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Hippo pathway effector YAP1 is a regulator of intestinal epithelial cell differentiation

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Résumé

Les cellules souches primordiales, qui sont situées à la base de la crypte dans l'intestin des mammifères, fournissent toutes les cellules épithéliales situées dans les cryptes et les villosités, comme les cellules absorbantes, les cellules caliciformes, les cellules de Paneth et les cellules entéroendocrines. La survie des cellules souches est associée à la niche qui est composée de divers signaux et facteurs provenant des cellules épithéliales et du mésenchyme environnant. Un signal important dont le rôle dans le maintien et la prolifération des cellules souches a été démontré est la voie de signalisation Hippo. Les signaux en amont, la cascade kinases et les gènes cibles en aval composent les trois parties de la voie de signalisation d'Hippo. Cette voie limite la croissance des cellules et la taille des organes par la régulation de YAP1 et TAZ qui sont les composants du cœur de la kinase. La voie active d'Hippo contribue à la phosphorylation et à l'inactivation des YAP1/TAZ, ce qui conduit à leur dégradation ou leur séquestration cytoplasmique. En l'absence de la voie Hippo, YAP1/TAZ actifs passent dans le noyau et induisent la transcription des gènes impliqués dans la croissance cellulaire. La présente étude vise à évaluer l'effet de YAP1 sur la différenciation des cellules épithéliales intestinales. Ainsi, l'expression de YAP1 a été neutralisée dans la cellule HT29 à l'aide de l'ARN interférant. Ensuite, l'immunoblot, l'immunofluorescence indirecte et l'analyse RT-qPCR ont été utilisées pour caractériser les cellules absorbantes et sécrétoires. Les résultats ont montré que l'expression des marqueurs de cellules absorbantes SI et DPPIV et des marqueurs de cellules caliciformes MUC2 et TFF3 a été augmentée de manière significative au niveau transcriptionnel et protéique par l'ablation de YAP1. La formation des cellules de type absorbante et de type caliciforme a été confirmée par immunofluorescence indirecte et microscopie électronique à transmission. De plus, l'expression de deux marqueurs de cellules souches, LGR5 et PROM1, a été réduite de manière significative dans la cellule HT29 déplétée en YAP1 par rapport au contrôle. Ensuite, le mécanisme par lequel YAP1 contrôle la différenciation de la cellule épithéliale intestinale a été étudié. Dans ce contexte, l'expression des facteurs de transcription CDX2, ATOH1, HES1, KLF4 et HNF1α a été mesurée à l'aide du qPCR. Les résultats ont montré une augmentation de CDX2 et ATOH1 ainsi qu'une légère augmentation du facteur de transcription HES1 dans la cellule HT29 déplétées en YAP1. L'augmentation de l'expression de la protéine CDX2 a été confirmée par immunobuvardage, tandis qu'ATOH1 n'était pas détectable. L'effet de CDX2 sur la différenciation des cellules absorbantes et caliciformes a été confirmé par l'ablation de CDX2 dans les cellules déficientes en YAP1. L'ablation de CDX2 dans ces cellules s'est accompagnée du retour à des niveaux de base d'expression de SI, MUC2 et TFF3 de même que de LGR5 et PROM1 comparables aux cellules contrôles. Cependant, l'expression de DPPIV est demeurée élevée suggérant l'implication d'autres facteurs. L'expression de MUC2 est également contrôlée par le facteur de transcription ATOH1. L'induction de l'expression d'ATOH1 dans la cellule HT29 a conduit à une augmentation de l'expression de MUC2. De plus, nos études d'inhibition des membres de la famille de kinase Src montrent une réduction de l'expression de YAP1 qui résulte à une augmentation de l'expression des marqueurs des cellules absorbante et à mucus et de CDX2. Nous pouvons donc conclure que YAP1 régule négativement la différenciation des cellules absorbantes et des cellules caliciformes par l'inhibition du facteur de transcription CDX2.

Mots clés : épithélium intestinal, voie Hippo, YAP1/TAZ, CDX2, différenciation, SI, MUC2, des membres de la famille de kinase Src

Summary

LGR5$^+$ crypt base columnar (CBC) stem cells that are located at the base of the crypt in the mammalian intestine, provide all the epithelial cells located in the crypts and villi including absorptive, goblet, Paneth and enteroendocrine cells. The maintenance of LGR5$^+$ stem cells is associated with the niche that is composed of various signals and factors originating from the epithelial cells and surrounding mesenchyme. One important signal which has been reported to have a role in stem cell maintenance and proliferation is the Hippo signaling pathway. Upstream signals, a kinase core and downstream target genes compose the three parts of the Hippo pathway. This pathway restricts cell growth and organ size through regulation of YAP1 and TAZ, which are kinase core components. An active Hippo pathway contributes to the phosphorylation and inactivation of the YAP1/TAZ which leads to its degradation or cytoplasmic sequestration. In the absence of the Hippo pathway, active YAP1/TAZ passes into the nucleus and induces the transcription of genes involved in cell growth. The present study is aimed at the investigation of the effect of YAP1 on intestinal epithelial cell differentiation. Thus, YAP1 expression was knocked down in HT29 cells using shRNA. Then Western blot, immunofluorescence and RT-qPCR analysis were used for the characterization of absorptive and secretory cells. The results showed that the expression of the absorptive cell markers SI and DPPIV, and goblet cell markers MUC2 and TFF3 was increased significantly at both transcriptional and protein levels by YAP1 ablation. The formation of absorptive-like and goblet-like cells was confirmed using indirect immunofluorescence and transmission electron microscopy. Furthermore, the expression of the two stem cell markers LGR5 and PROM1 was decreased significantly in YAP1 knockdown HT29 cells compared with the control. Then the mechanism by which YAP1 controls the differentiation of the intestinal epithelial cell was studied. In this context, the expression of the CDX2, ATOH1, HES1, KLF4 and HNF1α transcription factors was measured using qPCR. The results showed an augmentation of CDX2 and ATOH1, and also a slight increase of the HES1 transcription factor in YAP1 knockdown HT29 cells. An increased expression of CDX2 protein was confirmed by Western blot analysis while ATOH1 expression was not at a detectable level. The effect of CDX2 on absorptive and goblet cell differentiation was confirmed by CDX2 ablation in shYAP1-expressing HT29 cells. CDX2 ablation in shYAP1-expressing HT29 cells was accompanied by a return of SI, MUC2 and TFF3 as well as LGR5 and PROM1 to the expression levels of control HT29 cells. However, DPPIV expression remained high suggesting the involvement of additional transcription factors. The expression of MUC2 may also be controlled by the ATOH1 transcription factor. Induction of ATOH1 expression in HT29 cells resulted in increased MUC2 expression. Furthermore, inhibition of Src family kinases led to a decrease in YAP1 expression as well as an increase in absorptive and goblet cells markers and CDX2 expression. Altogether, this investigation showed that YAP1 negatively regulates the differentiation of both absorptive and goblet cells through the inhibition of the main transcription factor, CDX2.

Key words: intestinal epithelium, Hippo pathway, YAP1/TAZ, CDX2, differentiation, SI, MUC2, Src family kinases

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List of abbreviations

AKT	Protein kinase B
AMOT	Angiomotin
APC	Adenomatous polyposis coli
ASCL2	Achaete-scute complex homolog 2
ATOH1	Atonal homolog 1
BCL2	B-cell lymphoma 2
BM	Basement membrane
BMI1	B cell-specific Moloney murine leukemia virus integration site 1
BMP	Bone morphogenic protein
BSA	Bovine serum albumin
CBC	Crypt based columnar
CDK	Cyclin-dependent kinase
CDX	Caudal type homeobox
CHGA	Chromogranin A
CK1	Casein kinase 1
CRC	Colorectal carcinoma
CTGF	Connective tissue growth factor
CYR61	Cysteine rich angiogenic inducer 61
DAPI	4’,6-diamidino-2-phenylindole
DEFA	Alpha-defensin
DLL	Delta-like ligands
DMSO	Dimethyl sulfoxide
dNTP	Deoxynucleoside triphosphates
DPPIV	Dipeptidyl peptidase IV
ECL	Enhanced chemiluminiscence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EECs	Enteroendocrine cells
EGF	Epithelial growth factor
EGFR	Epidermal growth factor receptor
EPCAM	Epithelial cellular adhesion molecule
FAK	Focal adhesion kinase
GATA4	GATA binding protein 4
GFP	Green fluorescent protein
GI	Gastrointestinal
GPCR	G-protein coupled receptor
GSK3β	Glycogen synthase kinase 3 beta
HCL	Hydrochloric acid

HDAC	Histone deacetylase
HES1	Hairy enhancer of split 1
HH	Hedgehog
HNF1α	Hepatocyte nuclear factor 1-alpha
HNF4α	Hepatocyte nuclear factor 4-alpha
HRP	Horseradish peroxidase
IF	Immunofluorescence
JAG1	Jagged canonical Notch ligand 1
KLF4	Krüppel-like factor 4
LATS1	Large tumor suppressor kinase 1
LATS2	Large tumor suppressor kinase 2
LGR5	Leucine-rich repeat-containing G-protein-coupled receptor 5
LYZ	Lysozyme
MeOH	Methanol
MG132	Carbobenzoxy-l-leucyl-l-leucyl-l-leucinal
MMP7	Matrix metalloproteinase 7
MOB1	Monopolar spindle-one-binder protein 1
MST1	Mammalian STE20 kinase 1
MST2	Mammalian STE20 kinase 2
MTX	Methotrexate
MUC2	Mucin 2
NF2	Neurofibromatosis type II
NICD	Notch intracellular domain
OCT	Octamer-binding transcription factor 4
PBS	Phosphate buffer saline
PFA	Paraformaldehyde
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PP2	Pyrazolopyrimidine
PRC2	Polycomb repressive complex 2
PROM1	Prominin 1
qPCR	Quantitative polymerase chain reaction
REGIIIA	Regenerating family member 3 alpha
RNF43	Ring finger protein 43
RPLPO	Ribosomal protein large P0
RT-PCR	Reverse transcription polymerase chain reaction
RUNX3	Runt-related transcription factor 3
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SFKs	Src family kinases
SH3	Src homology 3 domain
SI	Sucrase isomaltase
SMAD	Small mothers against decapentaplegic

SMOC2	SPARC related modular calcium binding 2
SOX9	Sex determining region Y (SRY) box containing gene 9
sPLA2	Secretory phospholipase A2
SUZ12	Suppressor of zeste 12
TAZ	Transcriptional co-activator with PDZ-binding motif
TCF4	Transcription factor 4
TD	Terminal differentiation
TEADs	TEA domain family members
TEM	Transmission electron microscopy
TFF3	Trefoil factor 3
TGF-α	Transforming growth factor alpha
TGF-β	Transforming growth factor beta
VGLL4	Vestigial-like family member 4
VP	Verteporfin
WB	Western blot
WNT	Wingless/Int-1
WWTR1	WW domain-containing transcription regulator 1
YAP1	Yes-associated protein
ZNRF3	Zinc and ring finger 3

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1. Introduction

1.1. Mammalian gastrointestinal tract

From past to present, gastrointestinal (GI) epithelia have been interesting because of their regenerative properties. The GI tract which has a 200-300 m^2 mucosal surface area in the adult human includes the esophagus, stomach, small and large intestines with glands and other related tissues including the liver, gallbladder, pancreas, salivary glands and the oral cavity. The small and large intestines are important parts of the GI tract and are the focus of this study. The small intestine with a length of about 3 meters is composed of three parts including the duodenum, jejunum and ileum, and plays an important role in terminal digestion and absorption of nutrients, and protection against pathogens (S. Fallah *et al.*, 2020). The large intestine is the last segment of the GI tract, which has lost its ability for sugar, amino acid and lipid absorption and plays a pivotal role in the absorption of water, electrolytes and compaction of the feces. It consists of the cecum, appendix, colon, rectum and anal canal (Daulagala *et al.*, 2019; S. Fallah *et al.*, 2020). The mammalian intestinal wall is composed of four layers including the mucosa, submucosa, muscular layer and the adventitia or serosa. The mucosa consists of the epithelium, lamina propria and muscularis mucosa. The epithelial basement membrane (BM) or basal lamina which is in direct contact with the epithelium is composed of various components including laminin, type IV collagen, nidogen and proteoglycans. The BM plays an important role in proliferation, migration and differentiation of epithelial cells (Beaulieu, 1997). The lamina propria which is separated from the epithelium by the basal lamina, is composed of connective tissue and is located at the core of the villus and surrounds the crypt (Boudry, 2004). The muscularis mucosa is a continuous thin layer of smooth muscle which separates the lamina propria from the submucosa (Heath, 1999). The submucosa is mainly constituted of connective tissue which supports the mucosa and links the muscular layers to the mucosa. The muscular layers include the circular and longitudinal muscles and provide the peristalsis movement of the

gut. The adventitia that secretes serous fluid is composed of the visceral membrane and the parietal layer (Kong *et al.*, 2018).

1.2. The development of the gastrointestinal tract

The endoderm and mesoderm develop into the GI tract. Parenchymatous cells of the GI tissues originate from the endoderm. The endoderm is one of the three principal germ layers and its differentiation into epithelial cells is regulated by various soluble factors (Wells & Melton, 1999). The posterior endoderm, which provides epithelial cells for the small and large intestines, is patterned by the expression of the CDX2 transcription factor. However, the specific factors that are important in gastrulation remain unknown (Noah *et al.*, 2011). Smooth muscle cells, myofibroblasts, neurons, immune cells, endothelial cells, lymphatics and extracellular matrix molecules originate from the visceral mesoderm. Several signaling pathways such as the bone morphogenic protein (BMP), hedgehog (HH), platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β) and Wnt as well as the integrin-extracellular matrix (ECM) which participates in epithelial-mesenchymal interactions, play an important role in GI development (Beaulieu, 1997; McLin *et al.*, 2009; Ménard, 2005).

The first sign of differentiation can be observed at the time of the formation of the villus (Beaulieu, 1997; Ménard, 2005). During the formation of the mammalian intestinal epithelium, villi appear in both the small and large intestines. Before villus formation, scattered proliferating cells are observed throughout the endoderm but after formation of the villi, the proliferating zone is limited to the intervillus area (Beaulieu, 1997). The crypts of Lieberkühn are formed by the invagination of the intervillus regions between 16 and 18 weeks of gestation in the human and contain the proliferating cells. The epithelium of the large intestine is similarly organized until 30 weeks when the villi disappear and are replaced by the crypts. Therefore, the mucosa in the adult large intestine is composed of the crypts with depth of about 0.5 mm (Sepideh Fallah *et al.*, 2020; Ménard, 2005). The

BMP proteins which are expressed by the mesenchyme and bind to receptors on villus epithelial cells are effective in crypt formation. Ectopic crypt-villus units have been observed with an abnormal expression of the BMP inhibitor Noggin in the embryonic intestinal epithelium (Haramis *et al.*, 2004). Sonic hedgehog (SHH) and Indian hedgehog (IHH) which are expressed in the epithelium, bind to their receptors patched 1 and patched 2 in the mesenchyme (Madison *et al.*, 2005). HH signaling from the epithelium to the mesenchyme plays an important role in the development of connective tissue around of the gut and epithelium during embryonic gut development. Disruption of these two members of the hedgehog family is accompanied by several abnormalities in the mouse GI including a reduction in stem cell proliferation and differentiation, absence of villi, obstruction of the duodenum, malrotation of the gut and imperforate anus (Madison *et al.*, 2005; Ramalho-Santos *et al.*, 2000). PDGF which is also important in GI development, is expressed by epithelial cells and binds to its receptor PDGFR in the mesenchyme. Malformation and reduction of villi and loss of the pericryptal mesenchyme have been observed through PDGF-A or PDGFR-alpha abolition in the mouse (Karlsson *et al.*, 2000). It has been shown that canonical Wnt activity is barely detectable in intervillus cells, the precursors of crypt cells, in the fetal mouse and its activity only starts 3 days after birth. However, lack of TCF4, one of the Wnt effectors, results in the depletion of intestinal intervillus cells during mouse intestinal development (Ménard, 2005). The human intestinal BM, which separates the intestinal epithelium from the underlying connective tissue, contains several components. Type IV collagen, laminins and integrins, which are the BM components, play an important role during intestinal development (Ménard, 2005). The α5 and α6 collagen (IV) chains have been identified in the human epithelium and are generated by both the epithelium and the mesenchyme. The α6 chain is produced in all stages of intestinal development, while α5 chain expression is downregulated during adulthood (Ménard, 2005). Laminin-1, the predominant glycoprotein in the BM, has been detected in the intervillus zone from 8 to 10 weeks of gestation, and is replaced by laminin-2 after crypt formation. Various integrins have been identified to express at different parts of the intestinal epithelium during development. For example, α9β1 is detected in the lower part

of the crypt and laminin binding integrin α7B has been found to be expressed in the villus at 12 weeks, but is restricted to the upper part of the crypt and lower part of the villus after mid-gestation (Ménard, 2005).

1.3. Gastrointestinal epithelium

The mucosa that forms the inner layer of the intestinal tract is covered by a single layer of epithelial cells. The epithelium of the small intestine is divided into the crypts that are formed by their invagination into the underlying mesenchyme and villi that protrude into the lumen. As shown in Figure 1, the human intestinal crypt is divided into three sections including the lower stem/Paneth cell compartment, the middle transit amplifying (TA) zone and the upper terminal differentiation (TD) zone (Roostaee *et al.*, 2016). The small intestine contains about 10 million villi and 60 million crypts, which means that each villus is supported by six crypts (S. Fallah *et al.*, 2020). Furthermore each villus is covered by about 1000 microvilli (Pelaseyed & Hansson, 2020), approximately 1µM in length and 0.1µM in diameter, which provide the maximum surface for nutritional digestion and absorption (Ensari & Marsh, 2018).

The intestinal epithelium is composed of stem cells, proliferating cells and post mitotic differentiated cells, which are divided into absorptive and secretory lineages. Proliferating cells include absorptive (AP) and secretory progenitor (SP) cells. Goblet, Paneth and enteroendocrine cells have been identified as among the secretory lineages. It should be mentioned that Paneth cells are absent in the normal colon. About 80 percent of the total number of enterocytes (small intestine) and colonocytes (colon) are absorptive cells (S. Fallah *et al.*, 2020). In addition, there are other cell types named cup, tuft and M cells that have not yet been classified as secretory or absorptive cells. Tuft cells are chemosensory cells that are characterized by long apical microvilli and abundant apical vesicles (Ting & von Moltke, 2019). Cup cells contain a shorter brush border with linear arrays of particles in their microvillus membrane. The exact function of tuft and cup cells is unknown (Gerbe

et al., 2012). Tuft cells are effective against helminths and M cells that play a pivotal role in the presentation of pathogens to the immune system (Allaire *et al.*, 2018).

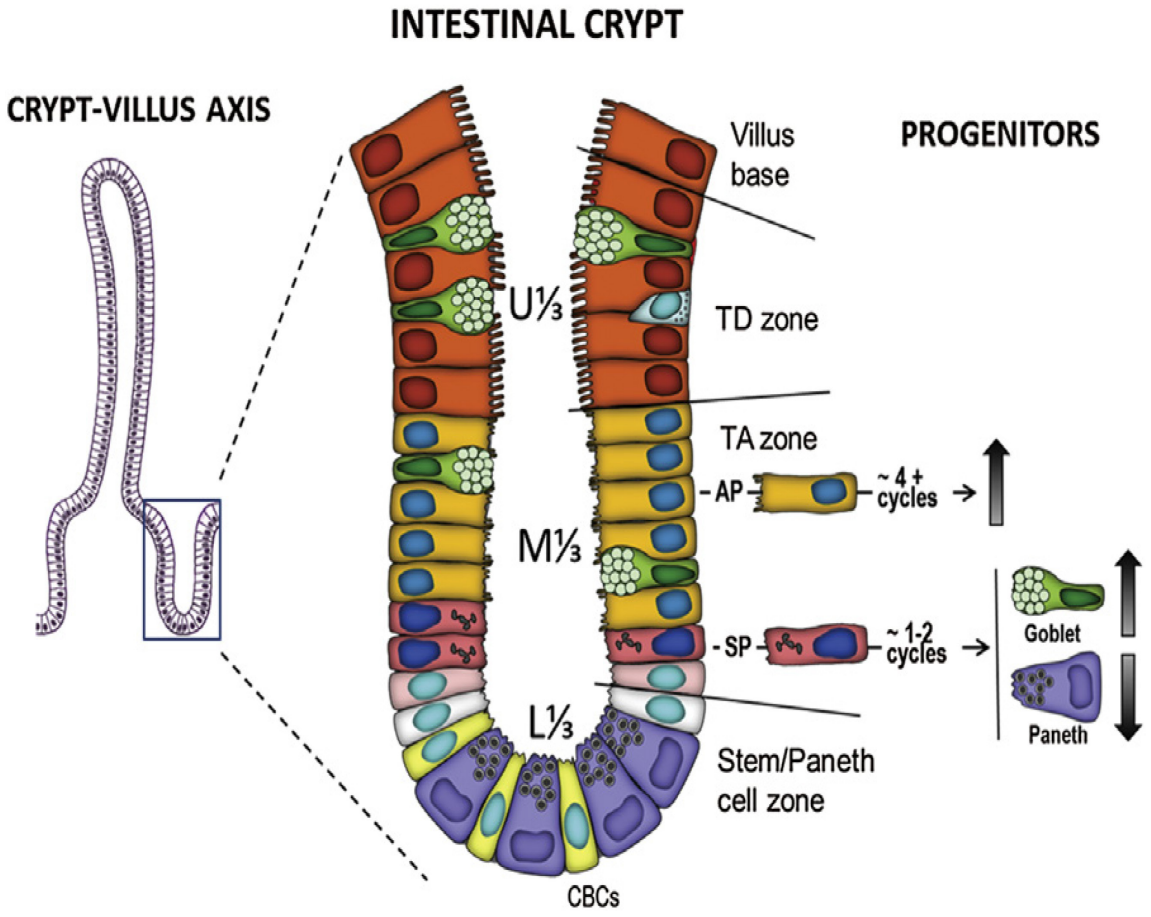


Figure 1. Stem, progenitor and differentiated cells in the human small intestine.

The crypt is divided into three parts named the stem/Paneth cell zone, the transit amplifying (TA) zone and the terminal differentiated (TD) zone. Crypt base columnar (CBC) stem cells (LGR5 positive) and +4 cells (BMI1 positive) are located at the base of the crypt in positions +1 to +4. Transit amplifying cells which include absorptive (AP) and secretory progenitor (SP) cells, are positioned above the stem cells and are capable of differentiating into absorptive, goblet, Paneth and enteroendocrine cells. Post-mitotic Paneth cells are intermingled between the stem cells and other differentiated cells, and are located along the villus. Used from (Roostaee *et al.*, 2016) with permission.

1.3.1. Goblet cells

Goblet cells are glandular epithelial cells which constitute 8%-10% of the total epithelial cells, and secrete highly glycosylated gel-forming mucins like MUC2 and trefoil factor 3 (TFF3) in the small intestine and colon (Umar, 2010). In the small intestine mucus covers the intervillus space and villus tips while in the colon mucus forms two inner and outer thick layers at the inner surface of the epithelium. The inner mucus layer of the colon, which is of 200-300 µm thick and firmly attached to the epithelium, is generated by surface goblet cells. The inner mucus layer with its compact structure is almost sterile, while the outer mucus layer has a looser structure and contains the intestinal bacteria. Bacteria in the colon degrade dietary substrates (plant polysaccharide) which cannot be degraded by the small intestine and produce absorbable amino acids and vitamins. Some species of bacteria can degrade mucus glycans and use them as an energy source (Li *et al.*, 2015). The outer mucus layer is not firmly attached and has a net-like structure. It is produced by host protease activity from the inner mucus layer. Mucus limit access of bacteria and other microorganisms to the epithelium and the Payer's patches (Pelaseyed *et al.*, 2014). It should be mentioned that mucus formation is slow and its accumulation in granules takes more than 4-5 hours (Pelaseyed *et al.*, 2014). TFF3 that is produced and secreted by goblet cells stabilizes the mucus layer by interacting with polysaccharide chains of MUC2 (Braga Emidio, Baik*, et al.*, 2020)s. In addition, it plays a role in protecting and healing the epithelium (Aihara *et al.*, 2017; Braga Emidio, Brierley*, et al.*, 2020). An increase in viscosity and elasticity of a mucin solution as well as formation of gel-like structures has been observed by the addition of the TFF3 dimer to a mucin solution (Thim *et al.*, 2002).

1.3.2. Enteroendocrine cells

Enteroendocrine cells (EECs) constitute less than one percent of the intestinal epithelial cell population. They produce and secrete various hormones at their base which reach the blood stream. EECs are divided into three main types including pyramidal shaped EC cells that are the most common EEC cells and secrete serotonin (5-HT), D cells that produce

somatostatin and finally flask shaped L cells that secrete proglucagon-derived peptides and peptide YY. Two kinds of secretory vesicles have been found in EECs, the large dense-core vesicles and the smaller synaptic-like microvesicles. Intestinal EECs are characterized by chromogranin A (CHGA) which is localized in secretory vesicles (Gunawardene *et al.*, 2011).

1.3.3. Paneth cells

More than century ago, columnar epithelial cells filled with prominent granules were identified in the small intestine by Gustave Schwalbe and Josef Paneth. These pyramid-shaped epithelial cells were termed Paneth cells. Their anatomical specification shows a short brush border (microvilli) on the apical surface with an extensive endoplasmic reticulum and Golgi network and apical clustering of secretory granules. Paneth cells are localized between the stem cells at the base of the crypt. The number and function of Paneth cells in the human intestine is at a maximum in early adult life and decreases after 70 years of age (Sandow & Whitehead, 1979). Several proteins and peptides with antimicrobial, antiviral and antifungal activity are generated and secreted by Paneth cells. Some of these significant peptides in the human include lysozyme (LYZ), secretory phospholipase A2 (sPLA2), regenerating family member 3 alpha (RegIIIA) and the enteric α-defensins (DEFA5 and DEFA6 also known as HD5 and HD6). It has been shown in mouse that several stimulants including acetylcholinergic agonists, bacterial cell surface products and toll-like receptor agonists stimulate the release of these peptides into the intestinal lumen through exocytosis (Ayabe *et al.*, 2002; Satoh *et al.*, 1995; Yokoi *et al.*, 2019). Some researchers believe that a biphasic increase in cytosolic Ca^{2+}, which is regulated by calcium-activated potassium (KCNN4) channels, is involved in this process in mouse (Ayabe *et al.*, 2002; Satoh *et al.*, 1995). DEFAs known as cryptdins in the mouse, are the most important antimicrobial peptides, and are effective in the formation of antimicrobial composition and protection against pathogens in the intestine. DEFA5 and DEFA6 kill pathogens using different methods. DEFA5 molecules interact with microbial membranes through their special structure, which is composed of positively charged amino-acid side chains and hydrophobic amino-acid side chains. Their interaction with the microbial membrane leads

to the formation of pores and membrane disruption that results in microbial death (Ganz, 2003). DEFA6 surrounds and entangles bacteria through stochastic binding to their surface proteins and formation of fibrils and nanonets (Chu *et al.*, 2012). DEFAs are produced as a premature form and need proteolytic processing to be mature and activated. This process in mice is different than in humans. In mice pro-α-defensins which are named pro-cryptdins are cleaved and activated by matrix metalloproteinase7, while in humans pro-α-defensins are processed by trypsin enzymes. Besides of the releasing of trypsinogen by pancreas, trypsin enzymes are also expressed and stored as inactive form by Paneth cells and are activated after secretion in the human intestinal lumen, which is associated to maturation of DEFA5. Therefore, the maturation and activation of DEFA5 and DEFA6 occur after their release from granules (Bevins & Salzman, 2011; Chairatana & Nolan, 2017; Ghosh *et al.*, 2002). In the mouse the mechanism of cryptdin activation is like human. Cryptdins are cleaved and activated after secretion into the intestinal lumen (Sankaran-Walters *et al.*, 2017). Lysozyme is an enzyme that has an antimicrobial function by cleaving the link between N-acetylglucosamine and N-acetylmuramic acid in the peptidoglycan component of bacterial cell walls, which results in bacterial death (Oliver & Wells, 2015). The secretory PLA2 enzymes catalyze the hydrolysis of phospholipids in the bacterial envelop (Weiss, 2015).

1.3.4. Absorptive cells

Absorptive cells are simple columnar epithelial cells which are characterized by a brush border on their apical surface to increase the surface for additional membrane-associated digestion and absorption of nutrients, vitamins, ions and water. They also form most of the physical barrier between the lumen and the interior (S. Fallah *et al.*, 2020; Kong *et al.*, 2018). Absorptive cells produce sucrase isomaltase (SI) and maltase glucoamylase enzymes that are used as markers for absorptive cell characterization (Beaulieu, 1997). SI is an absorptive cell specific α-glucosidase enzyme that cleaves disaccharides and oligosaccharides to monosaccharides to make them absorbable (Gericke *et al.*, 2017). Dipeptidyl peptidase IV (DPPIV)/CD26 is also expressed on the luminal surface of absorptive cells and cleaves

dipeptides from the N-terminus of polypeptides that contain proline or alanine in their penultimate position (Lammi *et al.*, 2018). In the human intestinal epithelium, some absorptive cell markers including DPPIV, aminopeptidase N and the immature form of SI have been detected in the crypt epithelium (Beaulieu *et al.*, 1989; Gorvel *et al.*, 1991; Quaroni *et al.*, 1992), suggesting the presence of immature absorptive cells in the crypt.

1.3.5. Stem/progenitor cells and the niche

With the exception of Paneth cells which have an at least 3-week turnover rate, all of the other differentiated cells are replaced every 3-5 days. Self-renewal of the intestinal epithelium is supported by continuous division of the intestinal long-lived multipotent crypt based columnar (CBC) stem cells, which are located at the base of the crypt and intermingled with Paneth cells (Andersson-Rolf *et al.*, 2017; Spit *et al.*, 2018; van der Heijden & Vermeulen, 2019). The most significant marker of the CBC stem cell is the leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5) and other markers include olfactomedin 4 (OLFM4), promonin 1 (PROM1), SPARC related modular calcium binding 2 (SMOC2), pleckstrin homology-like domain family A member 1 (PHLDA1), octamer-binding transcription factor 4 (OCT4), cluster of differentiation 44 (CD44), epithelial cellular adhesion molecule (EPCAM) and achaete-scute complex homolog 2 (ASCL2) (Guezguez *et al.*, 2014; Roostaee *et al.*, 2016). Progenitor or transit amplifying cells and new stem cells are the progeny of symmetric and asymmetric division of stem cells. Therefore intestinal stem cells have the potential to self-renew, and generate stem cells and all of the differentiated and undifferentiated intestinal epithelial cells (Baker & Graham, 2014; Sei *et al.*, 2019). Progenitor cells that undergo additional proliferation cycles before differentiation and maturation are divided into the two populations of secretory and absorptive progenitor cells. Absorptive progenitors proliferate and give rise to absorptive lineages, while secretory progenitors have limited divisions (around 1-2) and finally differentiate into secretory lineages (Roostaee *et al.*, 2016). During differentiation and maturation enterocytes, goblet and enteroendocrine cells migrate upward to the villus and

finally undergo anoikis upon reaching the villus tip but Paneth cells migrate to the bottom of the crypts of Lieberkühn and undergo degeneration and phagocytosis by mononuclear cells (Sandow & Whitehead, 1979). Another population of intestinal stem cells is the quiescent or label-retaining stem cell that is situated in zone +4 of the crypt above the Paneth cells. They are recognized by BMI1 as a prominent marker. It is believed that +4 stem cells act as reserve stem cells, however their identification and action is not completely clear and further studies are needed. In response to injury caused by radiation, chemotherapy or pathogens, and loss of $LGR5^{+}$ stem cells, this reserve stem cell population is activated to repair the epithelium and maintain epithelial integrity (Santos *et al.*, 2018). Renewal of stem cells is supported by the niche, which is a microenvironment located everywhere stem cells reside. Niche signals originate from epithelial cells and the surrounding mesenchyme. Several researchers have demonstrated that Paneth cells also provide the niche for stem cells. They have shown that the ablation of Paneth cells results in a reduction of stem cells showing a stem cell dependency on Paneth cells (Schuijers & Clevers, 2012). Wnt3A, epithermal growth factor (EGF), transforming growth factor alpha (TGF-α) and the Notch ligands, delta-like ligands 1 and 4 (Dll1 and Dll4), are produced by Paneth cells and act as niche factors for stem cells. However some of these factors including Wnt also are expressed by extra epithelial sources (Schuijers & Clevers, 2012). The niche is important in maintaining the balance of stem cell proliferation and differentiation in order to prevent neoplastic overgrowth created by disturbing turnover balance (Umar, 2010). Therefore the niche supports stem cell function by providing soluble, adhesive and physical signals which originate from the stem cells themselves, the neighboring niche cells, the ECM and other tissues (Beaulieu, 1997; Chacón-Martínez *et al.*, 2018). Once the stem cells leave the niche, they receive signals for differentiation and maturation.

1.4. Transcription factors and signaling pathways involved in intestinal epithelial cell proliferation and differentiation

Previous studies have introduced various molecular pathways and transcription factors involved in stemness and differentiation in the intestinal tract. Wnt/β-catenin and Notch signaling are two well-known reported pathways in stem cell maintenance and differentiation in the crypt-villus axis of the intestinal epithelium (Spit *et al.*, 2018; Tian *et al.*, 2015). The Hippo signaling pathway has been recently identified as an important pathway in the regulation of intestinal epithelial cell proliferation and regeneration. However, their exact role in stemness and the differentiation of intestinal epithelial cells is not completely understood. The role of some transcription factors in epithelial cell differentiation has also been studied previously. The function of some of these pathways and transcription factors is reviewed here briefly.

1.4.1. Transcription factors

CDX2 is a caudal homeobox 2 transcription factor that regulates the proliferation and differentiation of intestinal epithelial cells. Ectopic expression of CDX2 in undifferentiated adult rat crypt IEC-6 cells resulted in impaired proliferation and formation of absorptive-like and goblet-like cells (Suh & Traber, 1996). Conditional expression of CDX2 in normal human intestinal crypt HIEC-6 cells leads to a reduction in cell proliferation and an increase in DPPIV expression (Escaffit *et al.*, 2006). In addition, CDX2 plays a role during embryonic development by activating the expression of trophectoderm genes and repressing the pluripotent program and OCT4 expression during blastocyst formation (Huang *et al.*, 2017). In the adult mouse, CDX2 is expressed in most epithelial cells throughout the crypt-villus except the epithelial cells located in the stem/Paneth cell compartment (James *et al.*, 1994). CDX2 regulates the absorptive cell marker SI promoter and induces the expression of SI (Boudreau *et al.*, 2002b). Down regulation of CDX2 in colorectal cancers and poor prognosis of colorectal cancer patients with low expression of CDX2 have been reported (Sandberg *et al.*, 2019). Krüppel-like factor 4 (KLF4) is a zinc finger transcription factor which is mostly

detected in differentiated intestinal epithelial cells (T. Yu *et al.*, 2012). Its roles in the reprogramming of embryonic stem cells, homeostasis of the intestine and suppression of colorectal cancer have been reported previously (T. Yu *et al.*, 2012). It plays an important role in goblet cell differentiation in the colon while it has a minimal effect on small intestinal goblet cells (Katz *et al.*, 2002). Hepatocyte nuclear factor 1-alpha (HNF1α) is a homeobox transcription factor that is expressed in the liver, kidney, pancreas and intestine. Knockout of HNF1α in mice results in increased crypt proliferation, a decreased number of enteroendocrine cells, altered Paneth cell maturation and finally a disturbance of intestinal epithelial cells during adulthood (Lussier *et al.*, 2010). Hepatocyte nuclear factor 4 alpha (HNF4alpha or NR2A1) is a member of the nuclear receptor family of ligand-dependent transcription factors, which is provided by the HNF4A gene. HNF4α which is expressed in certain organs such as the liver, kidney, pancreas and intestine, plays an important role in differentiation and development (Garrison *et al.*, 2006; Lussier *et al.*, 2008). HNF4α is required for the development of the colon in mouse models and its ablation leads to the aberration of crypts, disruption of goblet cell maturation and disruption of the genes required for normal function of the colon (Garrison *et al.*, 2006). This transcription factor is expressed in the human colon crypt and is associated with epithelial cell proliferation and differentiation. HNF4α has 12 isoforms that are divided into two isoform classes, P1 and P2. P1 isoforms are connected to epithelial cell differentiation, however P2 isoforms are connected to epithelial cell proliferation at the lower part of the human colon (Babeu *et al.*, 2018). Another transcription factor that has been reported as a significant factor in intestinal epithelial cell differentiation is GATA binding protein 4 (GATA4). GATA4 is a zinc finger transcription factor that was initially identified in the heart. It is also expressed in the reproductive system, lung and liver as well as the small intestine (Molkentin, 2000). GATA4 is expressed in the villus in the mouse small intestine, however its expression in the colon has not been detected (Boudreau *et al.*, 2002b). CDX2 with GATA4 and HNF1α participate in the regulation of the absorptive cell marker SI during differentiation (Benoit *et al.*, 2010; Boudreau *et al.*, 2002b).

