PI(3,5)P2 or cyclic adenosine 5'-ribose (Ogunbayo, et al., 2018). In addition, short hairpin RNA (shRNA) knockdown of TPC2 ablates NAADP evoked calcium signals from acidic stores (Zhu, et al., 2009).

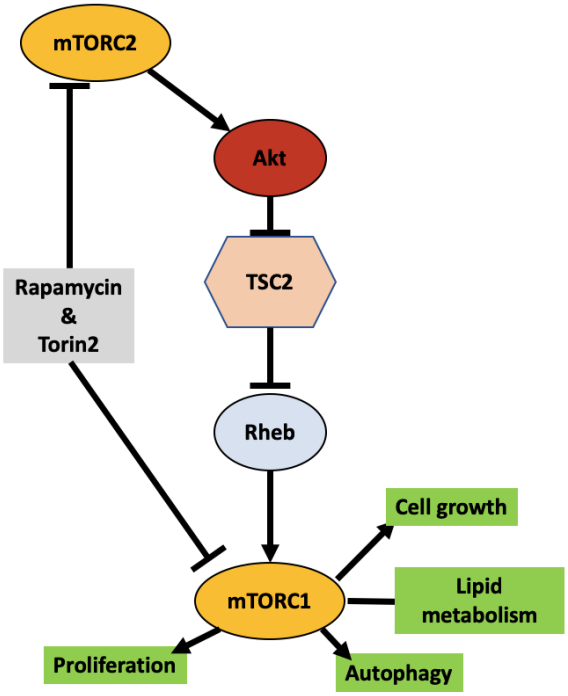


Figure 4.4.1- Schematic diagram of the mTOR/Akt signalling pathway
Akt pathway is situated downstream of mTORC2 but upstream of mTORC1 and is activated by mTORC2 inhibiting the inhibitory activity of the GTPase TSC2. Subsequently Rheb remains in an active GTP bound state and activates mTORC1 which regulates a number of different cellular processes through many different downstream signalling pathways. The activity of these mTOR complexes are inhibited by Rapamycin and Torin2 (Adapted from: (Technology, 2014)

Unlike previous data we observed no significant difference in calcium responses within colonic crypts when pre-incubating them in Rapamycin or Torin2 before cholinergic calcium activation with 10 µM Cch. In HEK 293 cells (HEK 293s) with overexpression of human TPC2 (hTPC2), Rapamycin provoked calcium signals from acidic stores in both an mTOR and TPC2 dependent manner. However, in wild-type HEK 293s only small transient calcium responses were observed (Ogunbayo, et al., 2018). Working concentrations of 3 µM and 30 µM Rapamycin were used because previous work demonstrated considerable calcium responses at 3 µM and multiphasic calcium transients at 30 µM Rapamycin (Ogunbayo, et al., 2018). It was also noted that the concentration-response curve of Rapamycin is bell-shaped and concentrations higher than 30 µM result in progressive desensitisation of HEK 293s (Ogunbayo, et al., 2018).

As 30 µM Rapamycin is at the peak of the Rapamycin concentration-response curve and there were no alterations in cholinergic calcium responses we used a lower concentration of 3 µM. Unlike Ogunbayo, *et al.,* 2018 we observed no calcium transients to either 30 µM or 3 µM Rapamycin **(Figure 3.4.1 & 3.4.2 a)**. These data could be a result of mTOR being over activated by components within organoid culture media and HBS, such as essential and non-essential amino acids. These components are important for maintaining ISC activity in culture but could have significant impact on Rapamycin and Torin2 function, potentially desensitising mTOR to the activity of these two inhibitors. As mentioned, TPC-mediated calcium release from acidic stores can have local effects if calcium is mediated by TPC1 or global effects if via TPC2 that

