Understanding the regulation of transcription factor SP1 via Wnt and Hippo signaling pathways in colon development and cancer

विद्या वाचस्पति की
उपाधि की अपेक्षाओं की आंशिक पूर्ति में प्रस्तुत शोध प्रबंध

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Dedicated to my family and friends who have supported me throughout

CERTIFICATE

Certified that the work incorporated in the thesis entitled "**Understanding the regulation of transcription factor SP1 via Wnt and Hippo signaling pathways in colon development and cancer**" submitted by **Ankita Sharma** was carried out by the candidate, under my supervision. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other university or institution.

Date: 31-10-2023 Sanjeev Galande

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LIST OF ABBREVIATIONS

APC	Adenomatous Polyposis Coli
ARID	AT-rich Interaction Domain
ATG	Autophagy-related
ATM	Ataxia-Telangiectasia Mutated
AXIN	Axis Inhibition
BCL	B-cell Lymphoma
BEX	Brain-Expressed X-linked
BMP	Bone Morphogenetic Protein
BUB	Buddi ng Uninhibited by Benzimidazoles
CASP	Caspase
CAV	Caveolin
CBP	CREB Binding Protein
CCN	Cyclin
CCSC	Colorectal Cancer Stem Cells
CENP	Centromere protein
CK	Casein Kinases
COAD	Colorectal Adenocarcinoma
CRC	Colorectal Cancer
DAPK	Death-Associated Protein Kinase
DE	Differentially Expressed
DEPP	Decidual Protein induced by Progesterone
DKD	Double Knockdown
DKK	Dickkopf-related protein
DNMT	DNA Methyltransferase

Dvl	Disheveled
EGF	Epidermal Growth Factor
EMT	Epithelial to mesenchymal transition
ESR	Estrogen Receptor
FGF	Fibroblast growth factor
Fzd	Frizzled
GI	Gastrointestinal
GSK3β	Glycogen Synthase Kinase β
H3K4me1	Histone 3, Lysine 4, mono-methylation
H3K4me3	Histone 3, Lysine 4, tri-methylation
H3K27ac	Histone 3, Lysine 27, acetylation
GTF	General Transcription Factors
HDAC	Histone deacetylases
hESC	Human Embryonic Stem Cells
HM	Histone Modification
ISC	Intestinal Stem Cells
ISWI	Imitation switch
KEGG	Kyoto Encyclopedia of Genes and Genomes
LATS	Large Tumor Suppressor Kinase
LEF	Lymphoid Enhancer Binding Factor
LGR	Leucine-rich repeat-containing G-protein coupled Receptor
LIPA	Lipoprotein(a)
LRH	Liver Receptor Homolog-1
LRP	Low-density-lipoprotein-receptor-Related Protein
M cells	Microfold cells
MCM	Minichromosome Maintenance Complex

MED	Mediator of RNA polymerase II transcription
MLL	Mixed Lineage Leukemia
MST	Macrophage Stimulating
MT-A	Mithramycin-A
NF	Neurofibromatosis protein
PCLAF	PCNA-associated factor
PCNA	Proliferating Cell Nuclear Antigen
POL2	Polymerase II
Porc	Porcupine
PYGO	Pygopus
ROR	Receptor tyrosine kinase-like Orphan Receptor
RYK	Receptor Tyrosine Kinase
SAV	Salvador
Ser	Serine
sFRP	secreted Frizzled Related Protein
SKD	Single Knockdown
SMAD	Suppressor of Mother Against Decapentaplegic
SSTF	Sequence Specific Transcription Factor
TAD	Transactivation Domain
TAZ	Transcriptional co-activator with PDZ-binding motif
TBP	TATA box-Binding Protein
TCF	T-cell-specific transcription Factor
TEAD	TEA Domain Transcription Factor
TF	Transcription Factors
Thr	Threonine
TRRAP	Transformation-transcription domain-associated protein

WDC	Wnt Destruction Complex
WIF	WNT Inhibitory Factor
WISH	Whole mount in situ hybridisation
WT	Wild Type
WWTR	WW domain containing transcription regulator
YAP	Yes-associated protein
β-TrCP	β- transducin repeat-containing protein

ABSTRACT

Wnt/β-catenin signaling is a highly conserved pathway found in various multicellular organisms. It plays a crucial role in a myriad of cellular processes necessary for both development and homeostasis during adulthood. Dysregulation of Wnt signaling causes diseases, with colorectal cancer (CRC) being one of the most common outcomes. Previously, we demonstrated that the transcription factor SP1 is an integral component of the Wnt/β-catenin pathway and interacts with β-catenin in the context of CRC. In order to gain a deeper understanding of the broader implications of this complex in relation to development and disease, we performed transcriptome analysis upon transient depletion of both SP1 and β-catenin. Remarkably, the set of genes co-regulated by the SP1:β-catenin complex was particularly enriched in functions related to cell cycle and DNA replication. These functions are essential for normal cell proliferation, which, when disrupted, can contribute to the development of cancer. In CRC patient datasets, we also identified a similar set of target genes displaying dysregulation, reinforcing our initial findings. Furthermore, through experiments involving a xenograft model in mice as well as ectopic overexpression in zebrafish larvae, we observed a synergistic effect driven by the SP1:β-catenin axis. This suggests a conserved role of the SP1:β-catenin complex in promoting cell proliferation during development as well as intestinal homeostasis in adults. To delve deeper, we explored the regulation involving SP1 and Hippo pathway effector YAP. YAP is a component of the Wnt destruction complex and has the ability to regulate the stability of β-catenin. We show that SP1 and YAP can physically interact and positively regulate each other. Notably, the stabilisation of YAP upon Wnt activation is dependent on SP1. In summary, our findings underscore the crucial role of SP1 as a vital regulator essential for the stabilising Wnt-dependent effector proteins, namely β-catenin and YAP.

KEYWORDS

Wnt pathway, Wnt destruction complex, SP1, β-catenin, Hippo pathway, YAP, colorectal cancer, colon development, cellular proliferation

SYNOPSIS

In **Chapter I**, we provide a brief overview of colon development and homeostasis, as well as the signaling network involved in these processes. The gastrointestinal lining consists of essential and interconnected components from all three germ layers. The interaction between these three layers is vital for the proper functioning of the digestive system. The epithelial lining, which derives from the endoderm, is responsible for digestive and absorptive functions. However, it relies on signals and support from the underlying mesenchymal and smooth muscle layer, which originates from the mesoderm, for regeneration. Additionally, the enteric nervous system, derived from the ectoderm, plays a crucial role in safeguarding the sterile environment of the body by managing microbes and potential pathogens. This intricate process relies on a precise network governed by the intercellular and intracellular signaling circuitry. This network ensures the regular formation of the epithelial lining while simultaneously maintaining an optimal number of stem cells. While several pathways are associated with this process, our primary focus has been on the Wnt pathway, which plays a pivotal role in maintaining the homeostasis of the intestinal lining. Wnt serves as the primary signal necessary for the stem cells to undergo proliferation and maintain their identity. Conversely, the absence or reduction of Wnt signals encourages differentiation. We have further narrowed our focus on the interplay between the components of the Wnt pathway and other proteins that fine-tune its effects. In a study published by our laboratory, we identified SP1 as a crucial regulator of the Wnt response. Further, we demonstrated that SP1 accomplishes this by interacting with the Wnt effector β-catenin and influencing its stability. Notably, the stability of the SP1 protein itself is subject to regulation by the Wnt pathway, suggesting that SP1 may play a role in the Wnt response.

We aimed to further this study by performing a comprehensive analysis of the genes influenced by SP1 and β-catenin. **Chapter II** is dedicated to this task and involves the analysis of the transcriptome data generated after depletion of SP1 and β-catenin in the HCT116 human cell line. HCT116 is a colon adenocarcinoma cell line characterized by

its aggressive nature and constitutively high levels of both SP1 and β-catenin. It offers an excellent *in vitro* system for investigating their gene targets. Depletion studies have demonstrated the essential roles of SP1 and β-catenin in promoting cell proliferation and preventing cell death. A significant proportion of these genes were found to be direct targets of SP1 and β-catenin, whether or not they also required TCF7L2, the primary DNA binding partner of β-catenin. The quantification of changes in gene expression for TCF7L2-dependent and independent targets revealed a reliance on both β-catenin and SP1. Additionally, a synergistic effect on their transcriptional activity was observed. This pattern was consistent with the results concerning cell viability and the cell cycle, providing further validation of their involvement in the regulation of cell proliferation and survival, as confirmed by the transcriptome analysis.

As all prior investigations have been conducted in cell line systems, our goal was to examine whether the SP1-β-catenin axis remains applicable in living organisms. To address this question, we chose to explore disease and developmental models. In **Chapter III**, we employed a xenograft model in mice to investigate the implications of SP1 and β-catenin working in tandem during tumor development. Cancer cells often exploit developmental pathways to promote growth and minimize differentiation. Therefore, we sought to determine if there was any interaction between SP1 and β-catenin during development, using zebrafish as our model system. In addition to the technical advantages of zebrafish, we were inclined to use this system because it completes the entire developmental process within a day while following the same basic developmental program. However, the development of the gut takes slightly longer than the rest of the organ systems, with a primitive tube forming at the 3- -day post-fertilization (dpf) stage. By the end of the 5dpf stage, the gastrointestinal system is fully developed. In both these experimental paradigms, we observed a positive interaction between SP1 and β-catenin. We also identified a set of genes that were regulated in a consistent manner in both paradigms, indicating that this complex is evolutionarily conserved and plays a crucial role in development, potentially contributing to the onset of colorectal tumorigenesis.

We then shifted our focus to another key player and an indispensable component of the Wnt destruction complex, which is YAP. YAP, along with TAZ, serves as the effector protein for the Hippo pathway, but it is also a crucial component of the Wnt destruction complex, making it an integral part of the Wnt pathway. Given the similarities in the regulatory mechanisms of β-catenin, SP1 and YAP, we embarked on an investigation to explore potential interactions between SP1 and YAP within the context of the Wnt destruction complex in **Chapter IV**. Our findings reveal a direct interaction between SP1 and YAP. YAP, in this context, exerts direct control over the transcription of SP1. Conversely, SP1 stabilises the YAP protein without any effect on its transcript level in a Wnt-dependent manner. Using a strategy akin to our approach for elucidating the functions of SP1 and β-catenin, we generated transcriptome data in cells in which SP1 and YAP were depleted. These depletion studies revealed a co-regulation by SP1 and YAP in various processes critical for driving the cell cycle, including DNA replication, metabolism, and chromosome segregation. However, the most intriguing observation was the enrichment of genes associated with autophagy and quiescence when SP1 and YAP were simultaneously depleted. Furthermore, we explored the extent of their transcriptional cooperation by considering the TEAD4-bound targets. We showed that the levels of the levels of SP1 in the system significantly impact TEAD4-bound targets, which are directly involved in cell growth. Consequently, we established that SP1 and YAP work in concert to co-regulate the cell cycle and development. Additionally, their simultaneous depletion triggers a program of dormancy, maintaining the cells in a state of low proliferation yet high metabolic activity.

In **Chapter V,** we conclude by presenting an overview of the potential implications stemming from the findings across all the chapters, specifically concerning SP1. Historically, SP1 has been regarded as a generic transcription factor primarily responsible for activating housekeeping genes. Nevertheless, several studies have revealed its cell-type-specific and context-specific functions. SP1 can interact with a variety of transcriptional regulators. It is, therefore, essential to study its interactions and how these can fine-tune gene regulatory programs. Motif analysis for SP1 genomic binding sites shows enrichment of factors, such as ESR1, components of the AP1

complex, NFY proteins, etc. Reportedly, several of these factors bind with SP1 for optimal or synergistic effects on the rate of transcription. However, a low (but significant) percentage of these sites show enrichment of motifs for TCFs and TEADs. Based on our transcriptome studies, we believe that β-catenin and YAP might influence the chromatin binding of SP1 to increase the transcription of their respective targets upon Wnt activation and/or Hippo inactivation. Future studies should address the possibility of competition and cooperation between all the proteins to provide a global picture of this network.

CHAPTER I

Colon Development, Organisation, and the Regulatory Machinery

The human intestinal tract is one of the primary organs responsible for the absorption of nutrients. It also houses the largest number of microorganisms in the human system, which aid in digestion and also release some beneficial substances. The small intestine has a large surface area to perform digestion and absorption of nutrients. It has a 'brush-border' lining due to the presence of protrusions known as villi, which can increase the surface area of intestinal lining up to 16-fold (Helander and Fändriks, 2014). Since the intestine has to face a harsh environment due to continuous exposure to extreme pH, microbes and mechanical abrasions, it needs to have a lining that is sturdy enough to withstand these pressures and prevent any kind of injury and leakage into the sterile compartment but also be permeable for the absorption of nutrients (Gehart and Clevers, 2019). This unique combination of functions is performed by the crypts-villi units, which undergo continuous proliferation. Intestinal stem cells (ISC) reside in the base crypts and start moving towards the tip of the finger-like villus upon initiation of differentiation. This system makes sure that the lining is replaced by new cells after every few days, thereby minimizing the chances of injury (Gehart and Clevers, 2019). Disruption of this system causes diseases with systemic implications, making it important to study the physiology of this system.

Colon development and maturation

Embryonic development initiates from a single cell, which undergoes rapid divisions required later for the diversification of cells into the three germ layers- ectoderm, mesoderm and endoderm. These layers undergo gastrulation movements and differentiate further to form all the organ systems of the adult organism (Gerri *et al.*, 2020). Interestingly, the gastrointestinal (GI) tract has contributions from all three germ layers. Endoderm forms the epithelial lining of the GI tube. Mesoderm gives rise to the connective tissues and smooth muscles, which form the outer musculature surrounding

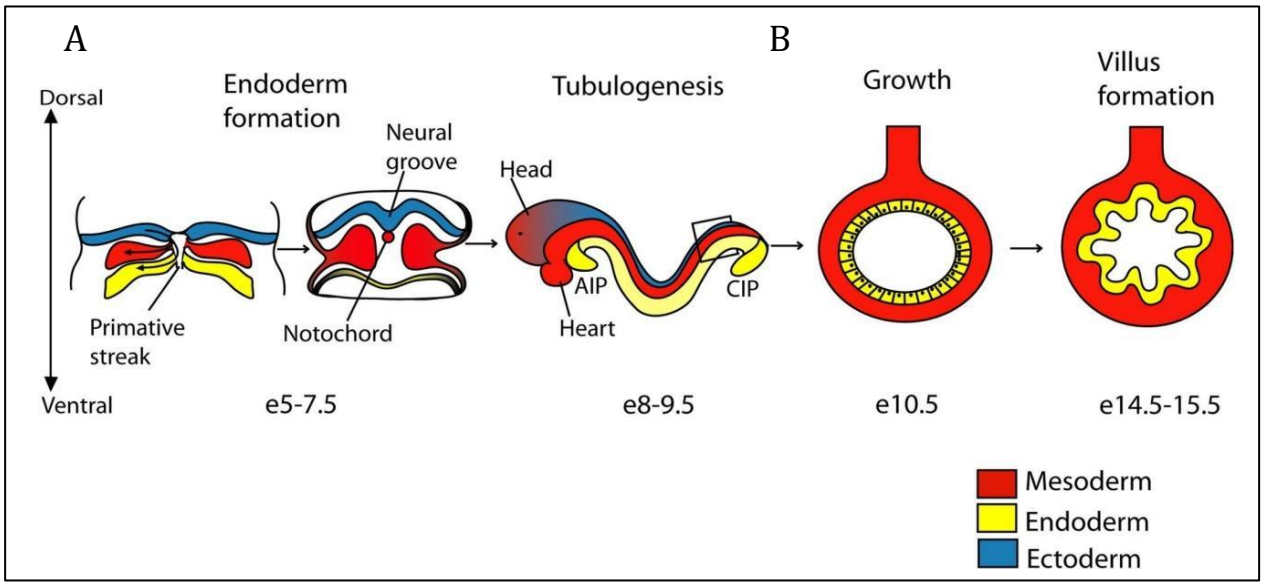

Figure 1.1. Development of the gastrointestinal tract. A. Formation of the three germ layers is followed by migration of mesendoderm from the primitive streak. Subsequently, the endoderm forms a flat sheet underlying the mesoderm. The process of tubulogenesis begins with the formation of the anterior and the posterior protons bend to form the Anterior Intestinal Port (AIP) and the Caudal Intestinal Port (CIP). **B.** The entire sheet eventually folds into a tube which remains attached to the main body through the mesoderm. The lining develops folds in the later stages known as the villi. (Modified from Noah *et al*., 2011).

the gut epithelium. The ectoderm forms the enteric nervous system (Noah, Donahue and Shroyer, 2011).

In the initial phase of development, the endoderm forms a flat epithelial sheet underlying the mesodermal layer. The anterior and the posterior portions of this sheet close to form pockets. These give rise to the future anterior and posterior gut respectively. When the anterior and posterior pockets continue to grow, the middle region of the sheet (which will form the midgut) eventually folds and closes the tube which completes the process of tubulogenesis (Noah, Donahue and Shroyer, 2011) (Figure 1.1.A). At this stage, it is enveloped by the mesodermal layer which connects the gut tube to the body wall. In the later stages, the gut epithelium reorganises itself and the villus morphogenesis begins (Figure 1.1.B). Initially, when the gut tube is growing and expanding, the entire sheet of the epithelium has proliferating cells but eventually the localisation of these cells starts to restrict in the intervillus regions. The proliferation also confines to these intervillus regions which later form the ‘Crypts of Lieberkühn’ in the mature gut epithelium. These crypts house the ISCs while the villi house the differentiated cell types (Lieberkuehn, 1745; Noah, Donahue and Shroyer, 2011).

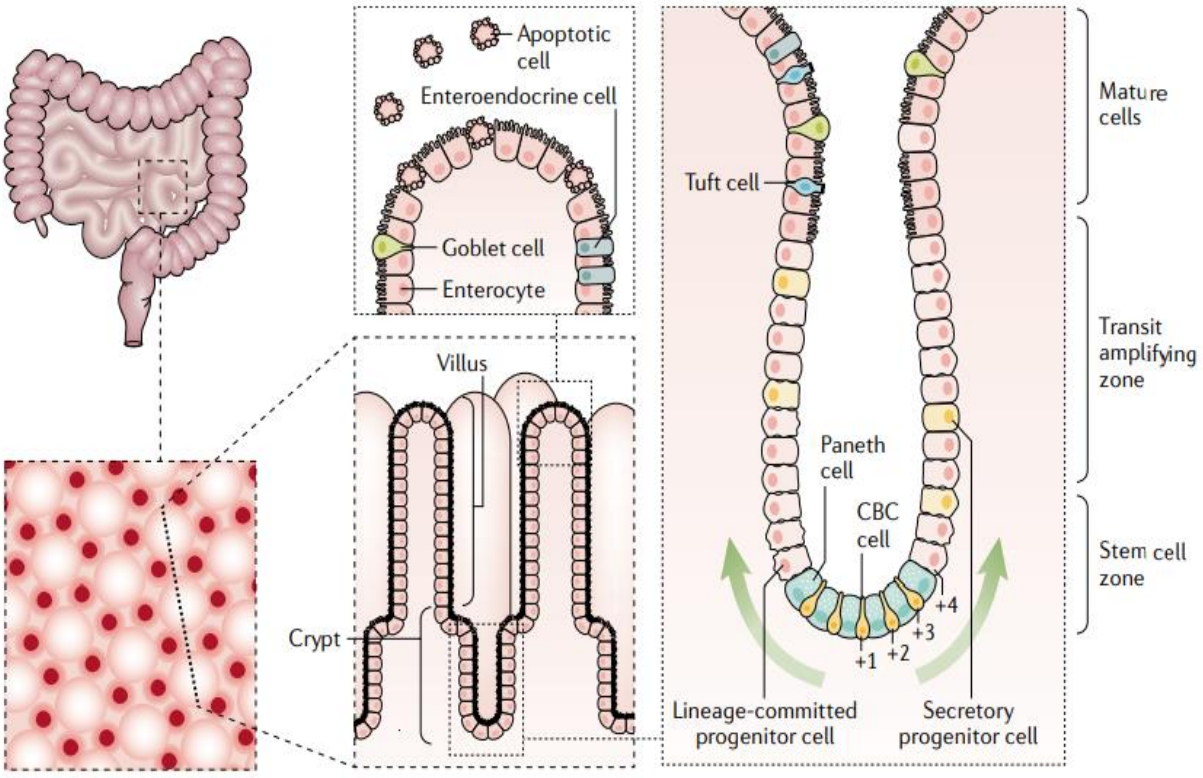

Figure 1.2. The crypt-villus organisation of the intestines. The intestinal lining is arranged into the functional units of crypt and villus. Crypts are present in the bases of villi and are enriched for the basal columnar cells (CBC) which are the stem cells giving rise to all the different types of intestinal cells. CBCs are surrounded by Paneth cells which nourish and protect the CBC. Stem cells divide and give rise to the progenitors also known as the transit-amplifying cells. These progenitors keep moving upwards while getting differentiated into either the absorptive or the secretory cells. (Adapted from Gehart and Clevers, 2019).

Crypt-villus structure: As mentioned previously, when the intestinal lining matures, it develops finger-like projections towards the lumen. These protrusions aid in expanding the surface area available for the absorption of nutrients. These projections are called villi and comprise of various types of differentiated intestinal epithelial cells (Figure 1.2). These post-mitotic cells have a short life span to minimize exposure to extreme environments. Since the intestinal lining undergoes continuous wear and tear, it is renewed every 3-5 days (Darwich *et al.*, 2014). Each villus is surrounded by multiple "crypts" at the base which house the ISCs. These crypts lie in the depths of the epithelium which protects them from the outer material with a very small opening. The crypts are continuously flushed with fluid to remove any kind of contaminants. Additionally, a physical barrier is created via the mucus secretion towards the lumen

which is also sensitive to any kind of bacterial invasion (Gehart and Clevers, 2019). ISCs divide continuously to give rise to progenitor cells and also self-renew. The progenitor cells further divide and move towards the upper part of the villus while getting differentiated into mature intestinal epithelial cells. The intestinal epithelium contains millions of these crypt-villus units (Winton, Blount and Ponder, 1988).

The mature epithelial cells are broadly of two types- absorptive and secretory type. This differentiation starts as soon as the cells leave the crypt base and move upwards. Absorptive types are further divided into enterocytes and M cells. Enterocytes comprise the majority of the intestinal lining and are responsible for the absorption of nutrients (Ross and Pawlina, 2011). M cells or microfold cells lie over the lymphoid follicles of the intestine (Peyer's patches). They can transport microbes from the lumen towards the mucosal layer as a part of the immune response (de Lau *et al.*, 2012). Secretory cells include Paneth cells, goblet cells, enteroendocrine cells and tuft cells. Paneth cells, unlike other mature cell types, move downwards upon differentiation. They nurture and protect the stem cells by secreting antimicrobial peptides into the lumen and providing the signals necessary to maintain the stem cell population (Gassler, 2017). Goblet cells secrete mucin required for the formation of the protective mucus layer over the epithelium and are usually the most abundant among the secretory types (Johansson, Larsson and Hansson, 2011). Enteroendocrine cells are further divided into multiple types depending on the kind of hormone they secrete. These hormones act as messengers between the gut and the rest of the system. Tuft cells are involved in immune regulation and are the rarest kind. They secrete IL-25 in response to parasitic helminths which activate the immune system and increase the production of both goblet cells and tuft cells (Gerbe *et al.*, 2016; von Moltke *et al.*, 2016). The majority of the lining is composed of the enterocytes with the secretory cells interspersed in between but depending on the cue received, the percentage of the specialised cells can increase.

Intestinal stem cells: Intestinal stem cells are enriched in the crypts and give rise to all the lineages of the intestinal epithelium. The crypt basal columnar cells (CBCs) are the stem cells under normal conditions (Barker *et al.*, 2007). The CBCs are protected by

Paneth cells with each CBC being surrounded by Paneth cells in an alternate fashion (Figure 1.2). Paneth cells move towards the crypt upon differentiation and protect the CBCs by secreting antimicrobial peptides, such as lysozyme and phospholipase A2 (Gassler, 2017). They also provide useful nutrients and growth factors to the stem cells. In the colon, there are $REG4^{+}$ crypt secretory cells which perform functions similar to the Paneth cells (Sasaki *et al.*, 2016). CBCs are not the only cells which can produce the intestinal lining. Due to the sensitivity of this system, there are several "backup modules" in place. During regeneration in the event of injury, the +4 cells can also give rise to all the lineages, including the CBCs. +4 cells are named according to their position within the crypt along the Paneth cells (Figure 1.2). These cells have higher resistance to injury and therefore they might be acting as a reserve in case of severe injury which requires a quick response (Sangiorgi and Capecchi, 2008; Takeda *et al.*, 2011; Sasaki *et al.*, 2016).

There have been several reports which show that other cell types can dedifferentiate to replenish the stem cell population when the CBCs are artificially depleted. Both the secretory and the absorptive progenitors have the ability to "fall back" into the crypt to repopulate it with stem cells (van Es *et al.*, 2012). Interestingly, it has also been shown that even the differentiated enteroendocrine cells have the ability to dedifferentiate (Yan, Gevaert, *et al.*, 2017). It is unclear however whether they are indeed differentiated or are slightly less differentiated than the mature enteroendocrine cells (Gehart and Clevers, 2019).

Pathways of the crypt

The intestinal fate specification is a result of the strength as well as the combination of the major pathways including Wnt, Notch, EGF, and BMP signaling. Figure 1.3 summarises the signal combination resulting in various intestinal epithelial cell types.

Wnt signaling is essential to maintain intestinal stem cell identity and proliferation. Wnt pathway activity is dependent on the stabilisation and nuclear accumulation of β-catenin

(Nusse and Clevers, 2017). Wnt ligand usually works on short ranges and is provided to the stem cells through the Paneth cells and the mesenchyme layer beneath the epithelium (Sato *et al.*, 2011; Stzepourginski *et al.*, 2017). Earlier, it was believed that Paneth cells are the sole source of the signals required to maintain the ISCs in their continuous proliferative state. However, recently it has been shown that intestinal-specific WNT3A gene knockout doesn't affect the epithelial function thereby showing the presence of alternate sources for Wnt (Farin, Van Es and Clevers, 2012). Wnt ligand remains attached to its receptor on the plasma membrane but keeps getting diluted during cellular divisions. This creates a gradient of Wnt signaling with the highest concentration in the crypt and progressively diluted towards the villi. The gradient of Wnt forms an important feedback loop for the stem cells as well. In case of a low proliferation rate, the Wnt ligands remain concentrated towards the crypt and aid in the continuous division. In case of hyperproliferation, the gradient range increases and the concentration at the crypt reduces which slows down the rate of proliferation (Farin *et al.*, 2016). The stem cells also carry the Lgr5 and Rnf43 receptors which help in further amplification of Wnt signaling by inhibiting the negative feedback (de Lau *et al.*, 2011; Glinka *et al.*, 2011). Functionally, loss of β-catenin's DNA binding partner TCF4 has been shown to result in loss of stem cells both during development and during later stages (Korinek *et al.*, 1998). In fact, the inactivation of APC, which is involved in the degradation of β-catenin, is enough to cause adenomas (Su *et al.*, 1992). Nearly 70% of colorectal cancers have a mutation in *APC* which demonstrates the importance of this pathway in the context of intestinal homeostasis (Vogelstein *et al.*, 1988).

Notch signaling is dependent on cell-cell contacts with one cell expressing the Notch ligand (ISC) and the neighboring cell (Paneth cell) expressing the Notch receptor (Sato *et al.*, 2011). Therefore, its range is even shorter than Wnt signaling. Notch signaling also promotes stem cell proliferation and identity. However, it is also involved in the differentiation of transit-amplifying population into secretory or absorptive types. Inhibition of Notch signaling promotes absorptive fate whereas Notch activation promotes the secretory fate (Shroyer *et al.*, 2007; VanDussen and Samuelson, 2010). This decision is made in conjunction with Wnt activation. Wnt effector β-catenin

promotes Notch by driving the expression of Notch ligand leading to continuous activation of Notch. If the Wnt signaling is not active then the Notch activation starts oscillating due to its feedback inhibition (Yamamizu *et al.*, 2010; Kay *et al.*, 2017). This phenomenon might be acting when the CBCs divide and one of the daughter cells move towards the other region with lower levels of Wnt ligand (Gehart and Clevers, 2019).

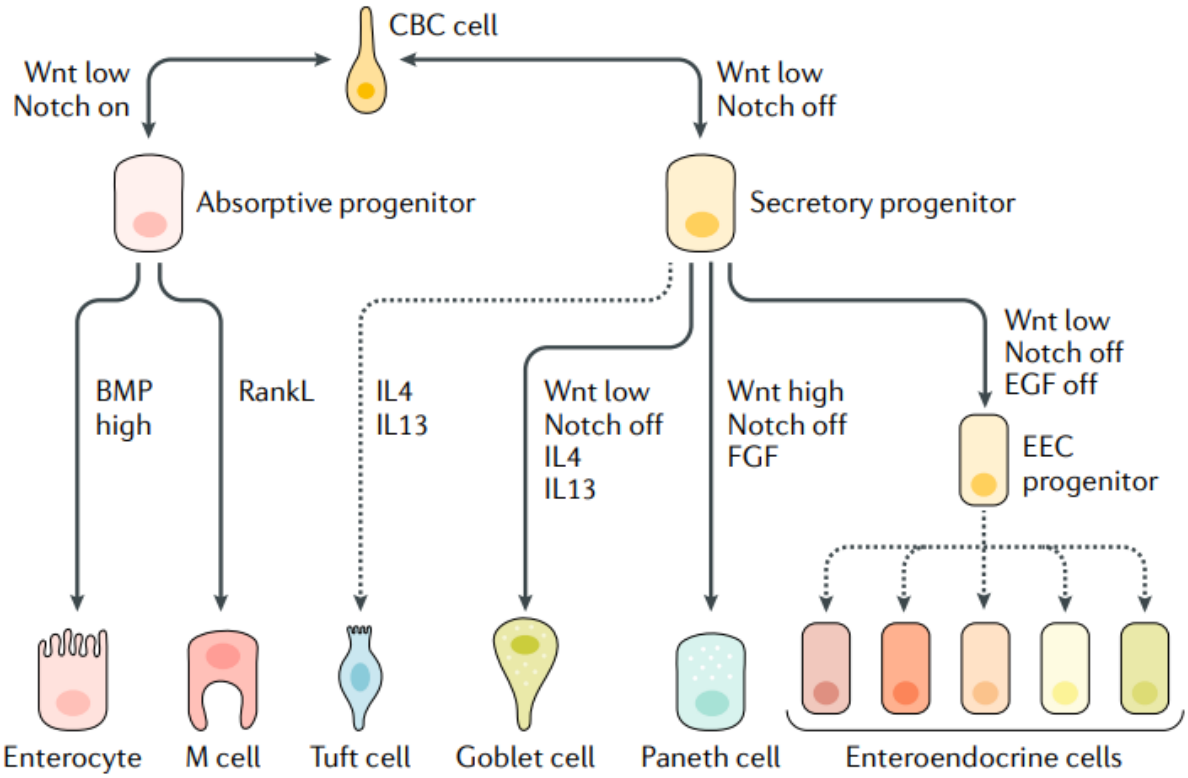

Figure 1.3. Pathways involved in the specification of intestinal epithelial cells. Stem cells (CBC) proliferate and maintain their stemness due to the presence of the Wnt ligand within the crypt region. Upon division, the first major decision towards differentiation into either the absorptive or the secretory progenitor is made by the Notch pathway. Notch and BMP activation favours the formation of the most abundant cell type i.e., the enterocytes. The presence of another ligand, RANKL pushes the differentiation path towards M cell fate. In case of secretory cell types, the goblet cells are the default cell type dependent on the presence of IL4 and IL13. High Wnt and FGF signaling favour Paneth cell formation. Enteroendocrine cells are formed when Wnt signaling is low and EGF & Notch signaling are absent. The dotted lines represent the absence of complete literature regarding the cues required for the differentiation of a particular cell type. (Adapted from Gehart and Clevers, 2019).

EGF pathway is mainly involved in maintaining the proliferation of ISCs (Schepers *et al.*, 2012). Stem cells carry the EGF receptor and the EGF ligand is secreted by Paneth cells (Sato *et al.*, 2011). Like the Wnt ligand, there are other sources for the EGF ligand as well. EGF signal is kept under check via the negative regulator LRIG1. Ablation of

LRIG1 has been shown to cause expansion of the crypts (Wong *et al.*, 2012). Although EGF signaling promotes proliferation, it doesn't play a role in the maintenance of stem cell identities like the Wnt and Notch pathways. This is demonstrated by the fact that when the EGF signal is depleted from the culture medium, it stops the stem cell proliferation but doesn't affect the stem cell identity (Basak *et al.*, 2017).

BMP signaling acts via the SMAD proteins and exerts an overall inhibitory effect on the intestinal epithelium proliferation. Deletion of BMP results in hyperproliferation of both stem cells and transit-amplifying cells causing polyp formation in the intestines (He *et al.*, 2004). BMP is thus required to keep the proliferation under check and promote differentiation. BMPs are secreted via the intercrypt as well as intervillus mesenchymal cells (Haramis *et al.*, 2004; Hardwick *et al.*, 2004). Since it can hamper the ISCs proliferation, the BMP pathway is kept inactive in the crypt region by the secreted BMP antagonists, such as Gremlin 1 and Noggin (He *et al.*, 2004; Kosinski *et al.*, 2007). These are provided by the fibroblasts and the smooth muscles lying below the crypt regions thereby creating a gradient of BMP activation in a manner opposite to the Wnt gradient (He *et al.*, 2004; Stzepourginski *et al.*, 2017).

Hippo signaling has gained attention fairly recently for its role in intestinal homeostasis but its exact role is not completely understood. Hippo effectors YAP/TAZ are directly involved in inducing the degradation of Wnt effector β-catenin in the cytoplasm and therefore can negatively regulate Wnt signaling (Azzolin *et al.*, 2014). Strikingly, the removal of YAP/TAZ also results in reduced stem cell regeneration (Imajo, Ebisuya and Nishida, 2015). The current hypothesis is that Hippo signaling keeps Wnt signaling under an optimal range during normal conditions but in the event of an injury, it promotes crypt expansion (Cai *et al.*, 2010; Barry *et al.*, 2013; Gregorieff *et al.*, 2015). Further studies are required to elucidate the exact mechanism for its action as well as its interplay with all the other signaling pathways.

Wnt/β-catenin signaling and its regulation

(Modified from the published review: ***Sharma A****, Mir R, Galande S. Epigenetic Regulation of the Wnt/β-Catenin Signaling Pathway in Cancer. Front Genet. 2021 Sep 6;12:681053. doi: 10.3389/fgene.2021.681053. PMID:34552611; PMCID:PMC8450413.)*

Discovered in the 1980s, the Wnt pathway remains one of the most exclusively studied signaling pathways to date (Nusse and Varmus, 1982). It has two modes of action, canonical and non-canonical. Canonical Wnt signaling is dependent on the activation of its effector, β-catenin leading to transcriptional alterations (Macdonald, Semenov and He, 2007). It is involved in regulating cellular proliferation, differentiation and/or self-renewal, etc. Non-canonical Wnt signaling acts via the Planar Cell Polarity (PCP) and Ca^{+} signaling and is associated with both transcriptional and cytoskeletal changes required for processes such as migration and cell polarity (Semenov *et al.*, 2007). Here, I will focus on the canonical or the Wnt/β-catenin pathway for the sake of my study.

Canonical Wnt signaling: As mentioned previously, it depends on the transcriptional activation of the Wnt target genes triggered by the nuclear translocation and subsequent binding of β-catenin to its DNA binding partner TCF/LEF. This, in turn, is driven by a large cytoplasmic destruction complex (or the Wnt destruction complex - WDC) comprising multiple proteins. Adenomatous Polyposis Coli (APC) is the largest protein in this complex and aptly acts as a scaffold, along with Axis inhibition protein (Axin), for the rest of the components to bind and assemble in one place. Additionally, it contains two important kinases- Casein Kinase 1 (CK1) and Glycogen Synthase Kinase 3 (GSK3), which sequentially phosphorylate β-catenin at Ser45 (via CK1) and Thr41, Ser37, and Ser33 (via GSK3). These phosphorylations act as a priming event for the binding of E3 ubiquitin ligase, β-TrCP. The ubiquitinated form of β-catenin eventually undergoes degradation via the proteasomal pathway (Aberle *et al.*, 1997). In a Wnt-Off condition, the WDC remains active, leading to continuous degradation of β-catenin (Nusse and Clevers, 2017) (Figure 1.4).

Wnt signaling is activated when the Wnt ligands (Wnt3a, Wnt1 or Wnt5) bind to the Wnt receptor complex. This transmembrane receptor complex consists of Frizzled (Fzd) and Lipoprotein-related protein (LRP5/6) (Cruciat and Niehrs, 2013). When not bound by the Wnt ligand, Fzd and LRP remain dissociated and in an inactive state. Wnt ligand binding causes dimerisation of these proteins, which allows the recruitment of Axin to LRP owing to a conformational change in their structure (Bhanot *et al.*, 1996; Janda *et al.*, 2012). It also causes the recruitment of another regulator, Dishevelled (Dvl). Dvl aids in the recruitment of the WDC by forming a platform between Fzd and Axin. When the scaffold protein itself gets engaged with the receptor complex, it leads to the inactivation of the entire WDC (Chen *et al.*, 2003). β-TrCP is unable to ubiquitinate, and thus, the proteasomal degradation of β-catenin halts (Aberle *et al.*, 1997). At this point, the already phosphorylated form of β-catenin remains bound and eventually saturates the entire pool of WDC (Li *et al.*, 2012). The rest of β-catenin becomes stabilised and is free to translocate inside the nucleus.

β-catenin does not have a DNA binding domain of its own, and therefore, whatever transcriptional changes are brought about by it requires binding to a transcription factor. The major DNA binding partner for β-catenin is the TCF/LEF (T cell factor/lymphoid enhancer-binding factor) family of transcription factors. When inactive, TCF/LEF is bound to an inhibitor, Groucho, which prevents its transcriptional activity (Cavallo *et al.*, 1998; Roose *et al.*, 1998). However, upon Wnt activation, nuclear β-catenin displaces Groucho and forms a transcriptional activator complex with TCF at promoters as well as enhancer elements (Behrens *et al.*, 1996; Molenaar *et al.*, 1996). Additionally, it binds to chromatin modifiers, majorly CBP, to bring about transcriptional activation (Hecht *et al.*, 2000).

Natural modulators of Wnt/β-catenin signaling

The stimulation and inhibition of Wnt signaling depend on multiple factors apart from the Wnt ligands. Wnts have a palmitoleic acid, which is added by palmitoyl transferase

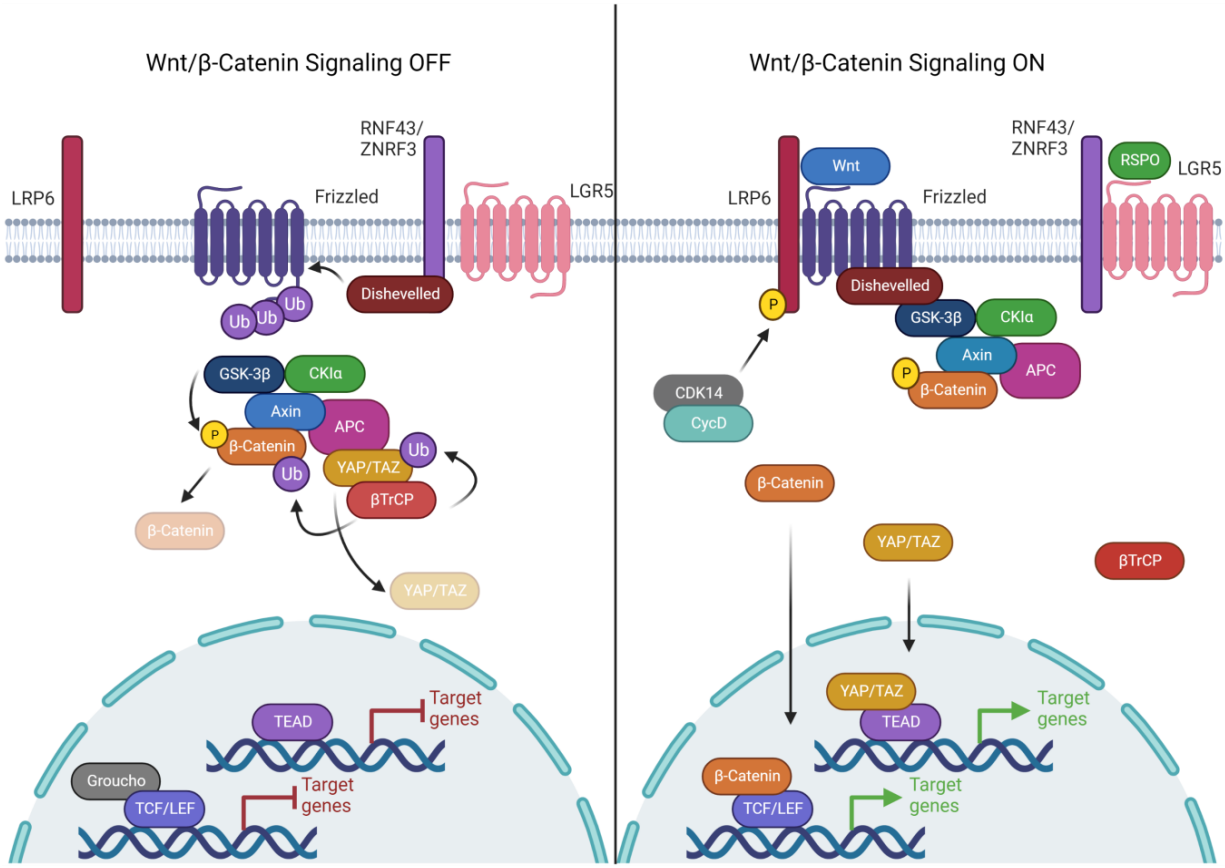

Figure 1.4. Components of the canonical Wnt pathway. Activation of Wnt targets is dependent on the activity of the Wnt DC. In the absence of the Wnt ligand, the Wnt DC, comprising the scaffold proteins (Axin, APC) and the kinases (GSK3β, CK1α), sequester and phosphorylate β-catenin which primes it for ubiquitination via βTrCP. The ubiquitinated form of β-catenin gets degraded within the cytoplasm, thereby preventing its nuclear translocation. When the Wnt ligand binds to the LRP-Fzd receptor complex, it changes the conformation of Fzd such that it now binds to the Wnt DC, resulting in the inhibition of its activity. In this state, β-catenin remains bound to the DC in its phosphorylated form without undergoing ubiquitination and subsequent degradation. Subsequently, the transmembrane receptor complex bound DC becomes saturated with phosphorylated β-catenin. Thus, the newly synthesized form of β-catenin remains free in the cytoplasm, which further allows its nuclear translocation and activation of its target gene via the β-catenin/TCF complex. (Adapted from Sharma *et al.*, 2021).

Porcupine (Porc). These are then transported to the plasma membrane in the form of vesicles with the help of transmembrane protein Wntless/Evi (WI) (Kadowaki *et al.*, 1996; Takada *et al.*, 2006). Wnt ligands are not capable of activating signaling at long distances due to the presence of lipid moiety. They can usually perform autocrine and paracrine signaling for nearby cells. The ligand may remain bound to the receptor in dividing cells but gets diluted during repeated divisions (Hofmann, 2000). There are certain alternative receptors for Wnt ligands as well, including ROR, RYK and GPR124, that can either activate some other signaling pathways or act via the non-canonical

pathway (Nusse and Clevers, 2017). It is unclear how these receptors cooperate along with Wnt signaling. There are a few proteins which can act as Wnt antagonists. Notum, which has been recently reported, inactivates Wnt by removing the palmitoleate moiety (Kakugawa *et al.*, 2015; Zhang *et al.*, 2015). Dickkopf (DKK) and Sclerostin/SOST family of proteins can antagonise Wnt signaling by binding to the Fzd receptor, thereby preventing Wnt receptor complex formation (Glinka *et al.*, 1998; Semënov *et al*., 2005). Secreted FZD-related proteins (sFRPs) and Wnt inhibitory protein (WIF) family of proteins antagonise by directly binding to the Wnt ligand (Niehrs, 2012).

Rnf43 and Znrf3 are transmembrane E3 ligases which act as the negative feedback regulators of Wnt signaling. They mediate ubiquitination of Fzd, causing endocytosis followed by lysosomal destruction of the Wnt receptor complex. These are countered by the Lgr5/Rnf43/R-spondin module, which is a key mechanism involved in the self-renewal of stem cells (including ISC) (Hao *et al.*, 2012; Koo *et al.*, 2012) (Figure 1.4). R-spondin family proteins interact with the Leucine-rich repeat-containing G-protein coupled (LGR) family of receptors. LGR receptors are enriched specifically in the adult stem cell population. In the absence of R-spondins, Rnf43 and Znrf3 target Fzd for degradation. However, in the presence of R-spondins, Rnf43 and Znrf3 interact with LGR4-6, leading to the inhibition of feedback and further potentiation of Wnt signaling even in low doses (Carmon *et al.*, 2011; de Lau *et al.*, 2011; Yan, Janda, *et al.*, 2017).

Biological functions and disease implications

Different cell types and/or developmental stages allow Wnt signaling to exert its effect in multiple biological contexts. The differential binding partners of β-catenin also mediate this. It is required for the initiation and/or maintenance of basic cellular processes, such as proliferation, differentiation and migration and therefore has a significant role in multiple biological processes. Wnt signaling comes into play right from the early events of development, such as cell-fate specification (formation of definitive endoderm) and late events, such as axis formation (dorsoventral and anteroposterior) and organogenesis (e.g., intestinal epithelial formation) (Kitajima *et al.*, 2013; Plaks *et al.*,

2013; Huang and Niehrs, 2014). Studies in *Hydra* and *Planaria* have shown its function for regeneration as well (Technau *et al.*, 2000; Guder *et al.*, 2006; Gurley, Rink and Sánchez Alvarado, 2008; Iglesias *et al.*, 2008). Recently, it has been shown to regulate innate and adaptive immune responses (Staal, Luis and Tiemessen, 2008; Malladi *et al.*, 2016; Trotter *et al.*, 2023). The most extensively studied role of Wnt signaling is with respect to maintaining the adult stem cell niches (including stem cells of the intestine, bone, and hair follicles) and, therefore, tissue homeostasis during the adult lifespan (Korinek *et al.*, 1998; Boyden *et al.*, 2002; Shimomura *et al.*, 2010).

Since Wnt signaling plays a crucial role in the maintenance of adult stem cell niches, it has been implicated in multiple aspects of cancer biology, such as initiation, malignant transformation, and metastasis. Colorectal cancer (CRC) is the most frequently caused cancer type upon perturbation of Wnt pathway components. Inactivation mutation in *APC* has been identified as the most frequent cause of inherited cases of adenomatous polyposis (also known as Familial Adenomatous Polyposis or FAP) (Su *et al.*, 1992). In general, mutation in the gene of any component (*CTNNB1, AXIN, RSPO, LGR5, LRP6, TCF7L2, GSK3B, RNF43,* etc.) which promotes hyper activation of the Wnt pathway has been linked to cancer (Cunningham and Boardman, no date; Lammi *et al.*, 2004; Bass *et al.*, 2011; Giannakis *et al.*, 2014; Morin, Kinzler and Sparks, 2016; Rismani *et al.*, 2017). Besides CRC, Wnt pathway has been linked to other gastrointestinal cancers including gastric, liver and pancreatic ductal adenocarcinoma (Clements *et al.*, 2002; Lustig *et al.*, 2002; Wang *et al.*, 2016). Others include various leukemia types (acute myelogenous leukemia, acute lymphoblastic leukemia, and chronic lymphocytic leukemia), melanoma, and breast cancer (Luis *et al.*, 2012; Shetti *et al.*, 2019; Gajos-Michniewicz and Czyz, 2020).