1.4.2. The Wnt/β-catenin signaling pathway

The Wnt/β-catenin pathway, which is activated by binding to the frizzled and lipoprotein receptor-related protein-5/6 receptors (LRP5/6), has its highest activity at the base of the crypt (Tian *et al.*, 2015). B-catenin is the key effector responsible for transduction of signals to the nucleus in the canonical Wnt signaling pathway, which undergoes phosphorylation, ubiquitination and proteasomal degradation in the absence of the Wnt ligand. The phosphorylation of β-catenin is associated with the AXIN destruction complex, which is composed of scaffolding protein AXIN, casein kinase 1 (CK1), adenomatous polyposis coli (APC) and glycogen synthase kinase-3 beta (GSK3β). Phosphorylated β-catenin is recognized by a component of an E3 ubiquitin ligase beta-transducin repeats-containing proteins (β-TrCP) for ubiquitination that makes it susceptible to proteasomal degradation. When the Wnt pathway is active, the destruction complex is kept inactive by binding to frizzled and LRP5/6 receptors, β-catenin transfers to the nucleus, forms a complex with transcription factor 4 (TCF-4) and finally activates the transcription of Wnt target genes (Czerwinski *et al.*, 2018) (Figure 2). The Wnt/β-catenin signaling pathway is negatively regulated by APC and the E3-ubiquitin ligase zinc/RING finger protein 3 (ZNRF3) and RING finger protein 43 (RNF43) (Czerwinski *et al.*, 2018). A key component of the β-catenin destruction complex APC regulates the stability of β-catenin coactivator that is required for the transcriptional activity of Wnt target genes. In most colorectal cancers, mutation in APC results in APC truncation and loss of multiple β-catenin binding sites (Parker & Neufeld, 2020). ZNRF3 and RNF43 are Wnt target genes that constitute the negative Wnt feedback loop. These two transmembrane E3 ligases induce the degradation of the Wnt receptor Frizzled by multiubiquitination of lysines in the cytoplasmic loops of the 7 transmembrane (7TM) domain which leads to the downregulation of Wnt signaling (Koo *et al.*, 2012). It has been shown that ZNRF3 and RNF43 are expressed in $LGR5^+$ stem cells and their knockdown in the mouse intestine results in the formation of adenomas containing a high number of Paneth and $LGR5^+$ stem cells (Koo *et al.*, 2012). On the other hand, the activity of the ZNRF3/RNF43 is negatively regulated by R-spondins that are agonists of Wnt signaling. LGR5 and its homologs LGR4 and LGR6 act as receptors for R-spondins and their binding with R-spondins

induces Wnt signaling activity. R-spondins simultaneously bind to the extracellular domains of ZNRF3/RNF43 and LGR4/5/6 receptors, and induce auto-ubiquitination of ZNRF3/RNF43, which leads to the removal of the ZNRF3/RNF43 transmembrane proteins and an increase in the surface level of the Frizzled receptors (Hao *et al.*, 2016). The Wnt pathway plays a significant role in the proliferation and homeostasis of the intestine and the differentiation of Paneth cells (Gregorieff & Clevers, 2005; Pin *et al.*, 2012). The Wnt ligands are mainly provided by Paneth cells and underlying stromal cells surrounding the progenitor/stem cell compartments and bind to a complex receptor to promote the proliferation of cells by transcriptional activation of Wnt target genes (Zou *et al.*, 2018). A low level of Wnt signaling results in a rise of stem cells towards absorptive, goblet and enteroendocrine cells while Paneth cell differentiation needs a high level of Wnt activity (Pin *et al.*, 2012). Interestingly, Wnt stimulation triggers CBC stemness marker expression in HIEC-6 confirming the importance of this pathway for CBC cells (Guezguez *et al.*, 2014).

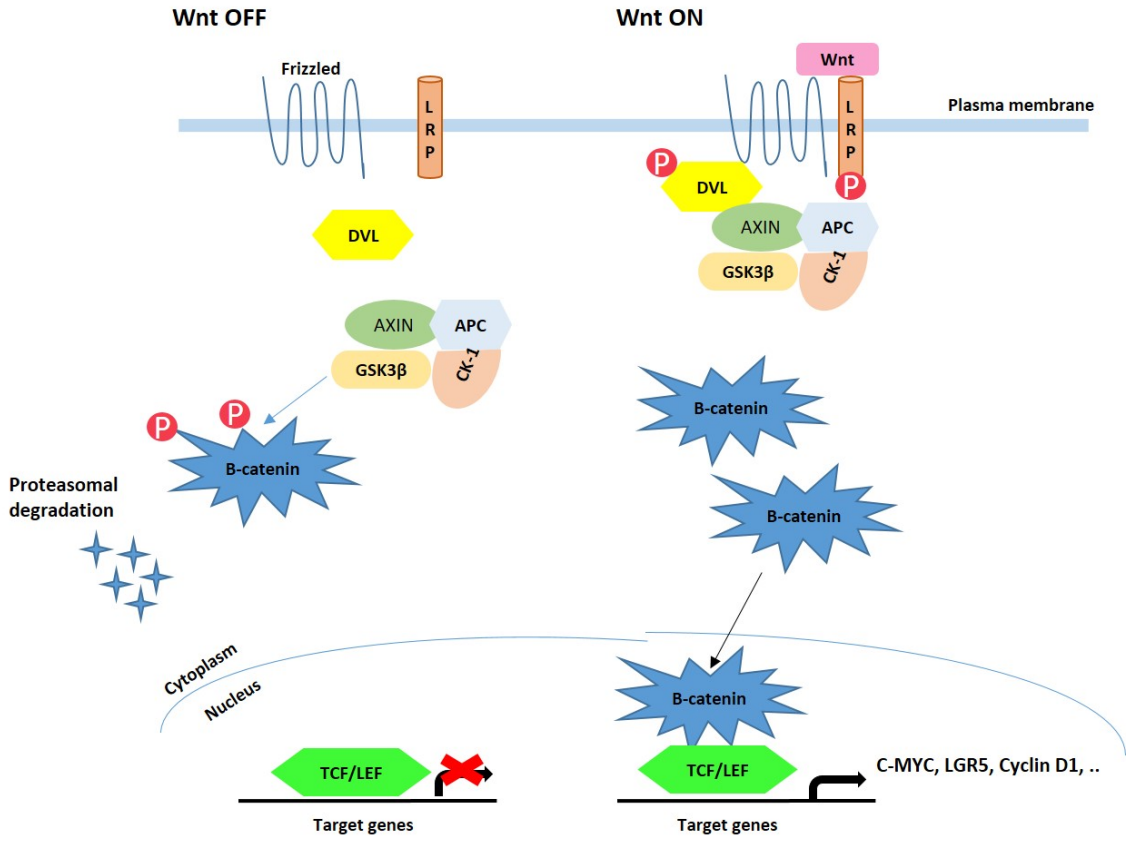


Figure 2. Wnt/β-catenin signaling pathway

A: In the absence of Wnt activity, β-catenin is phosphorylated by a destructive complex that is composed of Axin, APC, DVL, CK1 and GSK3β, and is recognized by β-TrCP for ubiquitination and proteasomal degradation. **B:** In the presence of the Wnt ligand, CK1 and GSK3β bind and phosphorylate the LRP receptors. Therefore, free β-catenin accumulates in the cytoplasm, transfers to the nucleus and activates the transcription of Wnt target genes.

1.4.3. The Notch signaling pathway

Another pathway participating in stemness and epithelial cell differentiation is the Notch signaling pathway, which is activated by the binding of Notch receptors on one cell to the jagged 1/2 (JAG1/2) and delta like ligands (DLLs) on adjacent cells (expressed by secretory cells) and induces hairy enhancer of split 1 *(HES1*) expression. In the Notch pathway, the four Notch1, 2, 3 and 4 receptors, and the five ligands DLL1, 3 and 4 and JAG1 and 2 have been identified in vertebrates. It has been reported that DLL1 and DLL4 are expressed by ATOH1 positive intestinal epithelial cells located adjacent to HES1 positive cells. Furthermore, DLL1 and 4 positive cells express the goblet cell marker MUC2 (Shimizu *et al.*, 2014). In addition, a high level of DLL4 is expressed by Paneth cells in a mouse model (Sato *et al.*, 2011). Notch1, and Jag1 and 2 ligands are expressed in the lower third of the crypts, however Notch2 is expressed in a few crypt cells (Sander & Powell, 2004). HES1, a basic helix-loop-helix transcription factor, induces differentiation of the absorptive cell lineage via inhibition of ATOH1 expression (Goodell *et al.*, 2015). HES1 expression suppresses the ATOH1 gene through directly binding to the 5’ promoter region of ATOH1 (Zheng *et al.*, 2011). Therefore the Notch pathway plays a significant role in the determination of a progenitor cell’s fate towards the absorptive or secretory lineages (Shroyer *et al.*, 2005) (Figure 3). Previous studies have reported a role for Notch in stem cell self-renewal and intestinal epithelial cell proliferation and homeostasis. They have shown that the deletion of the Notch receptors Notch 1 and Notch 2 in the mouse resulted in a reduction of proliferative stem/progenitor cells in the intestinal epithelium (Carulli *et al.*, 2015). In addition, a decrease in stem cell marker OLMF4 expression and the number of $LGR5^{+}$ stem cells has been observed through Notch pathway inhibition (Carulli *et al.*, 2015).

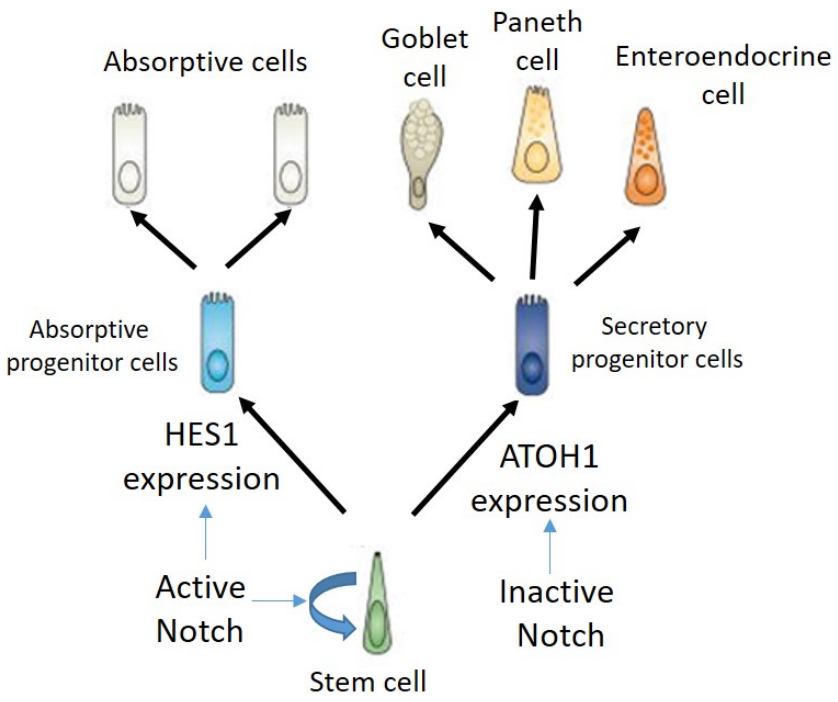


Figure 3. Notch signaling pathway.

When CBC stem cells leave the niche, they receive the signals involved in cell differentiation. One of these signals is Notch signaling which is activated through cell-cell contact. Notch activation induces HES1 expression that stimulates the differentiation of the absorptive cells. In the absence of Notch, ATOH1 expression leads to the differentiation of secretory cells. Adapted from (Goodell *et al.*, 2015).

1.4.4. The Hippo signaling pathway

The Hippo pathway, which is involved in the control of tissue growth and apoptosis was first identified in *Drosophila melanogaster* and later its function in mammals was reported. The Hippo pathway is mostly conserved between Drosophila and mammals (Hong *et al.*, 2016).

1.4.4.1. The Hippo pathway in Drosophila melanogaster

The core of the Hippo pathway in *Drosophila melanogaster* is composed of several proteins and its activation is initiated by Hippo (Hpo) kinase. Hpo kinase binds to the serine/threonine protein kinase Warts (Wts) through its binding partner adaptor protein Salvador (Sav). Then Wts through its binding partner MOB kinase activator-like 1 (Mats) binds to the Yorkie (Yki) co-activator. Finally, Yki binds to the Scalloped (Sd) transcription factor, which promotes the transcription of genes involved in cell proliferation and growth. Originally the Hippo pathway in *Drosophila melanogaster* was identified through an overgrowth of its organ size. Wts was the first component of the core kinase which was identified as a factor involved in cell growth and proliferation (Justice *et al.*, 1995). Mutation or loss of Wts, Hpo, Sav or Mats was identified as an important factor of tissue growth in *Drosophila melanogaster* (Justice *et al.*, 1995; Lai *et al.*, 2005; Udan *et al.*, 2003) .

1.4.4.2. The Hippo pathway in mammals

Hippo pathway components are highly conserved in mammals (Pan, 2007). The pathway is composed of three parts including upstream signals, a kinase core and downstream target genes. This pathway restricts aberrant cell growth through the phosphorylation and inactivation of the Yes associated protein (YAP1) and its paralog transcriptional co-activator with PDZ-binding motif (TAZ) or WW domain containing transcription regulator 1 (WWTR1). Some structural differences have been detected between YAP1 and TAZ. One WW domain is detected in the TAZ structure, however YAP1 has two WW domains. Also, the Src homology 3 (SH3) binding motif and N-terminal proline rich region in the YAP1 structure are not found in TAZ (Kaan *et al.*, 2017). The WW domain in YAP1/TAZ facilitates binding of YAP1/TAZ to the PPxY motif of LATS1 (Hao *et al.*, 2008). Upstream signals like cell-cell contact, cell polarity, mechanical signals such as stiffness and ECM composition, hormonal signals through the G-protein-coupled receptors (GPCRs) and growth factors regulate the Hippo pathway and YAP1/TAZ activity (Meng *et al.*, 2016). Kinase core activity is initiated through mammalian STE20 kinase 1/2 (MST1/2) activation, which phosphorylates and

activates the large tumor suppressor kinase 1/2 (LATS1/2) with the help of the scaffolding protein WW domain-containing adaptor 45 (WW45). According to some researchers, MST1/2 kinases are phosphorylated and activated by TAO kinases (TAOK1/2/3) (Boggiano *et al.*, 2011) and others report auto-phosphorylation of the MST1/2 kinases (Praskova *et al.*, 2004). Phosphorylated LATS1/2 with the regulatory protein MOB1 phosphorylates and inactivates the transcriptional co-activators YAP1 and TAZ (Meng *et al.*, 2016). In addition, some components of the apical-basal polarity complex like angiomotin (AMOT) family proteins can affect YAP1/TAZ activity. YAP1 and TAZ could be sequestered to the tight junction through binding of their WW domain to the PPXY motif of AMOT, which restricts their transcriptional activity (Zhao *et al.*, 2011). Furthermore AMOT proteins can promote LATS1/2 activity to phosphorylate and inactivate YAP1 (Zhao *et al.*, 2011). YAP1 and TAZ are phosphorylated on serine 127 and serine 89 by LATS1/2, respectively, which leads to the cytoplasmic retention of YAP1 (Lei *et al.*, 2008; Zhao *et al.*, 2007). Furthermore, YAP1/TAZ can also be phosphorylated at the S127/S89 residue through protein kinase B (AKT) that leads to their inactivation (Basu *et al.*, 2003). The 14-3-3 proteins, which are specific phosphoserine/phosphothreonine binding proteins, bind to phosphorylated YAP1/TAZ (S127/S89), which leads to its cytoplasmic retention (Dong *et al.*, 2007; Kanai *et al.*, 2000). The 14-3-3 proteins are small scaffolding proteins and are categorized to seven isoforms including β, γ, η, ε, τ, ζ and σ. These proteins are widely expressed in human tissues including the intestine and bind to phosphorylated serines or threonines in target proteins through their conserved RXXp(S/T) XP motif. Different isoforms of 14-3-3 protein exhibit structural similarity and functional overlap (Pennington *et al.*, 2018). The isoforms of the 14-3-3 protein which bind to YAP1/TAZ have remained unspecified. However, it has been reported that transcriptional activity of YAP1 is inhibited through cytoplasmic retention by 14-3-3ζ in gastric cancer. On the other hand, YAP1 overexpression leads to the ubiquitination and subsequent reduction in 14-3-3ζ expression (Zhang *et al.*, 2017). Besides LATS1/2 kinases, casein kinase 1 delta/epsilon (CK1δ/ε) phosphorylates YAP1/TAZ, which has been already phosphorylated by LATS at the S397 and S311 residues, respectively, that contributes to the recognition and ubiquitination of YAP1/TAZ by SCF $^{\beta\text{-TrCP}}$ E3 ubiquitin ligase and proteasomal

degradation (Liu *et al.*, 2010; Zhao *et al.*, 2010). SCF (β-TrCP) recognizes phosphodegron which is a DSGXXS motif in which the serine residues are phosphorylated. Inactivation of the Hippo pathway leads to the entrance of active YAP1/TAZ into the nucleus. Since YAP1/TAZ does not have a DNA binding domain, it binds to DNA through a TEA domain family member (TEADs) transcription factor. The YAP1/TAZ-TEAD complex activates the transcription of genes involved in cell growth and survival including cysteine-rich angiogenic inducer 61 (CYR61), connective tissue growth factor (CTGF) (Hong *et al.*, 2016; Ohgushi *et al.*, 2015), survivin (Dong *et al.*, 2007) and the EGF family member amphiregulin (Yang *et al.*, 2012; J. Zhang *et al.*, 2009) (Figure 4). In the absence of YAP1/TAZ in the nucleus, TEAD binds to vestigial-like family member 4 (VGLL4) in the nucleus and acts as a transcriptional repressor. Active YAP1/TAZ enters the nucleus and competes with VGLL4 for TEAD binding thereby activating TEAD-mediated gene transcription involved in cell growth (Jiao *et al.*, 2014). Besides TEAD, other transcription factors including T-box transcription factor 5 (TBX5), RUNT-related transcription factors (RUNX1/2/3), small mothers against decapentaplegic (SMADs), paired box gene 3 (PAX3), Erb-B2 receptor tyrosine kinase 4 (ErbB4) and P73 also can have an interaction with YAP1/TAZ (Boopathy & Hong, 2019). Knockdown of any member of the kinase core including MST1/2, LATS1/2 and/or MOB1 leads to increased YAP1/TAZ activity, hyper-proliferation and tissue overgrowth (Boopathy & Hong, 2019; D. Zhou *et al.*, 2011).

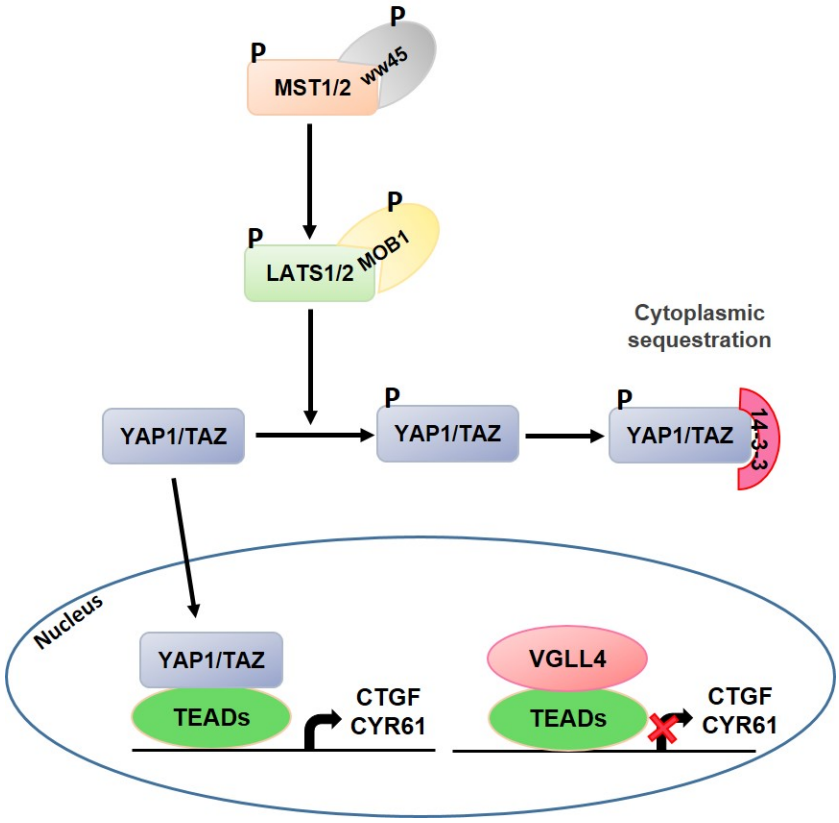


Figure 4. Hippo signaling pathway.

The kinase cascade in the core of the Hippo signaling pathway contains two kinds of serine/threonine kinases, MST1/2 and LATS1/2. Active MST1/2 kinases phosphorylate and activate the LATS1/2 kinases, which phosphorylate and inactivate the transcriptional co-activators YAP1/TAZ. Phosphorylated YAP1/TAZ is recognized by the 14-3-3 protein and is degraded by proteasomes. In the absence of YAP1/TAZ in the nucleus, TEADs bind to VGLL4 and repress the transcription of YAP1/TAZ target genes. When the Hippo pathway is off, active YAP1/TAZ enters the nucleus, forms a complex with TEAD transcription factors and induces transcription of the genes involved with cell growth.

1.4.4.3. Upstream regulatory signals of the Hippo pathway

The Hippo pathway is regulated by several upstream mechanisms and signals. Most of these signals regulate the kinase core phosphorylation cascade, while some regulate YAP1/TAZ directly without affecting the LATS kinases (Meng *et al.*, 2016). Increased cell-cell contacts and increased adherens and tight junctions at cell confluency results in LATS1/2 activation

and YAP1/TAZ inactivation while overexpression of YAP1 results in loss of contact inhibition in a mouse embryonic fibroblast cell line (Zhao *et al.*, 2007). ECM stiffness activates YAP1/TAZ through the FAK–Src–PI3K or Rho-GTPase pathways (Boopathy & Hong, 2019). Mitogen-activated protein kinase kinase kinase kinase (MAP4K) inactivates YAP1/TAZ through the phosphorylation and activation of LATS1/2 (Boopathy & Hong, 2019). MAP4K regulates the phosphorylation of LATS1/2 kinases and YAP1/TAZ in parallel with the MST1/2 kinases. Overexpression of MAP4K4 in wild type, LATS1/2 knockdown and MST1/2 knockdown HEK293A cells showed increased YAP1/TAZ phosphorylation in WT and MST1/2 cells, which indicates that MAP4K4 phosphorylates YAP1/TAZ through an MST1/2-independent manner (Meng *et al.*, 2015). Energy stress caused by a lack of glucose results in LATS1/2 activation and YAP1/TAZ phosphorylation (Meng *et al.*, 2016). In addition, YAP1/TAZ activity can be regulated by several signals mediated by G-protein coupled receptors (GPCRs). The GPCRs represent the largest class of plasma membrane receptors which respond to various physiological ligands and external stimuli. GPCRs are composed of the seven segmented glycoproteins located in the plasma membrane, extracellular and intracellular loops that interact with G proteins in the plasma membrane. It has been reported that active GPCRs via G12/13, Gq/11 and Gi/o-coupled receptors stimulate cell proliferation (Dorsam & Gutkind, 2007). Indeed, GPCRs can inhibit LATS1/2 and activate YAP1/TAZ through G12/13-coupled receptors while activating LATS1/2 and inhibiting YAP1/TAZ through the Gs-coupled receptor (F. X. Yu *et al.*, 2012). It has been reported that YAP1/TAZ can be activated by serum. Lysophosphatidic acid (LPA) and Sphingosine-1-phosphate (S1P) are two components of serum which prominently activate YAP1/TAZ through binding to GPCR and activating the Rho GTPases. They act via G12/13 or Gq/11 coupled receptors which inhibit LATS1/2 kinase activity and activate YAP1/TAZ coactivator. However, serum stimulation has no effect on the protein level of MST1/2 and LATS1/2 kinases (F. X. Yu *et al.*, 2012). In the presence of serum, active and dephosphorylated YAP1/TAZ is localized to the nucleus. However following serum deprivation, YAP1/TAZ is phosphorylated in HEK293A cells except LATS1/2KO HEK293A cells (Plouffe *et al.*, 2018). In the contrary, glucagon and epinephrine inhibit the activity of

YAP1/TAZ by activating of LATS1/2 kinases through the stimulation of Gs-coupled receptor (F. X. Yu *et al.*, 2012). YAP1/TAZ activity is also stimulated by prostaglandin E2 receptor (EP2), a GPCR, which is associated to Gq/11 coupled receptor (Tocci *et al.*, 2021). Furthermore, kidney and brain expressed protein (KIBRA also known as WWC1), a scaffold or adaptor-like protein, and neurofibromatosis type 2 (NF2), a membrane-cytoskeleton adaptor protein regulate the activity of YAP1. KIBRA and NF2 inactivate YAP1 through the phosphorylation of the LATS1/2 kinases without affecting the MST1/2 kinases (Moleirinho *et al.*, 2013; Yin *et al.*, 2013) (Figure 5). In addition, the activity of YAP1/TAZ can be negatively controlled by other regulators that act independently of the Hippo signaling pathway. YAP1 activity is restricted by direct binding of the PPxY domain of non-receptor tyrosine phosphatase 14 (PTPN14) to the WW domain of YAP1 (Liu *et al.*, 2013). On the other hand, PTPN14 can also inactivate YAP1 through an indirect mechanism. PTPN14 in cooperation with KIBRA by binding to its WW domain activates LATS1 which leads to YAP1 phosphorylation and cytoplasmic sequestration (Wilson *et al.*, 2014). Furthermore, AMOT proteins can prevent YAP1/TAZ transcriptional activity through directly binding and localizing it to the tight junctions or through inducing LATS1/2 kinase activity (Zhao *et al.*, 2011). It has been reported that cytoplasmic localization of YAP1 is stimulated by interacting with α-catenin, a key protein of adherens junctions, in keratinocytes (Silvis *et al.*, 2011).

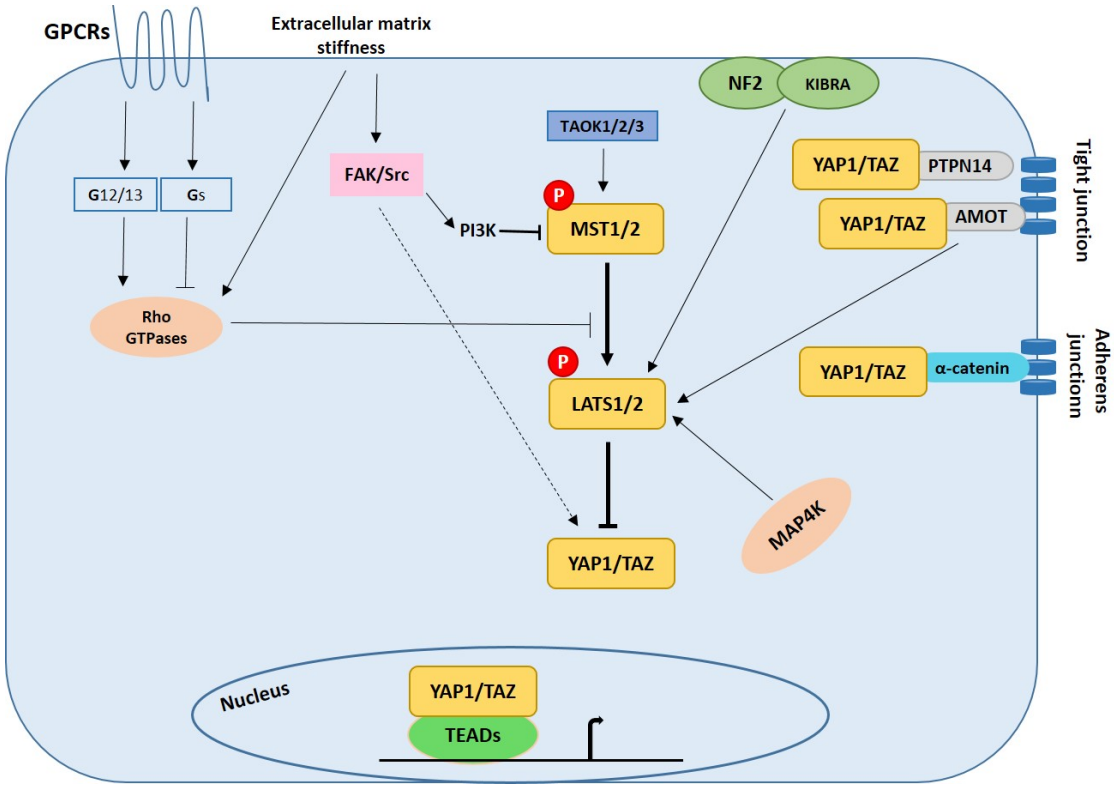


Figure 5. Hippo pathway is regulated by several upstream signals.

The LATS1/2 can be activated or inhibited by GPCRs through Gs and G12/13 proteins, respectively. High stiffness of the extracellular matrix promotes the activity of YAP1/TAZ through inactivation of LATS1/2 by Rho GTPase and/or FAK/Src. NF2, KIBRA, MAP4K and cell junctions regulate the phosphorylation and activation of LATS1/2 which leads to the inactivation and degradation of the YAP1/TAZ coactivator. YAP1/TAZ activity is restricted by sequestration into the tight and adherens junctions which is mediated by PTPN14, and AMOT as well as alpha catenin, respectively.

1.4.4.4. Association of YAP1/TAZ activity with Src family kinases

Src family kinases (SFKs) are membrane-associated non-receptor protein tyrosine kinases which contain nine members including Src, Fyn, Yes, Lyn, Lck, Hck, Fgr and Blk in vertebrates and Frk in invertebrates. In mammals, Src, Fyn and Yes are expressed in most tissues, however other members are expressed in certain cell types, mostly hematopoietic cells (Thomas & Brugge, 1997). SFKs play a key role in intestinal cell proliferation, migration, anoikis and differentiation (Bouchard *et al.*, 2007; Seltana *et al.*, 2013; Thomas & Brugge,

1997). In addition, the inducing role of Src in tumorigenesis and metastasis of various cancers has been reported previously (Irby & Yeatman, 2000). Src activity and protein abundance are elevated in neoplastic ulcerative colitis epithelia and have been reported at a high level in severely dysplastic epithelia (Cartwright *et al.*, 1994). Src is activated through different ways including dephosphorylation of phosphorylated tyrosine 527 (pY527) by a tyrosine phosphatase which results in intramolecular autophosphorylation of tyrosine 416 (Y416) (Roskoski, 2004). It has been shown that the peak of Src activity in the colorectal cancer Caco-2/15 cell line, which has the ability to differentiate spontaneously to absorptive cells at post confluence, occurs between 0 to 3 days of post confluence (Seltana *et al.*, 2013). Increased SI expression and accelerated absorptive cell differentiation has been observed by the inhibition of Src activity in Caco-2/15 cells using Src family kinase inhibitor Pyrazolopyrimidine compounds (PP2). SFKs inhibit differentiation of the absorptive cells through the regulation of CDX2 and HNF1α transcription factors (Seltana *et al.*, 2013). It also has been reported that treatment of HT29 cell (a colorectal cancer cell line) with PP2 is accompanied by an increased level of E-cadherin/catenin protein expression and strong cell-cell contact (Nam *et al.*, 2002). Src interacts with various tyrosine kinase receptors like EGFR, FGFR, IGFR, PDGFR and VEGFR, and other transmembrane receptors like integrin/FAK, GPCRs to promote their activity (Hsu *et al.*, 2020; Westhoff *et al.*, 2004). The association of Src with YAP1/TAZ has been reported by several researchers. Between three ubiquitous expressing SFK members (SRC, YES and FYN), SRC is a predominant regulator of the Hippo signaling pathway but YES and FYN have a minimal effect on YAP1 nuclear localization in MCF-10A cells (Kim & Gumbiner, 2015). It has been shown that the activity of YAP1/TAZ and its target genes is promoted by the activation of SRC in breast cancer and melanoma cells. Activation of SRC through integrin-mediated adhesion resulted in LATS1/2 repression and in consequence leads to YAP1/TAZ activation (Lamar *et al.*, 2019). It has been reported that SRC phosphorylates LATS1 in tyrosine residues which is accompanied by the suppression of LATS1 via inhibition of its active phosphorylated sites including serine 909 and threonine 1079. Thus, inhibition of SRC activity using dasatinib treatment rescues LATS1 activity (Si *et al.*, 2017). Partial knockdown

of YAP1/TAZ in mice injected with active expressing SRC (SRC^{Y527F}) A375 cells, resulted in a reduction of Src-mediated tumor growth and metastasis, and extended mouse survival (Lamar *et al.*, 2019). Nuclear localization of the YAP1 was prevented in MCF-10A cells using Src family kinase inhibitor PP2. In addition, YAP1 phosphorylation on LATS1/2 phosphorylation site serine 127 (S127), was increased by PP2 treatment and the effect of PP2 was abolished by LATS1/2 knockdown, which indicates the inhibitory effect of SRC on LATS1/2 kinase (Kim & Gumbiner, 2015). The association of YAP1/TAZ activity and rigidity of the extracellular matrix has been reported previously. Binding of the cell with the extracellular matrix via integrin signaling leads to the recruitment of focal adhesion and SRC protein into the attachment site that promotes Rho GTPases/ROCK activation and actin polymerization. Rigidity of the extracellular matrix can affect the behavior of the cell through alteration of integrin signaling and remodeling of the actin cytoskeleton. Therefore extracellular matrix stiffness plays a significant role in cell proliferation, differentiation, migration and invasion (Gkretsi & Stylianopoulos, 2018). Cytoplasmic localization and inactivation of YAP1/TAZ are observed by cell culturing on soft ECM, however rigid ECM promotes the nuclear localization and target gene expression of the YAP1/TAZ coactivator (Cobbaut *et al.*, 2020; Lachowski *et al.*, 2018). Fibronectin or laminin binding to integrin leads to FAK auto-phosphorylation on the Y397 residue which provides a high affinity for binding of the SH2-containing protein Src, which resulted in the prevention of MST1/2 and induction of YAP1/TAZ activity (Kim & Gumbiner, 2015).

