The role of Pygo2 during intestinal tumor initiation and progression *in vivo*

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I, Suranand Babu Talla, would like to confess that this entire project since the beginning has been carried out only by me with the guidance and supervision from Felix Brembeck. Any assistance from everybody else towards the successful completion of this study has been acknowledged with higher gratitude.

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List of abbreviations

Ac	Acetylation
AOM	Azoxymethane
APC	Adenomatous Polyposis Coli
AS	Antisense
Ascl2	Achaete-scute complex homolog 2
BCL9	B-cell CLL/lymphoma 9 protein
BCL9-2	B-cell CLL/lymphoma 9-like protein
BD	Binding domain
Bmi1	B lymphoma Mo-MLV insertion region 1 homolog
bp	Base pairs
BrdU	Bromo-deoxyuridine
BSA	Bovine Serum Albumin
β-TrCP	β-transducin repeat containing protein
CTNNB	β-catenin
CBC cells	Crypt Base Columnar cells
cDNA	Complementary DNA
CK1	Casein kinase 1
CSCs	Cancer stem cells
DCLK1	Doublecortin like kinase 1
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DSS	Dextran sulfate sodium

E	Embryonic day
EDTA	Ethane-1,2-diyldinitrilo tetraacetic acid
EGF	Epidermal growth factor
EtOH	Ethyl alcohol
FAP	Familial adenomatous polyposis
FBS	Fetal bovine serum
gDNA	Genomic DNA
GSK3	Glycogen Synthase Kinase 3
H	Histone
HAT	Histone acetyltransferase
H&E	Hematoxylin & Eosin
HD	Homology domain
HMT	Histone methyltransferase
HRP	Horse radish peroxidase
HPRT	Hypoxanthine phospho ribosyl transferase
IHC	Immunohistochemistry
ISCs	Intestinal stem cells
K	lysine
kb	Kilo base pair
kDa	Kilo Dalton
Lgr5	Leucine-rich repeat G protein-coupled receptor 5
LRP	Low Density Lipoprotein Receptor-related Protein
me	Methylation
me3	Trimethylation
min	Minutes

Min	Multiple intestinal neoplasia
MLL	Mixed lineage leukemia
mRNA	messenger RNA
NHD	N-terminal homology domain
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PHD	Plant homeo domain
PFA	Paraformaldehyde
Prox1	Prospero homeobox protein 1
Pygo	Pygopus
qRT-PCR	Quantitative real time PCR
RNA	Ribonucleic acid
RT	Reverse transcriptase or room temperature
S	Sense
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
sec	Seconds
siRNA	Short interfering RNA
Sox9	SRY (sex determining region Y)-box 9
TCF/LEF	T cell factor/lymphoid enhancer factor
TGFß	Transforming growth factor ß
TLE	Transducin-like Enhancer
Wg	Wingless

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1). **Introduction**

1.1). An introduction to the canonical Wnt/ß-catenin signaling

Canonical Wnt/ß-catenin signaling pathway is highly conserved in vertebrates and invertebrates, which controls the levels of cytosolic and nuclear ß-catenin (Armadillo in drosophila); maintains many aspects of embryonic development, and regulates the continuous homeostasis in many adult tissues. Cell proliferation, differentiation, migration, adhesion, and cell death are dependent on canonical Wnt/ß-catenin signaling pathway. Hence, any germ line and sporadic mutations of genes that express components of this pathway have been shown to result in an aberrant activation of pathway and subsequent deregulation of all these highly regulated and fine-tuned molecular and cellular processes which depend on it (Clevers, & Nusse, 2012; Clevers, 2006; Collu, 2014).

In the center of this pathway is ß-catenin, a main nuclear mediator; its nuclear translocation and elevation is the crucial hallmark for the activation of this signaling cascade, however ß-catenin is also described to be a key component in cadherin mediated cell-cell adhesion (Miller & Moon, 1996).

In the absence of Wnt ligands cytoplasmic β-catenin is engulfed and phosphorylated by a destruction complex, which comprises of scaffolding proteins Axin, APC (adenomatous polyposis coli), kinases GSK3β (glycogen synthase kinase 3) and Casein kinase 1 (CK1) (Kishida *et al.,* 1998; Hart *et al.,* 1998; Yost et al., 1996; Amit *et al.,* 2002; Liu *et al.,* 2002; Yanagawa *et al*., 2002). Once β-catenin is bound to the destruction complex, it is primarily phosphorylated by CK1α creating a binding site for GSK3β, which subsequently phosphorylates three further Ser/Thr residues. Phosphorylated β-catenin interacts with the E3 ubiquitin ligase β-TrCP (β-transducin repeat containing protein) which directs its degradation by proteasomal complex (Aberle *et al.,* 1997; Latres *et al.,* 1999). In unstimulated cells majority of the endogenous β-catenin is found at the membrane, bound to E cadherin, α-catenin and the cytoskeleton, regulating cell–cell adhesion (Peifer *et al.,* 1992; Heuberger *et al.,* 2010). In the nucleus, in the absence of β-catenin, the TCF/LEF family of transcription factors interacts with Groucho/TLE proteins and together acts as transcriptional repressors to halting the Wnt mediated gene output (Brannon *et al.,* 1997; Cavallo *et al.,* 1998).

In contrast, in the presence of secreted Wnt ligands, a receptor complex that is made up of Frizzled and LRP5/6 is formed at the plasma membrane. A still intact destruction complex associates with phosphorylated LRP. After binding to LRP this complex binds to ß-catenin, and subsequent

phosphorylation of it by CK1 and GSK3 β takes place, however ubiquitination by β-TrCP is blocked, and subsequent ß-catenin nuclear translocation is inevitable (Li *et al.,* 2012). In the nucleus, ß-catenin interacts with TCF/LEF family of transcription factors (Molenaar *et al.,* 1996; Van *et al.,* 1997). This ß-catenin/TCF-LEF transcriptional complex physically displaces Groucho (Daniels *et al.,* 2005), and recruits transcriptional co-activators, including Pygopus and BCL9/Legless which lead to the expression of downstream Wnt target genes (Jho *et al.,* 2002; He *et al*., 1998; Kramps *et al.,* 2002; Parker *et al.,* 2002; Thompson *et al.,* 2002).

1.1.1) Role of Pygopus and BCL9 co-factors in Wg/Wnt signaling pathway

The role of Pygopus and BCL9 co-factors during the transcriptional regulation of Wnt target genes by nuclear complex of ß-catenin/Lef-Tcf has been initially described in Drosophila studies (Kramps *et al.,* 2002; Thompson *et al.,* 2002; Townsley *et al.,* 2004; Hoffmans *et al.,* 2005).

Marian Bienz and colleagues identified Pygopus as a member of Wg pathway in Drosophila (Thompson *et al.,* 2002). Though B-cell CLL/lymphoma 9 protein (BCL9) is reported to be overexpressed in B-cell lymphomas due to the trans-location of the *B-cell lymphoma* gene locus (Willis *et al.,* 1998), only in 2002 Kramps *et al* first described that the BCL9 orthologue, Legless, is absolutely required for Wg signaling in Drosophila. Indeed, Legless and Pygopus deletion in Drosophila induce a set of developmental defects that is seen in Drosophila ß-catenin/armadillo null mutants as well (Kramps *et al.,* 2002; Thompson *et al.,* 2002).

This demonstrates that these proteins have a very crucial role in Wg signaling pathway in Drosophila. EGF (Epidermal Growth Factor) and Hedgehog target genes in Pygopus or Legless mutant flies are unaltered, indicating that these proteins are dedicated exclusively to the Wg/Wnt signaling cascade (Thompson *et al*., 2002; Kramps *et al.,* 2002; Belenkaya *et al.,* 2002; Parker *et al*., 2002). Studies in Xenopus revealed that xPygo depletion lead to embryonic defects, particularly in embryonic brain patterning, and a reduction in Wnt target gene expression (Lake, B. & Kao, 2003). Another experiment again in Xenopus has shown the co-dependency of xBcl9-xPygopus for body axis formation (Kennedy *et al.,* 2009). Apart from their transcriptional co-activating ability, Pygo proteins are supposed to be required for nuclear export of β-catenin. They have two distinct conserved domains, an N-terminal homology domain (NHD) and a C-terminal PHD (plant homeo domain) zinc finger motif (Parker *et al.,* 2002). Evidences suggested that both domains are important for the function of Pygo proteins. Several studies provided evidence that the NHD domain of Pygo has transactivation ability, whereas the PHD

finger has been shown to interact with legless/BCL9, which in turn interacts with N-terminal domain of β-catenin (Städeli, R. & Basler, 2005; Hoffmans *et al.,* 2005; Thompson *et al.,* 2004). Therefore, the function of legless/BCL9 is suggested to act like an adaptor between β-catenin and Pygopus (Townsley *et al.,* 2003).

Plant homeo domains are implicated in epigenetic regulations and bound to methylated residues on lysine 4 of histone H3 (H3K4me), which is strongly associated with active transcription (Santos-Rosa *et al.,* 2002; Bienz *et al.,* 2005; Aasland *et al.,* 1995). Pygopus PHD zinc finger domain in Drosophila is reported to link Bcl9/ß-catenin, but not to H3 domain of histone (Kessler *et al.,* 2009), In contrast mammary progenitor cell expansion is facilitated by Pygopus mediated methylation of H3 at K4 (Gu *et al*., 2009). This might be a primitive evolutionary exception in Drosophila. Further an increasing number of studies showed the Pygopus involvement in chromatin remodeling, which include its function in spermatogenesis, and association with MLL2 histone methyltransferase (HMT) and GCN5 histone acetyltransferase (HAT) complexes to induce Wnt target gene expression and breast cancer stem-like cell expansion (Nair *et al.,* 2008; Chen *et al.,* 2010). Hence, altogether these experimental output ratifies that Pygopus proteins augment ß-catenin/Tcf-Lef and complex with HMT, and HAT.

1.1.2) BCL9 and Pygopus homologues in mammalians

In mammalians the role of BCL9 and Pygopus homologues is rather divergent and complex compared to Drosophila. Vertebrate BCL9 homologue, BCL9-2, has been first identified by yeast two-hybrid screen experiments (Brembeck *et al.,* 2004). Structure of BCL9 mammalian homologues and the functional relevance of domains within it are extensively studied. Structural domains of BCL9 proteins are highly conserved in mammalians compared to the Drosophila Legless; share up to 90% of amino acid homology including in all of seven evolutionarily conserved domains (Brembeck *et al.,* 2006). The N-terminal consists of N-terminal homology domain (N-HD), a Pygopus binding region (PyBD), ß-catenin binding region (ß-cat-BD) and a Nuclear Localization Signal (NLS). In addition, C-terminal is a group of three homology domains, namely C-HD1, C-HD2 and C-HD3. On contrary to BCL9, BCL9-2 is demonstrated to have also a functional nuclear localization signal (NLS), and truncation of this region is also shown to cause migration of BCL9-2 to cytoplasm (Adachi *et al.,* 2004; Brembeck *et al.,* 2004 ;). This unique capability of BCL9-2 is not observed in BCL9 and Legless, which require Pygopus binding to its N-terminal Pygopus binding domain (PyBD) for its nuclear translocation (Adachi *et al.,* 2004; Brembeck *et al.,* 2004; Townsley *et al.,* 2004). Thus, BCL9-2 alone is sufficient for the ß-catenin translocation into the nucleus to induce target gene expression. Apart from this, BCL9-2 is as well reported to modulate the ß-catenin

adhesive and transcriptional capability through phosphorylation of tyrosine at 142 of ß-catenin protein. This made ß-catenin in favor of binding to BCL9-2, instead of α-catenin (Brembeck *et al.,* 2004).

Extensive Pygopus screenings in mouse revealed some surprising findings regarding requirement for mammalian Pygopus in canonical Wnt signaling (Li *et al*., 2007; Song *et al*., 2007; Schwab *et al*., 2007). Unlike in Drosophila, there exist two homologues of Pygopus, Pygo1 and Pygo2 in mammalians (Kramps *et al*., 2002; Li *et al*., 2002). Pygopus genes are expressed spatially where Wnt signaling is known to be prominent for development as well as where Wnt signaling has no demonstrated function (Li *et al*., 2007; Song *et al*., 2007; Schwab *et al*., 2007). Pygopus homologues are reported to be important for the kidney development in mammalians, in which they found the requirement of Pygopus proteins for the development of normal branching morphogenesis of the ureteric bud (Schwab *et al*., 2007). Another study in mammals revealed that Pygo2 deletion affected development of some but not all Wnt requiring tissues (Li *et al*., 2007). In contrast to this, germ line mutations in Pygo2 in mice resulted in microophthalmia, a defective lens development by reduced levels of Pax6 expression which decided lens fate, which is strikingly a Wnt independent function of Pygo2 (Song *et al*., 2007). Expression analysis of Pygo1 and Pygo2 in different tissues in mice revealed that, whereas pygopus1 is expressed only in heart tissue, Pygo2 is predominantly ubiquitous (Li *et al*., 2004). Previous experiments from our lab stated that in intestinal epithelium only Pygo2 is expressed along the crypt villus axis but not Pygo1 (Brembeck *et al*., 2011). In addition, intestinal stem cell formation and hair follicle development, two well-established processes that required active Wnt signaling are not or only minimally affected by loss of Pygo2 expression (Korinek *et al*., 1998; Alonso *et al*., 2003; Andl *et al*., 2002; Brembeck *et al*., 2011; Li *et al*., 2007). These studies clearly demonstrated that mammalian Pygopus genes are not always essential for all Wnt-requiring processes. All these clear observations indicated that Pygopus gene homologues play a context dependent role in canonical Wnt/ß-catenin signaling.

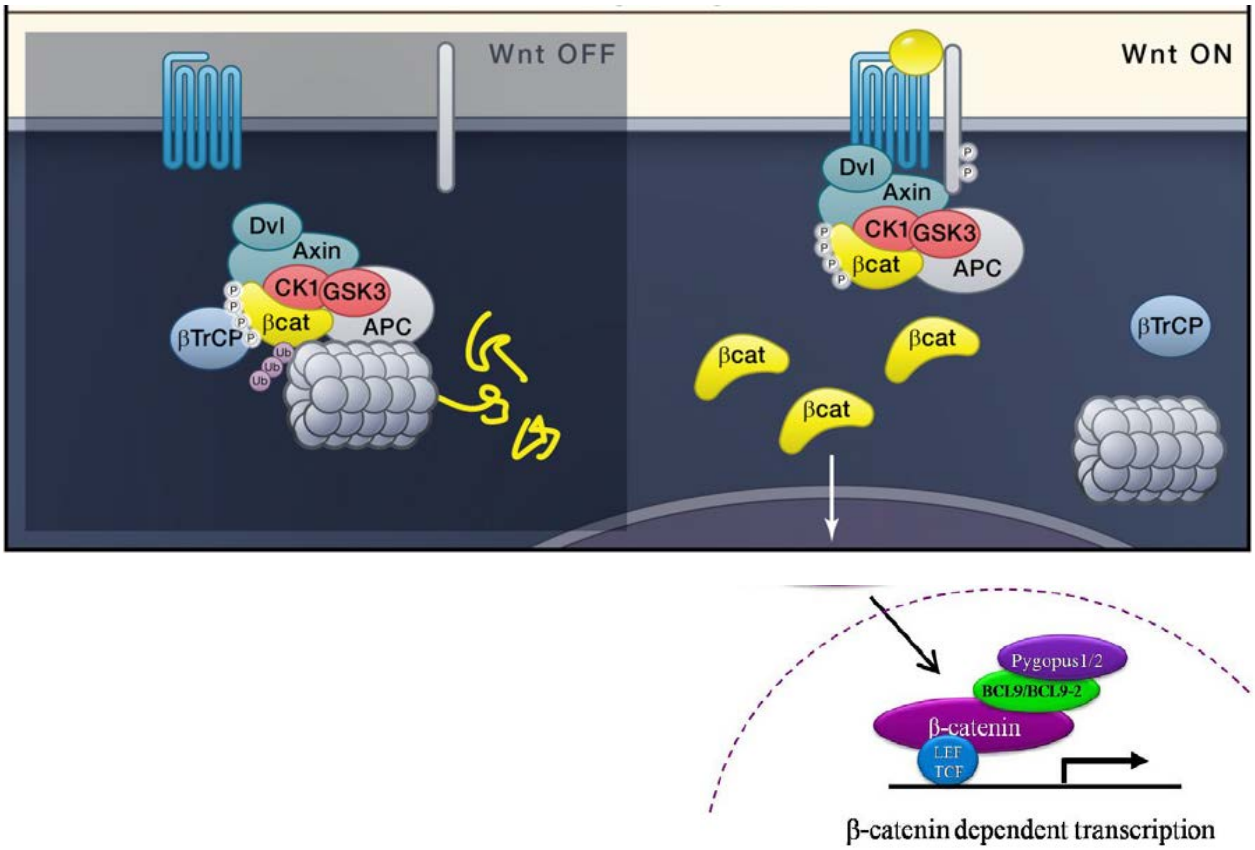

Figure 1. Revised model for canonical Wnt/ß-catenin signaling mechanism. In the absence of Wnt ligand destruction complex associates with ß-catenin for its Ser/Thr phosphorylation, and subsequent ubiquitination by β-TrCP that result in the degradation through proteasomal complex. However, when Wnt ligand binds to the Frizzled/LRP receptor, in contrast to current opinion, destruction complex is still intact and able to phosphorylate ß-catenin, nonetheless, ubiquitination by β-TrCP does not take place thereby promoting ß-catenin nuclear translocation where it forms an active transcription complex with Lef/Tcf for the downstream Wnt target gene cascade activation (Adapted from Clevers & Nusse, 2012; Brembeck *et al*., 2005).

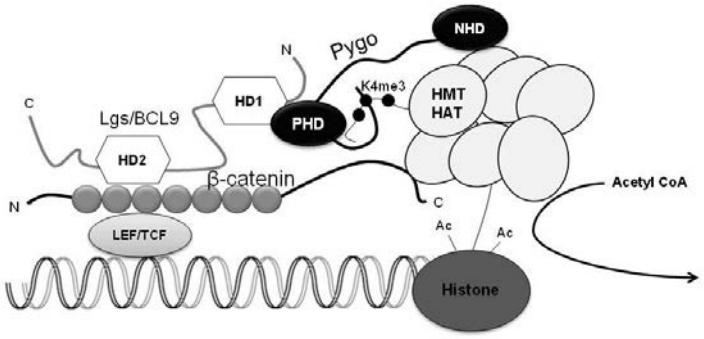

Figure 2. Schematic illustration of current knowledge regarding the binding pattern of BCL9 and Pygopus co-factors to ß-catenin during Wnt signaling pathway.
Pygopus1/2 binds to BCL9/BCL9-2 N-terminal HD1 domain through its C-terminal PHD zinc finger domain, and BCL9/BCL9-2 by its N-terminal HD2 domain binds to N-terminal armadillo first two repeats of ß-catenin. Pygopus chromatin remodeling functions are demonstrated by PHD zinc finger domain binding affinity to Histone 3 trimethylated at lysine 4 (k4). And Pygopus NHD (N-terminal homology domain) of Pygopus associates with histone methyltransferases (HMT) and histone acetyltransferases (HAT).
(Adapted from Jessen *et al*., 2008; Chen *et al*., 2010; Andrews *et al*., 2009).

1.2) Wnt/ß-catenin signaling dependent intestinal epithelial development

Wnt signaling has been investigated for its role in gut development of ascidian embryos in which ß-catenin is demonstrated to be crucial for the endoderm formation (Imai *et al*., 2000). Later on, the development of almost all endoderm originated organs are shown to exclusively rely on the active Wnt signaling pathway and in fact Wnt signaling controls proliferation and differentiation during gut organogenesis (Verzi *et al*., 2008). Previous in vivo investigations have concluded that Wnt signals are vital for the induction of intestinal epithelial progenitor cell division (Bienz & Clevers, 2000; Booth *et al*., 2002; Kinzler *et al*., 1996). Embryonic gut development in mouse starts between embryonic day E7.5 and E9.5 simultaneously in the anterior and posterior parts of the developing embryo. During this period, partially matured intestine mainly consists of the epithelium that is covered by outer layer of splanchnic mesoderm. However, splanchnic mesoderm eventually differentiates into a smooth muscle layer; simultaneously inner highly proliferative epithelium evolves into specific regions of intestine throughout the anterior and posterior axis approximately at E14.5 (Kedinger *et al*., 1998).

Maturation of intestinal epithelium completes around E18.5 with apparent highly differentiated numerous finger-like projections of villi that cover the entire intestinal lumen. This unique intestinal architecture dramatically increases the efficiency of absorption of nutrients by small intestinal epithelium. On the contrary, no villi are formed in large intestine; rather it has a flat surface of epithelium throughout the inner layer. Thus, the main function of colon is to compact the remaining after the active absorption of nutrients in small intestine. Villi in small intestine basically consist of terminally differentiated cells. Base of each villus region through invagination forms a crypt of Lieberkühn during the early weeks after the birth (Santa *et al*., 2003; Wells *et al*., 1999).

1.2.1). Intestinal epithelium architecture and two pools of stem cell models

Intestinal single layer of inner epithelium is undoubtedly a fascinating organ to study the self-renewal capacity in mammalians, which self-replicates entirely every 3-5 days in mice. Crypt-villus junction is considered a structural and functional unit of this epithelium (Leblond & Stevens, 1948; Schepers & Clevers, 2012).

Persistent renewal of gut epithelium is shown to be driven by multipotent intestinal stem cells (ISC), which are located in the crypt region. These stem cells divide into a group of rapidly proliferating progenitor cells called transit-amplifying cells (TA), which eventually differentiate into various kinds of

stem cell phenotypes, as they migrate towards the end of villi (Clevers, 2013). Thus, each villus only consists of highly matured and functionally distinctive cells, including enterocytes, enteroendocrine cells, and goblet cells. Enterocytes key role is transportation, enteroendocrine cells are hormone secreting cells, while goblet cells release mucin into the inner surface of the epithelium. Apart from these cells, ISC also differentiate into another minor pool of M cells, which are sparsely populated compared to remaining others. These M cells in fact sit just above the Payer's patches and absorb any antigen ingested with food from intestinal lumen and transport them to Payer's patches (Lau, de *et al*., 2012). In addition, another distinctive manifestation of intestinal stem cell is Tuft cell. These tuft cells secrete opioids and enzymes related to prostaglandin production to sense the intestinal lumen (Gerbe *et al*., 2011). Paneth cells are rather different form of lysozyme, cryptidin and defensin secreting cells because they migrate usually into the crypts to reside among stem cells in contrast to other differentiated cells, which migrate to the tip of the villi (Clevers, 2013).

To date, two distinct pools of intestinal stem cells (ISC) have been identified in the intestinal epithelium. Investigations by the group of Leblond first identified crypt stem cells. Indeed, they have empirically demonstrated the localization of crypt base columnar cells (CBC), and lineage tracing experiments gave the evidence that all differentiated cells were individual phenotypes of these stem cells (Cheng & Leblond, 1974). This model is also called as "stem cell zone" model. Crypt base columnar stem cells mature into four distinctive intestinal phenotypes, while they migrate towards the villus region (Bjerknes & Cheng, 1981). Clevers group first discovered a reliable marker to recognize these stem cells, a leucine rich orphan G protein coupled receptor (Lgr5), which is also a prominent Wnt target gene (Barker *et al*., 2007).

A second distinctive set of stem cells are called +4 stem cells, which is supported by early work of the Chris Potten research group. They have provided evidence that label retaining cells are present at this location in crypt. In addition, these stem cells also have demonstrated sensitivity to radiation that in turn protects them from genetic aberration (Potten *et al*., 1978). However, this model varies from stem cell zone one, because stem cells in this model are localized at +4 position that is just right above the Paneth cells towards the villus region. And Bmi1 is shown to be one marker gene that is specially expressed by these stem cells (Yan *et al*., 2012). Through lineage tracing experiments Bmi1 expressing cells are found to be rapidly dividing, self-renewal and able to differentiate into all stem cell phenotypes (Sangiorgi *et al*., 2008). In addition, previous examination had proved that mutations in TCF4 lead to the complete disappearance of stem cells and thereby impaired tissue regeneration (Korinek *et al*., 1998).

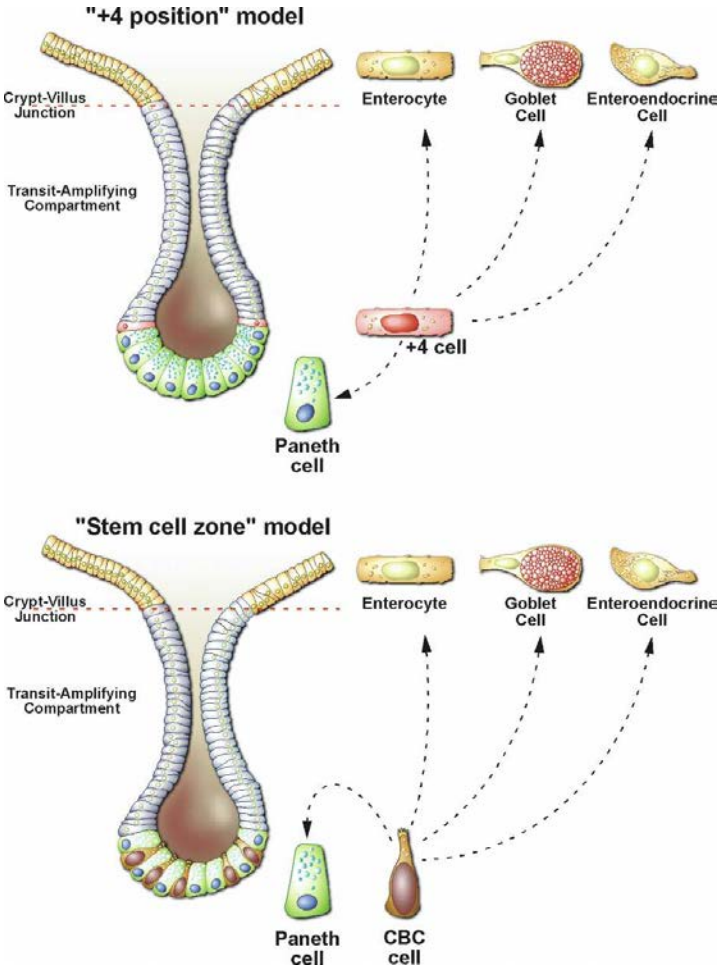

Figure 3. Two popularly accepted stem cell models based on intestinal stem cell location: +4 position model (above), and stem cell zone model (below) It is proposed in +4 cell model that since crypt is totally covered by Paneth cells stem cells should reside just above them at +4 position. Whereas crypt base columnar model stem cells are intermingled within the Paneth cells in the crypt base, which are believed to rapidly divide and replenish the villus compartment (Adapted from Barker *et al.,* 2008).

1.3). Deregulation of Wnt/ß-catenin signaling: A primary hallmark of Intestinal tumor initiation

In normal homeostasis of intestinal epithelium, there is a tight fine-tuned balance of proliferation, differentiation, anoikis type of apoptosis, and migration to ensure the consistent renewal. When this tight regulation is disrupted, this results in intestinal hyperproliferation at the cost of differentiation, and the pathological manifestation of this in humans is colorectal cancer in later stages (CRC) (Beauséjour *et al*., 2013; Pinto & Clevers, 2005). The earliest studies of CRC are reported by Marson in 1984. This primary morphologic change in colon or rectum is epithelial hyperplasia that becomes predominantly dysplastic which are called “aberrant crypt foci” (Cheng & Lai, 2003). These persistently dividing cells

evolve into benign tumors and after acquiring additional genetic alterations they become malignant tumors called carcinomas, which are invasive tumors.

In a famous study by Fearon and Vogelstein, neoplastic transformation of colon epithelium was demonstrated to be a sequential multistep process of different genetic events (Fig. 4). They illustrated mutations in adenomatous polyposis coli gene (APC) as a driver of primary polyp formation, and due to the accumulation of other gene mutations, for instance of K-ras these polyps progress into highly proliferative advanced tumors (Fearon & Vogelstein, 1990).