Mutation in Wnt pathway components is also associated with degenerative diseases of the skeleton (hereditary osteoporosis), retina (familial exudative vitreoretinopathy); and developmental defects (bone: Robinow syndrome, tooth: oligodontia) (Robitaille *et al.*, no date; van Bokhoven *et al.*, 2000; Baron and Kneissel, 2013).

Crosstalk with other signaling pathways

Signaling pathways typically do not function exclusively. Interaction between signaling pathways is an essential property of any biological system which helps in fine-tuning the gene expression in a context-specific manner. The Wnt pathway intersects with multiple pathways, such as Hippo, TGF-β, Hedgehog, FGF, and Notch signaling (Figure 1.5). The Hippo pathway has been of particular interest in the field because of the direct involvement of the Hippo effectors YAP/TAZ with the WDC. YAP/TAZ can bind to the destruction complex and help in the recruitment of βTrCP, thereby promoting the degradation of β-catenin and YAP/TAZ themselves (Azzolin *et al.*, 2014). Additionally, the phosphorylated form of β-catenin can bind to TAZ and initiate its ubiquitination (Azzolin *et al.*, 2012). The balance between β-catenin and YAP levels is crucial for maintaining the ISC niche. An elaborate interplay between the two pathways has been discussed in Chapter IV.

Co-operation between TGF-β and Wnt pathways has been reported in multiple contexts, including, promoting the expression of mesendodermal (ME) related genes in human embryonic stem cells (hESCs), myofibroblasts differentiation and epithelial-mesenchymal transition (EMT) in alveolar epithelial cells (Carthy *et al.*, 2011; Zhou *et al.*, 2012; Estarás, Benner and Jones, 2015). There is a direct interaction between the components of these pathways as well. For example, inhibitory SMAD7 promotes β-catenin recruitment to the plasma membrane through E-cadherin and prevents its degradation in cancer epithelial cells. SMAD7's interaction with AXIN can prevent the interaction of β-catenin with GSK3β (Tang *et al.*, 2008). Although this stops the degradation of β-catenin but increases its localization on the membrane instead of the nucleus. It, therefore, plays a role in the regulation of cell-cell adhesion. AXIN has been shown to mediate the binding of SMAD3 (not SMAD2) to GSK3β and promotes SMAD3 phosphorylation and degradation in the absence of TGF-β signaling (Guo *et al.*, 2008).

The inhibitory feedback between Wnt and Hedgehog pathways regulates developmental processes. Canonical Hedgehog signaling is an inhibitor of Wnt signaling especially in

the context of intestinal homeostasis. Hedgehog signaling suppresses Wnt signaling by promoting the expression of Wnt inhibitor sFRP1 via its effectors- Gli1 and Gli2 (He *et al.*, 2006). Activation of Hedgehog signaling also prevents the nuclear accumulation of β-catenin in liver cancers (He *et al.*, 2006). However, the non-canonical Hedgehog signaling has been shown to positively regulate Wnt signaling in intestinal tumors by promoting the undifferentiated state of cancer stem cells through Patched1 (PTCH1) (Regan *et al.*, 2017).

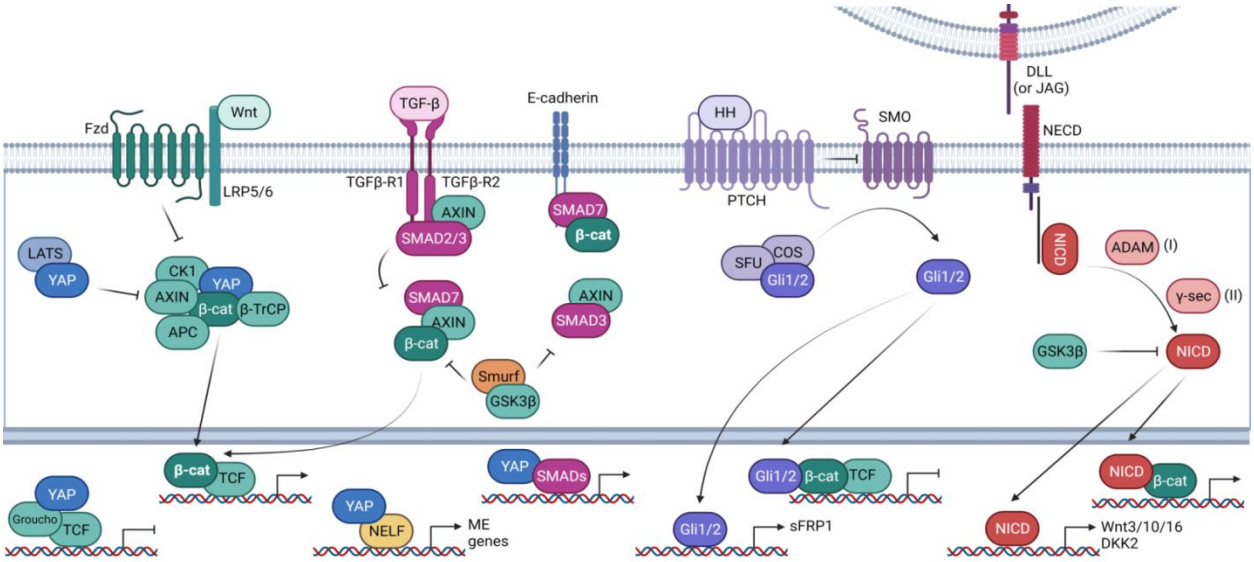

Figure 1.5. Crosstalk of Wnt pathway with other signaling pathways. The Wnt pathway has multiple points of crosstalk with other pathways, including β-catenin as well as the component of the Wnt DC, for example, Axin, GSK3β. Interaction of β-catenin with the effectors of other signaling pathways can govern the combination of downstream targets getting expressed upon activation of different pathways. Crosstalk via the DC components can influence the overall levels of the effectors from other pathways. Components from each pathway are depicted in a single color: Wnt pathway (green) Hippo pathway (blue), TGF-β pathway (magenta), Hedgehog pathway (purple), and Notch pathway (red). (Adapted from Sharma *et al*., 2021).

The Wnt-FGF gradient paradigm has been studied extensively, especially in the context of axis formation during development (Dyer *et al.*, 2014; Oginuma *et al.*, 2017). Additionally, high Wnt and FGF signaling together, via Wnt1 and Fgf3, have been shown to promote mitochondrial biogenesis which helps in maintaining stemness in breast cancer (Lamb *et al.*, 2015). Similar cooperation has been reported in ISCs via Wnt7b and Fgf10 (Volckaert *et al.*, 2017).

Direct transcriptional regulation exists between Wnt and Notch signaling pathways. Notch activation drives the expression of Wnt3, 10, and 16 and Wnt activates the expression of Notch ligand Jagged 1 (JAG1), both of which maintain the stem-cell characteristics (Chakrabarti *et al.*, 2018; Ma *et al.*, 2019). However, Wnt inhibitor DKK2 is also a target of Notch signaling in the ISCs, indicating negative feedback for Wnt by Notch signaling (Katoh and Katoh, 2007).

Alternative transcriptional effectors of Wnt signaling

β-catenin can interact with a variety of transcriptional regulators, including transcription factors (TCF/LEF, LRH-1, AR), chromatin modifiers (CBP, MLL1, Brg-1, ISW1) and the transcriptional co-activators (TBP, MED12, Parafibromin) (Valenta *et al.*, 2012). The TCF family is the major DNA binding partner of β-catenin and transcriptional activity with other factors may require the presence of TCF. However, there have been several studies which have shown that β-catenin can associate with other transcription factors without TCF leading to the activation of a differential set of genes. A study by Doumpas *et al.* in HEK293T cells shows that a set of “ghost genes” exist which exhibit transcriptional activation upon Wnt activation even in the absence of TCF/LEF family members. One of the factors identified in this study was FOXO4 which can interact with β-catenin and drive the transcription of genes independent of TCF (Doumpas *et al.*, 2019). Another study done by Mukherjee *et al.* using human pluripotent stem cells showed that SOX transcription factors can recruit β-catenin on lineage-specific enhancer elements majority of which are not occupied by TCF. While SOX17 and SOX2 recruit it to endodermal and neural enhancers respectively. TCF, on the other hand, recruits β-catenin to mesodermal enhancers (Mukherjee *et al.*, 2020). Essentially, it showed context-specific binding of β-catenin to other transcription factors.

In recent years, multiple members of the Specificity Protein (SP) family of zinc finger proteins have been shown to work along Wnt signaling. A study published by Galande laboratory (details described in the following section) showed that SP1 interacts with β-catenin and binds to a few Wnt-responsive elements. Further, this study also showed

that SP1 and β-catenin positively regulate each other's stability upon Wnt activation (Mir *et al.*, 2018). In contrast, SP5 has been shown to act as a feedback inhibitor of Wnt signaling. Interestingly, Huggins *et al.* hypothesized that SP5 might be competing with SP1 for binding to a limited set of loci upon Wnt activation. These loci are mainly the Wnt target genes which are negatively regulated by SP5 (Huggins *et al.*, 2017). Another study has shown that SP5 and SP8, both help in the amplification of Wnt target gene expression by recruiting enhancers to β-catenin bound LEF1 via looping. However, it occurs for only a subset of Wnt target genes (Kennedy *et al.*, 2016).

SP1 is regulated by the Wnt signaling pathway

*(Part of published research article: Mir R, **Sharma A**, Pradhan SJ, Galande S. Regulation of Transcription Factor SP1 by the β-Catenin Destruction Complex Modulates Wnt Response. Mol Cell Biol. 2018 Oct 29;38(22):e00188-18. doi: 10.1128/MCB.00188-18. PMID: 30181396; PMCID: PMC6206460.*

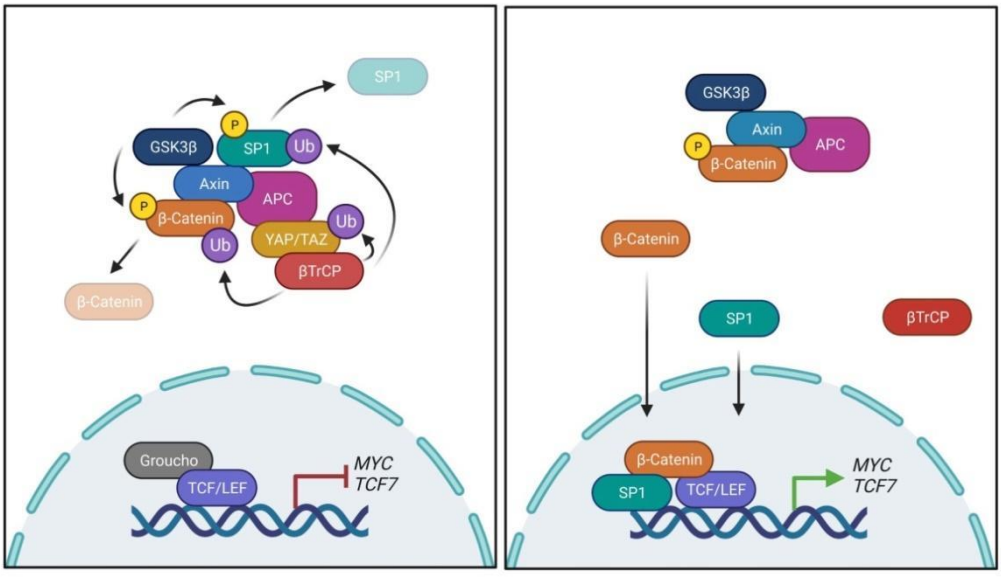

Figure 1.6. Model for the regulation of SP1 via Wnt/β-catenin pathway. SP1 stability is regulated by the Wnt pathway. In the absence of Wnt activation, SP1 can get degraded via the activity of Wnt DC. Upon Wnt activation, SP1 gets stabilised along with the Wnt effector β-catenin. It also allows the interaction of SP1 and β-catenin which further aids in their stabilisation. Translocation of these proteins promotes the expression of their common Wnt targets among others.

Specificity protein 1 (or SP1) is the first transcription factor to be identified (Dynan and Tjian, 1983). It has a propensity for binding to the GC-rich regions of the genome and therefore plays an integral role in driving transcription of the genes which do not have the conventional TATA box-containing promoters (Gidoni *et al.*, 1985). It belongs to the SP family of zinc-finger containing transcription factors which consists of 9 members in humans. The ubiquitous expression of SP1 and its involvement in regulating the expression of housekeeping and cell-cycle related proteins made an impression of it being a generic transcription factor. However, further studies identified that SP1 can perform tissue-specific functions as well. Such differential activity of SP1 is facilitated by the multitude of PTMs (acetylation, phosphorylation, ubiquitination), interaction partners (basal transcriptional machinery including TBP, TAF4; transcription factors such as c-Jun, c-Myc; and chromatin modifiers p300, CBP) which influence its DNA-binding activity, making it sensitive to multiple cues. SP1 is required for cellular processes such as cell proliferation, differentiation, apoptosis, angiogenesis (Chen *et al.*, 1994; Emili, Greenblatt and Ingles, 1994; Chiang and Roeder, 1995; Roos *et al.*, 1997; Doetzlhofer *et al.*, 1999; Suzuki *et al.*, 2000; Zhang and Dufau, 2002; Gueven *et al.*, 2003; Estève, Chin and Pradhan, 2007; Tapias *et al.*, 2008; Aiello *et al.*, 2010; Li *et al.*, 2011). It is therefore essential for the progression of normal development and adult homeostasis and has been implicated in multiple diseases such as cancers, Huntington's, and Alzheimer's disease (Black, *et al.*, 2001; Citron *et al.*, 2008; Wang *et al.*, 2012). Increased levels of SP1 have been linked to poor prognosis in multiple cancer types including gastric, pancreatic and liver (Beishline and Azizkhan-Clifford, 2015). Numerous studies have reported its specific PTMs and interacting partners associated with these functions. However, there is relatively little knowledge about the interplay with different signaling pathway components governing the biological functions of SP1 protein.

In the study by Mir *et al.* (2018), it was reported that SP1 is under the regulation of Wnt signaling pathway. A report published prior to this showed that the C terminus of SP1 carries the phosphodegron motif (^{727}DSGAGS^{732}) for β-TrCP (DSGX_nS, n can be 2-4 amino acids) which also encloses the recognition site for GSK3β (SXXXS) through

which it can undergo proteasomal degradation in LNCaP cells (prostate cancer) in response to glucose starvation (Wei *et al.*, 2009). Since canonical Wnt signaling requires the inactivation of GSK3β, it was hypothesized that GSK3β-β-TrCP regulation of SP1's stability is linked to the activity of Wnt signaling. Colorectal cancer cell lines (which have hyperactive or continuously active Wnt signaling) and HEK293 cells (which are Wnt responsive) were used as the model system to address this question.

SP1 exhibited higher levels of expression in cancerous cells as compared to primary colon cell line CRL1790. Additionally, Wnt activation in HEK293 cells increased the expression of SP1. Interestingly, it was observed that SP1 stabilisation occurred at the protein level but not at the transcript level thereby hinting towards a direct protein level regulation via Wnt signaling. Direct physical interaction between SP1 and the canonical Wnt signaling effector protein β-catenin was also observed. In fact, depletion of β-catenin led to a reduction in SP1 protein, with no effect at the transcript level. The absence of β-catenin prevented Wnt-mediated stabilisation of SP1 in HEK293T upon Wnt treatment as well. In the same system, overexpression of GSK3β-resistant form of β-catenin (S37A) was sufficient to increase SP1 protein in the Wnt-off state showing that Wnt-mediated stabilisation of SP1 requires the presence of β-catenin.

The study further investigated the effects of perturbation of the WDC components on SP1. SP1 was found to interact with GSKβ, Axin1 and β-TrCP in a Wnt-dependent manner. While its interaction with Axin1 and β-TrCP reduced upon Wnt treatment, however, it remained unaffected with GSK3β. Inhibition of GSK3β and Axin1 promoted stabilisation of SP1 protein. Additionally, SP1 showed reduced ubiquitination upon Wnt activation. This effect is similar to what is observed in the case of β-catenin stabilisation during Wnt activation. Since the presence of active β-catenin also stabilizes SP1, it was further checked if this mechanism can be replicated by overexpressing β-catenin S37A. Overexpression of β-catenin S37A did phenocopy the effect of Wnt activation on SP1. Both conditions were able to reduce the interaction between SP1 and β-TrCP thereby showing that β-catenin protects SP1 by impeding its interaction with β-TrCP.

Interestingly, interaction with SP1 benefitted β-catenin itself. There was a significant reduction in the protein levels of β-catenin upon SP1 depletion in HCT116 cell line even though it harbors mutation in one of the alleles for β-catenin gene *CTNNB1*, rendering it to be continuously active irrespective of Wnt activation. Absence of SP1 also prevented stabilization of β-catenin upon Wnt activation. Consequently, there was reduced expression of Wnt targets, Axin2 and TCF7. Further, overexpression of constitutively active SP1 abrogated the interaction of β-catenin with β-TrCP, GSK3β, Axin1 even in the absence of Wnt stimulation. Interestingly, the effects of the constitutively active β-catenin were also influenced by SP1. When the active form of β-catenin was introduced in HEK293 cells, it led to an increased expression of Wnt targets Axin2 and TCF7 along with SP1 stabilisation. However, simultaneous depletion of SP1 abolished these effects. Subsequently, the dose of the active form of β-catenin was increased even further to rescue this effect. The expression of Wnt targets could still not be rescued due to the absence of SP1. This highlighted the importance of SP1 in driving the expression of a subset of Wnt-responsive genes, if not all. Since SP1 and β-catenin exert their effect by binding to the DNA (directly or indirectly), it was checked if SP1 shows binding to any of the Wnt target genes. Upon analysis of ChIPseq data for SP1 in HCT116 cells, it was observed that SP1 did occupy the upstream regulatory regions for Wnt targets, *MYC* and *TCF7*. Further, the co-occupancy of these two proteins was observed at the promoters of *MYC* and *TCF7*. Expression of MYC and AXIN2 was also reduced at the transcript level upon SP1 depletion, thereby showing direct transcriptional regulation by SP1.

To summarise, it was shown that SP1 is under the positive regulation of Wnt signaling and can act as an effector protein for it (Figure 1.6). Inactive Wnt signaling promotes the interaction of SP1 with the WDC components- Axin1, GSKβ and β-TrCP leading to its degradation. Activation of Wnt signaling promotes stabilisation of SP1 which is aided by its association with β-catenin. SP1 itself can help β-catenin evade the degradation machinery. Their interaction with each other impedes the interaction with WDC thereby promoting nuclear translocation to carry out transcriptional activation. Additionally, SP1 directly affects the expression levels of some Wnt-responsive genes. Perturbation of

SP1 was sufficient to reduce their expression even in the presence of the main effectors β-catenin and TCF7L2. This study raised an important question of the global transcriptional regulation by β-catenin and SP1 together upon Wnt activation which we have tried to address in the subsequent study.

CHAPTER II

Wnt stabilised SP1:β-Catenin complex enhances cellular proliferation in colorectal cancer

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Introduction

The discovery of the Wnt-mediated interaction between SP1 and β-catenin has raised a multitude of inquiries concerning the underlying mechanism and the biological significance of this interaction. A prior investigation revealed that SP1 and β-catenin have the ability to engage with each other, mutually stabilizing one another and thus preventing degradation via the Wnt destruction complex (WDC). Furthermore, these two proteins co-occupy the promoters of Wnt target genes, such as *MYC* and *TCF7* (Mir *et al.,* 2018). Given that SP1 functions as a transcription factor, and β-catenin serves as a transcriptional regulator, the logical progression was to investigate the types of cellular processes that are co-regulated by these two proteins.

β-catenin can perform diverse biological functions owing to its structure which enables interactions with various types of proteins. Interestingly, β-catenin was discovered in two separate studies to perform two different functions (Valenta, *et al*., 2012). The first one identified it as a regulator of segmentation during *Drosophila* development, and the second showed it to be one of the interactors of E-cadherin, the adhesion regulator at the plasma membrane (Nüsslein-Volhard, Wieschaus and Kluding, 1984; Ozawa, Baribault and Kemler, 1989). In the central region of β-catenin, there exists a large domain composed of stacked Armadillo (ARM) repeats, which collectively create a rigid scaffold for interacting with other proteins. The N-term and C-term are more flexible than the middle domain (Huber, Nelson and Weis, 1997; Xing *et al.*, 2008) (Figure 2.1). The majority of the interactors of β-catenin binds via these ARM repeats and hence might compete with each other for the same. Notably, the most pivotal partners of β-catenin, such as E-cadherin at the cellular membrane, APC in the cytoplasm, and TCF/LEF in the nucleus, share common binding domains within the ARM repeats. This

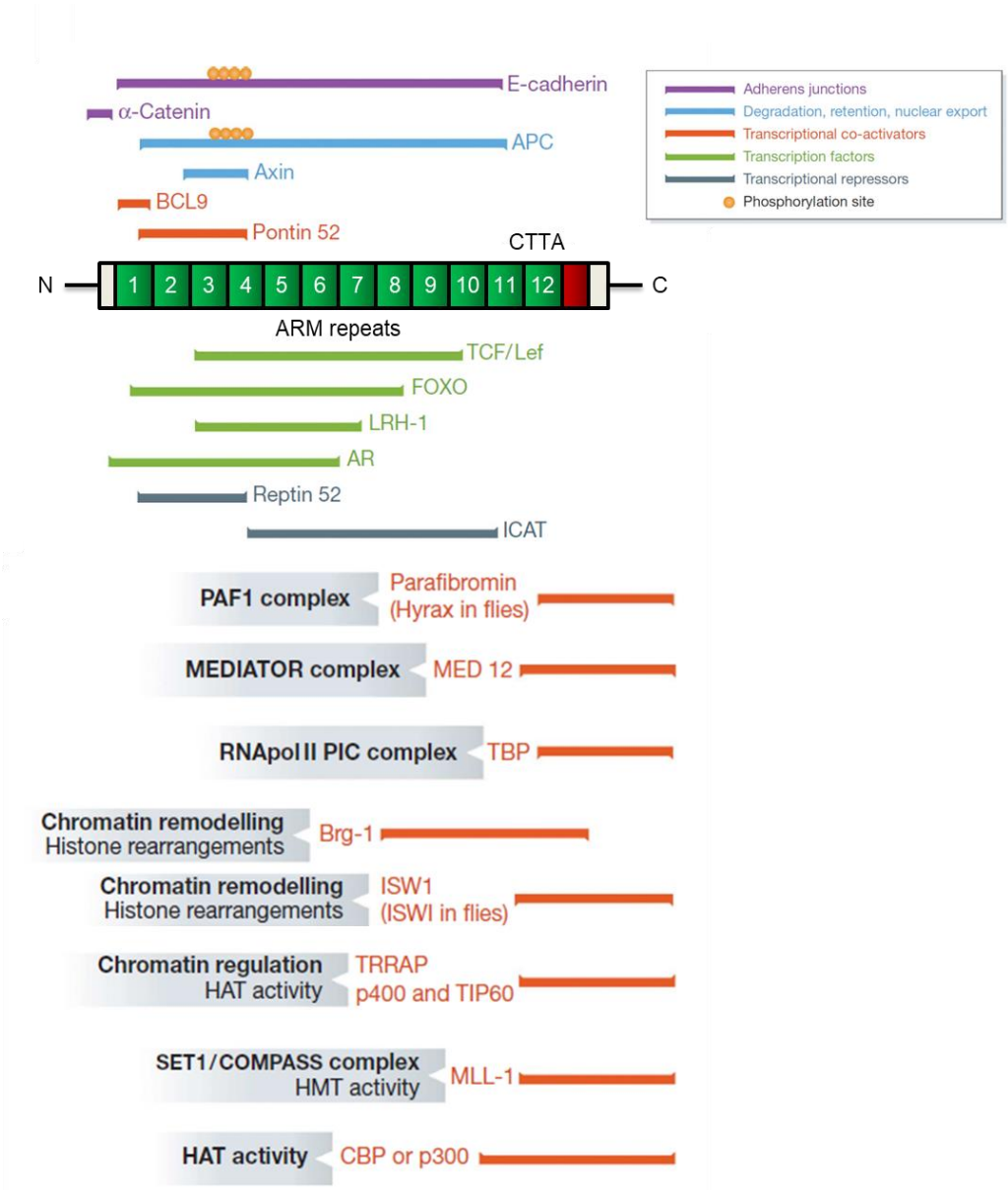


Figure 2.1. Structure and interactors of β-catenin. The known functional domains and interactors of β-catenin are summarised in this schematic representation. β-catenin has binding partners at the cell membrane (E-cadherin, α-catenin), cytoplasm (APC, Axin) as well as the nucleus (BCL9- B-cell lymphoma-9, Pontin52, TCF/LEF, FOXO- Forkhead box O, LRH1- Liver Receptor Homologue-1, AR-Androgen Receptor, PAF1- Polymerase-associated factor-1, MED12- Mediator Complex Subunit 12, TBP- TATA-box Binding Protein, BRG1- Brahma-related gene-1, ISW1- ISWI chromatin-remodeling complex ATPase, TRRAP- Transformation/transcription domain associated protein, p400, TIP60- Tat-interactive protein, MLL1- Mixed Lineage Leukaemia-1, CBP/p300, Reptin 52, ICAT- Inhibitor of β-catenin and TCF). The biological function is mentioned according to the color code in the box. The region of interaction is aligned along the domain structure of β-catenin (Adapted from Valenta et al., 2012).

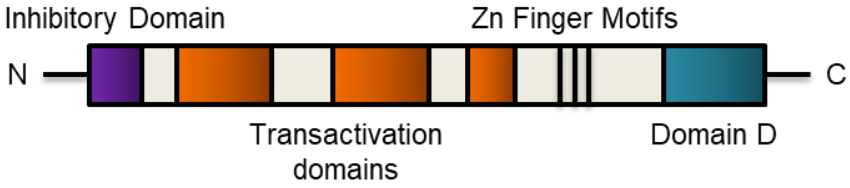
mode of interaction is favourable for β-catenin's functioning as these proteins are separated by cellular compartmentalisation (Valenta *et al*., 2012). Other binding partners for β-catenin include components of adherent junctions, transcriptional factors,

and other regulators. A few of the important interacting proteins are listed in Figure 2.1. Factors such as the core transcriptional machinery (e.g., TBP) and chromatin regulators (e.g., p300, PAF1, BRG1, etc.) form a larger assembly known as the Wnt-enhanceosome (Lyou *et al.*, 2017). Recruitment of the Wnt enhanceosome is essential for activating transcription from the Wnt-responsive elements (WREs). While TCFs primarily drive the recruitment of β-catenin to these elements, recent studies have shown that this might not be entirely true (Doumpas *et al.*, 2019; Mukherjee *et al.*, 2020). Interaction with other transcription factors is crucial for regulating β-catenin target gene expression in a context-dependent manner. Recently, Mukherjee *et al.* demonstrated how switching the DNA binding partners for β-catenin can alter the Wnt response and, ultimately, the cell fate. The authors showed that during the specification of human pluripotent stem cells (hPSCs) into endoderm, SOX17 directs β-catenin towards endoderm-specific enhancers. Similarly, differentiation towards neuromesoderm required the interaction between β-catenin and SOX2. Notably, many of these regulatory regions were not occupied by TCFs (Mukherjee *et al.*, 2020, 2022). Apart from the SOX proteins, other transcription factors have also been reported that can act with or without the presence of TCFs upon Wnt stimulation (Notani *et al.*, 2010; Kennedy *et al.*, 2016; Mir *et al.*, 2016; Doumpas *et al.*, 2019; Gu *et al.*, 2020).

Similar to β-catenin, SP1 can interact with multiple proteins, primarily those falling under the category of transcriptional regulators. Nevertheless, its ability to either activate or inhibit gene transcription hinges on its post-translational modifications (PTMs), binding partners, and external stimuli. SP1 possesses three zinc finger domains located in its C-terminus, which are essential for its DNA binding capacity. SP1 also possesses three transactivation domains (A, B, and C) in the middle which are necessary for protein-protein interactions (Pavletich and Pabo, 1991; Kingsley and Winoto, 1992; Suske, 1999) (Figure 2.2A). Prominent interactors of SP1 include components of the basal transcriptional machinery including TAF4, TAF7, and TBP; chromatin remodelers such as p300, DNMT1, HMGA1/2, and HDAC1/2; and DNA repair machinery proteins Rad51, ATM etc. (Chen *et al.*, 1994; Emili, Greenblatt and Ingles, 1994; Chiang and Roeder, 1995; Roos *et al.*, 1997; Doetzlhofer *et al.*, 1999; Suzuki *et al.*, 2000; Zhang and Dufau,

2002; Gueven *et al.*, 2003; Estève, Chin and Pradhan, 2007; Tapias *et al.*, 2008; Aiello *et al.*, 2010; Li *et al.*, 2011) (Figure 2.2B). Interaction with other transcription factors is also known including p53, c-MYC, E2F1, SMAD2 etc (Moustakas and Kardassis, 1998; Ohlsson *et al.*, 1998; Doetzlhofer *et al.*, 1999; Gartel *et al.*, 2001). While most of these interactions lead to transcriptional activation, the interaction with p53 seems to vary with the target gene. There are reports of both transcriptional activation and repression by SP1 and p53 (Ohlsson *et al.*, 1998; Lagger *et al.*, 2003; Innocente and Lee, 2005).

A



B

Function	Protein	Reference No.
General transcription factor	TBP, TAF4, TAF7	1,2,3
Chromatin Remodelers	P300, DNMT1, HMGA1, HMGA2, HDAC1, HDAC2, BRG1	4,5,6,7,8,9,10
DNA repair mechinery	Rad51, ATM	11,12
Transcription factors	p53, cMYC, E2F1, SMAD2, STAT6, Brca2	13,14,15,16,17,18

Figure 2.2. Structure and interactors of SP1. A. Domain structure of SP1. **B.** Table summarising the interactors of SP1 (TAF4/7-Transcription initiation factor TFIID subunit 4/7, DNMT1- DNA methyltransferase1, HMGA1/2- High-mobility group AT-hook 1/2, HDAC1/2- Histone deacetylase 1/2, ATM-ataxia telangiectasia mutated, E2F1- E2 promoter binding factor 1, SMAD2- Mothers against decapentaplegic homolog 2, STAT6- Signal Transducer and Activator of Transcription 6, Brca2- BReast CAncer gene 2). (1: Chen et al., 1994; 2: Emili et al., 1994; 3: Chiang and Roeder, 1995; 4: Roos et al., 1997; 5: Doetzlhofer et al., 1999; 6: Suzuki et al., 2000; 7: Zhang and Dufau, 2002; 8: Gueven et al., 2003; 9: Estève, Chin and Pradhan, 2007; 10: Tapias et al., 2008; 11: Aiello et al., 2010; 12: Li et al., 2011; 13:Ohlsson et al.,1998;14:Gartel et al., 2001;15: Lin et al. 1996;16:Moustakas et al., 1998;17:Wei et al., 2018; 18:Abramovitch et al., 2003).

To further explore the significance of the interaction between SP1 and β-catenin, our investigation began with a transcriptome analysis conducted on the previously utilized colorectal cancer (CRC) cell line, HCT116. This analysis involved depleting these

proteins individually and in combination. Subsequently, we integrated the resulting data with their respective chromatin occupancy profiles to assess potential transcriptional cooperation among them. We observed that SP1 and β-catenin promote cellular proliferation while preventing apoptosis, with the most pronounced impact observed when both proteins were simultaneously disrupted. Notably, SP1 was found to enhance the expression of genes targeted by both the TCF-dependent and TCF-independent pathways. Together, these results underscore SP1's importance as an interacting partner of β-catenin, critical for facilitating an optimal response to Wnt signaling.

Material and methods

Human cell culture and transfection

HEK293T and HCT116 cells were obtained from the European Collection of Cell Cultures (ECACC). Cells were cultured in DMEM (Gibco, ThermoFisher) supplemented with 10% heat-inactivated Fetal Bovine Serum (Gibco, Thermofisher) and 1% Anti-Bacterial, Anti-Mycotic (Gibco, Thermofisher). For transfections, 0.3 x 10^6 cells were seeded in a 6-well culture dish 16 hours prior to transfection. Each well was transfected with either 2 µg of plasmid or 10 pmol of siRNA using Lipofectamine 3000 or Lipofectamine RNAimax (Invitrogen, Thermofisher) respectively, according to the protocol provided by the manufacturer. The cells were harvested 48 hours post-transfection for RNA and protein isolation. A list of the siRNAs and plasmids sequences used is provided in Appendix 1 and 2, respectively.

RNA isolation, quantitative RT-PCR and analysis

The cells were lysed in 1 ml RNAiso Plus (Takara) followed by standard extraction protocol. Briefly, the cells were homogenised using Pellet Pestle Motor Kontes. 200 µL chloroform was added followed by centrifugation at 12000 rpm at 4℃, 15 minutes. The aqueous layer was collected in a fresh tube, followed by another chloroform wash. Precipitation was performed by adding 10% of 3 M sodium acetate solution and an

equal volume of 100% isopropanol containing Glycoblue coprecipitant (Invitrogen) and incubating at -20℃, overnight. RNA was pelleted at 12000 x g at 4℃ for 1 hour, followed by two washes of 75% ethanol at 12000 x g, room temperature (RT), for 5 minutes. cDNA synthesis was performed using the PrimeScript RT Reagent Kit (Takara) using the protocol provided by the manufacturer. qRT-PCR was performed using TB Green Premix Ex Taq II (Takara) using the protocol provided by the manufacturer in the ABi ViiA 7 Real-Time PCR System. qRT-PCR analysis was performed by subtracting Ct values for the control gene, 18S rRNA, from the gene of interest to obtain ΔCt. ΔΔCt values were obtained by subtracting the ΔCt value for the control sample from the treated sample. Internal normalisation was performed by dividing all the ΔΔCt values by the ΔΔCt of the control sample. A list of the primers used for western blotting is provided in Appendix 1.

Protein extraction, western blotting and analysis

Protein extraction was performed by lysing the cells in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulphate) for 30 minutes on ice followed by centrifugation at 14000 rpm for 30 minutes, 4℃. The supernatant obtained was quantified using the Pierce BCA Protein Assay kit (Thermo Scientific). 25 µg of total protein was boiled in SDS loading dye (Cold Spring Harbor Protocols) for 5 minutes at 98℃ and loaded for western blotting unless mentioned otherwise. ImageJ software was used for all the densitometric analyses: https://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j/. All values were normalised with the loading control, followed by internal normalisation by the control condition. A list of the antibodies used for western blotting is provided in Appendix 3.

Analysis for Chromatin Immunoprecipitation (ChIP)-seq datasets

The following datasets were used for analysis: SRA012054, ENCSR000BMK, ENCSR000BSF, ENCSR000BVT, ENCSR161MXP, ENCSR333OPW, ENCSR661KMA,

ENCSR000EUV (ENCODE Project Consortium, 2012). The sequencing reads were trimmed using Trimmomatic-0.39 and aligned to GRCh38 genome assembly for humans using the BWA alignment program (Li and Durbin, 2009; Bolger, Lohse and Usadel, 2014). Peak calling was performed using MACS2 with default parameters and q value 0.05 (Zhang *et al.*, 2008). Consensus peaks from the biological replicates were extracted using a custom R script from Roman Cheplyaka (https://ro-che.info/articles/2018-07-11-chip-seq-consensus). deepTools 3.3.2 and Integrative Genomics Viewer (IGV) were used to create the representative figures for the performed analysis (Robinson *et al.*, no date; Ramírez *et al.*, 2016). BigWig files were generated using bamCoverage (deepTools). Peaks were annotated to the nearest gene using Homer and classified into promoter (±5 Kb) and non-promoter regions. Clusters were annotated using Homer. Gene ontology analysis was performed using Metascape and WebGestalt (Wang *et al.*, 2017; Zhou *et al.*, 2019).

mRNA sequencing and analysis

RNA isolation was performed as described previously and processed for library preparation. Total RNA was resuspended in nuclease-free water (Ambion) followed by quantification using Qubit RNA HS system (Thermo Fisher Scientific) and RNA integrity determination using RNA 6000 Nano Kit on Bioanalyser 2100 (Agilent). RNA samples with RIN values greater than 8 were used for library preparation. 500 ng of total RNA was subjected to mRNA purification using NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs) according to the manufacturer's instructions. The purified mRNA was used for library preparation using the NEBNext Ultra II RNA Library Prep Kit for Illumina (New England Biolabs) using the protocol provided in the kit. The final libraries were purified using the HighPrep PCR Clean-up System (MagBio Genomics, USA). The libraries were quantified using the Qubit 1X HS DNA system (Thermo Fisher Scientific). All the libraries were pooled in equimolar ratios and subjected to 75 bp PE chemistry on Nextseq550 sequencer (Illumina) aiming at least 30 million reads per sample. The bcl files obtained from sequencing were demultiplexed and converted to fastq files for further analysis. The sequencing reads were trimmed

using Trimmomatic-0.39 and aligned to GRCh38 genome assembly for humans using the HISAT2 alignment program (Bolger Lohse and Usadel, 2014; Kim *et al*., 2019). Gene feature counts were calculated using the FeatureCounts package from Rsubread (Liao, Smyth and Shi, 2019). EdgeR was used to perform differential expression analysis for 3 replicates per condition (Robinson and McCarthy *et al.,* 2010). Graphpad prism was used for creating the representative plots for the performed analysis. Metascape was used for performing the Gene Ontology analysis (Zhou *et a*l 2019).

Annexin V/Propidium Iodide staining and quantification for cell viability and cell cycle analysis

Cell viability assay was performed using Annexin V/FITC kit (Abcam) according to the protocol provided by the manufacturer. Briefly, cells were trypsinised and washed with 1X PBS. The cells were resuspended in 250 µL 1X Annexin V binding buffer, followed by the addition of 2.5 µL Annexin V-FITC and 2.5 µL propidium iodide. The suspension was incubated in the dark for 5 minutes at RT. The Annexin V-FITC signal was then analysed via BD FACSCelesta™ Cell Analyzer (Ex=488 nm, Em=530 nm) using a FITC 0signal detector and a phycoerythrin emission signal detector to detect PI staining. The results were analysed using FlowJo™ v10.8.1 Software (BD Life Sciences). The debris was removed by defining an area of the FS vs. SS, followed by gating the single cell population using an FSC height vs. FSC Area plot. The singlet data was further classified as live (Annexin V^+PI^-), early apoptotic (Annexin V^+PI^-), late apoptotic (Annexin V^+PI^-), or 'necrotic' (Annexin V^-PI^+) using a quadrant gate. Cell cycle assay was performed using Propidium iodide (Merck). Cells were trypsinised and washed with 1X PBS, followed by fixing in ice-cold 70% ethanol overnight. The cells were subsequently washed with 1X PBS twice and incubated with 0.4 mg/ml RNase A overnight. The samples were then incubated with 50 µg/mL Propidium iodide for 5 minutes at RT. Data for the PI fluorescence was collected to a total count of 10,000 nuclei via BD FACSCelesta™ Cell Analyzer using phycoerythrin emission signal detection. The cell cycle fractions for G0/G1, S, G2/M, and sub-G0/G1 were analysed using the BD FACSDiva Software program (BD Biosciences).

Results and discussion

2.1 β-catenin and SP1 expression and perturbation show a positive correlation with each other in colorectal cell line HCT116

It was shown previously by Mir *et al*. that the depletion of either β-catenin or SP1 from colorectal cancer cell lines leads to the downregulation of the other protein (Mir *et al.*, 2018). The depletion strategy used for this study was transient, utilising either shRNA or siRNA followed by western blotting (WB) or immunofluorescence assay (IFA). Both experiments showed a significant decrease in overall protein levels (Figures 2.3A & B). Since this strategy doesn't ensure uniform depletion across all the cells, therefore, we analysed the IFA data to check if the depletion in one protein correlates with the other. When we plotted the nuclear staining intensities that were quantified individually in Figure 2.3B, a positive correlation was observed between the expression levels of β-catenin and SP1 in both the control group and siRNA-treated cells (Figure 2.3C & D). However, it is noteworthy that in one condtion (siSP1 treatment), the correlation was slightly weak (Figure 2.3E). This could be attributed to some differences in the regulatory machinery getting affected in reponse to SP1 depletion as opposed to β-catenin depletion. Overall, this demonstrates that their protein levels correlate with each other under normal conditions as well as with the degree of their depletion.

To gain knowledge of the global changes associated with this complex, we generated transcriptome data for HCT116 cells upon siRNA-mediated depletion of β-catenin alone, SP1 alone, and β-catenin + SP1 (Schematic shown in Figure 2.3F). For this, we checked the efficiency of each siRNA separately at both RNA and protein levels (Supplementary Figure S2.1A-D.). Since the efficiencies of all the siRNAs used were more or less the same, we selected siRNA #1 for β-catenin and siRNA #1 for SP1. Additionally, we kept the amount of siRNA identical in all the conditions; therefore, the amount of each siRNA used in the case of double knockdown (DKD) was half of what was used in the case of single knockdown (SKD).

2.2 β-catenin perturbation dysregulates the expression of genes required for cellular proliferation

Our analysis commenced with the examination of the perturbation of individual factors in HCT116. Upon depleting β-catenin from HCT116 cells, we observed a noticeable reduction in cell growth when compared to the control cells (Supplementary Figure S2.2A). We also assessed the efficiency of the knockdown, revealing a significant decrease in the counts of β-catenin transcripts (Figure 2.4A, left panel). Consistent with our earlier observations, we did not detect any significant alterations in the transcript levels of SP1 (Figure 2.4A, right panel). Differential gene expression analysis revealed an upregulation of 1304 genes and a downregulation of 1260 genes (Figure 2.4B). We proceeded to investigate the specific categories of genes that are dysregulated upon the depletion of β-catenin. To achieve this, we conducted a Gene Ontology (GO) analysis. Notably, the genes exhibiting elevated expression levels were associated with metabolic processes, encompassing lipid catabolism, fatty acid and lipid metabolism, as well as lipid localization (Figure 2.4C). It is worth noting that the Wnt/β-catenin pathway has been previously implicated in the direct activation of lipogenic genes in adipocytes (Tian *et al.*, 2019; Bagchi *et al.*, 2020). However, as our study did not focus on this particular aspect of β-catenin biology, we did not delve further into this area. Instead, our emphasis was on genes positively regulated by β-catenin, i.e., those that showed downregulation upon siβ-catenin treatment. These genes were primarily enriched for terms related to DNA replication, repair, and cell cycle (Figure 2.4D). Additionally, the direct target genes of TCF:β-catenin complex were also enriched, validating the results from our dataset. Remarkably, upon conducting the 'Transcriptional Regulatory Relationships Unveiled by Sentence-Based Text-Mining' (TRRUST) analysis for all the genes displaying differential expression, we observed an enrichment of targets for both collaborative and opposing transcriptional regulators involved in regulating Wnt/β-catenin target genes (Figure 2.4E). Factors such as E2F1, ESR1, TP53, and SRY are known to inhibit Wnt/β-catenin targets(Bernard *et al.*, 2008; Kim *et al.*, 2011; Wu *et al.*, 2011; Bhat *et al.*, 2021). Whereas factors including SMAD3, MYC, and JUN act positively in conjunction with β-catenin (DiRenzo *et al.*, 2016; Huang *et al.*, 2016;

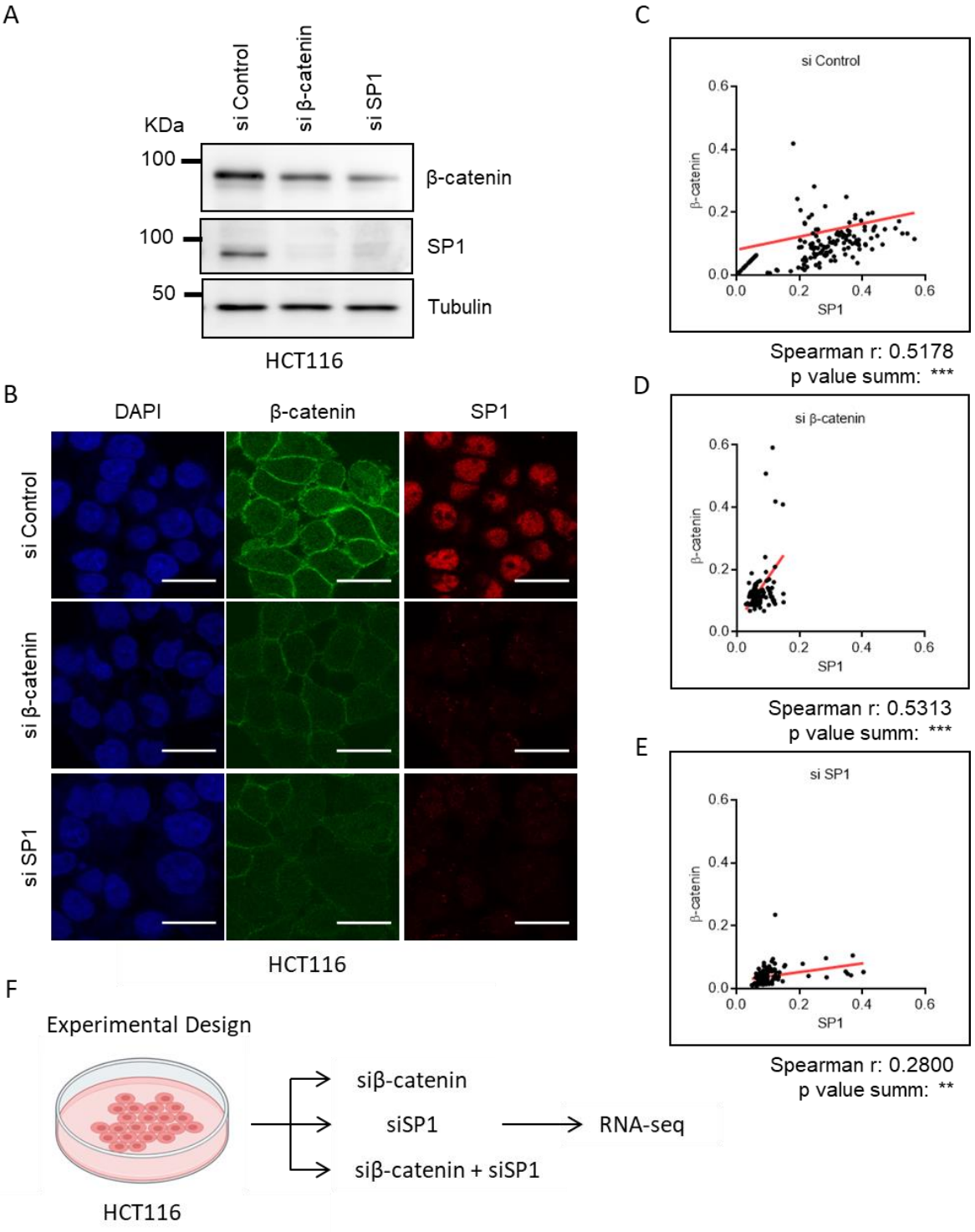


Figure 2.3. β-catenin and SP1 expression and perturbation show positive correlation in HCT116. A. Immunoblot for β-catenin and SP1 upon siβ-catenin and siSP1 treatment. **B.** Immunofluorescence image for β-catenin and SP1 upon siβ-catenin and siSP1 treatment. (Scale bar = 20 μM). **C.** Scatter plot for nuclear intensities for β-catenin and SP1 in cells treated with control siRNA. A regression line is depicted in red. **D.** Scatter plot for nuclear intensities for β-catenin and SP1 in cells treated with β-catenin siRNA. A regression line is depicted in red. **E.** Scatter plot for nuclear intensities for β-catenin and SP1 in cells treated with SP1 siRNA. A regression line is shown in red (Paired t-test performed to calculate significance). **F.** Schematic for the generation of transcriptome data.