1.4.4.5. The effect of the metabolic pathway on YAP1/TAZ activity

The metabolic pathways especially aerobic glycolysis and mevalonate synthesis regulate the activity of the YAP1/TAZ coactivator. The role of the mevalonate pathway is the production of the isoprenoid precursors including cholesterol, bile acids, steroid hormones, and heme from acetyl-coenzyme A (Goldstein & Brown, 1990). The process of prenylation in the mevalonate pathway is mediated by the two enzymes farnesyltransferase and geranylgeranyl transferase. Prenylation is accompanied by covalent attachment of hydrophobic molecules (C-15 isoprene farnesyl or the C-20 isoprene geranylgeranyl groups)

to the c-terminal of Rho GTPase proteins and in consequence, attachment of these proteins to the internal cell membranes. On the other hand, Rho GTPase promotes the activity of YAP1/TAZ through the inhibition of LATS1/2 activity. Therefore, the mevalonate pathway promotes the nuclear localization and activation of YAP1/TAZ via induction of Rho GTPase activity (Sorrentino *et al.*, 2014). Some pharmacological mevalonate pathway inhibitors like statins interfere with certain enzyme activities in the first steps of the HMG-CoA reductase pathway, which result in the inhibition of YAP1/TAZ activity (Sorrentino *et al.*, 2014). The energy and carbons which are important for growing cells are provided by glucose metabolism. In the absence of oxygen, cells mostly use glycolysis to produce energy and lactate, however in the presence of the oxygen, oxidative phosphorylation is preferred to produce more energy and water. Cancer cells perform another phenomenon named aerobic glycolysis or the Warburg effect. They consume a high level of glucose by transforming glucose to lactate even in the presence of high concentrations of oxygen (Warburg, 1956). It has been reported that glycolysis regulates YAP1/TAZ activity and the disruption of glycolysis metabolism leads to YAP1/TAZ impairment. Phosphofructokinase 1 (PFK1) which plays an important role in the initiation of glycolysis, binds to the TEADS, and stabilizes the interaction of YAP1/TAZ and the TEAD transcription factors (Enzo *et al.*, 2015). In breast cancers, there is a link between YAP1/TAZ expression, glucose metabolism and aerobic glycolysis. It has been reported that deprivation of glucose in the medium of MCF10A (breast cancer) cells resulted in YAP1 phosphorylation and inactivation which may be mediated by AMP activated protein kinase (AMPK) in response to energy stress (DeRan *et al.*, 2014; Wang *et al.*, 2015). However, the mechanism by which AMPK regulates YAP1/TAZ activity remains unclear. It has been reported that AMPK directly phosphorylates YAP1 at serine 61 (S61) which suppresses its transcriptional activity (Wang *et al.*, 2015). Another study has suggested that AMPK induces the phosphorylation and stabilization of AMOT and then activated AMOT phosphorylates and activates LATS1/2 which phosphorylates and inactivates the YAP1/TAZ coactivator (DeRan *et al.*, 2014).

1.4.4.6. Interaction of the Hippo pathway with other signaling pathways

The Hippo pathway interacts with various other signaling pathways including the Wnt/β-catenin, Notch, BMP and EGFR pathways (Alarcón *et al.*, 2009; Hong *et al.*, 2016). Both the Hippo and Wnt signaling pathways are known to be essential for tissue growth and homeostasis through cell self-renewal, proliferation and differentiation. Several studies have shown that aberrant activation of YAP1/TAZ and β-catenin occurs in the development of multiple tumor types. There is a complicated relationship between YAP/TAZ and Wnt pathway signaling in the intestinal epithelia. It has been reported that in the presence of an active Hippo pathway, phosphorylated YAP1/TAZ directly binds to β-catenin and prevents its nuclear translocation, which leads to the suppression of Wnt target gene expression (Imajo *et al.*, 2012). Another study showed that YAP1 inhibits Wnt pathway activity independently of the APC-Axin-GSK3β destruction complex through the inhibition of the Wnt effector DVL (Barry *et al.*, 2013). A number of studies have indicated that the Wnt/β-catenin pathway regulates the Hippo pathway. In colorectal cancer cells, the β-catenin/TCF4 complex binds a DNA enhancer element within the first intron of the *YAP1* gene to induce *YAP1* expression. Furthermore, β-catenin abolition results in a decreased expression of YAP1 at the mRNA and protein levels (Konsavage *et al.*, 2012a).
During homeostasis in the normal intestine, the role of YAP1/TAZ in stem cell maintenance is dispensable (Cai *et al.*, 2010). However, Notch is essential for adult intestinal homeostasis. The Notch pathway is also regulated by the Hippo pathway. Overexpression of YAP1 induces JAG-1 expression that activates the Notch pathway leading to increased proliferation in a hepatocellular carcinoma cell line (Tschaharganeh *et al.*, 2013). Increased expression of the HES1 gene which is a Notch pathway target gene has been observed through YAP1 overexpression in mice directly or through MST1/MST2 knockdown (Camargo *et al.*, 2007; D. Zhou *et al.*, 2011). BMP signaling also plays a role in intestinal homeostasis by controlling the proliferation of intestinal stem cells (Haramis *et al.*, 2004; Qi *et al.*, 2017). The activation of the BMP pathway is initiated by the binding of BMP ligands belonging to the transforming growth factor-β (TGF-β) superfamily to type I and II

serine/threonine receptors (BMPRI and BMPRII). Activated BMPRs phosphorylate and activate SMAD1, 5, and 8, which form a complex with SMAD4 and translocate into the nucleus to activate gene expression (Qi *et al.*, 2017). Increased YAP1 and especially TAZ activity, induced BMP expression, ATOH1 downregulation and consequently a reduction in the number of goblet and enteroendocrine cells were observed through ablation of MOB1A/B in the mouse (Bae *et al.*, 2018). Another study exhibited that YAP1 with SMAD1 induces the expression of BMP target gene DNA-binding protein inhibitor (ID) family members, which leads to stem cell maintenance in mouse embryonic stem cells (ESCs) (Alarcón *et al.*, 2009). It has been demonstrated that EGFR induces YAP1 and its target gene activity while the effect of YAP1 on EGFR is not significant (Liu *et al.*, 2018; Reddy & Irvine, 2013). On the contrary, the upregulation of EGFR activity through YAP1/TAZ has been reported by other researchers (Song *et al.*, 2015). Therefore, the Hippo pathway interacts with various signaling pathways including Wnt and Notch, which are important in stem cell proliferation and differentiation in the intestine. The full understanding this pathway's interactions, will require the clarification of the individual functions of the Hippo pathway in stemness and differentiation of the intestinal epithelium.

1.4.4.7. YAP1/TAZ in tumorigenesis

The genetic screens that led to the discovery of Hippo pathway factors in Drosophila relied upon single recessive mutations to drive overgrown phenotypes. Mutation in some negative regulators of the Hippo pathway including Hpo, Sav, Wts and Mats, resulted in increased cellular proliferation and organ overgrowth phenotypes (Hong *et al.*, 2016). Dysregulation of the Hippo pathway contributes to a number of cellular processes associated with cancer progression including increased cellular proliferation, inhibition of apoptosis, and the dysregulation of cellular differentiation. Besides cancer cell lines, some researchers have shown Hippo pathway impairment and YAP1/TAZ activity in a wide range of human cancers. High expression and nuclear localization of YAP1 in human colorectal cancer (CRC) tissues compared with normal tissues has been observed using YAP1 staining

and a microscopy approach (Liang *et al.*, 2014; Wang *et al.*, 2013). Hippo pathway impairment and YAP1 overexpression have been reported in a variety of human tumors including lung cancer, ovarian cancer, kidney cancer, CRC, breast cancer, prostate cancer, pancreatic carcinoma, skin basal cell carcinoma and liver cancer (Konsavage *et al.*, 2012a; Thompson, 2020; Wang *et al.*, 2010; Xu *et al.*, 2009). YAP1/TAZ expression in cancer stem cells resulted in tumor initiation, cell plasticity, metastasis and drug resistance (Nguyen & Yi, 2019; Zanconato *et al.*, 2016). The Hippo pathway induces apoptosis through the inactivation of YAP1/TAZ (Zanconato *et al.*, 2016). It has been reported that active YAP1/TAZ upregulates B-cell lymphoma 2 (BCL2) family members, which leads to the repression of the apoptosis pathway and tumor initiation (Rosenbluh *et al.*, 2012). Western blot and qPCR analysis focused on Hippo pathway components in CRC tissue have shown reduced expressions of MST1 and LATS2, and increased expressions of YAP1, TAZ and TEAD1 mRNA and protein compared with adjacent non-tumor tissue or colorectal adenoma (Liang *et al.*, 2014). Both YAP1 and TAZ are prognostic indicators of CRC outcome, indeed CRC patients who co-express YAP1 and TAZ have been predicted to have the lowest survival rates (Wang *et al.*, 2013). Removing both YAP1 and TAZ in a CRC cell line is more effective in the restriction of migration and invasiveness than ablation of each one individually as measured in vitro by Transwell assays using HCT116 cells (Wang *et al.*, 2013). On the contrary, some researchers have reported that YAP1 acts as a tumor suppressor in human colorectal and breast cancers (Barry *et al.*, 2013). Ablation of YAP1 in a breast cancer cell line led to the progression of tumor growth, which was accompanied by enhancement in colony formation, migration and invasion (Yuan *et al.*, 2008). Ectopic expression of YAP1 using inducible YAP1 wild type or mutated YAP1 (S127D) in a human colorectal carcinoma cell line DLD-1 with silenced YAP1, resulted in a reduction in tumor growth in a nude mouse (xenograft assay) (Barry *et al.*, 2013). Therefore, it seems that YAP1 can act either as an oncoprotein or as a tumor suppressor under different cellular contexts. Different YAP1 behaviours in different cells can be related to the availability of YAP1 binding partners. YAP1 binding to RUNX3 instead of TEADs leads to a reduction in tumorigenicity (Jang *et al.*, 2017). RUNX3 is a member of the runt domain-containing family of transcription factors and has

been reported to be associated with carcinogenesis. Inactivation of RUNX3 has been shown in several solid tumors including gastric, breast, lung and colorectal cancer, which can be associated to the hypermethylation of the CPG island in the RUNX3 promoter (Ito *et al.*, 2015). Ectopic expression of RUNX3 in gastric cancer cell lines with downregulated RUNX3, resulted in decreased tumor growth in injected nude mice (Jang *et al.*, 2017). Furthermore, overexpression of RUNX3 in CRC cells HT29 inhibits metastasis and invasion, which may be related to the reduction of MMP-2 and MMP-9 expression (Xue *et al.*, 2020). YAP1 can also bind to P73, which is a tumor suppressor protein and induces cell apoptosis. P73 acts as a tumor suppressor by inducing cell cycle arrest and apoptosis. During differentiation, the YAP1-P73 transcriptional program is activated by RASSF1A that leads to cell differentiation in ESCs (Papaspyropoulos *et al.*, 2018). YAP1 can act as an oncogene by binding to TEAD transcription factors. The N-terminal domain of YAP1 binds to the C-terminal domain of TEADs (Chen *et al.*, 2010). YAP1/TAZ-TEADs induces transcription of the genes involved in cell growth and proliferation including CTGF and CYR61 (Hong et al., 2016; Ohgushi et al., 2015). It also has been reported that the well-established tumor marker mesothelin is upregulated by TEADs (Ren *et al.*, 2012).

1.4.4.8. The Hippo pathway in proliferation, differentiation and homeostasis

A number of previous studies have shown a role for YAP1/TAZ in cell proliferation, regeneration and re-programming (Gregorieff *et al.*, 2015; Panciera *et al.*, 2016; Yui *et al.*, 2018). The role of YAP1/TAZ in stem cell maintenance in several tissues including the liver, skin, nervous system and intestine has been reported (Mo *et al.*, 2014). Overgrowth of mammalian organs including an increase in liver size, intestine and epidermis thickness in skin was initially observed by YAP1 activation in the mouse (Camargo *et al.*, 2007). Enlargement of the liver was related to an increased number of cells rather than the size of the cells which is associated with the augmentation of cell proliferation. In addition, resistance to Fas-mediated apoptosis has been shown to be induced by YAP1 overexpression (Camargo *et al.*, 2007). Increased YAP1 protein expression and nuclear

localization have been reported in hepatocellular carcinoma in the human (Xu *et al.*, 2009). YAP1/TEAD overexpression in the neural tube promotes cyclin D1 expression and neural progenitor cell expansion while suppressing neural differentiation. Thus, YAP1 knockdown led to the reduced neuronal tube thickness which is likely caused by a reduction in proliferation and increase cell death (Cao *et al.*, 2008). In skeletal muscle, YAP1 blocks the myogenic differentiation program and promotes proliferation of myoblasts (Judson *et al.*, 2012). It has been shown that active YAP1 is expressed in the nucleus of stem/progenitor cells and an inactive or phosphorylated form of YAP1 is expressed in the cytoplasm of differentiated skeletal muscle cells (Watt *et al.*, 2010). Panciera and colleagues have observed that induced YAP1 expression converts the differentiated mammary gland, pancreatic exocrine and neuron cells into stem/progenitor cells (Panciera *et al.*, 2016). On the other hand, knockdown of YAP1/TAZ expression leads to the differentiation of insulin-producing β cells (Rosado-Olivieri *et al.*, 2019). It has been reported that TAZ overexpression in HBE135 immortalized human bronchial epithelial cells resulted in increased proliferation, which was restored to its original level by knockdown of TAZ (Z. Zhou *et al.*, 2011). Lian et al. showed that YAP1 is expressed in embryonic stem cells at a high level and its expression is downregulated during differentiation (Lian *et al.*, 2010). Therefore, YAP1 and TAZ are required to maintain pluripotency in embryonic stem cells (Lian *et al.*, 2010; Varelas *et al.*, 2008). The Hippo pathway has also been shown to have critical functions in the mammalian intestine during both development and regeneration. In the intestine, endogenous expression of YAP1 and TAZ has been reported in the crypt compartment (Camargo *et al.*, 2007). However, the YAP1/TAZ regulator MST1/2 is mostly expressed in the cytoplasm of proliferative cells and in the nucleus of cells in the differentiated zone of the mouse intestine. It has been shown that MST1/2 is associated with the terminal differentiation of developing epithelial tissue (Lee *et al.*, 2008). A mouse model generated with a Tet-On YAPS127A allele in which the YAP1 phosphorylation residue serine 127 is replaced by alanine showed a high proliferative rate of undifferentiated cells and dysplasia in the intestine (Camargo *et al.*, 2007). In addition, Cai and colleague reported that removing the Hippo kinase core component SAV1 in the mouse intestinal epithelium

was accompanied by increased epithelial cell proliferation, crypt hyperplasia, and the development of sessile serrated colonic polyps. Furthermore, acceleration in polyp formation was observed by mouse treatment with dextran sodium sulfate (DSS) to induce chemical colitis (Cai *et al.*, 2010). Overexpression of YAP1/TAZ through knockdown of MST1/2 in the mouse was accompanied by a disruption in the villus, dysplastic epithelia and adenomas in the colon and expansion of undifferentiated cells (D. Zhou *et al.*, 2011). Imajo and colleagues reported a dual role for YAP1/TAZ in the intestinal epithelium. They showed that active YAP1/TAZ promotes the proliferation of stem/progenitor cells by binding to the TEAD transcription factor and on the other hand induces the differentiation of goblet cells by binding to the KLF4 transcription factor (Imajo *et al.*, 2015). It must be noted that this group of studies established a method named iGT, which enables rapid gene delivery into the intestinal epithelium of the mouse. They believe that using iRNA in this method leads to the moderate reduction of MST1/2 or LATS1/2 and moderate elevation in YAP1 and TEAD activity which makes their results different from those of other knockdown experiments (Imajo *et al.*, 2015). Therefore, it has been shown that nuclear accumulation of YAP1 is sufficient to stimulate the proliferation of undifferentiated intestinal stem cells. However, YAP1 ablation in a mouse model resulted in no observable changes in the mouse intestine that indicates a dispensable role for YAP1 in the mouse intestine under normal conditions (Cai *et al.*, 2010). Therefore, it has been suggested that a lack of YAP1 could be compensated by endogenous TAZ protein, whereas other studies have reported that ablation of both YAP1 and TAZ under normal conditions has no significant effect on intestinal epithelial cells (Azzolin *et al.*, 2014; Cai *et al.*, 2015; Yui *et al.*, 2018). Therefore, there is a complicated relationship between YAP1 and intestinal homeostasis in YAP1 knocked down animal models. Contrary to the normal intestine, YAP1 knockdown in the injured intestine has led to a severe regeneration phenotype. DSS treatment of YAP1 knockout mice was accompanied by loss of the intestinal crypt, a decrease in cell proliferation and an increase in apoptotic cells and mortality (Cai *et al.*, 2010). Therefore, YAP1 is required during intestinal regeneration, while its presence is dispensable in the adult intestine under normal conditions. During colonic epithelium repair in the intestine,

fetal-like tissue is formed through the remodeling of the ECM that leads to YAP1/TAZ activity. The high level of collagen type 1 in the ECM of repairing epithelium results in increased activity of FAK/Src signaling that controls YAP1/TAZ activity. Thus, dedifferentiation and modification of committed cells into stem/progenitor cells has been observed in ectopic YAP1/TAZ activation (Yui *et al.*, 2018). During regeneration of the irradiated intestine in mice, YAP1 participates in intestinal stem cell maintenance through temporary suppression of Wnt signaling (Gregorieff *et al.*, 2015). It has been shown that Wnt signaling activity and Paneth cell differentiation are restricted by active YAP1 and removal of YAP1 leads to a higher expression of lysozyme which is a Paneth cell marker (Gregorieff *et al.*, 2015).

As summarized above, the effect of the Hippo pathway and its effector YAP1/TAZ on various functions of the intestinal epithelial cell including proliferation and regeneration has been well documented using knockdown approaches. Increased expression of YAP1/TAZ has been observed by removing Hippo pathway components including MST1/2 in mouse models which indicates that it acts as an upstream regulator of YAP1/TAZ (D. Zhou *et al.*, 2011). Therefore, the role of YAP1/TAZ in the intestine has mostly been investigated in the context of proliferation and regeneration (Gregorieff *et al.*, 2015) using genetic mouse models. Furthermore, little is known about their role on intestinal epithelial cell differentiation. More investigations are required to determine the exact role of the Hippo pathway downstream main effector YAP1 on the regulation of differentiation of intestinal epithelial cells. The YAP1/TAZ coactivator is the final target of the Hippo pathway kinase core which is inactivated by MST1/2 and LATS1/2 kinases. Therefore, studying the effect of the Hippo pathway on epithelial cell differentiation can be approached by direct inactivation of the YAP1/TAZ coactivator.

1.5. Cell model for studying intestinal epithelial cell differentiation

There is no normal cell model to study intestinal epithelial cell differentiation. This is why most laboratories use CRC cell lines. The two best characterized cell lines that have a potential to differentiate are HT29 and Caco-2.

There are many reasons for choosing these cells first described by Jørgen Fogh in 1975 (Fogh, 1975). First, HT29 is a non-polarized, undifferentiated colorectal cancer cell line which grows as a multilayer under standard growth conditions, and like other cancer cell lines, contains stem cells (Vázquez-Iglesias *et al.*, 2019). Secondly, HT29 has the potential to differentiate into both the secretory and absorptive lineages leading to a formation of a polarized monolayer with developed junctional complexes and brush borders (Zweibaum *et al.*, 1985). This can be performed by changing the growth conditions including the replacement of glucose by galactose and sodium butyrate (Augeron & Laboisse, 1984; Kitamura *et al.*, 1996; Martínez-Maqueda *et al.*, 2015; Robine *et al.*, 1993; Zweibaum *et al.*, 1985). However, they grow as a multilayer of unpolarised undifferentiated cells under normal growth conditions with DMEM medium containing 25mM of glucose and 10% FBS (Augeron & Laboisse, 1984). Thirdly, we found HT29 cells to be a proper model for YAP1 investigation since they express the YAP1 protein at a high level but the TAZ protein remains below the detectable level. It has been demonstrated that the Hippo pathway kinase core components MST1 and LATS1/2 are expressed in the HT29 cell line. In addition, expression of the active form of LATS1 (pLATS1^{S909}) and phosphorylated YAP1 (pYAP1^{S127}) indicates the activity of the Hippo pathway in HT29 cells (Q. Wang *et al.*, 2018). Therefore, a high level of YAP1 expression in HT29 cells can be related to impairment of the Hippo pathway in YAP1/TAZ regulation (Konsavage *et al.*, 2012b) and/or regulation of YAP1/TAZ independently of the Hippo pathway through other signals. Finally, the high density of cells in CRC cell lines has no effect on the expression of the YAP1 protein (Konsavage *et al.*, 2012b). Caco-2/15 is another CRC cell line that has the potential to differentiate to absorptive cells. Therefore, it could be a proper model to study the effect of YAP1 on cell differentiation. However, the first limitation of using Caco-2/15 cells in this study is that

they are unable to differentiate into secretory cells. The second is the expression of both YAP1 and TAZ which may have overlapping effect. Nevertheless, the Caco-2/15 cell line was used in certain experiments to confirm some observation in HT29 cells.

1.6. Hypothesis and objectives

Most of studies agree that YAP1/TAZ plays a significant role in proliferation, adhesion, regeneration and tumorigenesis. However, the question about the involvement of YAP1/TAZ in cell differentiation, especially intestinal epithelial cells, remains open.

My hypothesis was that the Hippo pathway regulates differentiation of intestinal epithelial cells through restriction of its downstream effector YAP1.

My first objective in testing this hypothesis was to investigate the implication of YAP1 on differentiation using a knockdown approach targeting YAP1 in HT29 cells.

My second objective was to search out the molecular mechanisms by which YAP1 regulates differentiation of intestinal epithelial cells.

2. Materials and Methods

2.1. Materials

2.1.1. Antibodies

The primary antibodies used in this study are listed in Table 1. Mouse anti-bromodeoxyuridine-fluorescein (1/50, Sigma-Aldrich, Oakville, ON, Canada) was used for BrdU staining. The secondary antibodies used in this study included AlexaFluor 488 or 594 goat anti-mouse (A11017, A11072, 1/400, Thermo Fisher Scientific, Ottawa, ON, Canada) and goat anti-rabbit (A11070, A11072, 1/400, Thermo Fisher Scientific), ECL HRP-linked anti-mouse (NA931 V, 1/4000, GE Healthcare, Mississauga, ON, Canada) and anti-rabbit (NA934 V, 1/4000, GE Healthcare).

Table 1. Primary antibodies

Antibodies	SPECIFICITY	ORIGIN	USAGE
Mouse mAb (ab11197)	MUC2	Abcam,Toronto, ON, Canada	WB: 1/ 500 IF: 1/ 100
Rabbit mAb (ab108559)	TFF3	Abcam	WB: 1/ 1500
Mouse mAb (hsi-4/34 or caco-3/73)	SI	(Beaulieu *et al.*, 1989)	WB: 1/ 100
Rabbit polyclonal Ab (ab129060)	DPPIV or CD26	Abcam	WB: 1/ 2000
Mouse mAb (dao7/219)	DPPIV	(Daniele & Quaroni, 1990)	IF: 1/ 100
Rabbit mAb (d24e4)	YAP/TAZ	Cell Signaling, Danvers, MA, USA	WB: 1/ 1500 IF: 1/ 50

Mouse mAb (mu392a-uc)	CDX2-CD88	BioGenex, Freemont, CA, USA	WB: 1/ 700
Mouse mAb (umab212)	LGR5	Origene, Rockville, MD, USA	WB: 1/ 100 IF: 1/ 100
Mouse mAb (mab1501)	β-actin	Millipore, Etobicoke, ON, Canada	WB: 1/ 100,000
Rabbit polyclonal Ab (ab137534)	ATOH1	Abcam	WB: 1/ 2000

2.2. Methods

2.2.1. Cell cultures

The human CRC cell line HT29, available from the American Type Culture Collection (Manassas, VA, USA), as well as the Caco-2/15 cell line (Beaulieu & Quaroni, 1991) were obtained from A. Quaroni (Cornell University, Ithaca , NY, USA). HT29 and Caco-2 identities were confirmed by short-tandem repeat profiling cell authentication. HT29, Caco-2/15 and HEK293T cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Burlington, ON, Canada), supplemented with 10% fetal bovine serum (Wisent, Saint-Jean-Baptiste, QC, Canada), 2 mM GlutaMAX (Life Technologies) and 10 mM HEPES (Wisent). The HIEC cells used herein as normal control cells in some experiments were derived from a normal small intestine and grown as described elsewhere (Beaulieu & Menard, 2012). All cells were maintained in a 5% CO_2-humidified atmosphere at 37°C. Regular monitoring was performed to insure the absence of mycoplasma contamination.

2.2.2. Tissues

Normal adult human intestinal tissues (jejunum and ileum) were provided by Transplant Quebec (Québec, Canada) according to a protocol approved by the Institutional Human Subject Review Board of the Centre Hospitalier Universitaire de Sherbrooke.

2.2.3. Cell signaling inhibitor treatments

HT29 and Caco-2/15 cells were grown as described previously. The incubation of the cells with 20 µM PP2 (Abcam) and 10 µM dasatinib (Abcam), and specific SFK inhibitors, was started at day -2 of confluence (80% confluent). Stock solutions of PP2 and dasatinib were prepared in 100% of DMSO (Sigma-Aldrich). Controls consisted of DMSO only. The inhibitors and DMSO were added to the medium and medium was renewed daily for 5 days. Finally, cells were harvested for total RNA and protein extraction.

2.3. Generation of short hairpin RNA, lentivirus production and cell infection

2.3.1. Preparation of the lentivirus

For the knockdown of YAP1 and CDX2 expression, lentiviruses were prepared with MISSION shRNA (Sigma-Aldrich) plasmids containing shRNA targeting YAP1 and CDX2 obtained from Addgene (Watertown, Massachusetts, USA) and Sigma-Aldrich. The sequence of shYAP1#1 was 5'-GCCACCAAGCTAGATAAAGAA-3' and the sequence of shYAP1#2 was 5'-CCCAGTTAAATGTTCACCAAT-3'. The two shRNAs were a gift from William Hahn (Rosenbluh *et al.*, 2012) (Addgene plasmids # 42540 and 42541). The shCDX2 was the construct TRCN0000013687 (Sigma-Aldrich) targeting the sequence 5'-AGCCCTTGAGTCCGGTGTCTT-3'. The sequence of shRNA against TAZ was acquired from Sigma-Aldrich. The shTAZ#1 (TRCN0000019469 construct) with 5'-GCGATGAATCAGCCTCTGAAT-3' sequence was used as a negative control for all experiments with HT29 cells. The control shRNAs used for Caco-2/15 cells were shGFP (Groulx *et al.*, 2014) and shLUC (Benoit *et al.*, 2012).

In order to knockdown YAP1 and CDX2, HEK293T cells were employed for the preparation of lentiviruses. These cells were plated in 100 mm dishes one day before transfection. The day after, 10 µg of plasmid containing the shRNA targeting the gene of interest and 15 µg of proteolipid protein 1 (PLP1), 7 µg of proteolipid protein 2 (PLP2) and 9.8 µg of vesicular

stomatitis virus G (pLP/VSVG) were mixed along with lipofectamine in serum-free Opti-MEM (Life Technologies, Burlington, ON, Canada). After 20 min, this mix was added to 50% confluent 293T cells and incubated for 48 h. Finally, the cell medium was harvested and filtered before infection. For induction of ATOH1 in HT29 cells, the pLVX-Puro (Clontech Laboratories, Mountain View, CA, US) vector containing the cDNA of ATOH1 and pLVX-Puro empty vector (as control) were transfected into HEK293T cells to produce viral particles as described above.

2.3.2. Lentivirus infection

One day before infection with lentivirus, HT29 or Caco-2/15 cells were plated in 100 mm dishes. The cells were infected with virus at fifty percent confluent (one day after passage). Briefly, 500 µl of filtered virus was mixed with 1 ml of DMEM and 8 µg/ml of polybrene. The HT29 or Caco-2/15 cell medium was removed, and the virus mix was added drop by drop. After 1h incubation, 3.5 ml of DMEM was added to the cells and incubated for 48h. Then, the infected cells were selected by 10µg/ml puromycin (shYAP1, shTAZ, shGFP, shLUC, pLVX and pLVX-ATOH1) or 1 mg/ml of G418 (shCDX2) for 9 days. Cells were then plated and grown to confluence and harvested at day 0 and 8 days post confluence for HT29 shYAP1 and HT29 shTAZ or 5 days post confluence for HT29 shYAP1+shCDX2 and HT29 shYAP1+shTAZ#1. The shTAZ#1 and shYAP#1+shTAZ#1 were used as controls for HT29 shYAP1 and HT29 shYAP1+shCDX2 cells, respectively. Since TAZ is expressed in Caco-2/15 cells, shGFP and shLUC were used as controls. Caco-2/15 cells were tested at 5 days post confluence. HT29 pLVX and HT29 pLVX-ATOH1 were harvested at 0 days post confluence.

2.4. Protein extraction and Western blot

2.4.1. Protein extraction

The cell dishes were washed with phosphate buffer saline 1x (PBS 1x) two times, then the cells were collected and divided for protein and RNA extractions. In order to extract the

protein, Laemmli 1X buffer (62.5 mM Tris-HCl pH 6.8, 2.3% SDS, 10% glycerol, 0.005% bromophenol blue and 5% β-mercaptoethanol) was added and cells were incubated on ice for 5 minutes to lyse. Then the lysed cells were sonicated and centrifuged at 13,000 RPM for 15 min at 4°C. Finally the supernatants were harvested and protein concentrations were determined using the Lowry method with Folin phenol reagent and with BSA as the protein standard (Lowry *et al.*, 1951).

2.4.2. Western blot analysis

Equal amounts (50 µg) of each reduced (5% β-mercaptoethanol) protein sample were heated at 95°C for 5 min. Then each sample was migrated through 10%, 12% or 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in running buffer (25mM Tris pH 8.3, 192mM glycine, 0.1% SDS (v/w)). Vertical agarose gel electrophoresis (2%) was performed for the separation of the MUC2 protein with a molecular weight of approximately 500kD. Briefly, agarose powder (Roche, Penzberg, Germany) was dissolved in nanopure water by heating in a microwave for 90 seconds. Then 20X gel TM running buffer (20.92% MOPs, 12.12% Tris and 2% SDS (v/w)) was added to make a 1X final solution. Melted agarose was poured into a vertical casting unit and a well comb was inserted. After 20 minutes, the gel was transferred to 4°C for 30 minutes. Finally, samples were migrated for 45 minutes at 100 volts and then transferred for 20 min at 20 volts followed by overnight at 30 volts. Transfer efficiency and quality was visualized using Ponceau S stain [0.1% Ponceau (w/v), 7% trichloroacetic acid]. For all WB experiments, nitrocellulose membrane (GE Healthcare, Mississauga, ON, Canada) was used for transferring and 5% non-fat milk was used for blocking nonspecific binding sites. The membranes were incubated overnight with primary antibodies at 4°C. Then the membranes were rinsed three times with 0.1% PBS-tween for 10 min and incubated with horseradish peroxidase-conjugated secondary antibodies for 1h at room temperature. After rinsing the membrane, the enhanced chemiluminescence (ECL) method was used to detect HRP positive bands according to the manufacturer's instructions (Millipore, Etobicoke, ON, Canada). All membranes were exposed to autoradiography films (Denville Scientific Inc., Saint-Laurent, QC, Canada) and

films were developed for the appearance of bands. Image J (Rasband, 1997-2018) (National Institute of Health, Bethesda, MD, USA) was used for scanning and band quantitation.

2.5. RNA extraction, reverse transcriptase and quantitative RT-PCR

2.5.1. RNA extraction and reverse transcriptase (RT)

The cells were washed with PBS 1x, then lysed with RiboZol (VWR Life Science, Solon, Ohio, USA). The extraction of RNA was performed according to the manufacturer's instructions (VWR Life Science). The RNeasy lipid tissue mini kit (QIAGEN, Maryland, US) was used for RNA extraction of intestinal tissues. Briefly, the tissues were cut or crushed into the very small pieces on ice. Ribozol (1ml for 100mg) was added and tissues were homogenized using a Polytron at 0 to 4°C. The lysates were transferred to new microcentrifuge tubes and were kept on the benchtop at room temperature (RT) for 5 min. After the addition of chloroform (200 µl/ml), the tubes were shaken vigorously, kept at RT for 2-3 min and centrifuged at 12000 x g for 15 min at 4°C. Then the upper colorless aqueous phases (about 600 µl) were transferred to new tubes and mixed with about 600 µl of 70% ethanol. Finally, up to 700 µl of each sample was transferred to RNeasy mini spin columns placed in 2 ml collection tubes for purification and elution of total RNA.

Reverse transcription of RNA was performed using the Omniscript RT kit (QIAGEN, Maryland, USA). Briefly, 1 µg of purified RNA was incubated with 1 µl 10x DNase I buffer and 1 µl DNAse I for 15 min at room temperature. Then 1 µl of 25mM ethylenediaminetetraacetic acid (EDTA) was added to the RNA mix and incubated at 65°C for 10 min. Finally, 10 µl of master mix containing 2 µl 10x buffer RT, 2 µl of 5mM dNTP mix, 2 µl of 10µM oligo-dT primer, 2 µl of random primer, 1 µl RNase inhibitor and 1 µl reverse transcriptase was added to the RNA mix which was then incubated for 1 h at 37°C.

2.5.2. Quantitative RT-PCR

Green-2-Go qPCR low ROX Master Mix (Bio Basic) was used for quantitative polymerase chain reaction (qPCR). A sample of cDNA (1 µl) was mixed with 0.4 µl of forward and reverse primers, 8.2 µl of RNase-free water and 10 µl of master mix (2X mix of dNTPs, Hotstart Taq polymerase, $MgCl_2$, fluorescent detection dye, reference dye and proprietary buffer components). The primers used for qPCR are listed in Table 2. Gene expression was calculated according to the Pfaffl equation (Pfaffl *et al.*, 2004) using RPLPO as a validated normalizer (Dydensborg *et al.*, 2006) relative to control groups consisting of a pool containing various CRC cell lines (Tremblay *et al.*, 2006) or shCtrl as specified in the text.

Table 2. Primers used in qPCR

GENE	SEQUENCE	PCR PRODUCT SIZE
YAP1	Forward 5'-TGCGTAGCCAGTTACCAAC-3' Reverse 5'-GGTTCGAGGGACACTGTAGC-3'	194 bp
WWTR1 (TAZ)	Forward 5'-TGCTACAGTGTCCCCACAAC-3' Reverse 5'-GAAACGGGTCTGTTGGGGAT-3'	114 bp
CTGF	Forward 5'-CCTGGTCCAGACCACAGAGT-3' Reverse 5'-TGGAGATTTTGGGAGTACGG-3’	194 bp
CYR61	Forward 5'-TCCCTGTTTTTGGAATGGAG-3' Reverse 5'-GAGCACTGGGACCATGAAGT-3’	240 bp
ATOH1	Forward 5'-TGAAGGAGTTGGGAGACCAC-3' Reverse 5'-TCCGGGGAATGTAGCAAATA-3'	220 bp
KLF4	Forward 5'-GCGGCAAAACCTACACAAAG-3' Reverse 5'-CCCCGTGTGTTTACGGTAGT-3'	149 bp
CDX2	Forward 5'-GAGTGGTGTACACGGACCAC-3' Reverse 5'-TTTCCTCTCCTTTGCTCTGC-3'	161 bp
HNF1A	Forward 5'-CCGCAGACTATGCTCATCAC-3' Reverse 5'-GCTGAGTCTGAGCTCTGGT-3'	258 bp

MUC2	Forward 5'-CATCACATTCATGCCCAATG-3' Reverse 5'-CAGCTCTCGATGTGGGTGTA-3'	297 bp
TFF3	Forward 5'-CTCCAGCTCTGCTGAGGAGT-3' Reverse 5'-GAAACACCAAGGCACTCCAG-3'	160 bp
SI	Forward 5'-GAGGACACTGGCTTGGAGAC-3' Reverse 5'-ATCCAGCGGGTACAGAGATG-3'	187 bp
DPPIV	Forward 5'-AAGTGGCGTGTTCAAGTGTG-3' Reverse 5'-CAGGGCTTTGGAGATCTGAG-3'	241 bp
DEFA5	Forward 5'-AAGCAGTCTGGGGAAGACAA-3' Reverse 5'-TGAATCTTGCACTGCTTTGG-3'	225 bp
CHGA	Forward 5'-CGGGAGGACAGCCTTGAG-3' Reverse 5'-CTGGTGGGCCACTTTCTC-3'	156 bp
ASCL2	Forward 5'-AGCAAGAAGCTGAGCAAGGT-3' Reverse 5'-GGATGTACTCCACGGCTGAG-3'	220 bp
OCT4	Forward 5'-TGCAGAAAGAACTCGAGCAA-3' Reverse 5'-GTGAAGTGAGGGCTCCCATA-3'	565 bp
LGR5	Forward 5'-TGCTCTTCACCAACTGCATC-3' Reverse 5'-CTCAGGCTCACCAGATCCTC-3'	193 bp
PROM1	Forward 5'-TTTGGTGCAAATGTGGAAAA-3' Reverse 5'-TTGAAGCTGTTCTGCAGGTG-3'	221 bp
CD44	Forward 5'-TAAGGACACCCCAAATTCCA-3' Reverse 5'-CCACATTCTGCAGGTTCCTT-3'	285 bp
EPCAM	Forward 5'-CACAACGCGTTATCAACTGG-3' Reverse 5'-CCAGCTTTTAGACCCTGCAT-3'	291 bp

2.6. Indirect immunofluorescent staining and confocal imaging

Before seeding the HT29 cells, cover slips were treated with fetal bovine serum overnight. The cells were plated on cover slips and then sub-confluent and 8 day post-confluent cells were fixed with methanol (MeOH) at -20°C (10min) for MUC2 detection or 2%

paraformaldehyde (PFA) at +4°C (10min) for DPPIV, LGR5 and YAP1/TAZ detection. Caco-2/15 cells were seeded on 8-well cell culture Lab-Tek chamber slides (Nalgen Nunc, Naperville, IL) and treated with PP2, dasatinib and DMSO (Ctrl). After 5 days of treatment, Caco-2/15 cells were fixed with 2% PFA for YAP1/TAZ determination. Then they were washed three times with PBS 1x (5min) or one time with PBS-glycine (15min). Healthy adult human intestinal tissues (ileum) provided by transplant Quebec were embedded in OCT. The 3 µm cryosections were fixed with 4% PFA for 45 min at +4°C. Then the slides were rinsed with PBS-glycine for 45 min at +4°C. The cells fixed with MeOH and PFA were rinsed 3x with PBS 1x for 5 min and PBS-glycine for 15 min at +4°C, respectively. Free fat milk (5%) for MUC2 and DPPIV, and goat serum (10%) for YAP/TAZ, LGR5 and DEFA5 were utilized to block non-specific sites. Primary antibodies were applied according to the instructions of the manufacturers. The slides or coverslips were washed 3x with PBS 1x then incubated with secondary antibodies for 1h at room temperature. After rinsing 3x with PBS 1x, DAPI was utilized for nuclear staining and Evans blue was used for background colour. After rinsing 3x with PBS 1x, mounting medium (Dako, Santa Clara, CA, USA) was used before observation under a microscope. Leica DM RXA and Reichert Polyvar 2 microscopes were used to observe the cells/tissues and for taking images. Images were acquired using MetaMorph software (Universal Imaging Corporation). Stained tissues were also viewed using an Olympus FV1000 SIM confocal microscope equipped with Fluoview for image acquisition.