CRCs are among the most common cause of cancer mortality (Pai *et al*., 2016). Approximately 15% of all CRCs occur due to the inevitable inherited genetic predisposition, which is termed as familial adenomatous polyposis coli (FAP); in contrast remaining 85% happen due to sporadic reasons (Lynch & de, 2003). All FAP driven colon carcinomas and almost 90% of sporadic cases are initiated by Loss-of-Function mutations in the key tumor suppressor gene, APC (Soravia *et al*., 1998).

Almost 5% of APC wild type bearing sporadic cases harbor with Gain-of-Function mutations in ß-catenin proto-oncogene, which is the key nuclear mediator (Morin *et al*., 1997). Hyperactive Wnt pathway is also reported to be a result of mutations in Axin1 and Axin2 genes, which encode proteins that are part of the destruction complex, a negative feedback loop (Shimizu *et al*., 2001; Liu *et al*., 2000). Alternatively, gene fusions involving Tcf4/Tcf7l2 and secreted Wnt agonist R-spondins have very recently been studied (Bass *et al*., 2011; Seshagiri *et al*., 2012). Nevertheless, in any of these cases nuclear translocation of ß-catenin is a crucial event to occur for the hyper activation of downstream target genes through its binding to Tcf4/Lef1 DNA binding domains by replacing Groucho (Daniels *et al*., 2005).

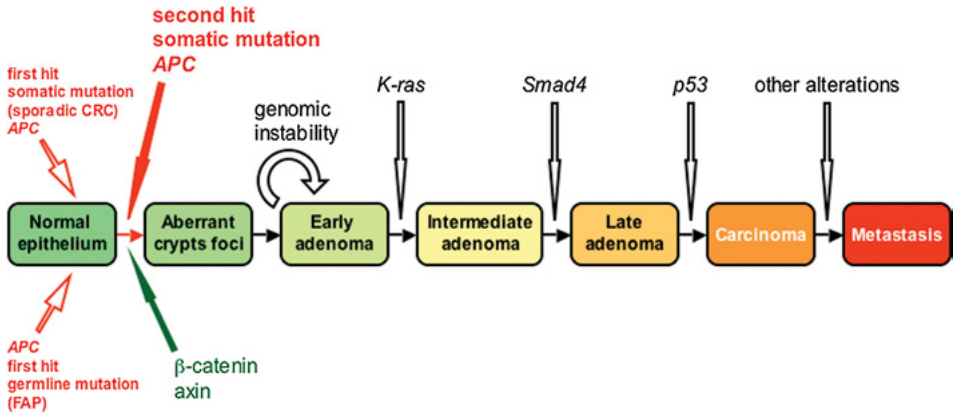

Figure 4. A genetic model illustrating the progressive mutations in CRC (Fearon & Vogelstein, 1990)

Inherited or sporadic mutations mainly in APC, ß-catenin, or Axin genes are required for tumor formation, and progression towards malignancy is attributed to reported sequential mutations in K-ras, Smad4, and p53, as well as numerous other unreported genes.

1.4). Involvement of BCL9/BCL9-2 and Pygo2 in Wnt dependent & independent cancer formation

Wnt signaling pathway remains active in many of the organs in adult mammals, among them intestinal epithelium, breast, hair follicles and blood are well studied (Krausova *et al*., 2014; Turashvili *et al*., 2006; Andl *et al*., 2002; Luis *et al*., 2011). As previously described that deregulation of Wnt signaling pathway is one of the major hallmarks in many cancers, including colon cancer, studies to discover Pygopus and BCL9 gene homologues functional relevance, concerning their role in Wnt signaling pathway, are under investigation.

BCL9 homologues are involved in various malignant formations. In an in vitro and in vivo analysis BCL9 has been shown to be essential for colon cancer progression as well as in multiple myeloma (Mala Mani *et al*., 2009; Marc de la Roche *et al*., 2008; Brembeck et al., 2011). In addition, targeted disruption of BCL9/ß-catenin complex has also resulted in the Wnt signaling suppression directed tumor growth arrest in xenograft models of colorectal carcinoma (Takada *et al*., 2012). However, a primary in vivo evidence for the evaluation of BCL9/BCL9-2 role in chemically induced colon cancer models showed that ablation of BCL9/BCL9-2 induced the suppression of Wnt target genes as well as epithelial to mesenchymal transition (EMT) and stem cell-like properties (Deka *et al*., 2010). In our examinations also BCL9-2 expression was significantly elevated in colon cancer patients; controlled Wnt gene transcription in colon cancer cells, and in vivo overexpression of BCL9-2 induced intestinal tumor progression and invasion in APC^{Min} mice (Brembeck *et al*., 2011). In accordance with our previous study, a recent examination by the group of Michel Aguet revealed that BCL9/BCL9-2, in the context of activated Wnt/ß-catenin signaling, has significantly influenced the epithelial to mesenchymal transition and stemness properties in colon cancer murine models and in colon cancer patients, which was eventually even linked to the poor survival of colon cancer patients (Moor *et al*., 2015).

Simultaneously, Pygo2, being a co-factor of Wnt signaling pathway, has also been found to be involved in various cancers. Pygo2 gene knockdown in colorectal cancer cells containing a mutant APC reduced Wnt reporter gene expression (Thompson *et al*., 2002), suggesting that endogenous Pygopus proteins modulated signaling output in these particular cancer cells. Pygo2 was also up regulated in some breast cancer cell lines, but after reducing the levels of Pygo2, there was a significant down regulation of CyclinD1, a known Wnt target gene (Andrews *et al*., 2007).

Even in epithelial ovarian cancers Pygo2 was overexpressed in both subtypes with Wnt active or not active (Popadiuk *et al*., 2006), and knockdown of Pygo2 resulted in the growth arrest in both subtypes. Non-small cell lung carcinomas also overexpressed Pygo2 and correlated with malignant phenotype; however, when Pygo2 was knockdown growth rate was severely inhibited (Fang *et al*., 2003). Previous reports in studies of glioblastomas found that Pygo2 knockdown decreased cell proliferation, invasiveness, and induced cell cycle arrest at the G1, and down regulation of cyclinD1 (Wang *et al*., 2010). In addition, Pygo2 was also correlated with the poor prognosis in patients with hepatocellular carcinoma (Zhang *et al*., 2014). Experiments conducted previously in our lab also revealed that Pygo2 knockdown in colon cancer cell lines including HCT116, and SW480 resulted in the reduction of a subset of Wnt/ß-catenin target genes (Brembeck *et al*., 2011). Further co-transfection of dPygo together with Legless increased the nuclear localization of ß-catenin as well as the TCF/ß-catenin mediated transcription in APC mutated cancer cells lines, suggesting that they may synergistically activate the Wnt signaling pathway in these cells (Townsley *et al*., 2004).

Aim of the current study

Intestinal epithelium homeostasis is strictly regulated by Wnt/ß-catenin signaling pathway. So far, the precise function of Pygo2, a nuclear co-factor and ß-catenin binding factor, is not studied in the context of intestinal tumor initiation and progression. Therefore, to investigate the function of Pygo2 chemically induced and conditional intestinal tumor mouse models have been analyzed in this study.

At first, Pygo2 function is studied in chemically induced intestinal tumor models. For this, constitutive Pygo2 deficient and control murine models are injected with azoxymethane for the induction of tumors in colon. Apart from this importance of Pygo2 is also investigated during the intestinal epithelial regeneration following the acute intestinal inflammation induced by dextran sodium sulphate. In each of these contexts, intestinal pathology of Pygo2 knockout and wild type mice are examined after the treatment. Further histochemical stainings are also performed on intestinal sections for the detection of specific markers to evaluate the tumor formation in colons as well as intestinal regeneration in inflammation induced mice. In addition, Wnt signaling target genes are as well studied in tumors of chemically induced mice and intestinal regeneration in inflammation induced mice.

Secondly, APC and ß-catenin conditional mouse models, with different Pygo2 genetic backgrounds, are taken to induce intestinal specific hyperproliferation to find a role for Pygo2 in the context of active Wnt signaling driven intestinal hyper-proliferation. To assess the Pygo2 function, survival period of intestinal tumor mouse models following the induction of genetic recombination, examination of intestinal tissue sections to assay the specific markers immunohistochemically, and further analyzing Wnt mediated transcription in these murine models are performed.

2). Materials

Cover Slips	Thermo Scientific
Filters for solutions (0.2 µm and 0.45 µm)	Sartorius
Gloves (nitrile, latex)	Sempermed
Hypodermic needle (23 G)	BBraun
Pasteur pipettes	Peske OHG
Petri dishes	Falcon
Pipettes (2, 5, 10 and 25 ml)	Eppendorf
Pipette tips (10, 200 and 1000 µl)	MbP
Pipette tips (10, 200 and 1000 µl with a filter)	Biozym
Plates for cell culture (96-well) TPP,	Nunc
Scalpels	Technic cut
SuperFrost®Plus Adhesion slides	Thermo Scientific
Tubes for cell culture (polystyrene, 15 and 50 ml)	Falcon, Sarstedt
Tubes for cell culture (polypropylene, 15 and 50 ml)	Falcon
Tubes for molecular biology, Safelock (1.5 and 2 ml)	Eppendorf, Sarstedt
Whatman paper	Whatman

2.1). Instruments and equipment

Camera	DC 300FX	Leica
Camera	DFC 290	Leica
Electrophoresis chambers for Agarose gels		Peqlab
Freezer (-20 °C)	PremiumNoFrost	Liebherr
Freezer (-80 °C)	Ultra low temperature freezer U725	New Brunswick Scientific GmbH
Fridge (+4 °C)	Electrolux SANTO	AEG
Gel analyzer	BioDocAnalyze	Biometra
Heating block	Thermostat plus	Eppendorf
Ice machine	ZBE 70-35	Ziegra
Incubator		Memmert

Micro centrifuge		Eppendorf
Micropipettes	(2, 10, 100, 200, 1000 µl)	Eppendorf
Microscope	DM 500	Leica
Microscope	inverted DM IRB	Leica
Microwave oven		Powerwave
Microtome cryostat	HM 355S	Microm
Modular tissue embedding center	EC 350-1; EC 350-2	Microm
PCR cycler	T3 Thermocycler	Biometra
Pipetting assistant	MATRIX	Thermo Scientific
Power supplier	EV231	Peqlab
Printer		Mitsubishi
Real Time PCR device	7900HT Fast Real-TimePCR System	Applied Bio systems
Refrigerated Micro centrifuge		Eppendorf
Rotator		GLW
Shaker	IKA-Shaker MTS4	W. Krannich GmbH+Co.KG
Spectrophotometer	ND-1000	Nano Drop
Transilluminator	UV Star	Biometra
UV lamp	EBQ100 isolated	Leica
Vortexer	IKA ® Vortex	IKA
Water purification system		Millipore

2.2). Chemicals and reagents:

Acetic acid	Roth
AOM	Sigma-Aldrich
Agarose	Invitrogen
Bromdesoxyuridin (BrdU)	Roche
Chloroform	Roth
D (+)-trehalosedihydrat	Roth
DAKO Envision Kit	DAKO
DAPI (4´,6-diamidino-2-phenylindole)	Sigma
DEPC (diethyl pyrocarbonate)	Roth
DNA Ladder	Fermentas

DNase	Roche
DSS	MP Bio medicals
EDTA	Roth
EtBr (ethidium bromide)	Roth
Ethanol	ChemieVertriebHannover
Eosin	Roth
Fast Start Taq DNA Polymerase (dNTPs pack)	Roche
GeneRulerTM 1 kb DNA ladder	Fermentas
HCl (hydrochloric acid)	Roth
Hematoxylin	Roth
HOT FIREPol DNA polymerase	Solis BioDyne
H_2O_2 (Peroxygen)	Roth
Immu-MountTM	ThermoScientific
Isopropanol	J.T.Backer
KCl (potassium chloride)	Sigma
KH_2P0_4	Roth
NaCl (sodium chloride)	Roth
NaHCO3 (sodium hydrogen carbonate)	Merck
Na2HPO4	Roth
NaOH (sodium hydroxide)	Sigma
PFA (paraformaldehyde)	Merck
Proteinase K	Roche
Protease & Phosphatase Inhibitor cocktail tablets, EDTA free	Roche
Random hexamer primers	IBA
RevertAid H Minus Reverse Transcriptase	Fermentas
RNA sample buffer	Fermentas
RNase A (Ribonuclease A)	Roche
RNase Inhibitor	Fermentas
Roti®-Histokitt	Roth
Roti®-Phenol/Chloroform/	Roth
Streptavidin-biotinylated HRP	GE Healthcare
SYBR GREEN I	Sigma Aldrich

TRI Reagent	Ambion
Tris	Roth
Xylol	Roth

2.3). Buffers

PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.47 mM KH2PO4)

Tail Lysis buffer (100mMTris-HCL, 5mM EDTA, 200mM NaCl, 0.2%SDS)

Citrate buffer (10mM Citric Acid, 0.05% Tween20, pH6.0)

2.4). qRT-PCR buffers:

component	Stock concentration	For 10ml	Final concentration
Tris-Hcl(pH8,8)	1,5M	5ml	750 mM
$(NH_4)_2SO_4$	1M	2ml	200mM
Tween-20	10%	100 µl	1%
H_2O		2,9 ml	

2.5). Master Mix for qRT-PCR:

component	Stock concentration	µl for 1 sample	Final concentration
10xbuffer	10x	2.5	1x
$MgCl_2$	25 mM	3	3mM
Cyber Green	1:100	0.0313	1:80000
dNTP´s	20 mM	0.25	0.2mM
Taq-Polymerase	5 U/µl	0.1	20U/ml

TritonX-100	10%	0.625	0.25%
Trehalose	1M	7.5	

Mouse model	Background	Source
β-catenin$^{fl(ex3)}$	Gain of function model; 3rd exon was flanked by loxP sites. Crossing with Villin-CreERT2 expressing mouse results in stabilization of ß-catenin.	Harada *et al.,* 1999
Pygo2$^{fl/fl}$	Loss of function model; deletion of 3rd exon by conditional mutation.	W. Birchmeier, unpublished
Apc15Lox	Loss of function mouse model; 15th exon of APC gene was deleted conditionally by crossing with Villin-CreERT2.	Robanus-Maandag EC *et al.,* 2010
Villin-Cre	Cre was expressed under the control of Villin promoter in intestinal epithelia specific manner.	el Marjou *et al.,* 2004
Villin-CreERT2	Cre-recombination conditionally activated up on the administration of Tamoxifen only.	el Marjou *et al.,* 2004

2.6). Human colon cancer cell lines:

The human colon cancer cell lines SW480, DLD1 and HCT116 were purchased from ATCC. All knockdown experiments, RNA extraction, and complementary DNA synthesis from cancer cell lines were performed by my previous colleague in the lab, Dr. Maria Weise. I am thankful to her.

Name	Source
SW480	Colon cancer cell line established from a primary adenocarcinoma of the colon of a 50 years old Caucasian male (Leibovitz *et al.*, 1976).
DLD1	Epithelial colorectal adenocarcinoma derived from an adult female (Dexter *et al.*, 1979).
HCT116	Colorectal carcinoma cell line obtained from an adult male (Brattain *et al.*, 1981).

Human colon cancer cell lines and their mutational status:

Summary of mutations in Wnt signaling pathway:

Cell line	**APC mutations**	**ß-catenin (CTNNB) mutations**
SW480	Mutant	Wildtype
DLD1	Mutant	Wildtype
HCT116	Wild type	Mutant (Exon3)

(M. Ilyas *et al.,* 1997)

Illustration of various deregulated signaling pathways in colon cancer cell lines:

Cell line	MSI status	KRAS	BRAF	PIK3CA	TP53
SW480	**MSS**	**G12V**	**WT**	**WT**	**R273H; P309S**
DLD1	**MSI**	**G13D**	**WT**	**E545K; D549N**	**S241F**
HCT116	**MSI**	**G13D**	**WT**	**H1047R**	**WT**

MSS: Micro Satellite Stable; MSI: Micro Satellite Instable (D Ahmed *et al*., 2013)

2.7). Oligonucleotides: Primers used for mice genotyping

Target gene	**Primer sequence (5'-3')**
Apc-lox3FW2	5'-TAGGCACTGGACATAAGGGC-3'
Apc-loxNot3BW	5'-CTTCGAGGGACCTAATAAC-3'
Apc-lox3R2	5'-GTAACTGTCAAGAATCAATGG-3'
Pygo2-lox-S	5'-CCT GGG TTG CTT GTC TTCTG-3'
Pygo2-ex3-AS	5'-GGA AGC AAA GGG ACA CAGAG-3'
deltaN-bCat483-S	5'-AGA ATC ACG GTG ACC TGGGTT AAA-3'
deltaN-Cat1051AS	5'-CAT TCA TAA AGG ACT TGGGAG GTG T-3'
Villin-Cre-S	5'-CAA GCC TGG CTC GAC GGCC-3'
Villin-Cre-AS	5'-CGC GAA CAT CTT CAG GTTCT-3'

2.8). Primers used for qRT-PCR on mouse models:

Target gene primer name	Sequence (5'-3')	Reference
Pygo2-S	GGTTGAGCAGAGCCATTCCT	Maria Wiese, Brembeck *et al.,* 2011
Pygo2-AS	CAGCCATGGGGCTATACAGG	Maria Wiese, *Brembeck et al.,* 2011
BCL9-2-S	AATCATGGCAAGACAGGGAATGGA	Maria Wiese
BCL9-2-AS	TCTTCAGACTTGAGTTGCTAGGTG	Maria Wiese
Axin2-S	GCTCCAGAAGATCACAAAGAGC	Sansom OJ *et al.,* 2007
Axin2-AS	AGCTTTGAGCCTTCAGCATC	Sansom OJ *et al.,* 2007
CMYC-S	GACCTAACTCGAGGAGGAGCTGGAATC	Besser, D. MDC, Berlin.
CMYC-AS	AAGTTTGAGGCAGTTAAAATTATGGCTGAAGC	Besser, D. MDC, Berlin.
Sox9-S	ACTCCCCACATTCCTCCTCC	Suranand Babu Talla
Sox9-AS	GGACCCTGAGATTGCCCAGA	Suranand Babu Talla
Prox1-S	GCTCCAACATGCTGAAGACCTA	Deka J *et al.,* 2010
Prox1-AS	GCTGCGAGGTAATGCATCTG	Deka J *et al.,* 2010
Lgr5-S	CCAATGGAATAAAGACGACGGCAACA	Suranand Babu Talla
Lgr5-AS	GGGCCTTCAGGTCTTCCTCAAAGTCA	Suranand Babu Talla
Lef1-S	AGAACACCCTGATGAAGGAAAG	Suranand Babu Talla
Lef1-AS	GTACGGGTCGCTGTTCATATT	Suranand Babu Talla
Tcf4-S	AAGACTTGAACATTAGCGAGAG	Suranand Babu Talla
Tcf4-AS	AAGAGCACAGGGCAGTTG	Suranand Babu Talla
DCMKL1-S	CAGCAAGTCTCCCAGAAGATAC	Suranand Babu Talla
DCMKL1-AS	AGGACTGGAGACCACACTAA	Suranand Babu Talla

Target gene primer name	Sequence (5'-3')	Reference
CyclinD1-S	TGCGTGCAGAAGGAGATTGT	Suranand Babu Talla
CyclinD1-AS	CCTCACAGACCTCCAGCATC	Suranand Babu Talla
Hes1-S	CATGGAGAAGAGGCGAAGGG	Suranand Babu Talla
Hes1-AS	GGAATGCCGGGAGCTATCTT	Suranand Babu Talla
Ascl2-S	AAG CAC ACC TTG ACT GGT ACG	Suranand Babu Talla
Ascl2-AS	AAG TGG ACG TTT GCA CCT TCA	Suranand Babu Talla
HPRT-S	CCTAAGATGAGCGCAAGTTGAA	http://www.rtprimerdb.org
HPRT-AS	CCACAGGACTAGAACACCTGCTAA	http://www.rtprimerdb.org
E-Cadherin-S	ATCAGCTGCCCCGAAAATGA	Suranand Babu Talla
E-Cadherin-AS	TGTCCCTGTTGGATTTGATCTGA	Suranand Babu Talla
Tcf1-S	TCTGCTCATGCCCTACCCA	Suranand Babu Talla
Tcf1-AS	TGTTATGCAGCGGGGGTTGAG	Suranand Babu Talla

All above oligonucleotides were synthesized by ©IBA GmbH, Göttingen, and Metabion AG, München.

2.9). Antibodies used for Immunohistochemical analysis:

Antibody	Source	Dilution	Catalogue No.	Manufacturer
Anti-ß-catenin	Mouse	1:500	610154	BD Transduction
Anti-ß-catenin	Rabbit	1:2000		Brembeck *et al.,* 2011
Anti-BrdU	Rat	1:100	ab6326	Abcam
Anti-Pygo2	Rabbit	1:1500		Brembeck *et al.,* 2011
Anti-BCL9-2	Rabbit	1:250		Brembeck *et al.,* 2011

Anti-BCL9	Rabbit	1:1500		Brembeck *et al.,* 2011
Anti-DCLK1	Rabbit	1:1000	ab31704	Abcam
Anti-Sox9	Rabbit	1:3000	AB5535	Millipore
Anti-Prox1	Rabbit	1:300	102-PA32AG	ReliaTech GmbH
		1:500	ab11941	Abcam
Anti-c myc	Rabbit	1:400	06-340	EMD Millipore
Anti-Lysozyme	Rabbit	1:2000	18-0039	Invitrogen
Anti-Tcf4	Rabbit	1:500	2565S	Cell signaling
Anti-CyclinD1	Rabbit	1:300	2978S	Cell signaling
Anti-Lef1	Rabbit	1:500	C12A5	Cell signaling
Anti - histoneH3k4me3	Rabbit	1:500	Ab8580	Abcam
Anti - histoneH3k4me2	Rabbit	1:500	Ab7766	Abcam
Anti-E cadherin	Mouse	1:300	610182	BD biosciences
Tcf1	Rabbit	1:400	C63D9	Cell signaling
Anti-Lgr5	Rabbit	1:500	AP03046U-N	Acris antibodies

3). Methods

3.1). Animal maintenance

All animal experiments were performed according to the German animal protection law (TierSchG), and approved by the animal maintenance department, University of Göttingen as well as local authority, Niedersäschsiches Landesamt. All mouse strains were grown and bred in European Neuroscience Institute, and Animal facility in University Medical Center, Göttingen.

3.2). Tamoxifen treatment

Dissolved 100 mg Tamoxifen (Sigma, T5648) in 1 ml 100% EtOH, it was further diluted in sun flower oil to 10 mg/ml. 6 to 8 weeks old mice had been injected intraperitoneally 1 mg Tamoxifen per mouse weighing approx. 20 g, for 5 continuous days. All APC homozygous mice were sacrificed on day 6. All ß-catenin heterozygous mice were sacrificed on day 6, 18, 30, 34 and 86. All APC heterozygous mice were sacrificed on day 70. During the course of survival study, all mice were monitored each day to record the date of death.

3.3). DSS treatment

For the inflammation related studies dextran sodium sulphate (DSS, MP Bio medicals, 216011050) was orally administered. Mice aged 6-8 weeks (Pygo2 wild type, and Pygo2 -/-) were fed with DSS, 1.5% (w/v), in drinking water for 5 consecutive days. Mice were monitored regularly for mortality and sacrificed on day 14 and day 28 for the histological examinations.

3.4). AOM & DSS treatment

Azoxymethane (AOM, Sigma, A5486) single intraperitoneal dose was injected to control and Pygo2 constitutive knockout mice. 10µl of azoxymethane (0.991mg/µl) was diluted in 990µl of sterile distilled water to bring the concentration to 9µg/µl; this was further diluted in 0.9% NaCl to get final concentration of ~1µg/µl and approx. 10µg of azoxymethane per gram weight of mouse was injected. 7 days after AOM treatment followed by DSS 1.5% (w/v) oral administration for five continuous days. Mice were sacrificed 6 months after the AOM treatment. Colon was cut open longitudinally and tumor number and their size were recorded.

3.5). BrdU for in vivo DNA labeling

BrdU (Roche, 10280879001) was injected (100µg/gm.) intraperitoneally 2 hours prior to sacrificing mice to facilitate the BrdU incorporation, and it was always brought to RT in PBS before intraperitoneal injection.

3.6). Epithelial cell extraction from small intestine and colon

Isolation of intestinal epithelial cells from mice has been performed by utilizing a modified protocol (Macartney *et al.,* 2000; Iwamoto *et al.,* 2011). Immediately after sacrificing mice, small intestines and colon were cut longitudinally before cleaning each of them separately in PBS. Further, they were rinsed in HBSS/EDTA (pH 7.4) solution, and incubated in a shaker at 37°C for about 15minutes to destabilize the outer epithelial layer of the intestine. After vortexing for 2 min to bring epithelial cells into HBSS solution, centrifugation for 10 min at 1600rpm was done. Finally, the pellet containing predominantly intestinal epithelial cells were stored in 1ml of TRIZOL for longer time at -80°C.

3.7). Histochemical analysis

Tissues were separated as quickly as possible to rule out the possibility of tissue degradation and stored in 4% paraformaldehyde in PBS over night to facilitate the permanent tissue fixation; for longer storage, after rinsing in cold distilled water, tissues were stored in 65%-70% ethyl alcohol. For dehydration and paraffinization tissue samples were incubated in 75% EtOH, 80% EtOH, 90% EtOH, 96% EtOH, 2 x 100% EtOH and 2 x Xylol for 1.5 h each, followed by incubation in Paraffin for up to 12 h before embedding them in fluid paraffin. Paraffin blocks were sectioned at three µm using a microtome before proceeding with immune analysis.

3.8). Hematoxylin and Eosin staining (H&E)

In order to perform H&E staining, paraffin sections (3 µm) on adhesion slides were dewaxed in Xylol (3 x 5 min), rehydrated in graded alcohol (2 x 100%, 96%, 80% and 70% EtOH for 3 min each step) and washed in dH2O. Slides were then treated for 2 min with Mayer's hematoxylin and rinsed with tab water for 5-10 min; slides were stained with eosin for 2 min and dehydrated in a rising EtOH-series (70% and 80% EtOH for 10 sec, 96% and2 x 100% EtOH for 3 min) and Xylol (3 x 3 min) and finally mounted with Roti®-Histokit.

3.9). Immunohistochemistry (IHC)

For Immunostaining on tissue sections slides were dewaxed and rehydrated. Subsequently antigen retrieval was performed by boiling in preheated antigen retrieval buffer (10 mM Tris, 1mM EDTA, and pH 9.0 or 10 mM trisodium citrate pH 6.0, 0.05 % Tween20 in case of anti-β-catenin) for 15 min. While remaining in antigen retrieval buffer the samples were cooled down in a cold water bath to RT for about 1 h. After 3x5 min washing in dH2O endogenous peroxidase was blocked by 10 min incubation in 1% H_2O_2 followed again by washing in dH_2O (5 min) and in 1 x PBS (2 x 5 min). Sections were then blocked for 30 min in IHC blocking solution (10% goat or horse serum, 1% BSA in 1xPBS) and incubated overnight

(4°C) with the specific primary antibody in a humidified atmosphere. Slides were washed in 1 x PBS (3x5 min) and the corresponding secondary HRP conjugated antibody (DakoEnVision Kit) was applied for 45 min at RT in a humidified atmosphere. After washing again in 1xPBS (3x5 min) staining was visualized with DAB according to manufacturer`s protocol and counterstained with Hematoxylin for 2 min followed by 10 min in continuous tab H2O. Stained sections were dehydrated and mounted.

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3.10) siRNA treatment of colon cancer cell lines

All RNA interference experiments of cultured colon cancer cells have been performed by Maria Wiese. HCT116 and DLD1 colon cancer cell lines were transfected with pools of 2 specific small interfering RNAs (siRNAs) for each gene (ß-catenin, BCL9, BCL9-2 and Pygo2) and a pool of 4 non targeting siRNAs as controls (Thermo Fisher Scientific Inc. Dharmacon, Fremont, CA). For further details, see methods as previously published (Brembeck *et al*., 2011).