induce CICR from the ER (Zhu, et al., 2009). These global responses are attenuated in HEK 293s when pre-incubating with the Calcium-ATPase inhibitor Thapsigargin before stimulation with 30 µM Rapamycin, suggesting mTOR has a role to play in acidic stores initiating a global calcium response (Ogunbayo, et al., 2018). Further evidence shows a close relationship between the inhibitors Rapamycin and Torin2 with TPC2. In *TPCN2* knockout mice rapamycin and Torin2 do not induce calcium signals while shRNA knockdown of mTOR in hTPC2 HEK 293s attenuated Rapamycin and NAADP calcium signalling (Ogunbayo, et al., 2018). Implying Rapamycin induces calcium signals in a TPC dependent manner through mTOR inhibition (Ogunbayo, et al., 2018), however, in our studies this was not observed.

As well as Rapamycin, we used another mTOR inhibitor, Torin 2 **(Figure 3.4.2 b & 4.4.1)**. Similar to Rapamycin there was no significant difference observed in calcium signalling with Torin2 present. Although no significant difference we observed at 1uM there appears no effect on calcium responses but at 100 nM Torin2 is amplifying the cholinergic calcium response **(Figure 3.4.2 b)**. As with Rapamycin experiments factors within extracellular solutions that could have a stimulatory effect on mTOR were reduced, removed or increased, for example essential and non-essential amino acids and L-glutamine. Even with these changes no significant effect was observed this may suggest that culture conditions are still too nutrient rich for mTOR to be sensitised to Torin2 inhibition, difficulty in Torin2 entering either epithelial cells or the endolysosomes. Possibly sequestering ATP or stripping out further nutrients such as glucose may have a positive impact on these studies in the future. However, a balance is needed because without all of these factors the health and morphology of these crypts would be lost. Future experiments using RT-PCR, immunohistochemistry could be employed to determine if mTORC complexes 1 and 2 are present within the epithelial cells of our *ex vivo* colonic crypt cultures. In hTPC2 HEK 293s Torin2 produces biphasic calcium transients similar to those produced by NAADP (Ogunbayo, et al., 2018). Both Rapamycin and Torin2 pre-incubation with hTPC2 HEK 293s or pulmonary arterial smooth muscle cells abolishes NAADP induced calcium signalling (Ogunbayo, et al., 2018).

Torin 2 effects TPC 2 activity in an mTOR-dependent manner rather than directly binding to TPC 2, further research demonstrated Torin 2 as a potent ATP-competitive inhibitor of mTOR. Torin 2 rapidly dephosphorylates rapamycin-sensitive mTORC1 substrates e.g. SK6, rapamycin-insensitive substrates e.g. 4EPB1 and mTORC2 substrates e.g. Akt (Liu, et al., 2013). With Torin2 being such a potent inhibitor of mTOR it has been investigated as a therapeutic agent for cancer. However, single agent treatment of Torin2 for KRAS driven lung tumours *in vivo* yielded no significant efficacy in tumour growth inhibition (Liu, et al., 2013). However, Torin 2 used in conjunction with MEL inhibitor AZD6244 yielded significant tumour growth inhibition. Demonstrating mTOR signalling has a pathogenic role in an oncological setting, in which Torin 2 would be a strong candidate for clinical use (Liu, et al., 2013). Furthermore, both Torin 2 and Rapamycin express bimodal effects on Akt activity, the acute inhibition of Akt S473 phosphorylation reduces the recruitment of Akt to the plasma membrane (Liu, et al., 2013). The site at which PDK1 phosphorylates T308, which is a site in the activation loop of Akt, directly regulating the activity of Akt (Liu, et al., 2013). Although, the prolonged inhibition of mTORC1 produces a de-repression of a feedback loop, that results with hyperactivation of PI3K (Wan, et al., 2007). The absence of Akt phosphorylation at S473 is believed to reduce Akt activity levels below the tumourigenesis threshold (Guertin, et al., 2009).