Chachoua *et al.*, 2022). This indicates that the decreased expression of Wnt/β-catenin target genes might be a combination of the absence of β-catenin co-operators and the recruitment of β-catenin antagonists on the regulatory regions of these target genes. SP1 targets were also enriched in both upregulated and downregulated sets of genes, hinting towards an interaction via their transcriptional activities. Expression values for selected genes are depicted in Figure 2.4F. These include the classical Wnt target genes (*AXIN2*, *TCF7* and *BMP4*), metabolism-related genes (*CYP4F3, LIPA* and *CAV1*), and regulators of DNA replication and cell cycle (*POL2RA, PCNA* and *DNMT1*). In essence, we successfully generate a dataset that not only validated previous discoveries but also provided a comprehensive overview of the transcriptomic alterations linked to the disruption of β-catenin.

2.3 SP1 regulates the genes required for cell cycle and cell death

Analysis of the transcriptome profile for SP1-depleted HCT116 cells revealed a significant reduction in SP1 transcript level confirming the effectiveness of the siRNA treatment, and no change in the case of β-catenin (Figure 2.5A). Gross morphological changes and cell death were observed in siSP1-treated cells, as depicted in Supplementary Figure S2.2A. We found 2052 and 2071 genes to show higher and lower expression, respectively, compared to the control cells (Figure 2.5B). When we analysed the differentially expressed genes using GO, we found upregulation of genes belonging to the TAP63 and P53 pathways and the genes related to programmed cell death, the release of cytochrome c from the mitochondria, and autophagy (Figure 2.5C). Since TAP63 and P53 are both involved in promoting cell death, it appears that depletion of SP1 does promote cellular death via P53. The genes involved in various cell cycle stages and related processes, such as DNA replication and repair, exhibited lower expression in the absence of SP1 (Figure 2.5D). TRUSST analysis revealed enrichment for targets of TP73, TP53 HDAC1, E2F1 and MYCN (Figure 2.5E). While TP53 and TP73 promote cell death, E2F1 and MYCN stimulate cell growth. This indicates that SP1 alone can confer a dual advantage to cancer cells by promoting cellular proliferation and inhibiting multiple pathways that trigger cell death. Expression

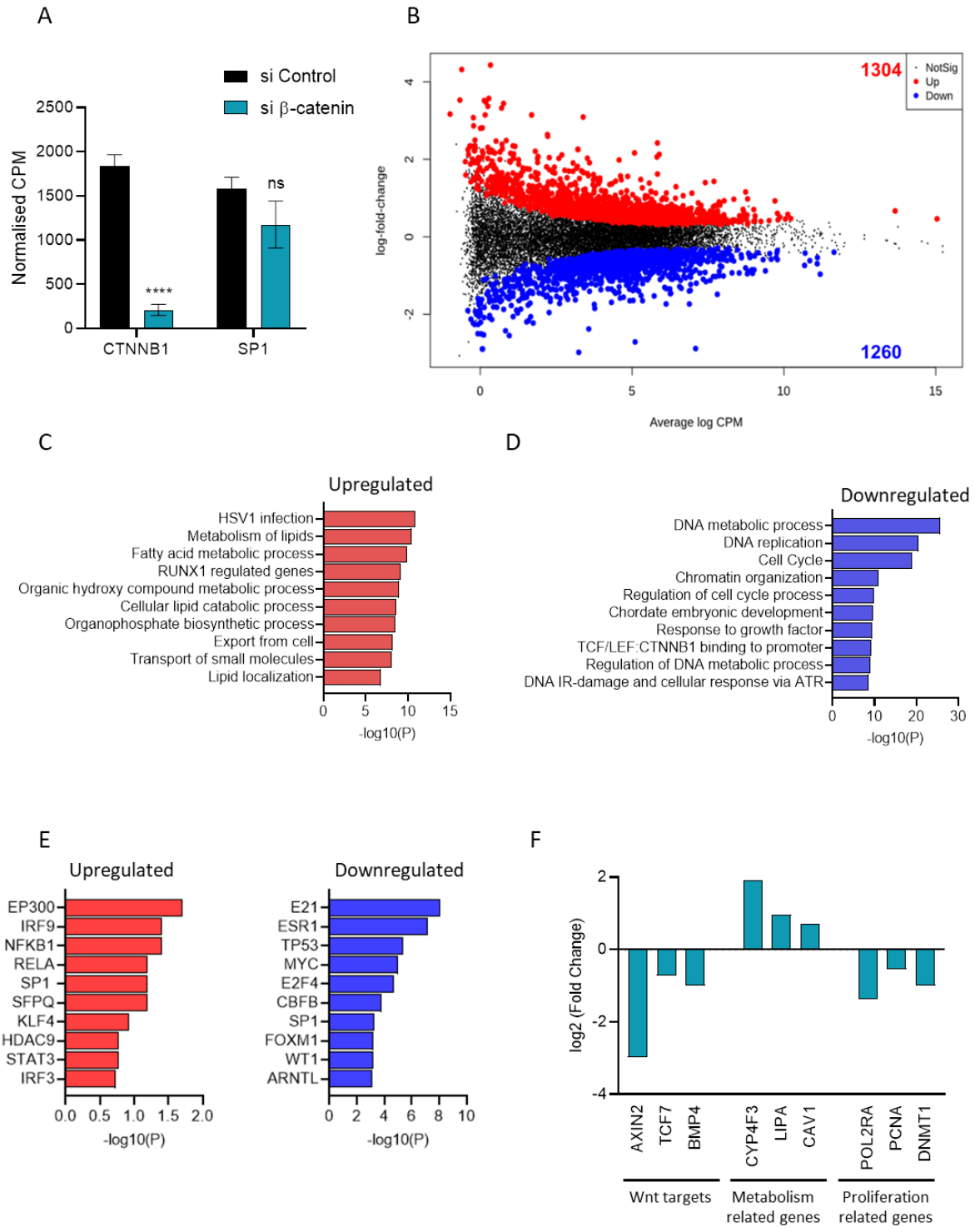


Figure 2.4. β-catenin perturbation dysregulates the expression of genes required for cellular proliferation. A. Expression of β-catenin and SP1 mRNA in control and β-catenin siRNA treated cells (Unpaired t-test was performed to calculate significance). **B.** Volcano plot for the differentially expressed genes. Upregulated genes are marked in red and downregulated genes are marked in blue. **C.** GO analysis for upregulated genes. **D.** GO analysis for downregulated genes. **E.** TRRUST analysis for upregulated genes (red) and downregulated genes (blue). **F.** log2 Fold change for selected targets.

values for selected target genes are shown in Figure 2.5F, encompassing cell death pathway-related genes (*DAPK3, BEX3, CASP4, ATG9B* and *DEPP1*) and cell cycle regulators (*CCNA2, BUB1B, MCM6 PCLAF* and *CENPO*). The role of SP1 in regulating the above-mentioned cellular processes in various cancer types has been well-documented. Furthermore, SP1 is notably enriched in the colorectal cancer stem cell (CCSC) population derived from multiple CRC lines, including HCT116 (Quarni *et al.*, 2019). Cancer stem cells are characterized by their self-renewal capacity and their contribution to tumor initiation, metastasis, and resistance to chemotherapy (Ayob and Ramasamy, 2018). Given that both SP1 and Wnt/β-catenin are implicated in driving CCSCs, we were prompted to investigate the transcriptional program initiated by SP1 and β-catenin in tandem.

2.4 β-catenin and SP1 have synergistic transcriptional activity

To investigate the common target genes for β-catenin and SP1, we performed transcriptome analysis of the cells treated with siRNAs against β-catenin and SP1 simultaneously. Combinatorial knockdown affected the cellular morphology and growth in manner similar to SKD (Supplementary Figure S2.2A). The quantification of both β-catenin and SP1 transcripts in this specific dataset revealed a decrease (Figure 2.6A). In comparison to the SKD cells, the number of dysregulated genes was slightly higher, with 2683 genes displaying increased expression and 2275 genes exhibiting decreased expression in comparison to the control condition (Figure 2.6B & Supplementary Figure S2.2B). For further analysis, we overlapped the complete set of differentially expressed (DE) genes from each of the transcriptome datasets created thus far (i.e., siβ-catenin, siSP1, siβ-catenin + siSP1) to identify a common set of genes (Figure 2.6C). This examination revealed that 1837 genes exhibited differential expression in all three datasets. Apart from these, 1591 genes were not picked up in any SKD datasets. We proceeded with the genes that were common in all three datasets. Our rationale was that since these genes exhibited altered expression even when one of the factors was removed from the system, their degree of expression change should be higher in comparison to the SKD condition. For this, we separately plotted the expression change

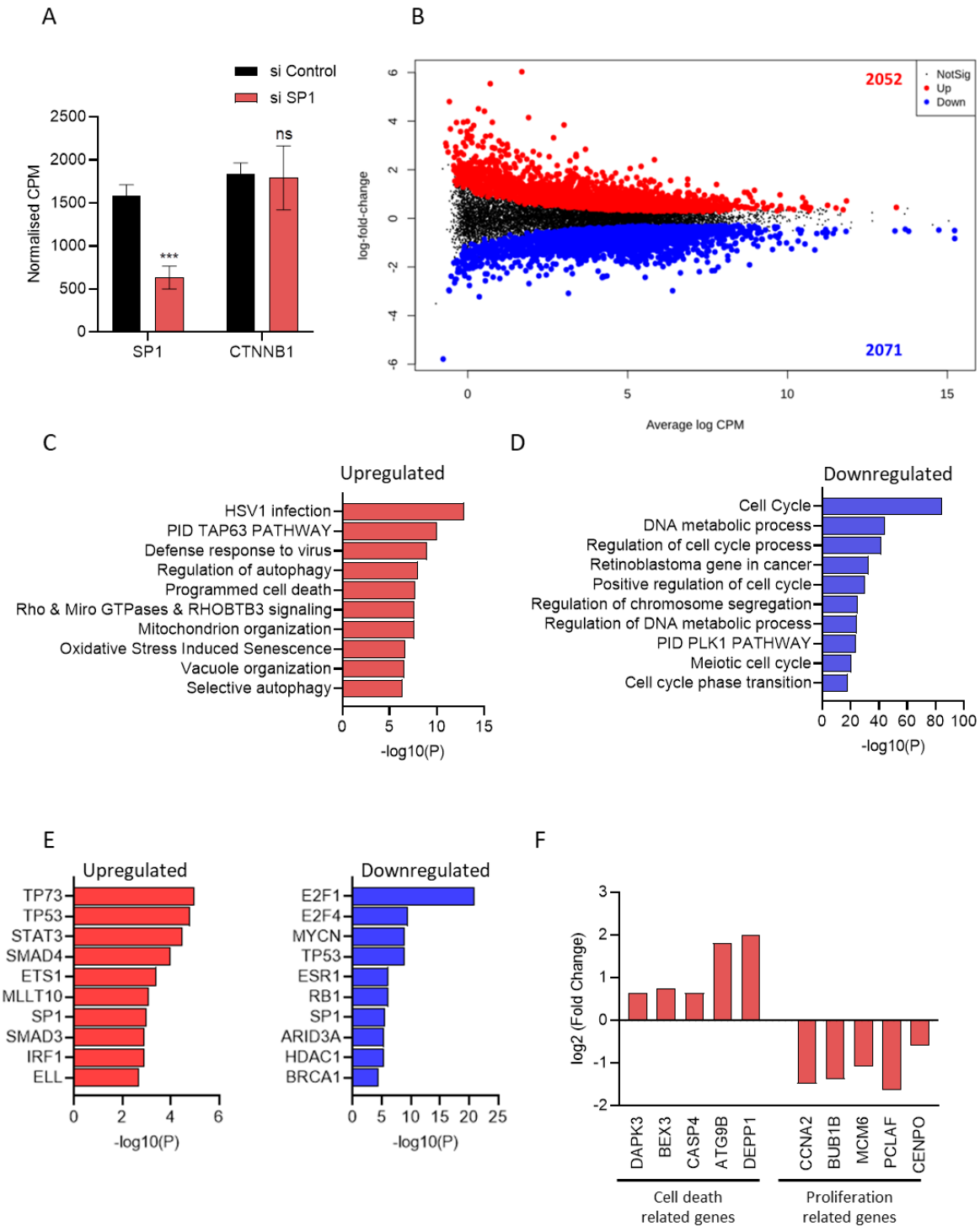


Figure 2.5. SP1 regulates the genes required for cell cycle and cell death. A. Expression of β-catenin and SP1 mRNA in control and SP1 siRNA treated cells (Unpaired t-test was performed to calculate significance). **B.** Volcano plot for the differentially expressed genes. Upregulated genes are marked in red and downregulated genes are marked in blue. **C.** GO analysis for upregulated genes. **D.** GO analysis for downregulated genes. **E.** TRRUST analysis for upregulated genes (red) and downregulated genes (blue). **F.** log2 Fold change plotted for selected target genes.

values (in terms of log2 Fold Change) of the genes under the positive and negative regulation of β-catenin and SP1. We did observe that the level of increase or decrease in expression was significantly higher in the DKD condition as compared to any of the SKD conditions, pointing towards a synergistic effect in their transcriptional activities (Figure 2.6D).

Subsequently, we subjected these genes to gene ontology (GO) analysis and found enrichment of cell proliferation and DNA replication in the case of downregulated set and cell death-related pathways (autophagy, P53, and TAP63 pathways) in the case of upregulated genes (Figure 2.7A&B). We also performed KEGG pathway enrichment analysis for all three datasets. There was a notable decrease in the activity of pathways associated with the cell cycle and DNA damage response. Furthermore, several marker genes for colorectal cancer stem cells (CCSC) displayed reduced expression (marker list obtained from Wang *et al.*, 2021) (Figure 2.7C). Conversely, genes linked to apoptosis and the p53 pathway exhibited increased expression. Of particular interest, we also observed an elevation in mTOR signaling (Figure 2.7C). The mTOR pathway typically fosters tumor growth and displays multiple points of mutual regulation and feedback with the Wnt pathway. It is conceivable that the simultaneous depletion of β-catenin and SP1 may drive the system towards an overly active mTOR signaling to support cell growth (Prossomariti *et al.*, 2020). However, it's worth noting that a few studies suggest that mTOR signaling can inhibit Wnt signaling, creating a feedback loop between these two pathways (Zeng *et al.*, 2018).

Based on the findings until here, it appeared that β-catenin and SP1 could be working in synergy to enhance cellular proliferation while simultaneously inhibiting cell death. To test this at the functional level, we performed staining for Annexin V and Propidium iodide in all three KD conditions. We observed reduced cell viability and increased apoptosis upon depletion of β-catenin and SP1, with the highest effect being observed in the case of the combinatorial knockdown (Figure 2.8A,B & Supplementary Figure S2.3A). Furthermore, we included serum deprivation as a control. While serum deprivation does not necessarily induce cell death, it does arrest cellular proliferation

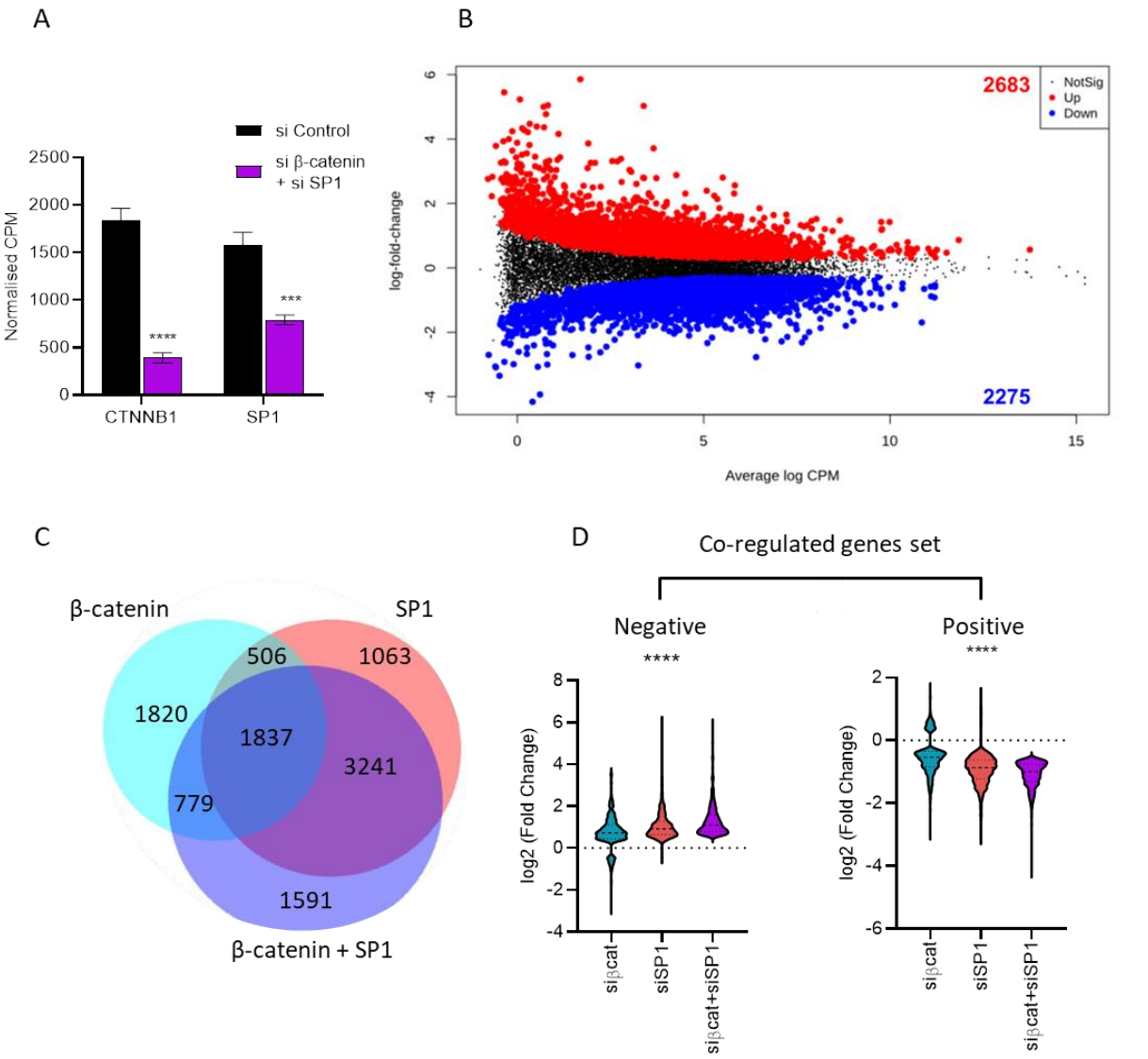


Figure 2.6. β-catenin and SP1 have synergistic transcriptional activity. A. Expression of β-catenin and SP1 mRNA in control and siSP1 siRNA treated cells (Unpaired t-test was performed to calculate significance). **B.** Volcano plot for the differentially expressed genes. Upregulated genes are marked in red and downregulated genes are marked in blue. **C.** Venn diagram showing overlap between the differentially regulated genes in cells treated with siβ-catenin only, siSP1 only and siβ-catenin + siSP1. **D.** Grouped log2 Fold change for common downregulated and upregulated genes upon SKD or DKD (2-way Anova test was performed to calculate significance).

(Chen *et al.*, 2012). As anticipated, there was no noteworthy alteration in overall cell viability during serum deprivation. We also performed cell cycle analysis and found an augmented accumulation of the G0/G1 population and a reduced G2/M population upon KD (Figure 2.8C & Supplementary Figure S2.4). A marked increase in the G0/G1 population was seen in the case of serum deprivation. Further, a significant reduction was observed in the factors required for DNA replication (PCNA) and repair (RAD51).

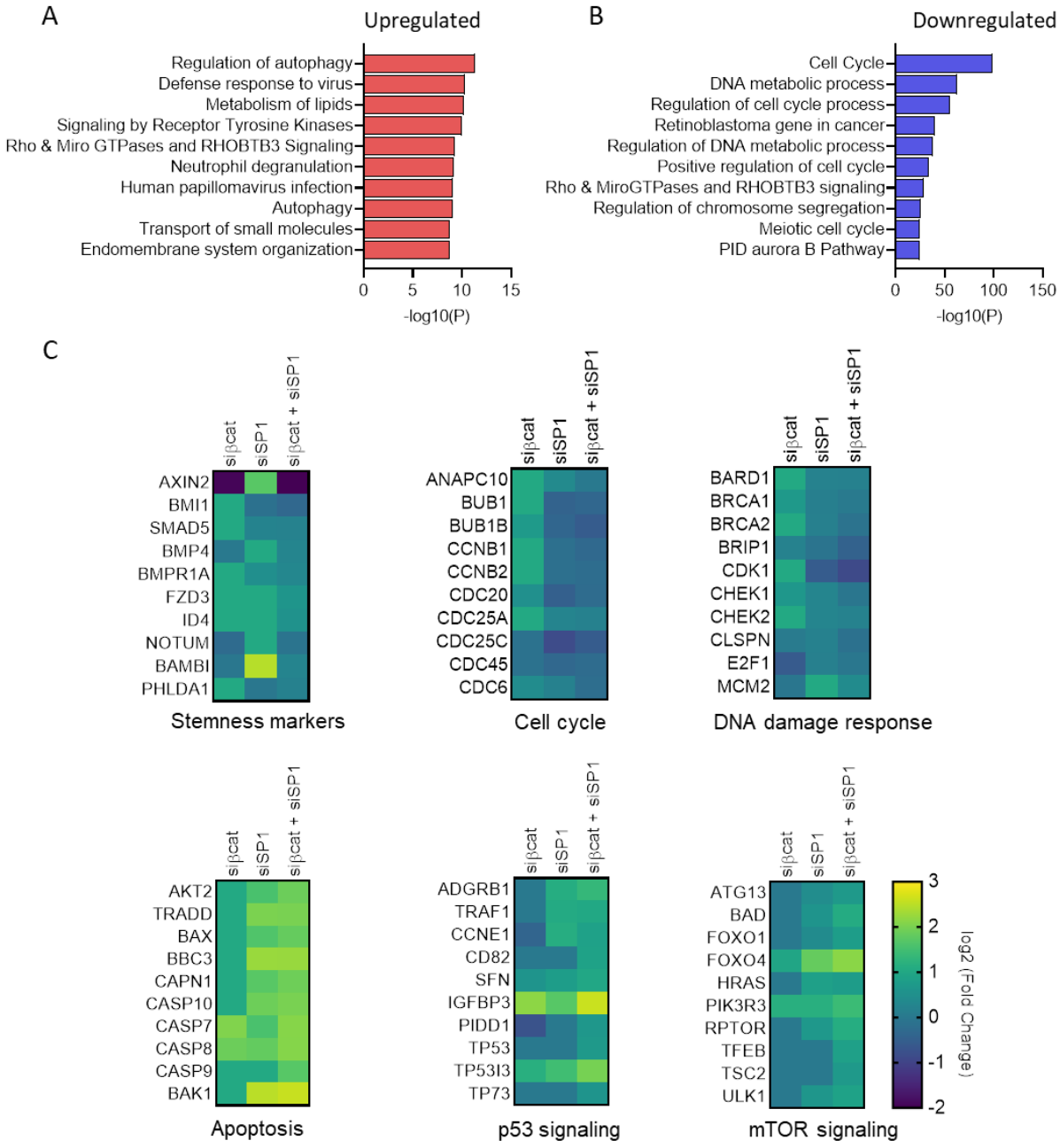


Figure 2.7. β-catenin and SP1 drive cell-cycle related pathways and inhibit cell-death related pathways. A. GO analysis for upregulated genes obtained from the common set in Figure 2.6C. **B.** GO analysis for downregulated genes obtained from the common set in Figure 2.6C. **C.** KEGG pathway enrichment analysis for the genes obtained from the common set in Figure 2.6C.

We also observed cleavage of PARP in all three KD conditions (Supplementary Figure S2.3B, right panel). Additionally, the changes associated with KD conditions were also replicated in serum deprivation (Supplementary Figure S2.3B, left panel), including

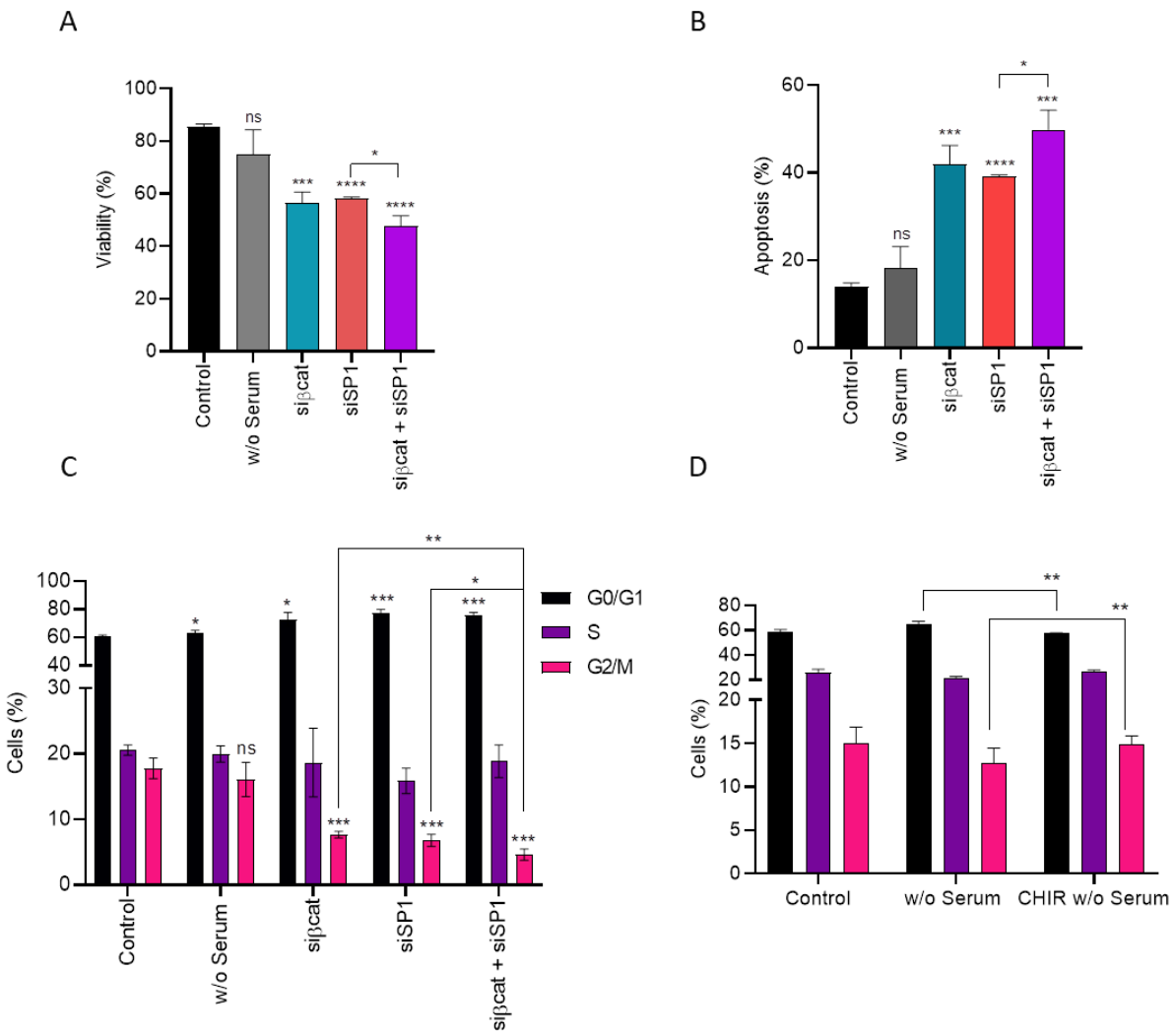


Figure 2.8. β-catenin and SP1 enhance cell survival and growth. A. Quantification of viable cells after Annexin V/PI staining after treatment with siRNA against β-catenin and/or SP1. Serum deprivation has been used as control. **B.** Quantification of apoptotic cells after Annexin V/PI staining after treatment with siRNA against β-catenin and/or SP1. Serum deprivation has been used as control. **C.** Cell-cycle analysis of cells treated with siRNA against β-catenin and/or SP1. Serum deprivation has been used as control. **D.** Cell-cycle analysis of cells treated with CHIR with serum deprivation. Unpaired t-test was performed to calculate significance for all

the decrease in β-catenin and SP1 proteins. One of the primary reasons for these changes could be the absence of MYC which is one of the immediate-early genes upon stimulation via serum mitogens. Ectopic expression of MYC alone is sufficient to initiate the cell cycle, even in the absence of serum or growth factors (Perna *et al.*, 2012). While MYC is one of the classical targets of the Wnt/β-catenin pathway, it is also known to act alongside SP1 for the expression of cell cycle-related genes. We monitored the expression of MYC under all the KD conditions, and observed that the expression of

MYC decreased in case of DKD and β-catenin SKD (Supplementary Figure S2.3C). Therefore, we performed a similar cell cycle experiment using serum deprivation along with CHIR treatment which inhibits the activity of GSK3β, thereby acting as a Wnt agonist (Ring *et al.*, 2003). Interestingly, the reduction in the G2/M population due to the absence of growth signals was rescued when the cells were treated with CHIR (Figure 2.8D & Supplementary Figure S2.5A). We also monitored if the ectopic expression of β-catenin and SP1 drives cell growth in the absence of serum. Although we did observe higher cell confluency in the cultures, there was no discernible difference in the cell cycle profile (Supplementary Figure S2.5A&B). Nevertheless, we identified an overlap of 267 out of the 590 genes (~45%) that are known to exhibit serum response (comprising both Myc-dependent and -independent serum response factors) (Perna *et al.*, 2012) (Supplementary Figure S2.5C). Collectively, these observations suggested that β-catenin and SP1 collaborate to enhance the expression of stemness markers, facilitating self-renewal in cells. They also independently support proliferative programs while hindering cell death, which can contribute to the maintenance of the CCSC population and overall increase in tumor growth.

2.5 β-catenin and SP1 show chromatin co-occupancy at promoter and enhancer regions

We wished to further investigate the mechanism via which β-catenin and SP1 act together at the level of chromatin. Mir *et al.* showed chromatin co-occupancy at the promoters of *MYC* and *TCF7*. For a comprehensive perspective, we examined the genome-wide chromatin occupancy profiles of β-catenin and SP1 by utilising the ChIP-seq datasets for β-catenin and SP1 in HCT116 cells (Bottomly *et al.*, 2010; ENCODE Project Consortium, 2012). As TCFs are the primary DNA-binding partners for β-catenin, we also included TCF7L2 into this analysis (ENCODE Project Consortium, 2012). Among all TCF/LEF members, TCF7L2 emerged as highly expressed while TCF7L1 and TCF7 displayed lower levels of expression and LEF1 didn't show of expression (Supplementary Figure S2.6A). A total of 5383 peaks were called for β-catenin, 6362 peaks for TCF7L2 and 19,673 peaks for SP1. Since motifs for SP1 are

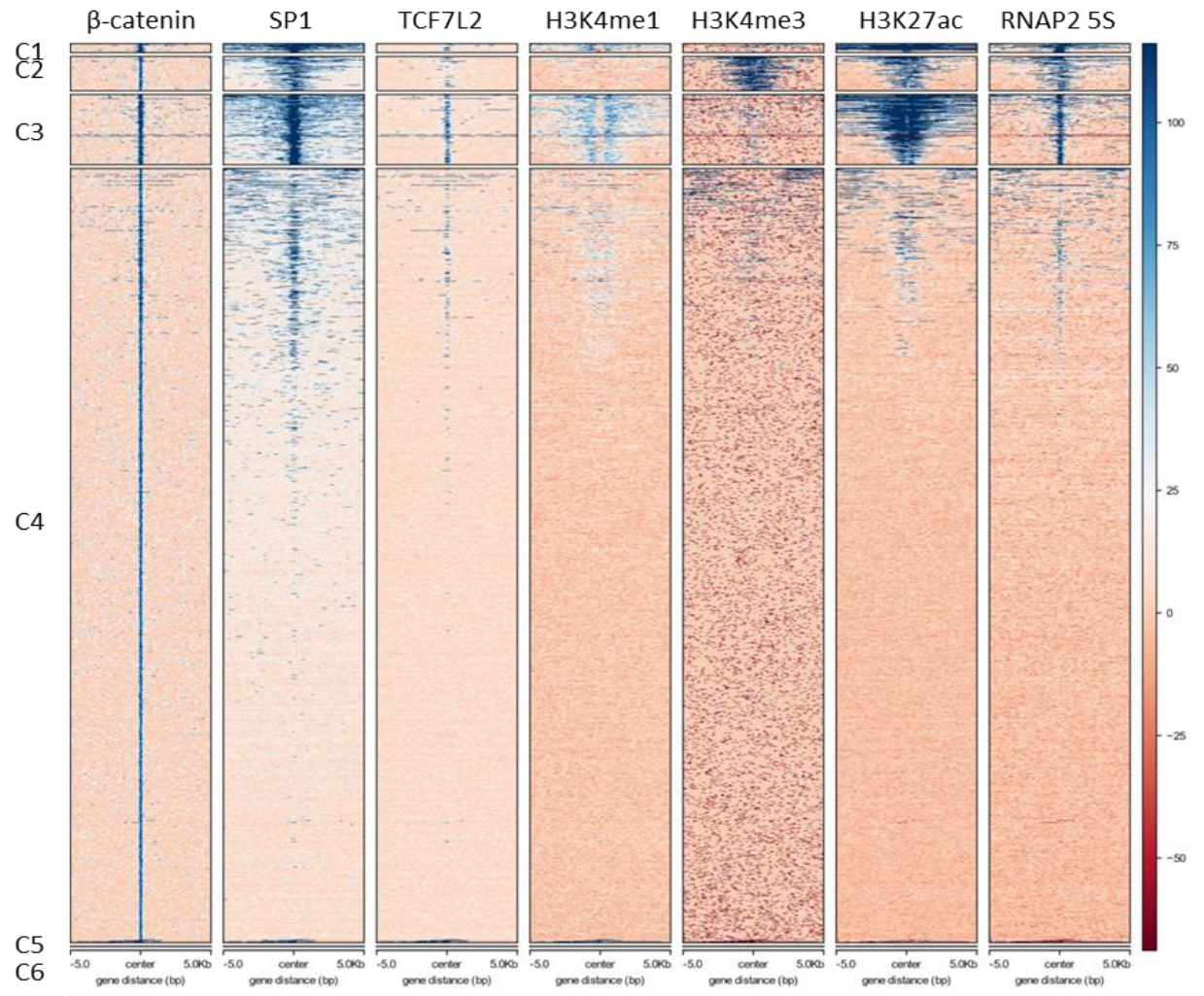


Figure 2.9. β-catenin and SP1 show chromatin co-occupancy at promoter and enhancer regions. Heatmaps for the ChIPseq profiles of β-catenin, SP1, TCF7L2, H3K4me1, H3K4me3, H3K27ac and RNA PolII 5S (Datasets: SRA012054, ENCSR000BMK, ENCSR000BSF, ENCSR000BVT, ENCSR161MXP, ENCSR333OPW, ENCSR661KMA, ENCSR000EUV).

much more abundant in the genome, therefore, the number of peaks is usually higher as compared to other transcription factors. Next, we overlapped these peaks to find the shared binding regions between the three proteins. Surprisingly, β-catenin shared ~86% (4631) of its peaks with SP1 as opposed to ~40% (2192) of its peaks with TCF7L2. Out of these, only a few were found to be devoid of SP1 occupancy (68 peaks) (Supplementary Figure S2.6B).

For a more comprehensive understanding of the types of regulatory regions that fall under their influence, we also incorporated the ChIP-seq profiles for H3K4me1 (mark for

enhancers), H3K4me3 (mark for promoters), H3K27ac (mark for active chromatin regions) and RNA Polymerase II 5S (an indicator for active transcription) (ENCODE Project Consortium, 2012). The co-occupancy of H3K4me1 and H3K27ac would suggest the presence of active enhancers, while a combination of H3K4me3 and H3K27c would indicate active promoter regions. Interestingly, we identified two distinct patterns of co-occupancy for β-catenin with SP1 and TCF7L2. In active enhancer regions (Cluster3 -C3), we observed co-occupancy of β-catenin, TCF7L2, and SP1. Conversely, in promoter elements (Cluster2- C2) β-catenin and SP1 exhibited co-occupancy, while TCF7L2 showed limited occupancy in these regions (Figure 2.9).

We obtained consistent findings when we plotted the average peak intensities for each of these two clusters separately (Figure 2.10A & E). Furthermore, we plotted the distance of these peaks to the nearest transcription start site (TSS). For cluster 2 (C2), the majority of the peaks fell within less than 1 Kb to the nearest TSS, whereas for cluster (C3), the peaks exhibited a broader distribution, with most of them being situated at distances greater than 1 Kb to the nearest TSS (Figure 2.10B & F). These observations further confirmed the presence of promoters in C2 and enhancers in C3. Next, we integrated the data from the RNAseq and the ChIPseq. We overlapped the genes obtained through these two methods and used the direct targets obtained for further analysis. We plotted the expression change for these direct targets upon the three KD conditions used earlier. Interestingly, both C2 and C3 genes showed the highest changes in the expression upon DKD as compared to any of the SKD conditions (Figure 2.10C & G). We subjected C2 and C3 genes to GO analysis to assess if there is any distinction between the two sets based on their biological functions. We observed that the majority of C2 genes belonged to RNA and protein processing (Figure 2.10D) while C3 genes were involved in signal transduction and cancer and development-related processes (Figure 2.10H & Supplementary Figure S2.7A). These results indicate that SP1 is involved in driving the expression of β-catenin direct target genes, which may or may not be dependent on TCF7L2. These findings also indicated a dosage-dependent regulation, suggesting that SP1 and β-catenin alone may be adequate to stimulate the expression of their shared targets, but the presence of both leads to higher levels of expression. Furthermore, there appears to be a functional differentiation

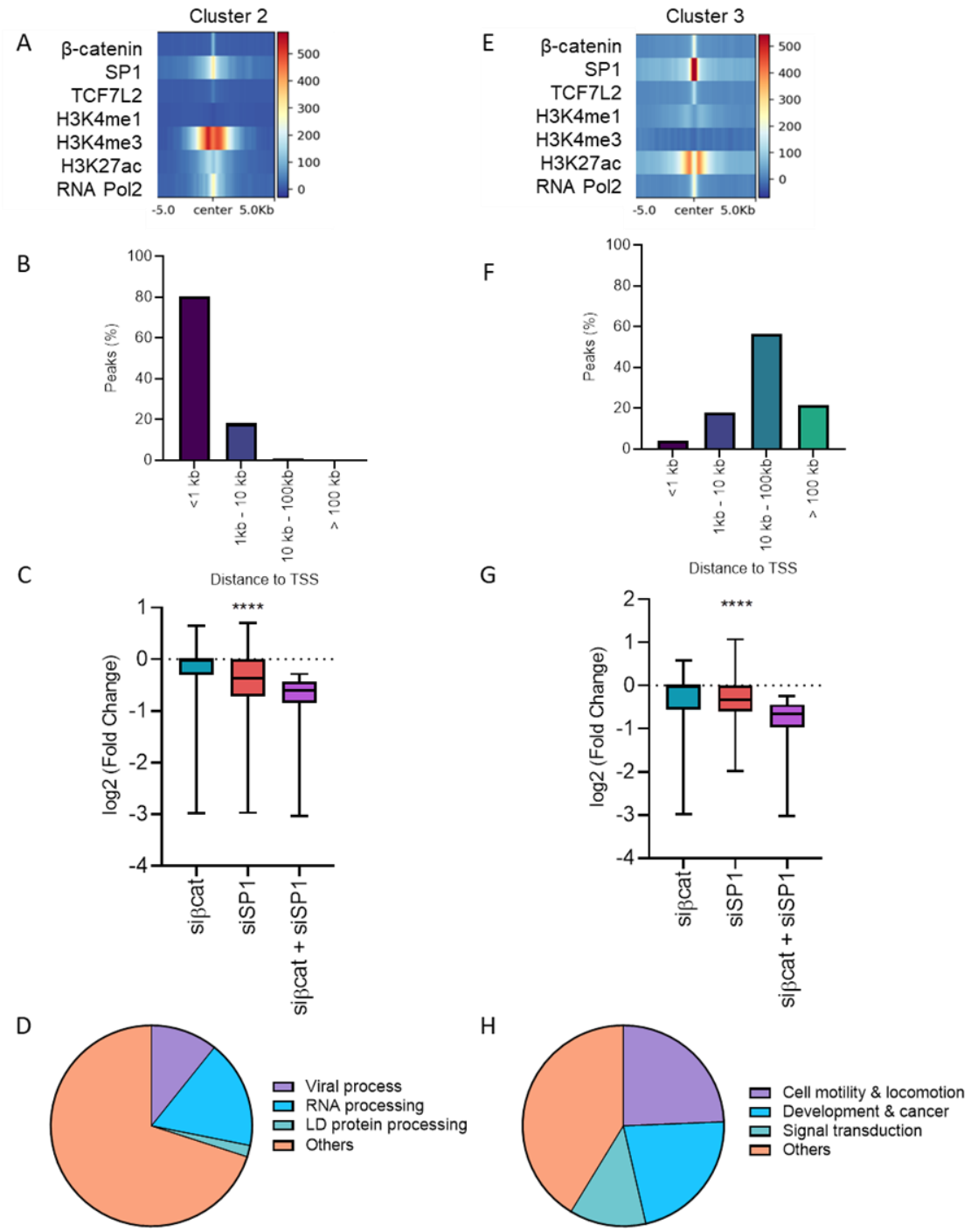


Figure 2.10. Cluster 2 and Cluster 3 genes correspond to distinct cellular functions. A. Peak intensities for all the ChIPseq datasets in C2. **B.** Percentage of peaks in C2 according to their distance to the nearest gene. **C.** Grouped log2 Fold change for common downregulated genes in C2 upon SKD or DKD (2-way Anova test was performed to calculate significance). **D.** GO analysis for common downregulated genes in C2 upon SKD or DKD. **E.** Peak intensities for all the ChIPseq datasets in C3. **F.** Percentage of peaks in C3 according to their distance to the nearest gene. **G.** Grouped log2 Fold change for common downregulated genes in C3 upon SKD or DKD (2-way Anova test was performed to calculate significance). **H.** GO analysis for common downregulated genes in C3 upon SKD or DKD.

among the target genes they control. The genes dependent on TCF7L2 (C3) are primarily enriched for canonical Wnt targets, while the TCF7L2-independent genes (C2) are predominantly involved in RNA processing, which adds to the understanding of context-specific interactions involving β-catenin.

2.7 SP1 co-localises with the components of the Wnt-enhanceosome

As mentioned previously, Wnt response requires the assembly of the multi-subunit Wnt enhanceosome (Figure 2.11A). Hence, we examined whether SP1 shares genomic binding sites with the components of the Wnt enhanceosome. To do so, we employed publicly available datasets for two of these components, BRG1 and LDB1 (Mathur *et al.*, 2017; Lai *et al.*, 2021). We used peaks for β-catenin as the reference and aligned the ChIPseq datasets for SP1, TCF7L2, BRG1 (SMARCA4) and LDB1. We observed co-occupancy of all these proteins except LDB1 (Figure 2.11B&C). This indicates that SP1 might be a part of the Wnt enhanceosome itself. We checked the literature for known direct interactions between SP1 and Wnt enhanceosome components and found that SP1 can interact with EP300, LDB1 and SMARCA4. The interaction network made using SP1 and all the components of the Wnt enhanceosome showed how SP1 and β-catenin can be engaged with the rest of the factors (Szklarczyk *et al.*, 2015) (Figure 2.11D).

We also aimed to investigate whether the expression of any of the regulators for the transcriptional activity through the WREs is dependent on either β-catenin or SP1. To accomplish this, we leveraged mRNAseq data to examine transcript levels for all the components listed in the Figure 2.11A. Our analysis revealed a significant reduction at the transcript levels for PYGO1, PYGO2, ARID5B, BCL9, TCF7L2 and HDAC1 (Supplementary Figure S2.8A-R). Notably, HDAC1, which typically functions as a repressor for WREs, was found to be under the positive regulation via β-catenin and SP1 indicating a feedback mechanism (Supplementary Figure S2.8A-R). While we observed occupancy of β-catenin and SP1 for nearly all the components we assessed, their perturbation did not lead to any significant change at the transcript levels (Supplementary Figure S2.8). Surprisingly, when we checked the protein expression for

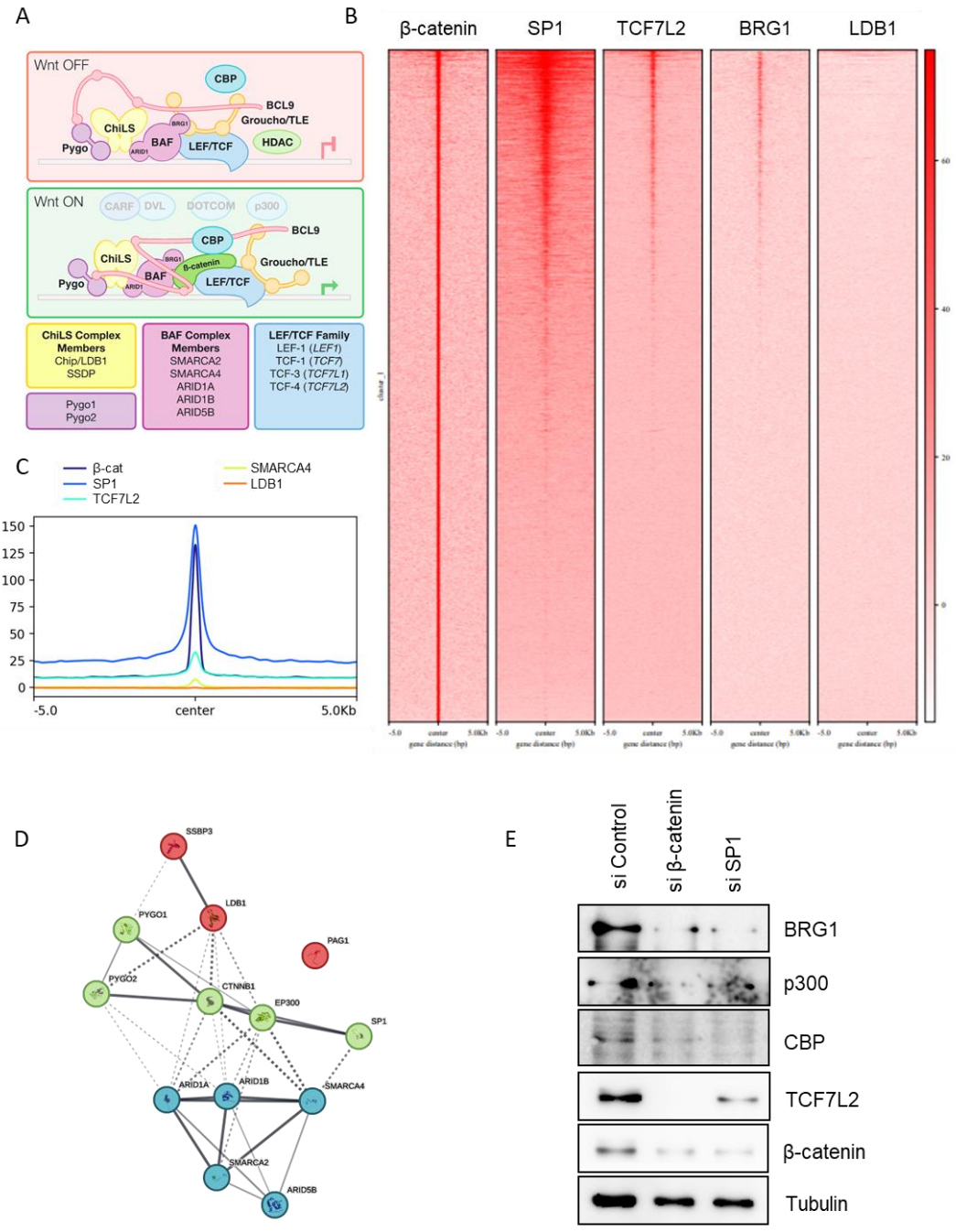


Figure 2.11. SP1 co-localises with the components of the Wnt-enhanceosome. A. Components of the Wnt enhanceosome and other transcriptional regulators (Adapted from Lyou *et al.*, 2017). **B.** Heatmap for ChIPseq for β-catenin, SP1, TCF7L2, BRG1 and LDB1. **C.** Peak summit for ChIPseq for β-catenin, SP1, TCF7L2, BRG1 and LDB1. **D.** STRING network for SP1, β-catenin and the Wnt enhanceosome. **E.** WB image for BRG1, p300, CBP, TCF7L2 and β-catenin under siβ-catenin and siSP1 conditions.

some of the components under β-catenin and SP1 KD conditions, reduced protein expression was observed for all the components analysed, including, BRG1, p300, CBP, and TCF7L2 (Figure 2.11E). Collectively, these findings suggest that SP1 engages with the constituents of the Wnt enhanceosome, as evidenced by their co-localization at shared target sites and their physical interactions. Moreover, both β-catenin and SP1 directly and indirectly regulate the expression levels of multiple Wnt enhanceosome components. Consequently, it can be inferred that the presence of these factors is crucial for the expression, and consequently, the assembly of the Wnt enhanceosome.