3.11). Mouse tail lysis for DNA extraction and genotyping

Tail biopsies from ~3 weeks old mice were incubated in 50µl of lysis buffer (100 mM Tris-HCl pH 8.5, 5 mM EDTA pH 8.0, 200 mM NaCl, 0, 2 % SDS) containing fresh Proteinase K (200µg/ml) overnight at 55°C on a shaker to facilitate the tissue digestion; genomic DNA was further diluted to 1:10 with dH20 and thermo denaturation of proteinase was done at 90°C for 10 Min before centrifuging for 2 min at maximum speed. The genomic DNA containing supernatant was used for PCRs.

3.12). Polymerase chain reaction (PCR)

PCR was performed on tail biopsy to confirm the genotype of each mouse before tamoxifen treatment, DSS and AOM administration. In addition, all mice which were utilized for histochemical and quantitative polymerase chain reaction analysis were re-genotyped to make sure about genetic background of each of mouse models. 1x Taq buffer without MgCl2, 0.6 units FastTaq DNA Polymerase, 0.5 µM primer each, 0.2 mM dNTPs each, 1.5-2.5 mM MgCl2 and 1 µl gDNA were mixed in a final volume of 15 µl per reaction. For comparison positive and H2O as a negative control were taken each time.

3.13). RNA extraction from mouse intestinal epithelial cells and colon tumors

Already TRIZOL stored epithelial cells or colon tumors were taken for homogenization, which was done for 4 times with 10 seconds each. Then samples were incubated at RT for 5 min before adding 0.1ml of chloroform per 0.5ml TRIZOL homogenate. All samples were incubated 10min at RT while shaking, and spin down at 10500 rpm at 4°C for 15min. Out of three phases formed by centrifugation, namely, organic

lower phase, protein inter phase, and aqueous RNA containing phase, aqueous phase from sample was carefully transferred to a fresh tube and 250µl isopropanol was added to precipitate RNA before incubating at RT again for 10min. Centrifugation again at 10500 rpm at 4°C to pull down the RNA as a pellet was performed; carefully after removing the supernatant pellet was washed with 0.5ml of 70% EtOH-DEPC-H2O and again spin down at 8000rpm at 4°C for about 10min. After removal of supernatant pellet was air dried to remove the residual alcohol, while not letting them to dry out completely. Each pellet was instantly re dissolved in DEPC H2O; to determine the concentration and purity of the RNA the absolute absorbance at 260 and 280 nm was measured using a Nano Drop ND-1000 Spectrophotometer.

3.14). DNase treatment of extracted RNA

To digest the residual DNA 20µg (10µl) of each sample RNA was treated with a mixture of 2.5µl of 10xDNase buffer, 1.25µl 20mM DTT, 0.5µl RNase Out, 0.2µl RNase-free DNase I, 10.6µl of nuclease free water, and final volume of 25µl was incubated at 37°C for 90 min. In order to make sure about complete digestion of genomic DNA another 0.2µl was added and incubated further for about 60 min. 74.8µl of nuclease free water was added to each of the sample for achieving the final volume of 100µl before the addition of 100µl of phenol: chloroform (20:1) for the RNA precipitation; vortexed for 15sec and spin down at 13000rpm for 10min before carefully transferring the aqueous phase, while avoiding the protein interphase, into a new tube. Ammonium acetate was added into each tube for the final 2.5M concentration, and further RNA was precipitated with 100% of 400µl of ethanol; briefly vortexed, and incubated at RT for 5min. Centrifugation of each sample at 13000 rpm for about 10mim was preceded to pull down the RNA before adding 200µl of 75% ethanol; after brief vortexing, all were centrifuged again for 5 min, and maximum of alcohol was removed. Each of pellets was dried at 37°C for about 2 min and resolved at least in 40µl of nuclease free water.

3.15). cDNA synthesis

Finally, genomic DNA free RNA was reverse transcribed into complementary DNA (cDNA) using MMLV reverse transcriptase and random hexamer primers. At first exactly equal amount (2-5µg) of each of the RNA sample was taken with 0.3µg random hexamer in a final volume of 35µl water, and incubated at 65°C for 5min on PCR machine, after that all samples were quickly transferred on to the ice to facilitate the rapid cooling. 30µl of master mix was added to each of the sample, which is a composition of 12µl of 5xRT buffer, 3µl of 20mM DTT, 1.5µl of RNase out, 3µl of 10mM dNTP mix, 9.75µl of nuclease free water, and 0.75µl of MMLV reverse transcriptase. After adding the master mix all samples were further incubated at 25°C for 10min followed by 37°C for 60min, and finally reaction was stopped by incubating at 72°C for 10min for the successful reverse transcription of sample RNA.

3.16). Quantitative Real Time Polymerase Chine Reaction (qRT-PCR)

A standard protocol for qRT-PCR was kindly provided by Prof. Steven Johnsen, Dept. of Molecular Oncology, University of Göttingen, although it was further adopted and optimized by our lab colleagues. SYBR Green assisted real time polymerase chine reaction examination of cDNA was performed to assess each gene expression level. To achieve this 5ng of cDNA with (0, 3 pmol/µl) of primer concentration was added to the 8µl of master mix, which includes (75 mM Tris-HCl pH 8.8, 20 mM $(NH4)_2SO_4$, 0.01% Tween-20, 3 mM $MgCl_2$, 0.2 mM dNTPs, 20 U/ml HOT FIREPol DNA Polymerase, 0.25% TritonX-100, 500 mM D (+)-Trehalose Dihydrate, Cybr Green (1:80000). Each of this 10µl of mixture was loaded in triplicates onto a 384 well PCR plate. Fluorescence was measured with an AB7300 Real-Time PCR System (Applied Bio systems). Each transgene expression was normalized to the level of HPRT house keeper gene using the $2^{-\Delta\Delta Ct}$ method. SDS Software 2.2 and Microsoft Excel were used for statistical analysis and graphical rendering.

3.17). Statistics

All out comes of qRT-PCR were analyzed using Microsoft excel. Two sided student t-test was used to find out the significance. Graph Pad Prism6 assisted Kaplan-Meier survival curve was used to compare and contrast the survival time of mice following tamoxifen treatment, in addition box plot analysis, for the comparison of tumor size and number between two mouse models was performed.

4). Results

4.1). Pygo2 ablation significantly reduces number and size of chemically induced colon tumors

Pygo2 was recognized to play a key role in various malignancies, which were either Wnt signaling dependent or independent (Andrews *et al.,* 2007; Popadiuk *et al*., 2006; Fang *et al.,* 2003; Wang *et al.,* 2010; Zhang *et al.,* 2014). In addition, in vitro experiments from our lab and Thompson B et al (Thompson *et al.,* 2002) also suggested that Pygo2 indeed was able to govern Wnt signaling gene cascade in colon cancer cell lines harboring mutations in APC or ß-catenin (Brembeck *et al.,* 2011). However, the precise role for Pygo2 in vivo in the context of intestinal tumorigenesis has not been studied yet. Therefore, the main goal of this entire project was to investigate extensively Pygo2 role during the intestinal tumor initiation and progression. Even though Pygo2 was shown to have a key function in many cancers including colorectal cancer (CRC), we demonstrated that intestinal specific constitutive ablation of Pygo2 two alleles (Vil^{Cre+}; $Pygo2^{-/-}$) in mice has not really affected epithelium development during embryogenesis as well as persistent epithelial homeostasis in adult mice (Schelp and Brembeck, unpublished). Hence, we have decided to study further this perplexing context dependency of Pygo2 role.

To investigate the Pygo2 role in intestinal tumorigenesis, at first constitutive Pygo2 deficient (Vil^{Cre+}; $Pygo2^{-/-}$) and control mice (Vil^{Cre-}; $Pygo2^{-/-}$) were treated with a single dose of chemical carcinogen, azoxymethane (De Robertis *et al.,* 2011; Aviello *et al.,* 2014). One week after the treatment all mice were fed with dextran sodium sulphate (DSS) in drinking water to induce the intestinal inflammation. We waited until six months after treatment to facilitate the tumorigenesis, during this time all mice were under inspection on daily basis. Anal bleeding was observed in most of mice regardless of Pygo2 expression, including considerable weight loss (data not shown). Eventually all mice were sacrificed exactly after six months, and we examined total small and large intestines carefully to find out that massive tumors were present only in large intestine and rectum, with higher tumor incidence in distal part of colon and rectum. Total number and size of all tumors in colon and rectum were counted in Pygo2 knockout and control mice. These data are presented in box plot for the better analysis of tumor induction in both mice. Indeed, box plot analyses illustrated that total number of tumors in colon and rectum were highly significantly reduced in Pygo2 deficient animals (Vil^{Cre+}; $Pygo2^{-/-}$) (n=30; P=0.0023), compared to control mice (n=25). No remarkable change in 2mm or lesser size tumors in both mice was observed. However, larger tumors (3mm or more) were significantly reduced in Pygo2 deficient mice (0.5 in Pygo2 knockout Vs 2.2 in control mice; P=0.0002) (Fig. 1A).

Further characterization of colon tumors was carried out by immunohistochemistry on tumor sections. RNA was extracted from total Pygo2 deficient and control tumors as well as from colons of untreated Pygo2 knockout and control animals to perform quantitative RT-PCR for detecting transcription of target genes.

First Hematoxylin and Eosin (H&E) staining was performed to evaluate the tumor histopathology that showed larger isolated tumors in colons of control and Pygo2 deficient animals. In addition, we also identified some tumors were indeed invasive in Pygo2 deficient and control tumors, which migrated into the submucosa (data not shown). BrdU positive cells throughout tumors sections suggested highly proliferative tumor cells in all sections examined (Fig. 1B). Elevated levels of nuclear ß-catenin in all tumors indicated deregulated Wnt signaling pathway. Thus, chemical carcinogen induced at least in part colon tumorigenesis by deregulating Wnt signaling. Pygo2 staining revealed that it was significantly nuclearly translocated in tumor cells in Pygo2 wild type animals however it was totally undetected in tumors of Pygo2 deficient tumors suggesting successful constitutive ablation of Pygo2. Nevertheless, Pygo2 RNA was not actually induced in control tumors though it was significantly downregulated in Pygo2 deficient tumors and untreated Pygo2 knockout animals. BCL9 was totally unaltered across all tumors, which was in line with our previous findings (Brembeck *et al.,* 2011). BCL9 homologue, BCL9-2, was only moderately upregulated in all tumors whereas it was highly significantly overexpressed in $APC^{Min/+}$ mice adenomas and colon cancer patients (Brembeck *et al.,* 2011). After this we checked a prominent Wnt target gene, Axin2, to find its massive upregulation, which clearly indicated hyperactive Wnt signaling pathway in all tumors. However, tumors from a sub-set of Pygo2 deficient animals showed a significant reduction of Axin2 expression transcriptionally (Fig. 1B, C).

These data clearly indicated that Pygo2 ablation was not able to completely rescue tumor formation in chemically induced colon tumors; however, it prolonged tumor evolvement, which was apparently linked to deregulated Wnt signaling pathway.

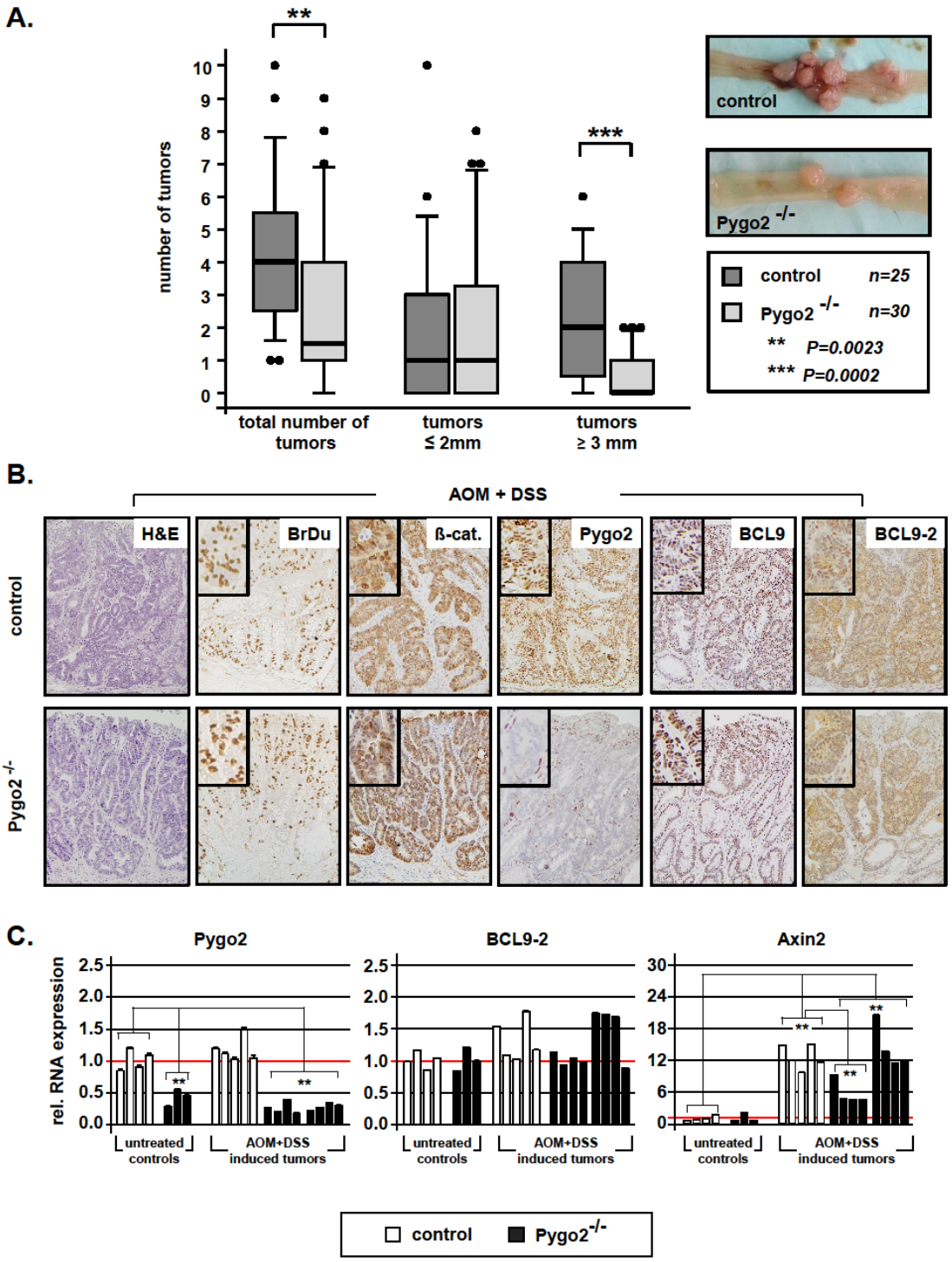


Figure 1: Pygo2 knockout delays the progression of chemically induced intestinal tumorigenesis.

(**A**) Box plot analysis and representative colon sections showing colon tumors in control (Vil^{Cre-}; $Pygo2^{-/-}$; n=25) and constitutive Pygo2 deficient mice (Vil^{Cre+}; $Pygo2^{-/-}$; n=30). The total number and size of tumors was determined after six month following treatment with a single dose of azoxymethane (AOM), and after seven days oral administration

of dextran sodium sulphate for five consecutive days (1.5% w/v DSS). Pygo2 deficient animals were compared to age-matched litter mate controls. Significant differences are indicated with ** for P<0.01 and *** for P<0.001.
(**B**) Representative H&E stains and immunostains on tumor sections from control and Pygo2 knockout animals with the indicated antibodies. (**C**) qRT-PCR analyses of RNA extracted from colon tumors of controls and Pygo2 deficient animals and from colon tissues obtained from untreated mice with the same genotype. Each bar represents the relative RNA expression of the indicated gene for one single animal; each result represents at least three independent experiments. All significant differences are marked with * for P < 0.05 and ** for P < 0.01.

4.2). Pygo2 is redundant for the intestinal epithelial regeneration following DSS treatment

We also investigated the role of Pygo2 in the context of intestinal tissue regeneration following DSS treatment. To achieve this, we have modified widely used DSS model by continuous oral administration of (2.5% w/v) dextran sodium sulphate in water for five consecutive days (Perše *et al.,* 2012; Puneet Kaur *et al.,* 2014) since usual 5% of DSS model was severe in our experiments, which caused death of all mice. However, after five days of DSS treatment Pygo2 knockout and control mice were sacrificed on 14^{th} and 28^{th} day and intestines were examined immunohistochemically. Hematoxylin stainings of intestines in Pygo2 deficient and control mice showed normal epithelium on day 14 and 28, which suggested total recovery of intestinal epithelium in both mice. In addition, BrdU stained cells in both mice were located only in crypts as well as no detectable Pygo2 was found in Pygo2 knockout animals. Hence, we concluded Pygo2 redundancy for the intestinal epithelial regeneration after DSS treatment. These observations were in agreement with our previous unpublished findings illustrating Pygo2 redundancy for normal intestinal epithelial homeostasis in the adult mice (Fig. 2A, B).

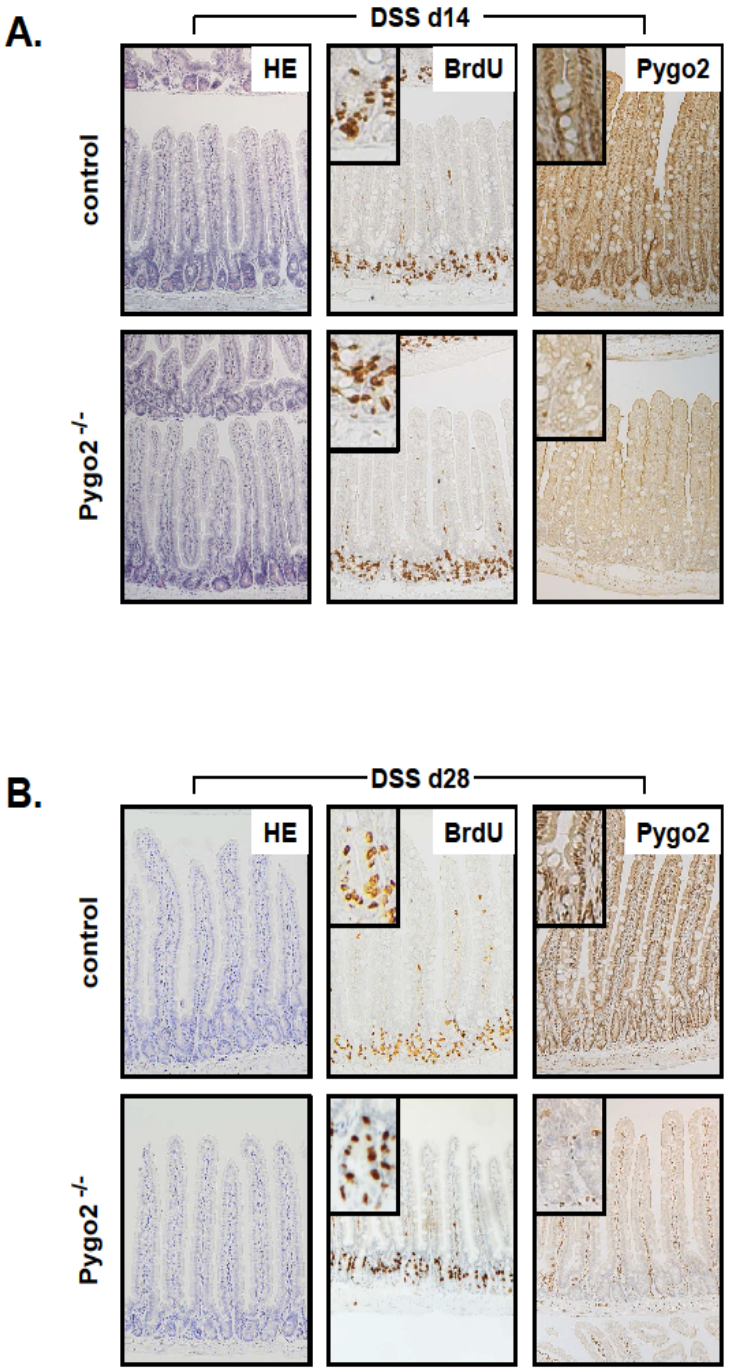


Figure 2: Pygo2 is dispensable for the intestinal epithelial regeneration following DSS assisted chemically induced inflammation.

(A). Representative of H&E, BrdU, and Pygo2 immunostains on intestinal tissue sections from Pygo2 wild type (Vil^{Cre-}; $Pygo2^{-/-}$) animals and constitutive Pygo2 deficient animals (Vil^{Cre+}; $Pygo2^{-/-}$) which were sacrificed on day 14 following five consecutive days of DSS oral administration (1.5% w/v DSS).

(B). Representative of H&E, BrdU, and Pygo2 immunostains on intestinal tissue sections from Pygo2 wild type (Vil^{Cre-}; $Pygo2^{-/-}$) animals and constitutive Pygo2 deficient animals (Vil^{Cre+}; $Pygo2^{-/-}$) which were sacrificed on day 28 following five consecutive days of DSS oral administration (1.5% w/v DSS).

All mutant mice lacking intestinal epithelial Pygo2 expression survived DSS induced inflammation at two time points as wild type animals did.

4.3). Pygo2 is dispensable for the expression of target genes in the normal intestine.

To study further the importance of Pygo2 for the normal intestinal epithelial homeostasis, we conditionally induced deletion of Pygo2 two alleles ($Vil^{Cre-ERT+}$; $Pygo2^{-/-}$) by tamoxifen administration in intestinal epithelium and extracted RNA from total intestines of Pygo2 conditional knockout and control animals. RT-PCR analysis was performed to analyze the expression Wnt signaling components, several classical Wnt target genes and Dclk1, a tuft cell marker (Nakanishi *et al.,* 2012); however, most of Wnt target genes were unaltered in Pygo2 loss animals. Nonetheless, we observed moderate but significant downregulation of Tcf4 and Lef1 in Pygo2 knockout animals including slight elevation of Dclk1 transcripts (Fig. 3). This minor alteration in endogenous levels of Lef1 and Tcf4 seemed to be insufficient to affect continual epithelial regeneration that was actively dependent on Wnt signaling. Altogether, these data indicated that Pygo2 was not needed for the regulation of Wnt target genes in normal intestinal epithelium. This in fact supported our previous histochemical analysis illustrating Pygo2 redundancy for intestinal epithelial homeostasis (Schelp and Brembeck, unpublished data).

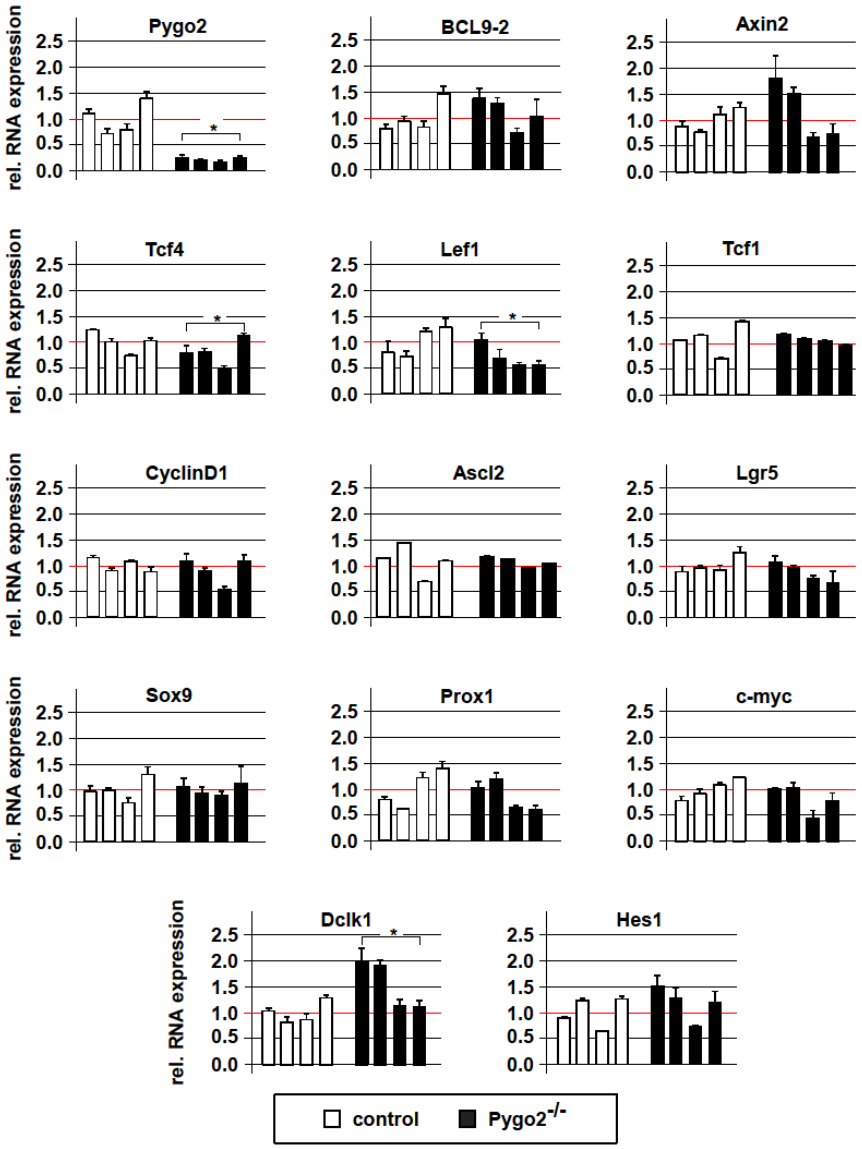


Figure 3: Pygo2 knockout does not affect the regulation of target gene expression in normal intestinal epithelium. QRT-PCR analyses of RNA extracted from epithelial cells of the intestine of controls ("control": $Vil^{Cre\text{-}ERT-}$; $Pygo2^{-/-}$) compared to homozygous Pygo2 deficient animals ("$Pygo2^{-/-}$": $Vil^{Cre\text{-}ERT+}$; $Pygo2^{-/-}$). Mice were analyzed on day 18 post induction with tamoxifen. Each graph shows the relative RNA expression of the indicated gene; each bar represents one animal. Significances were calculated for the mean expression level compared to the control group. The asterisk marks significant differences * for $P < 0.05$.

4.4). Deletion of Pygo2 rescues the initial phase of intestinal hyperproliferation induced by ß-catenin gain-of-function (GOF), but not hyperproliferation induced by APC loss-of-function (LOF).

Current study was aimed to investigate Pygo2 role further in genetic mouse models for intestinal tumor initiation and progression. To achieve our goal, conditional ablation of either one or two alleles of Pygo2 in mouse intestine was introduced ($Vil^{Cre\text{-}ERT+}$; $Pygo2^{+/-}$ and $Vil^{Cre\text{-}ERT+}$; $Pygo2^{-/-}$). Further these mice were crossed with conditional genetic mutants harboring homozygous truncated APC ($APC^{15exon\ -/-}$) (Robanus-Maandag *et al.,* 2010) or heterozygous stabilized ß-catenin (ß-$catenin^{ex3/+}$) (Harada *et al.,* 1999) to generate compound animals. For the recombination of Pygo2, APC and ß-catenin expressing genes tamoxifen was injected intraperitoneally for five consecutive days. This induced conditional deletion of Pygo2 expression in almost all epithelial cells of small and large intestine (data not shown) and concurrent APC or ß-catenin mutations.