4.5 Organoid growth rate enhanced through muscarinic receptor activation and potentially downstream intracellular calcium signals

Organoids are immortal 3D cultures that were used for measuring the effect of muscarinic receptor activation on colonic epithelial cell growth over the course of around a week. Organoids were cultured in 10% organoid culture media with or without the presence of the potent, highly specific muscarinic receptor Oxotremorine at 1 µM (Akk & Auerbach, 1999). Oxotremorine mimics the effects of *in vivo* production of ACh from non-neuronal $ChAT^+$ tuft cells and neuronal $ChAT^+$ myenteric neurons (Takahashi, et al., 2014) (Hayakawa, et al., 2017). A 10% culture media was selected rather than full culture media as at full culture media conditions are already more than optimal for ISC proliferation and therefore any impact of Oxotremorine would be insignificant. Dogma suggests that mAChR activation through ACh or its mimetics increases ISC proliferation. However, other data has challenged this dogma by demonstrating the presence of 10 µM Cch inhibits ISC driven growth in long-term cultured organoids (Takahashi, et al., 2014). Organoid growth was measured using two parameters, those being CSA and the number of organoid buds formed. CSA provides information on the overall size of individual organoids while the number of organoid buds is indicative of ISC activity as only cells undergoing rapid cell division give rise to these budding structures **(Figure 3.5.1 a-d)**. Oxotremorine organoids had a larger CSA **(Figure 3.5.1 a-b)** compared to control however, independent two-tailed t-tests assuming unequal variances determined there was no significant difference **(Figure 3.5.1 a-b)**. Even after grouping these analysed organoids into groups based on initial CSA size and observed a greater difference with small CSA organoids over nine days, there was no significant difference **(figure 3.5.1 c)**. There was a greater number of organoid buds per organoid in Oxotremorine culture and was significantly different.

Future experiments will aim to further investigate the effects of mAChR downstream signalling pathways on ISC activity. In the Williams laboratory factors present in organoid culture media that have been reportedly upregulated by mAChR activation are being removed. Prostaglandin E2 is present within our culture media and its production is upregulated through mAChR activation in colon cancer cells (Yang & Frucht, 2000). The high concentration of growth factors in organoid culture media almost recapitulates a rate of growth akin to cancer thus removal of factors upregulated in cancer will provide us with a larger difference between control and Oxotremorine. With control more closely resembling homeostatic conditions and Oxotremorine cultured organoids more closely resembling cancerous conditions. In rat bladders, intravesical injection of Oxotremorine affected cell proliferation in a mAChR dependent pathway (Kullmann, et al., 2008). In rat colonic crypts, the ISC niche is found to be the most ACh sensitive region due to the high expression of the mAChR3 subtype found there. Immunohistochemistry data within the Williams laboratory also finds a high expression of mAChR3 at the base of human colonic crypts (unpublished data). In mouse small intestinal organoids activation of muscarinic receptors are important for growth and differentiation of such organoids (Takahashi, et al., 2014). These data along with Oxotremorine data with Cch induced cholinergic-calcium signalling data in this report

could suggest a role for cholinergic-calcium signals in regulating ISC activity. Subsequently, regulating homeostasis of the gut epithelium.

Furthermore, these data potentially indicate that an upregulation of mAChR or an increase in its agonist ACh could result in increased cholinergic-calcium signals potentially upregulating ISC proliferation resulting in tumour formation. General dogma suggests muscarinic receptors are expressed within the normal colonic epithelium but are overexpressed in CRC (Xie & Raufman, 2016). With mAChR3 highly expressed in ~60% of cancer cell lines (Frucht, et al., 1999). Furthermore, ChAT and Dclk1 co-expression in tuft cells of rat colonic crypts contribute to early cancer growth and remodelling of the peritumoral environment (Hayakawa, et al., 2017). These data suggest an upregulation of ACh synthesis and mAChR expression and paired with our spatiotemporal data strongly imply intracellular calcium signals within ISCs are pivotal in regulating ISC activity and thus homeostasis.