Summary and Conclusions

β-catenin interacts with a variety of transcriptional regulators which ultimately aid in fine tuning the Wnt mediated transcriptional response. This strategy works in a context-dependent manner and plays an important role during crucial processes such as, development, differentiation and maintenance of different cellular functions. Hence, it is imperative to investigate these interactions and their functional implications comprehensively. Such an inquiry is essential for acquiring a thorough understanding of the regulation of Wnt signaling, considering its pivotal role in crucial biological processes (Nusse and Clevers, 2017). Here, we show that SP1 is an important interactor of β-catenin which helps in stabilizing it during Wnt activation and also helps in target gene expression. We show that together, these proteins regulate cell cycle and apoptosis (Figure 2.12). We also show that β-catenin and SP1 can autonomously support cell growth. This property has implications in both physiological and pathological scenarios. Early development requires fast and autonomous growth. Both β-catenin and SP1 are expressed right from the early stages of development. Embryonic lethality is also a common phenotype upon depletion of these two proteins (Marin *et al.*, 1997; Xie *et al.*, 2008; Theka *et al.*, 2019). This indicates that β-catenin-SP1 complex might be a crucial part of the machinery that drives this autonomous growth. Similarly, cancer initiation requires hyperproliferation before the epithelial to mesenchymal transition and chemoresistance come into play.

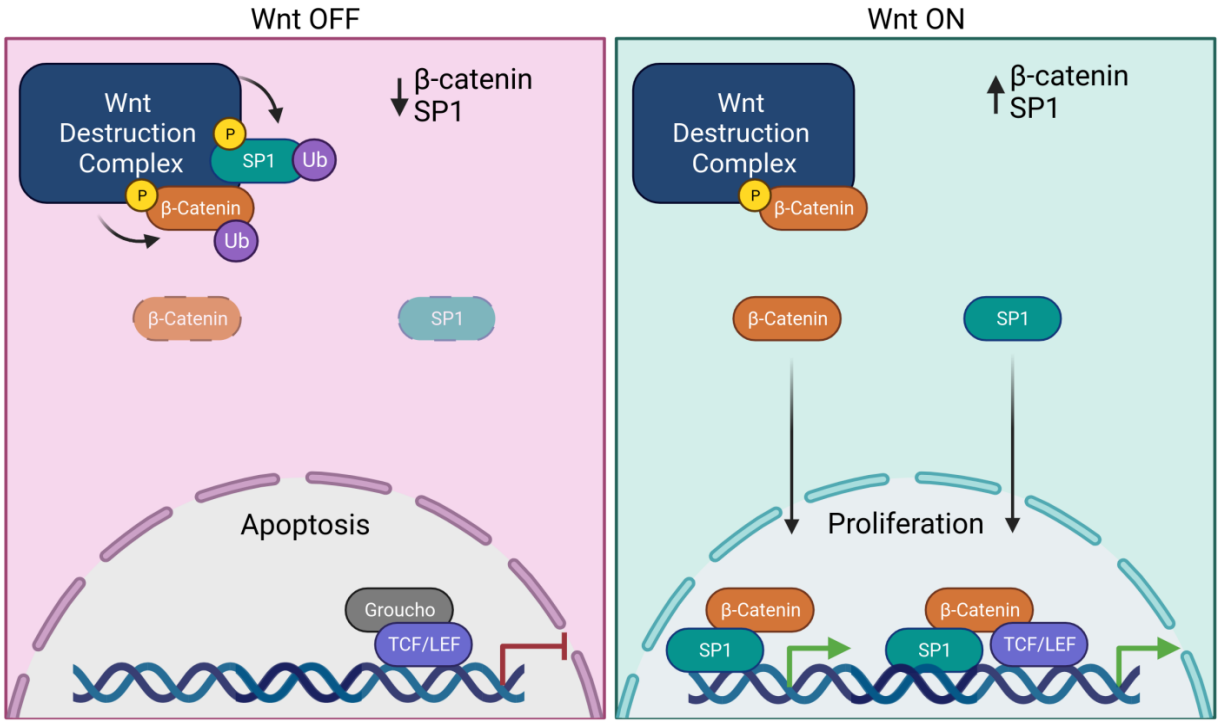


Figure 2.12. Model for co-operation between β-catenin and SP1. When the Wnt pathway is off, the Wnt destruction complex primes SP1 and β-catenin for degradation which in turn causes apoptosis. When Wnt signaling gets activated, the stabilised SP1 and β-catenin translocate inside the nucleus. Further, SP1 and β-catenin co-occupy active promoter and enhancer regions with and without TCF respectively and activate the expression of genes required for cell proliferation.

On the chromatin level, the β-catenin-SP1 complex demonstrates the ability to bind to DNA both in the presence and absence of TCF7L2. Interestingly, regions where β-catenin, SP1, and TCF7L2 co-occupied exhibited an enrichment of enhancer marks, whereas regions which showed co-occupancy of β-catenin and SP1 were enriched for promoter marks. Additionally, we observed a functional distinction between these two groups of targets. Various factors affect the binding kinetics of any transcriptional regulator which in turn affects the final transcriptional output. The ability of SP1 and β-catenin to bind to different regulatory regions might be dictated by their level of expression and presence of other proteins. For instance, SP1 shows higher affinity towards promoter elements and may be responsible for recruiting β-catenin to promoter elements (Hasegawa and Struhl, 2021). On the other hand, β-catenin may aid SP1's recruitment to Wnt responsive enhancers. Consequently, this will decide the kind of genes that will be transcribed upon Wnt stimulation. In the case of cancers, the high levels of all these proteins are enough to saturate the system which might result in a

uniform binding on all the elements. A time course analysis for the binding of SP1 and β-catenin upon Wnt stimulation should be able to answer this question in the future.

Lastly, we observed that SP1 co-occupies genomic loci with the Wnt enhanceosome components. Therefore SP1 might be a component of the Wnt enhanceosome, at least in this context. In addition to this, we observed that SP1 is involved in the expression of these components at either the transcript or the protein level. This suggests that SP1 is required for the expression and assembly of the Wnt enhanceosome. Though we did not quantify if the genomic co-occupancy of SP1 with BRG1 (SMARCA4) is strictly restricted to the TCF7L2-bound loci, but the ranked heatmap does indicate that a considerable number of loci which do not show binding of the BRG1 to β-catenin-SP1 bound loci. It will be interesting to study if these loci (probably promoters) have a different set of co-factors to active transcription. Another way in which SP1 can help in Wnt response is its ability to dimerise and cause looping. This might aid in the functioning of the Wnt enhanceosome since the WREs are majorly enhancer elements and might not be located immediately in the vicinity of the target gene. As mentioned previously, other members of the SP family, SP5 and SP8 are already known to loop the chromatin during Wnt-activated transcription (Kennedy *et al.*, 2016).

Limitations: We aimed to investigate whether the assembly of the Wnt enhanceosome is impacted in the absence of SP1, while simultaneously restoring β-catenin levels through ectopic expression. However, given that the depletion of SP1 resulted in the reduction of multiple components of the Wnt enhanceosome, we were unable to address this inquiry. Examining the role of SP1 in the assembly of the Wnt enhanceosome in various contexts is of utmost importance. Additionally, future studies should delve into the regulatory role of SP1 concerning Wnt target genes in the absence of TCFs. It would be intriguing to explore the impact on the expression of C2 and C3 genes when TCF7L2 is removed from the system.

Supplementary Figures

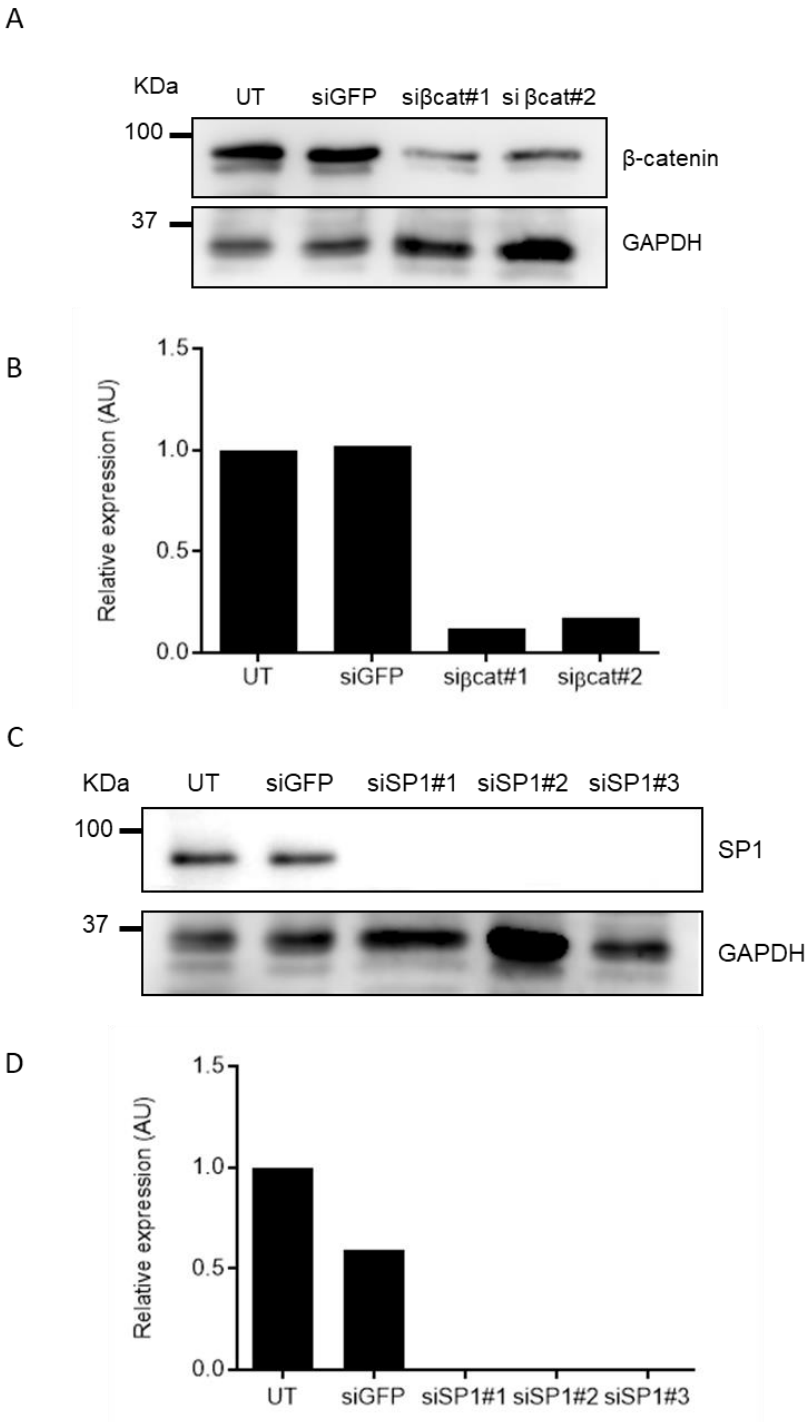


Figure S2.1. Knockdown efficiency of siRNAs used in the study. A. Immunoblot analysis to monitor the efficiency of siRNAs against β-catenin. **B.** Densitometric analysis for the WB in Figure S2.1A. **C.** Immunoblot analysis to assess the efficiency of siRNAs against SP1. **D.** Densitometric analysis for the Immunoblot in Figure S2.1C

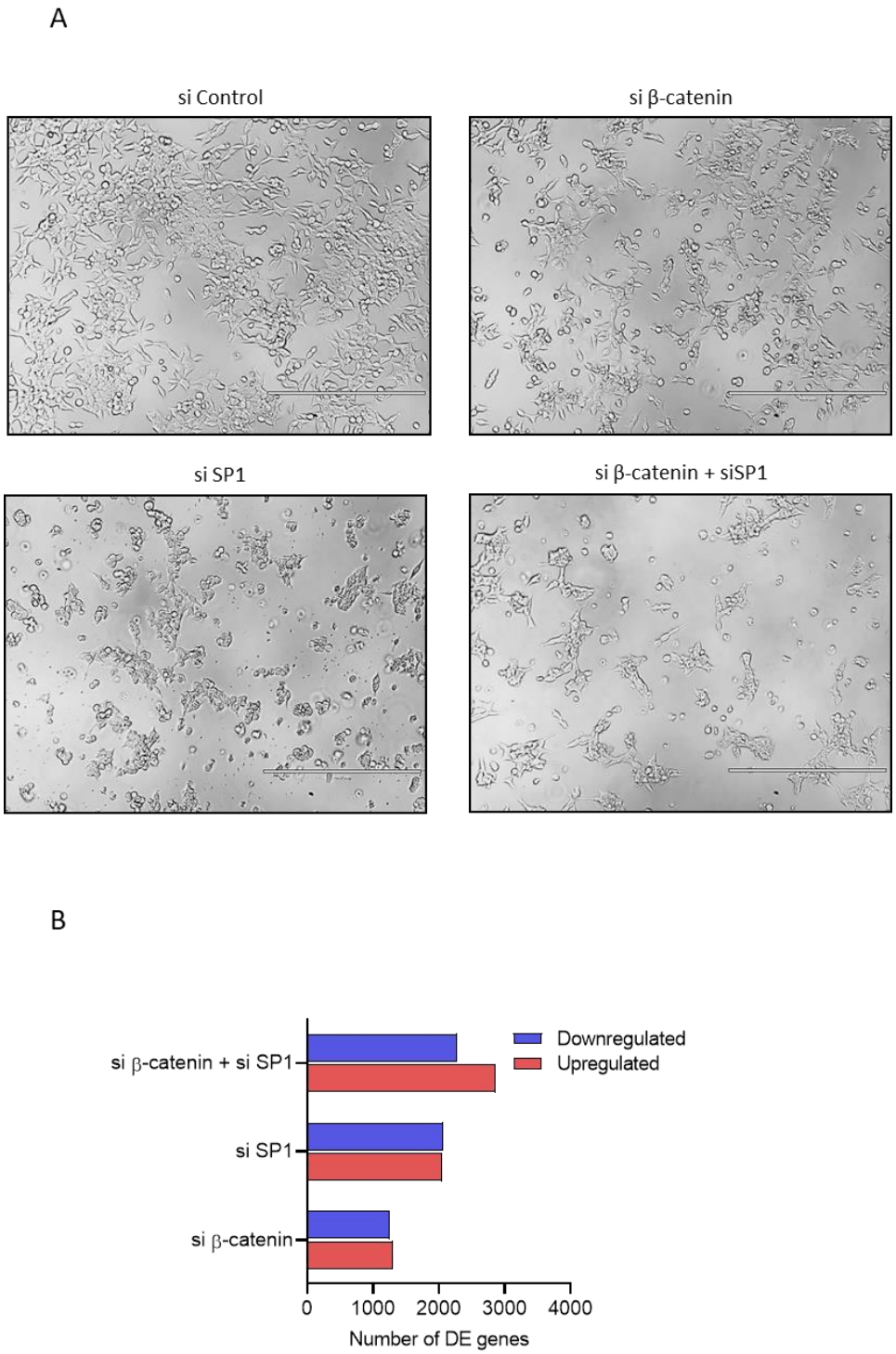


Figure S2.2. Morphological changes associated with depletion of β-catenin and SP1. A. DIC images for cells treated with control siRNA, siβ-catenin, siSP1 and combination of both. **B.** Total number of differentially expressed genes in control siRNA, siβ-catenin, siSP1 and combination of both.

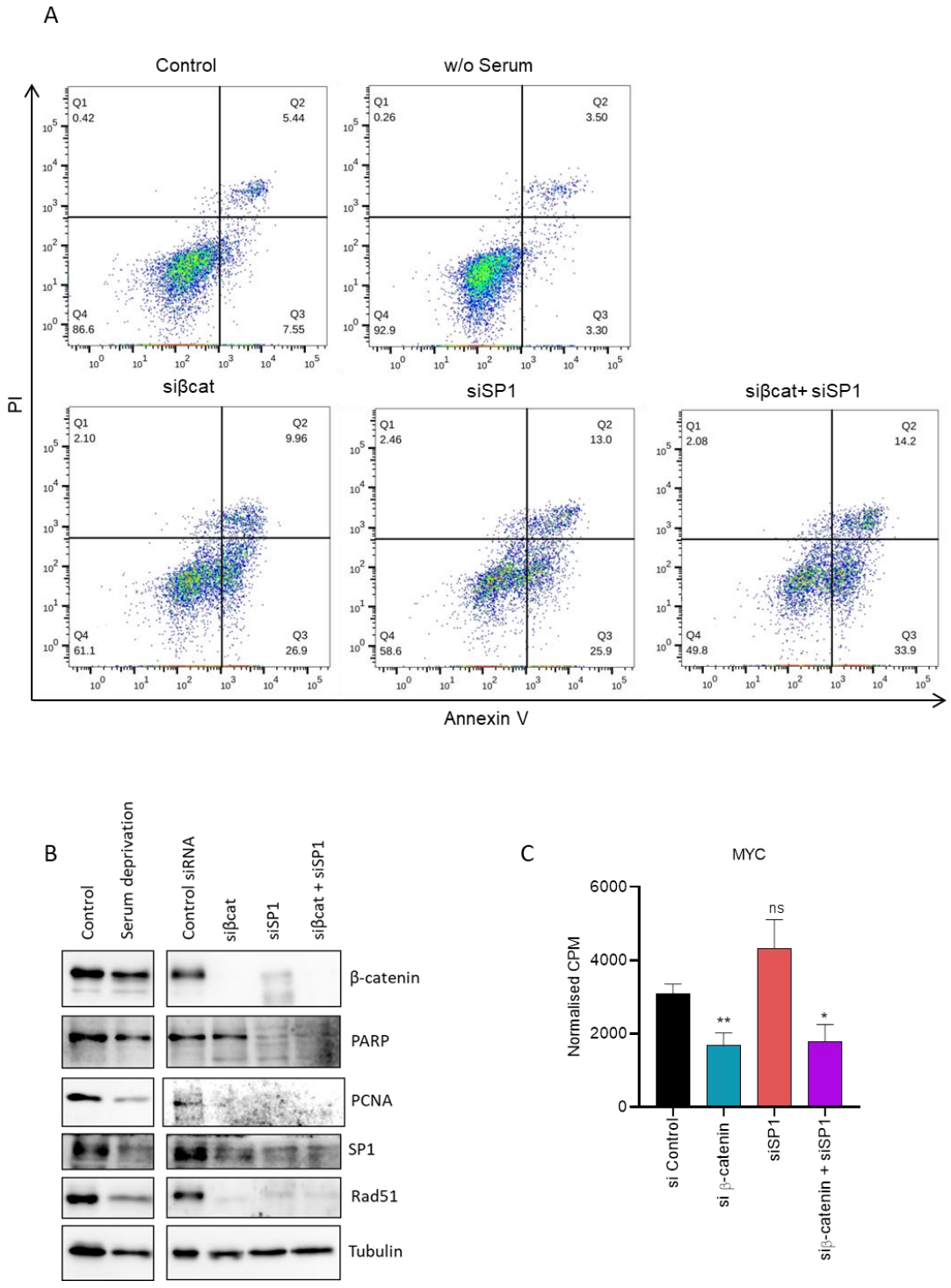


Figure S2.3. β-catenin and SP1 enhance cell survival and growth. A. Flow cytometry analysis for cells stained with Annexin V and PI after treatment with control siRNA, siβ-catenin, siSP1 and combination. **B.** WB image for β-catenin, PARP, PCNA, SP1, Rad51 in cells after treatment with control siRNA, siβ-catenin, siSP1, combination and serum deprivation. **C.** Expression of MYC mRNA in control, β-catenin, SP1 and β-catenin + SP1 siRNA treated cells (Unpaired t-test was performed to calculate significance).

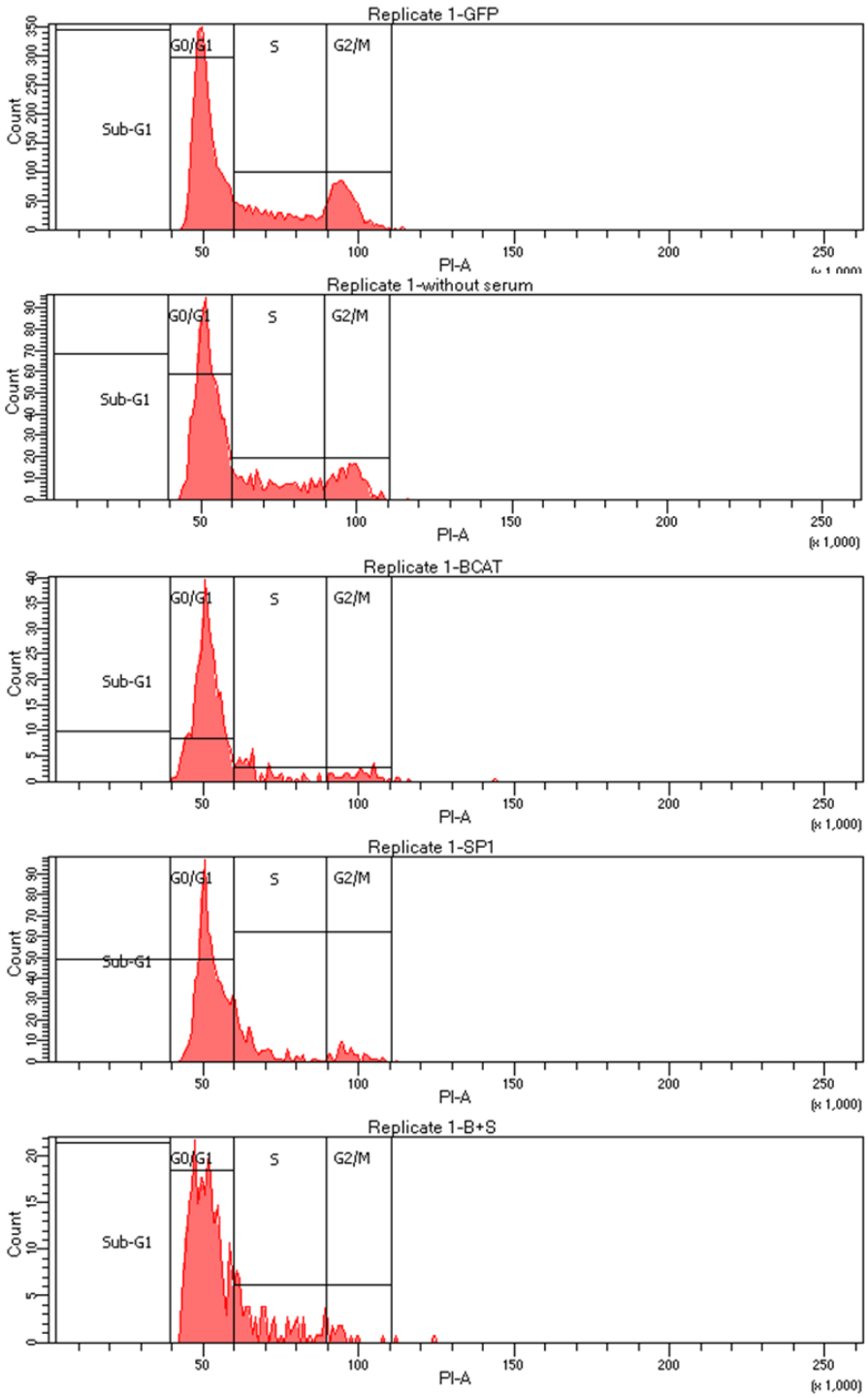
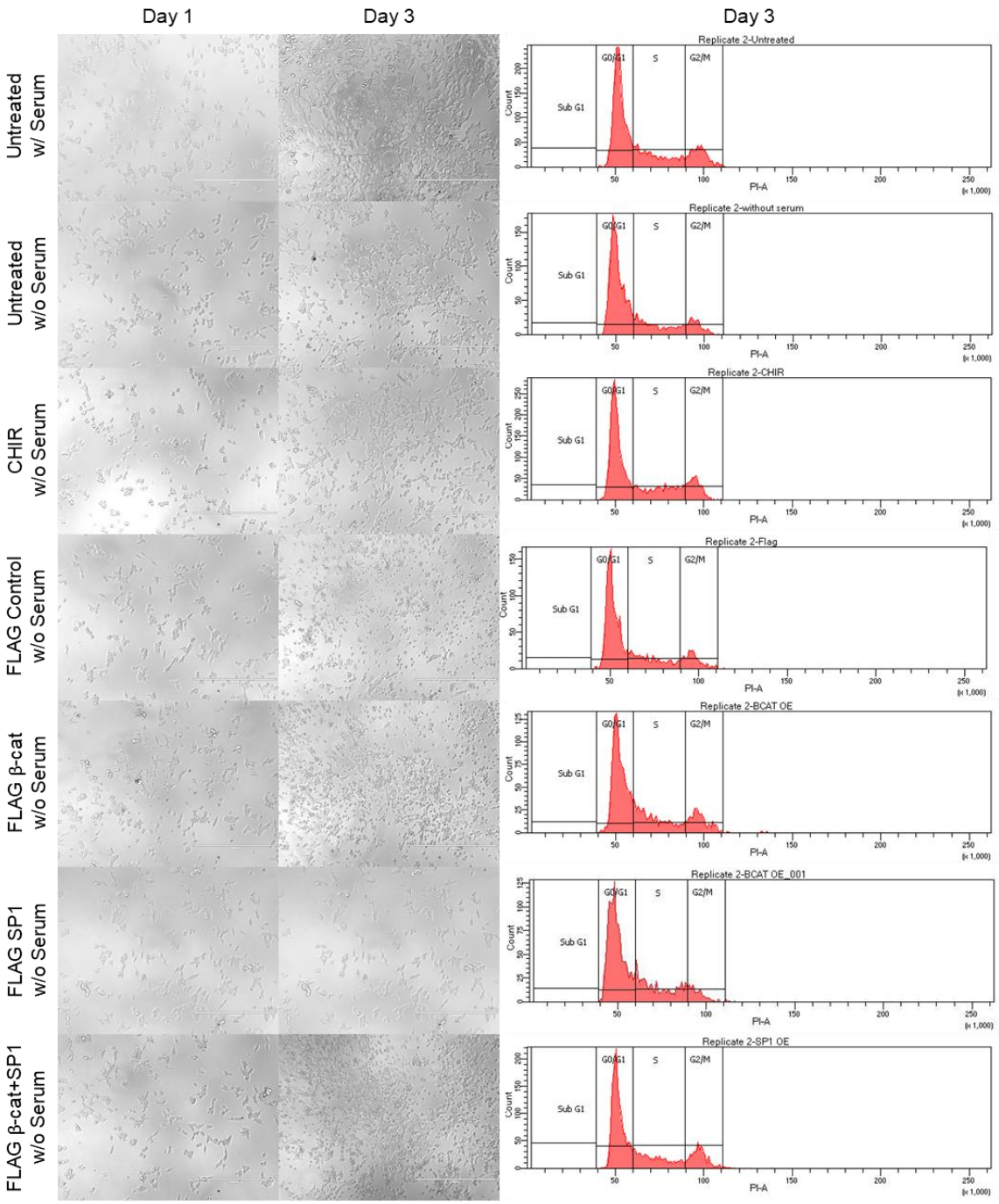


Figure S2.4. β-catenin and SP1 promote cell-cycle. Flow cytometry analysis after PI staining for cells treated with control siRNA, siβ-catenin, siSP1 combination and serum deprivation.

A


Day 1
Day 3
Day 3
Untreated w/ Serum
Untreated w/o Serum
CHIR w/o Serum
FLAG Control w/o Serum
FLAG β-cat w/o Serum
FLAG SP1 w/o Serum
FLAG β-cat+SP1 w/o Serum
Replicate 2-Untreated
Replicate 2-without serum
Replicate 2-CHIR
Replicate 2-Flag
Replicate 2-BCAT OE
Replicate 2-BCAT OE_001
Replicate 2-SP1 OE
G0/G1
S
G2/M
Sub G1
Count
PI-A

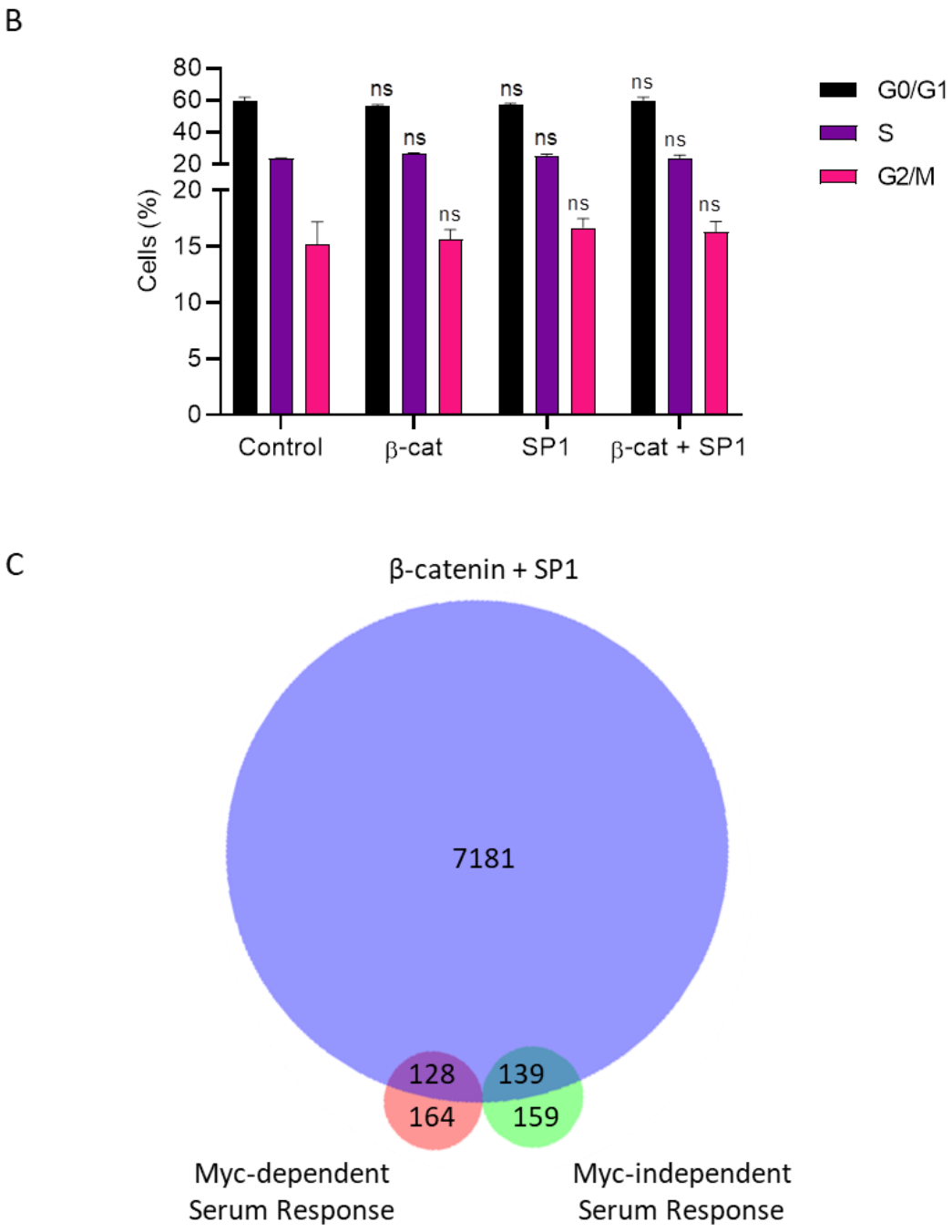


Figure S2.5. β-catenin and SP1 drive cell growth. A. DIC images and flow cytometry analysis after PI staining for cells in control medium, serum deprivation, CHIR treatment, overexpression of FLAG control, β-catenin, SP1 and β-catenin + SP1 in serum deprived medium. **B.** Cell-cycle analysis of cells upon overexpression of FLAG control, β-catenin, SP1 and β-catenin + SP1 in serum deprived medium. **C.** Overlap of all differentially regulated genes in DKD with Myc-dependent serum responsive factors and Myc-independent serum responsive factors.

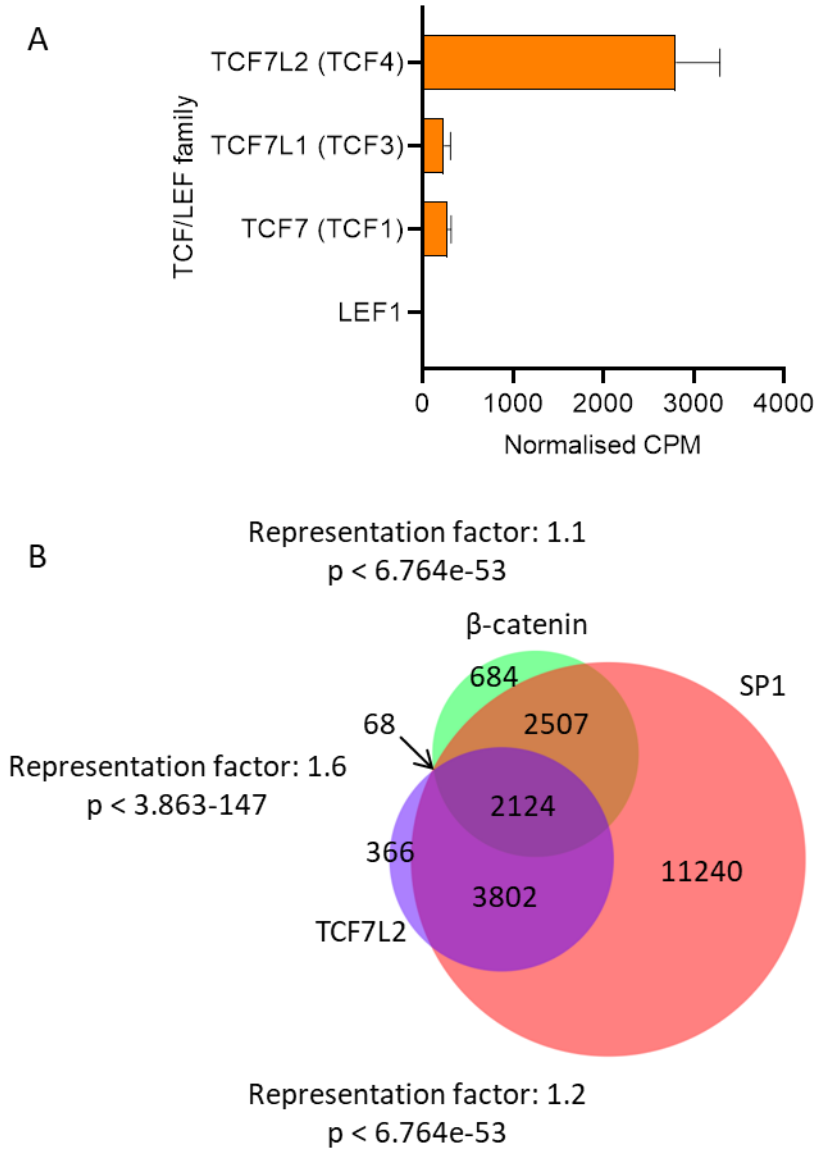
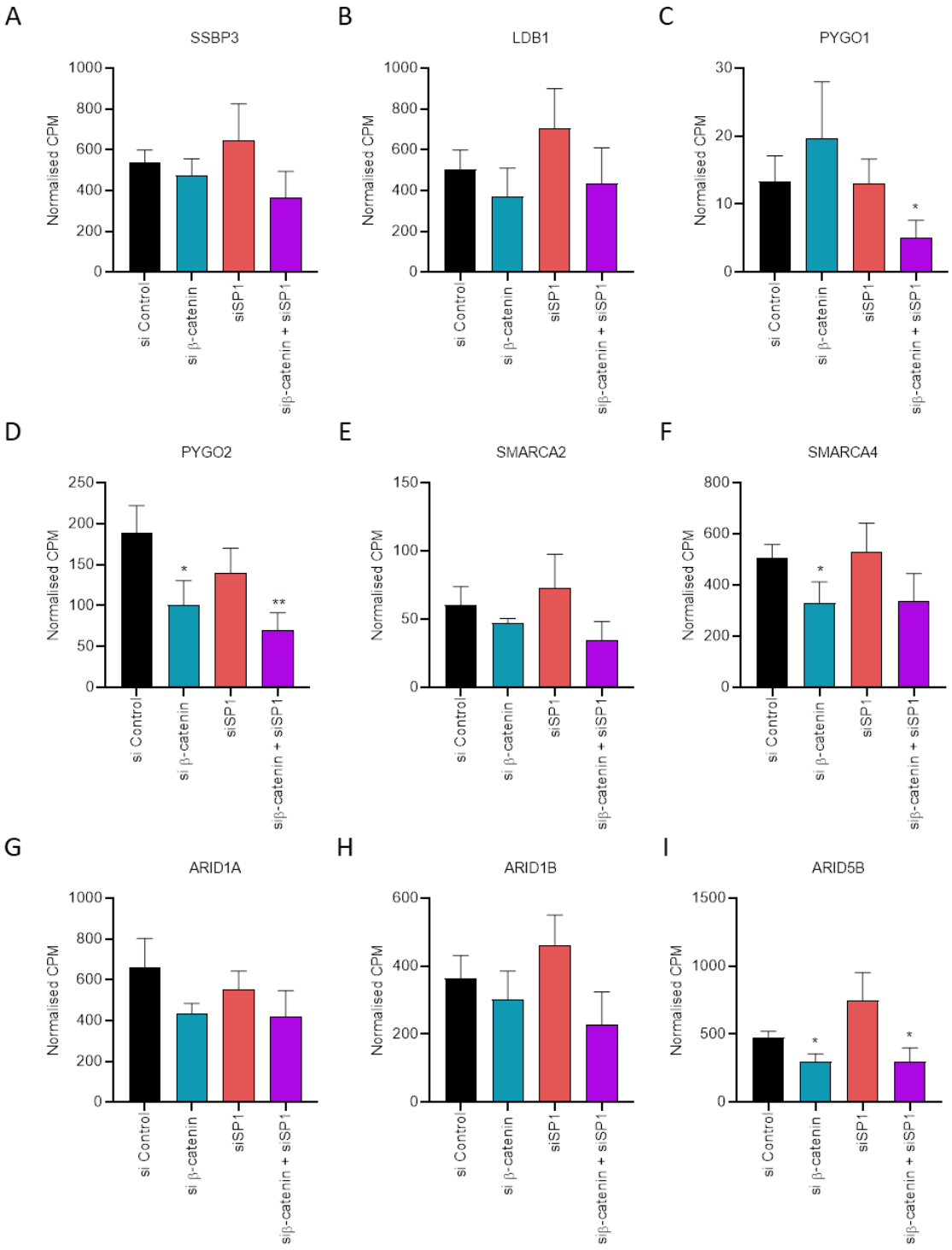


Figure S2.6. β-catenin and SP1 show chromatin co-occupancy. A. Expression of all TCF/LEF members in HCT116 cells in control conditions. **B.** Venn diagram to show overlap between the ChIPseq peaks for β-catenin, SP1 and TCF7L2.


A
SSBP3
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800
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siSP1
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B
LDB1
Normalised CPM
1000
800
600
400
200
0
si Control
si β-catenin
siSP1
siβ-catenin + siSP1
C
PYGO1
Normalised CPM
30
20
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si β-catenin
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siβ-catenin + siSP1
D
PYGO2
Normalised CPM
250
200
150
100
50
0
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si Control
si β-catenin
siSP1
siβ-catenin + siSP1
E
SMARCA2
Normalised CPM
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si Control
si β-catenin
siSP1
siβ-catenin + siSP1
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SMARCA4
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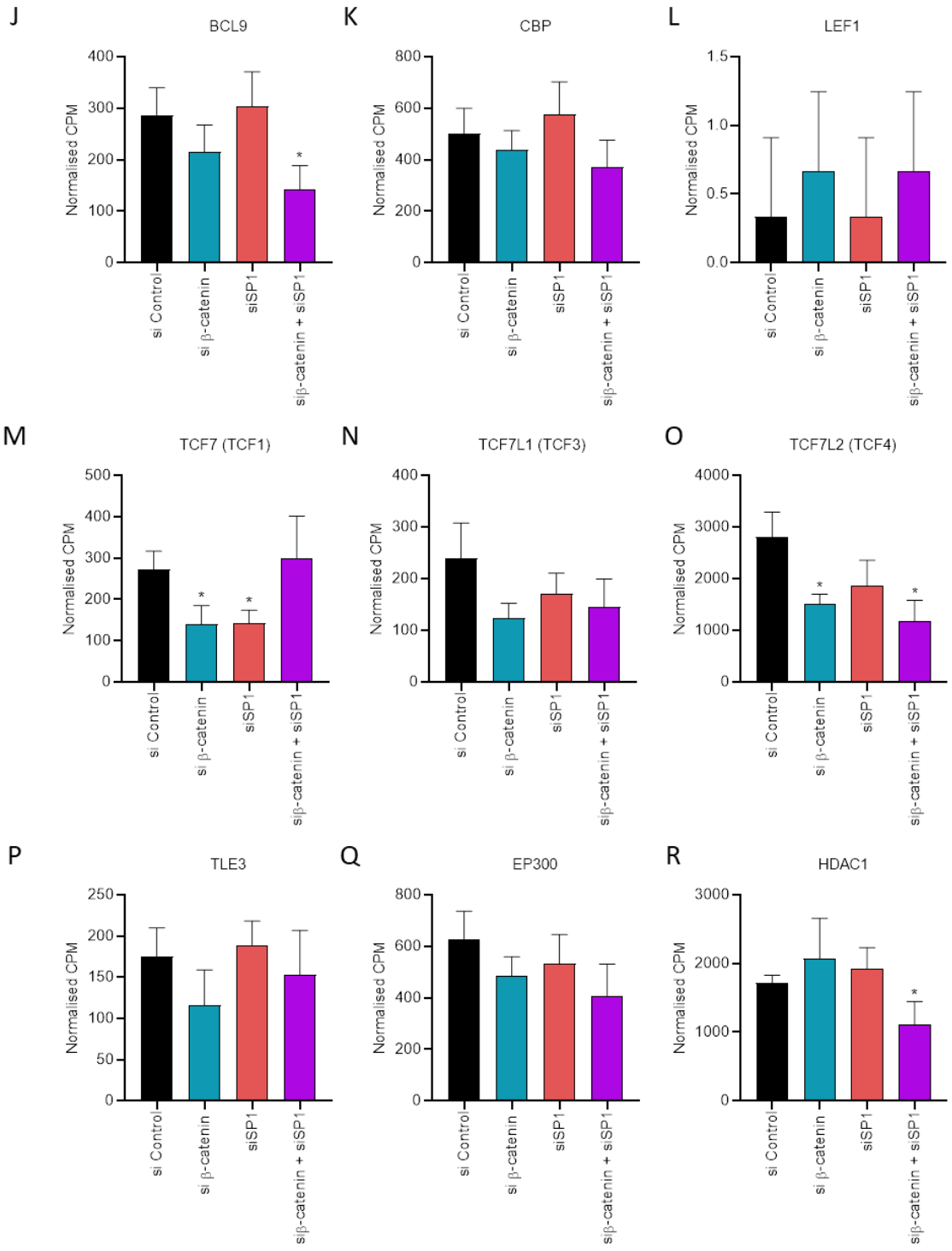

Figure S2.7. β-catenin and SP1 regulate the expression of the components of the Wnt enhanceosome. A-R: Expression of mRNA (name mentioned on top for each) in control, β-catenin, SP1 and β-catenin + SP1 siRNA treated cells (Unpaired t-test was performed to calculate significance, results not significant unless mentioned).