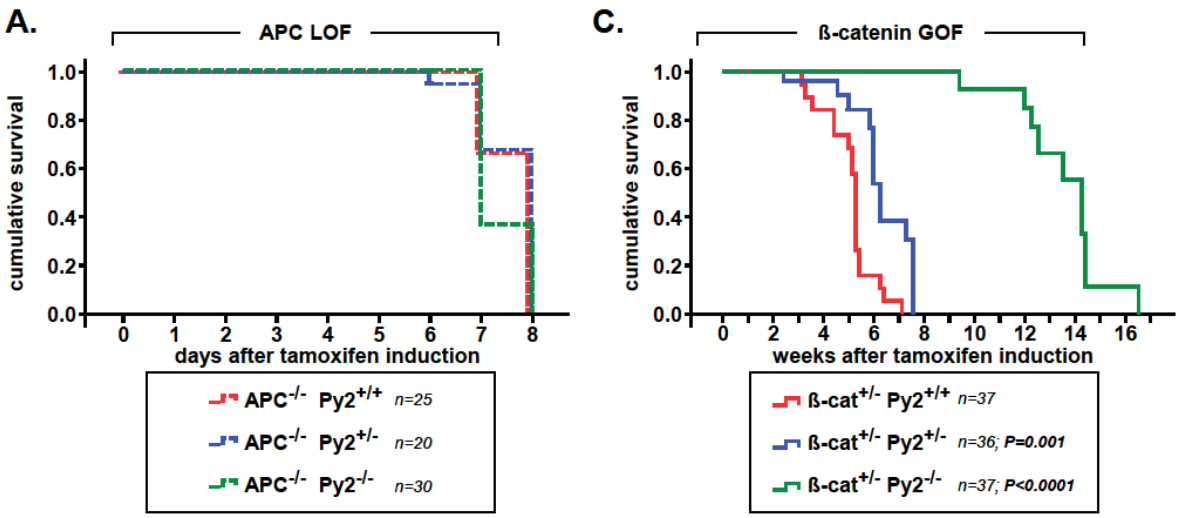
First we analyzed consequences of Pygo2 conditional loss of single or two alleles during the acute intestinal tumor initiation induced by homozygous truncated $APC^{15lox-/-}$ (APC LOF) and heterozygous stabilized ß-$catenin^{ex3+/-}$ (ß-catenin GOF) (Fig. 4).

APC two alleles truncation induced a severe intestinal phenotype within seven days post induction and all mutant mice regardless of Pygo2 expression died within eight days. Therefore, the survival of APC mutant animals was not altered by the introduction of conditional deletion of single allele or two alleles of Pygo2 (Fig. 4A). Histochemical analyses on intestinal tissues of APC mice sacrificed on day six after the beginning of induction showed massive intestinal hyperproliferation with enlarged crypts, proliferating cells along crypt-villus axes as identified by hematoxylin and eosin stain, BrdU stain, respectively. Upregulation of cytoplasmatic and nuclear ß-catenin demonstrated active Wnt signaling (Fig. 4B). Thus, knockout of Pygo2 did not rescue the intestinal hyperproliferation induced by APC LOF.

In contrast to this, induction of heterozygous ß-catenin GOF in mice led to a relatively less severe phenotype, however all mice with wild type Pygo2 expression (n=37) died between two to seven weeks after the induction. Strikingly, deletion of one single Pygo2 allele already statistically significantly prolonged the survival of ß-catenin GOF animals (n=36, p=0.001). Knockout of two alleles of Pygo2 highly significantly prolonged the survival of ß-catenin animals in the initial stage up to minimum of ten weeks to the maximum of over sixteen weeks (n=37; $p<0.0001$) (Fig. 4C).

We also analyzed the intestines of these compound mutant mice by immunohistochemistry (Fig. 4D, E). Whereas hyperproliferation was severe in APC LOF on day 6, only a mild hyperproliferation was observed in ß-catenin GOF animals on day 6; however, it was most prominent on day 18, the initial time point at which compound ß-catenin GOF-Pygo2 wild type animals began to die. Therefore, we decided to study the histopathology of ß-catenin GOF animals on day 6 and 18 for the better analogy of hyperproliferation in APC LOF and ß-catenin GOF mice at same time point. The intestines of ß-catenin GOF mutant animals on day 6 with the Pygo2 wild type background showed only a mild hyperproliferation as indicated by BrdU stain (Fig. 4D), whereas massive hyperproliferation was seen on day 18 as illustrated by BrdU positive proliferating cells along crypt-villus axes and overexpression of cytoplasmatic and nuclear ß-catenin. Remarkably, in ß-catenin GOF animals sacrificed on day 18 hyperproliferation was reduced with the ablation of one single Pygo2 allele. Most interestingly, deletion of two Pygo2 alleles completely rescued the phenotype of ß-catenin GOF animals, based on our finding that the intestinal architecture was identical to that of controls with normal crypt-villus axes and BrdU stained proliferating cells were present only in crypt compartment and ß-catenin was predominantly cytoplasmatic (Fig. 4E).

In conclusion, deregulated Wnt/ß-catenin signaling induced by conditional mutations of APC or ß-catenin initiated intestinal hyperproliferation, which resulted in highly divergent survival rates. Remarkably, Pygo2 conditional deletion effectively reversed intestinal hyperproliferation that was induced by ß-catenin GOF mutation whereas it failed to rescue in APC LOF mice.



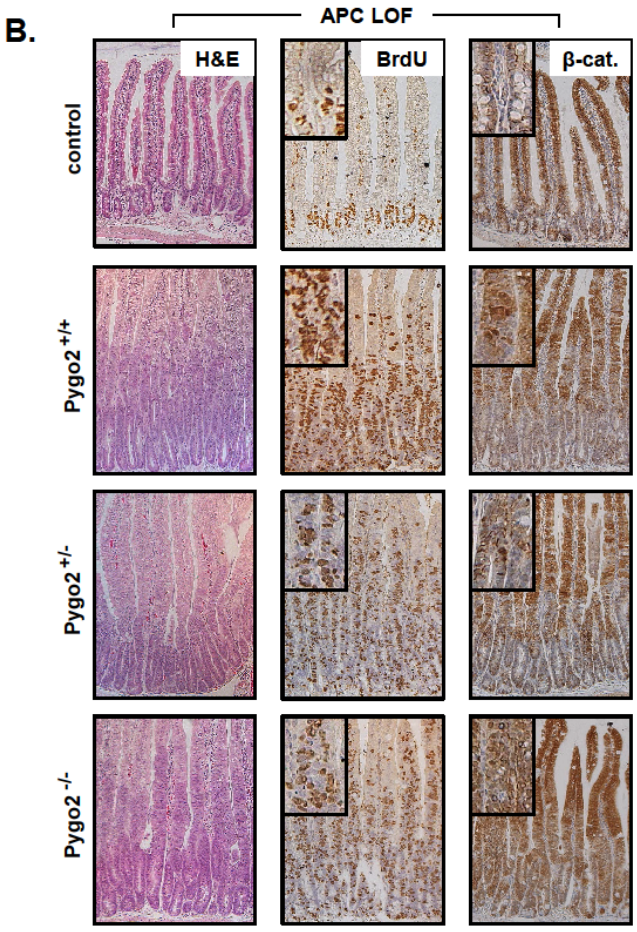


Figure 4: Pygo2 conditional knockout completely rescues the early intestinal hyperproliferation caused by ß-catenin gain of function (GOF), but not that is induced by APC loss-of-function (LOF).

(A) Kaplan Meier survival analysis shows the survival period of homozygous APC loss-of-function animals (LOF) with wild type Pygo2 background (Vil$^{Cre\text{-}ERT+}$; APC$^{ex15\text{-}/\text{-}}$; Pygo2$^{+/+}$, n=25) compared to APC homozygous mice (LOF) either with Pygo2 heterozygous (Vil$^{Cre\text{-}ERT+}$; APC$^{ex15\text{-}/\text{-}}$; Pygo2$^{+/-}$, n=20) or with complete Pygo2 deficient background (Vil$^{Cre\text{-}ERT+}$; APC$^{ex15\text{-}/\text{-}}$; Pygo2$^{-/-}$, n=30). Gene recombination was induced by tamoxifen intraperitoneal injections for five consecutive days, and survival was reported when a mouse was either severely sick or dead.

(B) H&E staining shows the massively enlarged crypts in all APC mutants. Immunohistochemical analysis for the detection of BrdU expressing cells and ß-catenin on intestinal sections from APC loss-of-function mice with wild type ("Pygo2 $^{+/+"}$), heterozygous ("Pygo2 $^{+/-"}$) and homozygous ("Pygo2 $^{-/-"}$) Pygo2 expression. Uppermost panel shows the immunostains from control mice. All animals were sacrificed on day six after beginning with tamoxifen treatment.

(C) Kaplan Meier survival analysis shows the survival period of heterozygous ß-catenin gain-of-function animals (GOF) with wild type Pygo2 background (Vil$^{Cre\text{-}ERT+}$; ß-catenin$^{ex3+/-}$; Pygo2$^{+/+}$, n=37) compared to heterozygous ß-catenin gain-of-function animals (GOF) either with Pygo2 heterozygous (Vil$^{Cre\text{-}ERT+}$; ß-catenin$^{ex3+/-}$; Pygo2$^{+/-}$, n=36) or

with complete Pygo2 deficient background (Vil$^{Cre\text{-}ERT+}$; ß-catenin$^{ex3+/-}$; Pygo2$^{-/-}$, n=37). Gene recombination was induced by tamoxifen intraperitoneal injections for five consecutive days, and survival was reported when a mouse was either severely sick or dead. Significance was calculated using the P Log Rank Test with p=0.001 for Pygo2 heterozygous mice and p<0.0001 for Pygo2 homozygous mice compared to wild type Pygo2 expressing animals.

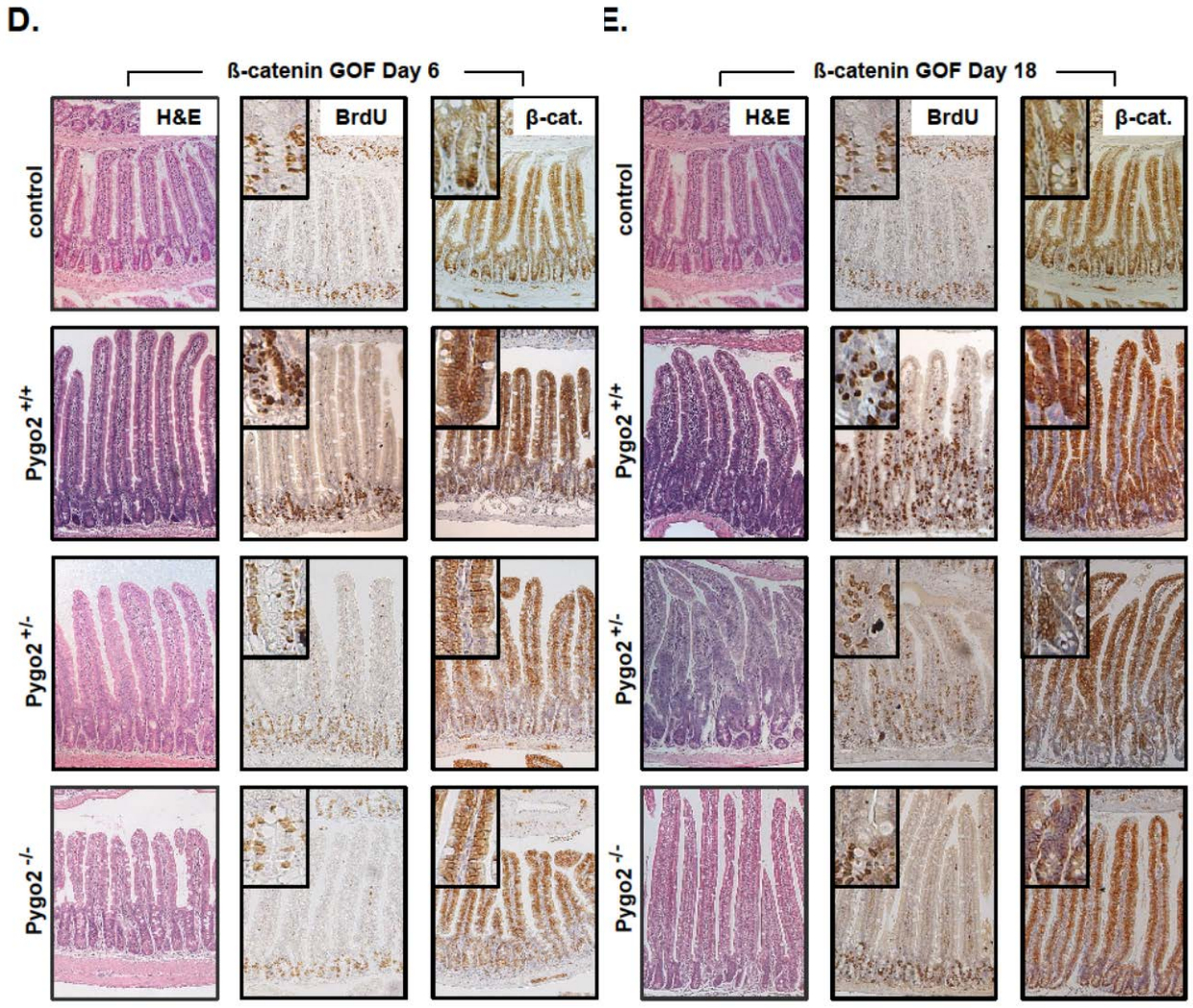


Figure 4: Pygo2 conditional knockout completely rescues the early intestinal hyperproliferation caused by ß-catenin gain of function (GOF), but not that is induced by APC loss-of-function (LOF).

(**D and E**) H&E and immunostains with indicated antibodies on intestinal sections from ß-catenin gain of function (GOF) mice that were sacrificed on day 6(D), and day 18(E) following the tamoxifen treatment for five consecutive days. H&E stains show enlarged crypts only on intestinal sections of ß-catenin GOF mice sacrificed on day 18, with Pygo2 wild type and heterozygous back grounds.

4.5). Pygo2 and related BCL9 co-factors in APC loss-of-function (LOF) and ß-catenin gain-of-function (GOF) mice during the intestinal hyperproliferation.

Next we used immunohistochemistry to analyze Pygo2 and the related BCL9 co-factors on tissue sections. RNA was extracted from epithelial cells enriched from intestinal tissues (see Material and Methods) to perform qRT-PCR analyses (Fig. 5A-D). Pygo2 was strongly expressed in the hyperproliferative cells of the intestine in both APC LOF on day 6 and ß-catenin GOF mutant animals on day 18 with Pygo2 wild type expression. Pygo2 transcription was slightly elevated only in ß-catenin GOF mice on day 18. In all mutants one allele of Pygo2 knockout led to the Pygo2 transcriptional reduction by almost half compared to Pygo2 wild type mutant animals, although protein expression was unaltered.

Pygo2 complete knockout caused significant downregulation of Pygo2 RNA across all mutants, and no detectable protein was found in all intestinal epithelial cells. In accordance with our previous results, BCL9 expression was unchanged in both mouse models. BCL9-2 protein was detected only in villi of control animals, which was in agreement with our previous findings (Brembeck *et al.,* 2011). In APC LOF mutant animals BCL9-2 transcription was not changed, however, protein was not found in hyper-proliferative regions of crypt-villus axes in all APC mutants regardless of Pygo2 expression. In ß-catenin GOF mutants on day 6 BCL9-2 proteins and transcription was not changed, while on day 18 BCL9-2 was only slightly increased on the RNA level in mice with Pygo2 wild type back ground, but was not detectable on the protein level in the hyperproliferative cells. These results are in contrast to high BCL9-2 expression in $APC^{Min/+}$ adenomas (Brembeck *et al.,* 2011), providing also in vivo evidence that BCL9-2 was not a target of deregulated Wnt/ß-catenin signaling during tumor initiation. However, transcription of Axin2, a prominent classical Wnt downstream target (Lustig *et al.,* 2002), was strongly induced in both APC and ß-catenin mutant animals. Remarkably, Axin2 was reduced even after the deletion of one allele of Pygo2 in both mouse models. In APC LOF compound mutant mice Axin2 was seen elevated even following the complete ablation of Pygo2. On the contrary, Axin2 was brought to the control levels in all ß-catenin GOF mice. These findings further supported that Pygo2 knockout can completely rescue the initial phase of intestinal hyperproliferation induced by stabilized ß-catenin, but not by truncated APC.

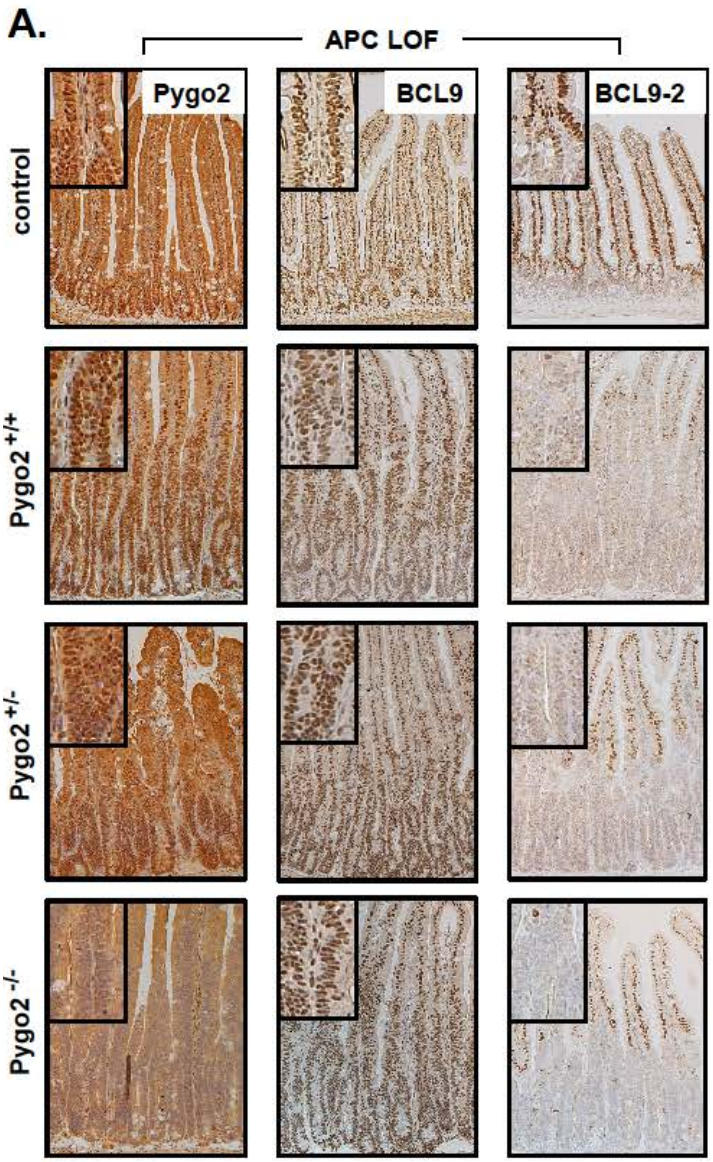


Figure 5: Analysis of Pygo2 and BCL9 family co-factors in APC loss-of-function (LOF) and ß-catenin gain-of-function (GOF) mice following the induction of intestinal hyperproliferation.

(A) Representative of immunohistochemical staining for the detection of Pygo2, BCL9, and BCL9-2 on intestinal tissue sections from APC LOF mice with wild type ("Pygo2 $^{+/+"}$), heterozygous ("Pygo2 $^{+/-"}$) and homozygous ("Pygo2 $^{-/-"}$) Pygo2 expression. Uppermost panel shows the immunostains from control mice. All animals were sacrificed on day six after beginning with tamoxifen treatment.

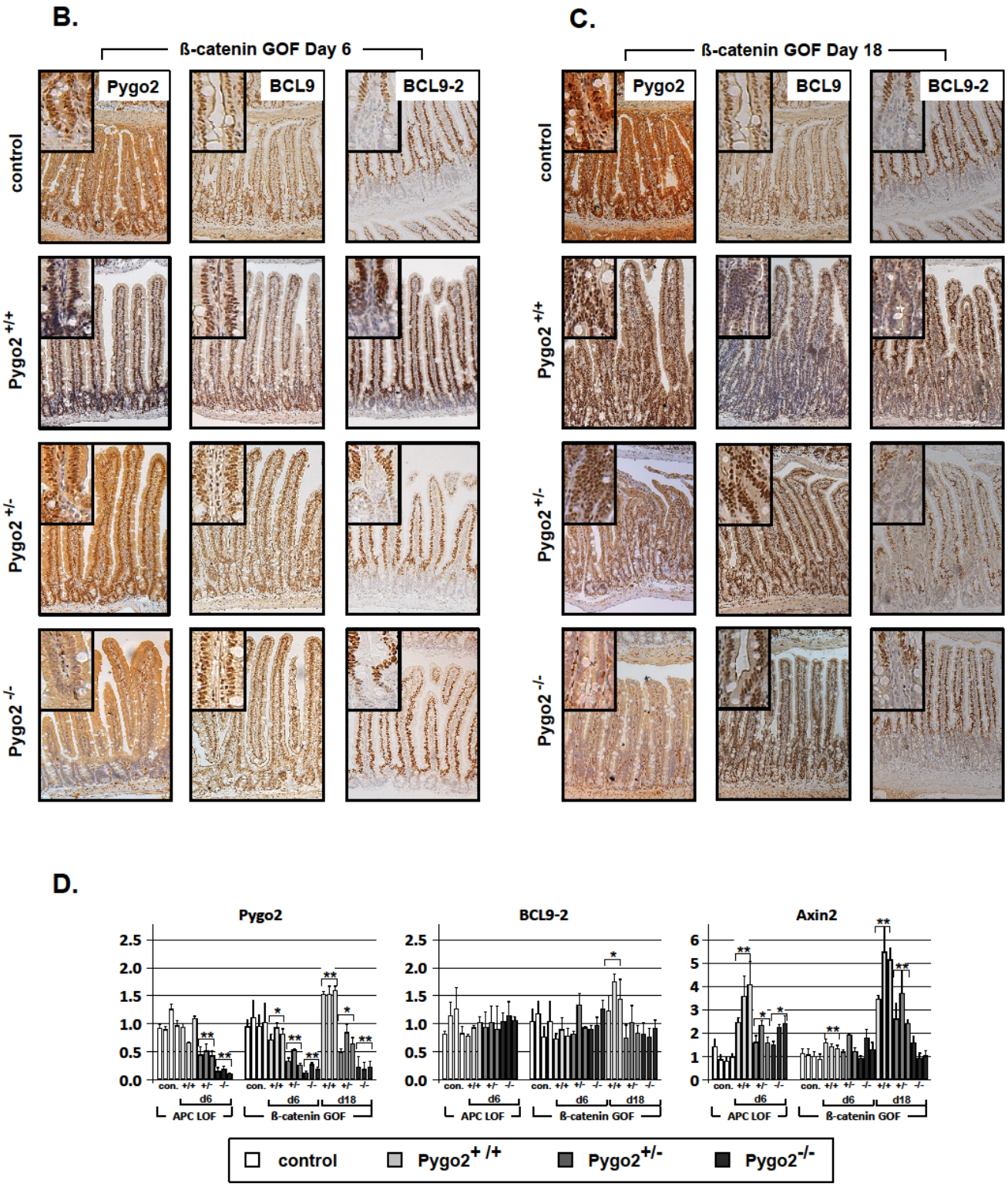


Figure 5: Analysis of Pygo2 and BCL9 family co-factors in APC loss-of-function (LOF) and ß-catenin gain-of-function (GOF) mice following the induction of intestinal hyperproliferation.

(B, C) Immunostains for detecting Pygo2, BCL9, and BCL9-2 proteins on intestinal epithelial tissue sections from ß-catenin GOF mouse models with Pygo2 wild type ("Pygo2 $^{+/+"}$), heterozygous ("Pygo2 $^{+/-"}$), and homozygous ("Pygo2 $^{-/-"}$) genetic backgrounds. **(B)** Animals were sacrificed on day six post tamoxifen induction for five consecutive days whereas **(C)** were sacrificed on day 18 post tamoxifen induction for five continuous days.

(D) qRT-PCR analyses show the expression of RNA extracted from epithelial cells of the control intestines, APC homozygous mice with three Pygo2 backgrounds ("Pygo2 $^{+/+"}$; Pygo2 $^{+/-"}$; "Pygo2 $^{-/-"}$) and ß-catenin heterozygous mice with three Pygo2 backgrounds ("Pygo2 $^{+/+"}$; Pygo2 $^{+/-"}$; "Pygo2 $^{-/-"}$). Each bar shows one animal, and each graph shows the relative expression of the gene indicated. All significant differences are marked with * for $P < 0.05$ and ** for $P < 0.01$.

4.6). Escapers of Pygo2 recombination in ß-catenin GOF Pygo2 knockout mice resulted in the formation of microadenomas.

At the time of survival analysis of ß-catenin GOF mice we identified ß-catenin GOF-Pygo2 homozygous knockout mice that lived longer than ten weeks after the induction of hyperproliferation compared to Pyo2 wildtype controls. Ultimately these mice also died within four months (Fig. 4C). Hence, we decided to perform immunohistological examinations on intestinal tissues of these mice, including ß-catenin GOF with Pygo2 wild type and heterozygous back grounds, at one-month post induction and at later time points (day 34, day 86). As expected, ß-catenin GOF mutants with Pygo2 wild type and heterozygous background had massive hyperproliferation as illustrated by BrdU stains, and high nuclear and cytoplasmatic ß-catenin. Notably, in H&E stains of ß-catenin GOF, Pygo2 knockout mice (day 34) we found hyper-proliferating areas with expression of Pygo2. At later time point (day 86), the intestines of these mice were covered with multiple small adenomas that showed high proliferation as indicated by BrdU stain and concomitant increased nuclear ß-catenin. Pygo2 was strongly expressed in these multiple adenomas, while it was not detected in surrounding epithelium where no visible hyper-proliferation was found. Remarkably, these small adenomas did not express BCL9-2 (Fig. 6). These examinations made us strongly suspect that these tumors might have emerged from single intestinal cells in which Pygo2 initial recombination deletion had failed. Thus, escapers of Pygo2 recombination promoted microadenoma formation in compound ß-catenin GOF, Pygo2 knockout animals at later stages.

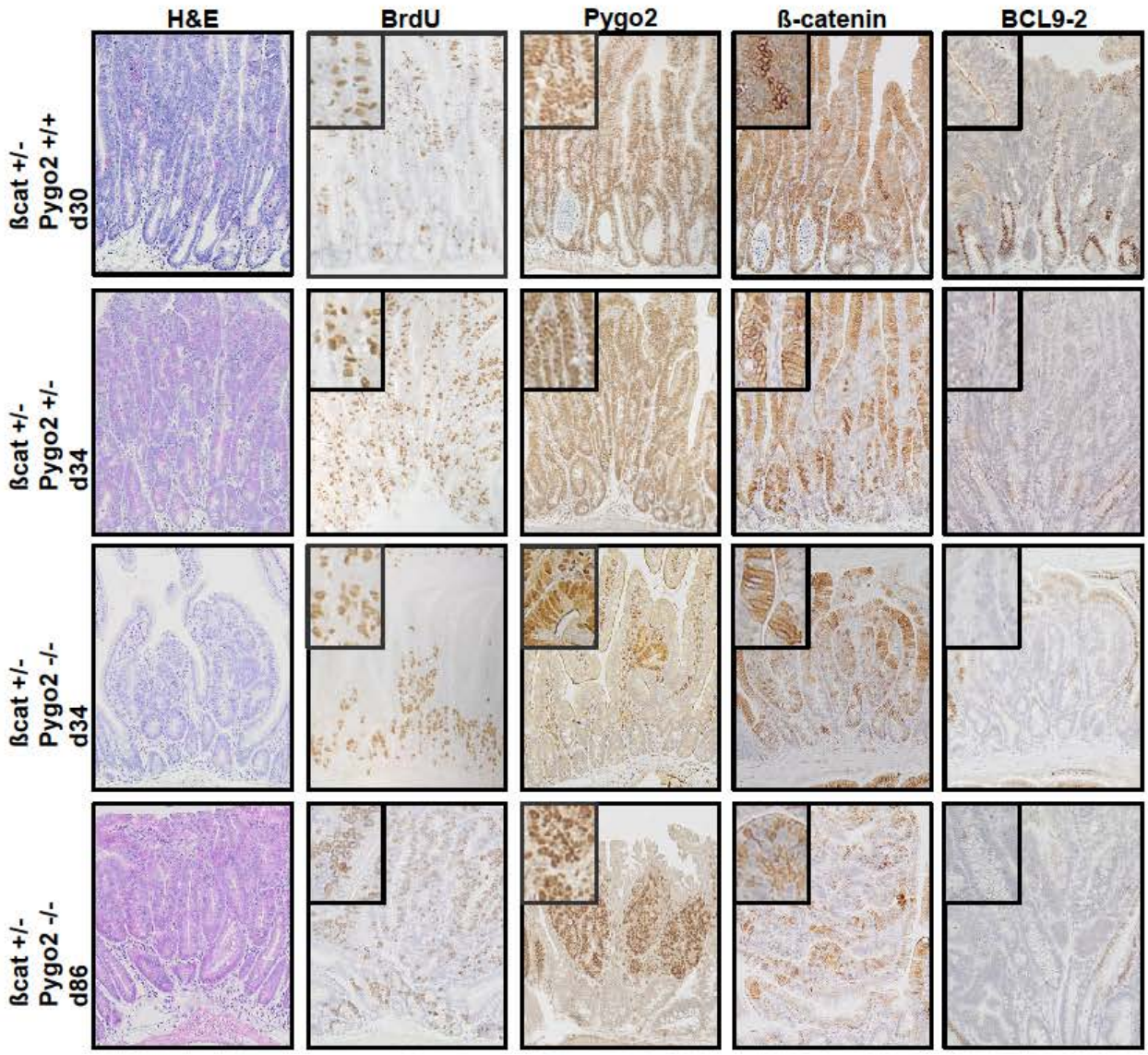


Figure 6: Failed Pygo2 knockout recombination resulted in the formation of micro adenomas in ß-catenin (GOF) Pygo2 homozygous compound mice.