4.6 Cholinergic calcium signalling status in colorectal cancer

Patient matched organoids and tumouroids allow comparison of calcium signalling in health and disease, similar to organoids, tumouroids are immortal and can be grown indefinitely. While organoids form budding structures over time, tumouroids continue to grow as spheroid structures. Initially the size of the calcium response in organoids and tumouroids was compared after stimulation with either 10 µM Cch or 50 µM UTP providing a rough estimate into the relative size of acidic and ER calcium stores respectively **(Figure 3.6.1)**. The size of cholinergic-calcium signals significantly differs whilst no significant difference is observed in the size of purinergic-calcium signals between organoids and tumouroids **(Figure 3.6.1)**. Subsequently, inhibitors affecting these two stores independently of one another were used to further investigate the size of these two calcium stores, GPN and Thapsigargin **(Figure 3.6.2 a)**. No significant difference in calcium signals was observed when evoked by either GPN or Thapsigargin in organoids and tumouroids **(Figure 3.6.2 a)**. However, these data were collected from organoids and tumouroids bathed in HBS solution with 1 mM calcium. Extracellular calcium may have affected the size of Thapsigargin induced calcium responses through increased SOCE (Gusev, et al., 2003). Thapsigargin mobilises calcium from the ER though the inhibition of the calcium-ATPase SERCA pump, preventing the sequestering of calcium to the ER (Doan, et al., 2015). The ER becomes calcium depleted and refills via activation of calcium release activated channels (CRAC) and SOCE from extracellular calcium. Prolonged SERCA pump inhibition induces a cascade reaction triggering apoptosis (Doan, et al., 2015). Although data does support the notion of the ER store as the largest intracellular calcium store, we repeated these experiments in calcium free media and 5-minute pre-incubation with the chelating agent EGTA before subsequent Thapsigargin or GPN stimulation **(Figure 3.2.6 b)**.

In healthy human mucosal cells, there is a large concentration of ER calcium with a small SOCE that is associated with the common release activated channel current (I_{CRAC}). This is regulated through ORAI1, STIM1 and 2 interaction **(Figure 4.6.1)** (Villalobos, et al., 2017) in which there is a higher expression of STIM 2 compared with STIM 1 (Villalobos, et al., 2017). In a CRC cell the ER calcium store is considerably smaller with an increase in SOCE and I_{CRAC} and an emergence of a non-selective store-operated calcium entry current (I_{SOC}). This is regulated by complexes formed from ORAI1, STIM1, TRPC1 and SK3 (Villalobos, et al., 2017) **(Figure 4.6.1)**.

Furthermore, in CRC cells there is a partial downregulation of STIM2 resulting in partial depletion of ER calcium store (Villalobos, et al., 2017) **(Figure 4.6.1)**.