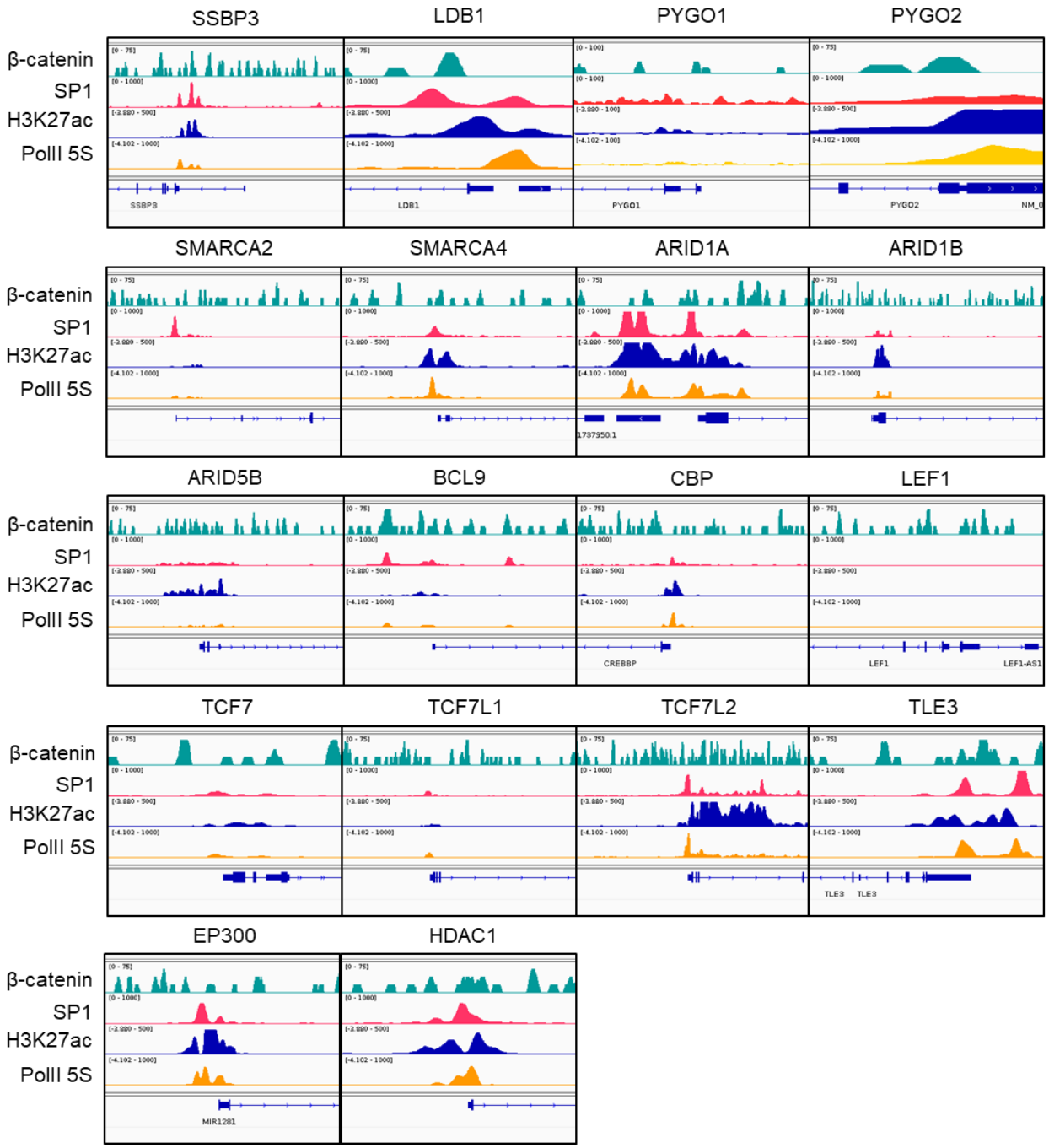

Figure S2.8. β-catenin and SP1 co-occupy the regulatory regions of the components of the Wnt enhanceosome. IGV tracks to show occupancy for β-catenin and SP1 on active promoter regions (enrichment for H3K27ac and PolII 5S) of the genes (name mentioned above each panel) for Wnt enhanceosome components

CHAPTER III

SP1:β-Catenin axis shares similar functions during colon development and cancer

Introduction

In the preceding chapter, we discussed the transcriptional partnership between SP1 and β-catenin. Given that the previous investigations were conducted in an *in vitro* setting, we aimed to explore this collaboration *in vivo*. Towards this, we leveraged colorectal cancer (CRC) as our model system, drawing upon publicly available transcriptome data from CRC patients. For studying developmental processes, we opted for zebrafish as the model organism. Zebrafish offer significant advantages for developmental studies due to the relatively brief duration of their developmental events compared to murine systems, along with the advantage of their externally observable development. Moreover, the presence of conserved developmental pathways and programs across humans, mice and fish, renders zebrafish a reliable system for the investigation of these processes.

Several mutations, more often *APC*, can cause hyperproliferation of the intestinal epithelium, leading to the formation of benign polyps (Su *et al.*, 1992). Eventually, these proliferated cells can acquire other mutations such as *KRAS* and *TP53*, which gives them the ability to grow even further and invade into the intestinal mucosa and turn malignant (Vogelstein *et al.*, 1988) (Figure 3.1A). Cancers of the GI tract are the most frequent among all cancer types owing to the high metabolic activity of the associated organs including stomach, liver, pancreas and colorectum. Owing to these reasons, the mortality rate in these cancers is equally high (Arnold *et al.*, 2020) (Figure 3.1B). Hence, it is crucial to investigate the genetic and epigenetic changes that underlie these types of cancers to devise precise and efficient targeted treatments. Several models have been put forth to explain the origins of CRC, with the most prevalent being the theory of initiation via a benign polyp, potentially driven by a genetic mutation in the *APC* gene.

Over time, the cells can develop a mutation in *KRAS*, which aids in cellular proliferation and survival. Subsequent mutations in the *TP53* gene, a critical tumor suppressor, can lead the initiation of tumorigenesis (Vogelstein *et al.*, 1988). Another mutation linked to the development of CRC is the *BRAF* mutation, often accompanied by aberrant promoter hypermethylation of genes involved in regulation of cell growth (Kambara *et al.*, 2004). Additionally, microsatellite instability, resulting from impaired DNA repair mechanisms, is another causal factor in CRC. It increases the frequency of genetic mutations associated with CRC (Kang, 2011). Recently, researchers have developed a new classification system for characterizing colorectal cancer tumors based on consensus molecular subtypes (CMS). This classification considers both the overall transcriptome profile and genetic alterations to provide a more comprehensive taxonomy for understanding the diversity of CRC tumors (Guinney *et al.*, 2015) (Figure 3.1C).

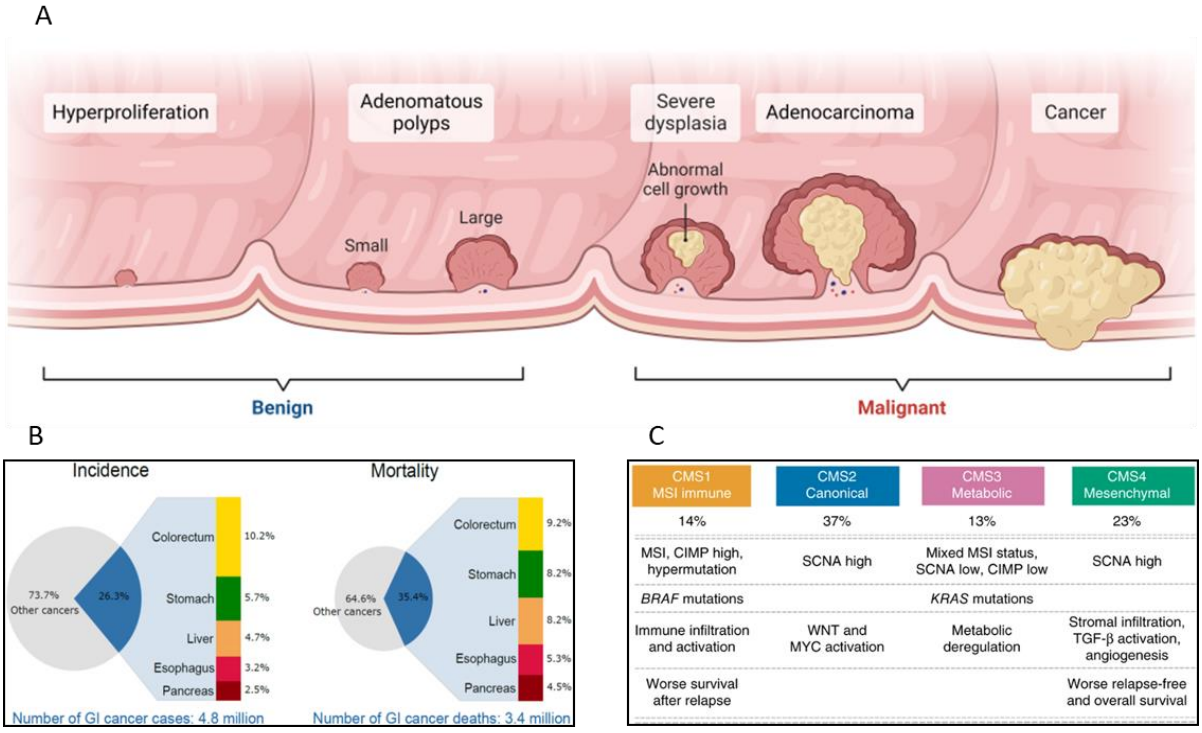

Figure 3.1. Disruption of intestinal homeostasis leads to colorectal cancer. A. Colorectal tumor can initiate as a benign polyp resulting from the hyperproliferation of the cells. Benign tumors turn malignant when the tumor cells acquire mutations, such as *KRAS*. **B.** Distribution of gastrointestinal cancers in the incidence and mortality causes due to cancers overall (Adapted from Arnold *et al.*, 2020). **C.** System for the consensus molecular signature for colorectal cancer. CIMP: CpG island methylator phenotype; MSI: microsatellite instability; SCNA: somatic copy number alterations (Adapted from Guinney *et al*., 2015).

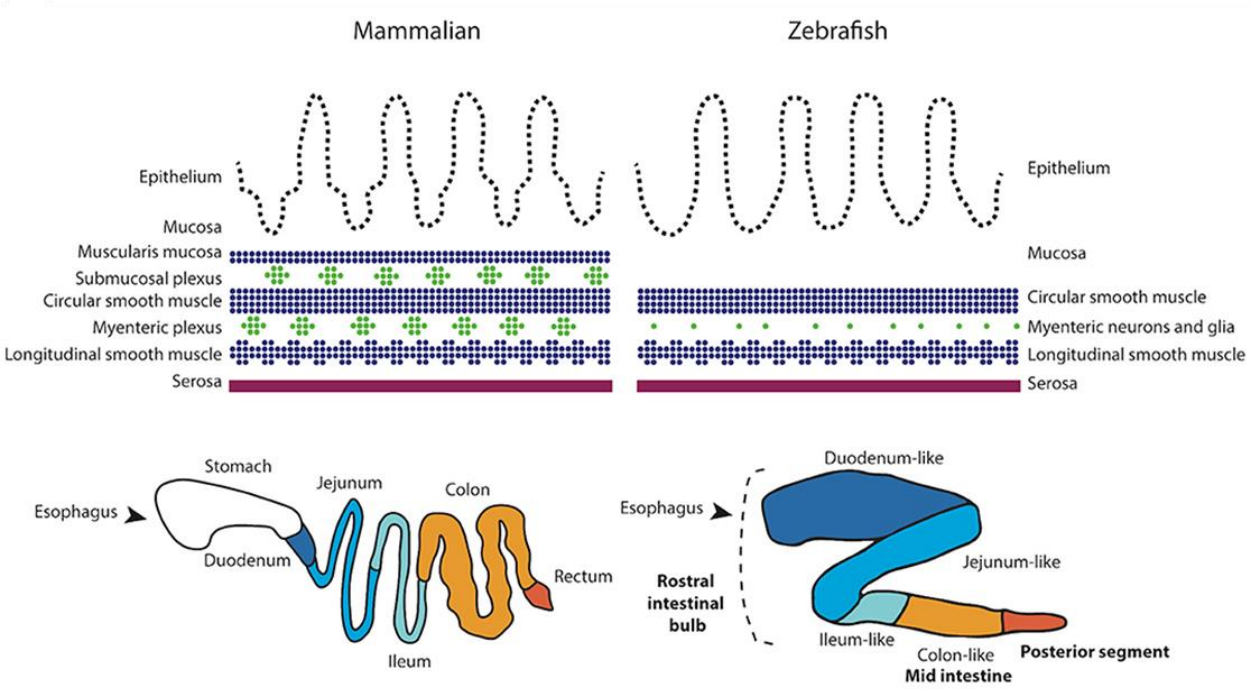

Figure 3.2. Zebrafish as a model system to study colon development. A. Different layers are present in the GI tract of mammals and zebrafish. **B.** Functional segments of the mammalian and zebrafish GI tracts. Different segments have been marked in colors depending on their conserved transcriptional profiles (Modified from Kuil *et al*., 2021).

To assess the relevance of β-catenin-SP1 complex in developmental processes, we employed zebrafish as our model organism. The basic developmental trajectory of zebrafish is similar to humans, i.e., the formation of the three germ layers and differentiation of the endoderm into the gut epithelium (Wallace and Pack, 2003). Additionally, zebrafish offer the advantage of completing their development, including gut development, within a relatively short period of 5 days post-fertilization (Wallace and Pack, 2003). Although there are some anatomical differences between the zebrafish and human digestive systems, such as the absence of mucosal layers and simpler enteric neuron organisation in zebrafish, the gut epithelium of zebrafish contains several intestinal cell types including enterocytes and goblet cells. It lacks the well-defined crypts of Lieberkühn found in the human gut but does feature finger-like projections toward the lumen, with their bases enriched in intestinal stem cells (Kuil *et al.*, 2020) (Figure 3.2, upper panel). It also has compartments like the duodenum, jejunum and colon based on their absorptive functions. Furthermore, through comparative transcriptome profiling, researchers have identified gut segments in zebrafish that bear

similarities to those in humans (Wang *et al.*, 2010). The upper panel of Figure 3.2 illustrates the similarities between multiple gut segments between the two systems (Kuil *et al.*, 2020). As mentioned previously, the developmental pathways in zebrafish closely resemble those in humans. The Wnt signaling pathway, for instance, plays a pivotal role in zebrafish, participating in various processes, including early development, differentiation of the three germ layers, and the establishment of both the dorsoventral and anteroposterior axes (Chan *et al.*, 2009; Hikasa and Sokol, 2013). It also regulates cellular proliferation within the zebrafish gut epithelium (Faro, Boj and Clevers, 2009; Cheesman *et al.*, 2011). Notably, *apc* mutants in zebrafish develop multiple benign polyps, further exemplifying the conserved phenotype associated with hyperactivated Wnt signaling (Kinzler and Vogelstein, 1996).

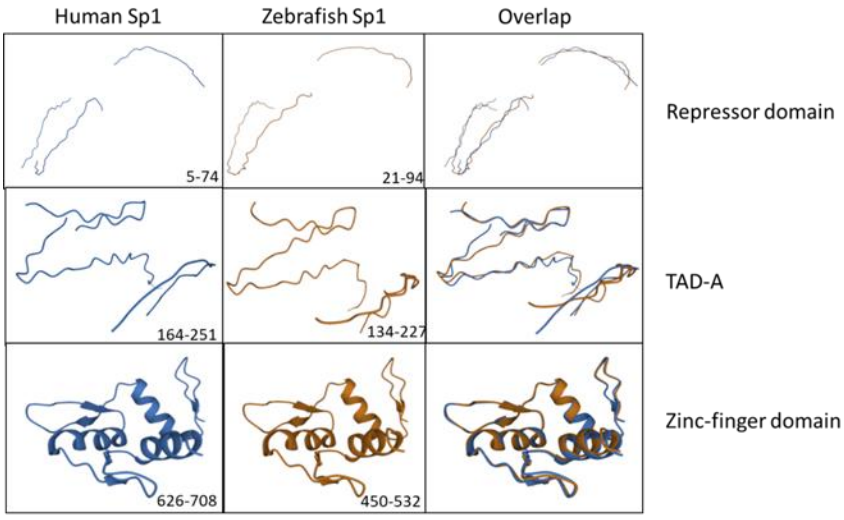

Figure 3.3. Functional domains are conserved between human and zebrafish Sp1 proteins. Predicted Repressor, TAD and Zn-finger domains of zebrafish Sp1 aligned with the corresponding domains of the human SP1 proteins (Modified from Mishra, 2021).

Sp1 has a conserved mode of functioning in zebrafish

(Part of a submitted MS thesis of a BS-MS student who worked under my guidance: Mishra S. Characterisation of Sp1 in zebrafish (Danio rerio) in the context of development, 2021, MS Thesis, IISER Pune, IISER Pune Digital Repository)

Information regarding Sp1 in zebrafish is relatively limited. There is a single study indicating that zebrafish Sp1 possesses conserved Zn-finger motifs and can induce transcription when expressed in a mammalian system (Lin *et al*., 2011). To explore a

potential cooperation between β-catenin and Sp1, we initiated our investigation by characterizing the fundamental expression and role of Sp1 during zebrafish development. Although the overall protein sequences of human and zebrafish Sp1 share a low similarity score (50.26%), the functional domains including Repressor domain, TAD and Zn-finger domain exhibit a high similarity of over 90% (Figure 3.3). Analysis of the Sp1 mRNA expression throughout various developmental stages revealed high expression in the early stages, which subsequently declined after the dome stage. This trend was consistent across both publicly available and in-house data (Figure 3.4A&B).

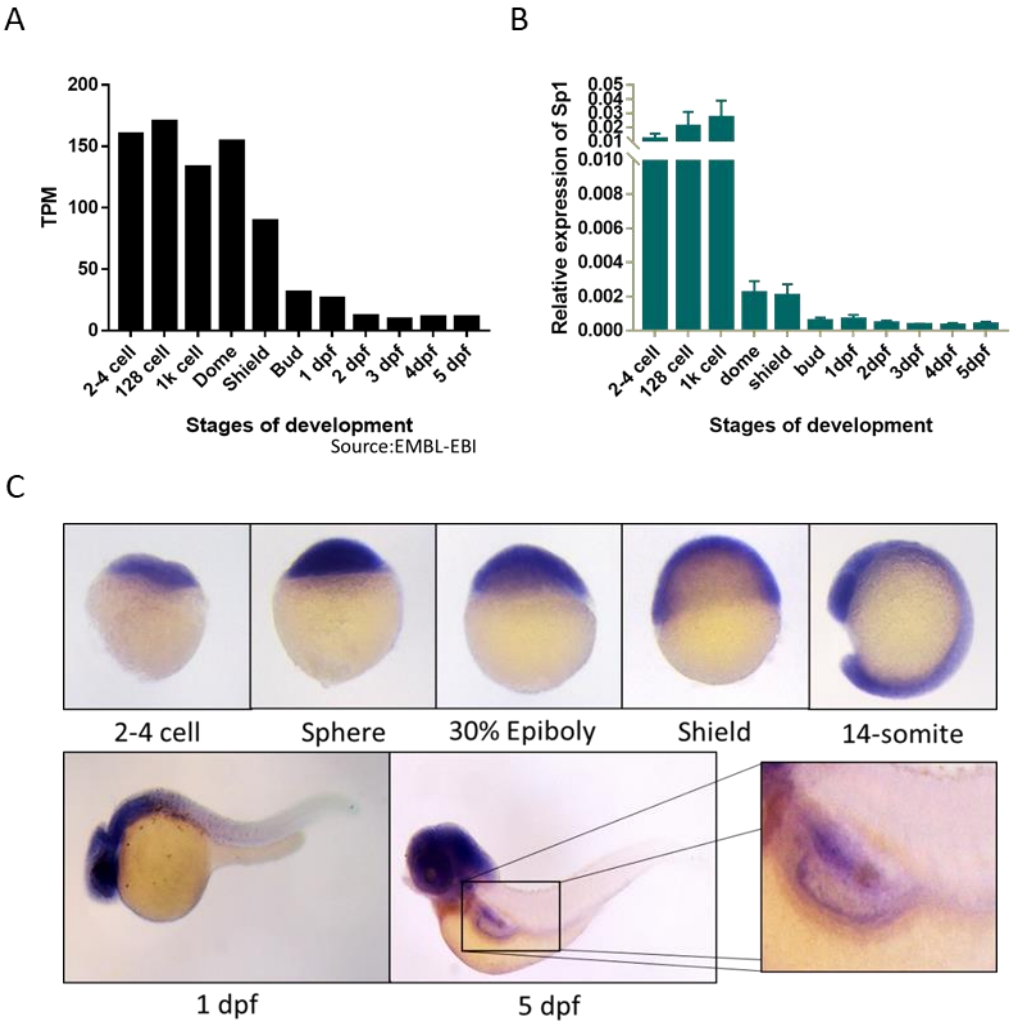

Figure 3.4. Expression profile of Sp1 at different developmental stages of zebrafish. A. Expression values for sp1 using the publicly available dataset through EBI-EMBL. **B.** Expression values of sp1 using qRT-PCR. **C.** Localisation of sp1 mRNA using whole mount *in situ* hybridisation (Modified from Mishra, 2021).

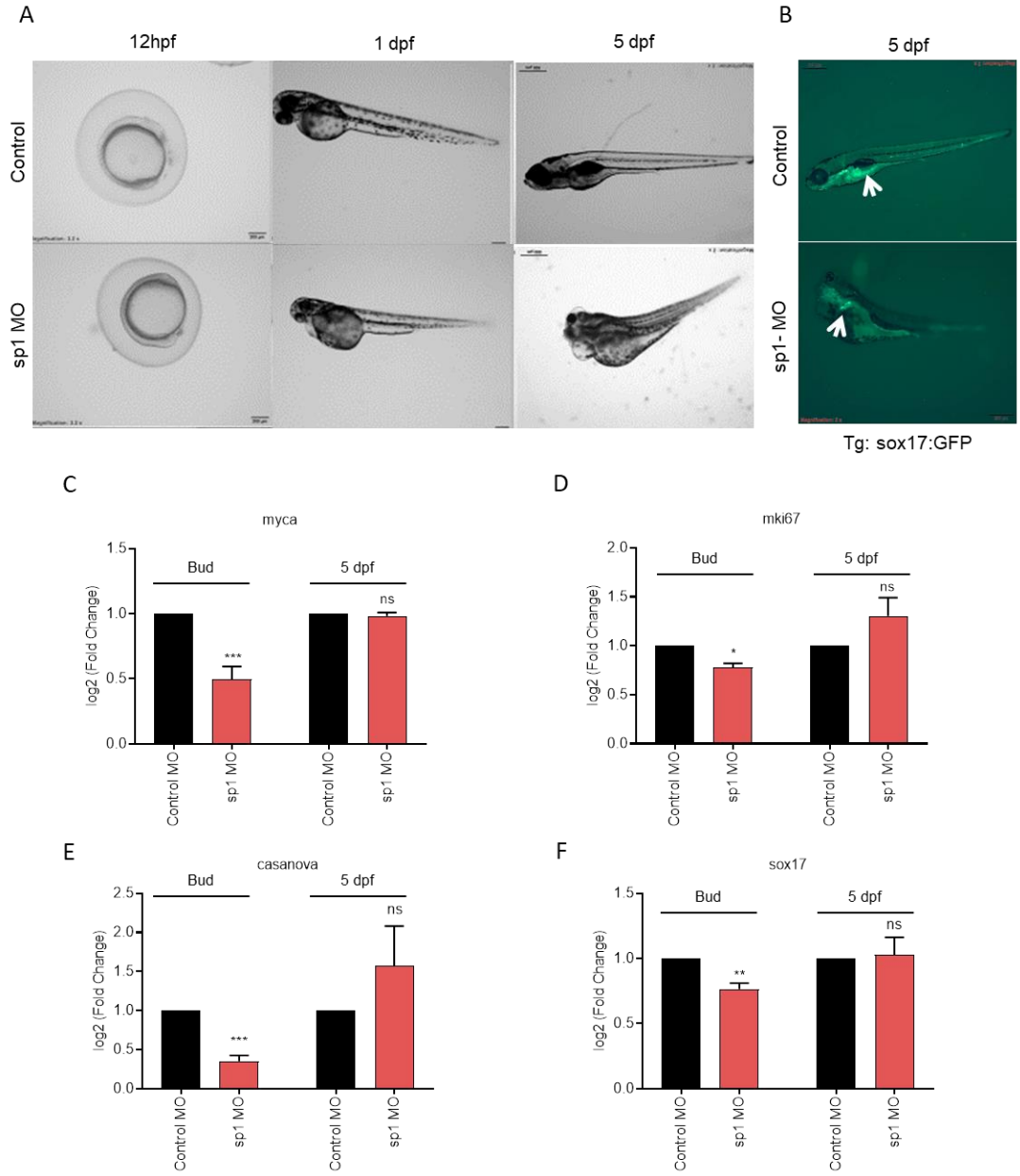

Figure 3.5. Sp1 perturbation causes developmental defects during larval stages. A. Bright field images of WT zebrafish at different stages injected with control or sp1 morpholino. Scale bar = 500 µm **B.** Confocal images of Tg:sox17 eGFP zebrafish injected with control or sp1 morpholino. Scale bar = 500 µm. qRT-PCR at Bud (10 hpf) and 5 dpf after injection with control or sp1 morpholinos for **C.** myca. **D.** mki67. **E.** casanova. **F.** sox17. (Modified from Mishra, 2021).

Notably, localisation of mRNA using whole mount in situ hybridization (WISH), revealed that in the later stages of development, Sp1 exhibited localized expression in the anterior region (visible from 1dpf) and the gut (visible from 4dpf). Conversely, the early stages of the embryos showed robust and widespread expression of Sp1 (Figure 3.4C).

Similarly, mouse embryos demonstrate ubiquitous expression of SP1 (Marin *et al*., 1997). Moreover, human SP1 is associated with neuronal processes and diseases (Citron *et al.*, 2008; Wang *et al.*, 2012). These findings provide additional confidence in the conserved role of SP1. Notably, morpholino-mediated depletion of Sp1 did not lead to any visible defects in the early stages of development, which was unexpected given that Sp1 exhibited maternal deposition, evident through its high expression from the 2-cell stage. However, the developmental defects became apparent at 1dpf. Severe edema was observed in the cardiac sac, alongside malformations in the heart and other anterior structures (Figure 3.5A). A similar experiment was conducted in the Tg: sox17 eGFP lines, which exhibit GFP expression in the endoderm and the gut. By 5 dpf, when the gut is fully formed, the Sp1 morpholino-injected larvae displayed severely malformed gut (Figure 3.5B). Interestingly, decreased expression of both the proliferation markers (*myca, mki67*) and endoderm markers (*casanova, sox17*) could be observed at 10hpf during the Bud stage but not at 5 dpf (Figure 3.5C-F). This discrepancy may be attributed to potential decrease in morpholino effectiveness at 5 dpf. However, it is noteworthy that cell proliferation significantly slows down during the larval stages, making the changes caused by Sp1 depletion not significant. In addition to these findings, transcriptome profiling of Sp1-depleted larvae at 3dpf indicated that several target genes exhibited similarities to those observed in the HCT116 cell line (Figure 3.6). These genes primarily regulated the cell cycle and cell death. Collectively, this demonstrates that zebrafish Sp1 fulfills the same fundamental functions as the human/mouse SP1 and is, therefore, a suitable model for studying its role during development.

Material and methods

Human cell culture and assays

HCT116 cells were cultured as described previously. Annexin V/Propidium Iodide staining and quantification for cell viability and cell cycle analysis were performed according to the protocol described previously.

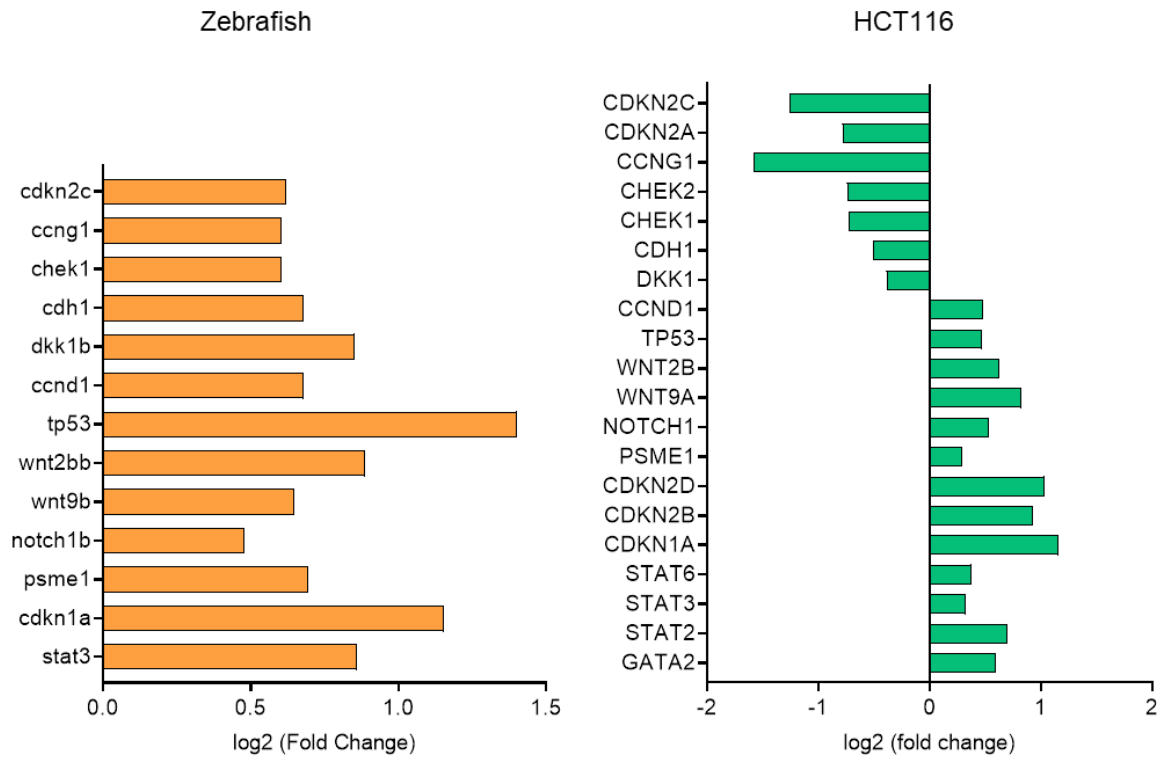

Figure 3.6. Zebrafish Sp1 and human SP1 regulate similar target genes. qRT-PCR for the common target genes in zebrafish embryos injected with control or sp1 morpholinos and HCT116 cells treated with control or SP1 siRNA.

Patient transcriptome data analysis

Gene counts for all the differentially expressed genes in colon adenocarcinoma were obtained from OncoDB (http://www.oncodb.org) with the parameters: FDR Adjusted p-value < 0.001, log2 Fold change > 1). Overlap between mRNAseq datasets was obtained using Venny 2.1.0 (https://bioinfogp.cnb.csic.es/tools/venny), and the Venn diagrams were generated using BioVenn (https://www.biovenn.nl/index.php). GO analysis for the common target genes was performed using the online version of Metascape (https://metascape.org/gp/index.html#/main/step1). Patient survival plots were generated using the online tool Kaplan-Meier Plotter (https://kmplot.com/analysis).

Mice rearing and xenograft assays

NOD/SCID strain of mice was used for the study and housed in a pathogen-free facility

according to the standard husbandry protocols at the National Facility for Gene Function in Health and Disease, IISER, Pune. For the cell-derived xenograft experiment, each NOD/SCID mouse was injected with 1 X 10^6 HCT116 cells subcutaneously in a 1:1 mixture with Matrigel (Corning). At the end of 2 weeks post-injection, mice were treated with either 1X PBS or PNU 74654 (PNU) (0.15 mg/kg) or Mithramycin-A (MT-A) (0.5 mg/kg) or a combination of PNU (0.15 mg/kg) and MT-A (0.5 mg/kg) once a week for a total of 6 weeks. At the end of the experiment, measurements were taken for the tumor weight and volume. Tumor volume was calculated using the formula of length × $width^2/2$.

Zebrafish husbandry, knockdown and overexpression assays

Wild-type strains of zebrafish, AB & TU; and transgenic sox17-eGFP lines were maintained according to the standard zebrafish husbandry protocols under the National Facility for Gene Function in Health and Disease, IISER, Pune. Embryos and larvae were grown at 28 ℃ in 1X E3 medium till harvesting. Bright field and fluorescence imaging were performed using the Olympus stereo microscope.

Morpholino for sp1 and control morpholino were obtained from Gene Tools. Morpholinos were resuspended in nuclease-free water according to the instructions provided by the manufacturer. Embryos were injected with morpholinos at 0.5 nM concentration at 1 cell stage and grown till the indicated stage and harvested depending on the downstream application. Capped mRNA for SP1 and β-catenin were synthesized using Ambion mMESSAGE mMACHINE™ SP6 Transcription Kit (Invitrogen, ThermoFisher). mRNA was injected at a total concentration of 3 ng/µl at 1 cell stage and grown till the indicated stage and harvested depending on the downstream application. For quantification of deformity and mortality, 100 embryos were injected per condition per replicate. For RNA isolation, 10 embryos were harvested per replicate and processed as described previously. A list of the morpholino sequences, plasmids and antibodies used for fish experiments are provided in Appendix 1, 2 and 3, respectively. Data analysis for the mRNAseq dataset used for sp1 morpholino injected larvae and *apc*$^{-/-}$ larvae was performed according to the protocol described previously.

Results and Discussion

3.1 β-catenin and SP1 regulated genes contribute towards colorectal cancer development

β-catenin and SP1 are well-established to be upregulated in multiple cancers, and their high expression is associated with reduced patient survival. These factors have also been independently shown to be enriched in Colorectal Stem-like Cancer Cells (CSCCs) (Li *et al.*, 2017; Quarni *et al.*, 2019). We sought to delve deeper into how the gene set coregulated by these factors plays a role in colorectal tumorigenesis. To accomplish this, we overlapped the differentially expressed (DE) genes from our mRNAseq data with the DE genes derived from patient samples of colorectal adenocarcinoma (COAD) (Tang, Cho and Wang, 2022). Notably, we observed a substantial overlap between the two sets (Representation factor: 1.2; $p < 2.960e-12$) (as depicted in Figure 3.7A). We then proceeded to compare the expression of these genes in normal and tumor samples. Our findings indicated that the genes under the negative control of β-catenin and SP1 displayed no significant change between the two conditions. However, genes under positive regulation displayed significant upregulation in the case of COAD (Figure 3.7B). Furthermore, these genes exhibited a strong enrichment for biological functions associated with the cell cycle and DNA replication, consistent with our previous observations (Figure 3.7C). Additionally, we assessed the impact of these shared targets on overall patient survival, revealing that several factors were negatively correlated with survival (Figure 3.7D). In summary, we established that the genes targeted by β-catenin-SP1 contribute to the development of CRC by promoting cell growth, consequently exerting a detrimental effect on patient survival.

3.2 β-catenin and SP1 synergize to promote tumorigenesis

To assess the impact of β-catenin and SP1 on tumorigenesis within an *in vivo* context, we performed a cell-derived xenograft assay using mice. The cell line used in our

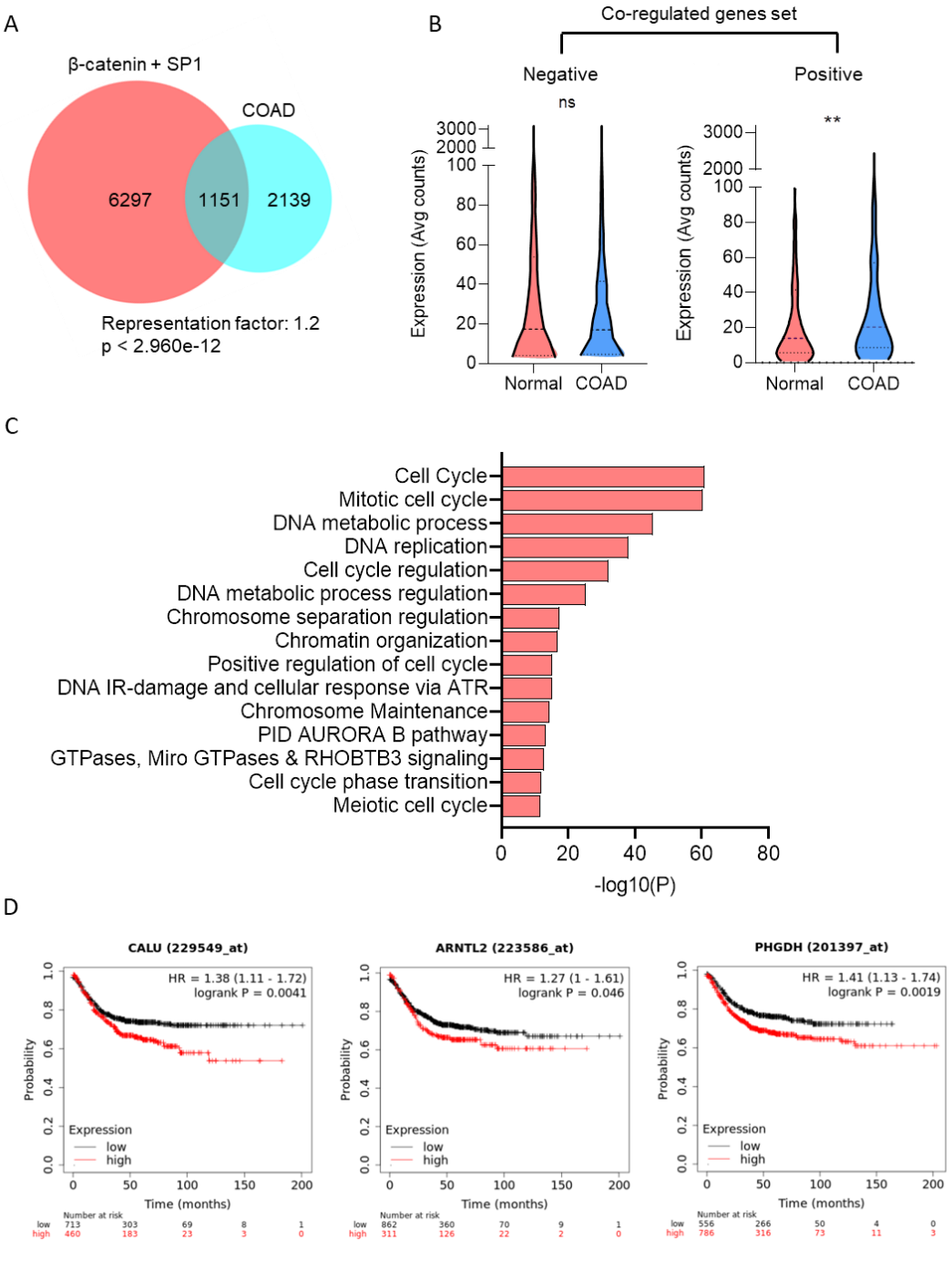

Figure 3.7. β-catenin and SP1 regulated genes contribute towards colorectal cancer development. A. Overlap between β-catenin and SP1 co-regulated genes obtained from mRNAseq in HCT116 and DE gene set for Colorectal Adenocarcinoma patient data from OncoDB using Tang et al., 2022(Tang, Cho and Wang, 2022). **B.** Expression counts for genes regulated by β-catenin and SP1 and overlapped with the COAD dataset (Paired t-test was performed to calculate significance). **C.** GO analysis for genes positively regulated by β-catenin and SP1 and overlapped with the COAD dataset. **D.** KM plots for selected targets (CALU: Calumenin; ARNTL2: Aryl Hydrocarbon Receptor Nuclear Translocator Like 2; PHGDH: Phosphoglycerate Dehydrogenase).

transcriptome analysis, HCT116, was utilized for this experiment. Following the induction of tumors, the mice were subjected to treatment with established inhibitors specific to either β-catenin, SP1, or both factors concurrently. The schematic outlining the experimental strategy is depicted in Figure 3.8A. PNU 74654 (PNU) was employed as an inhibitor to impede the activity of Wnt/β-catenin signaling by obstructing the interaction of β-catenin and the TCFs (Leal *et al.*, 2015). Mithramycin-A (MT-A) was used to hinder the transcriptional activity of SP1 by binding to the GC-rich regions of the DNA (Miller *et al.*, 1987; Blume *et al.*, 1991). Notably, our findings revealed that mice treated with both inhibitors concurrently exhibited significant reduction in tumor growth compared to those treated with either inhibitor alone (Figure 3.8B). This reduction was evident in the overall tumor volume and weight (Figure 3.8 C&D). It is important to note that the frequency and the dosage of the inhibitors used were lower than those reported in the literature (Takam Kamga *et al.*, 2020; Dutta *et al.*, 2021). The reason for reducing the dosage was to mitigate potential toxicity to the animals, given the unknown effects of the combined PNU and MT-A treatment. Nevertheless, this approach effectively demonstrated the synergistic effects of β-catenin and SP1 in the context of tumor development.

3.3 Combinatorial chemical inhibition of β-catenin and SP1 promotes cell death and inhibits cell cycle

Building upon the findings from the preceding section, we extended our investigation using the same treatment protocol to assess the impact of these inhibitors on cell survival and the cell cycle. We titrated the doses of the inhibitors to ensure they did not induce excessive cell mortality. Upon comparing the cell viability and the extent of apoptosis across all conditions, we observed a consistent pattern where the combinatorial treatment had a significantly more pronounced effect than single treatment (Figure 3.9A-C). Additionally, we examined the impact on the cell cycle profile and once again noted a drastic reduction in the G2/M population following the combinatorial treatment (Figure 3.10A&B).

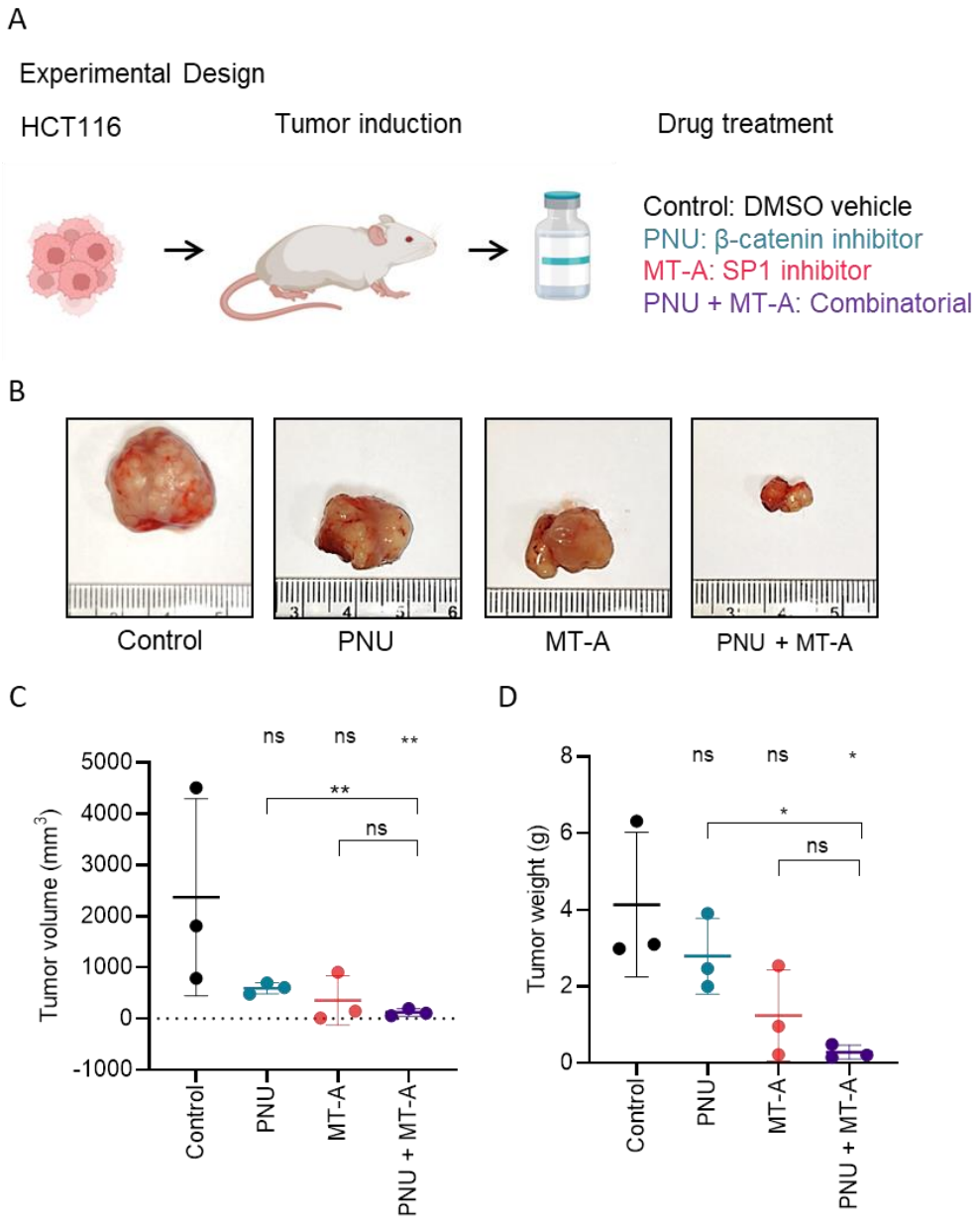

Figure 3.8. β-catenin and SP1 synergize to promote tumorigenesis. A. Schematic representation of the experimental design followed for the cell-derived xenograft study in mice. **B.** Representative images for the tumors harvested at the end of the treatments. **C.** Tumor volumes measured at the end of the treatments. Mean values: Control: 2371 mm^3; PNU: 593.6 mm^3; MT-A: 355.5 mm^3; PNU + MT-A: 120.1 mm^3. (Unpaired t-test used to calculate significance). **D.** Tumor weights measured at the end of the treatments. Mean values: Control: 4.137 g; PNU: 2.794 g; MT-A: 1.244 g; PNU + MT-A: 0.2844 g. (Unpaired t-test was used to calculate significance).