H&E stains and immunohistochemical analyses on intestinal tissues from compound Pygo2 deficient ß-catenin GOF mice that were sacrificed later than day18 after beginning with tamoxifen treatment. (Upper row) is the representative staining on intestinal tissue sections from ß-catenin (ß-cat$^{+/-}$) Pygo2 wild type (Pygo2$^{+/+}$) compound mice sacrificed on day 30. Immunostains on intestinal sections from ß-catenin (ß-cat$^{+/-}$) with heterozygous (Pygo2$^{+/-}$), and homozygous (Pygo2$^{-/-}$) Pygo2 expressing mice sacrificed on day 34 were shown on second and third row, respectively. Lower row immunostains represents the stainings on intestinal tissue sections from ß-catenin (ß-cat$^{+/-}$) Pygo2 complete knockout (Pygo2$^{-/-}$) compound mice which were sacrificed on day 86. Note that the Pygo2 staining in ß-catenin (ß-cat$^{+/-}$) Pygo2 knockout (Pygo2$^{-/-}$) compound mice (day 34+ day 86) revealed the re-expression of it

due to the failed Pygo2 recombination deletion. These ß-catenin Pygo2 knockout compound animals developed at later stages multiple microadenomas that express Pygo2, but not BCL9-2, with increased BrdU staining and nuclear ß-catenin, which was seen on sections in mice sacrificed at day 86 (fourth row).

4.7). Pygo2 conditional deletion did not rescue the intestinal adenoma formation in APC heterozygous mice.

Next, the effect of Pygo2 loss of function was studied in adenomas induced by conditional deletion of one functional allele of APC tumor suppressor gene. The phenotype was indeed similar to that of $APC^{Min/+}$ (Robanus-Maandag *et al.,* 2010). Before the induction of conditional genetic recombination by tamoxifen APC ($Vil^{Cre\text{-}ERT+}$; $APC^{ex15+/-}$) heterozygous mice were crossed with Pygo2 mutants to have Pygo2 heterozygous ($Vil^{Cre\text{-}ERT+}$; $APC^{ex15+/-}$; $Pygo2^{+/-}$) and Pygo2 homozygous ($Vil^{Cre\text{-}ERT+}$; $APC^{ex15+/-}$; $Pygo2^{-/-}$) backgrounds to compare these animals with Pygo2 wild type animals ($Vil^{Cre\text{-}ERT+}$; $APC^{ex15+/-}$; $Pygo2^{+/+}$) (Fig. 7A, B).

All mice regardless of Pygo2 expression lived minimum of 24 weeks post induction. However, Pygo2 conditional deletion of either one or two alleles did not significantly change the total survival of APC heterozygous mice (Fig. 7A). After sacrificing, overall inspection of intestines of all mice revealed that the intestines were fully covered by numerous small adenomas with no remarkable difference between different Pygo2 mutants.

Hematoxylin-eosin stainings confirmed the existence of adenomas in all mutant animals with BrdU stained cells covering whole adenomatous tissue. In addition, loss of Pygo2 was also seen by immune stainings on tissue sections from Pygo2 deficient animals. Along with overexpressed nuclear ß-catenin, adenomas also expressed upregulated BCL9-2 (Fig. 7B). Overall, this study was in agreement with our previous examination of $APC^{Min/+}$ mice in which adenoma formation was also not rescued by Pygo2 complete loss (Schelp and Brembeck, unpublished data). Finally, this study suggested that Pygo2 conditional deletion cannot prevent the adenoma formation in APC heterozygous mice.

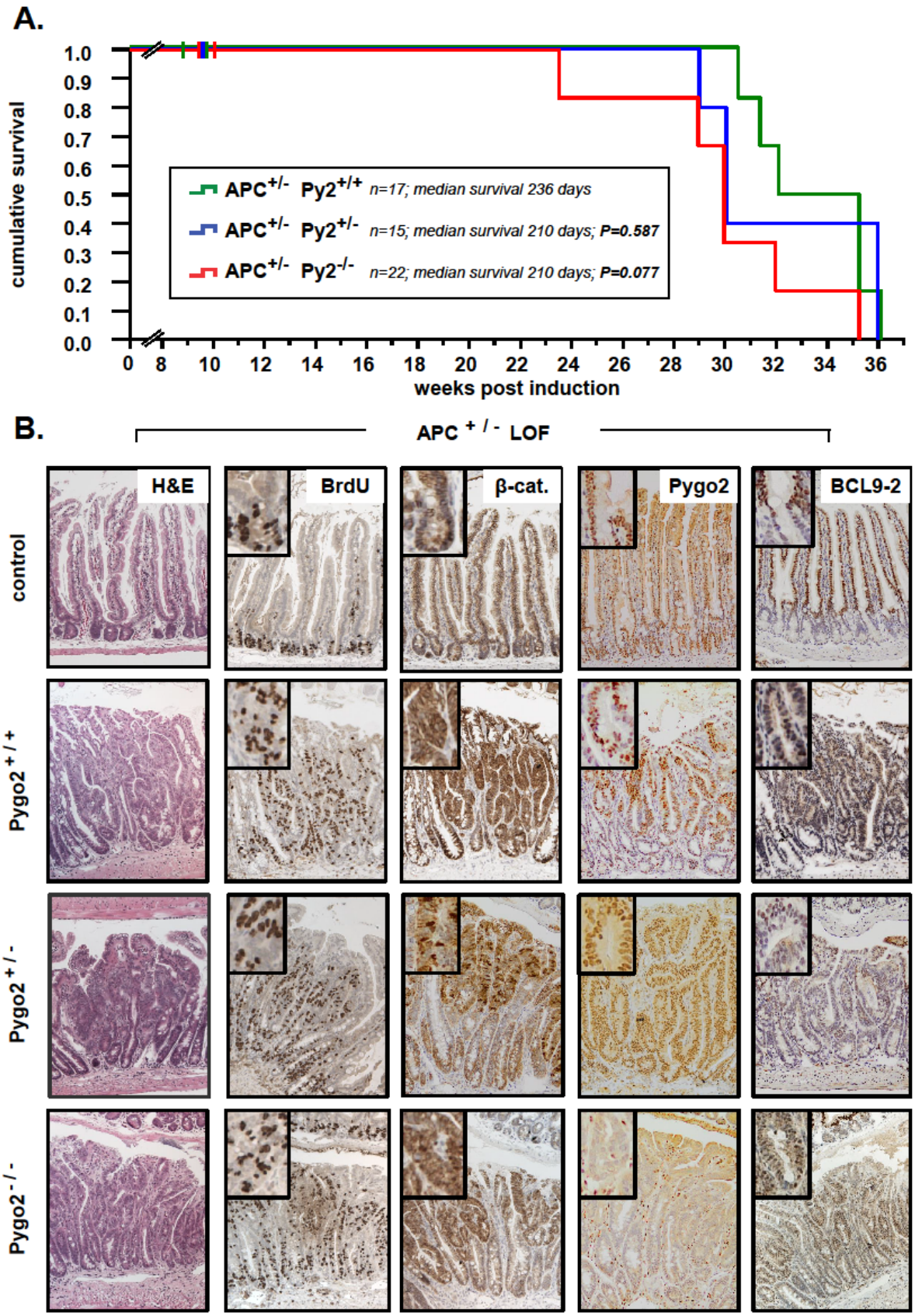

A.
cumulative survival
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
weeks post induction
APC+/- Py2+/+ n=17; median survival 236 days
APC+/- Py2+/- n=15; median survival 210 days; P=0.587
APC+/- Py2-/- n=22; median survival 210 days; P=0.077
B.
APC + / - LOF
H&E
BrdU
β-cat.
Pygo2
BCL9-2
control
Pygo2 + / +
Pygo2 + / -
Pygo2 - / -

Figure 7: Pygo2 knockout failed to rescue the adenoma formation induced by heterozygous APC loss-of-function.

(**A**) Kaplan-Meier survival analysis of compound heterozygous APC loss-of-function animals (LOF) with wild type Pygo2 expression ($Vil^{Cre\text{-}ERT+}$; $APC^{ex15+/-}$; $Pygo2^{+/+}$, n=17) compared to hetero- and homozygous Pygo2 deficient mice ($Vil^{Cre\text{-}ERT+}$; $APC^{ex15+/-}$; $Pygo2^{+/-}$ and $Vil^{Cre\text{-}ERT+}$; $APC^{ex15+/-}$; $Pygo2^{-/-}$; n=15 and 22 respectively). All mice were induced by tamoxifen injection for five consecutive days, and survival was monitored for the indicated time. Statistics were calculated by P Log Rank Test.

(**B**) Representative stains of intestinal sections from compound heterozygous APC LOF mutant animals with wild type Pygo2 expression ("$Pygo2^{+/+}$") compared to mice with hetero- and homozygous ablation of Pygo2 ("$Pygo2^{+/-}$" and "$Pygo2^{-/-}$"). Animals were analyzed on day seventy after beginning with tamoxifen treatment. The upper row shows intestinal tissues from control animals ($Vil^{Cre\text{-}ERT-}$; $APC^{ex15+/-}$; $Pygo2^{-/-}$). Tissue sections were stained by H&E and with the indicated antibodies.

4.8). The level of Wnt/ß-catenin target gene overexpression is dependent on Pygo2 during the intestinal hyperproliferation induced by APC LOF and ß-catenin GOF

Since survival of APC LOF and ß-catenin GOF mice was differentially regulated by Pygo2 expression we next analyzed any likely variation in the expression levels of Wnt target genes and genes reported to regulate intestinal tumor initiation and progression. Our previous studies already showed that the BCL9 related co-factors and Pygo2 in fact control the expression Wnt target genes in colon cancer cell lines (Brembeck *et al.,* 2011). Therefore, we hypothesized that Pygo2 may be also differentially controlling target gene expression in APC LOF and ß-catenin GOF animals, which might have resulted in their intestinal phenotypes and overall survival period after the induction of hyper-proliferation.

First we tested the expression of Lef/Tcf transcription factors on protein and RNA levels that direct downstream Wnt signaling events, and represent themselves targets of Wnt signaling pathway (Clevers, & Nusse, 2012) (Fig. 8A-E). Tcf4 was found to be expressed by all epithelial cells along the crypt villus axes in normal control tissue (Angus-Hill *et al.,* 2011). Whereas Tcf4 RNA levels were not induced in APC LOF and ß-catenin GOF mice on day 6 it was moderately but significantly increased in ß-catenin GOF-Pygo2 wild type mice on day 18. However, there was a marked downregulation of Tcf4 RNA in both mouse models when one allele or two alleles of Pygo2 was lost, which was even below to the levels of control animals. We observed this similar phenomenon also in Pygo2 complete knockout animals (Fig. 3) suggesting that Tcf4 transcription in general may partially rely on Pygo2. Unlike Tcf4, Lef1 protein was not expressed in control intestinal tissue but it was highly significantly upregulated in APC LOF on day 6 and ß-catenin GOF on day 18 in but not in ß-catenin GOF on day 6. However even deletion of a single Pygo2 allele significantly suppressed Lef1 expression in all mutants and Pygo2 complete deletion further

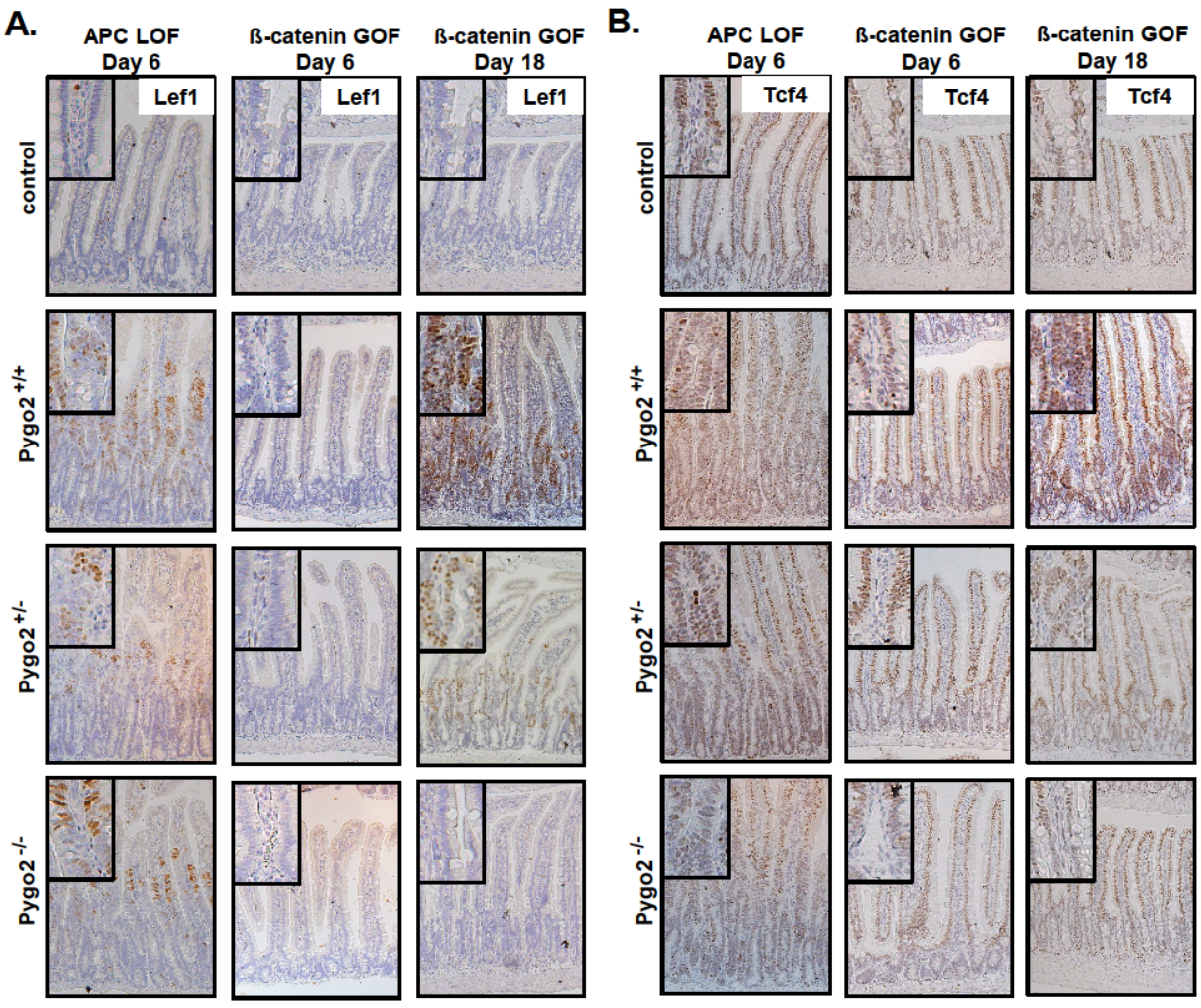
downregulated it. Nonetheless, Lef1 was still elevated in Pygo2 deficient intestines of APC mutant mice compared to controls.

Next we checked another Tcf family transcription factor, Tcf1. Tcf1 has been shown to be overexpressed in colorectal cancer cell as well as associated with tumor progression (Mayer *et al.,* 1997). Tcf1 was also elevated on protein and RNA levels only in APC LOF on day 6 and ß-catenin GOF animals on day 18. Pygo2 ablation was not able to suppress Tcf1 protein completely in APC LOF and Tcf1 RNA in APC LOF and ß-catenin GOF mutants. Thus Pygo2 deletion did not completely downregulate Tcf1 in APC LOF and ß-catenin GOF mice. Altogether, these data suggested that though Tcf1 was elevated in all mutants and regulated by Pygo2 it may not be crucial for intestinal hyperproliferation since ß-catenin GOF Pygo2 knockdown animals still expressed Tcf1.

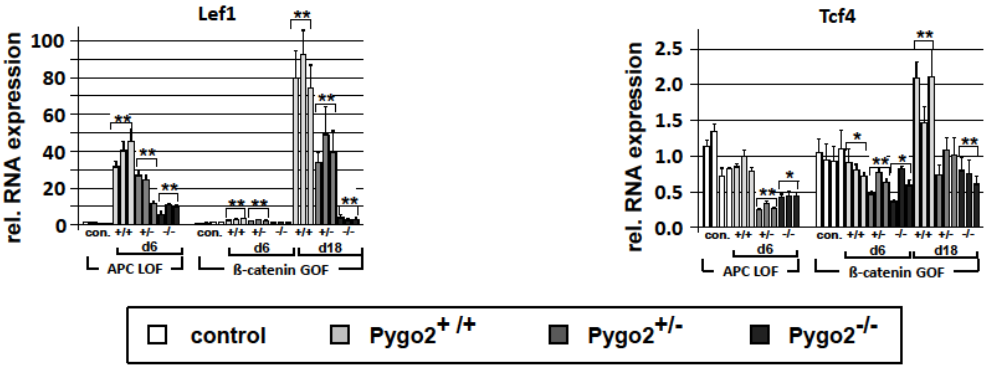
Together, we found that Lef1 was overexpressed in APC LOF and ß-catenin GOF mutants. However, Pygo2 loss did not completely downregulate Lef1 in APC LOF mutants, which might suggest Lef1 partial dependency on Pygo2 and a key role in mediating active Wnt signaling in hyperproliferative cells. On the other hand, although Tcf4 was deregulated in all mutants Pygo2 seemed to be essential even for its normal expression. Tcf1 was overexpressed in all mutants and its transcription may be independent of Pygo2.

Then other prominent Wnt target genes including cell cycle regulator Cyclin D1 and stem cell expressing genes such as Lgr5 and Ascl2 expression were analyzed (Fig. 8F-H). Cyclin D1, a known Wnt target gene, is crucial for cell proliferation during G1 phase of cell cycle (Sherr *et al.,* 1996; Arber *et al.,* 1997); Lgr5 and Ascl2 are Wnt inducible genes and are identified as intestinal stem cell markers (Barker *et al.,* 2009, Barker *et al.,* 2010 and Jaks *et al.,* 2008). Cyclin D1 protein and RNA levels were upregulated in APC LOF mice however no change was observed by Pygo2 ablation. Cyclin D1 transcription was not induced in ß-catenin GOF animals on day 6 and 18 but protein was expressed in hyperproliferative regions of intestinal epithelium in ß-catenin GOF on day 18 animals. However, Pygo2 knockout completely reversed Cyclin D1 expression to control level, restricting it only to crypts. Next, we studied the expression of intestinal stem cells markers, Lgr5 and Ascl2, which were reported to be expressed by crypt base columnar cells (CBC) (Lau *et al.,* 2006; Lips *et al.,* 2008). Lgr5 was overexpressed in both APC LOF on day 6 and ß-catenin GOF animals on day 18; Pygo2 knockout downregulated it completely only in ß-catenin animals on day 18 both at protein and RNA levels. Ascl2 transcription was also increased in both mouse mutant models however Pygo2 knockout only partially downregulated it in both mouse models.

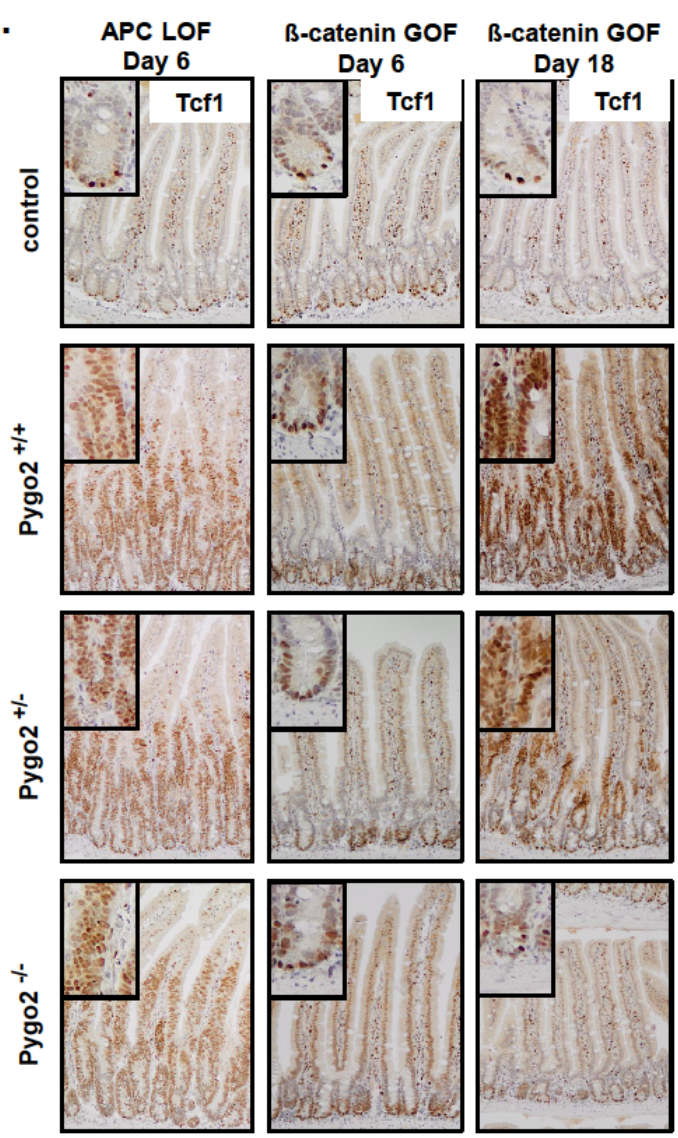
In summary, the Wnt/ß-catenin target genes Cyclin D1, Lgr5 and Ascl2 were induced in APC LOF mice as a result of hyperactive Wnt signaling. However, this deregulation cannot be completely rescued by knockout of Pygo2. On the contrary, Pygo2 ablation in ß-catenin GOF mice completely downregulated the overexpression of these target genes. In addition, these data also indicated that low degree of Wnt signaling due to ß-catenin GOF mutation might result in a milder intestinal phenotype in contrast to the severe intestinal phenotype of APC LOF mice. Consequently, Pygo2 complete deletion might be just sufficient to completely reduce deregulated Wnt signaling pathway induced by ß-catenin mutations, eventually eliminating intestinal neoplasia by reconstituting normal crypt-villus architecture. Hence, Pygo2 may modulate deregulated Wnt signaling only within a certain threshold.

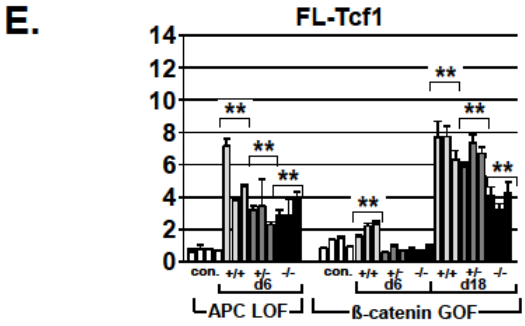


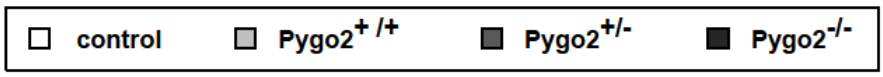
C.

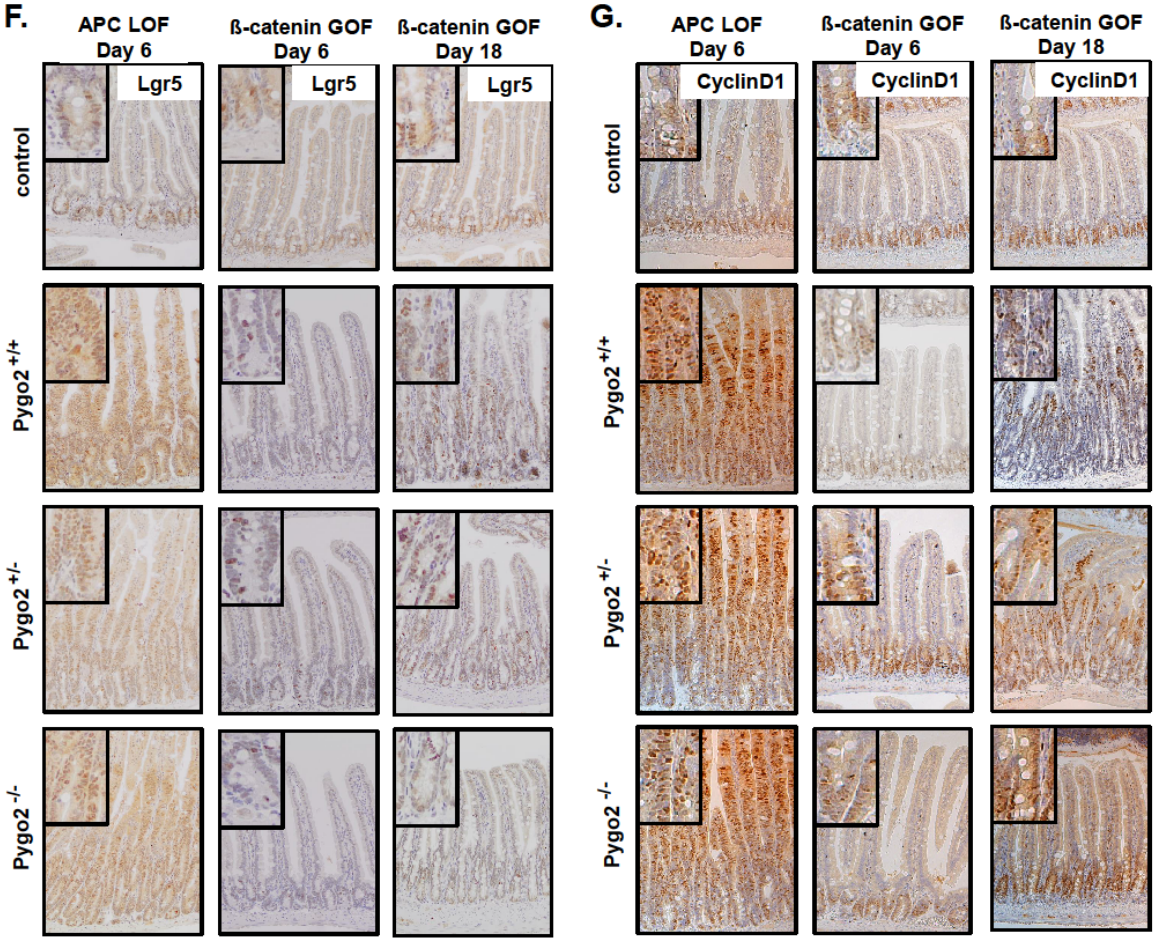

Lef1
rel. RNA expression
100
80
60
40
20
0
con. +/+ +/- -/-
d6
APC LOF
con. +/+ +/- -/-
d6
d18
ß-catenin GOF
Tcf4
rel. RNA expression
2.5
2.0
1.5
1.0
0.5
0.0
con. +/+ +/- -/-
d6
APC LOF
con. +/+ +/- -/-
d6
d18
ß-catenin GOF
control
Pygo2+ /+
Pygo2+/-
Pygo2-/-

D.