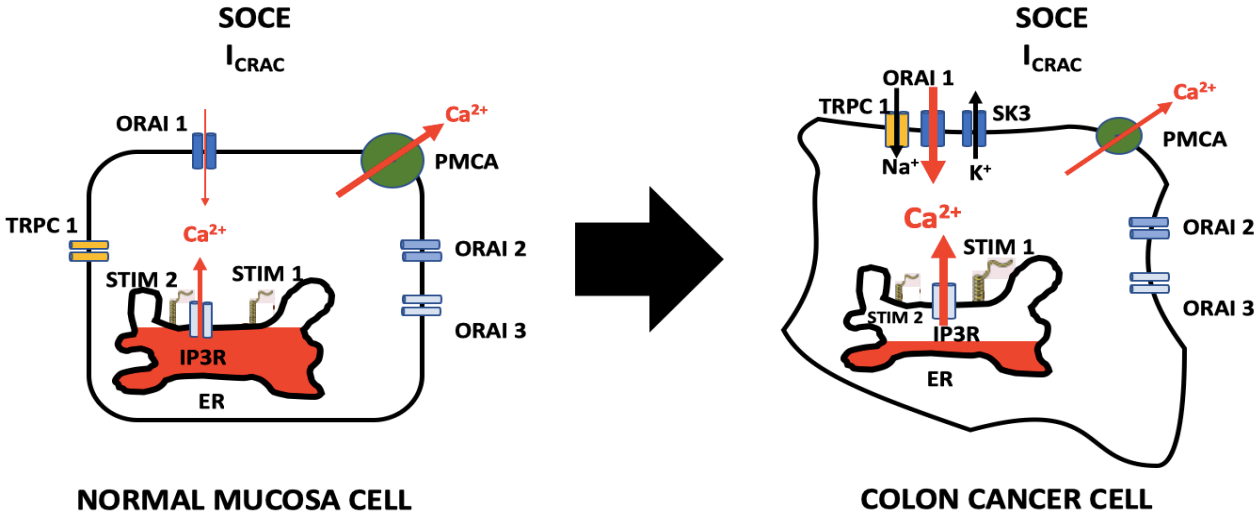


Figure 4.6.1- Schematic diagram, depicting the overall remodelling of SOCE in colorectal cancer cells. High STIM2 expression maintains a high concentration of calcium in the ER which is reduced in CRC. Under homeostasis a small SOCE is maintained through classic I_{CRAC}. However, in CRC, ER calcium is reduced along with increased SOCE associated with a larger I_{CRAC} contributing to

In calcium free EGTA conditions there was an expected decrease in amplitude of calcium responses to Thapsigargin and GPN, but not significant between organoids and tumouroids **(Figure 3.6.2 b)**. Even though no significant difference is observed in either standard HBS conditions or calcium free EGTA HBS conditions there is difference in trends between the conditions. Under standard conditions GPN induced calcium signals are larger in tumouroids while Thapsigargin induced calcium signals are larger in organoids **(Figure 3.6.2 a)**. However, in calcium free EGTA HBS Thapsigargin triggered calcium signals are almost identical in amplitude whilst GPN triggered calcium signals are now larger in organoids **(Figure 3.6.2 b)**.

Interestingly, preliminary data indicated when Thapsigargin or GPN are added followed by UTP or Cch respectively, there was no UTP/Cch calcium response. Similarly, when 10 µM Cch was added followed by GPN, there is a diminished GPN triggered calcium response **(Figure 3.3.1)**. These preliminary data suggest GPN is targeting endolysosomal stores, but also suggests that after Cch stimulation endolysosomal stores are not refilled, providing an interesting avenue to be explored in the future.

GPN and Thapsigargin induced calcium responses in health and disease are affected by more than just extracellular calcium. The expression levels of TPC sand IP_3Rs between organoids and tumouroids, mutations in these receptors could all impact the amplitude of calcium responses. Tumour suppressor genes (TSGs) regulate calcium signals, for example BRCA-1 associated protein 1 (*BAP1*) which regulates IP_3R3-mediated calcium flux between the ER and mitochondria which suppresses cell transformation (Bononi, et al., 2017). *BAP1* deubiquitylates and stabilises IP_3R3 at the ER membrane facilitating calcium release (Bononi,

et al., 2017). During carcinogenesis or in *BAP1*$^{+/-}$ cell lines, *BAP1* expression decreases subsequently decreasing IP_3R3 expression reducing ER-mitochondria calcium flux (Bononi, et al., 2017).. Such cells have a higher risk of accumulating DNA damage as they fail to trigger programmed cell death potentially leading to tumour formation (Bononi, et al., 2017). *PTEN* another TSG that is mutated in CRC, normally *PTEN* competes with the FBXL2 for IP_3R3 binding (Kuchay, et al., 2017). *PTEN* binding maintains IP_3R3 expression maintaining ER-mitochondria calcium flux, while FBXL2 binding targets IP_3R3 for degradation, limiting calcium flux from the ER to mitochondria (Kuchay, et al., 2017).