3.4 β-catenin and Sp1 overexpression cause similar developmental defects in zebrafish

We aimed to investigate whether the β-catenin:Sp1 complex plays a role in

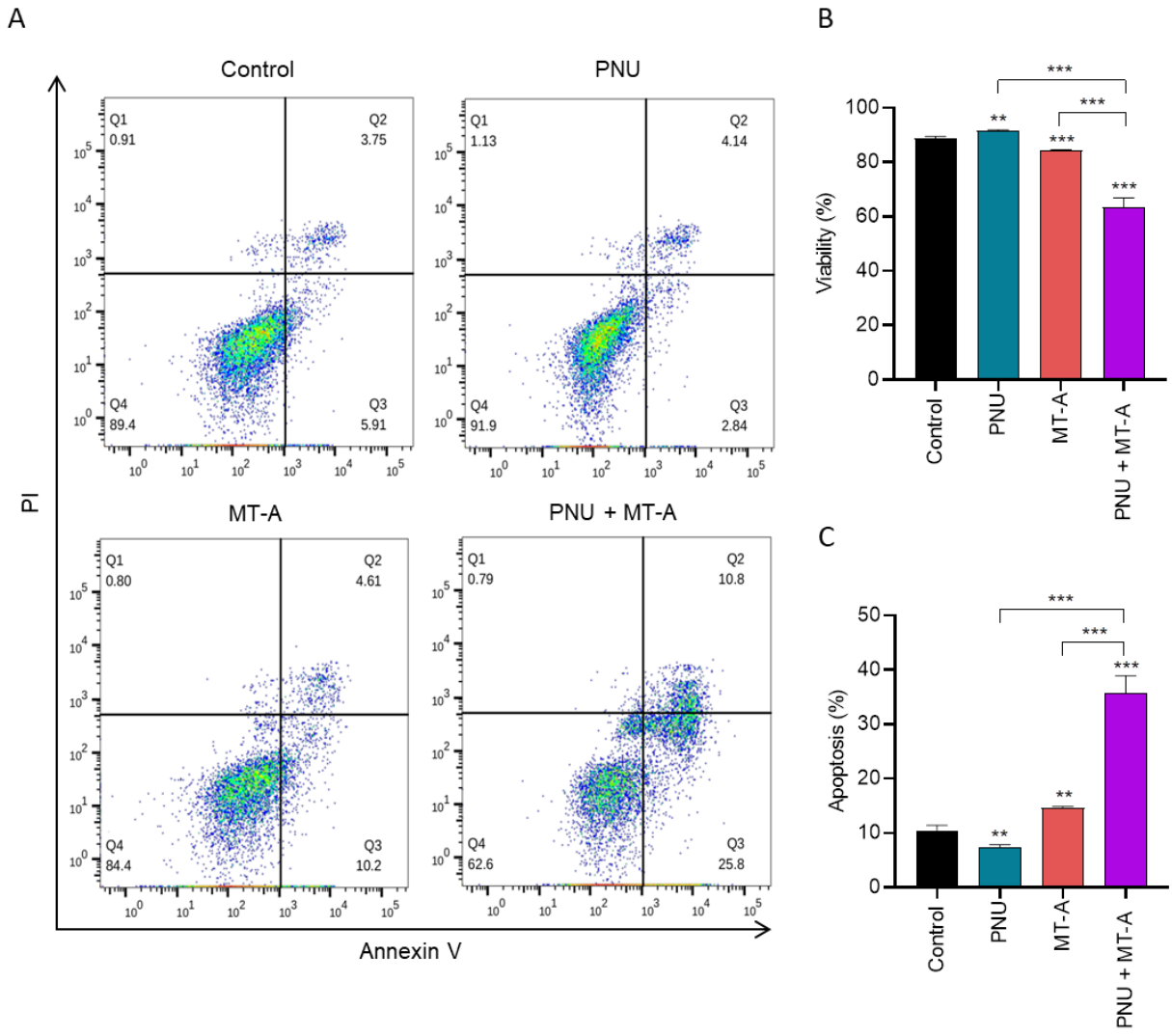

Figure 3.9. Combined inhibition of β-catenin and SP1 promotes cell death. A. Flow cytometry analysis of HCT116 cells stained with Annexin V and PI after treatment with PNU, MT-A and PNU + MT-A. **B.** Quantification of viable cells after Annexin V/PI staining after treatment with PNU, MT-A and PNU + MT-A (Unpaired t-test was used to calculate significance). **C.** Quantification of apoptotic cells after Annexin V/PI staining after treatment with PNU, MT-A and PNU + MT-A (Unpaired t-test was used to calculate significance).

development, using zebrafish development as our model system. We applied the same treatment approach as previously illustrated (Figure 3.11A). Briefly, we titrated 6 doses of both ctnnb1 and sp1 mRNA to identify doses that did not result in significant deformities or mortality. Subsequently, we injected ctnnb1 and/or sp1 mRNA in zebrafish at 1 cell stage and monitored the degree of mortality and phenotypic deformities until 2dpf. During the early stages, there were slight developmental delays associated with the overexpression of ctnnb1 and sp1, but most of these delays

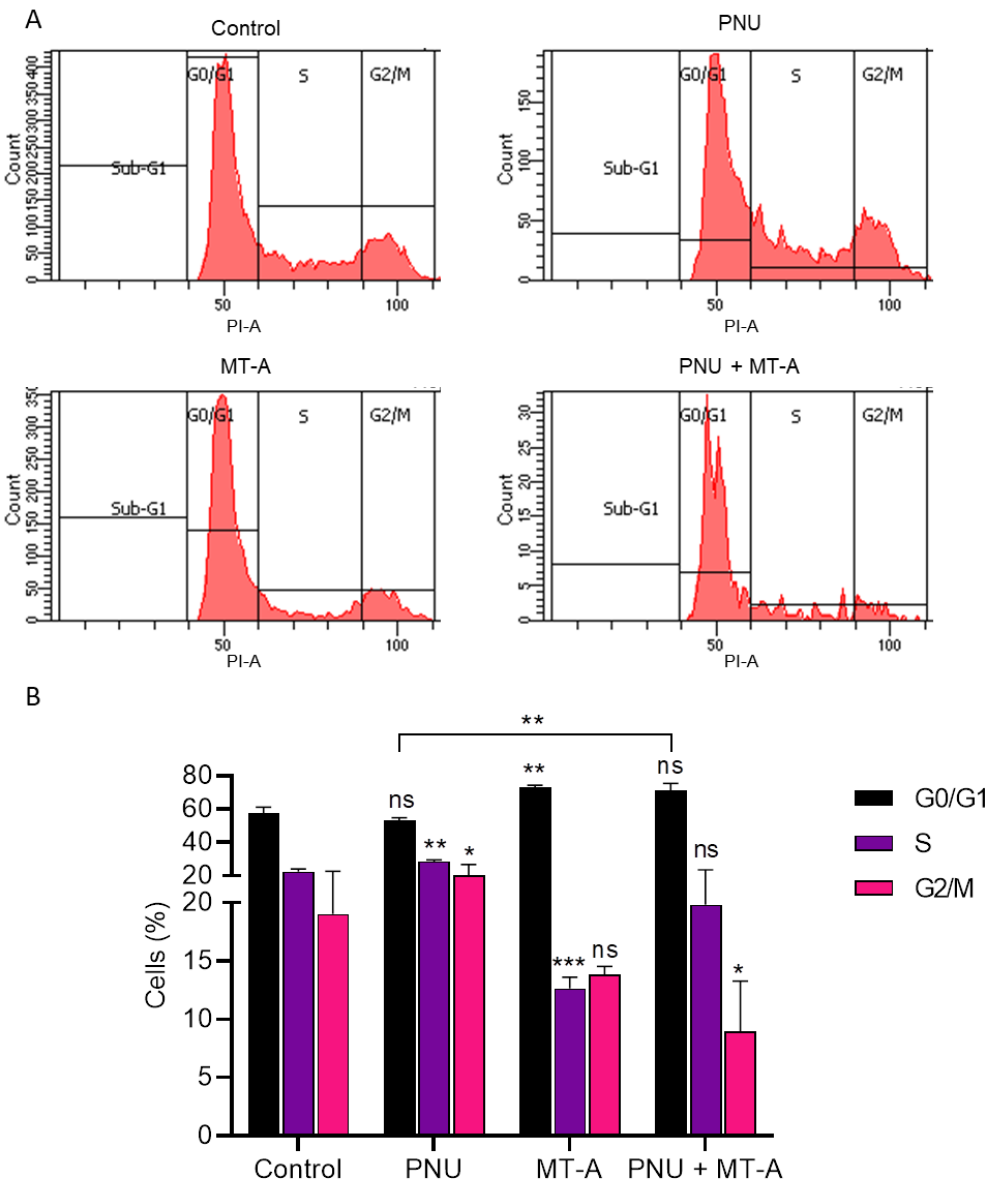

Figure 3.10. Combinatorial inhibition of β-catenin and SP1 affects the cell cycle. A. Flow cytometry analysis after PI staining for HCT116 cells treated with PNU, MT-A and PNU + MT-A. **B.** Cell-cycle analysis of HCT116 cells treated with PNU, MT-A and PNU + MT-A (Unpaired t-test was used to calculate significance).

resolved after the Shield stage (6hpf, i.e., post gastrulation). It is known that overexpression of ctnnb1 leads to anteriorisation due to its role in anteroposterior axis formation (Bellipanni *et al.*, 2006). Interestingly, we observed similar defects in the case of sp1 overexpression, suggesting their involvement in some developmental processes, if not all. Notably, there was a significant increase in the number of larvae displaying defects in their posterior structure (depicted in Figure 3.11B&C). Quantification of

mortality and deformities further revealed that the most pronounced effects were observed in the case of double overexpression (Figure 3.11D&E). The proliferation marker mki67 exhibited the highest expression levels when both β-catenin and Sp1 were overexpressed at 10hpf (Figure 3.11F). These observations strongly suggest that β-catenin and Sp1 jointly participate in specific developmental processes, such as cell proliferation and anterior-posterior axis formation.

3.5 Sp1 and Wnt regulator Apc have opposing effects on the genes required for regeneration after gut development

We integrated another major component of the Wnt signaling pathway in our study-Apc. Apc is a negative regulator of the Wnt pathway and promotes the degradation of β-catenin (Sierra *et al.*, 2006). We obtained the RNA isolated from *apc* mutants and WT siblings at 5dpf from our collaborators. We used these RNA samples to generate mRNAseq libraries for transcriptome analysis. Based on our hypothesis, if Sp1 collaborates with the Wnt signaling during zebrafish development, then depletion of Apc should result in the activation of Wnt target genes. Conversely, Sp1 depletion should result in the repression of Wnt targets (Figure 3.12A). To check whether the reduced levels of Apc or Sp1 affect the same set of genes, we overlapped the DE genes obtained from both datasets. Surprisingly, ~74% of Sp1 targets were found to be affected by *apc* deletion as well (Representation factor: 3.6; $p < 0.000e+00$) (Figure 3.12B). We proceeded with this common set of genes and analyzed the expression of a few relevant factors in both the depletion conditions. We noticed that the profiles obtained under both these conditions were completely contrasting to each other, aligning with our initial hypothesis. Several factors that are enriched in the differentiated compartment of the zebrafish gut epithelium exhibited an increase in the case of Sp1 depletion and a decrease with *apc* deletion. In contrast, certain markers associated with the proliferative compartment showed downregulation in response to Sp1depletion, with relatively little change in *apc* mutants (Figure 3.12C). However, we observed a consistent pattern in the case of gut regeneration markers. The expression of all regeneration markers was elevated in *apc* mutants but reduced in Sp1 morpholino-

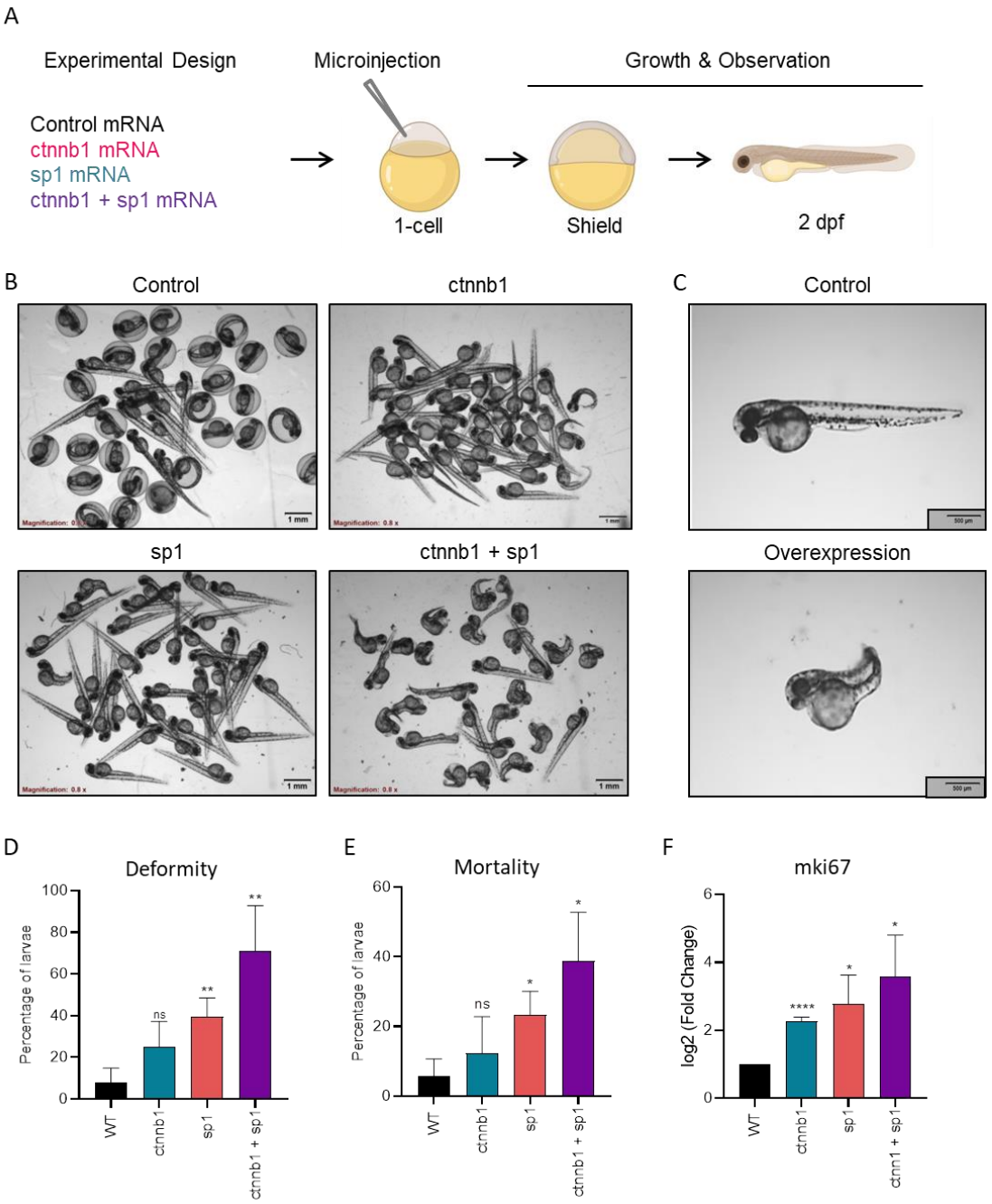

Figure 3.11. Overexpression of β-catenin and SP1 leads to similar developmental defects in zebrafish. A. Schematic representation of the experimental design followed for the overexpression study in zebrafish. **B.** Representative brightfield images for zebrafish injected with control (GFP), ctnnb1 mRNA, sp1 mRNA or both at 2dpf. Magnification = 0.8X, Scale bar = 500 µm. **C.** Representative higher magnification images for zebrafish for control and overexpression phenotype at 2dpf. Magnification = 2X, Scale bar = 500 µm. **D.** Quantification of larvae with deformities at 2dpf (Unpaired t-test was used to calculate significance). **E.** Quantification of mortality at the end of 2dpf (Unpaired t-test was used to calculate significance). **F.** Expression values of mki67 using qRT-PCR at Bud (10hpf) stage (Unpaired t-test was used to calculate significance).

-injected larvae (Figure 3.12D). In summary, our findings reveal the opposing roles of Apc and Sp1 during zebrafish development, hinting towards a potential collaboration between Sp1 and Wnt signaling facilitating the continuous regeneration and growth of the gut epithelium.

Summary and Conclusions

Through various assays, we have demonstrated that β-catenin and SP1 play a role in promoting cellular proliferation and preventing cell death. Under normal growth and development conditions, this partnership contributes to organ growth and maintenance of adult stem cells. However, excessive activation of this complex can lead to pathological conditions, notably cancer. We show that the gene signature of β-catenin and SP1 is remarkably conserved across multiple species. Notably, the same signature exhibits heightened activity in colorectal cancer. We further assessed the synergistic potential of β-catenin and SP1 in tumorigenesis through pharmacological inhibition, which yielded similar outcomes. The combined inhibition of both β-catenin and SP1 shows promise as a therapeutic approach for restraining the growth of colorectal tumors by simultaneously targeting both proteins.

Tumorigenesis and several other cancer-associated phenomena, such as EMT and angiogenesis, involve the re-activation of pathways such as Wnt, Notch and Hedgehog. These pathways play a pivotal role in regulating normal cell growth, motility, and differentiation during development. However, cancer cells exploit these developmental pathways to sustain their own population of cancer stem cells, promote cell growth, undergo EMT, and migrate to other organs (Kamdje *et al.*, 2017). Consequently, at the molecular level, cancer cells often exhibit similarities to normally developing cells. In the context of the gut epithelium, the Wnt and BMP signaling pathways, essential for endoderm formation during development as well as ISC maintenance during adulthood, have been implicated in the initiation and progression of CRC (Sharma *et al*., 2021). Given that genes co-regulated by β-catenin and SP1 are enriched for cellular proliferation and stemness; we aimed to determine if this signature is also vital during

normal development.

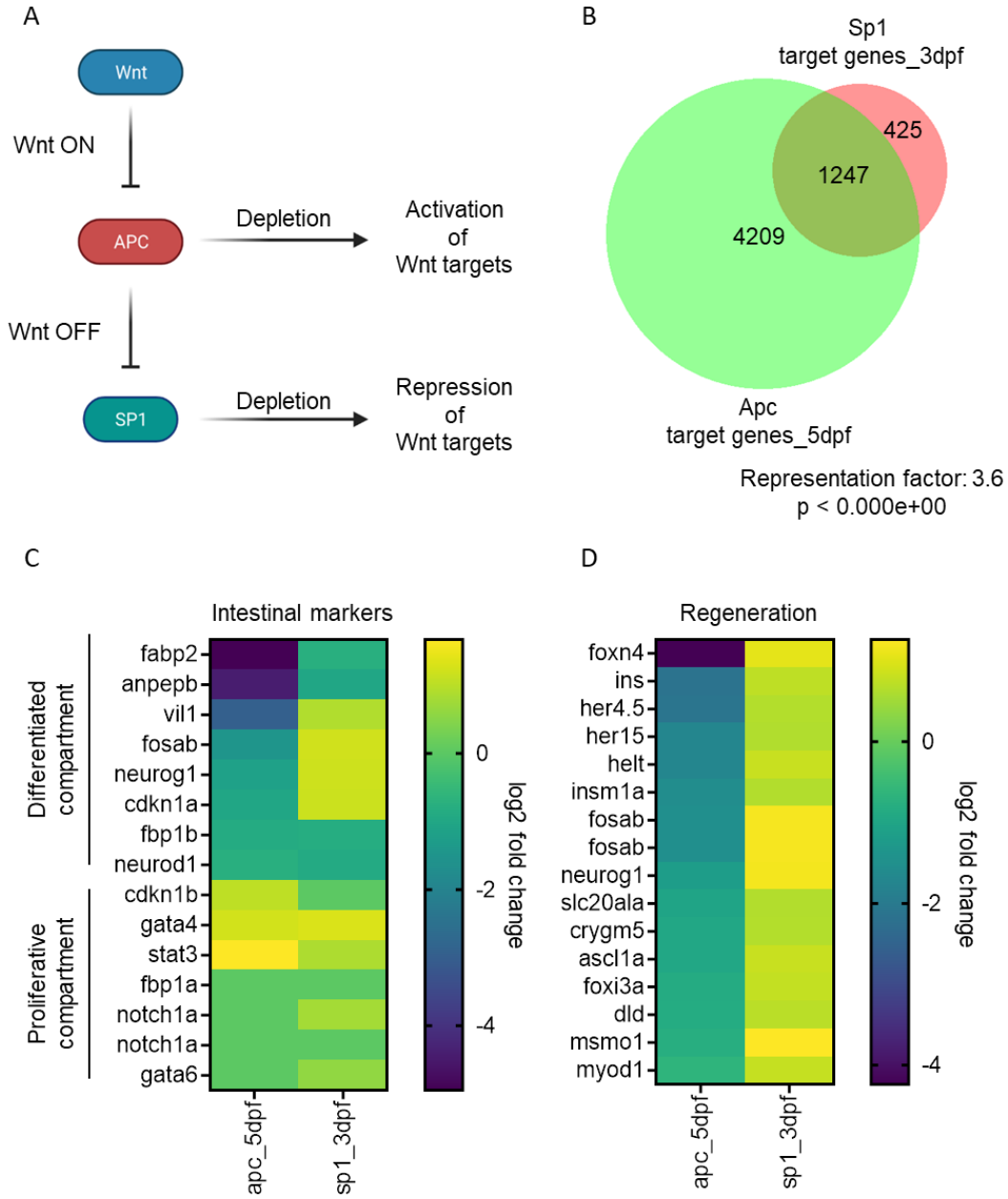

Figure 3.12. Sp1 and Wnt regulator Apc have contrasting effects on the genes essential for regeneration. A. Schematic representation of the hypothesis for the contrasting actions of Sp1 and Apc in zebrafish. **B.** Venn diagram depicting the overlap between the DE genes obtained after treatment with sp1 morpholino until 3dpf or *apc* deletion at 5dpf. **C.** Expression values for the intestinal epithelial markers (differentiated and stem cell markers) in *apc*$^{-/-}$ line at 5dpf and sp1 morpholino treatment until 3dpf. **D.** Expression values for the intestinal regeneration markers in *apc*$^{-/-}$ line at 5dpf and sp1 morpholino treatment until 3dpf.

As Sp1 has not been investigated in the chosen model organism, our initial focus was on characterizing its expression and role in zebrafish embryos. We observed that Sp1

displayed ubiquitous expression during the early stages of development but became more localised to the head and gut regions as development progressed. Based on this expression pattern, we hypothesized that Sp1 might be regulating fundamental cellular processes such as cell proliferation and differentiation during the early developmental phases. Indeed, we found that Sp1 played a role in the regulation of cell proliferation and endoderm formation, mirroring observations made in human cell line systems. In the later stages of development and adulthood, Sp1 might participate in the regulation of specific organ systems. This is supported by the enrichment of DE genes identified from the transcriptome of Sp1 morpholino-injected larvae, which showed associations with processes related to the nervous system, including synaptic signaling and neurotransmitter transport. Given that human SP1 is known to be involved in nervous system development and regulation, it is plausible that zebrafish Sp1 serves a similar conserved function (Citron *et al.*, 2008; García-Huerta *et al.*, 2012; Wang *et al.*, 2012).

When we combined our study of Sp1 with β-catenin, we observed remarkably similar phenotypic defects resulting from the overexpression of either β-catenin, Sp1, or both. During the early stages of development, we did not observe significant gross morphological changes. However, at 1dpf and in subsequent stages, we noticed anteriorization of the larvae. The Wnt/β-catenin signaling pathway is known to participate in the specification of the anteroposterior axis, with β-catenin promoting the formation of the anterior body structure. Consequently, its over activity leads to the development of an anterior structure at the expense of the posterior portion, disrupting the gradient necessary for posterior formation (Hikasa and Sokol, 2013). This phenomenon is well- documented and has been observed in various systems, including Xenopus, where overexpression of β-catenin results in axis duplication and the development of two heads (Funayama *et al.*, 1995). Given that Sp1 overexpression leads to a similar defect in zebrafish, it suggests a potential role for Sp1 in axis formation as well. The exponential increase in the frequency and severity of deformities observed with combined overexpression of β-catenin and Sp1 further supports the notion of these proteins working together.

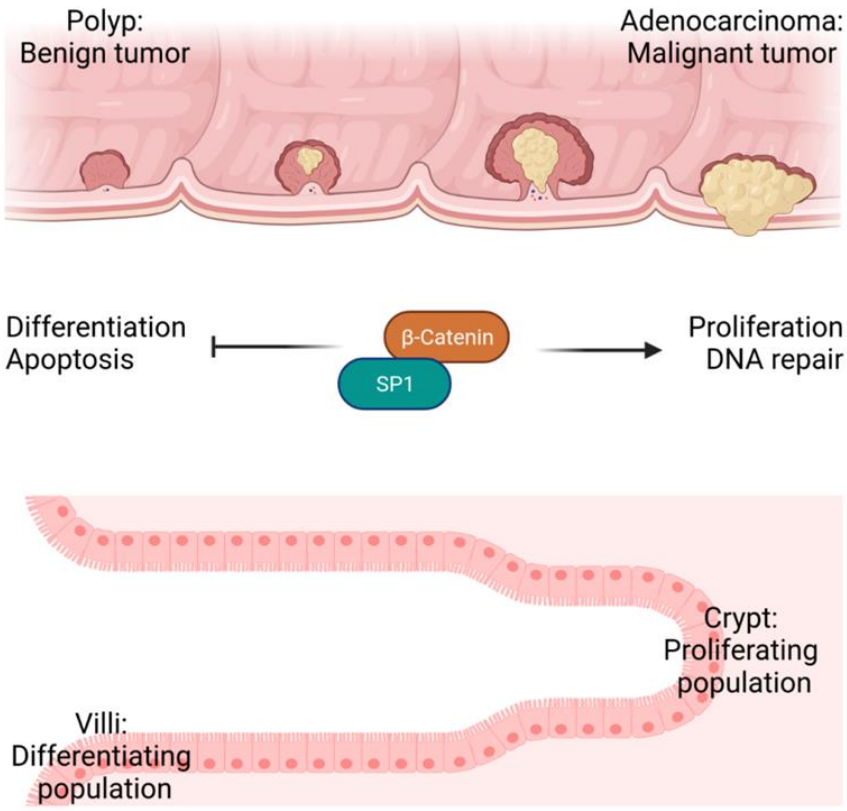

Figure 3.13. Model for the conserved function of the β-catenin and SP1 complex in development and disease. β-catenin and SP1 promote cellular proliferation and prevent cell death. During regular growth and development, it can help in organ growth and maintenance of adult stem cells (lower panel). However, in case of abnormal stimulation, it can contribute towards the aggressiveness of cancer.

Furthermore, our research reveals the antagonistic effects of Sp1 in comparison to the negative regulator of Wnt/β-catenin signaling, Apc. We found a significant overlap in the target genes regulated by Sp1 and Apc. Importantly, many of these shared targets, which play a role in intestinal epithelium regeneration, are regulated in opposing directions by these two proteins. This finding underscores the interplay between Sp1 and β-catenin in maintaining the normal turnover of the gut epithelium during later stages of development. In summary, our study demonstrates the existence of the β-catenin-SP1 axis *in vivo*, with relevance in regular cell proliferation and potential involvement in anterior-posterior axis formation. Furthermore, we show that in adulthood, this axis plays a crucial role in regulating the intestinal lining, and hyperactivation of this complex can lead to excessive cell proliferation, ultimately contributing to cancer.

Limitations: It will be useful to generate *sp1* mutant line to explore the consequences of sustained Sp1 depletion and investigate the potential for compensation at the later stages. Our previous attempts to generate a mutant fish were only partially successful, resulting in a non-functional mutation in the targeted Exon-3. It is conceivable that a more extensive alteration in the gene might yield a functional mutation. In the context of mice, SP1 deletion is shown to be lethal, even in the presence of SP3, a closely related protein with partially overlapping targets and functions. However, in zebrafish, compensation could potentially occur through the *sp1l* gene. Given that the function of the *sp1l* gene remains uncharacterized, it becomes an important avenue for exploration in future studies.

CHAPTER IV
Transcriptional Integration of Cellular Proliferation by SP1 and YAP in Colorectal Cancer

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Introduction

The Hippo pathway was first identified in *Drosophila*. Mutations affecting the Hippo pathway components resulted in developing abnormally large flies, hence the origin of its name. This pathway plays a significant role in regulating organ size during development (Justice *et al.*, 1995; Xu *et al.*, 1995; Jia *et al.*, 2003; Udan *et al.*, 2003). The Hippo pathway is not activated by a defined set of receptors. Instead, mechanosensory cues regulate the activation of a series of Hippo kinases, including cell adhesion, polarity and cell-cell junctions (Zhao *et al.*, 2007; Lei *et al.*, 2008). Various proteins have been identified that regulate this activation, e.g. Merlin and kidney and brain expressed protein (KIBRA) (Genevet *et al.*, 2010; Varelas, Samavarchi-Tehrani, *et al.*, 2010; Zhang *et al.*, 2010; Benham-Pyle, Pruitt and Nelson, 2015; Li *et al.*, 2016). Activation of the Hippo pathway ultimately results in the deactivation of its effector molecules, YAP and TAZ. This pathway comprises of a cascade of kinases that are activated upon activation of Hippo signaling. These kinases include Macrophage-stimulating protein1/2 (MST1/2), Salvador (SAV1), Large tumor suppressor (LATS1/2) and Mps one binder kinase (MOB1) (Figure 4.1). The phosphorylated and active form of MST1/2 forms a complex with phosphorylated SAV1 and this complex subsequently phosphorylates LATS1/2. Activation of LATS1/2 (along with MOB1) leads to the phosphorylation of YAP/TAZ. The phosphorylated form of YAP/TAZ can either remain sequestered in the cytoplasm (via 14-3-3) or undergoes degradation (Chan *et al.*, 2005; Callus, Verhagen and Vaux, 2006; Zhao *et al.*, 2007; Praskova, Xia and Avruch, 2008; Varelas, Samavarchi-Tehrani, *et al.*, 2010). The absence of Hippo signaling allows nuclear accumulation of YAP/TAZ. The YAP/TAZ proteins do not harbor a DNA binding domain. They bind to the transcriptional enhanced associate domain (TEAD) family of

transcription factors, leading to the transcription of their downstream targets (e.g., *MYC*, *FGF1*, and others) (Vassilev *et al.*, 2001; Mahoney *et al.*, 2005; Murakami *et al.*, 2019).

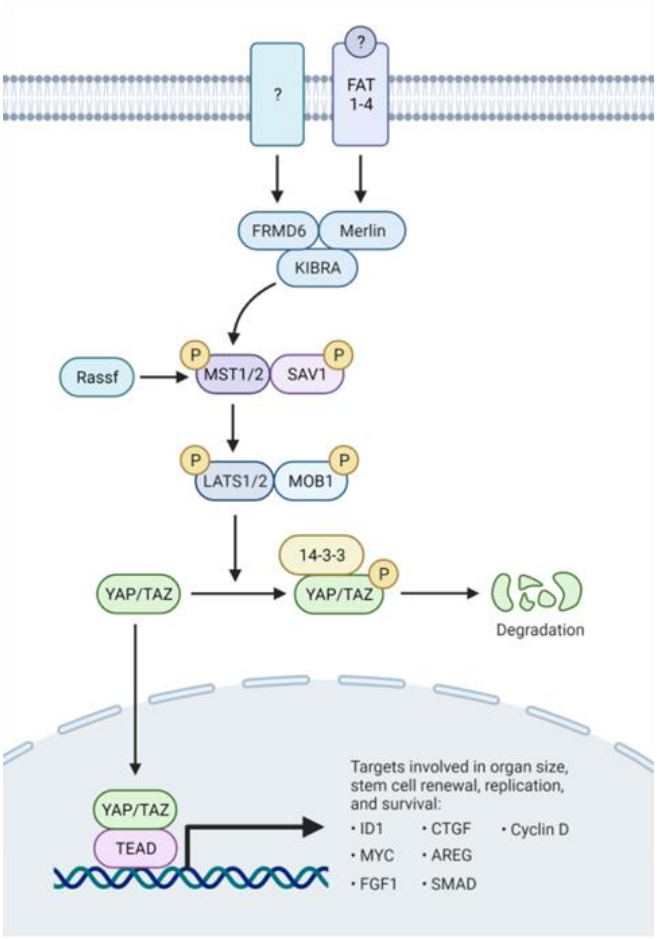


Figure 4.1. The components of the Hippo signaling pathway. The Hippo pathway contains a series of kinases which regulate the activation of the Hippo effectors YAP/TAZ. Physical cues for the Hippo pathway promote phosphorylation and activation of kinases MST1/2 and SAV1. This further leads to the phosphorylation and activation of LASTS1/2 and MOB1. LATS1/2 directly phosphorylates YAP/TAZ. The phosphorylated form of YAP/TAZ can either remain sequestered in the cytoplasm via the 14-3-3 complex or is degraded. In the state of inactive Hippo kinases, YAP/TAZ can translocate inside the nucleus and bind to the TEAD family of transcription factors to activate transcription.

Interplay between the Wnt and the Hippo signaling pathways

The first link between the Wnt and Hippo pathway was established in 2010 by Varelas *et al*., which demonstrated that TAZ is a negative regulator of the Wnt pathway. It was demonstrated that TAZ inhibits Dishevelled activation via its phosphorylation by CK1δ/Ɛ, leading to the inhibition of Wnt signaling. It was also demonstrated that the overexpression of MST1/2 and LATS1/2 resulted in increased cytoplasmic levels of TAZ and reduced Wnt reporter activity (Varelas *et al.*, 2010). However, a subsequent study revealed that the knockout of TAZ in mice kidneys led to only a nominal increase in β-

catenin levels (Makita *et al.*, 2008). Another piece of evidence supporting positive regulation through the Hippo pathway showed that the S127D mutant YAP (phosphomimetic form, constitutively cytoplasmic) inhibited the nuclear accumulation of β-catenin (Imajo, Ebisuya and Nishida, 2015). Some reports suggested the negative role of the Hippo pathway in regulating Wnt signaling. The knockout of Salvador in mice cardiomyocytes led to increased β-catenin expression. It was also revealed that both YAP/TEAD and β-catenin/TCF complexes cooperatively bind to some of their target genes, such as *Snail2* and *Sox2* (Heallen et al., 2013).

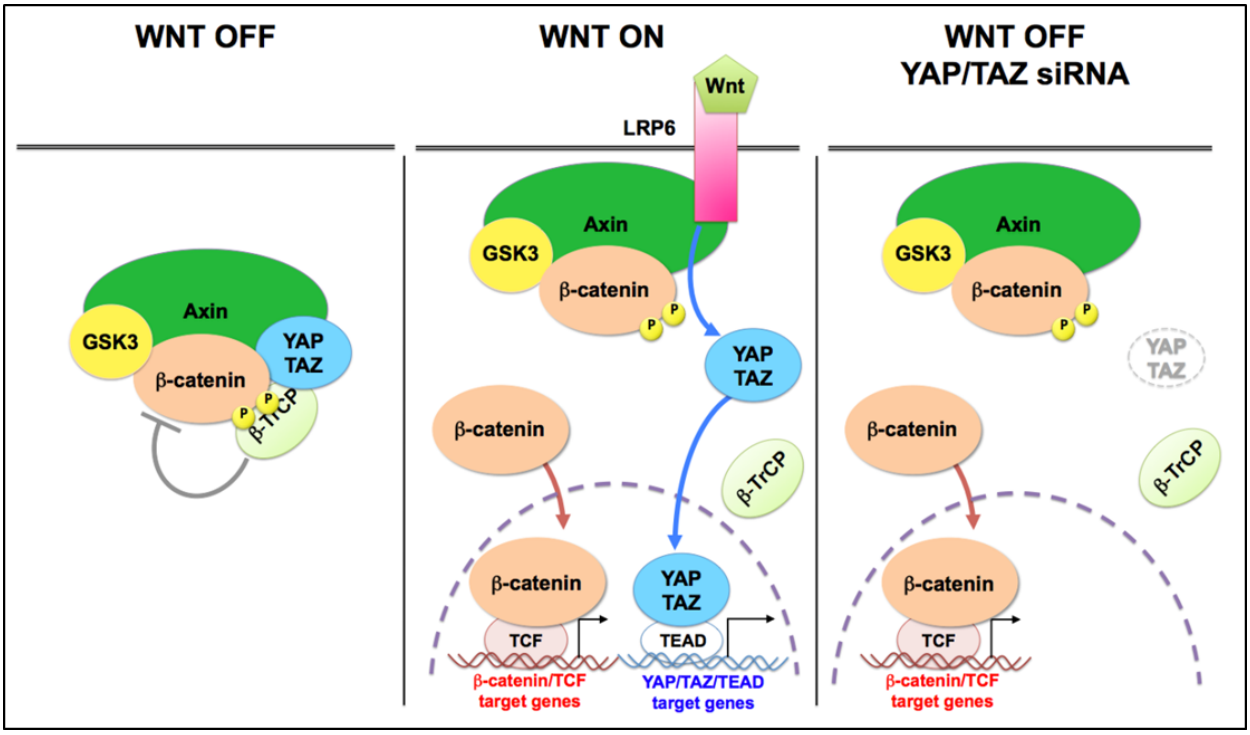


Figure 4.2. Crosstalk between the Wnt and the Hippo pathways. YAP/TAZ is an integral part of the WDC and regulates β-catenin. In the absence of Wnt activation, β-TrCP is recruited to the WDC via YAP/TAZ, thereby leading to the degradation of both β-catenin and YAP/TAZ (Left panel). Upon activation of Wnt signaling, β-catenin and YAP/TAZ are released to enter the nucleus and bind to their respective DNA binding partners (as depicted in Middle panel). Depletion of YAP/TAZ is adequate to enhance the stability of β-catenin, as it hinders β-TrCP from being recruited to the WDC (Right panel). (Adapted from Azzolin et al., 2014).

While all these studies identified Hippo as a regulator of Wnt signaling, two studies published by Piccolo's group demonstrated the regulation of Hippo effectors YAP/TAZ by β-catenin. It was demonstrated that phosphorylated β-catenin, which binds to β-TrCP, serves as a scaffold for TAZ to attach to the destruction complex, leading to its

degradation (Azzolin *et al.*, 2012). Another study demonstrated that YAP/TAZ is integral to the WDC. Further, YAP/TAZ is responsible for recruiting β-TrCP to the destruction complex, promoting the degradation of β-catenin (Figure 4.2). In fact, depletion of YAP/TAZ is sufficient to stabilise the newly formed β-catenin (Azzolin *et al.*, 2014). Thus, multiple pieces of evidence suggest the crosstalk between Hippo and Wnt signaling, primarily through the components of the destruction complex.

Earlier studies in our lab have shown that the Wnt/β-catenin pathway regulates SP1. In absence of Wnt signaling, SP1 interacts with the WDC, leading to its degradation, mediated by phosphorylation via GSK3β and ubiquitination via βTrCP. The activation of Wnt signaling leads to the stabilisation of SP1 by impeding its interaction with βTrCP. Furthermore, SP1 and β-catenin physically interact and mutually stabilise each other (Mir *et al.*, 2018). As SP1 and YAP interact with β-TrCP and are dependent on β-catenin for their stability, we aimed to investigate whether Hippo pathway exerts control over SP1 and whether SP1 and YAP interact with each other. This Chapter unveils the direct regulation of SP1 by the Hippo pathway. Furthermore, we demonstrate that SP1, reciprocally, plays a role in stabilizing YAP. Lastly, SP1 shares occupancy with TEAD4 at its target sites and governs the genes associated with cell cycle regulation and proliferation.

Material and methods

The following methods were used essentially as described in Chapter 2: Human cell line culture and transfection, RNA isolation, RT-qPCR and analysis; Protein extraction, western blotting and analysis; mRNA sequencing and analysis; Annexin V/Propidium Iodide staining and quantification for cell viability and cell cycle analysis.

Immunofluorescence analysis and quantification

Cells were cultured on fibronectin-coated glass coverslips and fixed using 4% paraformaldehyde (PFA) (Merck) in 1X PBS at RT for 15 minutes. Permeabilization was

performed using 0.25% Triton X-100 for 5 minutes, followed by blocking in 10% bovine serum albumin (BSA) in 1X PBS at RT for 1 hr. The cells were then incubated in primary antibody (1:100 in 5% BSA in 1X PBS) at 4°C for 16 hr, followed by four washes in 1X PBS at RT, incubation in secondary antibody (1:1000 2% BSA) for 1 hr, and four washes in 1X PBS. Imaging was performed using the Zeiss 710 LSM confocal microscope. Quantification of the nuclear intensity was performed using the ImageJ software. The nuclear area within each cell was selected using 4′,6-diamidino-2-phenylindole (DAPI) staining as the region of interest (ROI). Quantified nuclear intensity was normalized with the nuclear area and DAPI. At least 300 cells were used for quantification in each set. A list of the siRNAs and plasmids sequences used is provided in Appendix 1 and 2, respectively.

Analysis of Chromatin Immunoprecipitation-sequencing (ChIP-seq) datasets

The following datasets were used for analysis (https://www.encodeproject.org/): ENCSR000BVJ, ENCSR000BMK, ENCSR000BSF, ENCSR000BVT, ENCSR161MXP, ENCSR333OPW, ENCSR661KMA, ENCSR000EUV (ENCODE Project Consortium, 2012). The sequencing reads were trimmed using Trimmomatic-0.39 and aligned to GRCh38 genome assembly for humans using the BWA alignment program (Li and Durbin, 2009; Bolger, Lohse and Usadel, 2014). MACS2 was used for peak calling with default parameters and q value 0.05(Zhang *et al.*, 2008). Consensus peaks from the biological replicates were extracted using a custom R script from Roman Cheplyaka (https://ro-che.info/articles/2018-07-11-chip-seq-consensus). Representative figures depicting the performed analysis were created using deepTools 3.3.2 and Integrative Genomics Viewer (IGV) (Robinson *et al.*, no date; Ramírez *et al.*, 2016). BigWig files were generated using bamCoverage (deepTools). Peak annotation was performed using the nearest neighbour method using Homer and further classified into promoter (±5 Kb) and non-promoter regions. Gene ontology analysis was performed using Metascape and WebGestalt (Wang *et al.*, 2017; Zhou *et al.*, 2019).

Results and discussion

4.1 Hippo pathway negatively regulates SP1

We performed a cell density assay to investigate whether the Hippo pathway exerts control over SP1. While it is important to note that no dedicated receptors have been identified for the Hippo pathway as yet, it is responsive to various physical cues, with cell density being one of them (Zhao *et al.*, 2007). High cell density activates Hippo kinases, leading to inhibition of YAP nuclear translocation, whereas low cell density has the opposing effect, promoting YAP nuclear translocation via inhibition of Hippo kinases (Figure 4.3A). To determine if SP1 protein levels correlate with the cellular density, we performed this assay using multiple cell lines. In HEK293T and HeLa cells, we observed reduced SP1 protein levels at high cell density. However, HCT116 cells did not exhibit a significant change in SP1 protein levels (Figure 4.3B). Some cells respond to cell density through contact inhibition, but several aggressive cell lines do not respond to the cell density due to inactive Hippo activity. The HCT116 cell line has been reported to display this phenomenon due to constitutively high YAP expression, independent of the cell density. A similar trend was observed in SP1 levels. To further explore this connection, we overexpressed WT YAP and its active variants S127A and 5SA in HEK293T cells. The S127A variant includes a mutation converting a Serine to an Alanine in one of the active sites for LATS1/2, while the 5SA variant entails Serine-to-Alanine mutations in all the five LATS1/2 sites, rendering it highly active in comparison to S127A. Interestingly, all the forms of YAP upregulated SP1, consistent with our earlier findings (refer to Figure 4.3C-E). Notably, *SP1* promoter harbors a TEAD binding motif, and the overexpression of TEAD1 in HCT116 cells was found to enhance cell proliferation by increasing SP1 expression (Yu and Zhang, 2016). Collectively, these results indicate that SP1 is subjected to negative regulation by the Hippo pathway.

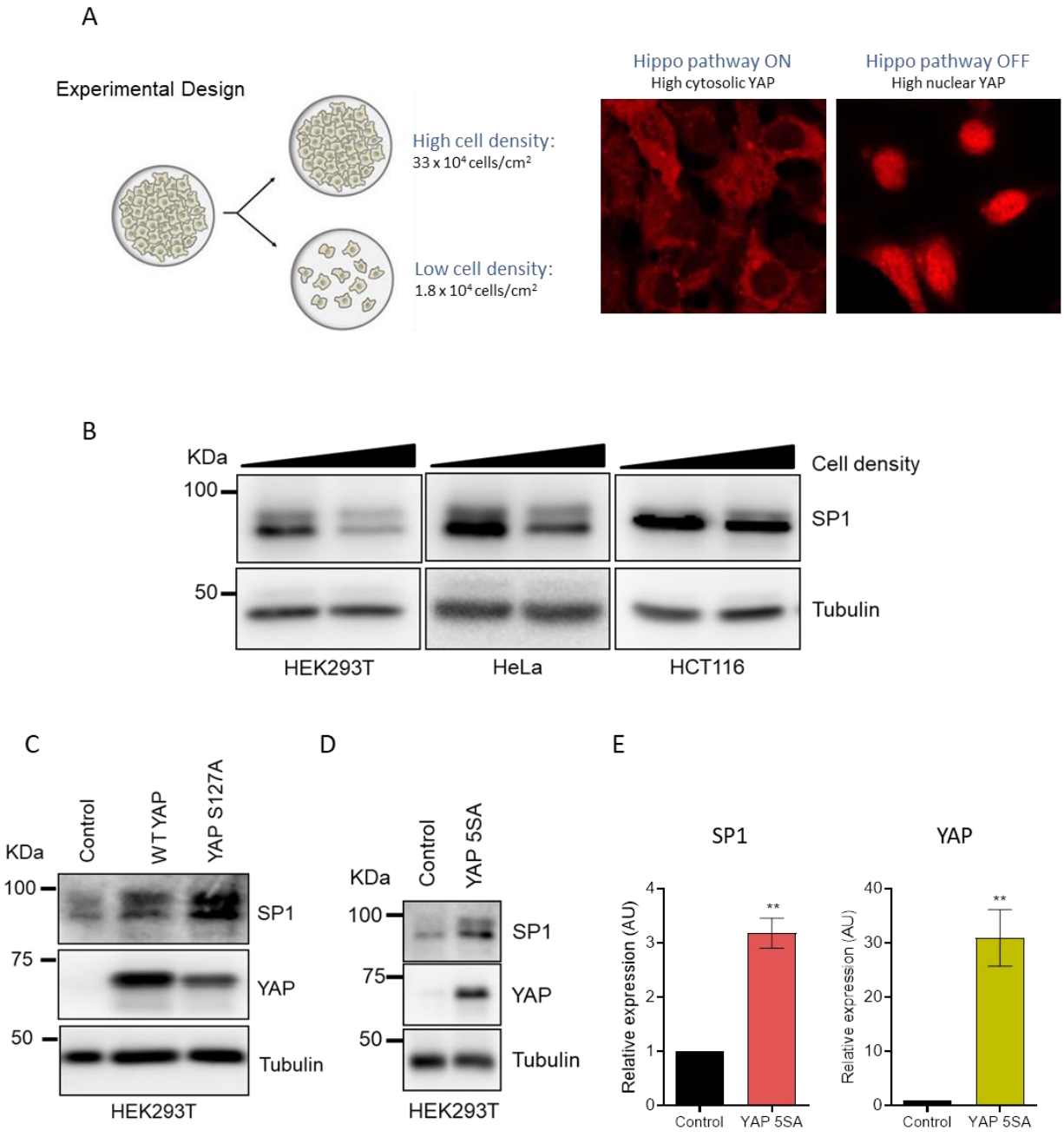


Figure 4.3. SP1 is regulated by cellular density. A. Experimental design for the cell density assay. **B.** Immunoblot analysis of SP1 and Tubulin expression at low and high cell densities in HEK293T, HeLa and HCT116 cell lines. **C.** Immunoblot analysis of SP1, YAP and Tubulin expression in control HEK293T cells and cells ectopically expressing WT and YAP S127A mutant proteins as indicated. **D.** Immunoblot analysis of SP1, YAP and Tubulin expression in control HEK293T cells and cells ectopically expressing YAP 5SA mutant protein. **E.** Densitometric analysis of immunoblots monitoring SP1 (left panel) and YAP (right panel) expression in control cells and YAP 5SA overexpressing HEK293T cells (Unpaired t-test was performed to calculate significance).

4.2 SP1 positively regulates the Hippo pathway effector YAP in a Wnt-dependent manner

Next, we aimed to assess whether SP1 can regulate the Hippo effector YAP itself. HEK293T cells exhibited both nuclear and cytosolic YAP under control conditions (Control siRNA at low density). However, when SP1 was depleted (using siSP1 at low density), it resulted in a noticeable reduction in the overall staining of YAP. Remarkably, the levels of nuclear YAP appeared to be highly affected (Figure 4.4A). We quantified the nuclear staining intensity for SP1 and YAP in both conditions, and a significant reduction in the overall nuclear intensity was observed for both (Figure 4.4B). We also conducted a correlation analysis between the intensity values of SP1 and YAP within each nucleus, revealing a positive correlation between these two proteins (Figure 4.4C). Furthermore, a slightly lower but significant positive correlation was evident when the cells were treated with siSP1 (Figure 4.4D). Likewise, the overexpression of SP1 led to increased YAP levels. Overexpression of both the wild-type (WT) and mutant (MT) forms of SP1 resulted in increased YAP protein levels (Figure 4.4E). Intriguingly, the increase in the YAP protein was slightly more pronounced in the case of MT SP1, even when the levels of overexpression for both the WT and MT SP1 were equivalent (as demonstrated in Figure 4.4F). As described previously, the MT form of SP1 is more stable than WT SP1 since it lacks the GSK3β phosphorylation site. Notably, our transcriptome data indicated that SP1 does not regulate YAP at the transcript level (Figure 4.5A), suggesting a direct role of SP1 in assisting the stabilization of YAP protein.