2.7. BrdU (5-bromo-2'-deoxyuridine) assay

One day before the BrdU immunofluorescence labeling experiment, the cells were counted and an equal number (1×10^4 cells/well) of YAP1 knockdown HT29 and control cells were seeded in 96-wells plates. After 24 h, cells were incubated with BrdU for two hours at 37°C and finally were fixed using methanol for 10 min at -20°C. The cells were rinsed 3x with PBS 1x. Then cells were incubated with Xigor (4M HCl) for 8 min at room temperature. Cells were rinsed 3x with PBS 1x. 0.5% BSA in 0.1% PBS-tween was used as a blocker. Cells were incubated with anti-BrdU antibody in the dark for 45 min at 37°C. After rinsing, the cells

were incubated with DAPI (1/50,000) for 3 min. Finally, after rinsing mounting medium was added and cells were observed and counted using a Leica DM IRBE microscope. The number of BrdU stained cells was calculated according to the total number of cell nuclei.

2.8. Transmission electron microscopy (TEM)

For the preparation of cell samples for ultra-structural analysis, equal amounts of HT29 shYAP1#1, HT29 shYAP1#2 and HT29 shTAZ#1 (control) cells were plated in 6 well plates. They were grown until 8 days post confluency and then washed 3x with PBS 1x. The cell samples were fixed in glutaraldehyde 1.5% for 30 min at room temperature, then glutaraldehyde 2.5% overnight at +4°C and post fixed in 1% osmium tetroxide. The samples were dehydrated with ethanol and then embedded in epoxy under vacuum. The sections were prepared on copper grids with Formvar/Carbon film on Copper 200 mesh and observed with a Hitachi H-7500 transmission electron microscope at 80 kV.

2.9. Statistical analysis

For data preparation and statistical analysis including the two tailed Student's t-test, Graph Pad Prism 8.3 (Graph Pad Software; San Diego, CA) was used. Data are presented as the mean ± SEM. A P value below <0.05 was considered significant in all analyses. All experiments were repeated independently at least three times.

3. Results

3.1. The expression of YAP1/TAZ protein in human intestinal crypt cells

Human adult small intestine specimens were analyzed to determine the location of YAP1/TAZ in intestinal epithelial cells. The expression of the YAP1/TAZ and DEFA5 proteins was detected in some crypt cells located in the stem cell zone (Figure 6A). A negative control without primary antibody is shown in Figure 6B. YAP1/TAZ expressing cells are located between the Paneth cells (DEFA5 positive) (Figure 7A, D) and their expression is localized in the nucleus (Figure 7A, C). In Paneth cells, YAP1/TAZ protein was below detectable levels in the nuclei (Figure 7A,C) which is in agreement with previous findings reporting an absence of YAP1 in Paneth cells (Gregorieff *et al.*, 2015).

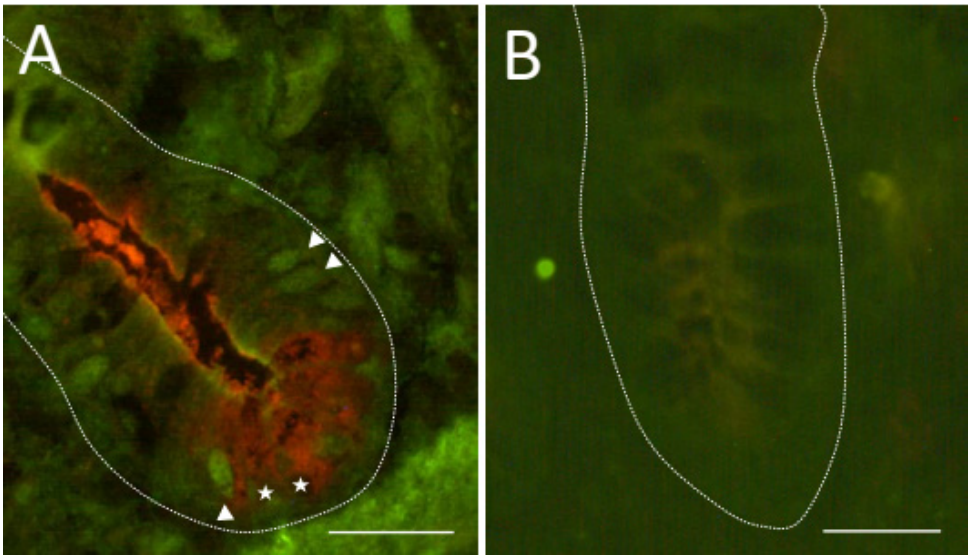


Figure 6. Staining of YAP1/TAZ and DEFA5 in human intestinal crypt cells.

A. Representative immunofluorescent staining for the detection of YAP1/TAZ (green) and DEFA5 (red) in the adult small intestine. The staining of YAP1/TAZ in some crypt cells (arrowheads) and of DEFA5 in a number of cells at the base of the crypt (stars) show the specificity of the antibodies. **B**. Corresponding staining of a control section incubated under the same conditions but without primary antibodies. Scale bars = 50 µm.

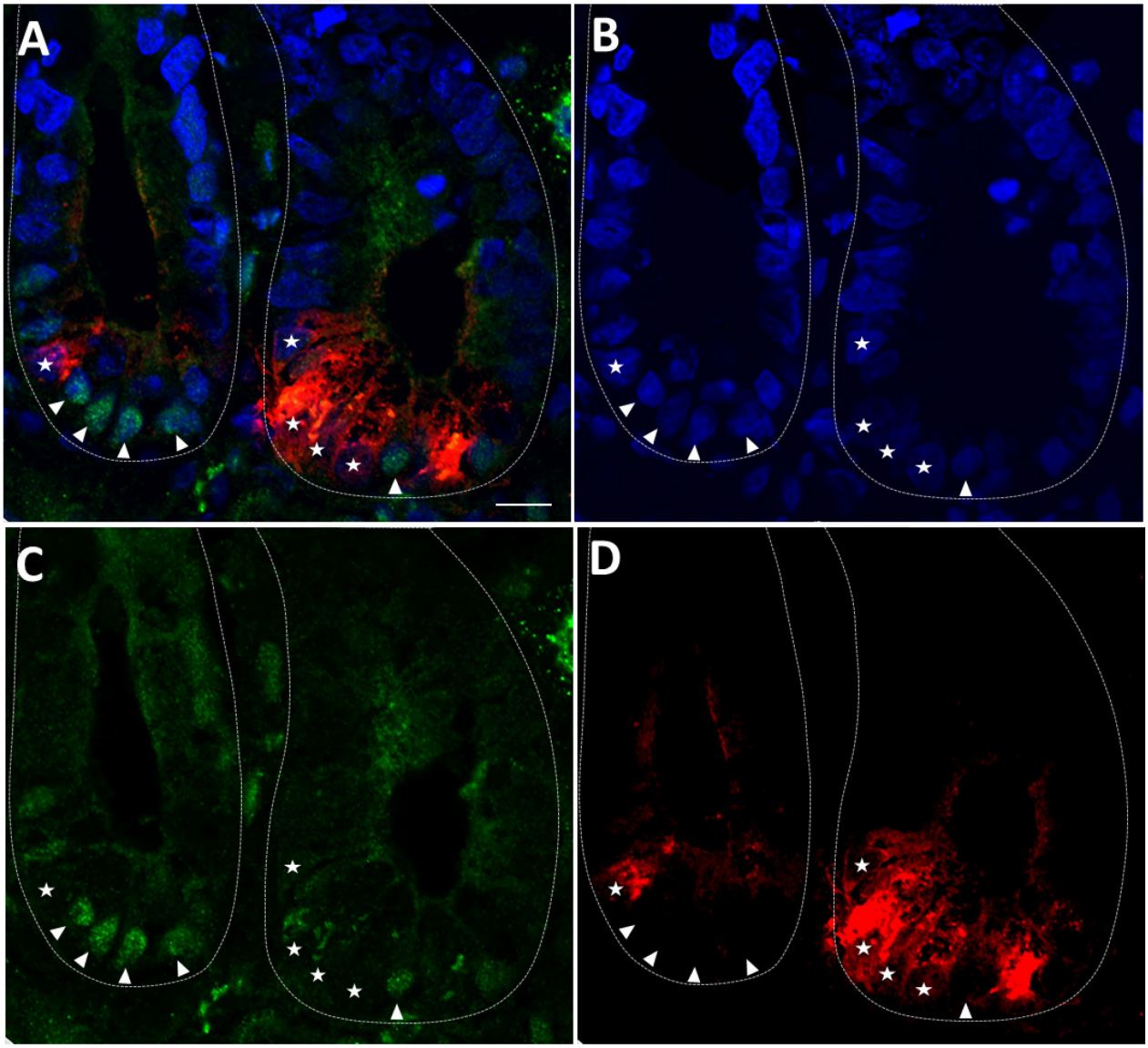


Figure 7. Nuclear localization of YAP1/TAZ in human intestinal crypt cells.

Representative confocal imaging for the detection of YAP1/TAZ (green; A, C), DEFA5 (red; A, D) and DAPI (blue; A, B) in the adult small intestine. Nuclear expression of YAP1/TAZ in some of the cells located at the bottom of the crypts was observed (arrowheads) except in Paneth cells (stars). Scale bar is equal to 10µm.

3.2. HT29 stem cell markers and YAP1 expression

HT29 is an undifferentiated CRC cell line which exhibits some multipotency since these cells express stem cell markers and can differentiate into both absorptive and goblet cells under certain conditions (Augeron & Laboisse, 1984; Kitamura *et al.*, 1996; Zweibaum *et al.*, 1985). The expression of the stem cell markers LGR5, CD44, PROM1/CD133, EPCAM/CD326, ASCL2 and OCT4, as well as the goblet cell marker MUC2 and absorptive cell marker SI was first evaluated in HT29 cells by qPCR analysis. The expression of these transcripts in HT29 cells was evaluated relative to a pool consisting of a mix of cancer cells including Caco-2/15, HT29, A549 and SKOV3. The results showed that five of the six stem cell markers are expressed at high levels in HT29 cells compared with the cancer cell pool. However, ASCL2 was detected at a low level in HT29 cells relative to the pool. YAP1 was detected at a comparable level to that of the pool but TAZ expression was at a low level. Despite a high expression of MUC2, SI expresses at a low level in HT29 cells compared with the pool (Fig. 8A). Western blot analysis confirmed a strong expression of YAP1 at the protein level in HT29 cells. However, in contrast with Caco-2/15 cells, another CRC cell line, the TAZ protein was not detected in HT29 cells. It is noteworthy that YAP1 and TAZ expression in a normal intestinal cell line was also distinct, HIEC expressing only TAZ protein (Figure 8B). These distinct patterns of YAP1 and TAZ expression were also observed at the transcript level for the three cell lines, as well as for the small intestine where relative mRNA levels of YAP1 appeared higher than those of TAZ (Figure 8C). Indirect immunofluorescent analysis demonstrated nuclear expression of the YAP1 protein in a large proportion of the HT29 cells (Figure 9). Also, consistent with LGR5 and MUC2 transcript expression, HT29 was found to constitutively express a subpopulation of stem-like and goblet-like cells (Figure 10) (Figure 11).

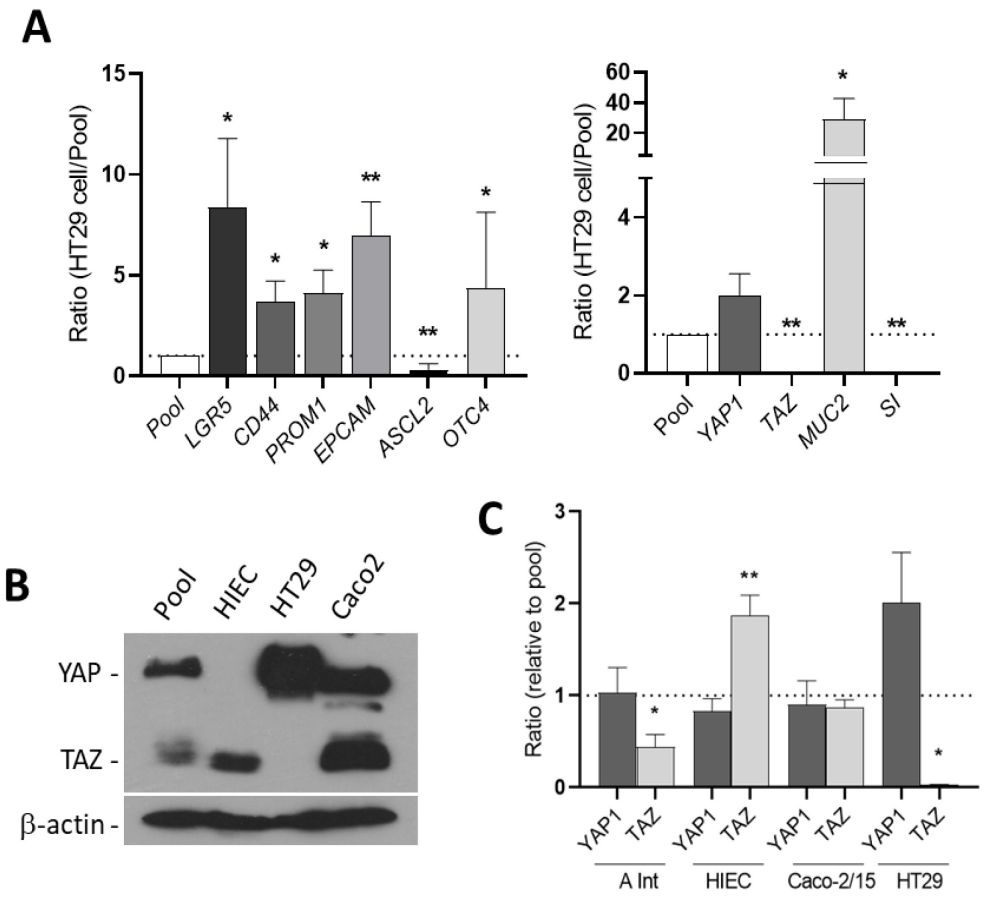


Figure 8. Expression of stem cell markers, Hippo effectors, goblet and absorptive cell markers in HT29 cells.

A. Expression of LGR5, CD44, PROM1, EPCAM, YAP1, TAZ, MUC2 and SI transcripts in HT29 cells relative to a pool of cancer cells. * $P < 0.05$, ** $P < 0.01$. **B.** Western blot analysis showing the expression of YAP1 protein in HT29 cells in which the TAZ protein was consistently below detectable levels. Both YAP1 and TAZ proteins were found to be expressed by Caco-2/15 cells while only TAZ was detectable in HIEC. β-actin was used as a loading control. **C.** The expression of YAP1 and TAZ was also investigated at the transcript level in the adult small intestine (A Int) and the intestinal cell lines relative to the pool. Statistical significance for YAP1 vs TAZ (paired T test): *$P < 0.05$, ** $P < 0.005$, n=3.

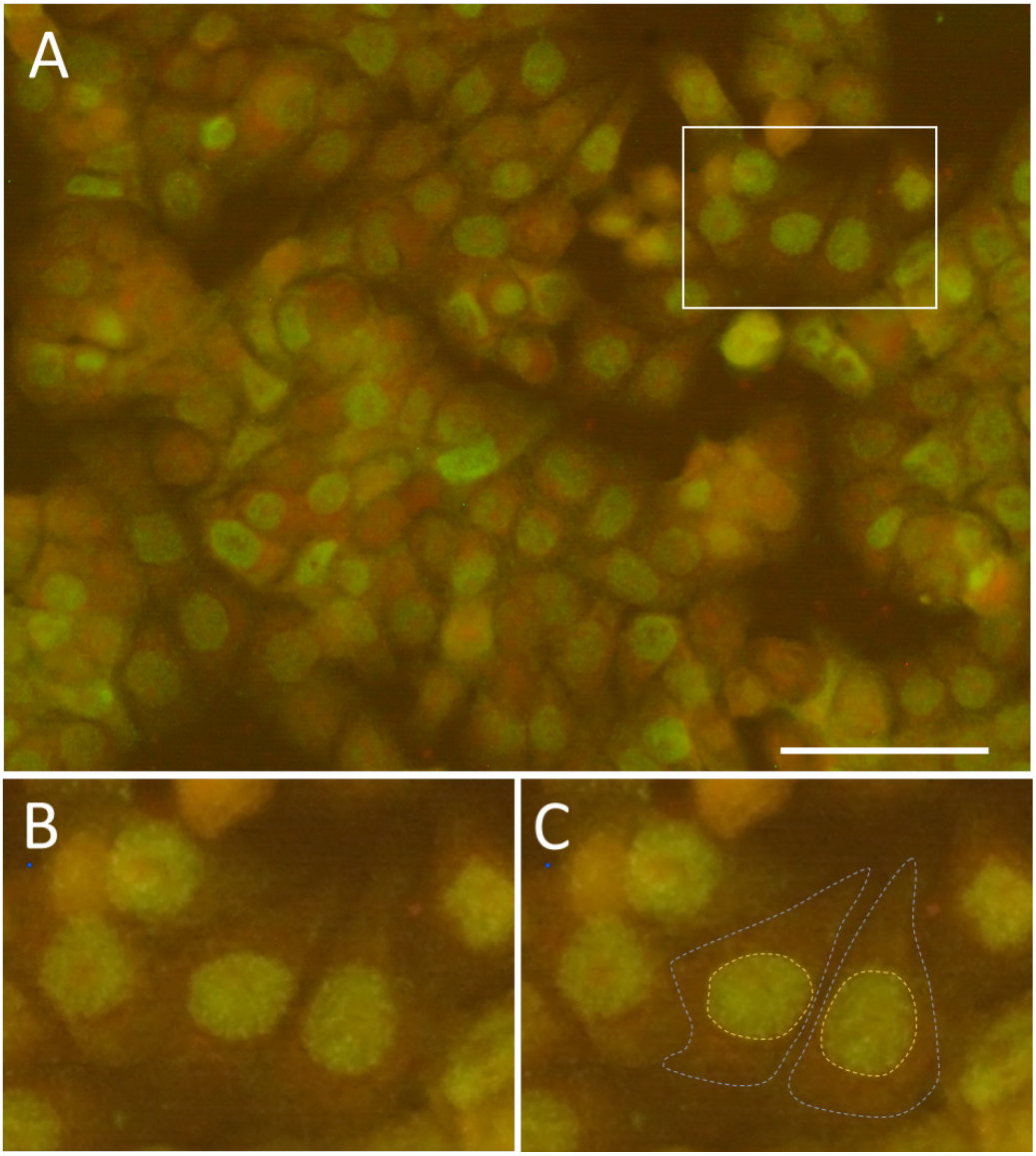


Figure 9. Nuclear expression of YAP1 protein in HT29 cells.

A. Indirect immunofluorescence of newly confluent HT29 cells confirmed the presence of the YAP1 protein in a large proportion of cells. Evans blue dye was used to give contrast and to highlight the cytoplasm, observed by red fluorescence in tissue sections by fluorescence microscopy. Therefore, cytoplasm was stained with Evans blue and is shown in red. The YAP1 protein, which is shown in green, is localized in the nucleus of HT29 cells. B, C. Higher magnification of the insert from panel A showing nuclear localization of YAP1. Scale bar in A = 50µm.

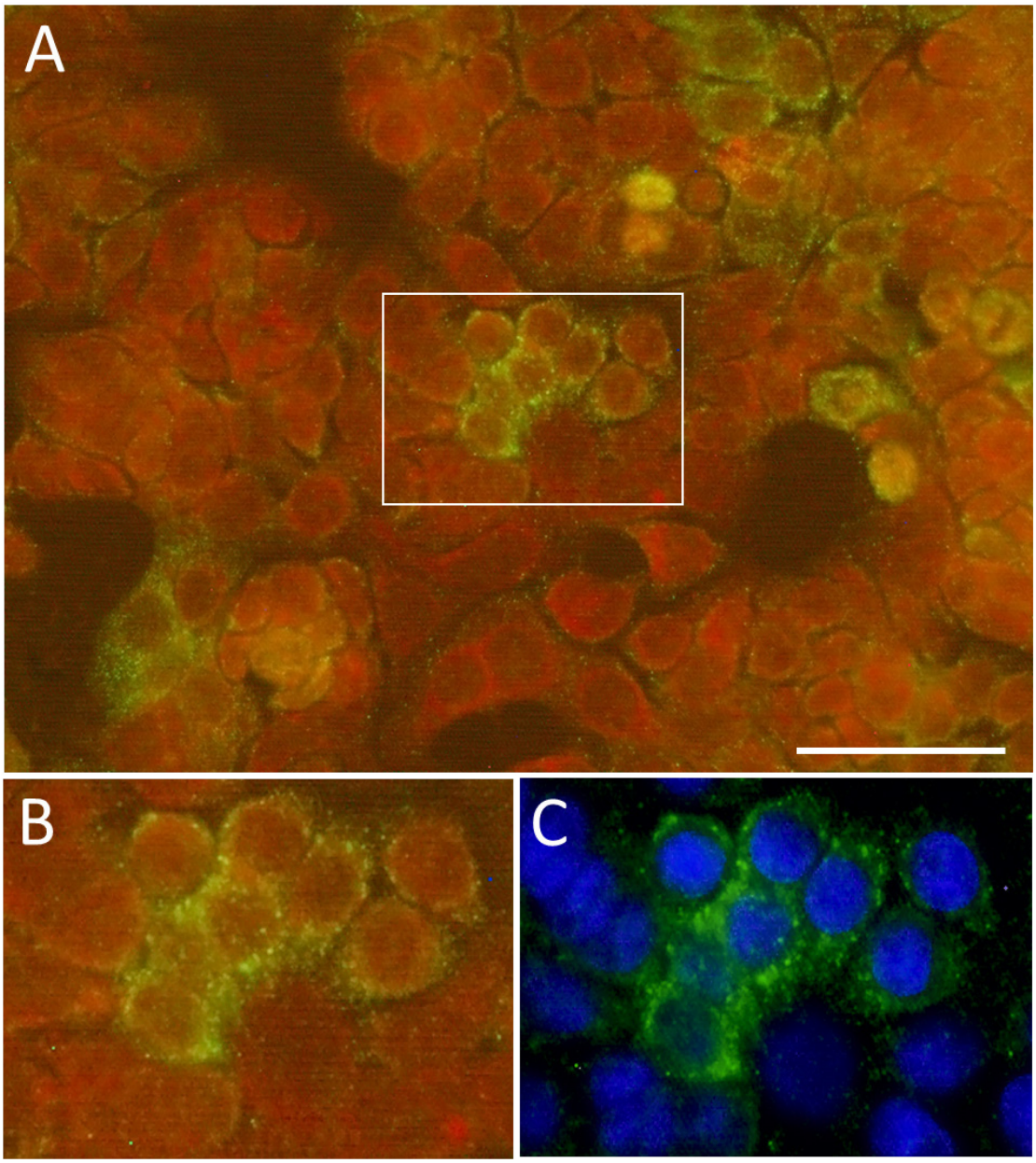


Figure 10. The expression of LGR5 protein in HT29 cells.

A, B. Indirect immunofluorescence representing newly confluent HT29 cells containing a few LGR5 positive cells. The red color is Evans blue dye used to highlight the cytoplasm and nuclei. The nucleus and cytoplasm can be observed in dark red and bright red, respectively. C. DAPI was used to confirm that dark red represents the nucleus. LGR5 staining can be observed in green and is localized in the cytoplasm of some HT29 cells. Scale bar in A = 50µm.

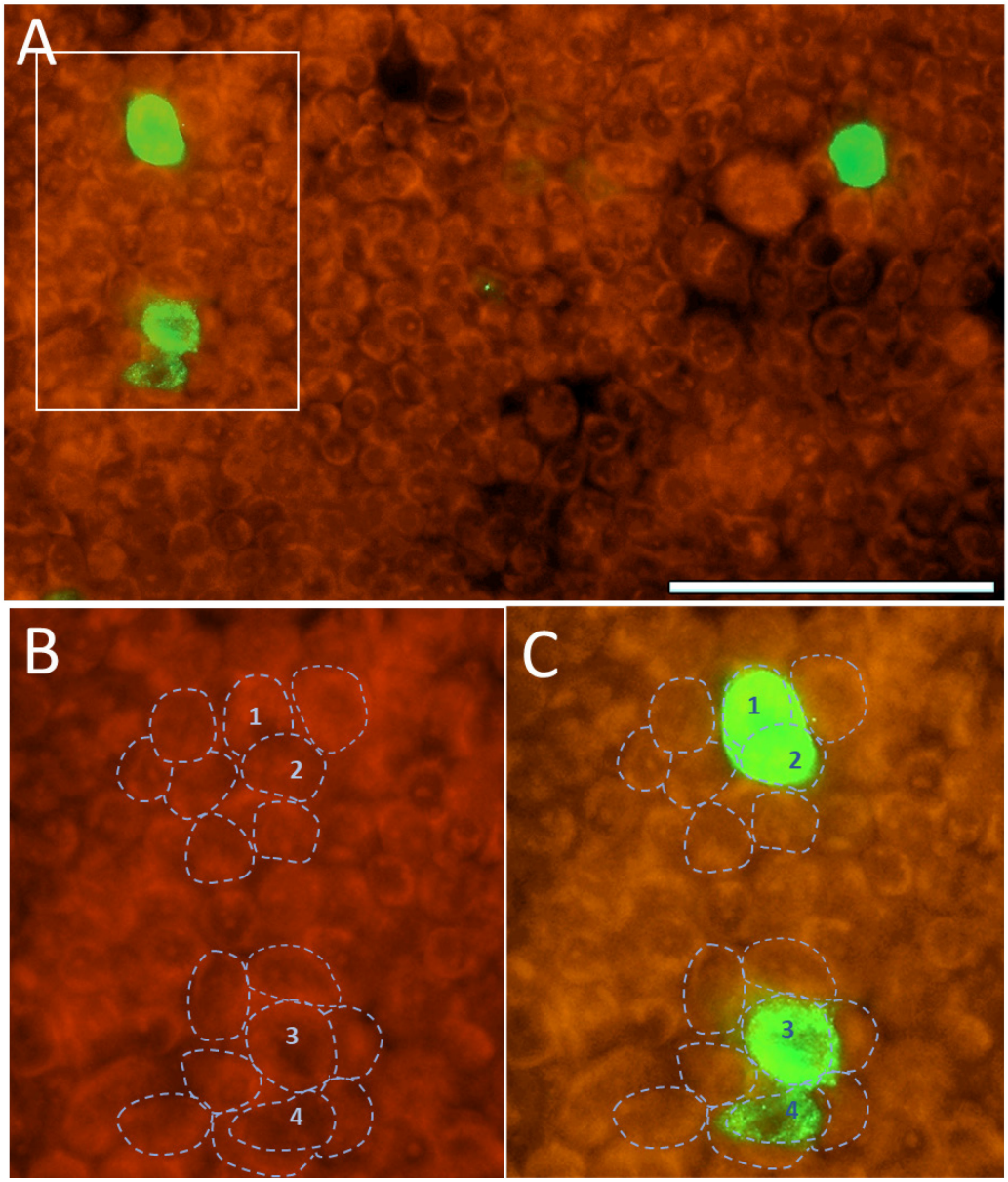


Figure 11. MUC2 expression in HT29 cells.

A. Indirect immunofluorescence in 3-day post confluent HT29 cells shows the presence of a few MUC2 positive cells. The green colour represents MUC2 and red represents the cytoplasm and nucleus. B. Higher magnification of the insert in A showing the nucleus and cytoplasm in dark red and bright red, respectively. C. Same region than B showing 4 MUC-2 positive cells. Scale bar in A = 50µm.

3.3. Knockdown of YAP1 expression using verteporfin

Verteporfin (VP) is a medication used as a photosensitizer which has been used in cancer therapy. Previous studies have reported the inhibitory effect of VP on the formation of the YAP1/TAZ-TEAD complex. It has also been exhibited that VP degrades YAP1/TAZ through up regulation of the 14-3-3 protein (Wang *et al.*, 2016). Thus, various studies have suggested that VP is a YAP1 inhibitor and could be a good treatment for limiting YAP1 activity. Therefore, VP was used in HT29 and Caco-2/15 cells to inhibit YAP1 in HT29 and YAP1/TAZ in Caco-2/15 cells. Several concentrations of VP (1µM, 5µM and 10 µM) were utilized for 5 days. Using VP at 5µM and 10 µM concentrations had a high cytotoxicity effect with the result of cell death. In spite of the low cytotoxicity effect of VP at 1µM, its efficiency for YAP1/TAZ inhibition was very low in both CRC cell lines (Figure 12). Therefore, another technique was performed to knockdown YAP1.

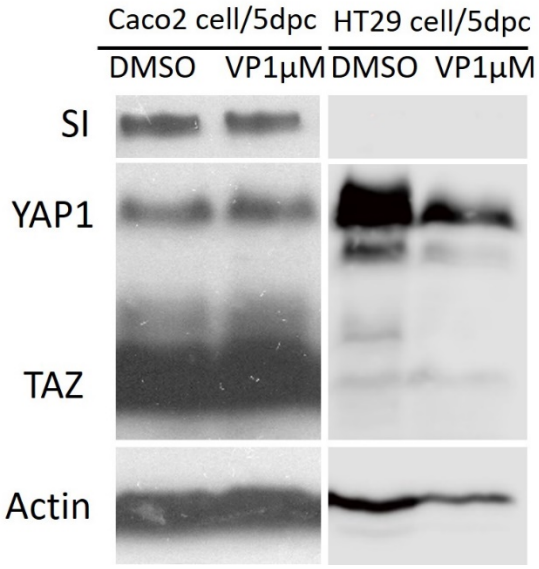
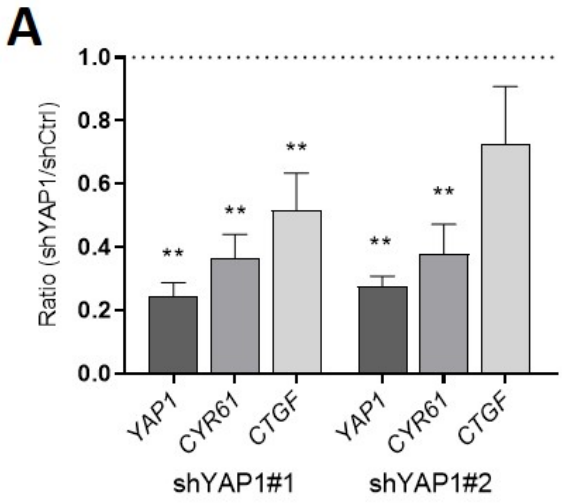


Figure 12. Effect of verteporfin on YAP1/TAZ activity.

Western blot analysis was utilized to show the YAP1, TAZ and SI expression in VP treated HT29 and Caco-2/15 cells compared with control cells (DMSO treated). β-actin was used as a loading control.

3.4. Knockdown of YAP1 expression by shRNAs

To determine whether YAP1 is involved in stemness and/or differentiation, sub-confluent HT29 cells were infected with shRNA, shYAP1#1 and shYAP1#2, in order to abolish YAP1 expression in HT29 cells. Since the TAZ protein is not expressed in HT29 cells, shRNA for TAZ was used as a control (shCtrl). The infected cells were harvested for RNA and protein at subconfluence (-2 d), 0 and 8-day post confluence. To determine the efficiency of YAP1 knockdown, the expression of YAP1 and YAP1 target genes including CTGF and CYR61 was first evaluated at the transcript level in newly confluent shYAP1 cells relative to shCtrl cells. YAP1, CYR61 and CTGF expression were all decreased significantly relative to shCtrl in both shYAP1#1 and shYAP1#2 cells (Figure 13A). Repression of YAP1 expression was confirmed at the protein level with both shYAP1 expressing HT29 cells while TAZ remained below the detection level (Figure 13B). YAP1 knockdown was also efficient in 8-day post-confluent shYAP1 cells (data not shown).



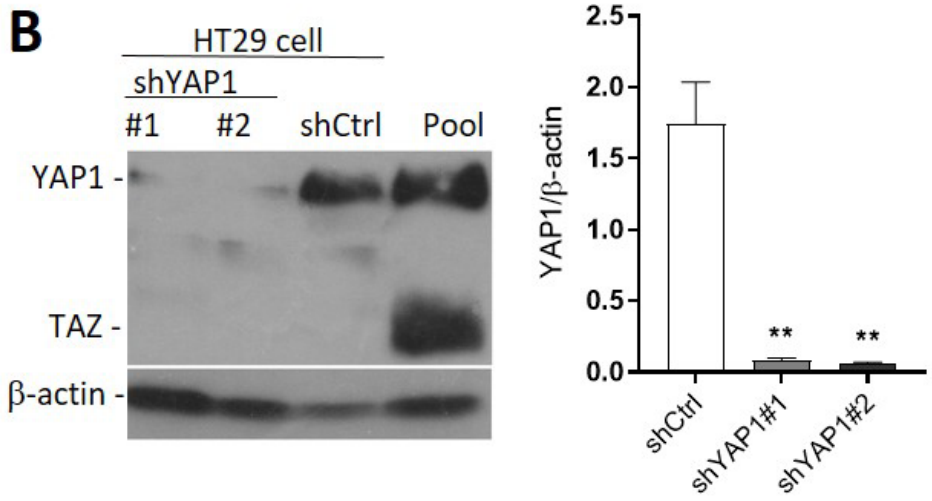


Figure 13. Efficiency of YAP1 knockdown in HT29 cells.