APC LOF
Day 6
ß-catenin GOF
Day 6
ß-catenin GOF
Day 18
Tcf1
Tcf1
Tcf1
control
Pygo2 +/+
Pygo2 +/-
Pygo2 -/-


E.
FL-Tcf1
14
12
10
8
6
4
2
0
**
con.
+/+
+/-
-/-
d6
d18
APC LOF
ß-catenin GOF


control
Pygo2+ /+
Pygo2+/-
Pygo2-/-


F.
APC LOF
Day 6
ß-catenin GOF
Day 6
ß-catenin GOF
Day 18
Lgr5
control
Pygo2+/+
Pygo2+/-
Pygo2-/-
G.
CyclinD1


H.
Lgr5
Cyclin D1
12
9
2.5
2.0
**

H.

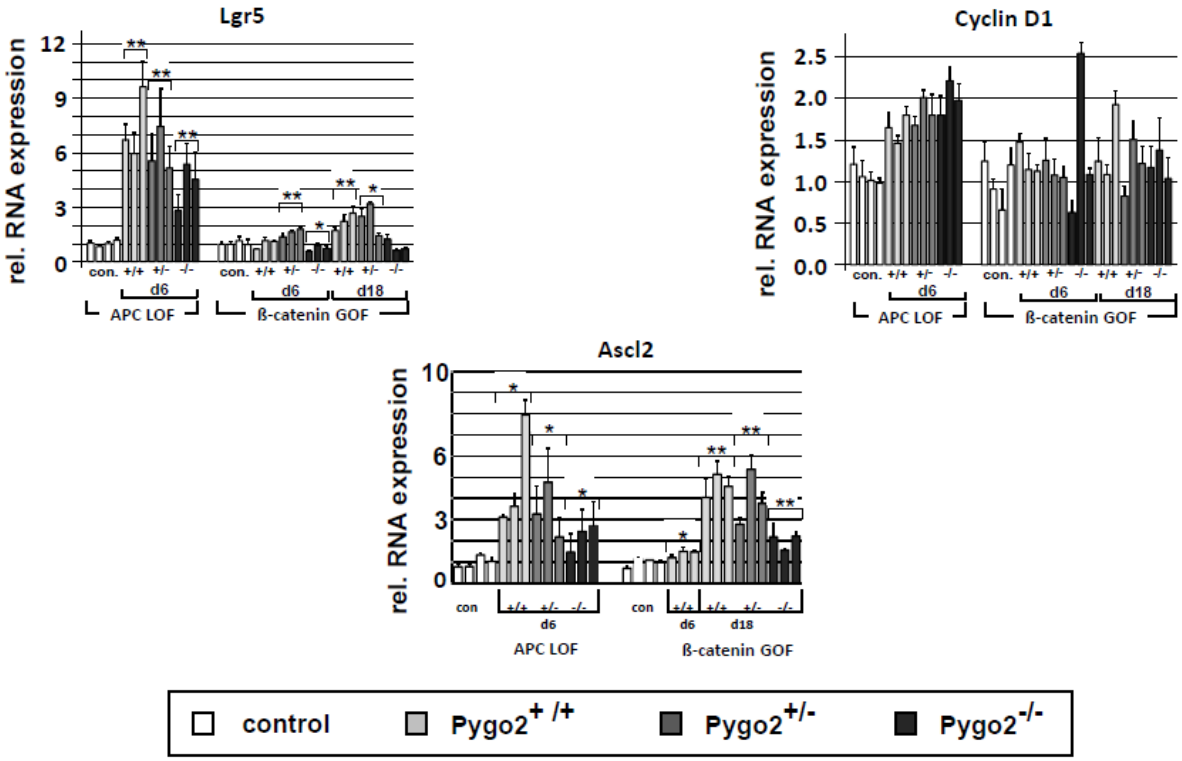


Figure 8: Pygo2 knockout is able to downregulate classical Wnt/ß-catenin target genes overexpressed during the intestinal hyperproliferation induced by APC LOF and ß-catenin GOF

(A, B, D, F and G) Immunostains for identifying Wnt target genes including Lef1, Tcf4, Tcf1, Lgr5 and Cyclin D1. Stainings were performed on intestinal sections from control mice, APC LOF mice, and ß-catenin GOF mice with indicated Pygo2 genetic back grounds, which were sacrificed on mentioned day.

(C, E, and H) qRT-PCR analyses show the RNA expression of Lef1, Tcf4, Tcf1, Lgr5, Cyclin D1 and Ascl2.

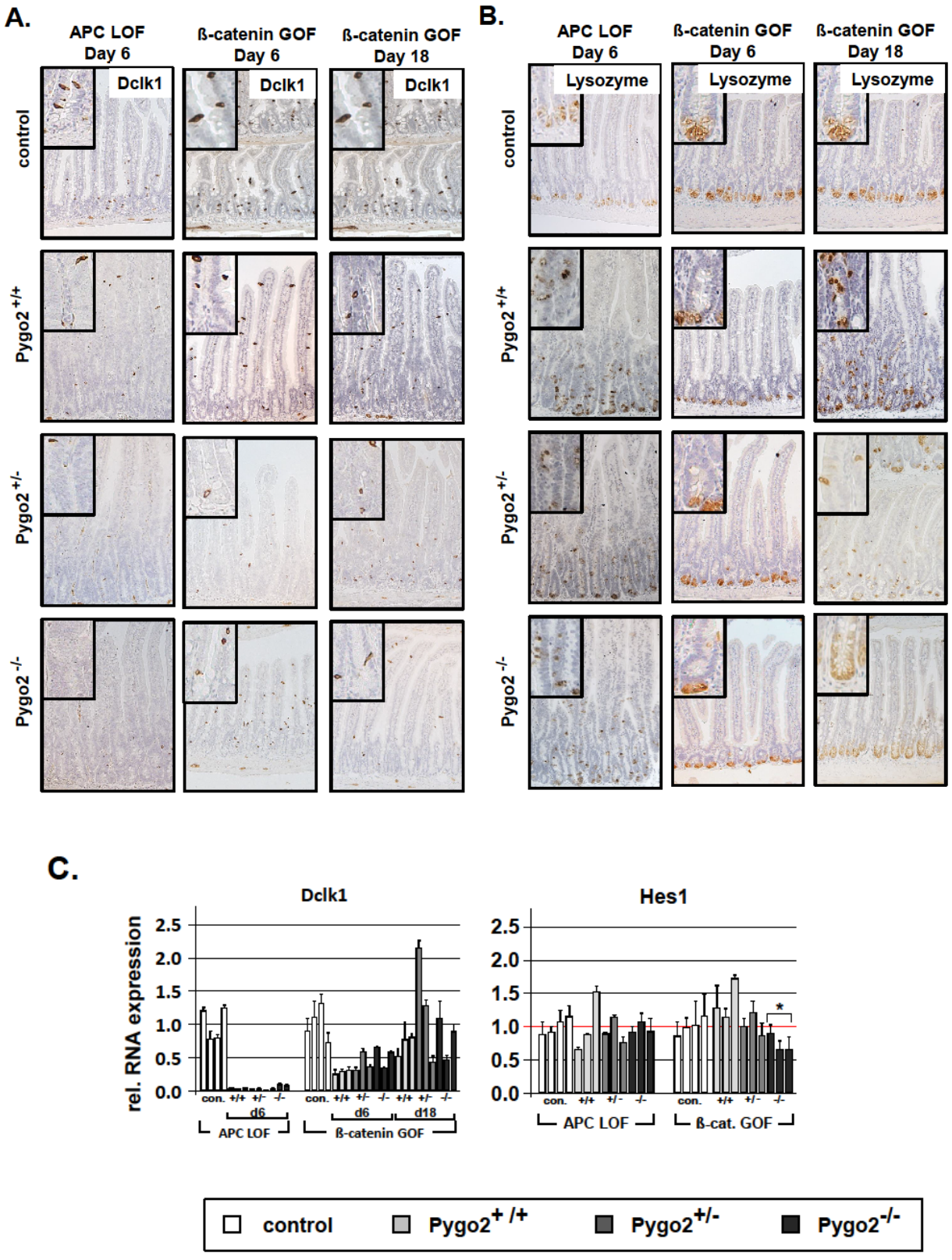
RNA was extracted from enriched intestinal epithelial cells of control mice, APC LOF and ß-catenin GOF mice with indicatedPygo2 back grounds. Each bar shows one animal, and each graph shows the relative expression of the gene indicated. All significant differences are marked with * for $P < 0.05$ and ** for $P < 0.01$.

4.9). Pygo2 knockout influences differentiation marker gene expression during the intestinal hyperproliferation induced by APC LOF and ß-catenin GOF

To investigate the effect of APC and ß-catenin genetic disruption on intestinal stem cell differentiation capacity and the role of Pygo2 we first tested the Paneth cell lineage. Paneth cells are generally localized at the crypt bottom and characterized by the expression of Lysozyme and of the Wnt/ß-catenin target

gene Sox9 (Bastide *et al.,* 2007; Mori-Akiyama *et al.,* 2007). In APC driven hyperproliferative intestine, Paneth cells were present throughout the crypt-villus axis as illustrated by lysozyme staining. In addition, Sox9 transcription was significantly induced and protein was expressed in all hyperproliferative cells whereas they were expressed only in Paneth cells in control crypts. However, Pygo2 ablation did not influence Sox9 expression and mislocalization of Paneth-like cells. In contrast to this, in ß-catenin GOF mutants on day 6 lysozyme and Sox9 expressing Paneth cells were still present in crypt compartment which once again explained the lower degree of induced Wnt signaling without apparent hyperproliferation as compared to APC LOF on same day 6. In contrast, ß-catenin GOF mutants on day 18 again demonstrated elevated Sox9 and mislocalized Paneth-like cells. However, Pygo2 knockout was not able to fully rescue these aberrations in both mutants; importantly Sox9 was still modestly expressed (Fig. 9B, 10A, C). In summary these data showed that APC and ß-catenin disruption induced hyperproliferation lead to differentiation defects with mislocalization of Paneth-like cells. Importantly, Pygo2 knockout prevented Paneth cells mislocalization only in ß-catenin GOF mutants.

After that we examined the expression of Dclk1, a tuft cell marker in the normal intestine and reportedly an intestinal tumor stem cell marker (Nakanishi *et al.,* 2012) (Fig. 9A, C). Notably, Dclk1 expressing cells were not induced in both mutant models, and were also not affected by Pygo2 loss. On the contrary, Dclk1 was entirely lost in APC mutants on protein and transcription levels, which might be due to the differentiation defects caused by severe hyperproliferation. In addition, E-cadherin, a cell-cell adhesion molecule, was also studied since it was previously shown to be deregulated in colorectal cancer (He *et al.,* 2013) (Fig. 9D, E). However, we did not find any marked change in E-cadherin expression in both mutants on protein and RNA levels nor it was affected by Pygo2 loss. Altogether, these data showed that lack of Dclk1 expression in APC LOF might indicate severe aberrations in intestinal stem cell differentiation. Although E-cadherin was expected to be downregulated in all mutants as it did in human colon cancer, we did not observe any such downregulation across all mutants (Khoursheed *et al.,* 2003; Dorudi *et al.,* 1993).


A.
APC LOF Day 6
ß-catenin GOF Day 6
ß-catenin GOF Day 18
Dclk1
control
Pygo2+/+
Pygo2+/-
Pygo2-/-
B.
APC LOF Day 6
ß-catenin GOF Day 6
ß-catenin GOF Day 18
Lysozyme
control
Pygo2+/+
Pygo2+/-
Pygo2-/-
C.
Dclk1
Hes1
rel. RNA expression
2.5
2.0
1.5
1.0
0.5
0.0
con. +/+ +/- -/- con. +/+ +/- -/- +/+ +/- -/-
d6
d6
d18
APC LOF
ß-catenin GOF
con. +/+ +/- -/- con. +/+ +/- -/-
*
APC LOF
ß-cat. GOF
control
Pygo2+ /+
Pygo2+/-
Pygo2-/-

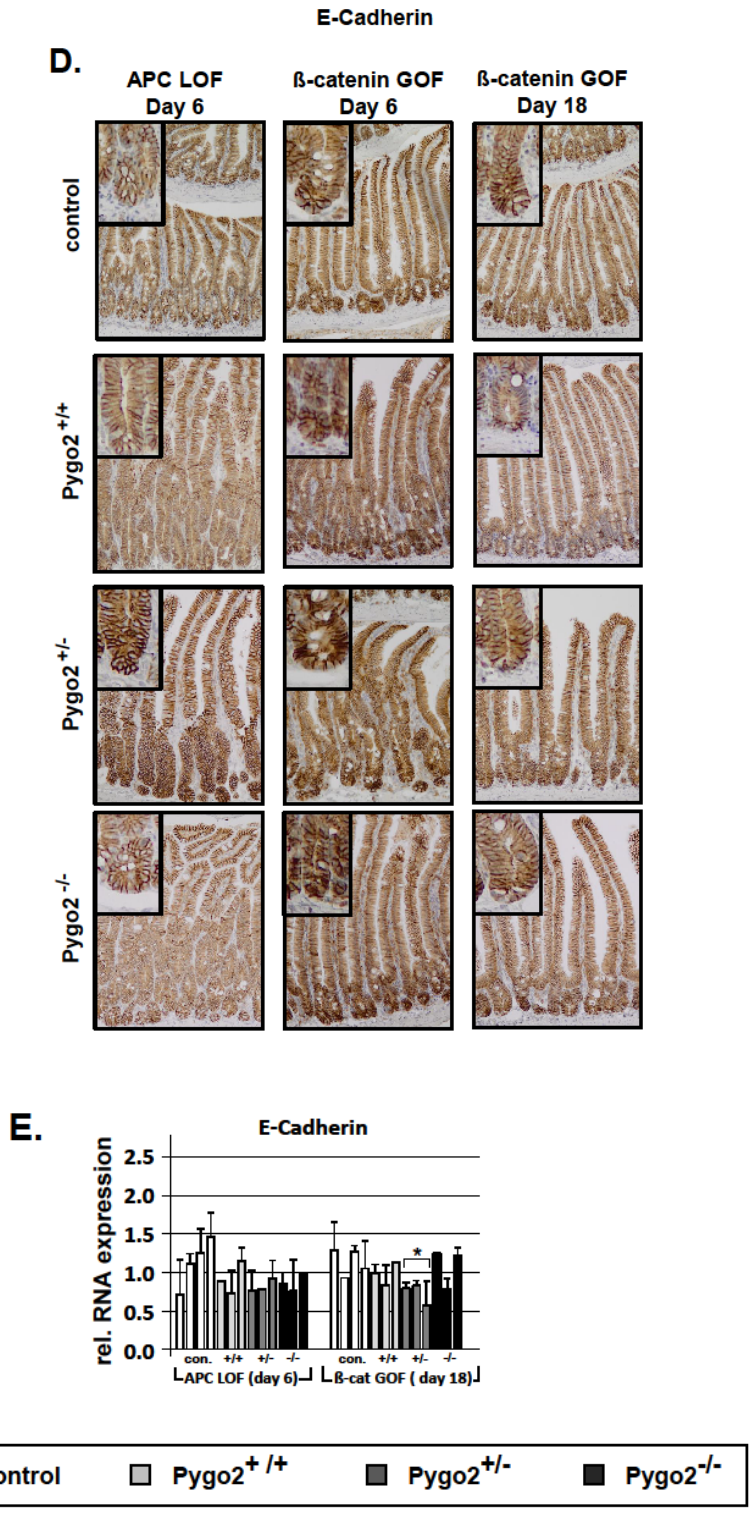


Figure 9: The effect of Pygopus2 ablation on differentiation marker expression during intestinal hyperproliferation induced by APC LOF and ß-catenin GOF.

(A, B and D) Histochemical analyses for detecting Dclk1, Lysozyme and E-cadherin. Intestinal sections were from control mice, APC LOF mice (first column, sacrificed day 6), and ß-catenin GOF mice (sacrificed on day 6 and day 18;

second and third columns, respectively) with indicated Pygo2 genetic back grounds. First row shows the histochemical stainings from control mice.

(C, E) qRT-PCR analyses on RNA extracted from epithelial cells of control mice, APC LOF mice and ß-catenin GOF mice. Each bar shows one animal, and each graph represents the relative expression of the gene indicated. All significant differences are marked with * for $P < 0.05$ and ** for $P < 0.01$.

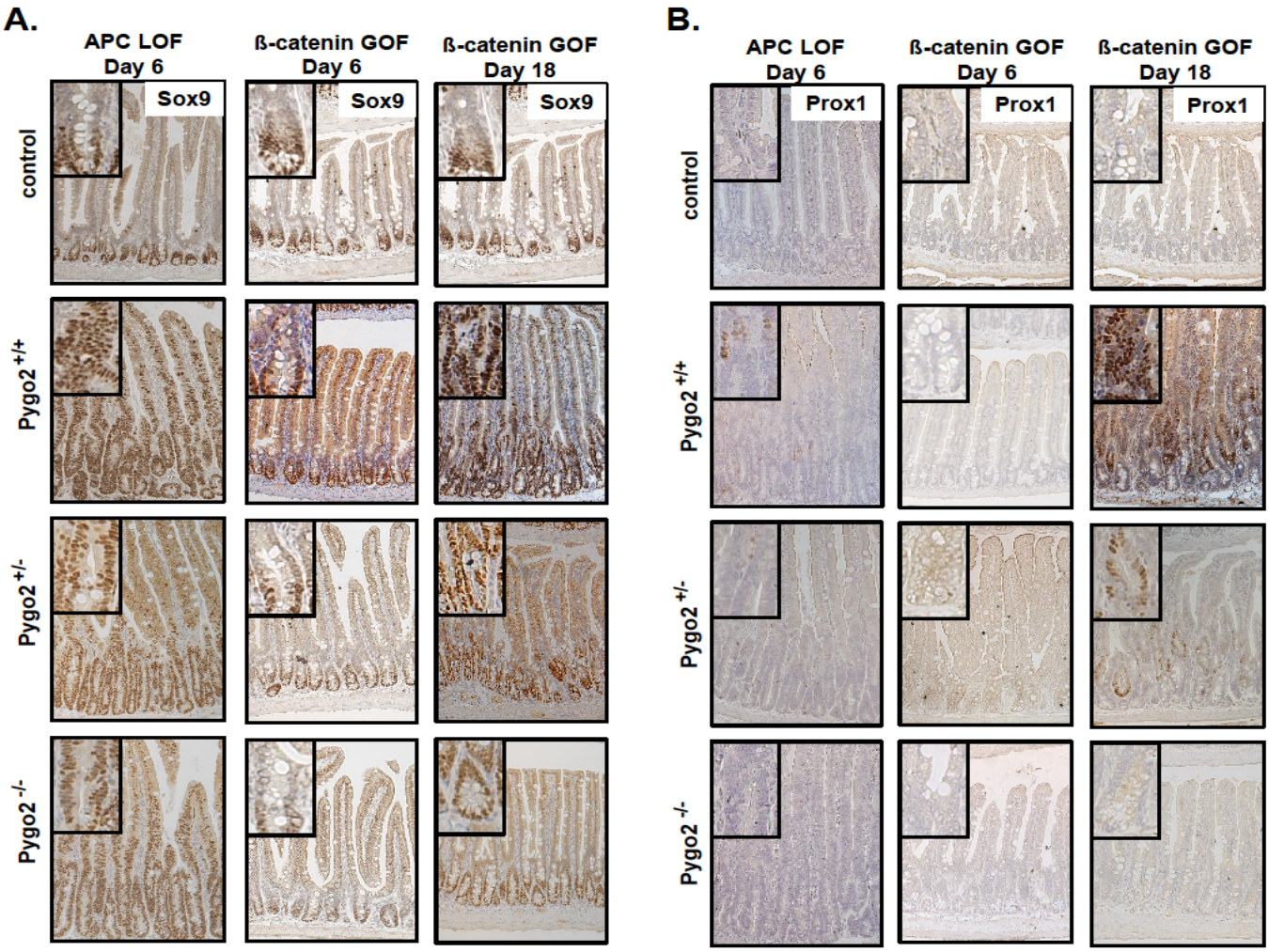
4.10). Pygo2 does not regulate the expression of Hes1, a Notch target gene

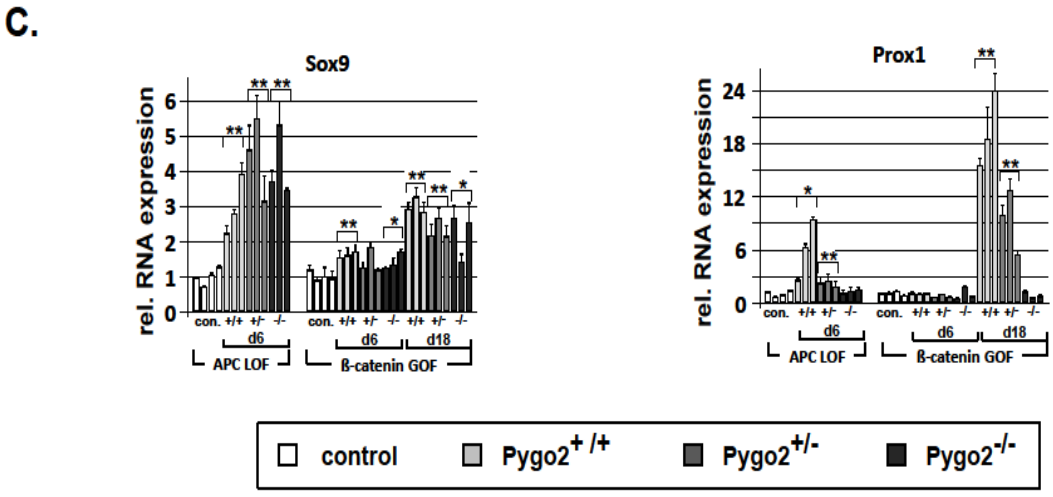
Pygo2 was recently found to be involved in Wnt and Notch signaling cross talk in mammary gland cells, considering its functions in chromatin remodeling (Gu *et al.,* 2013). Hence, we suspected any likelihood of Pygo2 functional involvement in regulating Notch signaling in our mouse models. To find this out, we tested Hes1 transcription regulation by Pygo2 in control and Pygo2 mutant animals. Hes1 is shown to be a Notch target gene that is expressed in highly proliferating crypt cells and is involved in self-renewal and tumorigenicity in colon cancer cell lines (Fre *et al.,* 2005; Fei Gao *et al.,* 2014). Nevertheless, Hes1 RNA levels were not upregulated in both APC LOF and ß-catenin GOF mice and in chemically induced tumors, which was on contrary to the previous reports stating that Hes1 was induced in APC LOF mice (Peignon *et al.,* 2011) (Fig. 9C, 12B). Genetic ablation of Pygo2 did only slightly downregulate Hes1 transcription in ß-catenin mutant animals, but not in APC LOF mice or in chemically induced tumors. In addition, Hes1 was also not affected by Pygo2 complete ablation in normal intestine (Fig. 3). Thus, Pygo2 might not be involved in the regulation Notch target gene expression in the intestine.

4.11). Overexpression of c-myc, a key player of intestinal hyperproliferation, is unaffected by Pygo2 loss in APC LOF mice in contrast to Prox1

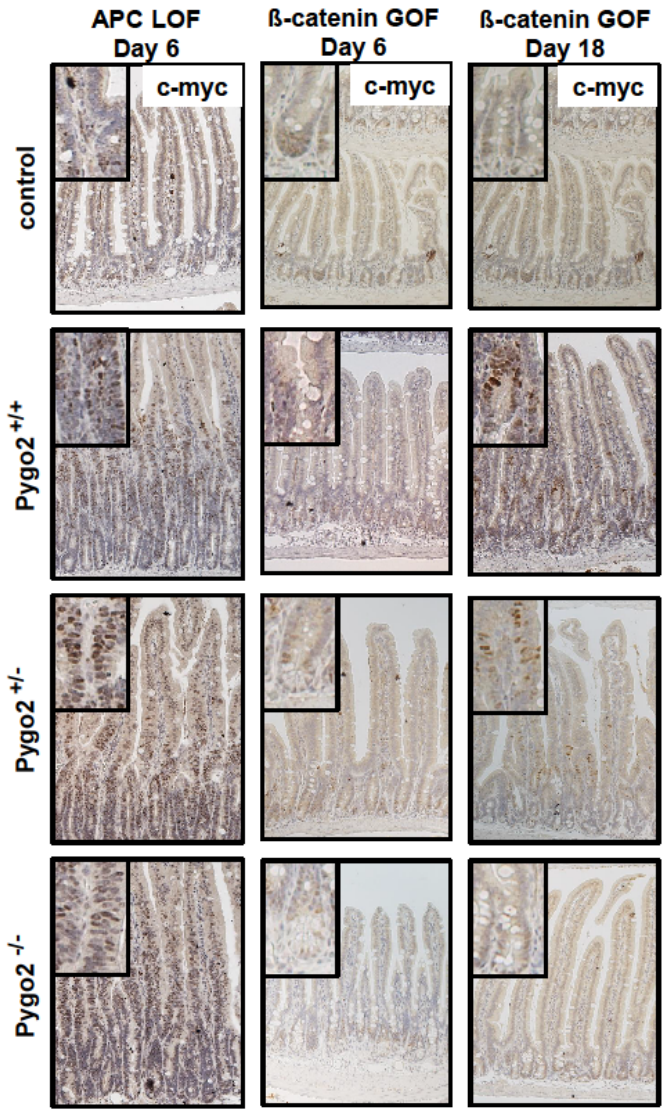
Transcription factors, which are also prominent Wnt target genes, were analyzed including Prox1 and c-myc, a global gene amplifier. Prox1 was reported to be important for the colon cancer initiation and evolution (Fig. 10B, C) (Petrova *et al.,* 2008; Ragusa *et al.,* 2014; Wiener *et al.,* 2014; Skog *et al.,* 2011). Despite active Wnt signaling Prox1 was not expressed in normal intestine, however, we observed massive upregulation in APC LOF, and even more prominently in ß-catenin GOF mice on day 18. At the same time, Prox1 was completely absent in ß-catenin GOF mice on day 6. Noticeably, Pygo2 total ablation fully downregulated Prox1 to that of control levels in both mutants. This indicated that though Prox1 was Wnt target gene it was completely regulated by Pygo2 expression, in addition persistent intestinal hyperproliferation in Pygo2 homozygous-APC LOF mice even after Prox1 loss indicated that Prox1 might not be absolutely required for early intestinal hyperproliferation.

In addition, c-myc, an important transcription factor during early stages of intestinal tumorigenesis and Wnt target gene (van, de, Lau, de & Clevers, 2002), was analyzed (Fig. 10D, E). Previous studies revealed that c-myc knockout completely rescued APC truncation induced intestinal phenotype suggesting its vital role in Wnt/ß-catenin signaling directed early hyperproliferation (Sansom *et al*., 2007). We found in fact a significant overexpression of c-myc protein and RNA levels in APC LOF mice but Pygo2 knockout failed to downregulate c-myc expression. In contrast, c-myc was not overexpressed in ß-catenin mutants on day 6; however, it was upregulated in day 18 mutants although relatively lower compared to APC LOF mutants. Importantly, c-myc expression was regulated by deletion of even one Pygo2 allele in ß-catenin GOF mutants on day 18 and after Pygo2 complete loss it was brought to control levels on protein and RNA levels. This clearly showed that Pygo2 loss totally downregulated c-myc expression only in ß-catenin GOF but not in APC LOF. These data indicated that c-myc overexpression in APC LOF mice was not regulated by Pygo2 expression, and may be a key player that contributed to hyperproliferation. Thus, Pygo2 might control Wnt signaling mediated gene expression only when it was under a certain threshold. In particular, Pygo2 might completely downregulate c-myc in ß-catenin GOF because of the lower degree of c-myc overexpression compared to APC LOF.