We investigated the possibility of a physical interaction between SP1 and YAP using HCT116 cells. Our observations revealed a robust nuclear co-localisation and interaction between these two proteins (Figure 4.5B&C). Subsequently, we performed a Wnt activation assay in HEK293T cells, which are sensitive to the Wnt ligands. As anticipated, the proteins β-catenin (serving as a control), SP1 and YAP were all stabilised upon treatment with Wnt3A (Figure 4.5D). However, when SP1 was depleted from the cells, this stabilisation was lost, even in the presence of Wnt activation

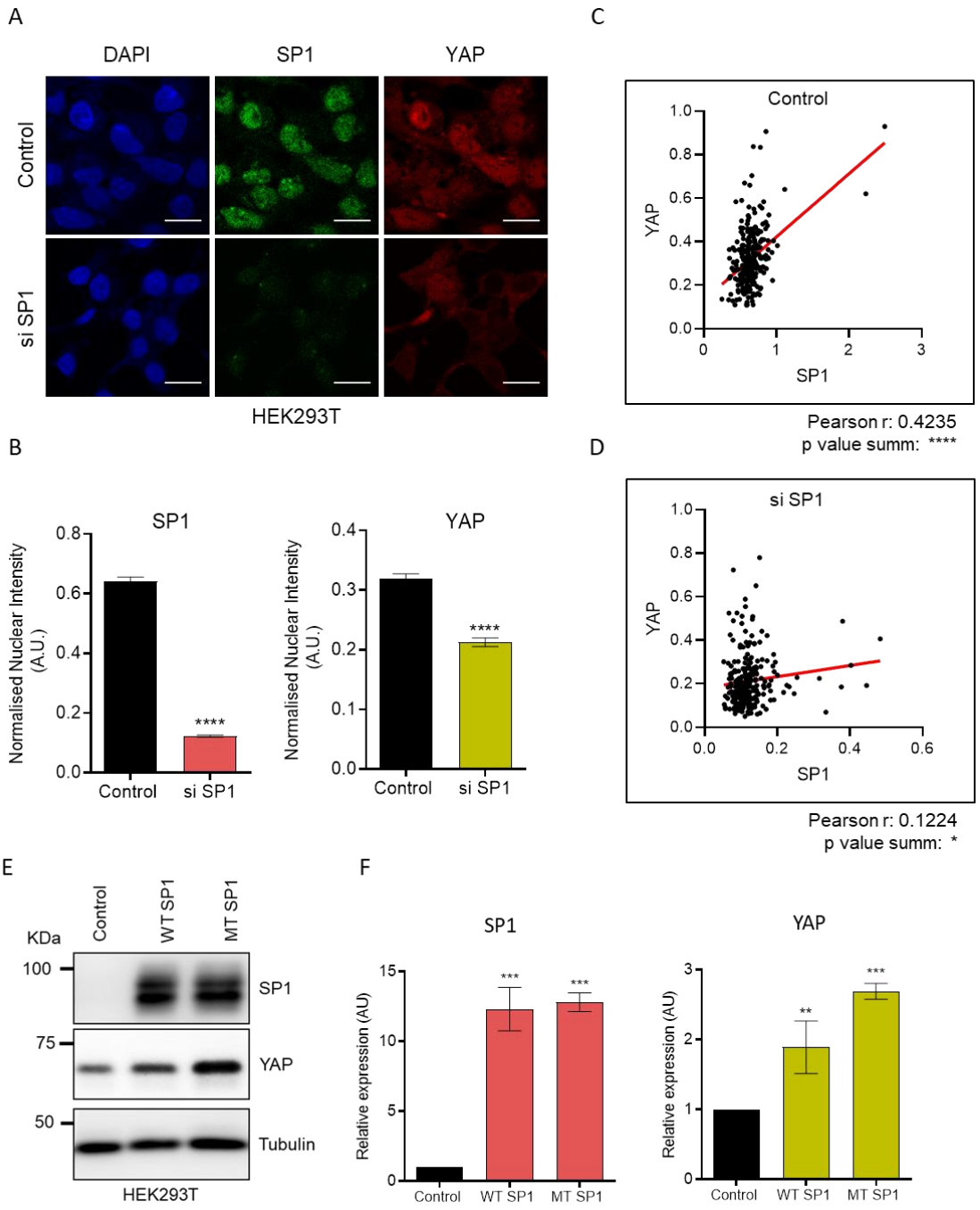


Figure 4.4. SP1 positively regulates YAP. A. IFA images for SP1 and YAP in HEK293T cells under control condition and siSP1 treatment. Scale bar = 20 µm. **B.** Quantification of the nuclear intensities for SP1 (left) and YAP (right) immunostaining in control and siSP1 treated HEK293T cells (Unpaired t-test was performed to calculate significance). **C.** Scatter plot for nuclear intensities for SP1 and YAP in control condition for HEK293T. A regression line is shown in red (Paired t-test was performed to calculate significance). **D.** Scatter plot for nuclear intensities for SP1 and YAP after siSP1 treatment of HEK293T cells. A regression line is shown in red (Paired t-test was performed to calculate significance). **E.** Immunoblot analysis to monitor the expression of SP1, YAP and Tubulin in control and ectopic expression of WT and mutant (MT) SP1 in HEK293T. **F.** Densitometric analysis of immunoblots for monitoring SP1 (left panel) and YAP (right panel) expression under control condition and ectopic expression of WT and mutant (MT) SP1 proteins (Unpaired t-test was performed to calculate significance).

(Figure 4.5D-G). In summary, our findings indicate that SP1 plays a role in stabilising YAP upon Wnt activation, which in turn can facilitate its nuclear translocation.

4.3 YAP regulates cell cycle and cytoskeletal machinery in HCT116

Given that SP1 and YAP, both are activated by Wnt and exhibit nuclear co-localisation, our objective was to investigate the specific cellular processes they jointly regulate. We initiated this study by generating transcriptome data in HCT116 cells upon depletion of YAP individually and in combination with SP1, similar to our approach with SP1 and β-catenin (Outlined in the schematic shown in Figure 4.6A). Interestingly, the morphological changes observed with siYAP treatment differed from those observed with SP1 and β-catenin. We did not observe any detached cells indicative of cell death; however, there was a noticeable alteration in cell spreading patterns, suggesting an effect on the cellular cytoskeletal machinery. When siSP1 and siYAP were used in combination, the combined effect included both a reduction in the number of cells and a constricted cell spreading pattern (Figure 4.6B).

Before delving further, we analysed the transcriptome of cells in which YAP was solely depleted. This analysis revealed a significant reduction in YAP mRNA levels in siYAP-treated cells, confirming the effectiveness of the knockdown (Figure 4.7A). Within this dataset, 2071 genes exhibited upregulation, while 2052 genes were downregulated (Figure 4.7B). When we performed Gene Ontology (GO) analysis for the DE genes, it revealed an enrichment of genes associated with morphogenesis and various signaling processes among the upregulated genes (Figure 4.7C). Conversely, the downregulated genes were linked to the processes such as the cell cycle and DNA replication (Figure 4.7D). Notably, YAP is known to play a role in promoting cellular proliferation, both in colorectal cancer and in the normal regeneration of the intestinal lining (E.g., via *CCNA2, ATM*, *PCNA* and *MCM4* genes). Furthermore, various genes involved in cellular cytoskeletal rearrangements displayed dysregulation, including *WNT3A*, *WNT7B* and the Hippo regulator *NF2*. Changes in expression levels of these target genes are presented in Figure 4.7E. Consequently, out findings enabled us to generate

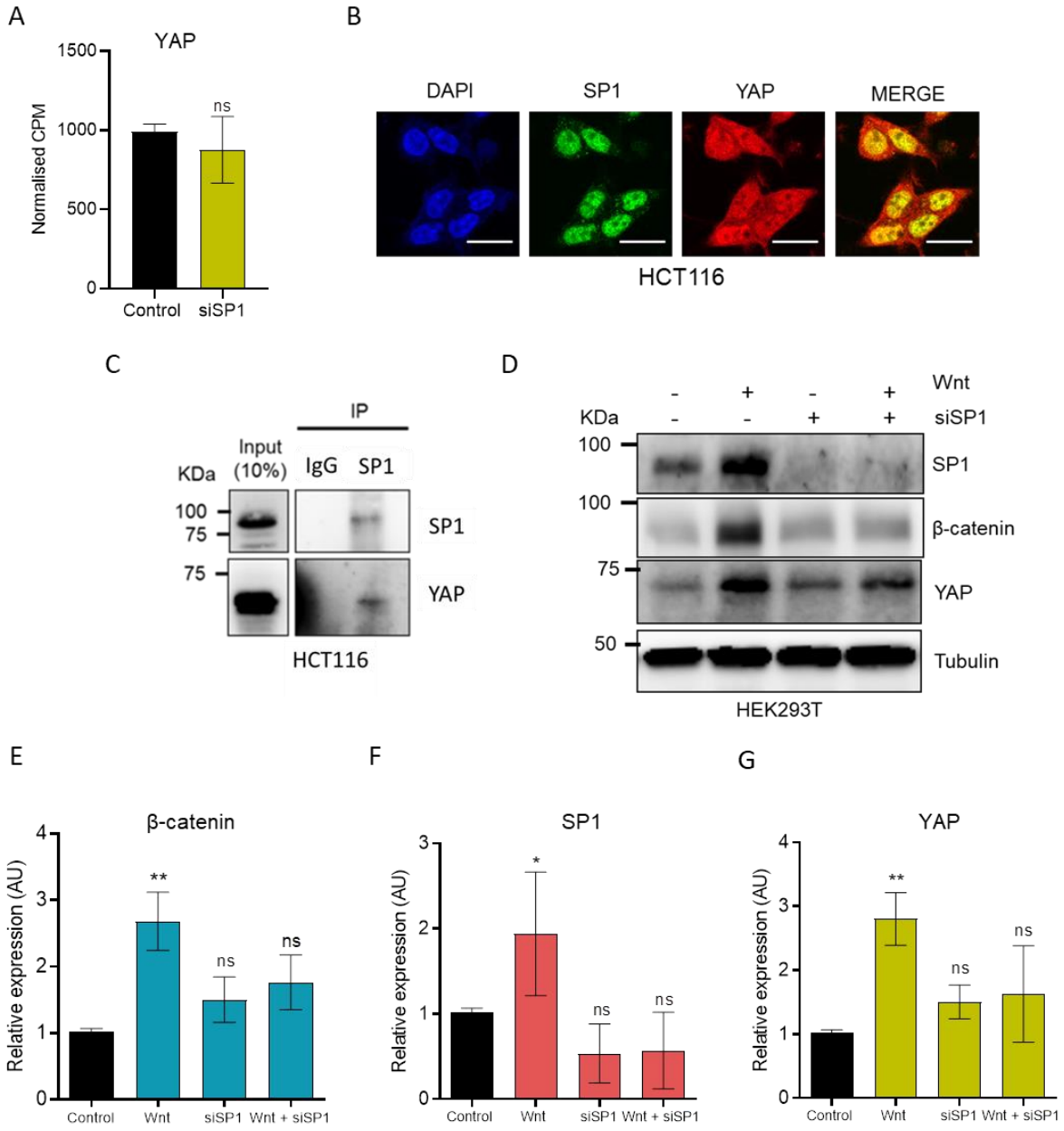


Figure 4.5. Wnt-mediated stabilisation of YAP is SP1-dependent. A. Expression of YAP mRNA in control and SP1 siRNA treated HCT116 cells (Unpaired t-test was performed to calculate significance). **B.** IFA images for SP1 and YAP in HCT116 cells. Scale bar = 20µm. **C.** WB image for Co-IP assay. Anti-SP1 antibody was used to perform IP, and anti-YAP antibody was used to perform IB in HCT116 cells. **D.** Immunoblot analysis of SP1, β-catenin, YAP and Tubulin expression in control condition, Wnt3a treatment with or without siSP1 treatment of HEK293T cells. **E.** Densitometric analysis of immunoblots from panel D for monitoring β-catenin expression (Unpaired t-test was performed to calculate significance). **F.** Densitometric analysis of SP1 expression from the immunoblots in panel D (Unpaired t-test was performed to calculate significance). **G.** Densitometric analysis of YAP expression from the immunoblots in panel D (Unpaired t-test was performed to calculate significance).

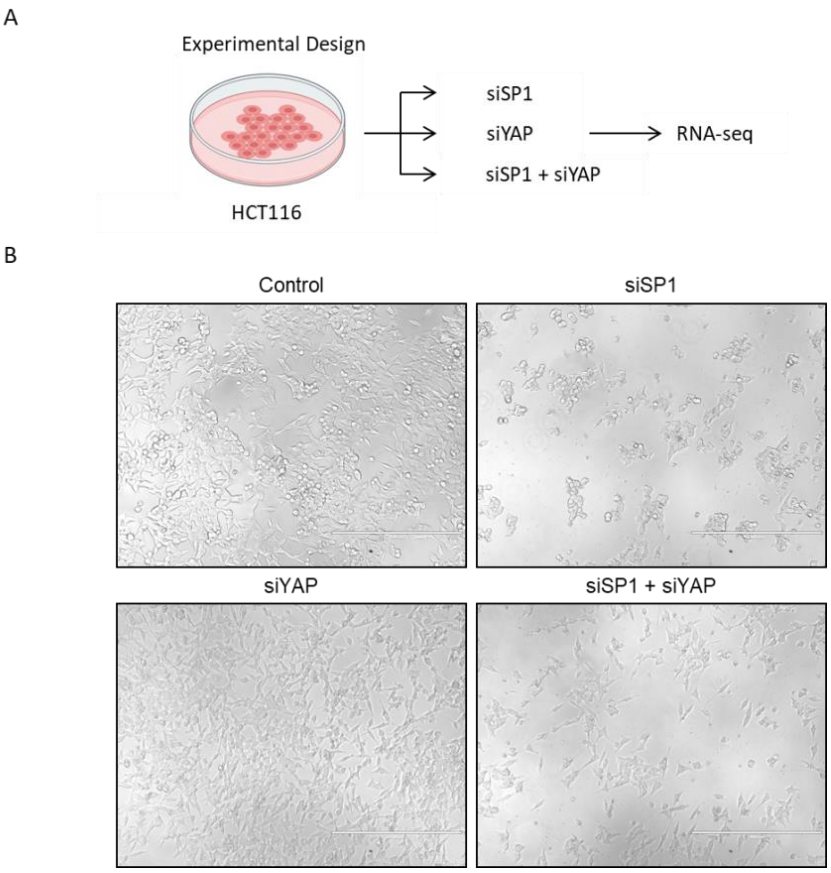


Figure 4.6. Combinatorial depletion of SP1 and YAP in HCT116. A. Schematic for the experimental strategy used to generate transcriptome data. **B.** DIC images for cells treated with control siRNA, siSP1, siYAP and a combination of both.

YAP-sensitive transcriptome data that aligns with YAP's known functions, particularly its involvement in regulating cellular proliferation and cytoskeletal dynamics.

4.4 SP1 and YAP coregulate cell cycle and related processes

The combined knockdown of SP1 and YAP yielded similar results in terms of their respective transcripts. However, an important observation to note here was that the levels of YAP transcript were the lowest in this dataset (Figure 4.8A). This observation is intriguing, given that the amount of siRNA targeting YAP was only half of what was used in the single knockdown (SKD) condition, and SP1 knockdown alone did not influence YAP transcript levels. It is possible that YAP and SP1 might directly regulate the expression of certain factors that, in turn, promote the expression of YAP. The presence of either SP1 or YAP (in conjunction with TEAD) may be adequate to maintain YAP expression, but the combined (Double) knockdown (DKD) might have contributed

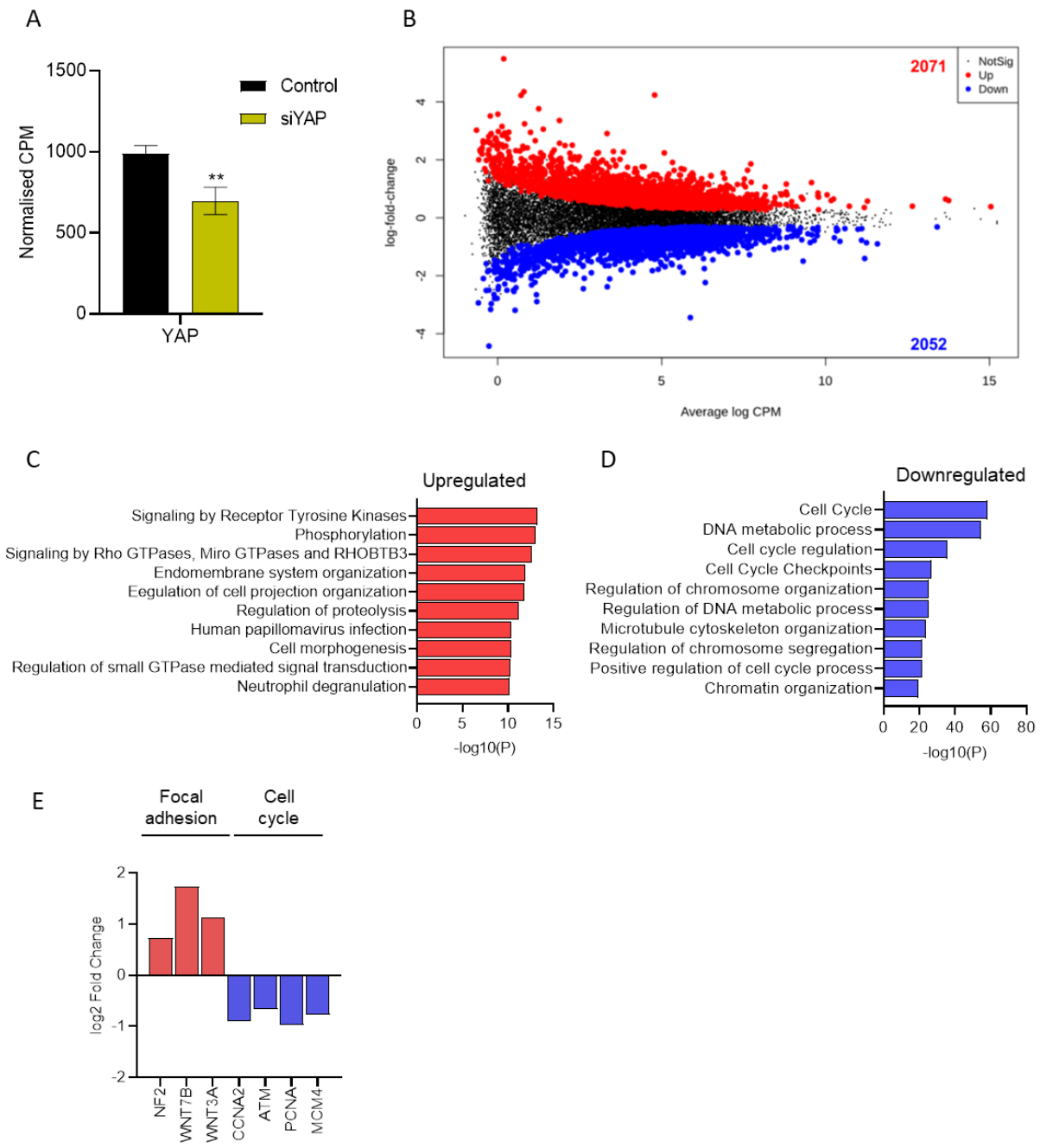


Figure 4.7. YAP depletion regulates cell cycle and cytoskeleton. A. Expression of YAP mRNA in control and YAP siRNA treated cells (Unpaired t-test was performed to calculate significance). **B.** Volcano plot for the differentially expressed genes. Upregulated genes are marked in red, and downregulated genes are marked in blue. **C.** GO analysis for upregulated genes. **D.** GO analysis for downregulated genes. **E.** log2 Fold change for selected targets: Focal adhesion regulators- *NF2, WNT7B* and *WNT3A* and cell cycle regulators- *CCNA2, ATM, PCNA* and *MCM4*.

to the observed reduction. Consistent with our previous findings, the SKD of SP1 still resulted in reduced YAP protein levels (Figure 4.8B). The total number of DE genes remained similar across all three transcriptomes, suggesting an additive effect by SP1

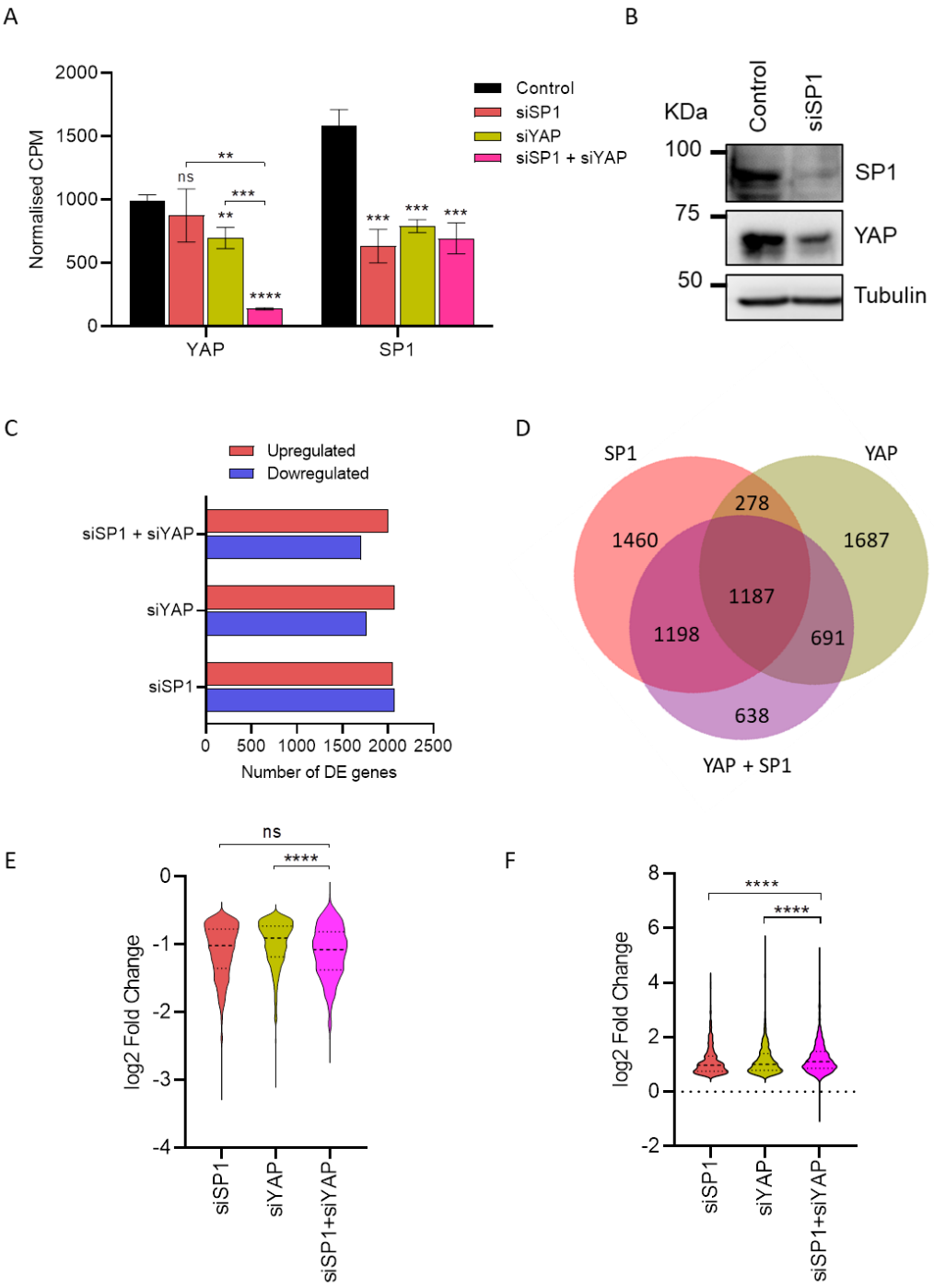


Figure 4.8. Transcriptional cooperation between SP1 and YAP as revealed by transcriptome analysis. A. Expression of YAP and SP1 mRNA (depicted as normalised read counts) in control siRNA, siSP1, siYAP and a combination of both siSP1 + siYAP (Unpaired t-test was performed to calculate significance). **B.** Immunoblot analysis to monitor SP1, YAP and Tubulin expression in control and siSP1 treated HCT116 cells. **C.** Total number of differentially expressed genes in control siRNA, siSP1, siYAP and combination of both. **D.** Venn diagram indicating the overlap between the differentially regulated genes in cells treated with siSP1 only, siYAP only and siSP1 + siYAP. **E.** Grouped log2 Fold change for the common downregulated genes upon SKD or DKD (Unpaired t-test was performed to calculate significance). **F.** Grouped log2 Fold change for the common upregulated genes upon SKD or DKD (Unpaired t-test was performed to calculate significance).

and YAP (Figure 4.8C). The overlap among all the DE genes also displayed a relatively even and statistically significant distribution among all datasets (Figure 4.8D). We further compared the overall fold change for the common set of DE genes and noted a slightly higher fold change in the case of DKD compared to SKD (Figure 4.8E&F). Specifically, for the positively regulated common gene set, the difference was significant compared to YAP KD but not SP1 KD. Given that YAP alone cannot directly bind to DNA and relies on TEAD (or other DNA binding proteins) to activate transcription, it is plausible that YAP might be dependent on SP1 (with or without TEAD) for the activation of certain target genes. In contrast, SP1 is capable of independently initiating transcription in conjunction with the basal transcriptional machinery and may not be entirely dependent on YAP (Figure 4.8E). For the negatively regulated common set of genes in DKD, there were significant different as compared to both the SKD conditions. Notably, some genes exhibited a completely opposite pattern, with a shift toward negative fold change (Figure 4.8F). This suggests that certain genes may have complex regulatory interactions involving both SP1 and YAP, leading to varying expression patterns in response to their single or combined knockdown.

Furthermore, we investigated the biological functions regulated by these two proteins. GO analysis revealed an enrichment of genes related to cell cycle and DNA replication in response to the depletion of SP1 and YAP. Notably, the autophagy-associated genes were found to be highly upregulated. The top 10 enriched processes in the case of the DKD are listed in Figure 4.9A and B. Figure 4.9C displays the expression patterns of selected target genes across all three KD conditions. Surprisingly, unlike the β-catenin-SP1 combination, we did not observe any impact on genes related to apoptosis. SP1 and YAP have previously been reported to have both negative and positive regulatory effects on apoptosis, leading us to anticipate the presence of apoptosis-related genes in the set of DE genes. To validate whether SP1 and YAP collectively affect cell viability, we conducted a viability assay using Annexin V-PI staining (Figure 4.10A). Intriguingly, despite a significant decrease in overall cell viability in the case of the DKD compared to the control, the percentage of viable cells was significantly higher than any of the SKD scenarios (Figure 4.10B). A similar trend was observed for apoptosis, where the

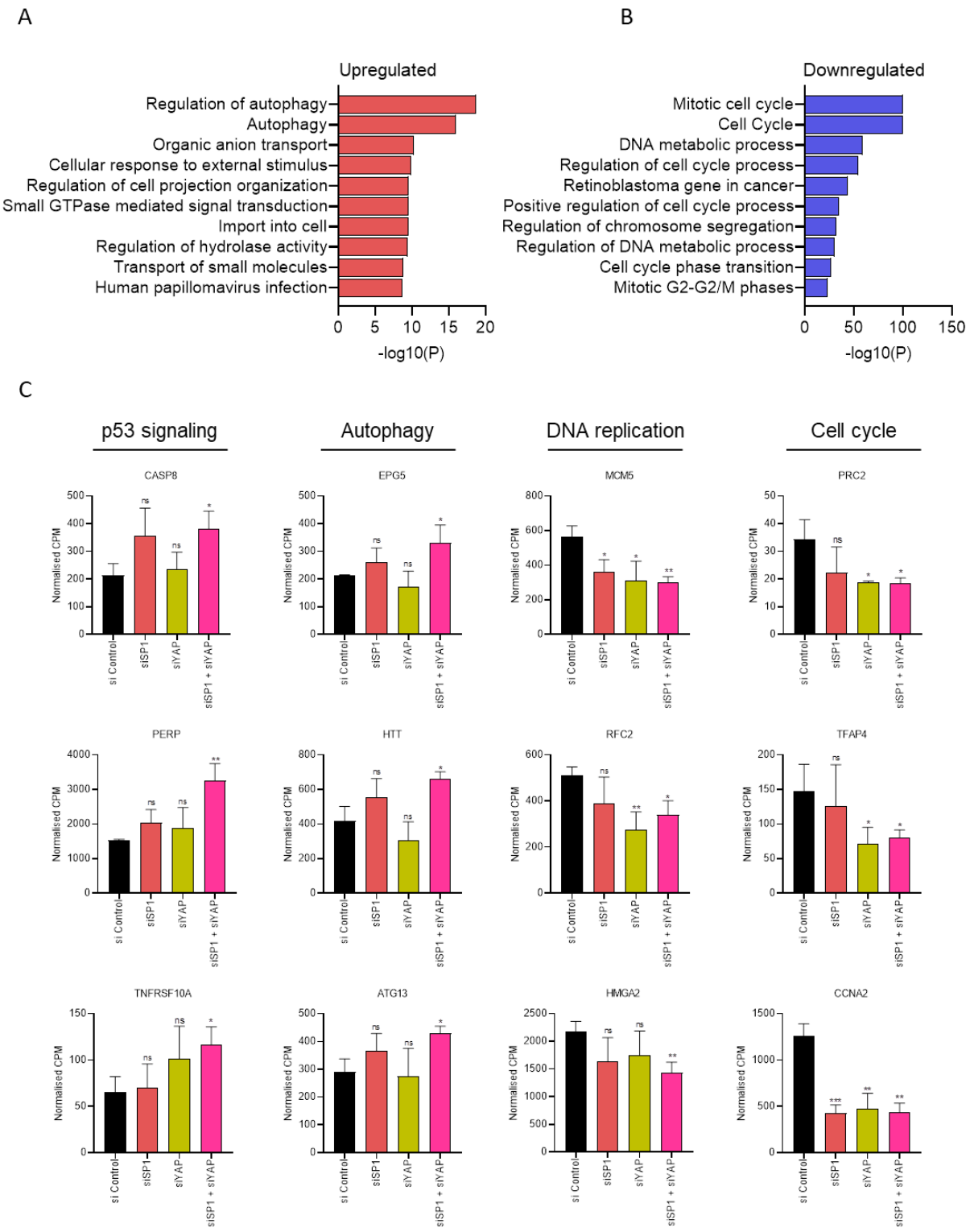


Figure 4.9. The biological processes regulated by SP1 and YAP. A. GO analysis for upregulated genes co-regulated by SP1 and YAP. **B.** GO analysis for downregulated genes co-regulated by SP1 and YAP. **C.** Expression of selected genes regulated by SP1 and YAP (depicted as normalised read counts) for p53 signaling & autophagy (negatively controlled) and DNA replication & cell cycle (positively controlled). Names of genes are indicated on top of each plot.

percentage of apoptotic cells was lowest in case of the DKD (Figure 4.10C). This suggests the absence of SP1 and YAP may be favoring the expression of a distinct set of genes or an altered pattern for the same set of genes, as observed in Figure 4.8F. We also explored the possibility of TAZ compensating for YAP as YAP's expression was affected by SP1-YAP DKD. TAZ (also known as WWTR1) was found to be significantly downregulated upon SP1 KD, but neither YAP KD nor the DKD had a significant impact on its levels (Figure 4.10D).

We also conducted a cell cycle analysis using PI staining (Figure 4.11A). In contrast to cell survival, we observed a cooperative relationship between SP1 and YAP concerning cell cycle progression. The percentage of G2/M cells was the lowest upon DKD, indicating their collective role to regulate cell cycle (Figure 4.11B). Revisiting our GO analysis, we found that terms related to autophagy and regulation of autophagy were the most prominent hits. In fact, the number of genes associated with any process related to autophagy was the highest in the case of SP1-YAP DKD (Figure 4.12A). This suggests that the level of autophagy may be the highest when SP1 and YAP are simultaneously depleted. Given the increase in autophagy and a decrease in proliferation, we also examined the expression of various markers for the quiescent state (Cheung and Rando, 2013; Bordi *et al.*, 2021). As expected, the positive markers for quiescence were upregulated, while the negative markers were downregulated in response to the DKD, confirming that the cells were entering a state of quiescence. In summary, we observed that SP1 and YAP together play a pivotal role in regulating the processes required for cellular proliferation. However, in absence of both these factors the cells can enter into a non-proliferative state, which prevents them from undergoing apoptosis.

4.5 SP1 and TEAD4 co-occupy direct target genes on chromatin

To assess the significance of SP1-YAP interaction in direct transcriptional control, we analysed the ChIPseq dataset for TEAD4, which is known to be a DNA binding partner of YAP. Despite TEAD1 having the highest expression among all the TEAD members in

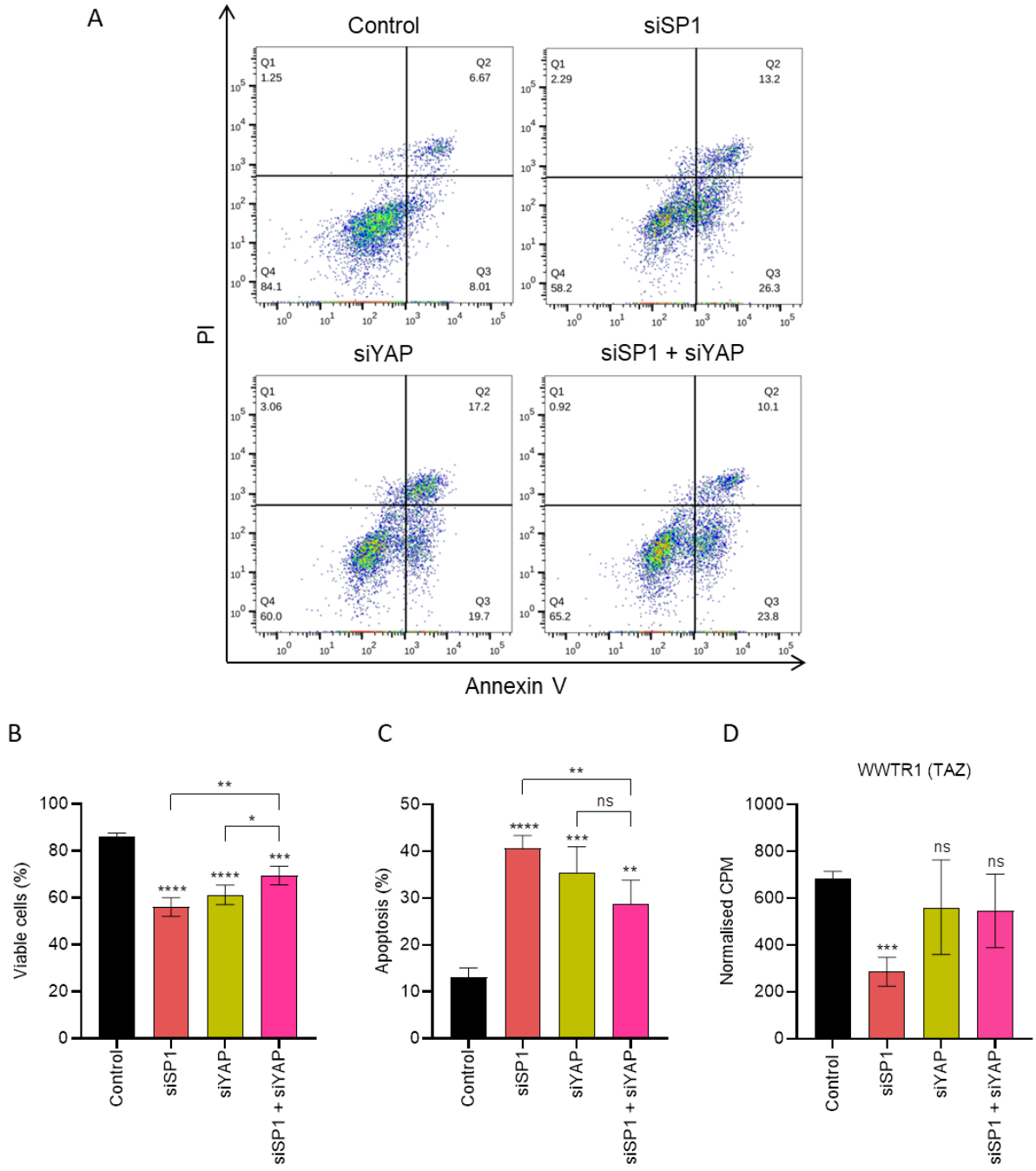


Figure 4.10. SP1 and YAP regulate cell survival. A. Flow cytometry analysis of HCT116 cells stained with Annexin V and PI after treatment with control siRNA, siSP1, siYAP and siSP1 + siYAP. **B.** Quantification of viable cells after Annexin V/PI staining after treatment with siRNAs for silencing the expression of SP1 and YAP individually or together (Unpaired t-test was performed to calculate significance). **C.** Quantification of apoptotic cells (right panel) after Annexin V/PI staining after treatment with siRNA against SP1 and YAP (Unpaired t-test was performed to calculate significance). **D.** Expression of TAZ (WWTR1) mRNA (depicted as normalised read counts) in control siRNA, siSP1, siYAP and cells treated with a combination of both (Unpaired t-test was performed to calculate significance).

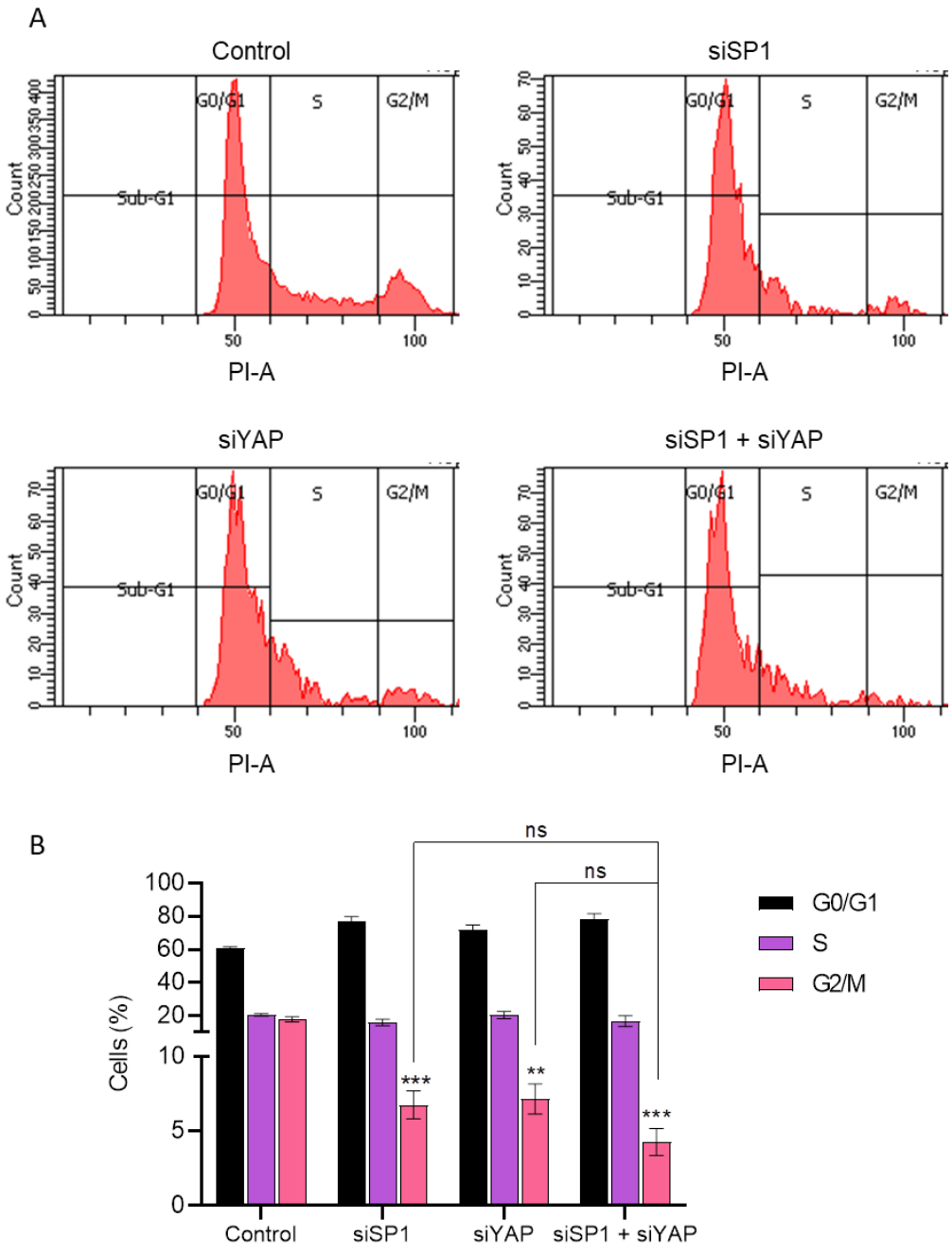


Figure 4.11. SP1 and YAP promote cell growth. A. Flow cytometry analysis of HCT116 cells stained with PI after treatment with control siRNA, siSP1, siYAP and siSP1 + siYAP. **B.** Cell-cycle analysis of cells treated with siRNAs for silencing the expression of SP1 and YAP individually or together (Unpaired t-test was performed to calculate significance).

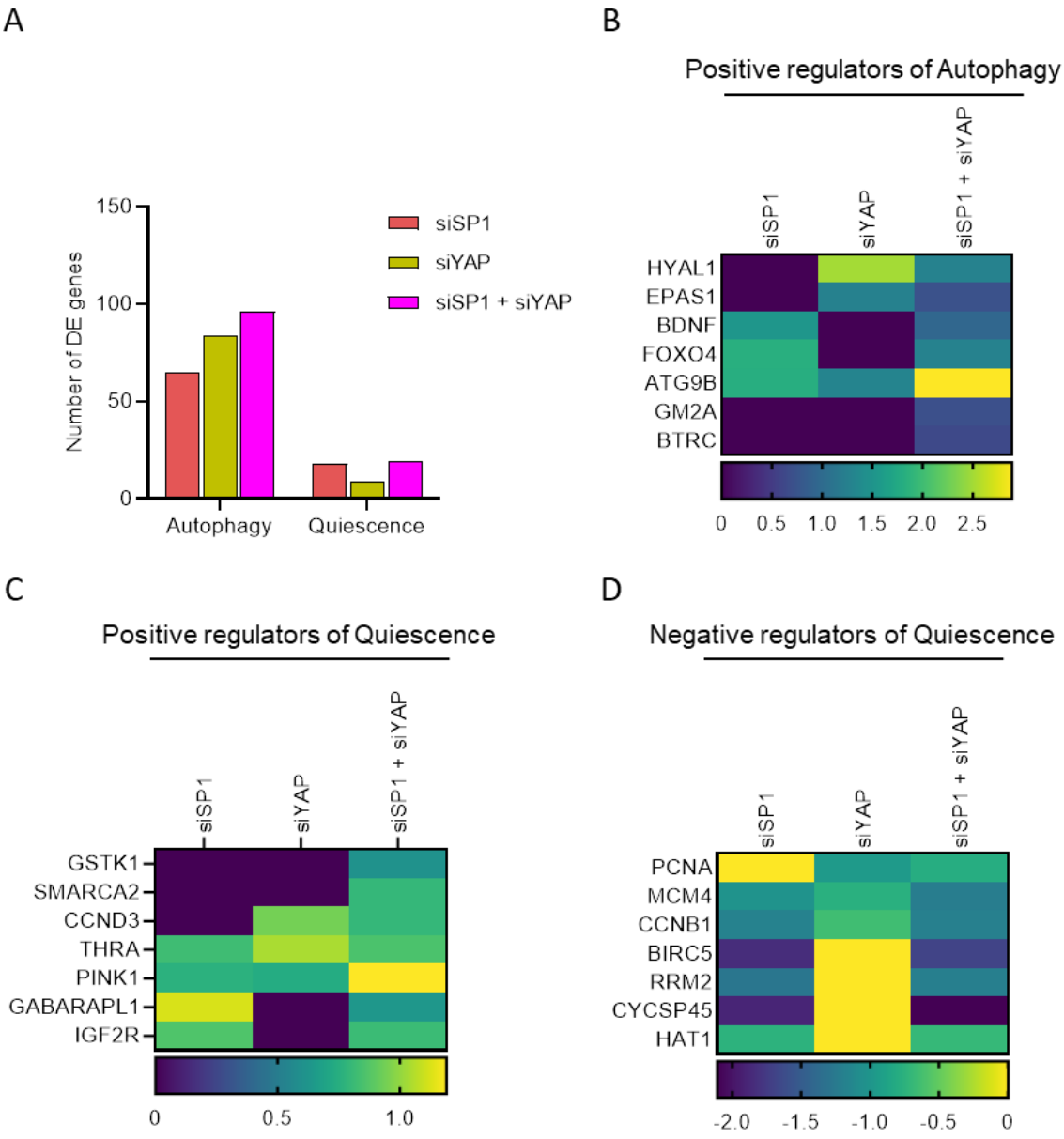


Figure 4.12. Simultaneous depletion of SP1 and YAP enhances autophagy A. Total number of DE genes for autophagy and quiescence upon depletion of SP1 and YAP. **B.** Heatmap for the log2 fold change for autophagy-related upregulated in siSP1, siYAP and combinatorial conditions. **C.** Heatmap for the log2 fold change for (positive markers) quiescence-related upregulated in siSP1, siYAP and combinatorial conditions. **D.** Heatmap for the log2 fold change for (negative markers) quiescence-related downregulated in siSP1, siYAP and combinatorial conditions.