A. qPCR analyses were performed to detect the expression of YAP1 and two of its target genes CYR61 and CTGF in shYAP1 stably expressing cells using two specific sequences (identified as shYAP1 #1 and #2) relative to shCtrl. **B.** Western blot analysis showing the expression of YAP1 protein in shYAP1#1, shYAP1#2 and shCtrl expressing cells. A cancer cell pool was used as reference for YAP1 and TAZ detection. β-actin was used as a loading control. * $P < 0.05$, ** $P < 0.005$, n=3.

3.5. The effect of YAP1 knockdown on intestinal stem cell marker expression

To evaluate the effect of YAP1 knockdown on stemness, the mRNA levels of the stem cell markers used for wild type HT29 cells were assayed in shYAP1 cells. As shown in Figure 14, a reduction of *LGR5* and *PROM1* was noted in shYAP1 cells compared to shCtrl cells. Western blot analysis confirmed a reduced expression of LGR5 protein in shYAP1 HT29 cells compared with the control.

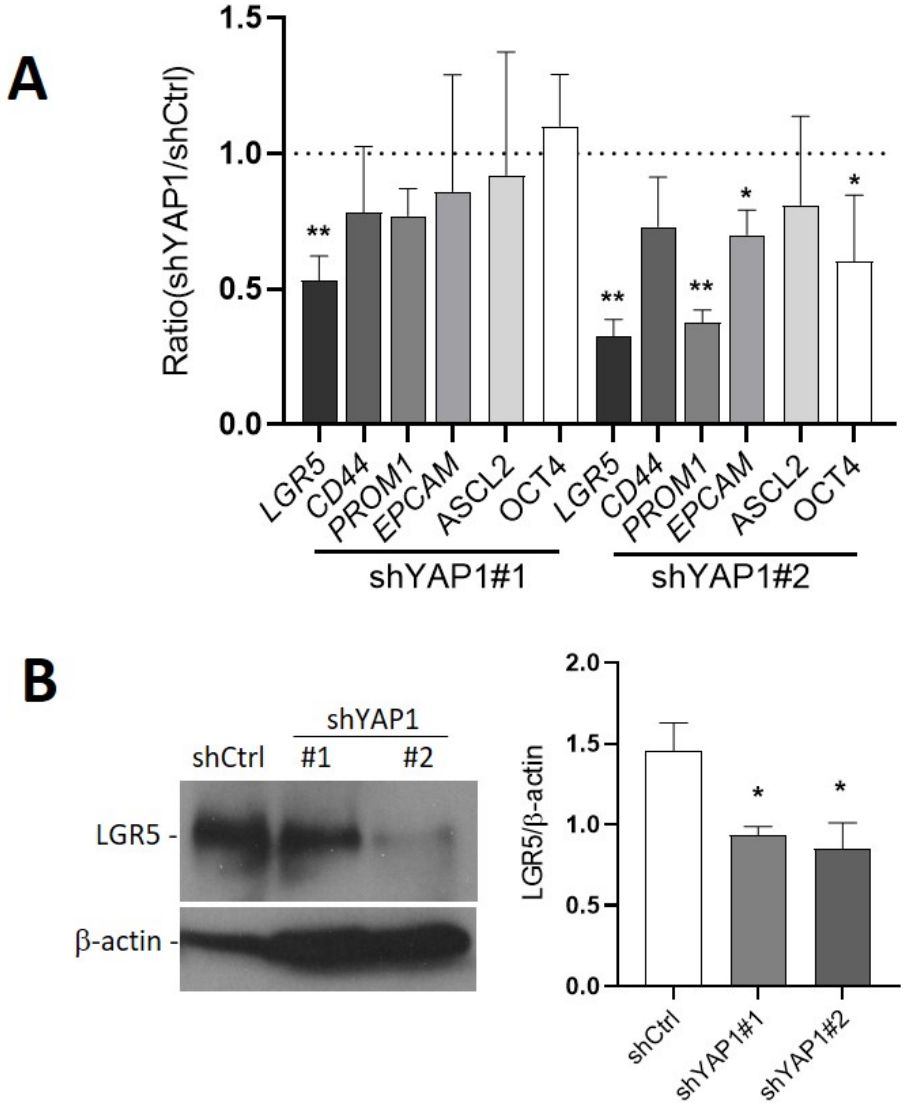


Figure 14. Effect of YAP1 knockdown on stem cell marker expression in subconfluent HT29 cells.

A. Transcript expression of the stem cell markers LGR5, CD44, PROM1, EPCAM, ASCL2 and OCT4 in YAP1 knockdown cells relative to shCtrl (dotted line). **B.** Reduction in LGR5 protein in both shYAP1 cell lines compared with shCtrl. β-actin was used as a loading control. * $P < 0.05$, ** $P < 0.005$, n=3.

3.6. The effect of YAP1 on the proliferation of intestinal epithelial cells

The BrdU (5-bromo-2'-deoxyuridine) assay was performed to determine the effect of YAP1 knockdown on the proliferation of HT29 cells. As shown in Figure 15, the number of proliferating cells was not changed significantly in YAP1 knockdown compared with control HT29 cells. Therefore, this result shows that YAP1 is not the only factor responsible for cell proliferation in HT29 cells.

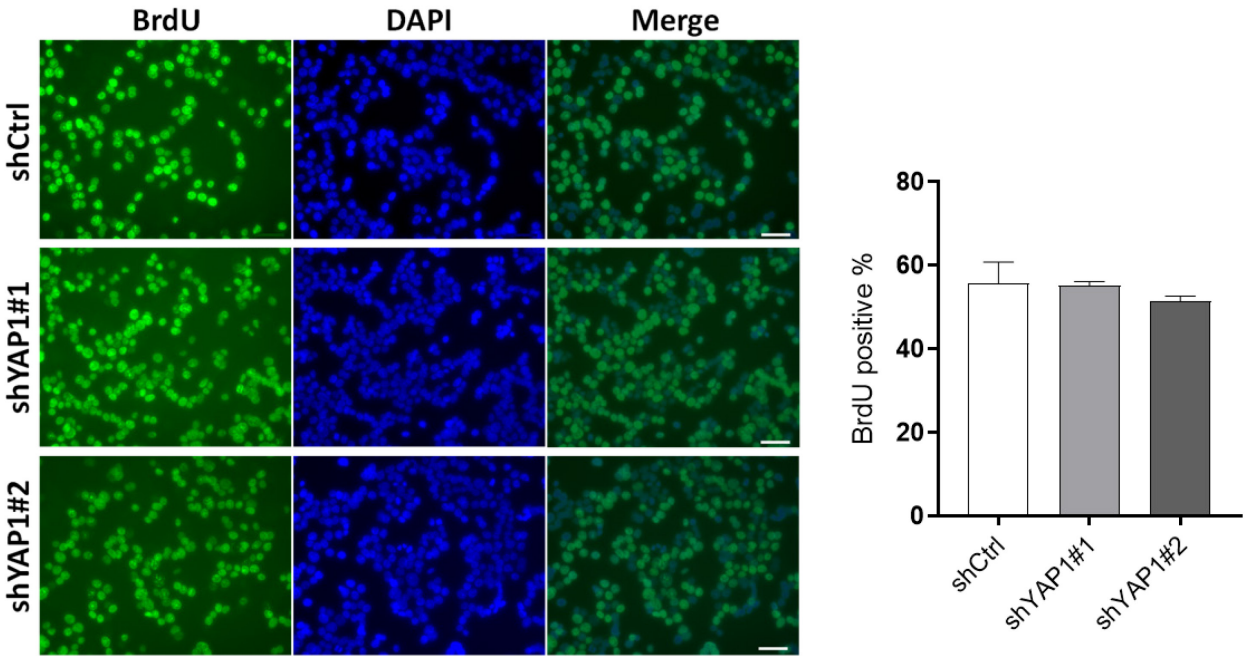


Figure 15. YAP1 knockdown has no effect on the proliferation of HT29 cells at sub-confluence.

Indirect immunofluorescent staining was used to show the number of proliferative cells which are stained with BrdU (green) and the number of total cells which are stained with DAPI (blue). The graph shows the percentage of BrdU positive cells compared with the total number of cells. Bar = 50µm, n=3.

3.7. The effect of YAP1 knockdown on intestinal epithelial cell differentiation

To investigate the effect of YAP1 on cell differentiation, qPCR analysis was first performed to detect the effect of YAP1 knockdown on the differentiation of absorptive, enteroendocrine, goblet and Paneth cell lineages. The results showed that the expression of the goblet cell markers, mucin 2 (MUC2) and trefoil factor-3 (TFF3) was increased significantly in YAP1 knockdown HT29 cells compared to shTAZ#1 used as control (Figure 16). In addition, expression of absorptive cell markers including sucrase-isomaltase (SI) and dipeptidyl peptidase-4 (DPPIV) was increased significantly at the transcription level in shYAP1 cells. However, removing YAP1 in HT29 cells had no effect on the expression of CHGA nor DEFA5, which are specific enteroendocrine and Paneth cell markers, respectively (Figure 16).

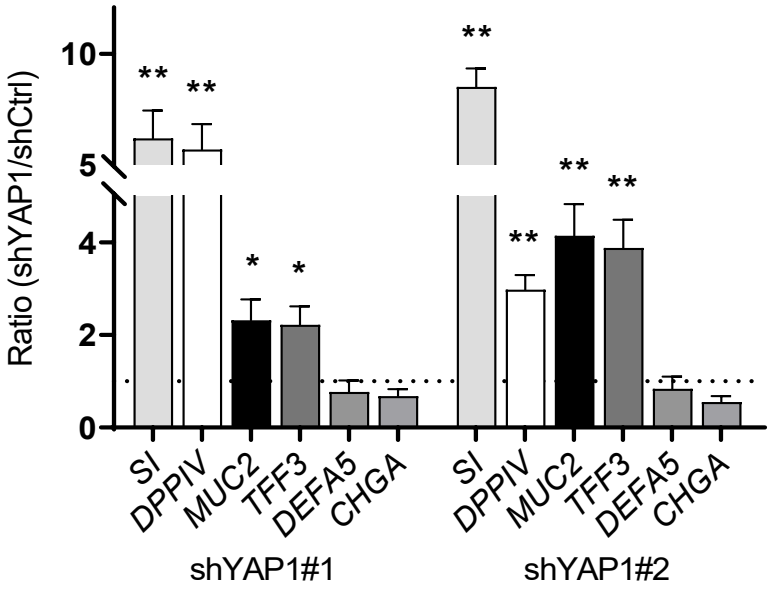
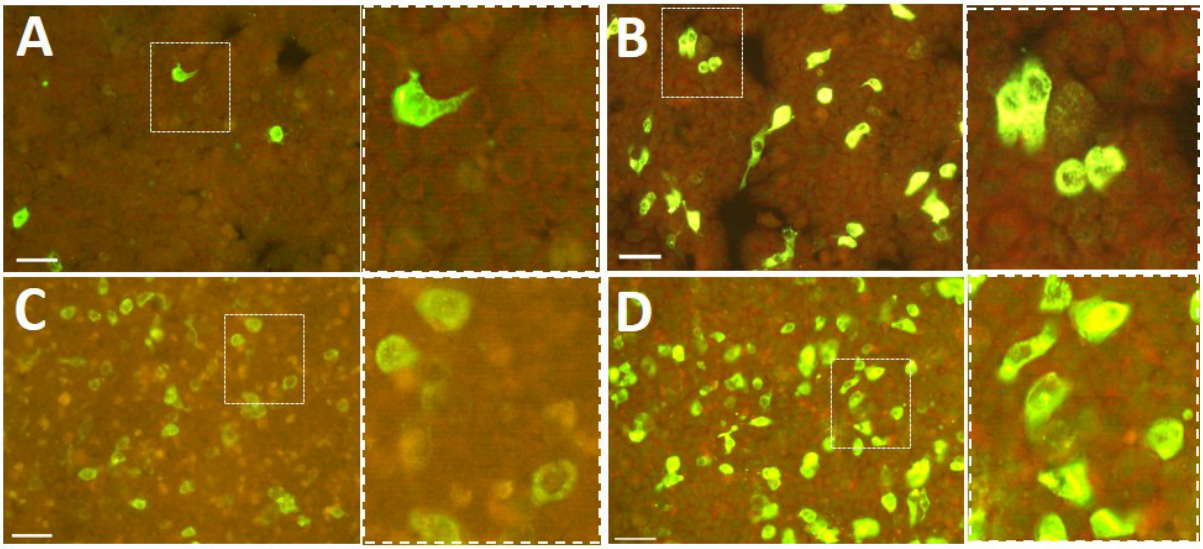


Figure 16. Expression of intestinal cell lineage markers in response to YAP1 knockdown.

Absorptive cell markers *SI* and *DPPIV*, goblet cell markers *MUC2* and *TFF3*, Paneth cell marker *DEFA5* and enteroendocrine cell marker *CHGA* were analyzed by qPCR in shYAP1#1 and shYAP1#2 expressing cells relative to shCtrl cells (dotted line). * $P < 0.05$, ** $P < 0.005$, n=3.

Indirect immunofluorescence on these cells revealed that goblet cell differentiation triggered by YAP1 abolition is not uniform in the monolayer but leads to a consistent apparent increase in the number of goblet cells in both newly confluent and 8-day post-confluent cells (Figure 17A-D). Western blot analysis confirmed that abolishing YAP1 expression in HT29 cells promotes goblet cell differentiation. As shown in Figure 17, the goblet cell markers MUC2 and TFF3 were consistently found to be increased in shYAP1 cells. Higher expression was observed in newly confluent (0PC) as well as in 8-day post-confluent (8PC) shYAP1 cells relative to shCtrl cells (Figure 17E). Absorptive cell differentiation was further investigated by indirect immunofluorescence and Western blot.



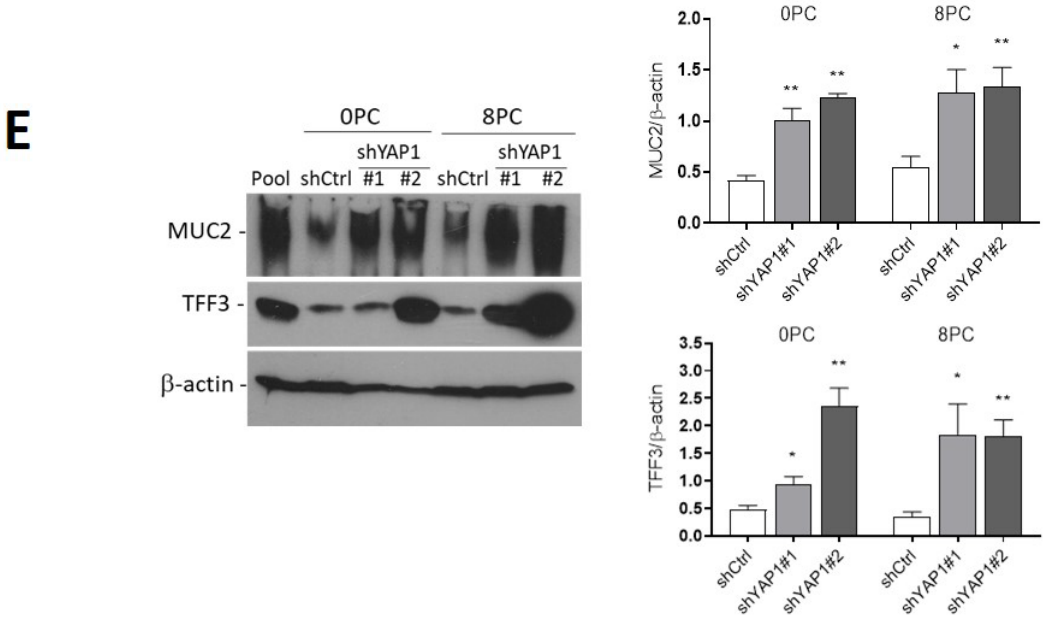
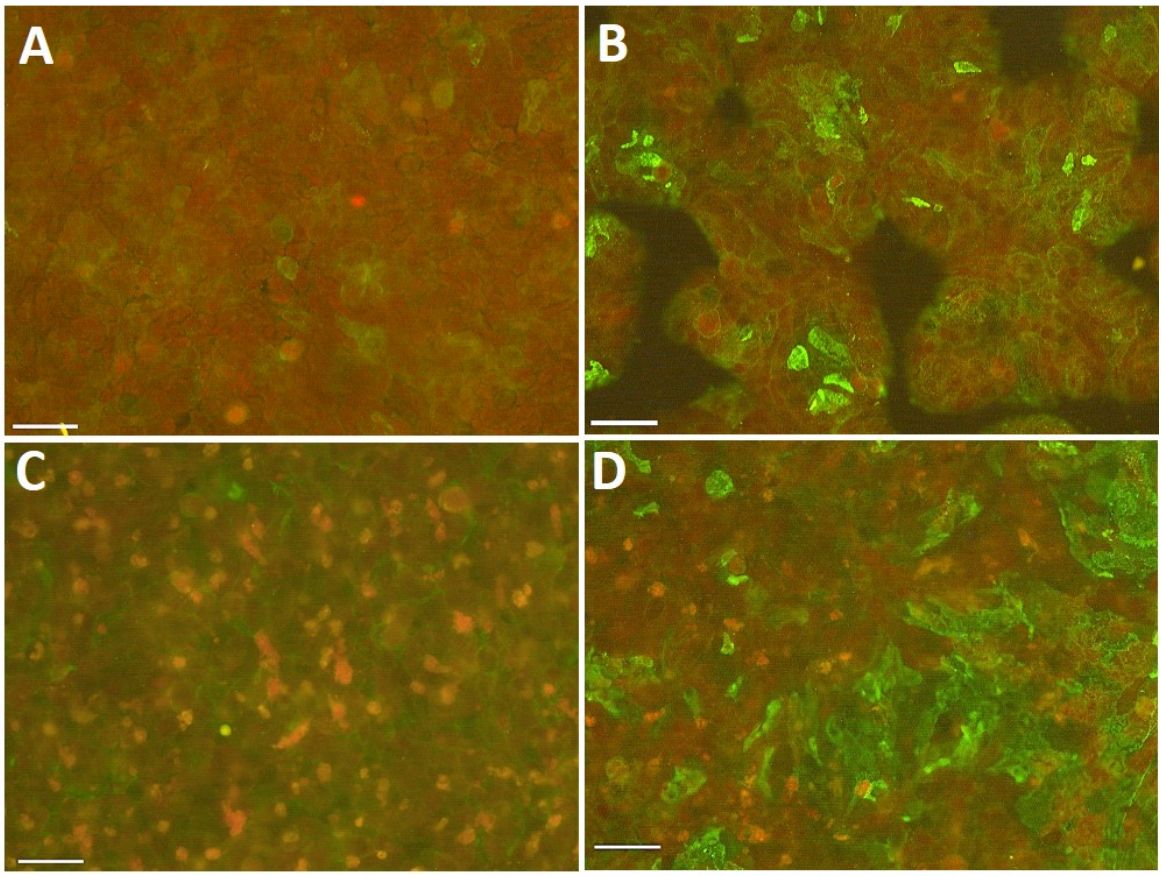


Figure 17. YAP1 knockdown stimulates goblet cell differentiation.

A-D. Indirect immunofluorescent analysis for the detection of MUC2 positive cells in shCtrl (A,C) and shYAP1#2 (B,D) at 0PC (A,B) and 8PC (C,D). Evans blue dye was used to give contrast and to highlight the cytoplasm. It can be observed in red while MUC2 positive cells can be observed in green in tissue sections by fluorescence microscopy. Bar = 50 µm. **E.** Representative Western blot analysis and data compilation from three separate experiments showing a higher expression of MUC2 and TFF3 in shYAP1#1 and shYAP1#2 expressing cells relative to shCtrl cells at both day 0 (0PC) and 8 days (8PC) post-confluence. β-actin was used as a loading control. *P< 0.05, ** P< 0.005, n=3.

As shown in Figure 18, Indirect immunofluorescence staining at sub confluence and 8 days post confluence showed that the apparent number of DPPIV positive cells was increased in YAP1 knockdown HT29 cells compared with the control (Figure 18A-D). It is noteworthy that as for goblet cells, only a subset of shYAP1 express DPPIV in the HT29 cell monolayer. To confirm this observation, Western blot analysis was used to evaluate the expression of the absorptive cell markers. As shown in Figure 18E, SI was barely detectable in control HT29 cells at all stages. The expression of SI protein was not at significant levels in newly confluent shYAP1 cells but was expressed at a high level in 8-day post-confluent cells (Figure 18E). DPPIV was found to be increased in both newly confluent and post-confluent shYAP1 cells relative to shCtrl cells (Figure 18E).


A
B
C
D

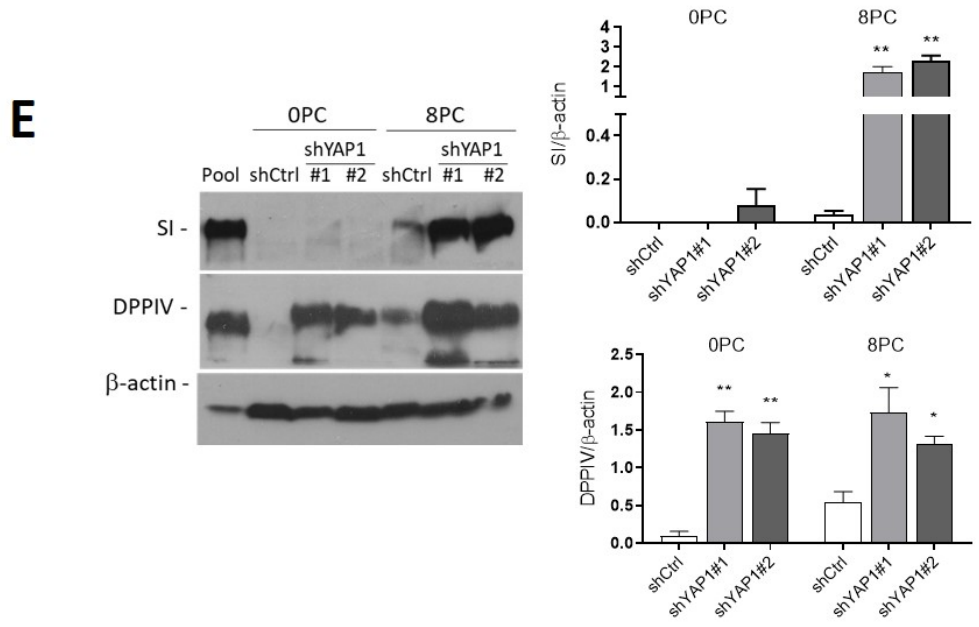

E
0PC
8PC
shYAP1
Pool shCtrl #1 #2 shCtrl #1 #2
SI -
DPPIV -
β-actin -
SI/β-actin
0PC
8PC
shCtrl
shYAP1#1
shYAP1#2
DPPIV/β-actin

Figure 18. YAP1 knockdown stimulates absorptive cell differentiation.

A-D. Indirect immunofluorescent analysis for the detection of DPPIV positive cells in shCtrl (A,C) and shYAP1#2 (B,D) at 0PC (A,B) and 8PC (C,D). Evans blue dye was used to give contrast and to highlight the cytoplasm. It can be observed in red while DPPIV positive cells can be observed in green in tissue sections by fluorescence microscopy. Bar = 50µm. **E.** Representative Western blot analysis and data compilation from three separate experiments showing higher expression of SI and DPPIV in shYAP1#1 and shYAP1#2 expressing cells relative to shCtrl cells at both day 0 (0PC) and 8 days (8PC) post-confluence. β-actin was used as a loading control. *P<0.05, ** P< 0.005, n=3.

Differentiation of goblet- and absorptive-like cells in YAP1 knockdown HT29 cells was confirmed by transmission electron microscopy (TEM) (Figure 19). The control cells are small and multilayered. They contain a few undeveloped granules and microvilli. However, shYAP1 expressing cells are larger and some display microvilli with features of absorptive-like cells while some contain mucinous granules with the appearance of goblet-like cells.

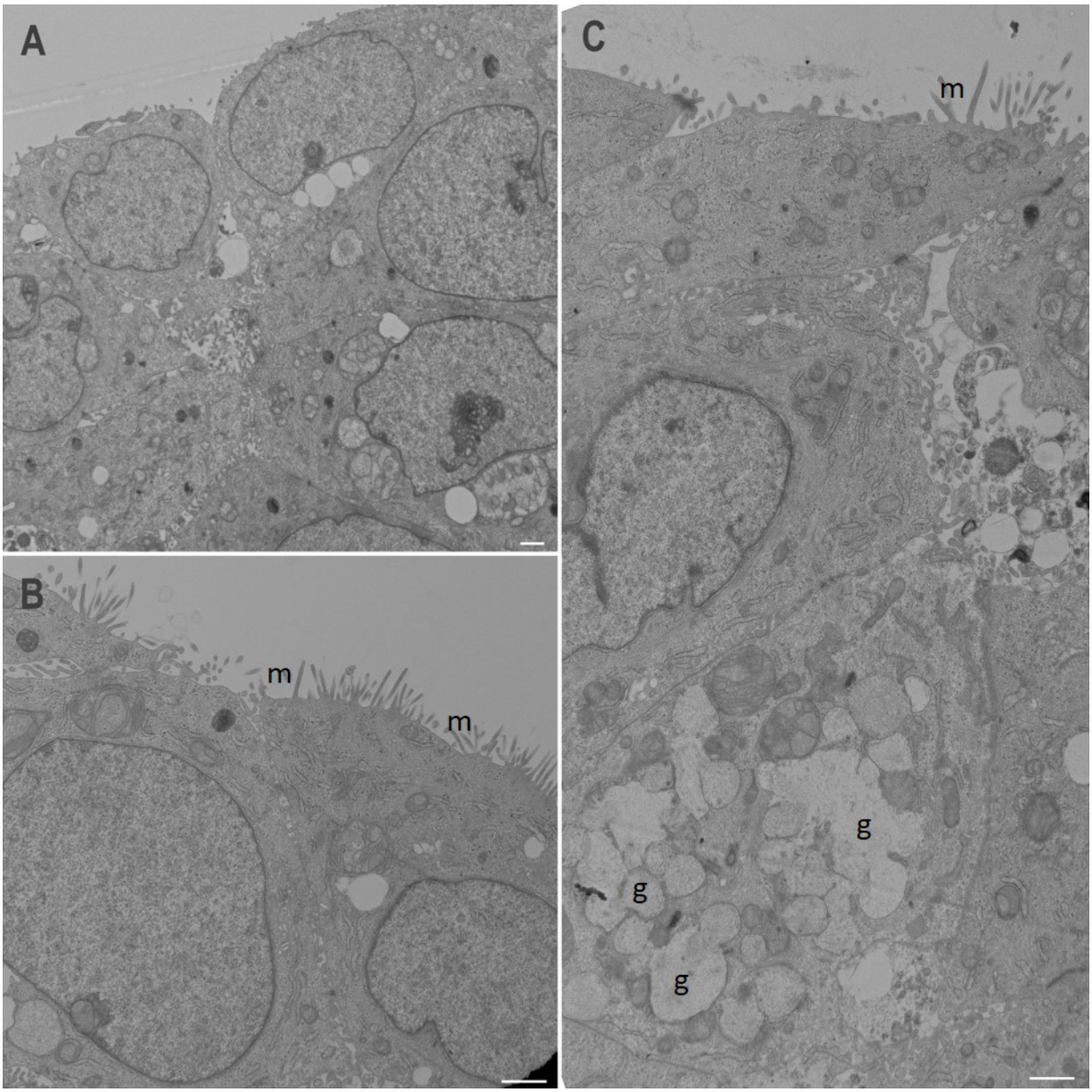


Figure 19. Transmission electron microscopy of HT29 stably expressing shCtrl or shYAP1 at 8 days post confluence.

A. Control cells expressing shTAZ are small and multilayered and express limited differentiation characteristics. **B-C**. Cells expressing shYAP1 are larger and exhibit some absorptive-like features such as regular microvilli (M) (B) and goblet cell-like features such as mucinous granule (G) forming goblet-like aggregates (lower part of C). Scale bars in A, B and C = 1 µm.

In order to further support our observations on the role of YAP1 in intestinal epithelial cell differentiation, YAP1 expression was knocked down in Caco-2/15 cells using shRNA, shYAP1#1 and shYAP1#2. Considering that TAZ1 is expressed by Caco-2/15 cells, shLUC was utilized as a control. Western blot analysis was performed to determine the efficiency of the YAP1 knockdown. Figure 20 shows that YAP1 expression was knocked down partially in both Caco-2/15 shYAP1#1 and shYAP1#2 compared with control. The expression of TAZ relative to β-actin in control versus shYAP1#1 and shYAP1#2 showed a slight but statistically significant increase (1.13 ± 0.41, 1.86 ± 0.54 and 2.3 ± 0.64, respectively, $P< 0.04$). Since Caco-2/15 only have the potential to differentiate to absorptive cells, the effect of YAP1 knockdown was investigated on the expression of the absorptive cell marker SI in 5-day post confluent cells using qPCR and Western blot analysis. A higher expression of SI mRNA was observed in Caco-2/15 cells expressing shYAP1 compared with control cells expressing shLUC (Figure 21A). A significant increase in SI protein was also observed in shYAP1 expressing cells as compared with controls (Figure 21B). Although not further investigated, these results support the finding that YAP1 exerts an inhibitory influence on intestinal cell differentiation.

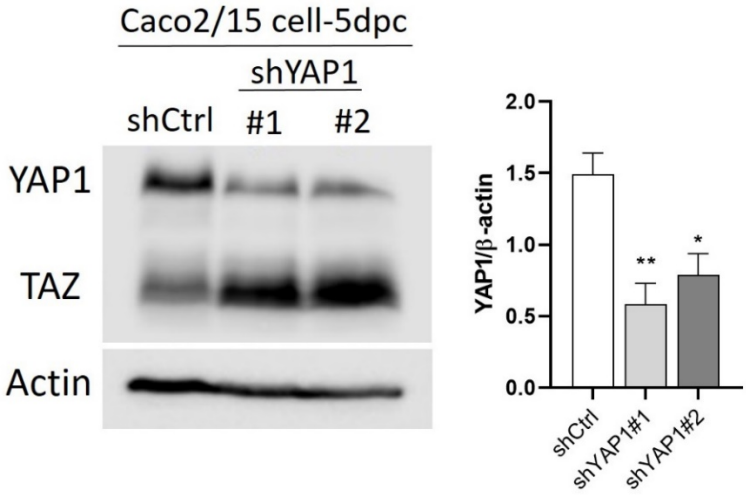


Figure 20. YAP1 knockdown in the Caco-2/15 cell line.

Representative Western blot analysis and data compilation from three separate experiments showing a reduction of YAP1 expression at the protein level in shYAP1#1 and shYAP1#2 relative to shCtrl. β-actin was used as a loading control. *$P< 0.05$, ** $P< 0.005$, n=3.

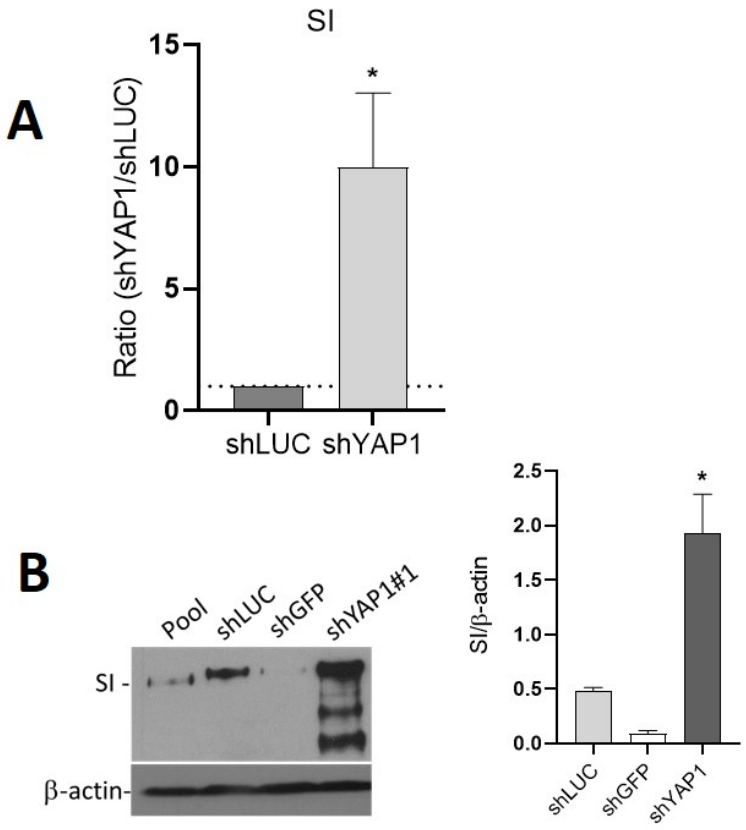


Figure 21. The effect of shYAP1 expression on Caco-2/15 cell differentiation.

A. Transcript expression of the absorptive cell marker SI in Caco-2/15 cells expressing shYAP1 relative to shCtrl (shLUC). **B.** Western blot analysis and data compilation showing higher expression of SI (upper band corresponding to 205 KD) in shYAP1 Caco-2/15 cells relative to shCtrl at 5 days post-confluence. β-actin was used as a loading control. Statistical comparison between shYAP1 vs shLUC: * $P < 0.05$, n=3.

3.8. Role of YAP1 on the expression of intestinal differentiation-regulating transcription factors

As a first step to investigate the mechanism by which the abolition of YAP1 leads to differentiation, the expression of specific transcription factors involved in goblet and absorptive cell differentiation was studied. *CDX2*, *ATOH1*, *HNF1α* and *KLF4* were analyzed by qPCR. As shown in Figure 22, the expression of *CDX2* and *ATOH1* was increased significantly in both sets of YAP1 knockdown cells compared with the control while the other tested transcription factors were not found to be significantly modulated (Figure

22A). Western blot analysis confirmed the important induction of CDX2 protein expression in shYAP1 expressing cells compared to shCtrl in which the CDX2 protein was barely detectable (Figure 22B). However, ATOH1 was found to be below the detection level in HT29 under all conditions and was therefore not further analyzed.

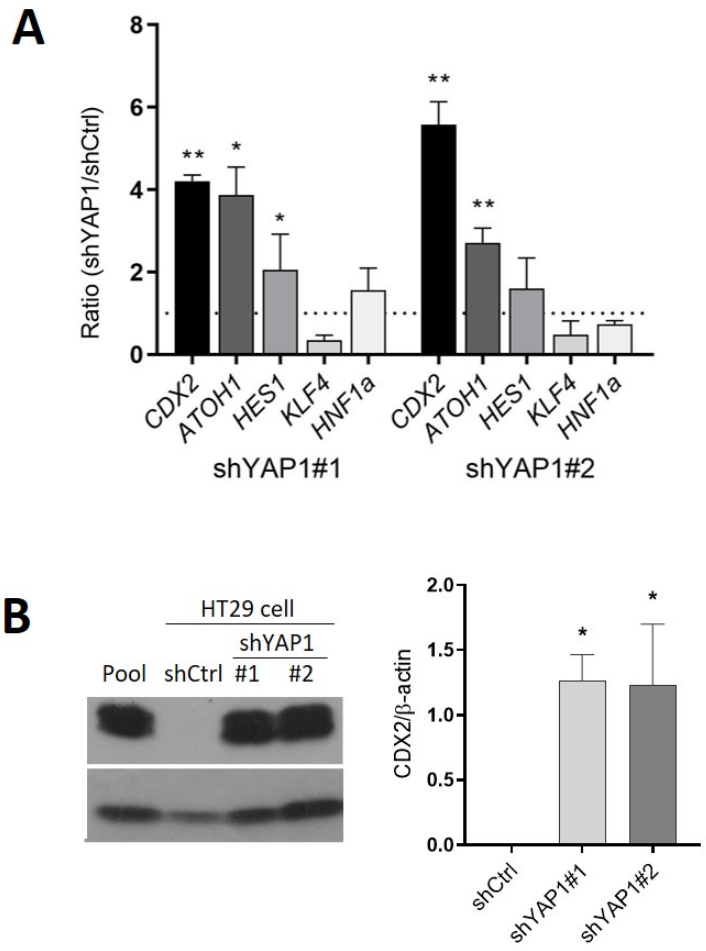
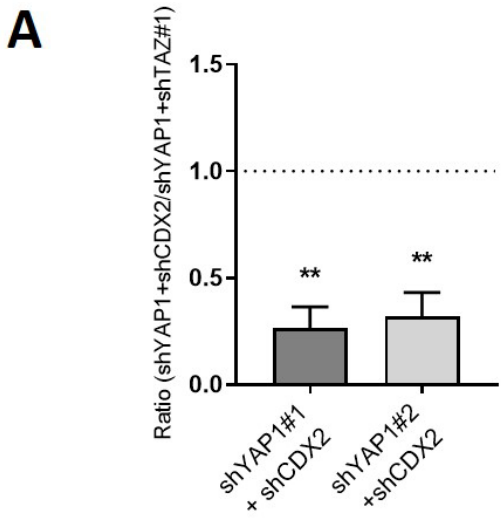


Figure 22. Expression of intestinal differentiation-regulating transcription factors in response to YAP1 knockdown.