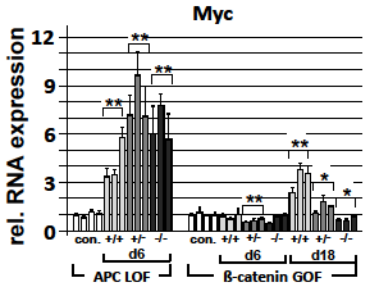

A.
APC LOF Day 6
ß-catenin GOF Day 6
ß-catenin GOF Day 18
Sox9
control
Pygo2 +/+
Pygo2 +/-
Pygo2 -/-
B.
APC LOF Day 6
ß-catenin GOF Day 6
ß-catenin GOF Day 18
Prox1
control
Pygo2 +/+
Pygo2 +/-
Pygo2 -/-


C.
Sox9
rel. RNA expression
0
1
2
3
4
5
6
con. +/+ +/- -/- con. +/+ +/- -/- +/+ +/- -/-
d6
d6
d18
APC LOF
ß-catenin GOF
Prox1
rel. RNA expression
0
6
12
18
24
con. +/+ +/- -/- con. +/+ +/- -/- +/+ +/- -/-
d6
d6
d18
APC LOF
ß-catenin GOF
control
Pygo2 + /+
Pygo2 +/-
Pygo2 -/-

D.


APC LOF
Day 6
ß-catenin GOF
Day 6
ß-catenin GOF
Day 18
c-myc
c-myc
c-myc
control
Pygo2 +/+
Pygo2 +/-
Pygo2 -/-

E.


Myc
rel. RNA expression
12
9
6
3
0
con. +/+ +/- -/-
d6
APC LOF
con. +/+ +/- -/-
d6
d18
ß-catenin GOF

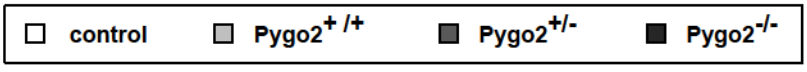

control
Pygo2+ /+
Pygo2+/-
Pygo2-/-

Figure 10: Pygo2 ablation completely inhibits the upregulation of Prox1, but fails to downregulate the overexpression of c-myc during early intestinal tumorigenesis in APC LOF mice.

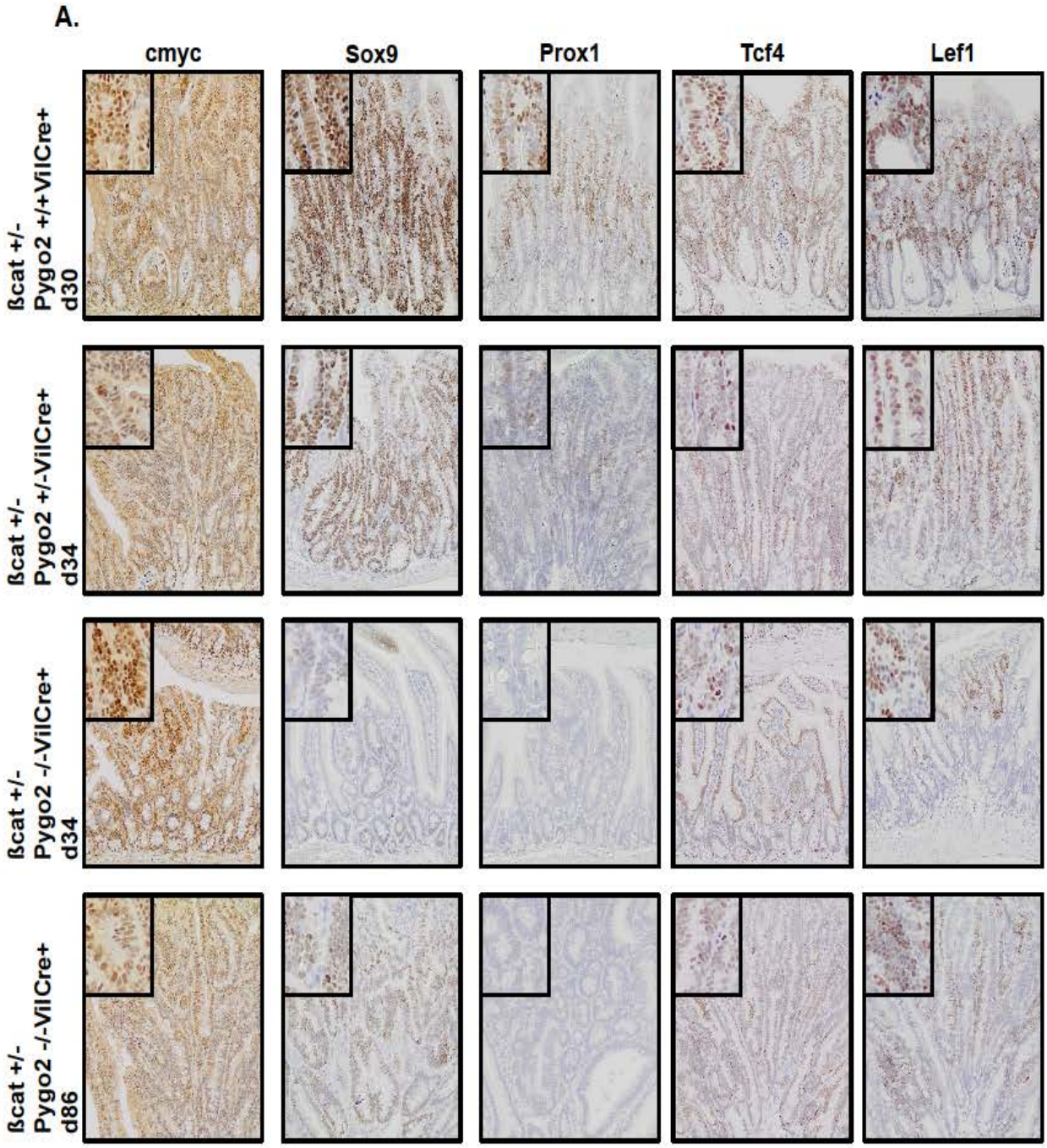
(A, B, and D) Pictures of immunostains for the detection of tumor progression markers Sox9, Prox1, and c-myc. Intestinal sections analyzed were from APC LOF mice (first column) sacrificed on day 6, ß-catenin GOF mice sacrificed on day 6 (second column), and day 18 (third column) following tamoxifen treatment, which have indicated Pygo2 genetic back grounds. Upper row shows the immunostains from control mice.

(C, E) qRT-PCR analyses show the expression of Sox9, Prox1 and c-myc. RNA was extracted from enriched epithelial cells of control mice, APC LOF mice sacrificed on day 6, ß-catenin GOF mice sacrificed on day6, and day 18. Pygo2 genetic back grounds were indicated. Each bar shows one animal, and each graph shows the relative expression of the gene indicated. All significant differences are marked with * for $P < 0.05$ and ** for $P < 0.01$.

4.12). Overexpression of Wnt target gene cascade in microadenomas generated due to failed Pygo2 deletion in ß-catenin GOF-Pygo2 knockout mice

Next we performed immunostains for the detection of various Wnt target genes on microadenomas that were induced due to the failed Pygo2 recombination deletion in ß-catenin GOF mice (see above, chapter 4.6). (Fig. 11A, B). Indeed, these adenomas overexpressed many target genes including Tcf4, Lef1, Sox9, Cyclin D1 and Tcf1. However, Prox1 was not expressed. Lgr5 expression was relatively low in all adenomatous cells.

Finally, c-myc was markedly overexpressed in microadenomas, which may have originated from cells that escaped recombination of Pygo2 deletion. This indicated that c-myc may exert a key role in hyperproliferation and tumor formation, and that c-myc was completely regulated by Pygo2 in ß-catenin GOF mice in contrast to APC LOF mice.


A.
cmyc
Sox9
Prox1
Tcf4
Lef1
ßcat +/- Pygo2 +/+VilCre+ d30
ßcat +/- Pygo2 +/-VilCre+ d34
ßcat +/- Pygo2 -/-VilCre+ d34
ßcat +/- Pygo2 -/-VilCre+ d86

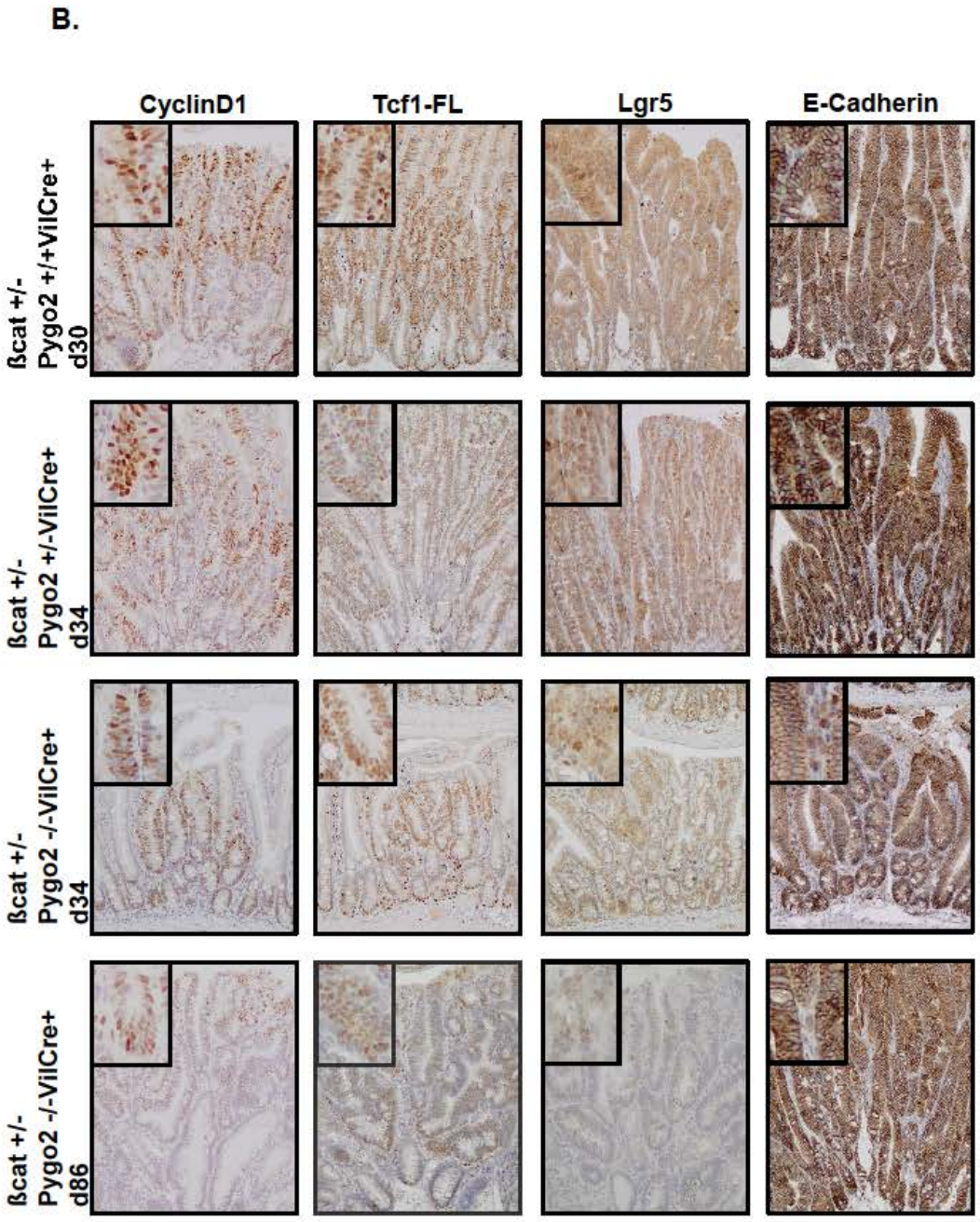


Figure 11: Pygo2 failed recombination resulted in the upregulation of Wnt target genes in microadenomas of Pygo2 deficient ß-catenin heterozygous stabilized mice.

(A-B) Immunohistochemical analyses with indicated antibodies on intestinal tissues from compound Pygo2-ß-catenin GOF mice, which were sacrificed later than day 18 after the beginning of tamoxifen treatment.

(Upper row) shows immunostains on intestinal sections from ß-catenin (ß-catenin$^{ex3+/-}$) Pygo2 wild type (Pygo2$^{+/+}$) compound mice sacrificed on day 30.

(Second row) shows immunostains on intestinal sections from ß-catenin (ß-catenin$^{ex3+/-}$) with heterozygous (Pygo2$^{+/-}$) Pygo2 expressing mice sacrificed on day 34.

(Third row) shows immunostains on intestinal sections with microadenomas from ß-catenin (ß-catenin$^{ex3+/-}$) Pygo2 homozygous (Pygo2$^{-/-;}$) knockout mice, which were sacrificed on day 34. Unsuccessful Pygo2 deletion by recombination in some regions of intestines here had resulted in the formation of microadenomas in intestines.

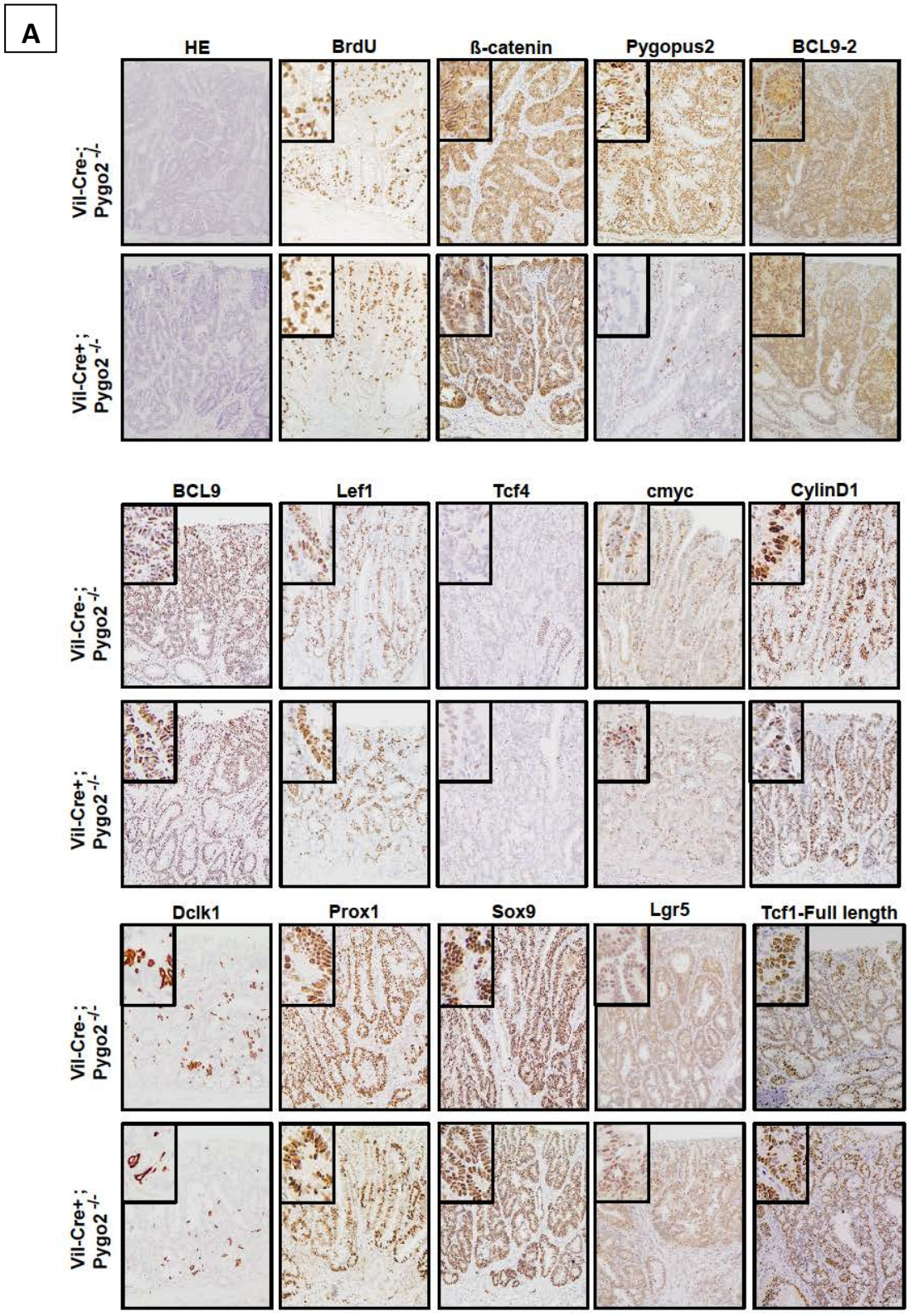
(Fourth row) shows immunostains on intestinal tissue sections from ß-catenin (ß-catenin$^{ex3+/-}$) Pygo2 complete knockout (Pygo2$^{-/-}$) compound mice which were sacrificed on day 86. Again due to the Pygo2 failed deletion intestinal sections of these mice had adenomas larger in size, compared to those in intestines from ß-catenin (ß-catenin$^{ex3+/-}$) Pygo2 homozygous (Pygo2$^{-/-}$) mice sacrificed on day 34.

4.13). Downregulation of elevated Wnt/ß-catenin target genes and tumor progression genes in tumors from a sub set of Pygo2 deficient chemically induced mice

Next, to elucidate whether these genes were also implicated in tumors of AOM/DSS treated mice, and especially regulated by Pygo2 we evaluated the expression of target genes by immunohistochemistry and qRT-PCR (Fig. 12A, B). As expected, all genes, except Tcf4, were highly upregulated in tumors of controls and Pygo2 deficient animals both on protein and RNA levels. However, tumors from a subgroup of Pygo2 deficient animals demonstrated significant downregulation of the transcripts of Lef1, Sox9, Lgr5, Prox1, Tcf1 and Ascl2 genes as compared to control tumors. Additionally, we found the moderate downregulation of Tcf4 RNA in both Pygo2 wild type and Pygo2 deficient tumors, which was in line with Tcf4 expression in APC and ß-catenin mutants. Surprisingly, Prox1 was still upregulated in Pygo2 deficient chemically induced tumors in contrast to its complete absence in Pygo2 deficient APC LOF and ß-catenin GOF mutants. E-cadherin was not downregulated in control and Pygo2 deficient tumors, however, previous studies linked E-cadherin downregulation to adenoma to carcinoma transition and differentiation defects in colon cancer (Dorudi *et al.,* 1993; Perl *et al.,* 1998). Most importantly, c-myc overexpression on protein and transcription level was totally unaltered in Pygo2 deficient chemically induced tumors, which was in accordance with its expression in APC LOF mutants in the context of Pygo2 knockout.

Taken together, we have identified elevated Wnt target genes confirming the hyperactive Wnt signaling in chemically induced tumors. In addition, Pygo2 deletion was partially able to downregulate most of them thereby modulating the degree of Wnt gene output. However, c-myc expression seemed to be totally independent from Pygo2 expression and might be also a major driver during the tumor

development. This could explain why Pygo2 cannot totally reverse chemically induced colon tumorigenesis, but was able to delay the tumor development in our mouse model.



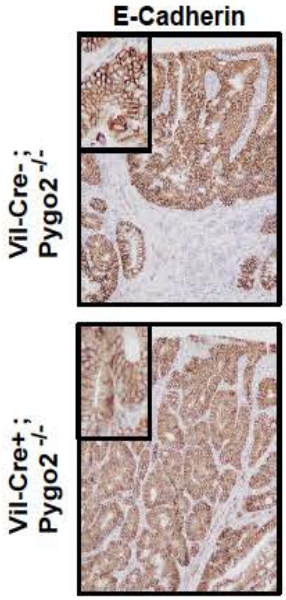
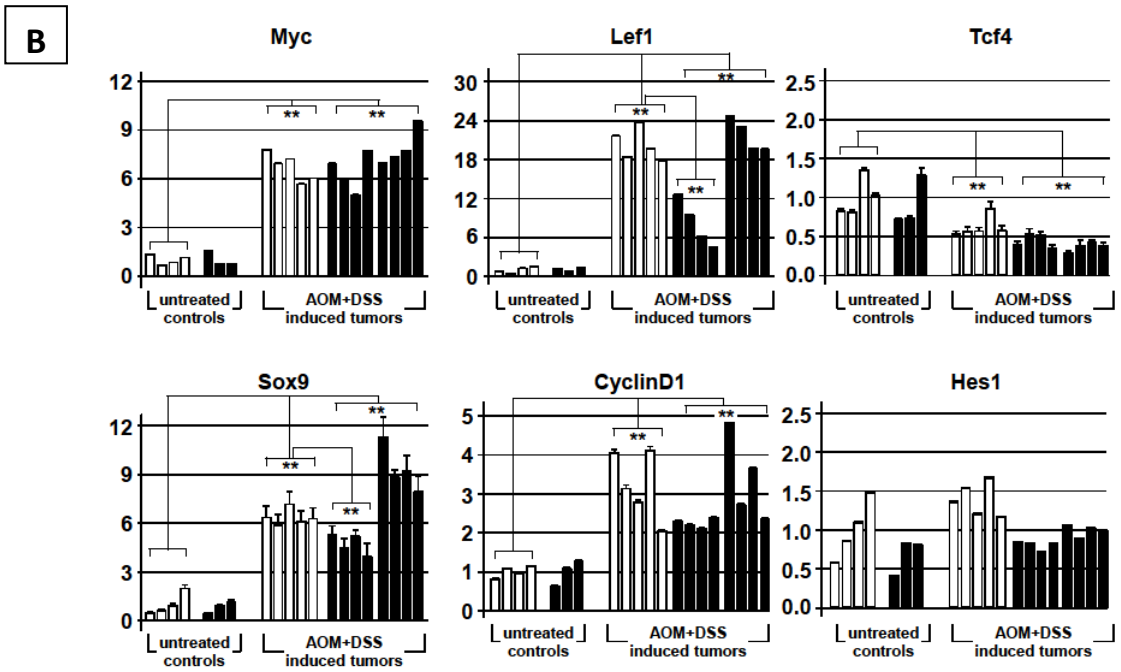


Figure 12: Ablation of Pygo2 downregulates the overexpression of Wnt/ß-catenin targets and tumor progression genes in a subgroup of chemically induced intestinal tumors.

(A) Representative Immunostains performed on intestinal tumor sections of AOM treated mice. (Upper row) shows all stainings, on Pygo2 wild type intestinal tumors (Vil^{Cre-}; $Pygo2^{-/-}$), for H&E and indicated antibodies. (Lower row) shows all stainings, on constitutive Pygo2 deficient intestinal tumors (Vil^{Cre+}; $Pygo2^{-/-}$), for H&E and indicated antibodies.



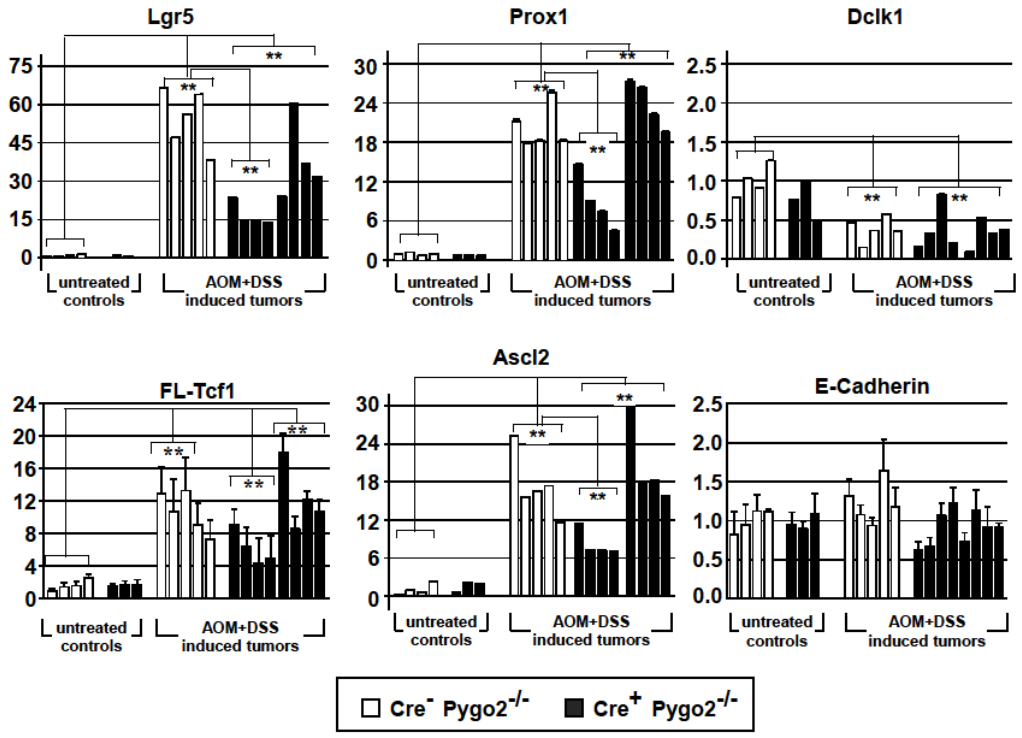


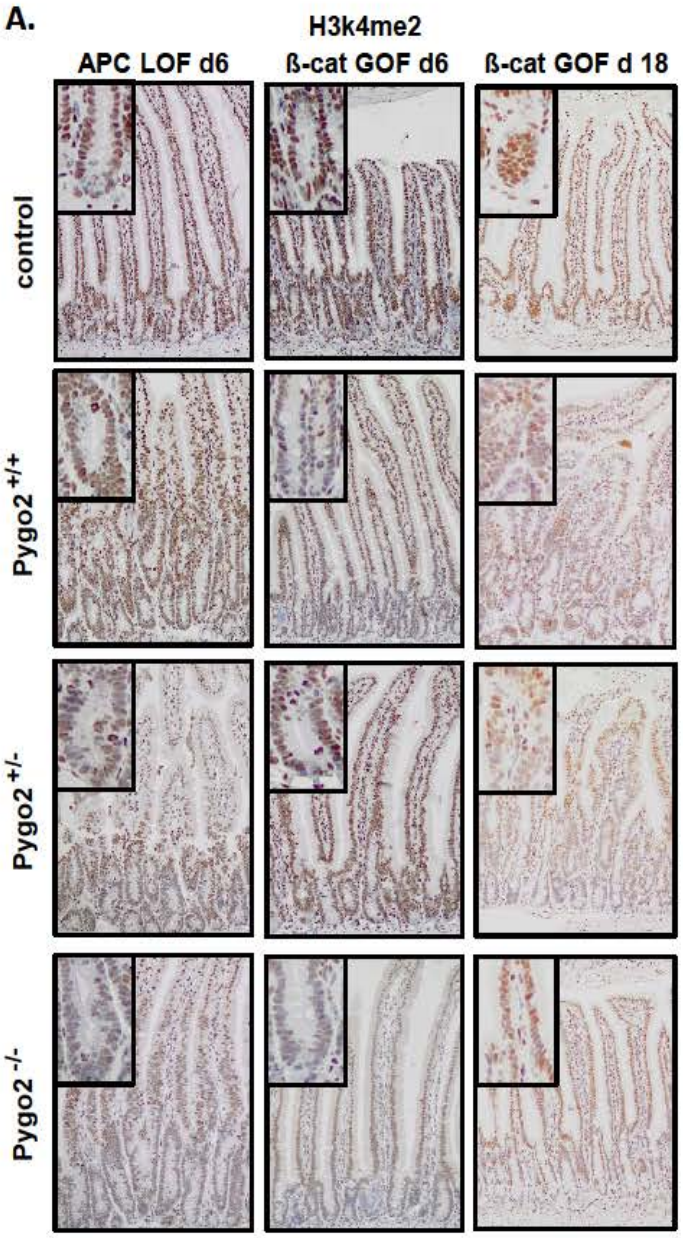
Figure 12: Ablation of Pygo2 downregulates the overexpression of Wnt/ß-catenin targets and tumor progression genes in a subgroup of chemically induced intestinal tumors.