HCT116 cells, we opted to utilise the factor with the second-highest expressed factor-TEAD4 as our dataset for analysis because of its substantial expression and prevalence in multiple colorectal cancer cell lines (Figure 4.13A). TEAD factors typically occupy 80-90% of YAP's direct target genes, serving as a proxy for the chromatin occupancy of YAP. Most of the TEAD4 binding sites were enriched with enhancer marks H3K4me1 along with H3K27ac. Interestingly, in this context, we observed co-occupancy of SP1 with TEAD4 at enhancer elements (C2&C4), but reduced occupancy for SP1 at the promoter elements (C3) (Figure 4.13B). To delve further into this co-occupancy, we explored the correlation between the loci occupied by SP1 and TEAD4. This analysis revealed a moderate but significant correlation between the two factors, reinforcing our earlier observations (Figure 4.13C). Cluster-specific peak summit further validated the patterns observed in the heatmap (Figure 4.14A). We also confirmed the presence of enhancer and promoter elements for each cluster by plotting the distance from the nearest TSS (Figure 4.14B). We annotated these loci to the nearest genes to gain insights into their associated biological functions. These target genes were predominantly involved cell growth and maintenance, encompassing functions related to proliferation, DNA damage response, as well as the regulation of the cytoskeleton, including processes such as cell organisation, polarity and Hippo pathway regulation (Figure 4.14C). Notably, we did not identify direct regulation of autophagy or related processes in these datasets, suggesting that autophagy may be regulated by an indirect mechanism. Surprisingly, when we examined the list of direct targets shared between SP1 and TEAD4, there was a significant overlap, with approximately 83% of TEAD4 target genes exhibiting occupancy by SP1 (Figure 4.14D). Although the peak correlation was moderate, it is possible that SP1 and TEAD4 may jointly regulate the regulatory regions of these genes, suggesting a potential cooperative role in their transcriptional control. Nevertheless, the presence of both YAP and SP1 is expected to have an impact on the expression of these shared direct targets. To assess this, we analysed the expression of genes coregulated by SP1-YAP (through transcriptome), which overlapped with TEAD4's direct targets (indicating TEAD4-dependence) as well as those that did not overlap with TEAD4 direct targets (indicating TEAD4-independence). In both scenarios, the reduction in the gene expression was significantly more

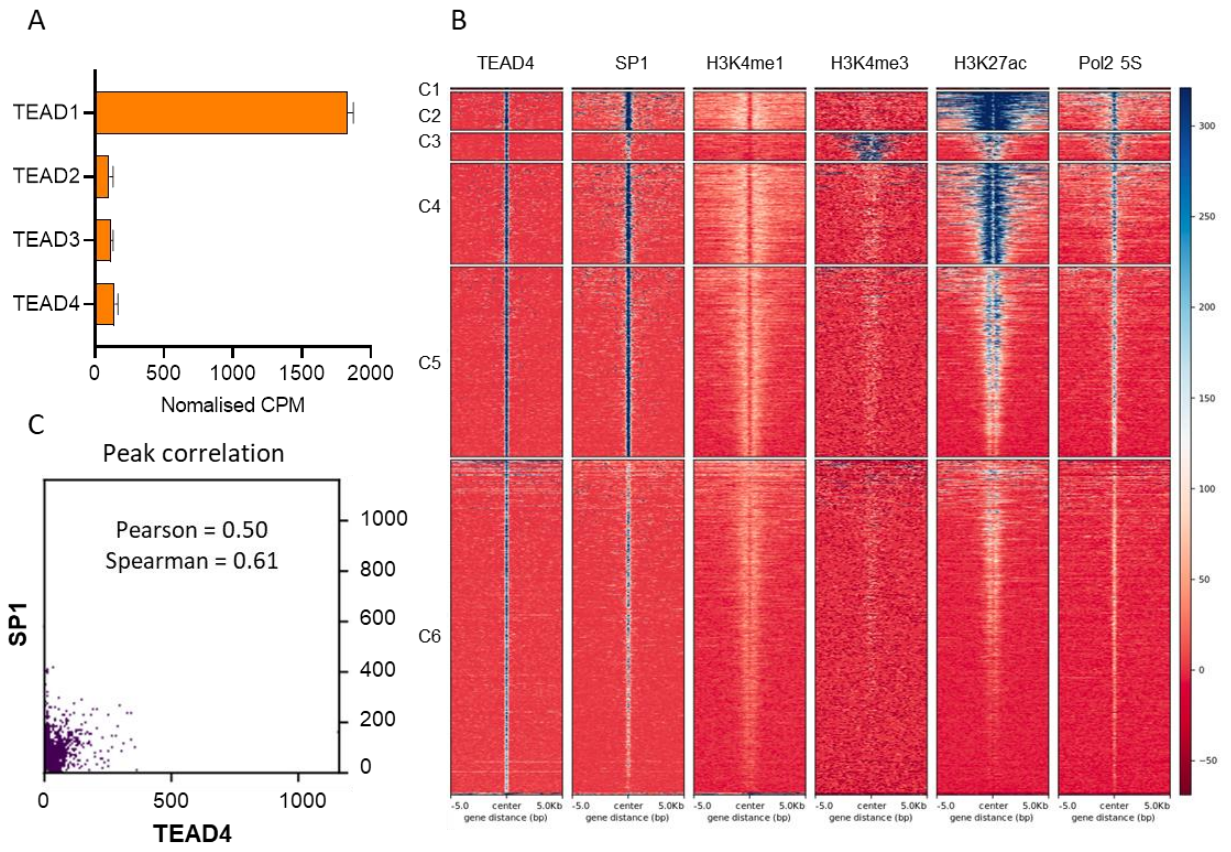


Figure 4.13. SP1 and TEAD4 co-occupy direct target genes. A. mRNA levels for TEAD1-4 in control HCT116 cells. **B.** Heatmap for the ChIPseq of TEAD4, SP1, TCF7L2, H3K4me1, H3K4me3, H3K27ac and RNA PolII 5S (Datasets: ENCSR000BVJ, ENCSR000BMK, ENCSR000BSF, ENCSR000BVT, ENCSR161MXP, ENCSR333OPW, ENCSR661KMA, ENCSR000EUV). **C.** Peak correlation for the ChIPseq signals for SP1 and TEAD4.

pronounced when both factors were absent, underscoring the critical role of SP1 and YAP working together to facilitate the optimal transcription of YAP target genes (Figure 4.14E&F). In summary, we have demonstrated that SP1 and YAP must act in conjunction to activate their direct target genes, which, in turn, drive cellular growth.

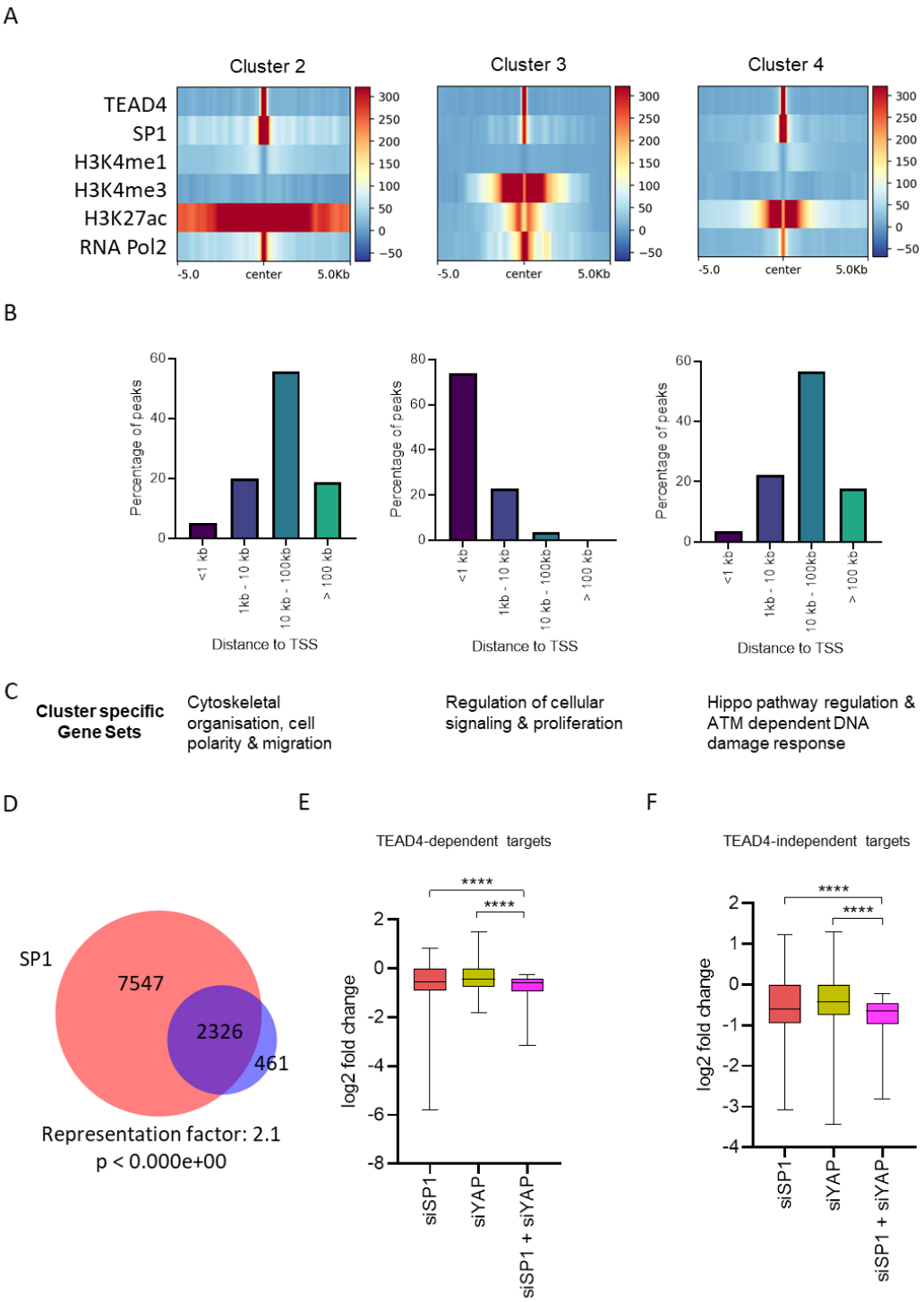


Figure 4.14. Biological functions associated with the direct targets of SP1 and TEAD4. A. Peak intensities for all the ChIPseq datasets in Cluster 2, 3, and 4. **B.** Percentage of peaks in Cluster 2, 3, and 4 according to their distance to the nearest gene. **C.** Major terms obtained after GO analysis for the annotated genes for Cluster 2, 3, and 4. **D.** Venn diagram illustrating the overlap for the ChIPseq peaks for SP1 and TEAD4. **E.** Grouped log2 Fold Change for common downregulated genes shared between YAP & SP1 and TEAD4 bound genes (Unpaired t-test was performed to calculate significance). **F.** Grouped log2 Fold Change for common downregulated genes shared between YAP & SP1 without TEAD4 occupancy (Unpaired t-test was performed to calculate significance).

Summary and Conclusions

YAP performs a dual role, participating in the activation of target gene transcription and controlling the stabilisation of proteins under the control of WDC. Consequently, the activation status of the Hippo pathway directly influences the Wnt response. Typically, when growth-promoting conditions are present, Wnt signaling is activated by Wnt ligands (Liu *et al.*, 2022). However, as cellular density reaches a specific threshold, the cell's mechanosensory machinery comes into play, preventing further growth. This process is primarily regulated by the Hippo/YAP(TAZ) pathway (Panciera *et al.*, 2017). YAP stimulates expression of genes related to proliferation, but activation of the Hippo kinases inhibits its nuclear localisation. Cytoplasmic YAP can remain unchanged by associating with the 14-3-3 complex, or undergo proteasomal degradation (Vassilev *et al.*, 2001). However, this cytoplasmic form of YAP can also interact with β-TrCP and transport it to the WDC, leading to the degradation of both β-catenin and YAP (Azzolin *et al.*, 2014). Conversely, the activity of nuclear YAP is mediated by the DNA-binding partner it engages with. For instance, YAP can act as an anti-apoptotic factor when paired with TEAD as its DNA binding partner or as a pro-apoptotic factor when associating with p73 as the DNA binding partner (Vassilev *et al.*, 2001; Zhang *et al.*, 2018). Other proteins that interact with YAP include AP1, MYC and RUNX3 (Zanconato *et al.*, 2015; Qiao *et al.*, 2016; Croci *et al.*, 2017). As a result, it is crucial to identify different DNA binding partners for YAP, as they fine-tune the transcriptional outcomes mediated by YAP.

In this study, we have identified SP1 as a critical regulator of YAP-mediated transcriptional processes. Previously, it has been demonstrated that SP1 is a target of TEAD1 and the inactivation of Hippo pathways lead to increased cell proliferation by enhancing SP1 expression (Yu and Zhang, 2016). We have confirmed a similar pattern through a cell density assay in cell lines that are responsive to these cues. However, the HCT116 cell line, which lacks contact inhibition, displayed no alteration in SP1 expression. This observation highlights how cancerous cells can overcome growth inhibition mechanisms. Likewise, the overexpression of nuclear forms of YAP led to an

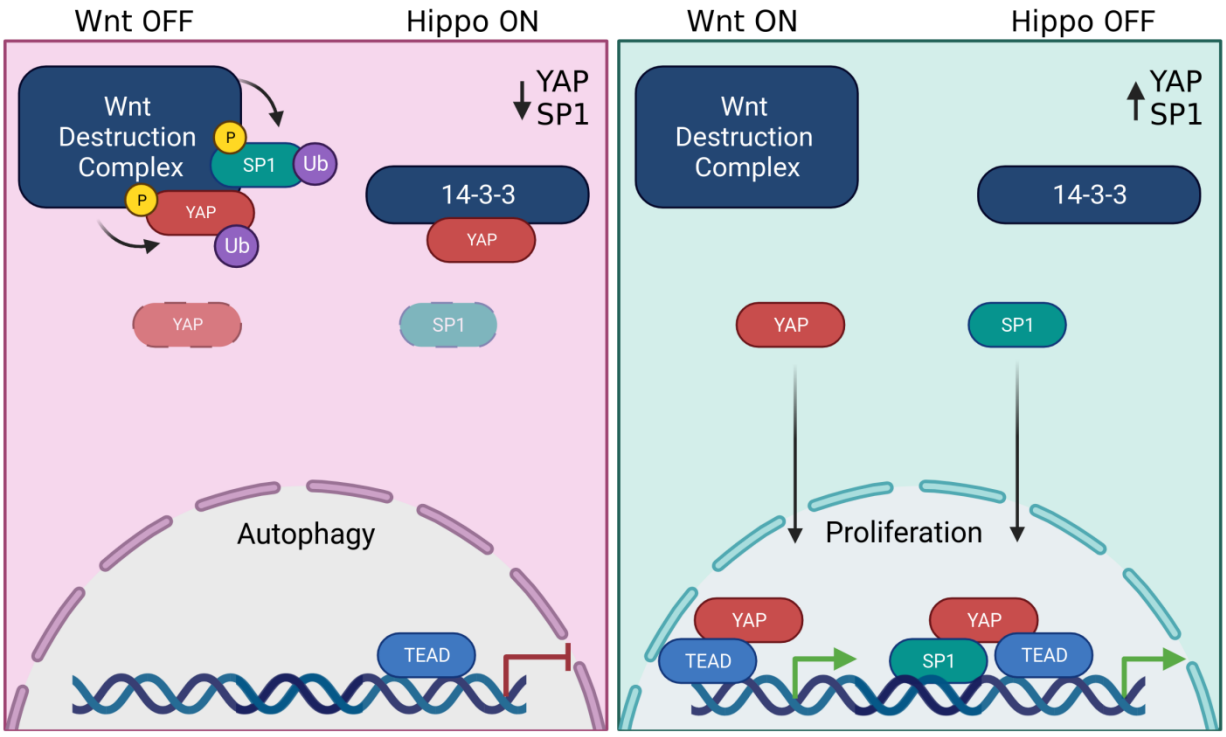


Figure 4.15. Model for cooperation between SP1 and YAP. When Hippo pathway is active and Wnt pathway is inactive, YAP recruits β–TrCP to the Wnt destruction complex leading to degradation of SP1 and YAP (shown in faded icons). Depletion of SP1 and YAP slows down cell proliferation and promotes autophagy. Upon Wnt activation and Hippo inactivation, SP1 interacts and stabilises YAP. Stabilised forms of SP1 and YAP translocate inside the nucleus and activate transcription. Several YAP/TEAD4-dependent genes are sensitive to SP1 and require the binding of all three factors for optimal expression.

increased SP1 expression. Additionally, we have shown that SP1 acts as a positive feedback regulator for YAP. Depletion of SP1 resulted in a reduction in YAP levels, particularly in the nuclear compartment, suggesting that SP1 may be essential for both stabilising and facilitating the nuclear translocation of YAP. Notably, overexpression of the active form of SP1 was significantly more effective in stabilising YAP compared to the WT form. Interestingly, we did not observe any evidence of transcriptional regulation of YAP by SP1 indicating a potential role in YAP protein stabilisation. Moreover, we observed a pronounced nuclear co-localisation and direct interaction between SP1 and YAP. Subsequently, we investigated whether SP1 plays a role in the regulation of YAP protein stability upon Wnt activation. Notably, when Wnt was activated, in the absence of SP1 it prevented the stabilisation of YAP. This finding underscores the essential role of SP1 in the Wnt-mediated stabilisation of YAP; indicating that while SP1 may not be directly involved in the upregulation of YAP mRNA, it enhances the stability of the YAP

protein through its direct interaction with YAP.

Given that both SP1 and YAP are transcriptional regulators and interactor with each other, we mapped the combined transcriptional changes these two proteins induce. To achieve this, we generated transcriptome data in HCT116 cells by depleting of SP1 and YAP individually as well as in combination. Notably, we identified a significant overlap in the set of genes regulated by SP1 and YAP, primarily encompassing various aspects of the cell cycle, DNA replication, and autophagy. Interestingly, individual depletion of these factors resulted in suppression of the cell cycle and activation of apoptosis, whereas a simultaneous knockdown had the opposite effect. We observed a rescue in terms of cell viability and reduction in the degree of apoptosis. However, the impact on the cell-cycle progression remained consistent with our previous observations. Essentially, the cells were transitioning into a state characterized by low proliferation, but they demonstrated enhanced survival due to increased autophagy. This state resembles a quiescent-like condition in which cells do not divide but remain metabolically active. To validate this, we examined the expression of quiescence markers and noted that several positive markers exhibited increased expression upon the simultaneous knockdown of SP1 and YAP. It is also noteworthy that many factors related to autophagy and quiescence were not picked up upon single knockdown, indicating that this pattern was specific to the simultaneous depletion of both SP1 and YAP.

From a physiological standpoint, this mechanism could function as a means to maintain the cells in a dormant state until their immediate response is necessary. For example, YAP typically does not play a role in the cellular regeneration and differentiation of the intestinal stem cells. However, it becomes active during regeneration process that follows tissue injury. Regeneration in response to an injury demands a swifter response, and the existing ISCs are insufficient to deliver an immediate response. In such scenarios, neighboring near-differentiated cells (+4 cells) come to the rescue. This wound-healing response is primarily orchestrated by YAP (Tetteh, Farin and Clevers, 2015; Deng *et al.*, 2018; Cheung *et al.*, 2020). As discussed previously, SP1 is also

enriched in colorectal cancer stem cells (CCSCs) (Quarni *et al.*, 2019). As these cells commence differentiation, their rate of proliferation decreases, and SP1 levels decrease accordingly. It is possible that YAP and SP1 are maintained at low levels to induce a dormant state in the cells, which can be later activated. However, from a disease perspective, cancer stem cells that enter quiescence and exhibit high levels of autophagy can remain dormant, displaying chemoresistance. These cells also contribute to cancer recurrence and relapse. This underscores the dual roles of the SP1-YAP complex, which can modify cellular responses in a context-specific manner within the same cell type.

To delve deeper into the direct transcriptional regulation by SP1 and YAP, we made use of the chromatin occupancy data for TEAD4. TEADs are the primary DNA binding partners for YAP and are found at the majority of YAP's target gene locations. Based on the peak correlation data, we found that SP1 and TEAD4 exhibited moderate level of co-occupancy. Furthermore, TEAD4 is known to be enriched at enhancer regions rather than promoter regions (Lai et al., 2020). We also noted that majority of the peaks for TEAD4 were located within the enhancer regions, with a few regions showing enrichment for promoter marks (Cluster 3). Interestingly, SP1 displayed a strong signal within enhancer regions and a weaker signal within the promoter regions within TEAD4-occupied areas. While SP1 can bind to both promoter and enhancer regions, it was surprising to see its enrichment specifically at enhancer regions, suggesting a potential co-regulation of these common targets primarily through enhancers. Given that SP1 regulates the stability of the YAP protein, it is possible that SP1 facilitates the recruitment of YAP to these shared targets. Moreover, although the peak correlation between TEAD4 and SP1 is moderate, the overlap between their target genes is quite extensive, suggesting that their binding motifs may not be closely aligned. It would be intriguing to map the chromatin occupancy of YAP itself to determine if YAP can bridge the gap between SP1 and TEAD4. This analysis would also verify whether YAP can directly bind to SP1 and activate transcription without the involvement of TEAD4.

In conclusion, our findings introduce SP1 as a pivotal partner and collaborator with YAP

in regulating cell proliferation. Investigating the combined effects of established key players in any biological process is of utmost importance. In this scenario, despite the known roles of both YAP and SP1 in governing cellular growth and death, we demonstrate that their simultaneous depletion can lead to an entirely different state of cellular dormancy.

Limitations: The precise mechanism behind SP1-mediated YAP stabilisation warrants a more comprehensive investigation. This can be achieved by investigating the interaction between YAP and the components of the WDC in the presence and absence of SP1. Examining YAP's chromatin occupancy can provide deeper insights into the transcriptional regulation involving SP1 and TEAD4. This approach will also elucidate whether SP1 alone has the capability to recruit YAP to promoter regions. Lastly, it is imperative to explore this interaction in an *in-vivo* context, as it will offer valuable insights into its physiological significance.

CHAPTER V

The specificity in the Specificity Protein 1

Transcription in eukaryotic organisms is primarily mediated by two types of transcription factors (TFs): the general transcription factors (GTFs) and the sequence-specific transcription factors (SSTFs). GTFs bind to core promoter elements typically located in close proximity to the transcription initiation sites. This interaction of the GTFs with the RNA polymerase subsequently initiates the transcription process. On the other hand, SSTFs bind to pre-defined DNA motifs located at varying distances from the target gene. These sequence-specific factors come in contact with RNA polymerase, either directly or through a mediator complex, ultimately leading to transcriptional activation (Müller, 2001). The utilization of SSTFs introduces an additional layer of complexity to the spatiotemporal regulation of gene expression at the transcriptional level.

Modes of gene expression control via SP1

One of the earliest SSTFs discovered was Specificity Protein 1 (SP1). It was initially isolated from HeLa cells due to its ability to bind to GC boxes and activate transcription from the SV40 early promoter and thymidine kinase promoter (Dynan and Tjian, 1983; Gidoni *et al.*, 1985; Kadonaga *et al.*, 1987). For a significant period, it was believed that SP1 might be the exclusive factor regulating gene expression through GC boxes. However, a few years later, SP1-related TFs, SP3 and SP4, were identified in Ishikawa cells, a human endometrial cell line, during a recognition-site screening using a GT-box motif from the uteroglobin promoter as a probe (Hagen *et al.*, 1992). Subsequently, several other members of this family were identified and included, expanding the group to a total of 9 members as of the present day (Scohy *et al.*, 2000; Treichel, Becker and Gruss, 2001; Nakashima *et al.*, 2002; Treichel *et al.*, 2003; Kawakami *et al.*, 2004).

The most highly conserved region across the SP family members resides in the C terminus, which includes the three zinc-finger motifs. The Cys2-His2 type zinc-finger

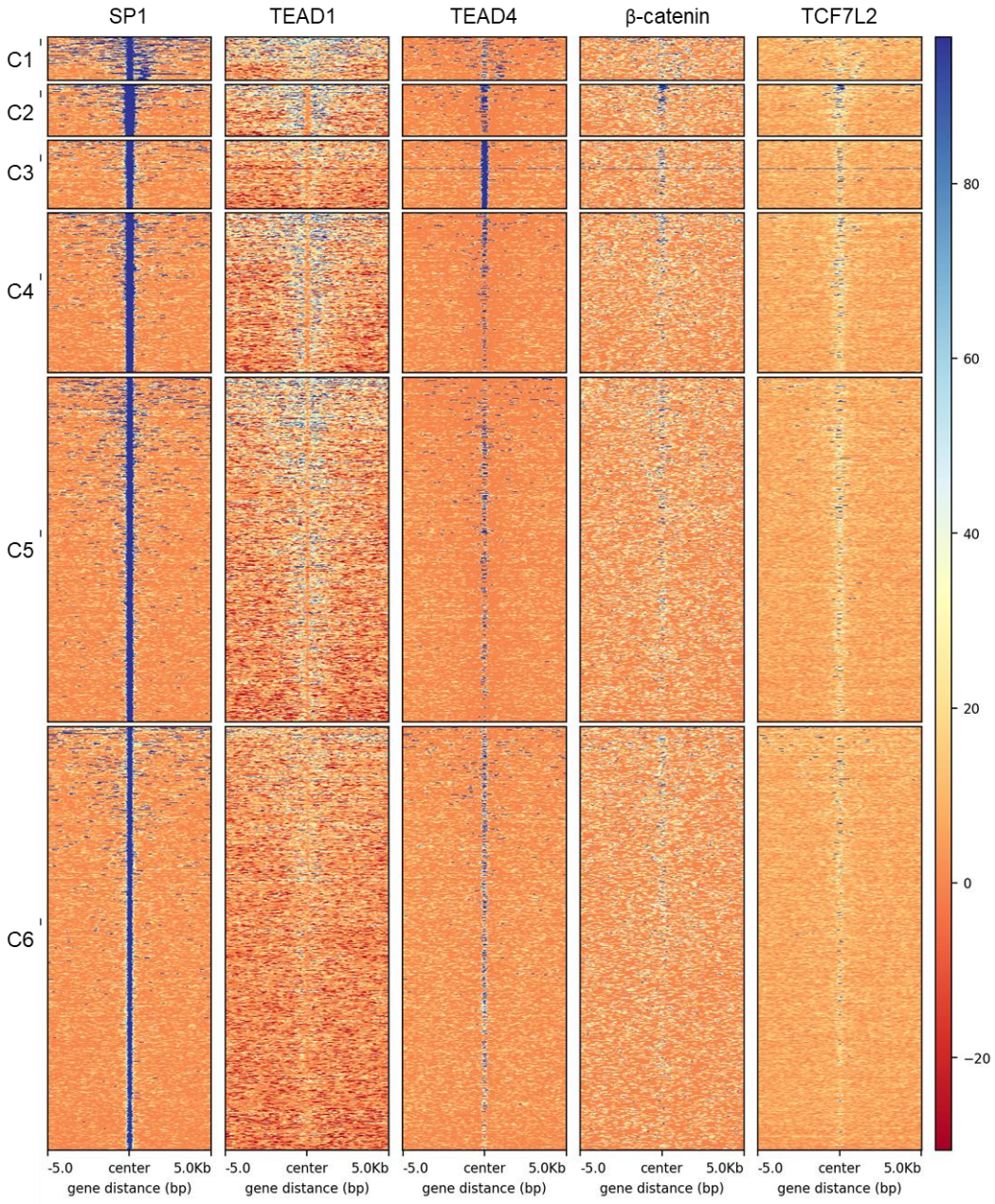


Figure 5.1. Genome-wide occupancy profiles of transcriptional partners of SP1. Heatmap for the ChIPseq datasets for SP1, TEAD1, TEAD4, β-catenin and TCF7L2, using SP1 binding sites as the reference for the datasets (SRA012054, ENCSR000BSF, ENCSR000BVT, ENCSR000EUX, ENCSR000EUV, ENCSR000BVJ, SRR64776373, SRR6477617)

domain spans approximately 81 amino acids (Suske, 1999). Detailed structural studies on zinc-finger DNA binding proteins have revealed the precise amino acids that interact with DNA: Lys-His-Ala in the first zinc-finger, Arg-Glu-Arg in the second zinc-finger, and Arg-His-Ala in the third zinc-finger (Pavletich and Pabo, 1991). All these tri-peptides are conserved in SP1, 3 and 4 but not in SP2. Consequently, SP1, SP3 and SP4 recognise

the classical SP1 binding site with equal affinity. In contrast, SP2 replaces histidine with leucine in its zinc finger and binds to GT-rich elements instead of GC box (Kingsley and Winoto, 1992).

SP1 functions as a transcriptional activator for a wide array of housekeeping genes. It is capable of stimulating transcription from both proximal and distal promoters (Courey *et al.*, 1989). As previously mentioned, SP1 establishes direct interactions with multiple components of the basal transcriptional machinery. Additionally, the transactivation domains (TADs) of SP1 form homotypic and heterotypic protein interactions, enabling the formation of tetramers of SP1. Such multimers lead to “superactivation”, where the presence of multiple binding sites results in synergistic transcriptional activity (Mastrangelo *et al.*, 1991; Su *et al.*, 1991). The transcriptional activity of SP1 is further regulated by its interaction partners. These interactions are contingent on the specific post-translational modifications of SP1 and the proteome unique to the cell type. SP3 is considered one of the most potent competitors for SP1. Similar to SP1, SP3 plays a dual role both as an activator and a repressor, depending on the isoforms and motif configuration (Udvadia, Templeton and Horowitz, 1995; Dennig, Beato and Suske, 1996; Ihn, LeRoy and Trojanowska, 1997; Majello, De Luca and Lania, 1997; Ding *et al.*, 1999; Galvagni, Capo and Oliviero, 2001). In the presence of a single SP1 motif, SP3 functions as an activator, but with multiple motifs, it acts as a repressor by forming stable complexes with the promoter. The repressive activity of SP3 (and SP2) can also employ recruitment of histone deacetylases to the promoter regions (Birnbaum *et al.*, 1995; Hagen *et al.*, 1995; Majello, De Luca and Lania, 1997; Yu, Datta and Bagchi, 2003). Certain isoforms of SP3 are inherently repressive (Kennett, Udvadia and Horowitz, 1997; Kennett, Moorefield and Horowitz, 2002).

A novel role has been discovered for SP1 in the regulation of gene expression. A recent study showed that SP1 functions as an RNA-binding protein, capable of binding to the 3’ untranslated regions (UTRs) of mRNA. This interaction leads to the recruitment of RNA cleavage machinery, resulting in the shortening of UTRs. These shortened UTRs serve to prolong the half-life of mRNA and enhance translation efficiency. The utilization

of these shorter UTRs has been linked to improved survival in cancer cells. Intriguingly, the RNA targets (approximately 2000) of SP1 do not non-overlap with the DNA targets (around 6000) of SP1, establishing it as the pivotal regulator of gene regulation (Song *et al.*, 2022). This highlights that despite our extensive knowledge of SP1's modifications and interaction partners, a significant gap remains regarding its molecular functions and the mechanisms governing its stability and activity, particularly with other signaling pathways

Transcriptional partners of SP1

The final transcriptional output is determined by the combinatorial binding of transcription factors at gene regulatory regions. These interactions can be additive, synergistic, or inhibitory in nature. While SP1 is capable of independently activating transcription, it often associates with other transcriptional regulators (Black *et al*., 2001). These partners vary not only based on the cell type and stimulus but also depend on the specific regulatory region to which SP1 is binding. To obtain a comprehensive view from the perspective of SP1, we aligned the chromatin occupancy profiles of all the transcriptional regulators included in our study, namely TEAD1, TEAD4, β-catenin and TCF7L2. Unlike these factors, which have fewer binding sites, the SP1 profiles did not exhibit a dominant association of any one factor (depicted in Figure 5.1). We did observe a strong correlation between SP1 and TEAD4, particularly within Cluster 3, which displayed the most robust signal. β-catenin and TCF7L2 demonstrated a similar pattern, with numerous loci exhibiting shared occupancy with SP1. While TEAD1's limited read counts made visualization less clear, it can be assumed that some loci do indeed share occupancy with SP1. In summary, we observed that SP1 shares genomic binding sites with all of these factors.

Furthermore, we conducted a motif analysis for all the SP1 binding sites in order to assess which other motifs are linked to our context (Figure 5.2). The top factors identified through this analysis align with the previous findings from other groups. JUN, FOSL2, KLF4, NFY and ETS1 are recognized for having motifs closely related to those

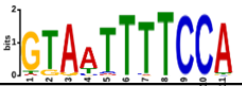
S.No.	Consensus	Name	No. of peaks	E-value
1		JUN, JUND, FOSL2	8587	2.9e-167
2		KLF4, KLF12, SP1, SP3	3946	7.6e-025
3		NFYA, NFYB, NFYC	2084	3.4e-019
4		ETS1, ETV2, ERG	4901	5.1e-015
5		RUNX1, RUNX3	1376	3.8e-007
6		ATF1, ATF2, ATF3	630	1.9e-005
7		TEAD1,TEAD4	1961	2.1e-005
8		IKZF1	195	1.7e-004
9		TCF7, TCF7L1, TCF7L2	1154	8.5e-004
10		ZNF76, ZNF143, THA11	132	1.4e-003
11		ATF4, CEBPB, CEBPG	339	1.5e-003
12		FOXO2, FOXO3, FOXO4	2456	2.3e-002
13		NFAC2, NFAC3, NFAC4	104	4.5e-002

Figure 5.2. Motif analysis for SP1 binding sites. Top 13 annotated motifs obtained after motif analysis for SP1 bound regions using online tool: MEME-Suite. The second column depicts the consensus sequence for the motif, the third lane contains the DNA binding proteins known to bind to the enriched binding sequences, the fourth lane consists of the frequency of occurrence for each motif type, and the fifth lane gives the respective E-values.

of SP1 (Hasegawa and Struhl, 2021). ETS1, in particular, has been noted to exhibit a synergistic effect along with SP1 (Gégonne *et al.*, 1993). NFY factors are often associated with SP1 in the context of basal transcription (Benfante *et al.*, 2005; Nylén *et al.*, 2018; Ventura *et al.*, 2022). The KLF family of transcription factors shares a similar

motif with SP1 due to their close relationship (Presnell, Schnitzler and Browne, 2015). We also observed an enrichment of SP3, which binds to the same motif as SP1. Additionally, motifs corresponding to TCFs and TEADs showed enrichment. The biological significance of these associations can also be further correlated with the actual binding of these factors and the measurement of transcriptional output. Both of these aspects are explored in Chapters 2 and 4, where we employed ChIPseq datasets for TCF7L2 and TEAD4, respectively, to demonstrate the actual binding of these factors alongside SP1. Furthermore, we quantified the changes in expression of these common targets upon depletion of SP1 and their respective partners, namely β-catenin for TCF7L2 and YAP for TEAD4. Both sets of observations revealed a quantitative relationship between SP1 with β-catenin/TCF7L2 and YAP/TEAD4.

A recent development in our understanding of SP1 biology involves its binding kinetics. SP1 has substantial number of binding sites within the genome. Recent research has identified that when SP1 translocates into the nucleus, it selectively accumulates at certain regions. It identified that SP1 exhibits the fastest binding at promoters while binding at enhancers occurs at a relatively slower rate. Furthermore, sites containing multiple SP1 motifs are favored over those with a single motif. Interestingly, it was shown that regions with promoters that exhibit fast SP1 binding and enhancers with slow SP1 binding ultimately result in lower transcriptional output, in contrast to regions where promoters have slow binding, and enhancers have fast SP1 binding after nuclear translocation. These findings were also associated with the enrichment of active histone marks such as H3K27ac and Pol II occupancy at regions where transcription rates are higher (Hasegawa and Struhl, 2021). Notably, both TCF7L2 and TEAD4 are proteins associated with enhancers. We observed the enrichment of H3K4me1 (an enhancer mark), the presence of H3K27ac, and the presence of active Pol II (5S). This indicates that β-catenin and YAP may facilitate the more rapid recruitment of SP1 at these common enhancer regions to prioritise increased transcription. However, further research is needed to address this hypothesis.

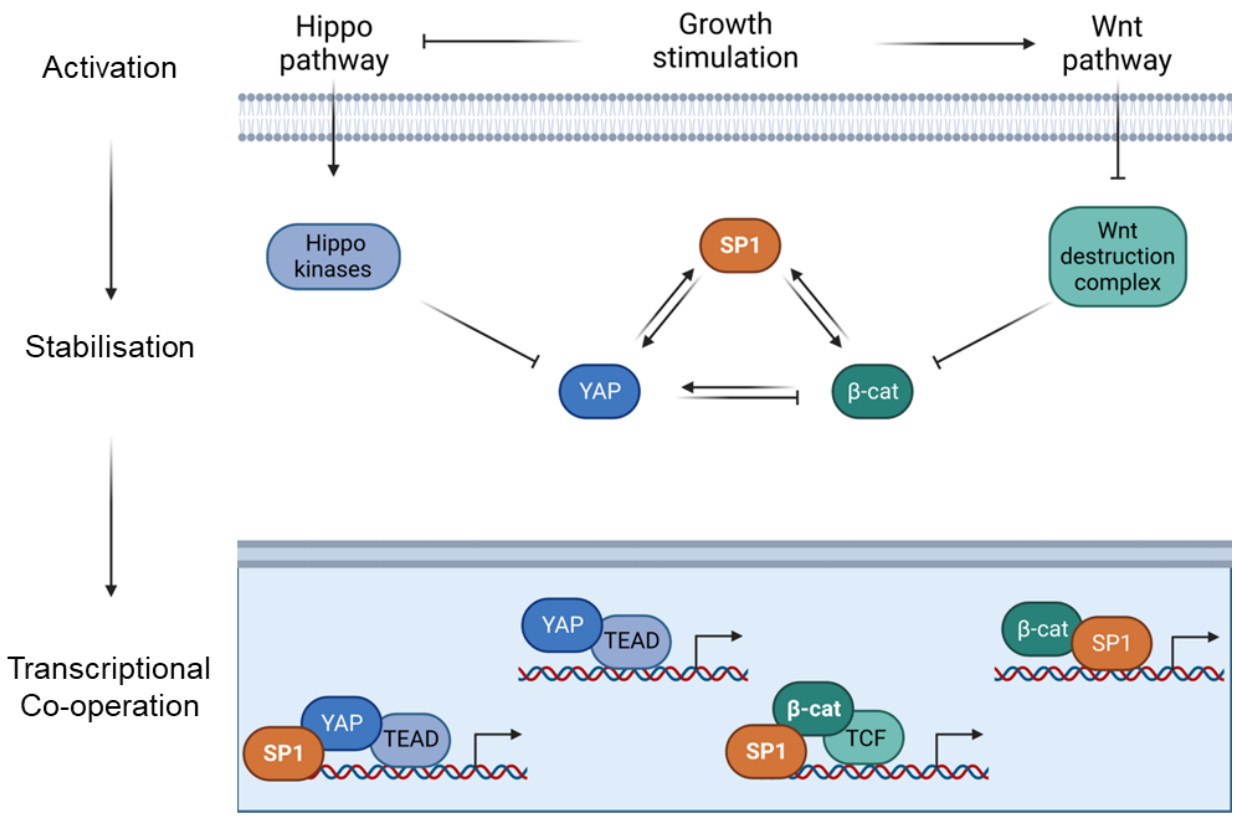


Figure 5.3. Final summary. Growth-conducive environment promotes the activation of Wnt signaling and inactivation of the Hippo pathway, which inactivates their respective kinases. Subsequently, SP1 and its interactors- β-catenin and YAP are stabilised to translocate inside the nucleus. The nuclear forms of β-catenin and YAP associate with their respective DNA binding partners, commonly TCFs and TEADs, respectively, to activate transcription. SP1 also binds to these complexes, further amplifying the transcriptional output. The combination of these proteins binding on the regulatory regions of target genes varies and thus helps in fine-tuning the gene expression.

Growth conducive environment promotes the stabilisation of SP1 and its interactors

From the findings of the studies conducted thus far, we can summarize that cells are subjected to growth stimulation through a variety of signals, including Wnt activation mediated by Wnt ligands and Hippo inactivation as a response to increased cellular rigidity (as illustrated in Figure 5.3). These upstream signals ultimately lead to the stabilisation of SP1, β-catenin and YAP proteins by preventing their interaction with the Wnt destruction complex and Hippo kinases. As the cytoplasmic fractions of these proteins increase, they can freely translocate inside the nucleus and bind to their respective genomic sites in various combinations. The specific factors influencing these

combinations may include the type of regulatory element (promoter or enhancer), the frequency of binding motifs (with SP1 demonstrating faster binding to regions with multiple motifs), and the availability of co-factors (DNA-bound activatory or inhibitory proteins). The ultimate outcome in terms of transcription and cellular phenotype hinges on the interplay of all these events. While these stabilisation processes support the rapid growth required during development and regeneration, the same molecular circuitry can be exploited in pathological conditions, leading to abnormal and uncontrolled growth.

Future perspectives

Exploring the intricate molecular mechanisms governing the mutual regulation of stability and transcription by SP1, β-catenin, and YAP holds significant interest. Although we have investigated these interactions in pairs (two factors at a time), it becomes crucial to understand the dynamics when all three proteins are involved. For example, it would be valuable to ascertain if there is competitive binding between YAP and β-catenin for SP1 or whether they can form a trimeric complex to enhance stability. Furthermore, investigating the binding dynamics within the nucleus is essential: does SP1 exhibit a preference for binding to specific sites based on the bound proteins, or does the ratio of these complexes influence it? Moreover, an in-depth analysis of the biological implications of this interplay, particularly in the context of intestinal maintenance and regeneration, is warranted.

APPENDIX 1

Reagent	Source	Sequence
siRNAs		
Control siRNA	Mir et al., 2018	AACGTACGCGGAATACTTC
β-catenin siRNA #1	Azzolin et al., 2012	GTAGCTGATATTGATGGAC
β-catenin siRNA #2	Azzolin et al., 2012	GCTTGGAATGAGACTGCTG
SP1 siRNA #1	Dharmacon	GCCAATAGCTACTCAACTA
SP1 siRNA #2	Dharmacon	GAAGGGAGGCCCAGGTGTA
SP1 siRNA #3	Dharmacon	GGGCAGACCTTTACAACTC
YAP siRNA #1	Azzolin et al., 2012	TCTCTGACCAGAAGATGTC
YAP siRNA #2	Azzolin et al., 2012	GCTTTGAGTTCTGACATCC
Morpholino		
Control	GeneTools	NA
Sp1	GeneTools	AAACAGTACCCCCGGACTGACC TGG
qRT-PCR primers		
mki67	This study	(F) AATGTGATGCGTTCAAGGCG
		(R) CCGCAAAGGTGTCTTTGGTT
sox17	Pradhan et al., 2021	(F) AAAACCCAGACCTGCACAAT
		(R) GGTCTTGCATATGTTTGACTC
casanova	Pradhan et al., 2021	(F) ACGAAAGAGGAGCGCAGA
		(R) CATTGCTTTCCATGTCTTGC
myca	This study	(F) CGTGACTCTGACGCCACTTA

		(R)TCACCGGCATTTTGACACTTG
18s	This study	(F) TTACCCCAGGCTCGGAAAAC (R) CGGGAAGGTCTTTGAACCCA
IVT primers		
sp1	This study	(F) GAGTAATACGACTCACTATAG ATGTCAGACTTGAAGAAGCGTG (R) CTAGAATCCGTTGCTGCCG
Public Datasets (in HCT116)		
ChIPseq Input for SP1	ENCODE Consortium	ENCSR000BVT
ChIPseq IP for SP1	ENCODE Consortium	ENCSR000BSF
ChIPseq Input for TCF4	ENCODE Consortium	ENCSR000EUX
ChIPseq IP for TCF4	ENCODE Consortium	ENCSR000EUV
ChIPseq Input for TEAD4	ENCODE Consortium	ENCSR000BVT
ChIPseq IP for TEAD4	ENCODE Consortium	ENCSR000BVJ
ChIPseq Input (HMs)	ENCODE Consortium	ENCSR000BMK
ChIPseq IP for H3K4me1	ENCODE Consortium	ENCSR161MXP
ChIPseq IP for H3K4me3	ENCODE Consortium	ENCSR333OPW
ChIPseq IP for H3K27ac	ENCODE Consortium	ENCSR661KMA
ChIPseq Input for POL25S	ENCODE Consortium	ENCSR000BMK
ChIPseq IP for POL25S	ENCODE Consortium	ENCBS389ENC

ChIPseq Input for β-catenin	Bottomly et al., 2010	SRA012054
ChIPseq Input for BRG1	Mathur et al., 2017	SRR2133624
ChIPseq IP for BRG1	Mathur et al., 2017	SRR2133616
ChIPseq for LDB1	Lai et al., 2020	SRR11969104
ChIPseq Input for TEAD1	Lai et al., 2020	SRR6476373
ChIPseq IP for TEAD1	Lai et al., 2020	SRR6476317

APPENDIX 2

Reagent	Source	Identifier
Experimental models		
Cell line HCT116	ECACC	91091005
Cell line HEK293T	ECACC	12022001
Cell line HeLa	ATCC	CCL-2
Mice NOD.SCID	Envigo Research	NOD.CB17-Prkdc^{scid}/NCrHsd
Zebrafish WT AB	Heisenberg lab	NA
Zebrafish WT Tübingen (TU)	Sonawane lab	NA
Zebrafish Tg: sox17 eGFP	Heisenberg lab	NA
Zebrafish *apc*$^{-/-}$ isolated RNA (5dpf)	Kirankumar lab	NA
Zebrafish WT sibling isolated RNA (5dpf)	Kirankumar lab	NA
Recombinant Constructs		
pCMV10 FLAG-SP1 WT	Mir et al., 2018	NA
pCMV9 FLAG-SP1 MT	Mir et al., 2018	NA
pCMV10 YAP WT	Kulkarni lab	NA
pBabe-Hygro-Myc-YAP S127A	Kulkarni lab	NA
pQCXIH-YAP-5SA	Addgene	33093
pSC2+ ctnnb1	Heisenberg lab	NA

APPENDIX 3

Reagent	Source	Identifier
Antibodies		
SP1	Cell Signaling Technology	9389
β-catenin	BD Transduction Labs	610153
YAP	Santa Cruz	sc-101199
Tubulin	BIO-RAD	MCA4403Z
BRG1	Santa Cruz	sc-10768
TCF7L2	Cell Signaling Technology	2569
p300	Santa Cruz	sc-48343
PARP	Cell Signaling Technology	9532
PCNA	Santa Cruz	sc-25280
Rad51	Santa Cruz	sc-8349
Normal Rabbit IgG	Cell Signaling Technology	2729
Normal Mouse IgG	Upstate	12-371
Proteins and chemicals		
Human Wnt3A ligand	R&D Systems	5036-WN
Human Fibronectin	Merck	F2006
DAPI	Merck	D9542
PNU-74654	Cayman Chemical	Cay16349-10
Mithramycin-A	Cayman Chemical	Cay11434-5

LIST OF PUBLICATIONS

A. Thesis work

1. **Sharma A**, Pradhan SJ, Mishra S, Dsilva GJ, Galande S. SP1-β-Catenin complex regulates cell proliferation in colorectal cancer in a Wnt-dependent manner. (Manuscript to be submitted shortly).
2. **Sharma A**, Dsilva GJ, Pradhan SJ, Mishra S, Galande S. Transcriptional integration of cellular proliferation by SP1 and YAP in colorectal cancer. (Manuscript to be submitted shortly).
3. **Sharma A**, Mir R and Galande S. 2021. Epigenetic Regulation of the Wnt/β-Catenin Signaling Pathway in Cancer. Front. Genet. 12:681053.

B. Other projects

1. Pradhan SJ, Reddy PC, Smutny M, **Sharma A**, Sako K, Oak MS, Shah R, Pal M, Deshpande O, Tang Y, Dsilva G, Mishra R, Deshpande G, Giraldez AJ, Sonawane M, Heisenberg CP, Galande S. 2021. Satb2 acts as a gatekeeper for major developmental transitions during early vertebrate embryogenesis. Nat Commun 12, 6094.
2. Shah R, **Sharma A***, Kelkar A*, Sengupta K, Galande S. 2021. A novel cis regulatory element regulates human XIST in CTCF-dependent manner. Mol Cell Biol 41:e00382-20. * Equal contribution
3. Mir R, **Sharma A**, Pradhan SJ, Galande S. 2018. Regulation of transcription factor SP1 by the β-catenin destruction complex modulates Wnt response. Mol Cell Biol 38:e00188-18.

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