A. qPCR analysis of the expression of various transcription factors involved in intestinal epithelial cell differentiation in shYAP1#1 and shYAP1#2 expressing cells relative to shCtrl (dotted line) showing the significant increase in expression of *CDX2* and *ATOH1* in YAP1 knockdown cells. **B.** Representative Western blot analysis and data compilation from three separate experiments showing higher expression of the CDX2 protein (both phosphorylated and nonphosphorylated forms) in YAP1 knockdown HT29 cells. The β-actin was used as a loading control. *P< 0.05, ** P< 0.005, n=3.

3.9. YAP1 controls the differentiation of intestinal absorptive and goblet cells through CDX2

To explore the involvement of CDX2 as a possible mediator of the pro-differentiation effect of YAP1 abolition in HT29 cells, the expression of CDX2 was knocked down in shYAP1 HT29 cells using shRNA. The shYAP1 cells stably expressing ± shCDX2 and shCtrl were harvested at 5 days post-confluence and mRNA and protein fractions were prepared. We first evaluated the efficiency of CDX2 knockdown by measuring CDX2 at transcriptional and protein levels using qPCR and Western blot analysis, respectively. As shown in Figure 23, the expression of CDX2 was significantly diminished in shYAP1 + shCDX2 expressing cells at both the mRNA and protein levels. In fact, Western blot analysis showed that the increase in CDX2 resulting from YAP1 abolition was completely neutralized in shYAP1 expressing shCDX2 (Figure 23B).



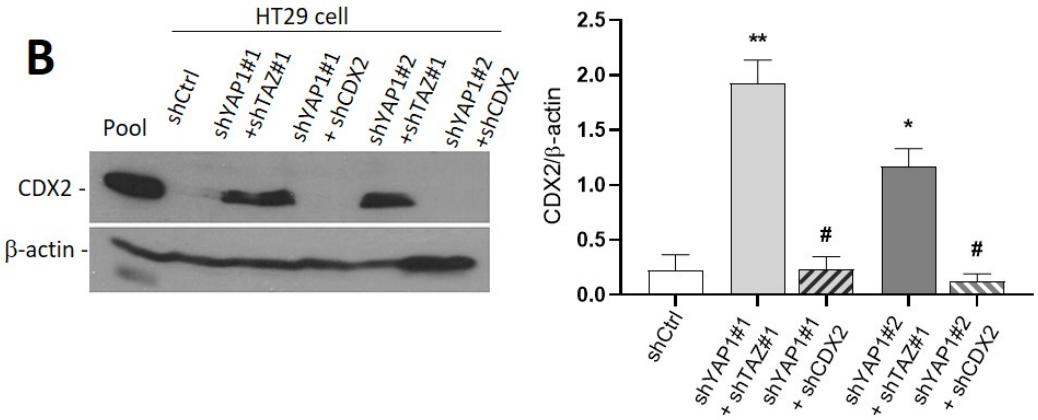
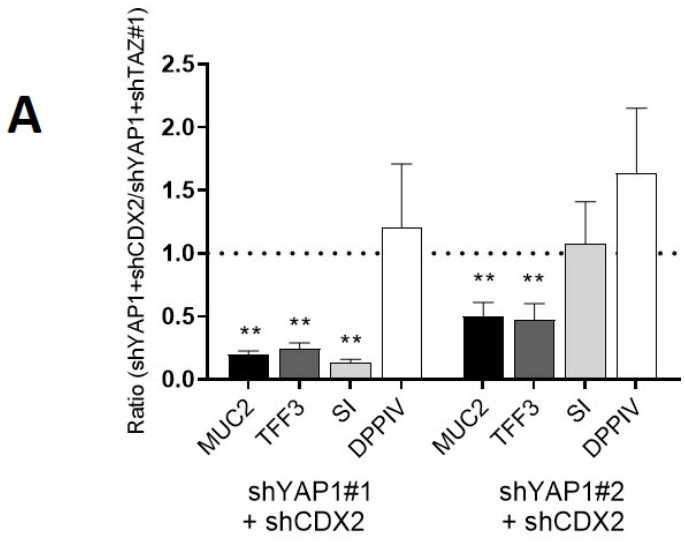


Figure 23. Efficiency of CDX2 knockdown in shYAP1 expressing HT29 cells.

A. qPCR analyses were performed to evaluate the expression of *CDX2* in shCDX2 stably expressing shYAP1#1 and #2 cells relative to shTAZ#1 expressing shYAP1#1 and #2 cells. **B.** Representative Western blot analysis showing the expression of CDX2 protein in shCtrl vs shYAP1#1 and shYAP1#2 + shCDX2 expressing cells and data compilation of three separate experiments. A cancer cell pool was used as reference for CDX2 detection. β-actin was used as a loading control. * Significant vs shCtrl; # significant vs shYAP1+shTAZ#1; */# $P < 0.05$, ** $P < 0.005$, n=3.

Further analysis of these 5-day post-confluent cells for the expression of absorptive and goblet cell markers showed a significant reduction of three of these markers at both transcript (Figure 24A) and protein levels (Figure 24B). Indeed, MUC2, TFF3 and SI were consistently repressed to comparable levels as those observed in shCtrl cells (Figure 24B) suggesting that the upregulation of these markers in YAP1 knockdown cells mainly results from CDX2 upregulation. One exception was DPPIV for which the expression was not altered by the abolition of CDX2 expression indicating that YAP1 may regulate other transcription factor(s) as well.


A
Ratio (shYAP1+shCDX2/shYAP1+shTAZ#1)
2.5
2.0
1.5
1.0
0.5
0.0
**
**
**
**
**
MUC2
TFF3
SI
DPPIV
MUC2
TFF3
SI
DPPIV
shYAP1#1
+ shCDX2
shYAP1#2
+ shCDX2

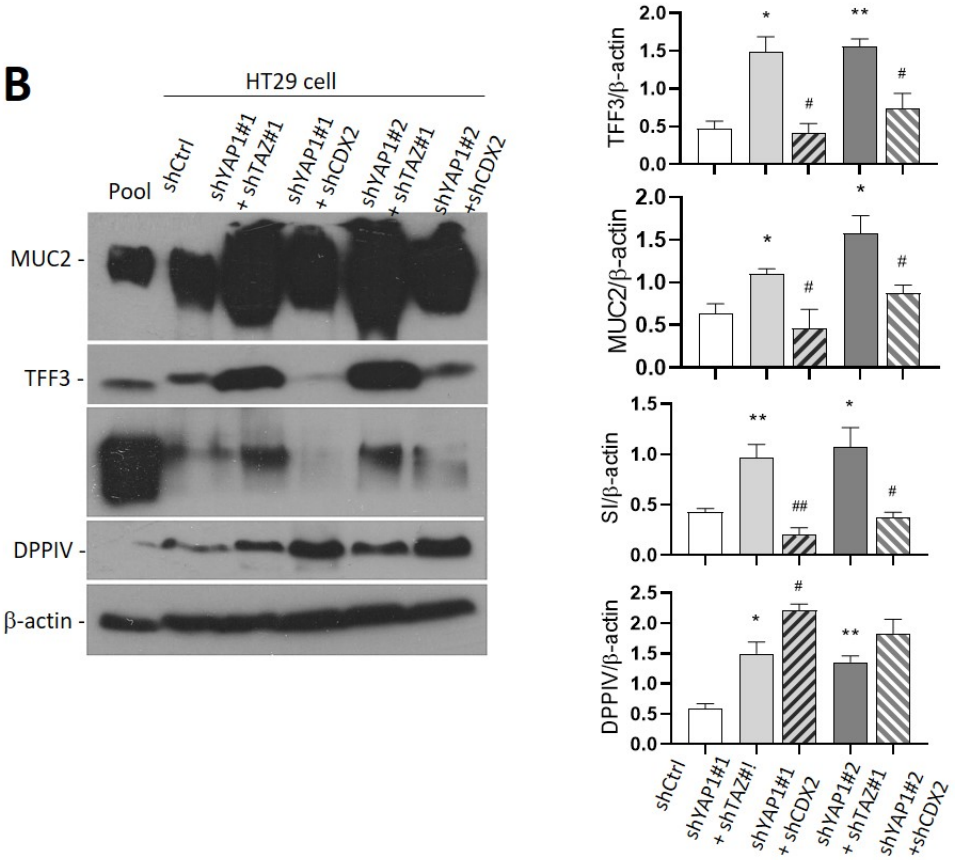

B
HT29 cell
Pool
shCtrl
shYAP1#1 + shTAZ#1
shYAP1#1 + shCDX2
shYAP1#2 + shTAZ#1
shYAP1#2 +shCDX2
MUC2 -
TFF3 -
DPPIV -
β-actin -
TFF3/β-actin
MUC2/β-actin
SI/β-actin
DPPIV/β-actin
shCtrl
shYAP1#1 + shTAZ#!
shYAP1#1 + shCDX2
shYAP1#2 + shTAZ#1
shYAP1#2 +shCDX2

Figure 24. CDX2 mediates the upregulation of most of the intestinal differentiation markers in shYAP1 cells.

A. qPCR analysis showing that the abolition of CDX2 in shYAP1 cells resulted in a significant reduction of *MUC2*, *TFF3* and *SI* expression while it remained without effect on *DPPIV*. **B.** Representative Western blot analysis showing the expression of MUC2, TFF3, SI and DPPIV in shCtrl (shYAP1#1 and shYAP1#2 + shTAZ#1) vs shYAP1#1 and shYAP1#2 + shCDX2 expressing cells and data compilation of three separate experiments. At the transcript level, the upregulation of these markers in shYAP1 cells appears to depend upon CDX2 since its abolition results in the restoration of HT29 basal levels for MUC2, TFF3 and SI while DPPIV appears to remain unaffected. A pool was used as a positive control. β-actin was used as a loading control. * Significant vs shCtrl; # significant vs shYAP1; */# $P < 0.05$, **/## $P < 0.005$, n=3.

Finally, expression of stem cell markers at 5 days post-confluence was significantly reduced in shYAP1 expressing cells as compared to shCtrl indicating that YAP1 still modulates stemness-like features even at confluence (Figure 25A). Interestingly, abolition of CDX2 expression in shYAP1 cells restored the levels of *LGR5* and *PROM1* expression observed in the wild-type HT29 population (Figure 25B).

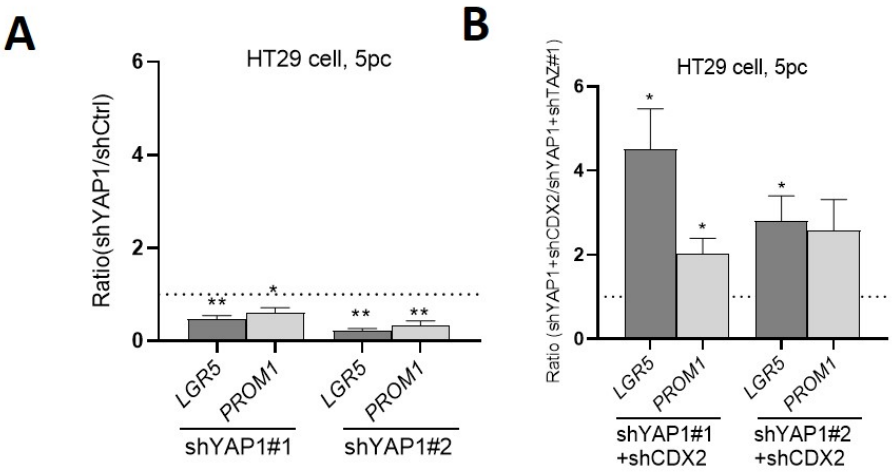
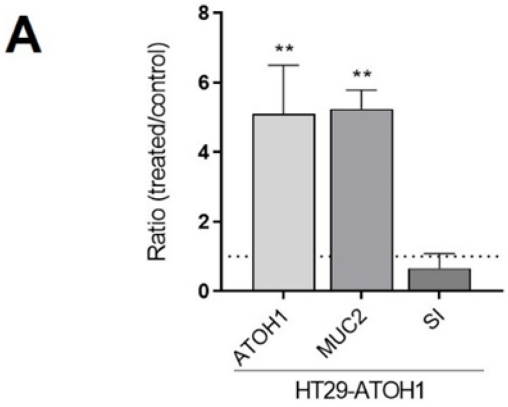


Figure 25. Abolition of CDX2 expression restores control LGR5 and PROM1 levels in 5-day post-confluent shYAP1 cells.

A. qPCR analysis showing that the abolition of YAP1 resulted in a significant reduction of *LGR5* and *PROM1* expression. **B**. qPCR analysis showing that the abolition of CDX2 in shYAP1 cells resulted in a significant increase in *LGR5* and *PROM1* expression relative to shYAP1 control cells. A: * significant vs shCtrl, B: * significant vs shYAP1; * $P < 0.05$, ** $P < 0.005$, n=3.

3.10. YAP1 controls MUC2 expression through the regulation of ATOH1

As shown earlier YAP1 abolition resulted in ATOH1 expression at the transcription level. To determine the role of ATOH1 in HT29 cell differentiation, the expression of ATOH1 was induced using viral strategies. To determine whether ATOH1 transduction into HT29 cells was successful, the expression of ATOH1 was measured by RT-PCR. As shown in Figure 26A, ATOH1 expression was increased compared with control cells. At the protein level ATOH1 is barely detectable by Western blot. According to previous studies, ATOH1 protein is phosphorylated by GSK3β and degraded via the proteasome in most colon cancer cells (Aragaki *et al.*, 2008). The level of ATOH1 protein can be increased using GSK3β or proteasomal inhibitors such as lithium chloride or carbobenzoxy-l-leucyl-l-leucyl-l-leucinal (MG132) in colon cancer cells (Aragaki *et al.*, 2008). Therefore, HT29 ATOH1 cells were treated with MG132 inhibitor (10µM) overnight (12 h). Treatment with this proteasomal inhibitor stabilizes ATOH1 protein which is then detectable by Western blot (Figure 26B). The mRNA levels of the goblet (MUC2) and absorptive (SI) cell markers were analyzed to evaluate the effect of ATOH1 expression on their differentiation. RT-PCR analysis of non-MG132 treated cells showed that MUC2 expression in the HT29 ATOH1 cell line was around 6-fold higher than in control cells while the expression of SI was the same as in control cells (Figure 26A). In addition, indirect immunofluorescent staining for MUC2 revealed that the number of MUC2 positive cell was increased in HT29 ATOH1 cells compared with control cells at 7-day confluence (Figure 26C).



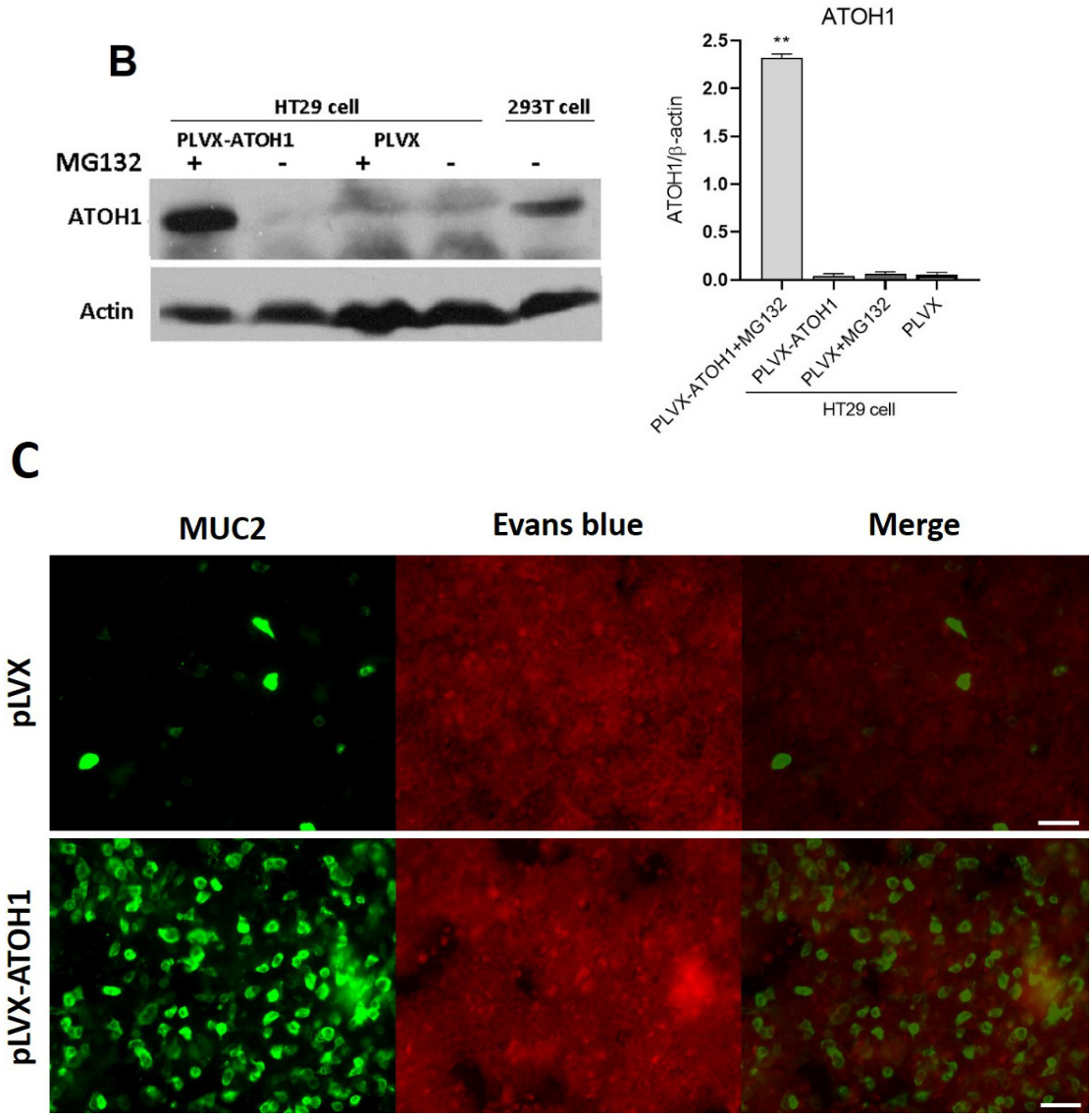


Figure 26. The effect of the ectopic expression of ATOH1 in HT29 cells.

A. The expression of ATOH1 mRNA was measured to detect the efficiency of ATOH1 induction in HT29 cells. The qPCR results show that MUC2 expression was increased in HT29 ATOH1 cells relative to the control while the expression of SI did not change. **B.** Western blot analysis showing the expression of ATOH1 protein in PLVX-ATOH1 HT29 cells treated with MG132 compared with untreated PLVX-ATOH1 HT29 cells and control (PLVX). 293T cells were used as a reference for ATOH1 detection. β-actin was used as a loading control. ** $P < 0.005$. **C.** Representative indirect immunofluorescent picture for the detection of MUC2 positive cells in pLVX-HT29 cells (Ctrl) and pLVX-ATOH1- HT29 cells at 7 PC. Red colour (Evans blue) represents the whole cell and green colour represents MUC2 expression. n=3, Bar = 50µm.

3.11. SFKs inhibition induces the differentiation of both intestinal absorptive and goblet cells

As discussed in the introduction, SFKs are involved in a number of different cellular activities such as regulation of cell proliferation, migration and anoikis (Bouchard et al., 2007; Thomas & Brugge, 1997). In addition, their association with the induction of the differentiation of Caco-2/15 cell has been reported previously (Nam *et al.*, 2002; Seltana *et al.*, 2013). Given that aberrant activation of SFKs is associated with tumor development and metastasis in various cancers (Irby & Yeatman, 2000), several FDA approved drugs have been developed to target SFK proteins including dasatinib, imatinib, SFK-1 and PP2 (Elias & Ditzel, 2015). Lamar and colleagues reported that the level of phosphorylated SRC family kinases especially SRC kinase is effectively decreased in breast cancer and melanoma cell lines by using dasatinib (Lamar *et al.*, 2019). In order to study the effect of the SFKs on intestinal epithelial cell differentiation, Caco-2/15 and HT29 cells were incubated with the SFK inhibitors PP2 at 20 µM (Bouchard *et al.*, 2007; Nam *et al.*, 2002; Seltana *et al.*, 2013), dasatinib at 10 µM (Honeywell *et al.*, 2020; Kim & Gumbiner, 2015) and DMSO as control. Treatment of the cells was started at -2 days of confluence (80% confluence) and continued for 5 days. Then, the cells were analyzed by qPCR to evaluate the effect of SFK inhibition on the expression of absorptive cell markers and both absorptive and goblet cell markers in Caco-2/15 and HT29 cells, respectively. The qPCR results showed that the expression of SI and DPPIV in Caco-2/15 cells (Figure 27A) and SI and MUC2 in HT29 cells (Figure 28A) was increased significantly in SFK treated cell compared with control cells. Western blot analysis confirmed that the inhibition of the SFKs in Caco-2/15 and HT29 cells promotes the differentiation of the absorptive and both absorptive and goblet cells, respectively. As shown in Figure 27B, the absorptive cell markers SI and DPPIV were consistently found to be increased at the protein level in SFK inhibited Caco-2/15 cells. In HT29 cells, higher expression of SI and DPPIV proteins as well as MUC2 and TFF3 proteins was observed in SFK inhibited cells relative to control cells (Figure 28B).

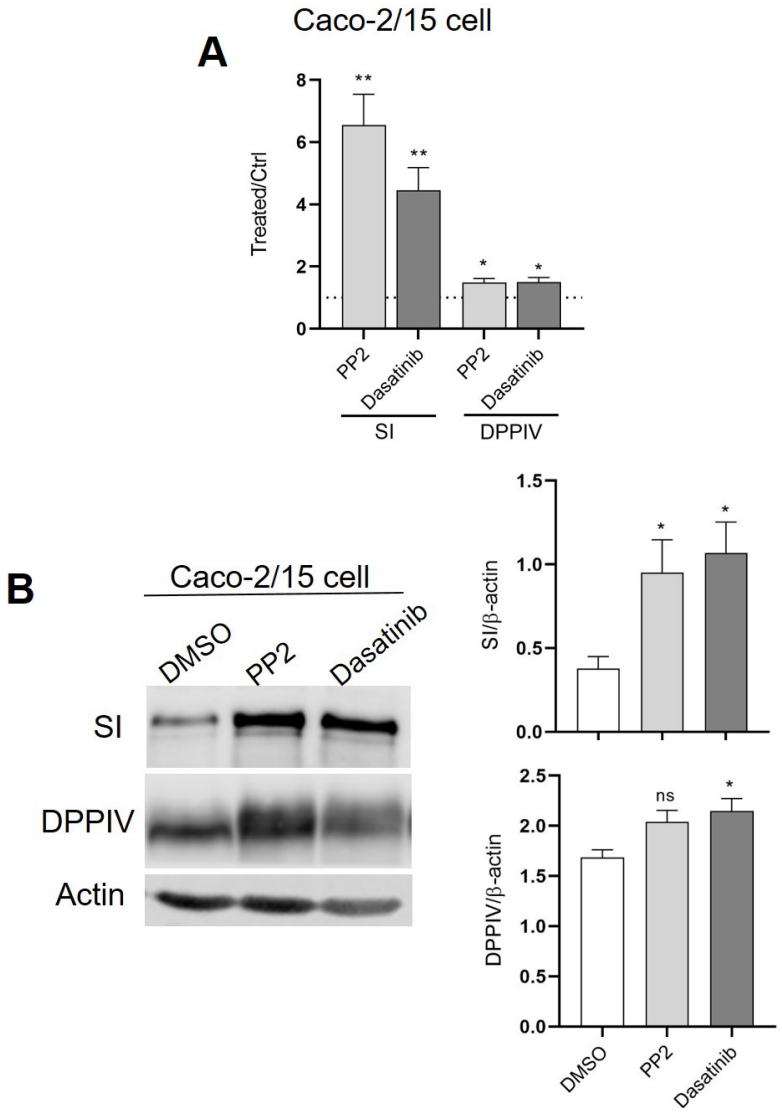


Figure 27. The effect of SFK inhibition on the differentiation of absorptive cells in Caco-2/15 cells.

A. Transcript expression of the absorptive cell markers SI and DPPIV in Caco-2/15 cells treated with SFK inhibitors (PP2 and dasatinib) relative to control (DMSO). **B.** Western blot analysis and data compilation showing higher expression of SI in PP2 and dasatinib treated Caco-2/15 cells relative to control. β-actin was used as a loading control. * $P < 0.05$, ** $P < 0.005$, n=3.

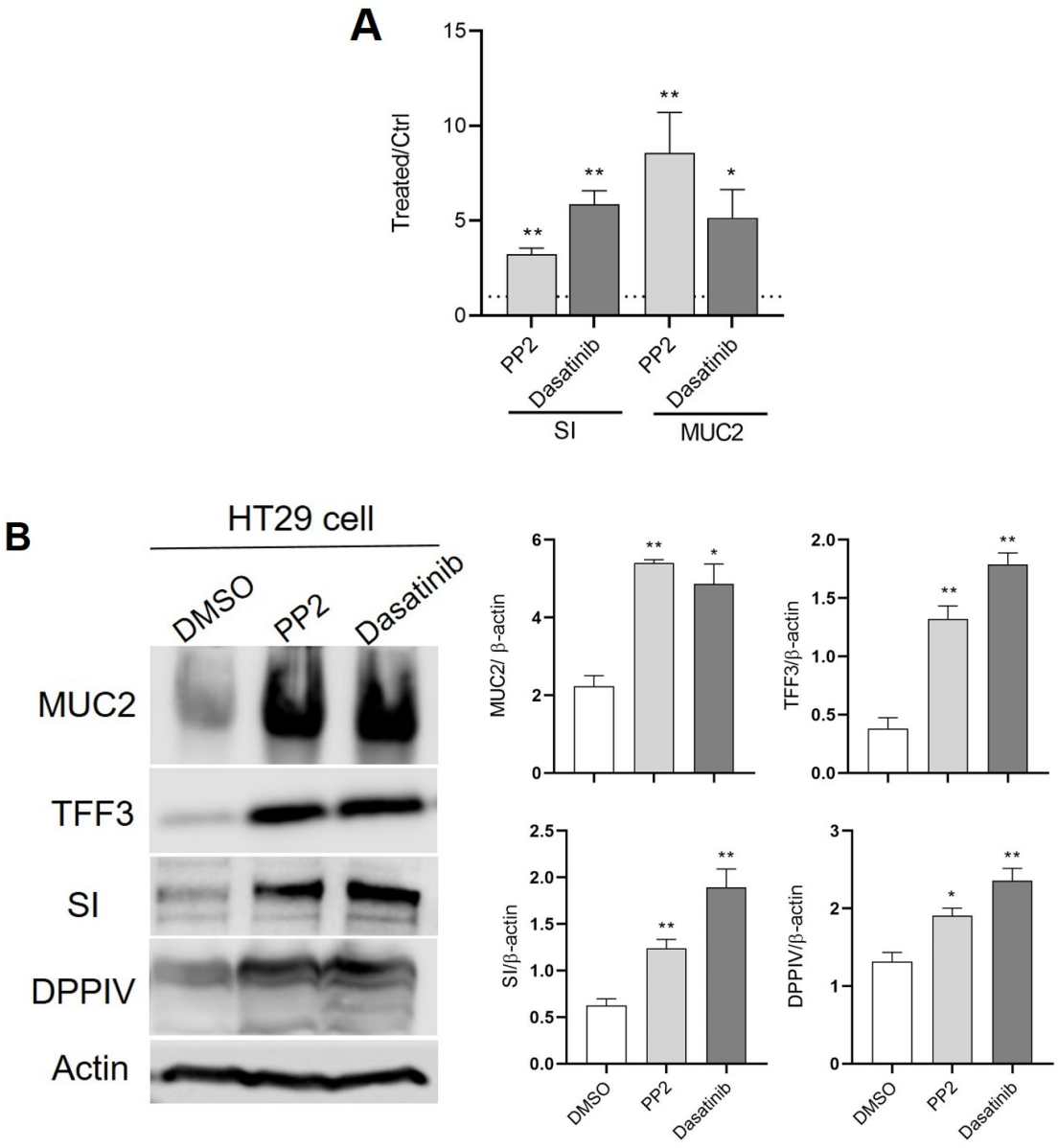
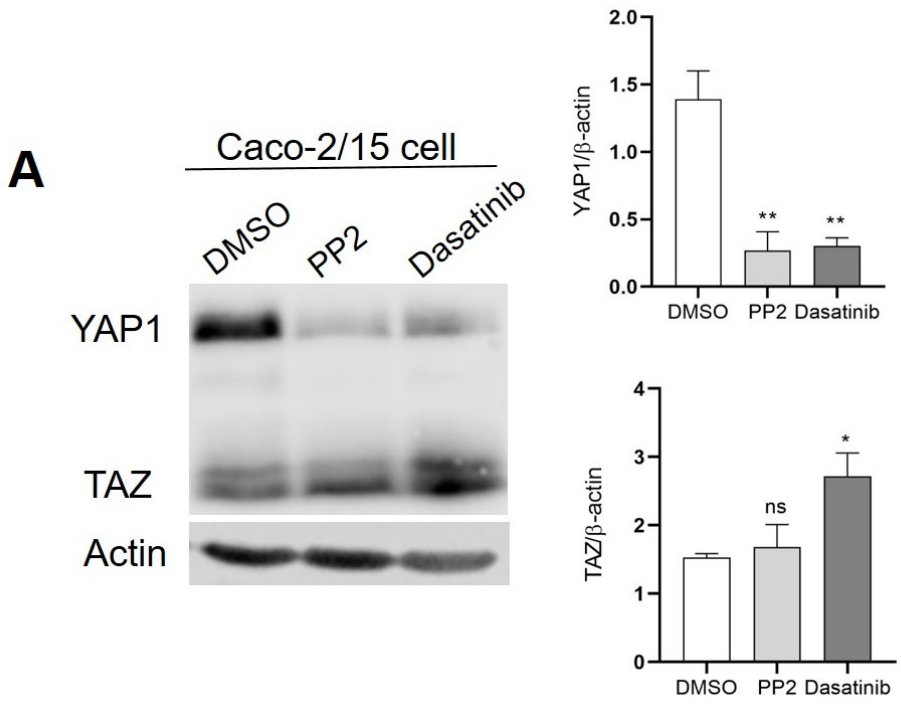


Figure 28. The effect of SFK inhibition on the differentiation of absorptive and secretory cells in HT29 cells.

A. Transcript expression of the absorptive cell marker SI and goblet cell marker MUC2 in HT29 cells incubated with SFK inhibitors (PP2 and dasatinib) relative to control (DMSO). **B.** Representative Western blot analysis and data compilation from three separate experiments showing higher expression of MUC2, TFF3, SI and DPPIV in PP2 and dasatinib treated HT29 cells relative to DMSO treated HT29 cells. β-actin was used as a loading control. *P<0.05, ** P< 0.005, n=3.

3.12. SFKs controls the differentiation of intestinal epithelial cells through YAP1 and CDX2 regulation

Previously, it has been reported that SRC induces YAP1/TAZ activity in several breast and melanoma cancer cell lines which plays an important role in tumor growth and metastasis. YAP1/TAZ transcriptional activity has been promoted by stable expression of the SRC^{Y527F} in human and mouse breast cancer and melanoma cell lines (Lamar *et al.*, 2019). Therefore, the effect of the SFK inhibition on YAP1/TAZ expression was evaluated by Western blot analysis in Caco-2/15 cells. The result showed significant reduction of YAP1 protein in PP2 and dasatinib treated cells compared with control cells whereas TAZ expression was not reduced by SFK inhibition and its expression was slightly increased in dasatinib treated Caco-2/15 cells (Figure 29A). Furthermore, the effect of SFK inhibitors on YAP1/TAZ expression was monitored in Caco-2/15 cells using indirect immunofluorescence. Cytoplasmic staining was observed in most PP2 and dasatinib treated cells compared with control cells (Figure 29B). Western blot analysis for HT29 cells treated with SFK inhibitors revealed a significant reduction of YAP1 protein which was shown in Figure 29C. In order to determine if differentiation of treated HT29 cells is associated with YAP1 inhibition and its regulatory effect on the CDX2 transcription factor, the expression of CDX2 was evaluated at the transcriptional and protein levels. The results revealed increased expression of CDX2 mRNA and protein in HT29 cells treated with SFK inhibitors compared with control (Figure 29D).