Similar mutations may have occurred in tumouroids analysed in this project. Future studies would need to increase N numbers for both Thapsigargin and GPN, use different inhibitors. Additionally, use genome wide analysis of patient matched organoids and tumouroids to identify any mutations in genes involved in the calcium signalling toolkit. For example, K-Ras is a common oncogenic mutation in CRC linked to remodelling of the calcium signalling toolkit. (Pierro, et al., 2014). In K-Ras^{G13D} knock-out cell lines there is increased IP_3R3 but reduced IP_3R1 expression while in K-Ras^{G13D} cell lines the opposite is seen therefore repression of IP_3R3 by K-Ras^{G13D} reduces ER-mitochondria calcium crosstalk, inhibiting apoptosis (Pierro, et al., 2014).

Overall, despite the development of novel targeted therapy, prognosis of metastatic CRC remains limited. Therefore, requiring production of efficient and novel chemoprevention therapies (Villalobos, et al., 2017). Aspirin is a promising adjuvant therapy, as *In vivo* Aspirin is rapidly deacetylated into a number of substrates one of which is Salicylate is a potent SOCE pathway inhibitor (Coyle, et al., 2016). The SOCE pathway has been identified as an important pathway in CRC progression (Gusev, et al., 2003) (Nunez, et al., 2006). Therefore, inhibition of SOCE through Aspirin substrates and other NSAIDs are important potential therapeutics.

Loss of key regulators within the calcium signalling pathway are also a prerequisite for CRC formation, such as SERCA3. Usually, SERCA3 expression increases up the crypt axis as cells differentiate (Brouland, et al., 2005). Loss of SERCA3 is an early event in colon carcinogenesis, a well-known multistep process (Brouland, et al., 2005). Mutations within the Akt pathway, upstream of mTORC1 but downstream of mTORC2, effects lysosomal calcium stores (Ballou & Lin, 2008) **(Figure 4.4.1)**. Akt pathway is activated by mTORC2 and PDK1, once active Akt inhibits tuberous sclerosis complex-2 (TSC2) expression. TSC2 is a GTPase and subsequent inhibition results in no further inhibition of Rheb, which now maintains an active GTP bound state activating mTORC1 (Francipane & Lagasse, 2013) **(Figure 4.4.1)**. The Akt pathway has important implications on the activity of TPCs through mTOR regulation. mTOR is important for cell survival, metabolism and proliferation and is therefore a target for novel anti-cancer therapies (Francipane & Lagasse, 2013). Due to the broad regulatory factors and effects of mTOR activity and thus TPC activity, further investigation is needed into lysosomal calcium mobilisation. Also, discrepancies in the outcomes of different mTOR inhibitors were identified in CSCs, because mTOR inhibitors vary in pharmacokinetic and pharmacologic properties. Data collected in this project provide a foundation for future work into the calcium signalling toolkit in health and cancer.

Overall, cancer cells use the same calcium signalling toolkit as their non-malignant counterparts. However, subtle alterations occur, whether that be down- or up- regulation, post-translational modification or gene mutation (Montieth, et al., 2012). Understanding these mutations can help develop key therapeutic drugs, such as, targeting ER-mitochondria calcium flux for novel anti-cancer therapy (Pedriali, et al., 2017).

5 Conclusion

This project has determined cholinergic signaling induces an intracellular calcium signal initiating at the apical pole of ISCs at the base of human colonic crypts. mTOR may have an important regulatory role in TPC mediated calcium release using mTOR inhibitors Torin 2 and Rapamycin. There may be a difference in calcium store size of the ER and endolysosomes and remodelling of the calcium signalling pathway in health versus cancer. Elucidated using GPN and Thapsigargin along with the muscarinic and purinergic agonists Cch and UTP respectively. However, more work is needed in the investigation of the roles that the endolysosomes and ER have in health and also CRC and what mutations occur that dive these differences. In the very near future, collaborative work between the Williams laboratory and the Macauley laboratory using multi-omic data on several paired organoids and tumouroids will provide the first insight into the main mutations that occur throughout CRC initiation and development. Coupled to these data, will form the basis for future research to assess the effects of known chemotherapeutic drugs on the treatment or prevention of colon cancer. Better yet, these data could form the basis for the development of novel therapeutic treatments.

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