A
Caco-2/15 cell
DMSO
PP2
Dasatinib
YAP1
TAZ
Actin
YAP1/β-actin
2.0
1.5
1.0
0.5
0.0
**
**
DMSO PP2 Dasatinib
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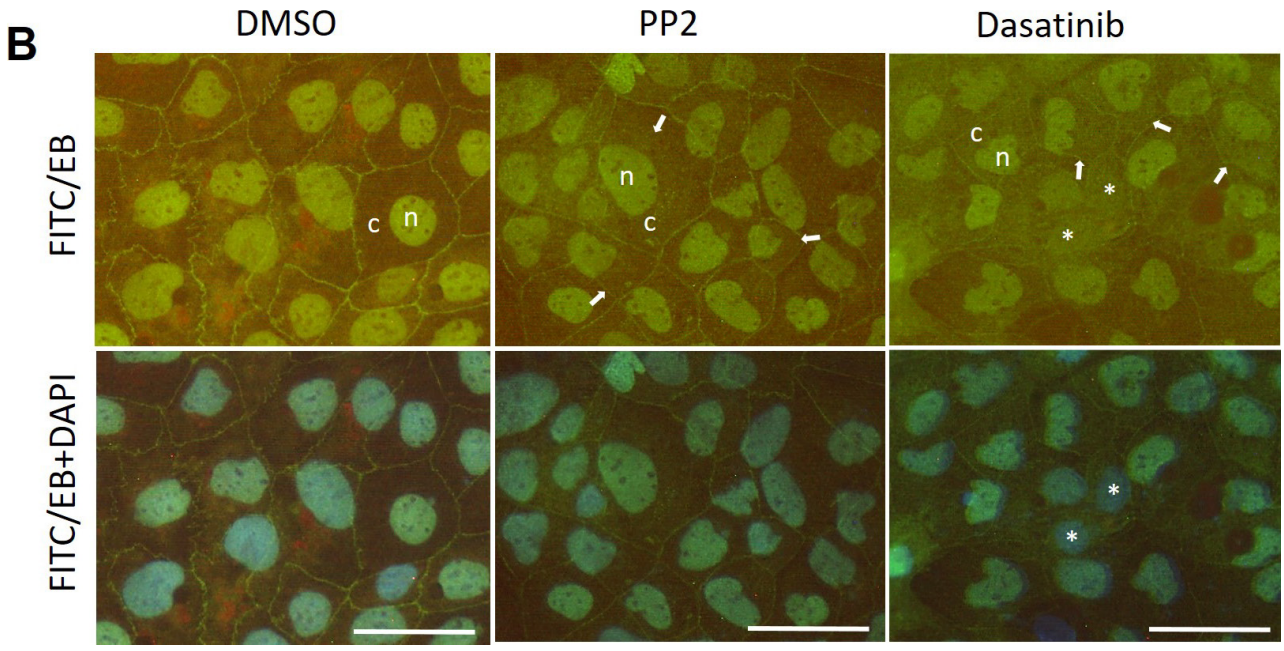
DMSO PP2 Dasatinib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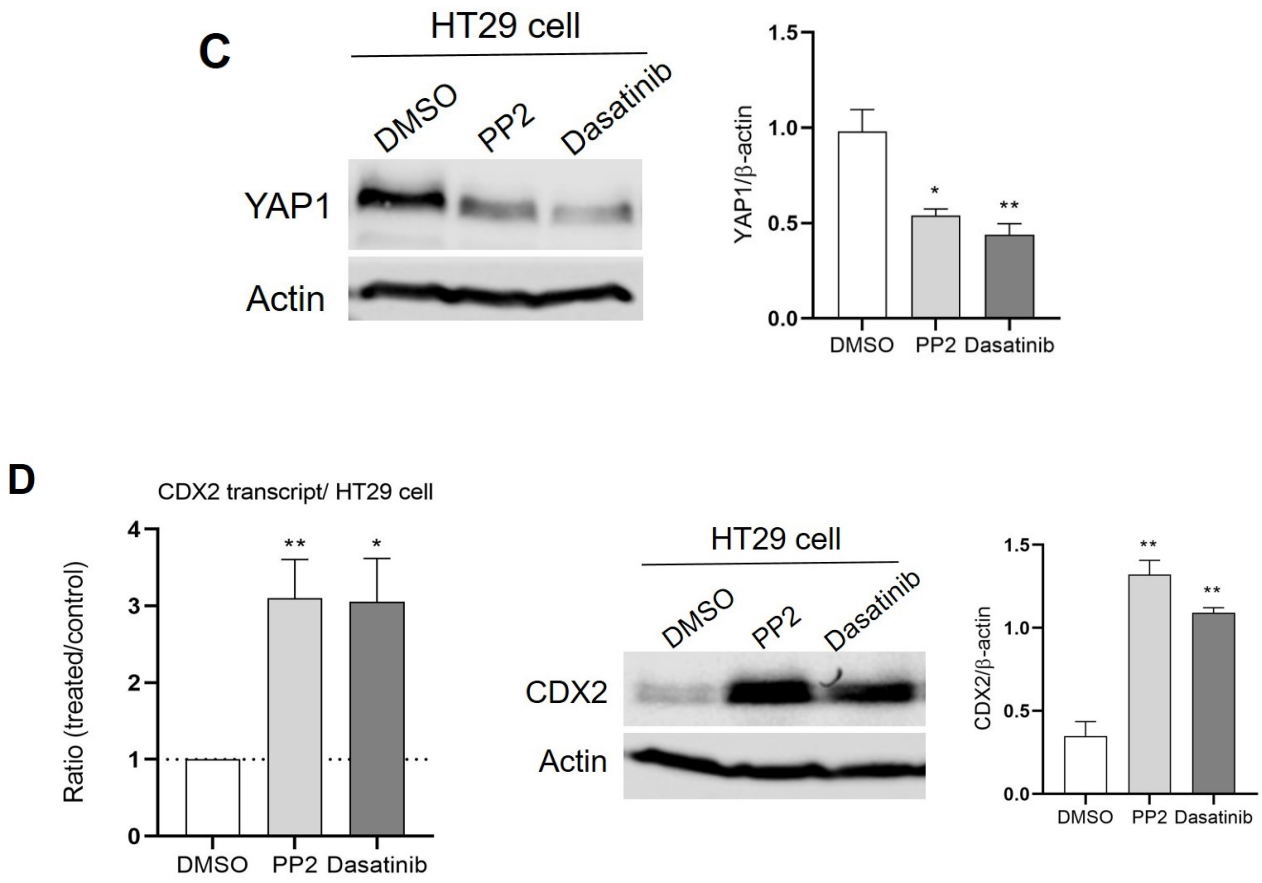

Figure 29. Reduction of YAP1 and increase of CDX2 expression in response to SFK inhibition.

A. Representative Western blot analysis and data compilation from three separate experiments showing a reduction of YAP1 and slight increase of TAZ expression at the protein level in PP2 and dasatinib treated Caco-2/15 cells relative to control. **B.** Indirect immunofluorescence of sub-confluent Caco-2/15 cells confirmed the presence of the YAP1/TAZ protein in the cytoplasm of a large proportion of PP2 and dasatinib treated cells. The YAP1/TAZ protein also was detected in the nucleus of all three groups of the experiment. Evans blue dye (EB) was used to give contrast and to highlight the cytoplasm which can be observed by red fluorescence. YAP1 protein and DAPI are shown in green and blue, respectively. n, nucleus; c, cytoplasm; arrow, cytoplasmic staining; asterisk, weaker nuclear staining, Scale bars = 50 µm. **C.** Western blot analysis showing the expression of the YAP1 protein in HT29 cells treated with PP2, dasatinib and DMSO. **D.** qPCR, Western blot and data compilation from three separate experiments showing a higher level of CDX2 transcription and protein in PP2 and dasatinib treated HT29 cells relative to the control. β-actin was used as a loading control. *P< 0.05, ** P< 0.005; ns, not significant, n=3.

4. Discussion

The necessity of the activity of the Hippo pathway effectors YAP1/TAZ has been shown in the development of embryonic tissue, growth of cancer tissue and the regeneration of injured tissue (Gregorieff *et al.*, 2015; D. Zhou *et al.*, 2011). The Hippo pathway plays a significant role in the control of tissue growth and homeostasis while inactivation of any member of the Hippo pathway kinase core could result in YAP1/TAZ activation (Gao *et al.*, 2019; D. Zhou *et al.*, 2011) .

The present study provides new insights into the role of the Hippo pathway in the regulation of intestinal cell functions by showing that YAP1 can specifically repress differentiation towards both absorptive and secretory cell lineages, and confirming its involvement in stemness maintenance (Fallah & Beaulieu, 2020).

One difficulty in assessing the involvement of the Hippo pathway was the need to consider YAP1 as well as its paralog TAZ since both transcriptional coactivators can combine with other factors to promote the expression of target genes (Hong *et al.*, 2016). As shown herein for Caco-2/15 cells, most colorectal cell lines express both YAP1 and TAZ resulting in limited effects in knockdown approaches unless double repression is performed (Wang *et al.*, 2013). Some medications including VP have been applied for YAP1/TAZ knockdown in certain cell lines (Lui *et al.*, 2019; Wang *et al.*, 2016). It has been shown that VP, a member of the porphyrin molecule family, binds to YAP1 directly and disrupts the YAP1/TAZ-TEAD interaction which leads to YAP1/TAZ sequestration into the cytoplasm (Wang *et al.*, 2016). However, the effective concentrations of VP for YAP1/TAZ knockdown have been found to be cytotoxic in Caco-2/15 and HT29 cells. In this context, the use of HT29 cells which only express YAP1 was a clear advantage. Interestingly, SFK pathways have been shown to be involved in the Hippo pathway by regulating its main kinase core components. Another difficulty for differentiation-based studies is that most available intestinal cell models display very limited differentiation potential and no multipotency, HT29 cells being the exception since these cells can give rise to both absorptive and goblet cells, the two main

intestinal cell types (S. Fallah *et al.*, 2020), under certain conditions. It has been shown that replacement of glucose by galactose in cell culture medium leads to the differentiation of HT29 cells to absorptive cells compared with cells grown in glucose containing medium in which the cells grew tightly packed without signs of differentiation (Zweibaum *et al.*, 1985). Mucus secreting cells morphologically similar to intestinal goblet cells are characterized by treatment of HT29 cells with sodium butyrate (Augeron & Laboisse, 1984). A mixed population of mucus-like and absorptive-like cells and a high concentration of mucus-like cells are formed by the treatment of HT29 cells with low and high concentration of methotrexate, respectively. Methotrexate, an anticancer drug that interferes with a metabolic pathway by decreasing the rate of aerobic glycolysis, leads to the selection of stable cells that are committed to goblet cell differentiation (Lesuffleur *et al.*, 1990). Therefore, HT29 cells undergo growth adaptation to these conditions which is followed by the appearance of differentiated cells. The exact mechanisms involved in metabolic adaptation and differentiation are not fully understood. In this study we used methotrexate (MTX) treatment in HT29 cells to understand the mechanism involved in intestinal epithelial cell differentiation. However, as evaluated by electron microscopy for mucus granules as well as the detection of MUC2 expression by indirect immunofluorescence, we found no significant increase after selection with increasing MTX concentrations (10^{-7}M, 10^{-6}M and 10^{-5}M).

HT29 cells are well characterized for their stem-like cell subpopulation. HT29 cells generate spheres when cultured in sphere formation medium which indicates the presence of cancer stem cells. Formation of new spheres from single cells acquired from dissociated spheres suggests the self-renewal feature of the HT29 cell. The expression of stem cell markers including EPCAM, CD44 and LGR5 in HT29 cells has been shown by Western blot analysis and indirect immunofluorescence staining (Vázquez-Iglesias *et al.*, 2019). $CD133^{+}CD44^{+}$ cells were isolated from HT29 cells using fluorescence-activated cell sorting and flow cytometry which indicates the presence of stem cells (Wei *et al.*, 2017).

Although cancerous in nature, HT29 and Caco-2/15 have been widely used as experimental models to study human small intestinal cell differentiation. The utility and limitations of these models are well documented (Kitamura *et al.*, 1996; Martínez-Maqueda *et al.*, 2015; Pageot *et al.*, 2000; Robine *et al.*, 1993; Tremblay *et al.*, 2006; Whitehead & Watson, 1997). In brief, they consume a high rate of glucose because of an impairment in glucose metabolism. Despite their origination from CRC, there are some limitations of using HT29 and Caco-2 cells because of their colonic origin albeit the fact that their morphological and functional characteristics mimic small intestinal absorptive cells. Furthermore, some receptors like neurotensin are found in HT29 cells but not in the intestinal epithelium. However, some receptors (neuropeptide Y) found in small intestinal epithelial cells are not detected in HT29 cells (Martínez-Maqueda *et al.*, 2015).

4.1. The association of YAP1/TAZ to stemness

Cancer stem cells are associated with tumor growth, resistance to chemotherapy and metastasis (Mo *et al.*, 2014). Thus, finding the participating factors and signals in cancer stem cell maintenance is very interesting in cancer therapy. It has been shown that YAP1 and TAZ play an important role in stem cell maintenance and proliferation in various tissues including liver, skin, nervous system and the intestine (Mo *et al.*, 2014). Also, in embryonic stem cells, YAP1 plays a role in pluripotency and maintenance of stem cells (Mo *et al.*, 2014). In skeletal muscle, the active form of YAP1 was detected in the nucleus of progenitor/stem cells and the inactive form (phosphorylated) was detected in the cytoplasm of differentiated mature cells (Watt *et al.*, 2010). Barry and colleagues have shown with a microscopy approach and cellular fractionation that YAP1 is predominantly cytoplasmic in the upper part of the crypt and throughout the villi, while, in the lower part of the crypt and CBC stem cells it is mostly nuclear. In addition, YAP1 localization is correlated with CD44 expression and TAZ is highly expressed in $CD44^+$ cells (Barry *et al.*, 2013). YAP1 protein has been detected at a higher level in the nucleus and cytoplasm of $CD133^+CD44^+$ HT29 and Caco-2 cells compared with $CD133^-CD44^-$ cells, however phosphorylated YAP1 is detected predominantly in the cytoplasm of $CD133^-CD44^-$ cells (Wei *et al.*, 2017). In the rodent small

intestine, endogenous YAP1 expression is mostly restricted to the crypt compartment in the progenitor/stem cell area (Camargo *et al.*, 2007). It has been shown that YAP1 inactivation is required for the differentiation phenomenon because YAP1 overexpression results in the expansion of stem/progenitor cells (Camargo *et al.*, 2007; Watt *et al.*, 2010). In the present study the expression of YAP1/TAZ was detected in the normal adult human small intestine. Through immunofluorescence and confocal microscopy, the endogenous restricted expression of YAP1/TAZ in a few non-Paneth cells located in the lower portion of the crypts in the human intestine was observed. This distribution of YAP1/TAZ in the intestinal crypt is in line with previous studies that have been reported in the intestine of animal models in which a functional association with stem cells was demonstrated (Gregorieff *et al.*, 2015; Yui *et al.*, 2018). High mRNA expression of stem cell markers including LGR5, PROM1, EPCAM, CD44 and OCT4 was detected in HT29 cells while the expression of ASCL2 was at a low level. According to indirect immunofluorescence images YAP1 protein is expressed in most populations of HT29 cells, however LGR5 is expressed in a limited number of the cells. Imajo et al. have shown that the expression of the dominant negative form of TEAD4 named TEAD4-EnR (EnR: the repression domain of Engrailed) in the mouse crypt is accompanied by a reduction in LGR5-eGFP^{+} stem cells. However, knockdown of LATS1/2 in the mouse crypt led to an increased number of LGR5-eGFP^{+} cells (Imajo *et al.*, 2015). Wei and colleagues have reported that the stem-like CD133+CD44+ cells isolated from HT29 cells express a higher level of YAP1 and lower level of MST1 compared with CD133^{-}CD44^{-} isolated cells. In addition more colonies and larger spheres were formed by CD133^{+}CD44^{+} HT29 cells compared with CD133^{-}CD44^{-} cells (Wei *et al.*, 2017). Hirsch and colleagues reported that LGR5 knockdown using shRNA in HT29 cells was accompanied by a reduction in colony formation (Hirsch *et al.*, 2014).

Thus using the HT29 cell model, we first assessed the influence of YAP1 on stemness properties by showing a reduction of two intestinal stem cell markers, LGR5 and PROM1, in cells knocked down for YAP1, which is consistent with the fact that YAP1/TAZ supports stem cell properties in the intestine (Gregorieff *et al.*, 2015; Imajo *et al.*, 2015) and is required

for stemness maintenance and colony formation in colorectal cancer cells (Yuen *et al.*, 2013; D. Zhou *et al.*, 2011). It has been reported that LGR5 and YAP1 expression is regulated by kinase C ζ (PKCζ) (Llado *et al.*, 2015). PKCζ is a protein kinase enzyme that is involved in controlling the function of other proteins through the phosphorylation of hydroxyl groups of serine and threonine amino acid residues. It is expressed in the apical borders of LGR5 positive crypt cells in the mouse (Llado *et al.*, 2015). In addition, the expression of LGR5 is upregulated in deficient PKCζ organoids compared with controls. PKCζ represses the stem cell function through the phosphorylation and downregulation of YAP1 and β-catenin. It has been shown that PKCζ knockdown in the CRC cell line SW480 was accompanied by increased YAP1 expression (Llado *et al.*, 2015).

YAP1 induces stemness in embryonic cells through TEAD transcription factors. It has been reported that overexpression of YAP1 and TEAD2 in neural, hematopoietic and embryonic stem cells induce stemness (Ramalho-Santos *et al.*, 2002). It also has been reported that YAP1 induces stemness indirectly via TGF-β/BMP signaling through the regulation of genes involved in stem cell maintenance. Upon stimulation by BMP, YAP1 binds to SMAD1 and promotes the expression of DNA-binding protein inhibitor (ID) family members that leads to stem cell maintenance in mouse embryonic stem cells (Alarcón *et al.*, 2009). In addition, it has been shown that CD44 expression is regulated by Wnt/β-catenin signaling in the normal and cancerous intestinal epithelium (Wielenga *et al.*, 1999) which explains the lack of the effect of YAP1 knockdown on CD44 expression. Further studies are required to understand the molecular mechanism by which YAP1/TAZ regulates stem cell maintenace. For this reason, the sorting of cancer stem cells presented in HT29 cells can be useful. The most common method that can be applied is the sorting of colorectal cancer stem cells based on the expression of surface markers including $LGR5^+$, $CD133^+$, $CD44^+$and $CD24^+$ cells (Atashpour *et al.*, 2015; Fan *et al.*, 2011; Leng *et al.*, 2018). In the present study, the expression of LGR5 and PROM1 or CD133 was reduced by YAP1 knockdown. Therefore, for further study, sorting of the $LGR5^+$ $CD133^+$ cell could be performed for more investigation about YAP1 and its effect on stemness.

4.2. YAP1 involvement in cell proliferation

YAP1/TAZ is expressed in the intestinal stem cell compartment suggesting a role in stem cell proliferation. YAP1/TAZ activity was found to be entirely dispensable for intestinal epithelial turnover under normal conditions in the mouse. However, following injury, YAP1/TAZ presence is necessary for intestinal regeneration (Cai *et al.*, 2010; Hong *et al.*, 2016; Yui *et al.*, 2018). Considering that YAP1 is expressed in the nucleus of a large proportion of HT29 cells, we were interested in determining the potential role of YAP1 in the proliferation of CRC cells. Interestingly, a BrdU assay performed for the detection of cell proliferation showed no change in proliferation of YAP1 knockdown HT29 cells. The proliferation of YAP1 knockdown HT29 cells as well as control cells is between 40 to 60 percent. This result is in agreement with previous studies which reported that the percentage of cell proliferation in CRC cells including HT29, Caco-2, T84 and SW480 cells is between 40 to 60 percent (Boudjadi *et al.*, 2017; Dydensborg *et al.*, 2009; Seltana *et al.*, 2010). Wang and colleagues have shown that individual knockdown of YAP1 and TAZ has a weak effect on the proliferation of HCT116 colon cancer cells while a slight reduction in cell proliferation was observed by knocking down YAP1 and TAZ together (Wang *et al.*, 2013). The lack of change in proliferation of YAP1 knockdown HT29 cells can be related to the involvement of other important factors in cell proliferation including epidermal growth factor receptor (EGFR) signaling. EGFR is a transmembrane glycoprotein with tyrosine-specific protein kinase activity, is expressed at a moderate level in HT29 cells and is required for cell growth and survival (Prewett *et al.*, 2002; Wu *et al.*, 2009). EGFR is activated by several polypeptide ligands including epidermal growth factor (EGF). Binding of the EGF ligand to the extracellular domain of EGFR leads to the phosphorylation and activation of the receptor which mediates and activates various downstream signaling cascades (Purba *et al.*, 2017). Moreover, EGFR ligands including epiregulin, EGF and transforming growth factor alpha (TGFα) are expressed in HT29 cells (Wu *et al.*, 2009). Pangburn and colleagues have documented that the activity of EGFR is increased by the addition of EGF into the HT29 growth environment (Pangburn *et al.*, 2005). Liu et al. showed that YAP1 knockdown has no significant effect on EGFR, however EGFR induces YAP1 and its target gene CYR61 activity

(Liu *et al.*, 2018). Therefore, EGFR also can affect the proliferation of HT29 cells and the removal of YAP1 may be ineffective on EGFR expression.

It has been documented that MYC is expressed at a high level in HT29 cells at all stages, exponentially growing, stationary, non-differentiated and differentiated cells (Forgue-Lafitte *et al.*, 1989; Trainer *et al.*, 1988). Zhang and colleagues have demonstrated that MYC knockdown inhibits HT29 cell proliferation and induces apoptosis (X. Zhang *et al.*, 2009). The MYC proto-oncogene has been identified as a Wnt target gene which is controlled through APC (He *et al.*, 1998). MYC also acts as a transcriptional repressor which suppresses the genes involved in growth arrest including the cyclin-dependent kinase inhibitors p21CIP1 and p15INK4B (Wanzel *et al.*, 2003). Given that the APC gene negatively regulates the activity of Wnt/β-catenin signaling, mutation of the APC gene in HT29 cells is accompanied by hyper-activity of Wnt/β-catenin (Parker & Neufeld, 2020). Therefore, one important factor in HT29 cell proliferation is Wnt/β-catenin activity. Several studies have reported the interaction between Wnt and YAP1/TAZ (Barry *et al.*, 2013; Imajo *et al.*, 2012). It was demonstrated that the β-catenin/TCF4 complex regulates YAP1 expression by binding to the DNA enhancer element within the first intron of the YAP1 gene in colorectal cancer cells. Also, YAP1 mRNA and protein levels have been reduced by abolition of β-catenin (Konsavage *et al.*, 2012a). On the other hand, Cai and colleagues suggested another mechanism by which APC regulates the activity of the Hippo pathway and YAP1. They showed that both β-catenin and YAP1 accumulate in the nucleus of neoplastic epithelia of APC deficient mice and human adenomas in spite of the neighboring nonneoplastic epithelia. They also observed the nuclear accumulation and increased YAP1 and TAZ expression in APC ablated mice. Stabilization of β-catenin in mice had no significant effect on YAP1 activity. Therefore, it has been concluded that YAP1 activity is directly regulated by APC, independent of the Wnt pathway effector β-catenin. However, TAZ expression was increased by β-catenin stabilization which suggests that TAZ could be regulated by APC through both Hippo and β-catenin pathways. According to their study in HEK293 cells, APC participates in Hippo pathway kinase core by interacting with SAV1 and LATS1 which leads

to LATS1 activation (Cai *et al.*, 2015). Such a discrepancy with the investigation of Konsavage and Cai could be explained by their use of different models of CRC cells and mouse/HEK293, respectively. In this respect, YAP1 knockdown may have no significant effect on β-catenin and proliferation of HT29 cells. In addition, Cotton and colleagues have studied the effect of YAP1/TAZ on Wnt signaling by removing YAP1/TAZ in GI tissue in a normal mouse model. They reported that YAP1 and TAZ knockout were ineffective on nuclear localization of β-catenin and expression of Wnt target genes including CD44 and SOX9 under normal conditions (Cotton *et al.*, 2017). Various stem cell markers including LGR5 are expressed in HT29 cells. An indirect immunofluorescence study for the detection of LGR5 positive cells confirmed the presence of a minor population of stem cells in HT29 cells. Therefore, an observed lack of significant change in cell proliferation in YAP1 knockdown HT29 cells appears to be the result of losing this relatively small stem cell population. It has been shown that $CD133^{+}CD44^{+}$ isolated HT29 and Caco-2 cells have higher proliferation rates compared with $CD133^{-}CD44^{-}$ isolated cells (Wei *et al.*, 2017).

4.3. YAP1 negatively controls epithelial cell differentiation

Both HT29 and Caco-2/15 cell lines are colorectal cancer cell lines which have been used extensively as differentiation models in various studies. Although derived from the colon, they are well characterized as models for the small intestinal epithelium as they maintain fetal intestinal characters (Pageot *et al.*, 2000; Seltana *et al.*, 2012; Vachon & Beaulieu, 1992; Zweibaum *et al.*, 1985). These cells have the potential to differentiate and express brush-border-associated hydrolases that are specific for small intestinal cells (Beaulieu & Quaroni, 1991; Tremblay *et al.*, 2011; Zweibaum *et al.*, 1985). It should be mentioned that during mid-gestation of human embryonic development, colon structure is similar to that of the small intestine containing the villus, brush border and digestive enzymes (Beaulieu *et al.*, 1989; Ménard, 2005). Therefore, HT29 and Caco-2/15 cells, although derived from colorectal lesions, exhibit morphological and functional characteristics similar to the small intestine which makes them proper models in studies focusing on intestinal epithelial cell differentiation.

The striking effect of YAP1 knockdown on HT29 cell differentiation was interesting. Indeed, YAP1 and TAZ knockdown performed in various colorectal cell lines has shown that the Hippo pathway can affect proliferation, apoptosis, migration and invasion (Wang *et al.*, 2013; D. Zhou *et al.*, 2011). However, the role of YAP1/TAZ on intestinal epithelial cell differentiation was less clear. Indeed, during differentiation of a mouse embryonic stem cell model, reduction of active and enhancement of phosphorylated YAP1 has been observed (Lian *et al.*, 2010). Previous studies have shown that overexpression of YAP1 directly or through elimination of Mst1 and Mst2 promotes crypt cell proliferation that was accompanied by a significant impairment of differentiation for all cell types in a mouse model (Camargo *et al.*, 2007; D. Zhou *et al.*, 2011).

In our study, YAP1 knockdown in HT29 cells resulted in increased expression of the absorptive (SI, DPPIV) and goblet (MUC2, TFF3) cell markers while Paneth (DEFA5) and enteroendocrine (CHGA) cell markers were not changed. It has been shown that YAP1/TAZ overexpression by MST1/2 kinase knockdown in mice, leads to the reduction in differentiation of secretory cells including goblet, Paneth and enteroendocrine cells (D. Zhou *et al.*, 2011). Furthermore, the lack of YAP1/TAZ expression in adult human and mouse Paneth cells has been exhibited by indirect immunofluorescence and immunohistochemistry, respectively (Fallah & Beaulieu, 2020; Gregorieff *et al.*, 2015). Furthermore, Gregorieff and colleagues have reported that Paneth cell differentiation is inhibited by YAP1 in a mouse model (Gregorieff *et al.*, 2015). Moreover, Wnt signaling pathway and sex determining region Y (SRY)-box containing gene 9 (SOX9) transcription factors are two important factors in Paneth cell differentiation that are expressed at high levels in HT29 cells (Blache *et al.*, 2004; Mori-Akiyama *et al.*, 2007). The high motility group (HMG)-box transcription factor SOX9, that is regulated by the Wnt signaling pathway, is expressed in the intestinal epithelium especially by stem cells, Paneth cells and progenitor cells (Bastide *et al.*, 2007). According to the literature, the possible inhibitory effect of YAP1 in Paneth cell differentiation in HT29 cells has been suggested. Upregulation of SI, DPPIV, MUC2 and TFF3 was confirmed at the protein level. As expected from the normal villus

epithelium (S. Fallah *et al.*, 2020), even at 8 days post-confluence, only a subset of cells exhibited goblet-like cell properties while a larger proportion of the cells displayed absorptive-like cell properties in immunofluorescence and electron microscopy. The preventive effect of YAP1 on cell differentiation was confirmed by YAP1 knockdown in Caco-2/15 cells which are widely used to study human intestinal cell differentiation (Pageot *et al.*, 2000; Whitehead & Watson, 1997). According to our result, the expression of the SI protein was undetectable at day 0 while its expression was increased at 5 and 8 days of confluence. Cohen and colleagues have reported that an increased number of cells exhibiting a brush border was observed by prolonging the period of HT29 cell growth. They showed that treating HT29 cells with forskolin, gradually increased the number of cells exhibiting a brush border. They reported that this phenomenon can be related to the reorganization of the actin cytoskeleton and a delay in the assembly of the molecules required for cell polarization and formation of the brush border at the apical domain of the cells (Cohen *et al.*, 1999). HT29 cells grown in the absence of glucose also need several days to express absorptive cell differentiation markers (Zweibaum, 2011). The knockdown of YAP1 in Caco-2/15 led to slightly increased TAZ protein compared with the control. Finch-Edmondson et al. showed that in the mouse, YAP1 negatively regulates the abundance of TAZ protein post-transcriptionally by proteasomal degradation, whereas TAZ expression has no effect on YAP1 abundance (Finch-Edmondson *et al.*, 2015). The TAD domain and PDZ binding motif of the YAP1 protein have been identified to be responsible for TAZ degradation. It has been reported that regulation of TAZ abundance through YAP1 is mediated by heat shock protein 90 (HSP90) and GSK3α/β, however the exact mechanism by which YAP1 regulates TAZ degradation is unclear (Finch-Edmondson *et al.*, 2015). A sharp increase in SI expression was observed in Caco-2/15 cells by removing YAP1. However, because Caco-2/15 cells express high levels of both YAP1 and TAZ and can only differentiate into absorptive cells, further experiments with this cell model were not pursued. This result is in line with the study of Camargo et al. which reported the absence of enterocytes as well as mature Paneth and goblet cells in the mouse intestine after 5 days of YAP1 activation (Camargo *et al.*, 2007).

In breast cancer cell lines, it has been reported that YAP1 transcriptionally represses growth differentiation factor-15 (GDF-15) through the recruitment of the polycomb repressive complex 2 (PRC2) into the H3K27me3 of GDF-15 promoter, which induces metastasis (T. Wang *et al.*, 2018). YAP1 physically interacts with the PRC2 core component EZH2 and represses the activity of GDF-15 by helping its transcription factor TEAD (T. Wang *et al.*, 2018). The inhibitory effect of YAP1/TAZ on cell differentiation has been shown in a breast cancer cell line (MCF10A). The YAP1/TAZ-TEAD complex acts as a transcriptional repressor by recruiting the NuRD (nucleosome remodeling and histone deacetylase) complex, which is required for lineage commitment of pluripotent cells (Kim *et al.*, 2015). Furthermore, deacetylation of H3K27-mediated by NuRD recruits PRC2 into the NuRD target promoters for further repressive action. Like NuRD, PRC2 is a transcriptional repressor complex required for gene silencing in embryonic stem cells. PRC2 acts by directing methyltransferase activity to di- and tri-methylate H3K27 (Reynolds *et al.*, 2012). In summary, by recruiting both NuRD and PRC2, YAP1/TAZ boosts transcriptional repression in pluripotent cells to maintain stemness and restricts lineage commitment. Interestingly, it has been demonstrated that the expression of the PRC2 core component SUZ12 is restricted to the undifferentiated cells of the crypt in the TA zone while it is absent in mature Paneth and absorptive cells (Benoit *et al.*, 2012). Therefore, PRC2 activity in the TA zone resulted in maintenance of proliferation and a delay in terminal differentiation. The expression of SUZ12 was also identified in Caco-2/15 cells and its expression was gradually decreased by differentiation of Caco-2/15 cells into absorptive cells. Early appearance and an increase in SI expression were observed by knockdown of SUZ12 in Caco-2/15 cells (Benoit *et al.*, 2012). In this context, considering that CDX2 is already expressed at a high level in Caco-2/15, it may be possible that increased SI in YAP1 knockdown Caco-2/15 cells is related to PRC2 repression. This suggests that YAP1/TAZ may negatively control the differentiation of intestinal epithelial cells through the recruitment of PRC2 and methylation of H3K27 leading to gene silencing. Further investigation is required to understand the association of YAP1 and PRC2 in the differentiation of colorectal cancer cells.

4.4. YAP1/TAZ downstream factors involved in epithelial cell differentiation

Several transcription factors that control epithelial cell differentiation could be regulated by YAP1. In order to understand which of these factors are involved in the induction of absorptive and goblet cell differentiation, some known regulators of intestinal cell differentiation were screened in YAP1 knockdown HT29 cells. Therefore, the expression of CDX2 and HNF1α, two master regulators of intestinal epithelial cells (Benoit *et al.*, 2010; Boudreau *et al.*, 2002a; Boyd *et al.*, 2010; Suh & Traber, 1996), ATOH1, a downstream effector of the Notch pathway for promoting secretory lineages (Demitrack & Samuelson, 2016; Tian *et al.*, 2015), KLF4 required for goblet cell differentiation (Katz *et al.*, 2002) and HES1, a Notch target gene required for absorptive cell differentiation were analyzed. Elimination of YAP1 was accompanied by a higher expression of CDX2 and ATOH1 at the transcriptional level. In addition, the expression of the HES1 transcription factor was slightly increased at the transcriptional level. Contrary to ATOH1 that was barely detectable at the protein level, the CDX2 protein was detected at a high level in YAP1 knockdown cells. According to the Western blot, the CDX2 band seems to be two attached bands. It has been reported that in the intestinal mucosa and colon cancer cell lines, a complex band pattern of CDX2 is observed around 36 KD which contains the phosphorylated form of the CDX2 protein (Gross *et al.*, 2005). CDX2 is phosphorylated by cyclin dependent kinase (CDK-2) at serine 281 in the 4S motif, which decreases its stability by making it susceptible for ubiquitination and proteasomal degradation. Moreover, phosphorylated CDX2 at S281 in the 4S motif and wild type CDX2 show the same behavior on downstream target promoters (Gross *et al.*, 2005).

CDX2 plays a significant role in the differentiation of small and large intestinal epithelial cells. The distal small intestine and proximal colon express the highest levels of CDX2, and its expression rises from the crypt to the lumen (Olsen *et al.*, 2012). Cell proliferation in colorectal cancer cells has been inhibited by overexpression of CDX2 and induced cell proliferation has been observed through CDX2 knockdown (Yu *et al.*, 2019). It has been demonstrated that CDX2 expression is repressed in wild type HT29 and some intestinal cell

lines (Hinoi et al., 2003). The involvement of CDX2 in absorptive and goblet cell differentiation in response to YAP1 ablation was then confirmed by observing a return to the basal level of SI, MUC2 and TFF3 expression in shYAP1 + shCDX2 transduced cells while DPPIV remained elevated. These results are consistent with the fact that CDX2 has been found to specifically interact with the promoters of SI (Boudreau *et al.*, 2002a; Krasinski *et al.*, 2001), MUC2 (Mesquita *et al.*, 2003; Yamamoto *et al.*, 2003) and TFF3 (Shimada *et al.*, 2007) to directly stimulate their expression in various intestinal cell models including HT29 and Caco-2 (Krasinski *et al.*, 2001; Mesquita *et al.*, 2003). Furthermore the expression of SI, lactase phlorizin hydrolase, E-cadherin and the integrin-β4 subunit was promoted by the overexpression of CDX2 in Caco-2 cells (Lorentz *et al.*, 1997). Also a reduction in SI expression and the goblet cell population by CDX2 ablation in mice confirms the regulatory effect of CDX2 on absorptive and goblet cells (Hryniuk *et al.*, 2012). The lack of effect of CDX2 knockdown on DPPIV at the transcriptional level suggests that other transcriptional factors may be stimulated by the ablation of YAP1. For instance, DPPIV depends on factors such as HNF1α, HNF1β, USF-1 and USF-2 (Erickson, Lai, & Kim, 2000; Erickson, Lai, Lotterman*, et al.*, 2000), which with the exception of HNF1α have not been investigated. In addition, it has been reported that DPPIV expression is regulated by PRC2 (Benoit *et al.*, 2012). An increase in DPPIV expression has been observed through PRC2 ablation in CDX2/HNF1α expressing HIEC cells (Benoit *et al.*, 2012). Considering the general pro-differentiation effect of YAP1 abolition noted on morphological differentiation, it is likely that other transcription factors involved in the regulation of epithelial cell polarization such as GATA4 (Benoit *et al.*, 2010; Escaffit, Boudreau*, et al.*, 2005) could be involved. CDX2 knockdown in shYAP1 HT29 cells resulted in a slight increase of DPPIV at the protein level. Therefore, the CDX2 may control DPPIV at the protein level. The exact mechanism that explains this phenomenon remains unclear. One explanation could be that CDX2 regulates the unknown factors which participate in DPPIV degradation. Therefore, CDX2 abolition leads to increased DPPIV stability.

In this context, it is interesting to note that the levels of LGR5 and PROM1 found to be repressed by the ablation of YAP1 were restored in shCDX2 expressing cells suggesting that this factor may act in the repression of stem cell-related genes in intestinal cells in agreement with previous work (San Roman, Tovaglieri*, et al.*, 2015). The lack of the effect of YAP1 knockdown on DEFA5 expression in HT29 cells can be the result of the induced expression of CDX2 (Crissey *et al.*, 2011). However, the removal of CDX2 in shYAP1 expressing cells had no significant effect on DEFA5 expression (result not shown). ATOH1, also known as MATH1 and HATH1 in the mouse and human respectively, participates in secretory cell differentiation (VanDussen & Samuelson, 2010). The expression of the ATOH1 transcription factor, which is negatively regulated by the Notch pathway, is suppressed in about 70% of colorectal tumors and in CRC cell lines including HT29 cells (Leow *et al.*, 2004). The reason for the lack of ATOH1 expression in CRC cell lines may be the involvement of genetic and/or epigenetic mechanisms (Bossuyt *et al.*, 2009) or may be due to proteasomal degradation of the ATOH1 protein initiated by GSK3β phosphorylation (Aragaki *et al.*, 2008). In the present study, we observed that YAP1 abolition contributed to an increased expression of ATOH1 mRNA while that of the ATOH1 protein was under the detectable level. The lack of the ATOH1 protein in this experiment can be related to the proteasomal degradation of ATOH1 in HT29 cells. Furthermore, the induction of ATOH1 expression in HT29 cells resulted in elevated MUC2 (Leow *et al.*, 2004) expression at both the transcriptional and protein levels, while SI expression stayed at a barely detectable level. It has been shown that MUC2 is a direct target of the ATOH1 transcription factor (Lo *et al.*, 2017). Increased MUC2 expression in ATOH1-HT29 cells without treatment with the proteasomal inhibitor indicates that ATOH1 is effective for triggering goblet/secretory cell differentiation even at low levels. In this context, a number of researchers have reported that the ATOH1 enhancer is regulated by CDX2 and a high expression of CDX2 in IEC-6 cells upregulates ATOH1 expression (Mutoh *et al.*, 2006). This suggests that YAP1 knockdown contributes to increased CDX2 expression which promotes ATOH1 expression. Furthermore, it has been reported that CDX2 induces the expression of the Notch ligand DLL1 by interacting with its promoter, but has no effect on DLL3/4 ligands (Grainger *et al.*,

2012). Shimizu and colleagues have been reported that DLL1 and DLL4 are expressed by ATOH1 positive intestinal epithelial cells located adjacent to HES1 positive cells. Furthermore, DLL1 and 4 positive cells express the goblet cell marker MUC2 (Shimizu *et al.*, 2014).

The increased expression of both absorptive and secretory cell differentiation can be related to both ATOH1 and HES1 activity in YAP1 knockdown HT29 cells. In the normal intestine Notch activity is regulated through several mechanisms including lateral inhibition. Through lateral inhibition, Notch activation in cells expressing the Notch ligand is inhibited by binding to the Notch receptor in neighboring cells. When one cell expresses HES1, the neighboring cell expresses ATOH1. Therefore it is possible that this phenomenon is activated in shYAP1 expressing HT29 cells specifically at post confluence, which results in the high expression of both absorptive and secretory cell markers (Figure 30) (Lewis, 1998). Some researchers reported that YAP1 overexpression can contribute to higher nuclear localization of the Notch intracellular domain (NICD) and upregulation of the Notch target gene HES1 (Camargo *et al.*, 2007; D. Zhou *et al.*, 2011). However, our results showed that HES1 was not only reduced in YAP1 knockdown HT29 cells but its expression was also slightly increased. This result is in agreement with Totaro and colleagues who have shown that YAP1/TAZ activation through rigidity of the extracellular matrix maintains the epidermal stem cells in an undifferentiated state through Notch inhibition, which is a key factor for epidermal differentiation (Totaro *et al.*, 2017). Therefore, YAP1 ablation resulted in increased HES1, which is required for absorptive cell differentiation.

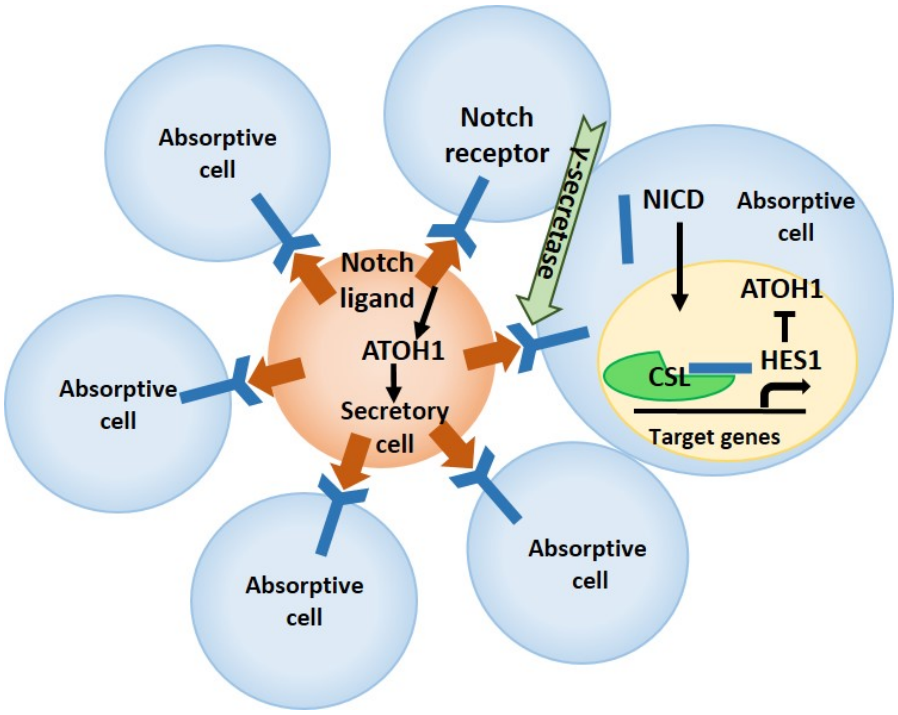

Figure 30. A schematic representation of lateral inhibition by a Notch ligand.

The cell with high Notch activity represses the expression of the Notch ligands in the activated cells and also limits Notch activity in neighboring cells. ATOH1 expressing cells express Notch ligands, which bind to Notch receptor, activate the Notch signaling and inhibit ATOH1 expression in neighboring cells.

4.5. The molecular mechanism by which YAP1 regulates CDX2 expression

While CDX2 has been shown to regulate YAP1 expression in intestinal cells (Larsen *et al.*, 2019a), the mechanism by which YAP1 represses CDX2 remains elusive. As shown previously, a lack of CDX2 expression in HT29 is obviously not the result of a somatic mutation nor does it appear to result from epigenetic silencing (Hinoi *et al.*, 2003; Lu *et al.*, 2008) although a recent study suggests that minimal expression of CDX2 could be restored in HT29 after treatment with a histone deacetylase 4/5 inhibitor (Graule *et al.*, 2018). It has been shown that β1 integrin suppresses the expression of CDX2 and its target gene SI in the CRC cell lines Caco-2/15 and DLD1 (Brabletz *et al.*, 2004). Furthermore, FAK overexpression

resulted in a sharp decrease in CDX2 promoter activity in HEK293 cells while FAK knockdown led to an increased expression of CDX2 at the transcriptional level in DLD1 cells (Brabletz *et al.*, 2004). It should be mentioned that high stiffness of ECM activates FAK through the β1 integrin. Subsequently, FAK activates the tyrosine kinase SRC, which in turn activates YAP1 (Rausch & Hansen, 2020). In this respect, YAP1 may repress CDX2 via the FAK/SRC signaling pathway. Further investigation is required to clarify the effect of FAK/SRC/YAP1 on CDX2 expression.

Another possibility is that YAP1 may repress CDX2 expression through the TEAD4 transcription factor. It has been reported that TEAD4 binds to the intron 1 and 3'UTR regions of the CDX2 locus by using ChIP-seq in mouse trophectoderm derived trophoblast stem cells (mTSCs). It must be noted that in a mouse embryo cell model, TEAD4 induces CDX2 expression, which is required for early development (Home *et al.*, 2012). In present study, TEAD4 may act as a repressor on the CDX2 promoter in HT29 cells.

Another factor that may be involved in the YAP1 inhibitory effect on CDX2 is HNF4α. HNF4α which is a member of the nuclear receptor family of transcription factors plays a role in enterocyte differentiation in the intestinal epithelium (San Roman, Aronson*, et al.*, 2015). The expression of CDX2 is regulated by HNF4α in the normal intestine and CRCs. Silencing of HNF4α in CRC cell lines and the mouse was accompanied by a reduction in CDX2 expression (Saandi *et al.*, 2013). It has been revealed that HNF4α binds and activates a reporter plasmid containing the CDX2 promoter in the colorectal cancer cell line Caco-2 (Boyd *et al.*, 2009). On the other hand, YAP1 negatively regulates HNF4α expression through ubiquitination and proteasomal degradation in hepatocellular carcinoma (HCC) cells (Cai *et al.*, 2017). Altogether, this suggests that YAP1 activation leads to the reduction of HNF4α and in consequence a reduction in CDX2 expression. It has been reported that HNF4α is expressed at a low level in HT29 cells, which can be related to the inhibitory effect of YAP1. However, in the Caco-2/15 cell line expression of YAP1, CDX2 and HNF4α (Babeu *et al.*, 2018) suggests another possibility. Larsen and colleagues reported that CDX2 and

HNF4α bind to the enhancer of the YAP1 promoter in Caco-2 cells. This group has mentioned that CDX2 only has a limited effect on basic YAP1 promoter activity because of the expression of YAP1 in some cells with no CDX2 expression (Larsen *et al.*, 2019b). Given that YAP1 could regulate HNF4α through their ubiquitination and degradation, it may also negatively regulate the CDX2 protein post-transcriptionally via the induction of their degradation. However, increased CDX2 expression at the transcriptional level in YAP1 knockdown HT29 cells suggests a transcriptional effect of YAP1 on CDX2 expression.

The antagonistic influence of Oct4 on Cdx2 transcription in early embryonic development (Niwa *et al.*, 2005) was also investigated based on the negative correlation reported between the expression of the two factors in CRC (Liang *et al.*, 2014) but no modulation of Oct4 expression was observed in response to YAP1 ablation in HT29 cells. Furthermore, the expression of Oct4 in the mouse intestine and embryonic cells was not affected by YAP1 induction (Camargo *et al.*, 2007). In another study, Shang et al. reported that the factor ASCL2 was overexpressed in a subset of colorectal cell lines and that its knockdown in HT29 cells resulted in approximately a 2X increase in CDX2 expression, through a transcription-dependent mechanism (Shang *et al.*, 2015). However, the very low level of ASCL2 in HT29 cells observed herein and the lack of modulation in its expression in response to YAP1 ablation indicates that this mechanism cannot account for the pro-differentiation effects observed herein. It has also to be pointed out that the identity of the HT29 cells used in the study of Shang et al. (Shang *et al.*, 2015) could be questioned considering that wild type HT29 do not express detectable levels of CDX2 protein as shown herein and elsewhere (Benahmed *et al.*, 2007; Hinoi *et al.*, 2003; Qualtrough *et al.*, 2002).

Therefore, the molecular regulatory mechanism of YAP1 on CDX2 expression in intestinal cells can be elucidated by analysis of CDX2 promoter.

4.6. SFKs restrict the differentiation of epithelial cell through YAP1 regulation

YAP1/TAZ as an effector of the Hippo pathway is also regulated by various upstream signals among which are the SFKs. On the other hand, activation of SRC is regulated by several factors like mechanical stress which include cell-cell contact, cell-matrix adhesion, extracellular stiffness, etc. (Boopathy & Hong, 2019; Zhao *et al.*, 2007). In the first place, YAP1 was cloned as an interacting protein of c-Yes which is a member of SFKs but later it was shown that it is phosphorylated at a tyrosine residue by SRC while YES has a minimal effect on YAP1 phosphorylation and nuclear localization (Kim & Gumbiner, 2015; Zaidi *et al.*, 2004). In the present study, inhibition of SFKs in Caco-2/15 cells resulted in higher expression of the absorptive cell markers SI and DPPIV, which indicates that SFKs negatively regulate epithelial cell differentiation. Previously, it has been shown that the highest level of SRC kinase activity occurs in 0 to 3 days post confluent Caco-2/15 cells. Thus, repression of SFK activity using the SFK inhibitor (PP2) in Caco-2/15 cells at confluency was accompanied by acceleration of absorptive cell differentiation and elevation of SI expression (Seltana *et al.*, 2013). We showed that in HT29 cells, inhibition of SFKs by PP2 and dasatinib resulted in increased expression of absorptive cell markers (SI and DPPIV) and goblet cell markers (MUC2 and TFF3). Nam and colleagues also have reported that increased level of E-cadherin, which is a marker of epithelial differentiation and α/β/γ catenin proteins was observed by treatment of HT29 cells with the PP2 inhibitor. In addition, cell adhesiveness was increased by inhibiting SRC activity (Nam *et al.*, 2002). E-cadherin is a component of the adherens junction in epithelial tissue. It is expressed strongly at the apical part of the crypt, however its expression is decreased toward the base of the crypt (Daulagala *et al.*, 2019; Escaffit, Perreault*, et al.*, 2005). Treatment of pancreatic adenocarcinoma cells with dasatinib also resulted in increased total and membranous E-cadherin/β-catenin levels. It has been suggested that SRC kinase downregulates the expression of E-cadherin through the pro-EMT transcription factor Slug, which is a negative transcriptional regulator of E-cadherin (Dosch *et al.*, 2019). Treatment of Caco-2/15 and HT29 cells with PP2 and dasatinib in this work was also accompanied by partial knockdown

of the YAP1 protein which suggests the regulatory effect of the SFKs on YAP1 activity. Recent studies have demonstrated high activity of SFKs and YAP1/TAZ in cancers. Lamar and colleagues have reported that increased SRC activity may be the driver of high activity of YAP1/TAZ in human cancers, however it is not the only cause of YAP1/TAZ activity in cancer. This group has shown that inhibition of SRC activity using dasatinib was accompanied by a reduction in YAP1/TAZ activity, tumor growth and metastasis. On the contrary, expression of constantly active SRC by the generation of mutant SRC (Y527F), led to elevated levels of YAP1/TAZ and its target genes in several human and mouse breast cancer and melanoma cell lines (Lamar *et al.*, 2019).

The mechanisms by which SFKs and SRC kinase regulate YAP1/TAZ activity, are considerably various. Several studies reported that SFKs and Src kinase can directly affect YAP1/TAZ activity by inducing their stability and transcriptional activity. SFKs and Src kinase phosphorylate YAP1 and TAZ at tyrosine Y357 and Y316, respectively, which promotes their nuclear localization and stabilization (Byun *et al.*, 2017; Li *et al.*, 2016; Taniguchi *et al.*, 2015). On the other hand, it has been shown that Src kinase phosphorylates LATS1 in several tyrosine residues which suppresses its activity. Therefore, Src induces YAP1/TAZ activity through repressing the activity of LATS1/2 kinases (Si *et al.*, 2017). YAP1 and TAZ are phosphorylated in serine 127 and serine 89 by LATS1/2 kinases, respectively which leads to its cytoplasmic restriction and inactivation (Lei *et al.*, 2008; Zhao *et al.*, 2007). Therefore YAP1/TAZ is regulated by LATS1/2 kinases. Furthermore, LATS1/2 kinases are phosphorylated in serine 909 and/or threonine 1079 by MST1/2 kinases or other regulators like MAP4K which leads to their activation (Ni *et al.*, 2015; Si *et al.*, 2017). Therefore, this suggests that SFK inhibition by PP2 and dasatinib leads to the increased activity of LATS1/2 kinases and increased YAP1/TAZ phosphorylation and their cytoplasmic sequestration. Our results for IF staining of YAP1/TAZ in Caco-2/15 cells showed observable cytoplasmic staining in cells treated with PP2 and dasatinib. This cytoplasmic staining may be related to increased activity of the LATS1/2 kinases and YAP1/TAZ phosphorylation as a result of SFK inhibition. Given that our antibody detects both YAP1 and TAZ proteins, specifying the

relation of cytoplasmic staining in the YAP1 and/or TAZ protein is difficult at this time. Therefore, the use of an antibody specific for the YAP1 protein would be needed to discriminate between the effect of the inhibition of SFKs on YAP1 and/or TAZ cytoplasmic sequestration. Kim and Gumbiner demonstrated that treatment of MCF-10A cells with PP2 was accompanied by abolishment of nuclear and elevation of phosphorylated YAP1 (S127). They showed that the effect of PP2 on YAP1 was abolished by knockdown of the LATS1/2 kinases (Kim & Gumbiner, 2015). Since Western blot analysis in the present study showed partial abolition of the YAP1 protein in both HT29 and Caco-2/15 cells, we suggest that SFKs are at least involved in the YAP1 degradation pathway. Besides LATS1/2 kinases, which lead to the cytoplasmic sequestration of YAP1, CK1δ/ε can phosphorylate the YAP1 that is already phosphorylated at S397 by LATS1/2, which leads to its recognition by SCF (β-TrCP), ubiquitination and proteasomal degradation (Zhao *et al.*, 2010). In IF staining, the border of Caco-2/15 cells were stained by the YAP1/TAZ antibody. This phenomenon can be related to the binding of α-catenin as well as AMOT and PTPN14 into the YAP1/TAZ protein and its localization into the adherens and tight junctions, respectively (Liu *et al.*, 2013; Silvis *et al.*, 2011; Zhao *et al.*, 2011) (Figure 31).

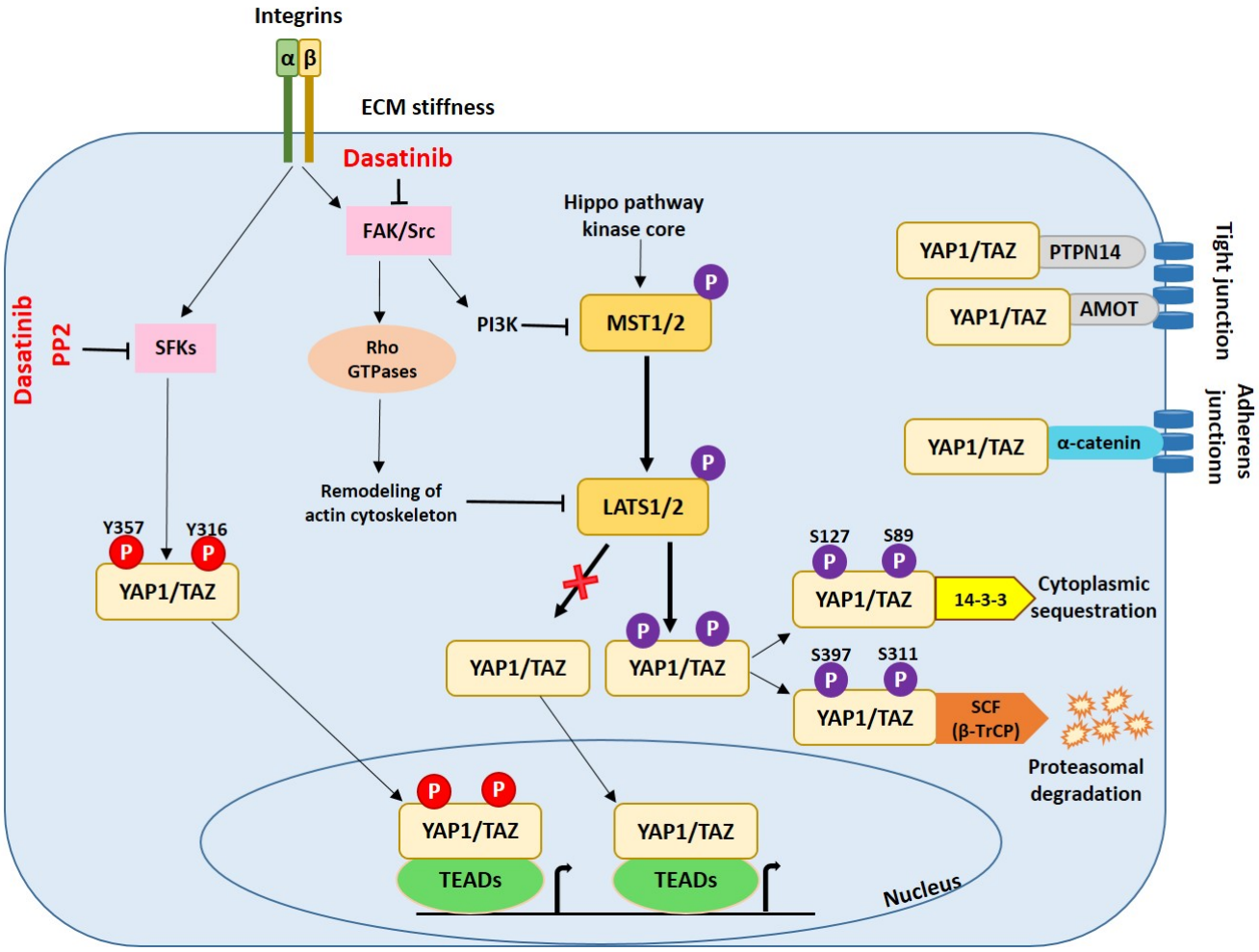

Figure 31. The regulatory effect of SFKs on downstream YAP1/TAZ coactivators.

This model summarizes the proposed mechanisms by which YAP1/TAZ is regulated by SFKs and how inhibitors may control YAP1/TAZ expression. The first mechanism suggests that SFKs can directly phosphorylate YAP1/TAZ in their tyrosine residue which leads to their activation and nuclear localization. The second mechanism proposes that Src kinase induces the activity of YAP1/TAZ through LATS1/2 kinase inhibition. SFK inhibition leads to an increasing of YAP1/TAZ phosphorylation, which depending on the phosphorylated serine residue is accompanied by cytoplasmic sequestration and/or proteasomal degradation. YAP1/TAZ activity can also be restricted by sequestration into the tight and adherens junctions which is mediated by PTPN14 and AMOT as well as α-catenin, respectively.

In the present study, inhibition of SFKs and reduction of YAP1/TAZ expression was accompanied by increased CDX2 expression, which is in line with our results in the direction of the role of YAP1 in CDX2 regulation and epithelial cell differentiation. Seltana et al. have reported that SFK inhibition in Caco-2 cell leads to the upregulation of CDX2 and HNF1α, two important transcription factors in the differentiation of intestinal epithelium. In addition, the expression of H3K27me3 is decreased by the inhibition of SFK activity (Seltana *et al.*, 2013). Moreover, it has been demonstrated that FAK overexpression in HEK293 cells results in a reduction of the activity of the CDX2 promoter, while FAK knockdown in DLD1 cells results in the upregulation of CDX2 mRNA (Brabletz *et al.*, 2004). FAK is a tyrosine kinase protein, and its phosphorylation and activation can be promoted by several stimuli including the interaction of integrin receptors with various ECM components and/or mechanical stretching across the plasma membrane. Phosphorylated FAK has a high affinity binding site for SH-2 containing proteins such as SFKs which leads to SFK stabilization and activation (Schaller *et al.*, 1994). Recruited Src kinase phosphorylates FAK in tyrosine residues that leads to the induction of its catalytic activity (Calalb *et al.*, 1995).

Overall, based on the literature, it can be suggested that in HT29 and Caco-2 cells, SFKs phosphorylate and inactivate the LATS1/2 kinases, which leads to the activation and nuclear localization of the YAP1/TAZ coactivator, and the suppression of CDX2 expression which is important in absorptive and goblet cells differentiation. Thus, SRC inhibition is accompanied by a reduction in YAP1/TAZ protein, increase in CDX2 expression, acceleration in absorptive and goblet cell differentiation, and increased SI, DPPIV, MUC2 and TFF3 expression.

5. Conclusions and perspectives

The work done during my PhD was aimed at determining the role of the Hippo pathway effector YAP1 in stemness, proliferation and differentiation of the human intestinal epithelium. The Hippo pathway plays a significant role in intestinal homeostasis and regeneration, while its effector YAP1/TAZ is known as a driver of tumor formation and progression in many cancers including CRC. Several experimental models have proposed that tumor growth and metastasis can be inhibited by restricting the aberrant activation of YAP1/TAZ. Therefore, YAP1/TAZ may be considered as a therapeutic target. On the other hand, considering that YAP1/TAZ plays an important role in normal tissues, preventing YAP1/TAZ activity in cancer patients may lead to adverse side effects. Therefore, knowing the mechanism by which YAP1/TAZ regulates the proliferation and differentiation of the cells and the mechanisms by which YAP1/TAZ activity is regulated, would enable the discovery of the correct cancer treatment strategy. In the present study we showed that YAP1 regulates stem cell maintenance and also negatively regulates the differentiation of intestinal epithelial cells. We demonstrate that YAP1 prevents the differentiation of goblet and absorptive cells. Our results revealed that this action of YAP1 was mostly mediated through the inhibition of CDX2, which is one of the main regulators of intestinal growth and differentiation. In cells knocked down for YAP1, the expression of the goblet and absorptive cell markers, as well as that of the CDX2 transcription factor were increased at both the transcriptional and protein levels. Furthermore, the expression of goblet and absorptive cell markers returned to the basal level by CDX2 ablation in YAP1 knockdown HT29 cells. Our results also suggest that the YAP1 inhibitory effect on goblet cell differentiation may also be partially mediated by the repression of ATOH1 expression. Indeed, increased expression of the goblet cell marker MUC2 was observed by ATOH1 overexpression in HT29 cells. Our results also showed that YAP1 is associated with stemness. A decrease in some stem cell markers (LGR5 and PROM1) was observed through YAP1 ablation in HT29 cells. Furthermore, LGR5 and PROM1 expression was restored by CDX2 ablation in shYAP1 infected HT29 cells suggesting an association of YAP1 with stemness through CDX2. We also

showed that SFKs which are Hippo pathway upstream signal, negatively regulate the differentiation of absorptive and goblet cells. Reduction in YAP1 and an increase in CDX2 expression by treatment with SFK inhibitors indicates that SFKs promote the expression of YAP1/TAZ, which negatively regulates CDX2 expression.

Besides YAP1/TAZ, some other repressor mechanisms including PRC2 and histone deacetylases have been identified which repress the differentiation of absorptive cells by different manners without affecting CDX2 expression (Benoit *et al.*, 2012; Roostaee *et al.*, 2016) as summarized (Figure 32).

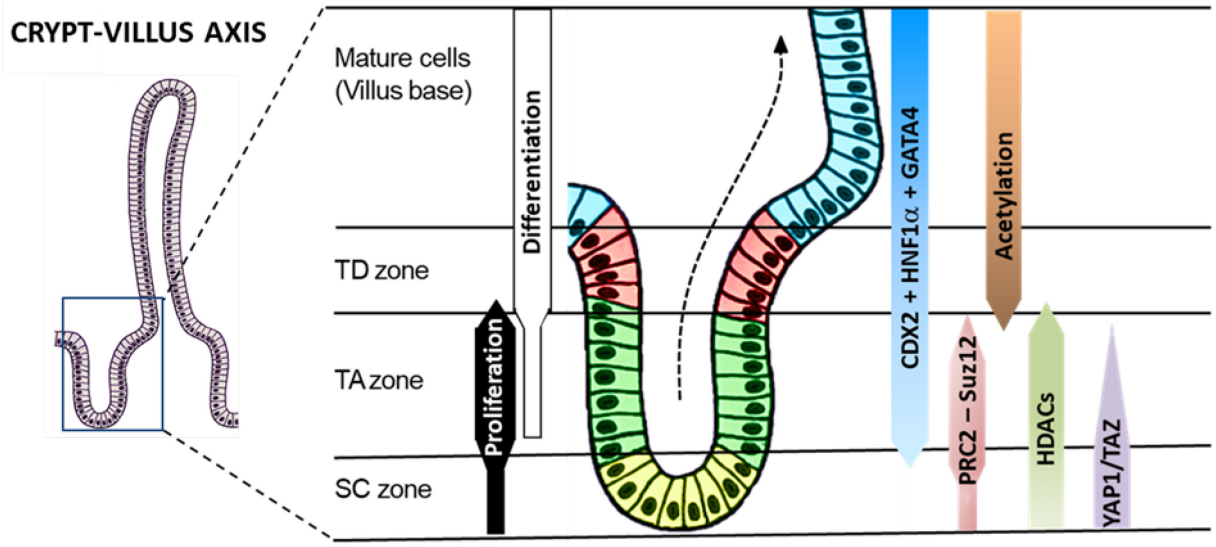

Figure 32. Integration of the molecular mechanisms regulating intestinal differentiation.

The differentiation of intestinal epithelial cells is regulated in the intestinal crypt at all stages while post mitotic mature cells reach the villus except for mature Paneth cells that are localized at the base of the crypt (S. Fallah *et al.*, 2020; Roostaee *et al.*, 2016). The mammalian intestinal crypt is composed of three zones involving stem/Paneth cell, transit amplifying (TA) and terminal differentiation (TD) zones. Certain factors are expressed in different parts of the crypt to regulate and initiate epithelial cell differentiation. CDX2, HNF1α and GATA4 are expressed in the TA and TD zones. The differentiation of goblet cells is initiated in the TA zone while differentiation of the absorptive cells in the TA zone is restricted by the PRC2 complex and histone deacetylase (HDAC). Activation of these two

repressors in the TA zone results in more cycles of proliferation of absorptive progenitor cells to ensure a larger proportion of absorptive cells in the TD zone. YAP1/TAZ appears to act at an earlier phase which involves stemness maintenance and prevention of both absorptive and secretory lineages through repression of pro-differentiation transcription factors such as CDX2. Adapted from (S. Fallah *et al.*, 2020; Roostaee *et al.*, 2016).

There are other signaling pathway and transcription factors which are important in intestinal epithelial cell differentiation, which may be affected by YAP1 expression. The Wnt pathway plays a significant role in proliferation and homeostasis of the intestine and differentiation of Paneth cells (Gregorieff & Clevers, 2005; Pin *et al.*, 2012). A low level of Wnt signaling results in a rise of stem cells towards absorptive, goblet and enteroendocrine cells while Paneth cell differentiation needs a high level of Wnt activity (Pin *et al.*, 2012). The Notch pathway plays a significant role in the determination of a progenitor cell's fate towards the absorptive or secretory lineages (Shroyer *et al.*, 2005). Notch activation induces HES1 expression that stimulates the differentiation of the absorptive cells. In the absence of Notch, ATOH1 expression leads to the differentiation of secretory cells (Goodell *et al.*, 2015). KLF4 plays a significant role in goblet cell differentiation (Katz *et al.*, 2002). HNF4α has also been reported as a regulator of intestinal epithelial cell differentiation (Babeu *et al.*, 2018). Bmps are generated primarily by the mesenchymal compartment. However, a BMP receptor (Bmpr1a) is strongly expressed in the upper part of the crypt and villus and is weakly expressed in the lower part of the crypt in a mouse model (Auclair *et al.*, 2007). It has been shown that BMP signaling plays a significant role in terminal differentiation of secretory cells, while it has no effect on absorptive cell differentiation (Auclair *et al.*, 2007) (Figure 33).

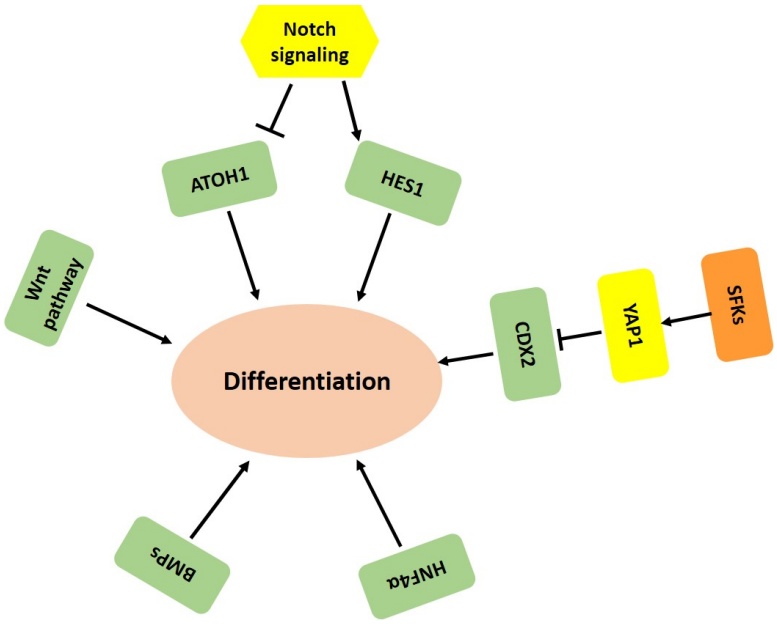

Figure 33. Regulatory factors in the differentiation of intestinal epithelial cells.

Notch pathway activity induces the differentiation of absorptive cells; however, secretory cell differentiation is promoted by ATOH1 expression in the absence of Notch signaling. Wnt signaling which is strongly active at the base of the crypt plays a significant role in Paneth cell differentiation. BMP signaling regulates the terminal differentiation of secretory cells. HNF4α along with CDX2 regulates the differentiation of absorptive cells (San Roman, Aronson*, et al.*, 2015). SFKs negatively control the differentiation of absorptive and secretory cells through the regulation of the YAP1 protein.

My research work has highlighted the importance of YAP1 and its upstream regulator SFK in the regulation of intestinal epithelial cell differentiation. However, additional questions remain regarding the role of SFKs and in turn YAP1/TAZ in differentiation and stemness. Particularly, it would be helpful to gain a better understanding of the context-dependent transcriptional events. The exact mechanism by which YAP1 inhibits CDX2 expression could be revealed by investigating the CDX2 promoter. In addition, these analyses would make it possible to validate whether YAP1 represses CDX2 expression indirectly through other still unidentified factors which bind to the *CDX2* promoter and act as transcriptional repressors. The first step would be to search for the shortest promoter that regulates CDX2 expression in YAP1 knockdown HT29 cells by preparing different constructs (such as 100, 200, 500 and 1212 bp corresponding to the CDX2 promoter (Boyd *et al.*, 2010)) generated using certain

restriction enzymes and testing by luciferase assays (Boudjadi & Beaulieu, 2017). Once identified, the shortest active promoter sequence would be analyzed by the online transcription factor database JASPAR (Boyd *et al.*, 2010) to identify all potential regulatory elements. Then, qPCR screening of YAP1 knockdown and control HT29 cells would be applied to investigate every modulated factor corresponding to regulatory element sequences identified in the CDX2 shortest active promoter, which would be confirmed by WB analysis. The next step would be the mutation of the sequence of candidate regulatory elements as described previously (Boudjadi & Beaulieu, 2017). Then transient transfection would be done and then the activity of the promoter would be analyzed using the luciferase assay. A reduction in promoter activity indicates that candidate regulatory elements activate CDX2 expression by inducing the activity of the CDX2 promoter.

Also, it would be interesting to study the effect of YAP1 on ATOH1 expression. Thus, abolition of the ATOH1 using shRNA in YAP1 knockdown HT29 cells would be helpful. Then the effect of ATOH1 on goblet cell differentiation in YAP1 knockdown cells could be verified by evaluation of MUC2 and TFF3 expression.

Xenografts could be used to study if shYAP1 expressing HT29 cells maintained or lost their tumorigenic capacity. To do so, an equal number of shYAP1 expressing HT29 and shCtrl cells would be subcutaneously injected into immunodeficient mice. Then, the size of the tumor would be monitored and measured, as done previously (Boudjadi *et al.*, 2017).

Another hypothesis that needs more investigation is the effect of YAP1 on HNF4α. It has been reported that the expression of HNF4α is restricted by YAP1 activity. In the human, HNF4α has 12 isoforms which exist as homo- and heterodimers divided into P1 and P2 promoter-driven isoform classes (Babeu *et al.*, 2018; Ko *et al.*, 2019). It has been observed that P1 which is associated with differentiation and P2 which is associated with proliferation are expressed at below detectable and low levels in HT29 cells, respectively (Babeu *et al.*,

2018). Therefore, it would be useful to measure the levels of both of HNF4α isoforms in YAP1 knockdown HT29 cells in comparison with a control.

Several factors have been reported as transcriptional suppressors in the differentiation phenomenon such as the suppressive epigenetic mechanisms including histone methylation/deacetylation. Deacetylation or methylation of certain lysines or arginines on the histone tail contribute to transcriptional repression. For example three methylations of lysine 27 on histone 3 in the intestinal crypt represse the terminal differentiation of absorptive cells (Roostaee *et al.*, 2016). In addition, histone deacetylases promote proliferation and repress differentiation through the removal of acetyl groups from certain lysines of histone (Roostaee *et al.*, 2015). Therefore, it would be useful to study the association of the YAP1/TAZ coactivator with epigenetics by measuring the level of certain methylated and deacetylated histones in YAP1 knockdown HT29 cells relative to controls. In addition, it has been shown that YAP1 recruits the transcriptional repressor NuRD to regulate the lineage commitment of pluripotent cells. On the other hand, PRC2 is recruited to the NuRD promoter to boost its repressive action. Therefore, it would be interesting to study the effect of YAP1 ablation on PRC2 and histone methylation.

The antibody used for indirect immunofluorescence of Caco-2/15 cells treated with SFK inhibitors detects both YAP1 and TAZ proteins. Therefore, identifying and using the antibody specific for YAP1 protein would help us to find out whether the cytoplasmic staining is related to phosphorylated YAP1 and/or TAZ protein. Furthermore, the detection of different forms of phosphorylated YAP1 (phosphorylated at tyrosine and serine residues) would help us to better understand the mechanism by which SFKs regulate the activity of YAP1/TAZ.

Finally, it would be very interesting to investigate the role of the YAP1 paralog TAZ on intestinal epithelial cell proliferation and differentiation using HIEC cells. According to our results, TAZ is expressed in HIEC cells at both the mRNA and protein levels while YAP1 is undetectable at the protein level.

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