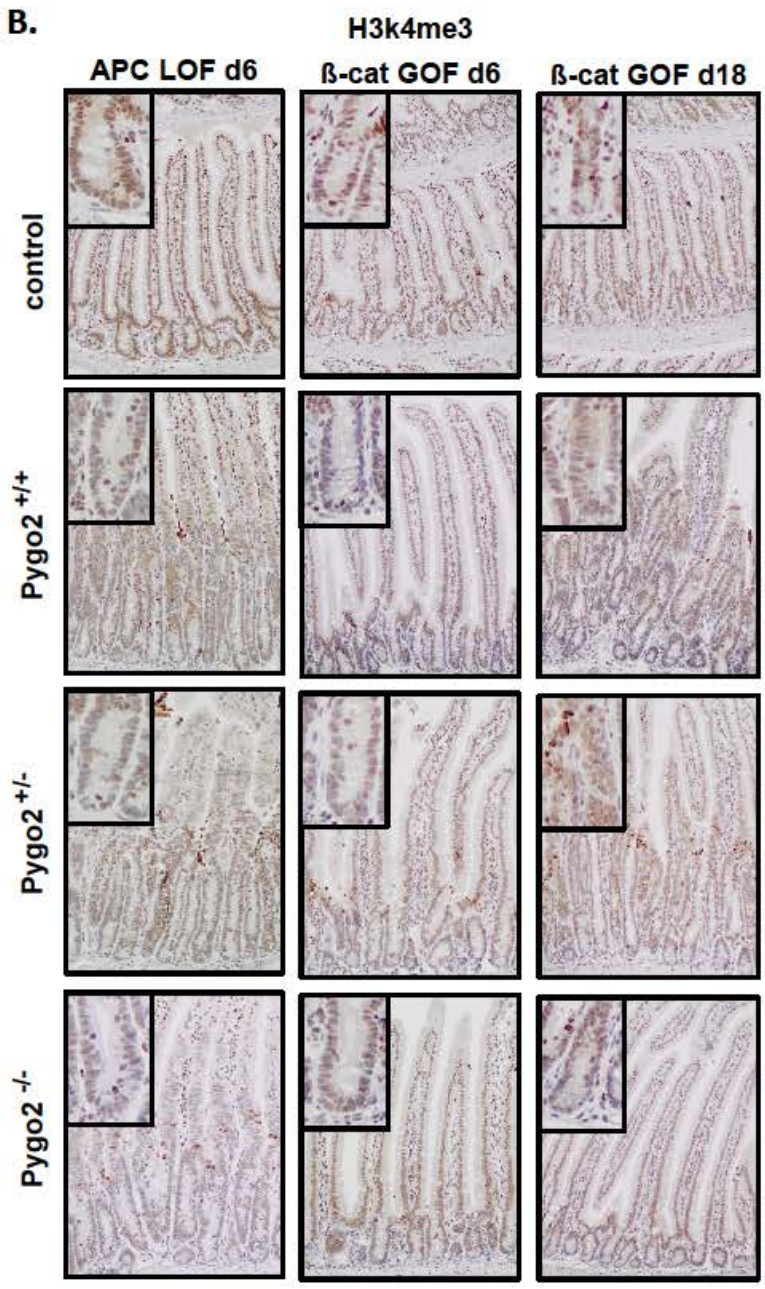
(B) qRT-PCR analyses of RNA extracted from tumors and from colon tissues of untreated mice, both of controls and Pygo2 deficient animals. Each graph shows the relative RNA expression of the indicated gene. Each bar represents one single animal. Significances were calculated for the mean expression level compared to untreated controls and relative to the expression levels of control tumors, respectively. All significant differences are marked with ** for $P < 0.01$

4.14). Tri/di methylation of H3K4 (Lysine4 of Histone3) is independent of Pygo2 expression, which is also not induced during intestinal tumorigenesis

Apart from its role in Wnt/ß-catenin signaling, Pygo2, was previously shown to be involved in chromatin remodeling as it bound to histone H3 tail methylated at lysine 4 (Gu *et al.,* 2009). Therefore, we studied the relevance of Pygo2 association with tri/di methylated histone 3 at lysine4 during intestinal hyperproliferation by immunohistochemistry (Fig. 13A, B). In fact, we observed the expression of tri/di methylated lysine4 of histone3 in all intestinal epithelial cells of control mice however it was not induced in intestinal hyperproliferative cells in APC LOF and ß-catenin GOF mice. Importantly, Pygo2 complete

knockout in APC LOF and ß-catenin GOF mutants did not affect the expression of tri/di methylated lysine4 of histone3. In addition, it was not also induced in tumor sections of AOM treated mice regardless of Pygo2 expression. Together, these data indicated that methylation of histone3 at lysine4 was not induced during intestinal tumorigenesis, and it was not dependent on Pygo2 expression.




B.
H3k4me3
APC LOF d6
ß-cat GOF d6
ß-cat GOF d18
control
Pygo2 +/+
Pygo2 +/-
Pygo2 -/-

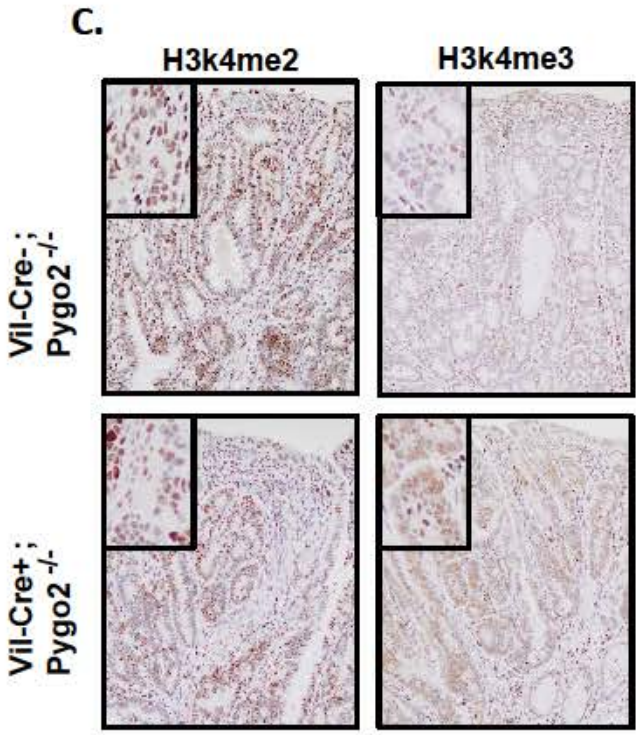


Figure 13. Tri/di methylation of H3k4 (Lysine 4 of Histone 3) is independent of Pygo2 expression, which is also not induced during intestinal tumorigenesis.

(A). Pictures of immunostains for the detection of H3k4me2 on intestinal tissue sections of APC LOF mice (first column) sacrificed on day 6, ß-catenin GOF mice sacrificed on day 6 (second column), and day 18 (third column) following tamoxifen treatment, which have indicated Pygo2 genetic back grounds. Upper row shows the immunostains from control mice.

(B). Representative immunostains for the detection of H3k4me3 on intestinal tissue sections of APC LOF mice (first column) sacrificed on day 6, ß-catenin GOF mice sacrificed on day 6 (second column), and day 18 (third column) following tamoxifen treatment, which have indicated Pygo2 genetic back grounds. Upper row shows the immunostains from control mice.

(C). Representative Immunostains performed for detecting H3k4me2 & H3k4me3 on intestinal tumor sections of AOM treated mice. Upper row shows all stainings on Pygo2 wild type intestinal tumors (Vil^{Cre-}; $Pygo2^{-/-}$) and lower row shows all stainings on constitutive Pygo2 deficient intestinal tumors (Vil^{Cre+}; $Pygo2^{-/-}$).

4.15). Effect of Pygo2 and BCL9 related co-factors knockdown on target gene expression in colon cancer cell lines

Our study has indicated that the complete conditional knockout of Pygo2 effectively downregulated several elevated Wnt/ß-catenin target genes in mice and rescued the early intestinal hyperproliferation in ß-catenin GOF mice, but not APC LOF mice. Next, we examined whether Pygo2 can also demonstrate the same function in human colon cancer cells. Indeed, our previous investigations in SW480 colon cancer cells that harbor APC mutations illustrated Pygo2 regulation of classical Wnt/ß-catenin target genes (Brembeck *et al.,* 2011). Therefore, in the current study we further examined the role of Pygo2 in other colon cancer cell lines including HCT116 that harbor ß-catenin mutations and DLD1 cancer cells with APC truncation mutations (M. Ilyas *et al.,* 1997). In addition to deregulated Wnt signaling mutations, these cells were also reported to have other genetic aberrations: microsatellite instability (MSI), K-ras oncogenic mutations (G13D in both cell lines), PIK3CA mutations (E545K; D549N in DLD1 and H1047R in HCT116 cell lines) and TP53 (S241F only in DLD1) (D Ahmed et al., 2013).

Therefore, we studied the consequences of Pygo2, BCL9 related co-factors, and ß-catenin knockdown in these cancer cell lines in vitro. siRNAs were transfected into colon cancer cells to knockdown the expression of Pygo2, ß-catenin, and BCL9 related genes. Knockdown efficiency and subsequent regulation of target genes was tested by qRT-PCR analysis. SiRNA treatment against ß-catenin caused a significant downregulation of ß-catenin expression. Although ß-catenin was induced by BCL9 knockdown, Pygo2 and BCL9-2 silencing resulted in its downregulation. Next Pygo2 knockdown was confirmed, and Pygo2 expression was regulated by ß-catenin only in DLD1 cells. Most interestingly, c-myc was downregulated by ß-catenin knockdown in both cell lines that indicated Wnt signaling directed c-myc expression; however, Pygo2 knockdown also controlled the c-myc expression in both cell lines. This was on contrary to Pygo2 regulation of c-myc in mouse models because c-myc in APC LOF mice was not regulated by Pygo2. This may be because of lower degree of Wnt signaling in DLD1 colon cancer cell lines, compared to acute Wnt signaling in APC LOF mice thus Pygo2 might still be able to control c-myc expression in DLD1 cancer cells. Therefore, investigations in this direction could reveal new findings that may explain this perplexing Pygo2 differential regulation of c-myc in the context of APC mutations. Similarly, Sox9 was downregulated following ß-catenin silencing in both cell lines, but it was more significantly downregulated by Pygo2 knockdown only in HCT116 cells. Axin2 was also controlled by ß-catenin expression although Pygo2 also partially regulated it. On the other hand, CyclinD1 and Tcf4 were not dependent on ß-catenin expression despite Pygo2 deletion was able to suppress them partly.

Altogether, these data clearly showed that Pygo2 was implicated in the regulation of Wnt/ß-catenin target genes in colon cancer cells lines.

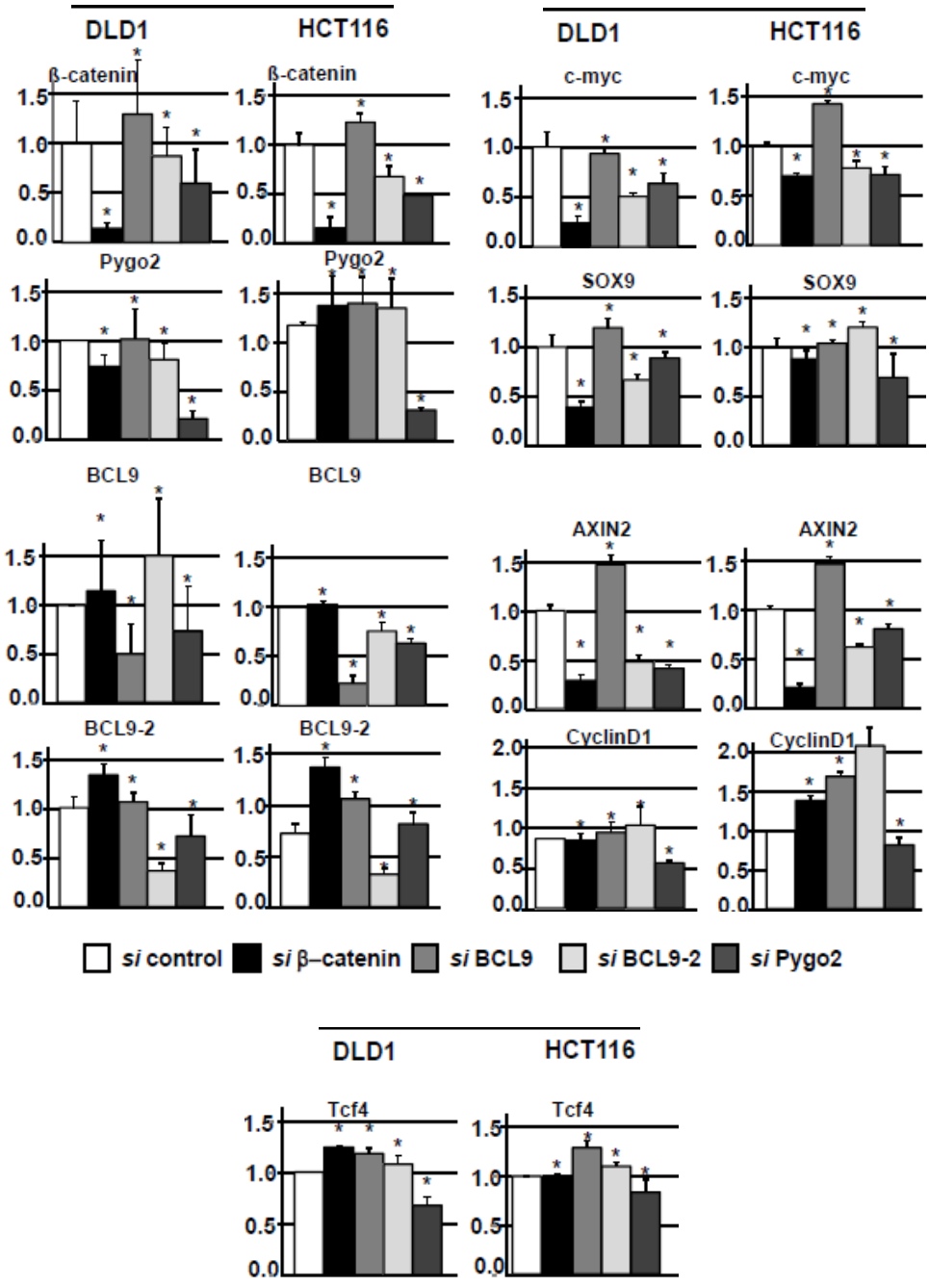


Figure 14: Pygo2 is implicated in the regulation of Wnt/ß-catenin target genes in colon cancer cell lines.

QRT-PCR analyses for the expression of Wnt signaling co-factors and Wnt/ß-catenin target genes following ß-catenin, Pygo2, BCL9 and BCL9-2 knockdown in DLD1 and HCT116 colon cancer cell lines. Each graph shows the

relative expression of gene indicated from at least three independent experiments. Actin was used as a house keeper gene. All significant differences are marked with * for $P < 0.05$.

5). Discussion

Colorectal cancer is a major public health concern in elderly (Pai *et al*., 2016). Numerous genetic mutations associated with different signaling cascades in the initiation and evolution of colorectal carcinogenesis has been discovered (Cancer Genome Atlas, 2012). Especially, the primary role of Wnt/β-catenin signaling pathway in intestinal homeostasis and tumor formation was well established (Gregorieff & Clevers, 2005; Tejeda-Muñoz *et al.,* 2015; Sebio *et al.,* 2014). Since the discovery of Pygopus as a co-factor in Wnt/ß-catenin signaling, several studies have disclosed its key role in the context of Wnt/ß-catenin dependent malignant development (Thompson *et al.,* 2002; Andrews *et al.,* 2007; Popadiuk *et al.,* 2006; Wang *et al.,* 2010; Brembeck *et al*., 2011). However, Pygopus key role in deregulated Wnt/ß-catenin signaling pathway induced intestinal tumorigenesis has not been studied extensively. In our previous studies we have examined Pygopus expression and its functional relevance. We found only one Pygopus homologue, Pygopus2, that was expressed ubiquitously by epithelial and mesenchymal cells throughout the intestine, whereas Pygopus1 was absent (Brembeck *et al.,* 2011). In addition to this, we indeed found Pygo2 upregulation in tumors of $Apc^{Min/+}$ mice and in colorectal cancer patients from previous experiments (Brembeck *et al.,* 2011), suggesting its feasible role during the intestinal tumor formation. Hence, in our current study we mainly focused on Pygo2 function during intestinal tumorigenesis that was achieved in mice by chemical carcinogen administration and conditional hyper activation of Wnt signaling by genetic mutation of APC and ß-catenin normal function. Of note, Pygo2 germ line knockout did not affect the normal development of mouse intestinal epithelium, and conditional Pygo2 deletion in adult mice failed to disturb normal epithelial homeostasis (Brembeck and Schelp, unpublished data).

5.1). Pygo2 ablation significantly reduces number and size of chemically induced colon tumors

Carcinogen induced colon cancer can recapitulate the phases of tumor progression in human colon cancer patients; hence it has been an ideal model to study the tumor evolvement (Wang *et al.,* 2013; An *et al.,* 201). We used colitis associated cancer model that was by sequential treatment with azoxymethane (AOM) single dose intraperitoneal injection followed by dextran sodium sulphate (DSS) oral administration. This model was proved to be a golden standard because of synergistic effects of DSS and AOM: DSS simultaneous pro inflammatory and tumor promoting role quickly accelerated the tumor induction by AOM in mice, compared to AOM or DSS alone models (Tanaka *et al.,* 2003; Kohno *et al.,*

2005; Rosenberg *et al.,* 2009). Whereas in mice treated only with AOM tumors at first were identified between six to seven month (Hata *et al.,* 2004; Bissahoyo *et al.,* 2005; Papanikolaou *et al.,* 2000). In contrast, combination of AOM and DSS treatment triggered colon tumor induction as early as 3 months after induction (Okayasu *et al.,* 1996; Becker *et al.,* 2004; Tanaka *et al.,* 2003; Kohno *et al.,* 2005; Rosenberg *et al.,* 2009). However, DSS dose dependent promoting effects and even mouse strains were shown to be important factors for tumor predisposition in AOM treated mice. AOM/DSS treatment triggered higher incidence of tumorigenesis in Balb/c mice, followed by C57BL/6N, C3H/HeN, and DBA/2N mice. (Suzuki *et al.,* 2005; Suzuki *et al.,* 2006). Of note, AOM/DSS treatment was shown to induce genetic mutations mainly by adding methyl group to guanine in liver and colon that triggered point mutations (Laqueur *et al.,* 1964; Delker *et al*., 1998; Ochiai *et al.,* 2001). Any reason for AOM organ specific mutagenicity that was apparently restricted only to larger intestine has not been known; however, a study demonstrated that AOM could also induce liver tumors (Nishihara *et al.,* 2008). Further studies may provide its organ specific potential of carcinogenicity. Nevertheless, AOM was reported to deregulate Wnt/ß-catenin signaling in colonic tumors in AOM/DSS treated mice (Cooper *et al.,* 2000; Tanaka *et al.,* 2004) despite the specific role of Pygo2, considering its Wnt dependent and independent functions, in chemically induced mouse tumor models (AOM/DSS) has not been studied yet.

Therefore, in our study intestinal epithelial specific constitutive Pygo2 deficient and control mice were administered with AOM/DSS for the tumor induction as described in methods. In accordance with previous reports (Rosenberg *et al.,* 2009), six-month post induction we observed relatively larger tumors restricted only to colon and rectum of control and Pygo2 deficient mice.

In addition, there was a significant difference between genetic mouse models and chemically induced AOM models: APC, ß-catenin or other genetic aberrations induced tumors were almost always predominantly in small intestine in contrast AOM/DSS model had better reflected human FAP (Familial adenomatous polyposis coli) patients because of colonic tumorigenesis (Corpet *et al.,* 2003). Invasiveness of tumors into submucosa was also seen in many of Pygo2 deficient and wildtype treated mice as it was reported previously (Ochiai *et al.,* 2001). Most importantly, reduced number of overall tumors and in specific larger tumors in Pygo2 deficient mice indicated that Pygo2 deletion was able to delay the colon tumor progression.

Elevated levels of nuclear ß-catenin in all colon tumors along with Axin2 up-regulation (Lustig *et al.,* 2002) illustrated the hyperactive Wnt signaling due to AOM/DSS treatment, which was also in line with

previous study (Deka *et al.,* 2010; Greten *et al.,* 2004). General overexpression of Axin2, as compared to normal controls, was reported in a very large cohort of colorectal cancer patients suggesting its pathogenic function (Schaal *et al.,* 2013). Despite being a tumor suppressor, Axin2 upregulation was demonstrated to induce the tumor invasion by promoting the activation of Snail1 thereby inducing epithelial to mesenchymal transition in colorectal cancer (Wu *et al.,* 2012). Presumably, colon tumor local invasion in Pygo2 deficient and control mice in our present study may be also linked in part to elevated levels of Axin2. Jürgen Behrens group also studied comprehensively about Conductin/axin2 expression and importance in colon cancer (Lustig *et al.,* 2002; Bernkopf *et al*., 2015).

Even though we did not examine for any specific mutations within Wnt/ß-catenin signaling, latest studies had proved deregulation of the Wnt pathway in AOM/DSS treated mice owing to mutations in ß-catenin gene at codons 32, 33, 34 and 41, which represents the glycogen synthase kinase-3ß phosphorylation motif, and loss of function mutations in APC expressing gene (Maltzman *et al.,* 1998; Koesters *et al.,* 2001; Takahashi *et al.,* 2000). Therefore, nuclear ß-catenin and elevation of Axin2 in tumors indicated the possibility of mutations in genes including APC and ß-catenin that express Wnt/ß-catenin signaling components. Apart from APC and ß-catenin mutations, AOM may also activate Wnt signaling by causing genetic mutations in other Wnt components. Therefore, genetic analysis of other Wnt signaling components in tumors of AOM treated mice can potentially reveal unknown mutations. In addition to deregulation of Wnt/ß-catenin signaling, aberrations in other signaling pathways were also reported. For instance, in a global gene expression analysis in AOM/DSS mice, TGFß3 was deregulated, which was linked to increased invasiveness of tumors at later stages of cancer development (Suzuki *et al.,* 2007; De Robertis *et al.,* 20011). Although we had not evaluated, presumably, deregulation of TGF-ß signaling along with Axin2 upregulation might have synergistically induced invasion of tumors in current mouse models. Another noticeable difference observed, in contrast to our conditional APC and ß-catenin mouse models, in our AOM/ DSS model was the presence of distinctively massive size tumors, which were located only in colon and rectum. This phenomenon might not be triggered only due to the dysregulated Wnt/ß-catenin cascade, because previous AOM/DSS model studies in fact demonstrated the prevalence of activating oncogenic mutation of K-Ras (Takahashi *et al.,* 2004) that associated with increased tumor size therefore we may not also rule out the oncogenic K-Ras mutations. Group of Jacoby showed that 1, 2-dimethylhydrazine (DMH), a precursor of azoxymethane (AOM), exposure induced K-Ras activating mutations (G to A) in 66% of colon mouse carcinomas (Jacoby *et al.,* 1991), which also indicated the differential mutagenic effect of carcinogen, AOM. Although we did not study the mutational status of K-Ras, previous studies in human colorectal cancer patients disclosed somewhat contradictory outcome

when Kazuhisa Shitoh team found in a small cohort of 64 patients 27% had K-Ras mutations and 11% had ß-catenin mutations, and patients with ß-catenin mutations never found to harbor K-Ras mutations (Kazuhisa *et al.,* 2004). Future studies are required to find whether this mutual exclusiveness of mutations of ß-catenin and K-ras is also present in AOM/DSS treated mice. In an interesting in vitro examination, K-ras oncogenic signaling activation in intestinal epithelial cells promoted Wnt/ß-catenin signaling via LRP phosphorylation (Lemieux *et al.,* 2015), which illustrated Wnt/ß-catenin and K-ras signaling cross talk. Thus, K-ras mutations might have also activated Wnt signaling by LRP phosphorylation in cases of only K-ras mutations by AOM/DSS.

Further studies in AOM/DSS treated mice are needed to identify mutations in K-ras and ß-catenin, which may reveal whether these mutations are indeed mutually exclusive like in human patients, and for the validation of whether KRAS signaling is also implicated in Wnt/ß-catenin pathway activation in chemically induced colon tumors.

Notably, Pygo2 was also overexpressed in the nuclei of control tumor cells. To the best of our knowledge, this was the first time to demonstrate the possible relevance of Pygo2 overexpression in the chemically induced model for colon cancer. Pygo2 was totally downregulated in Pygo2 deficient tumors, which indicated the efficient recombination of Pygo2 deletion in intestinal epithelial cells. We have previously correlated Pygo2 expression with early stages of colon cancer in human patients (Brembeck *et al.,* 2011).

In our chemically induced colon tumors, BCL9-2, a proto-oncogene, was moderately elevated, in contrast to our previous findings of BCL9-2 overexpression in $APC^{Min/+}$ mice and particular correlation of BCL9-2 overexpression to the degree of colorectal cancer advancement in human patients. In accordance with our study, a recent clinical examination also linked BCL9/BCL9-2 expression to stemness and overall survival of colon cancer patients (Andreas *et al.,* 2015). BCL9-2 overexpression was shown to induce invasiveness in tumors of $APC^{Min/+}$ mice (Brembeck *et al.,* 2011). Similarly, in our present study also colon tumor local invasion in chemically induced mice may be partly linked to its moderate BCL9-2 expression. In fact, Michel Aguet group showed that intestinal specific compound knockout of $BCL9^{-/-}$/$BCL9\text{-}2^{-/-}$ in AOM/DSS treated mice caused significant tumor size reduction compared to control littermates. In addition, they also demonstrated downregulation of a sub-set of Wnt target genes in BCL9/BCL9-2 mutants. Of note, identical to Pygo2 conditional deletion from our study (Brembeck and Schelp, unpublished), BCL9/BCL9-2 conditional knockout also did not affect intestinal epithelial homeostasis. (Deka *et al.,* 2010). Altogether, these data confirmed that although Wnt/ß-catenin signaling co-factors

including BCL9/BCL9-2 and Pygo2 were redundant for normal intestinal epithelial regeneration, they apparently dictated colon tumorigenicity in AOM/DSS induced human CRC model via controlling Wnt target gene expression.

One reason that Pygo2 knockout was not sufficient to inhibit colon tumor formation may be due to AOM induced additional genetic mutations in other signaling pathways, such as K-Ras and TGF-ß. Previous investigations provided evidence that different point mutations in a single gene and mutations in different Wnt/ß-catenin signaling components exerted different degrees of Wnt signaling gene output. In particular, different APC mutations were shown to exhibit varying predisposition to tumorigenesis because of different dosage of ß-catenin signaling (Gaspar *et al*., 2004). Similarly, AOM treatment may have driven varying degrees of Wnt signaling in each of mice because of random mutations in APC or ß-catenin or other unknown mutations in genes associated with Wnt signaling pathway, although treated with equal AOM concentration. This was clearly illustrated by dissimilar fold increase of each Wnt target gene examined, which might be reflecting Wnt signaling dosage effect in both Pygo2 deficient and wild type mice.

Second reason may be unknown additional functions of Pygo2 in different signaling pathways during tumor development. So far the role of Pygo2 role in Wnt signaling was well established (Thompson *et al.,* 2002). Additionally, Pygo2 was shown to exert a Wnt independent role as an epigenetic modifier in a Wnt- Notch cross-talk (Gu *et al.,* 2009). Therefore, in our model Pygo2 may be also involved in other signaling pathways that are needed for the tumor formation or may also activate other genes by direct or indirect interaction. Therefore, further studies which focus on Pygo2 role for the regulation of Wnt dependent and independent genes may disclose new insights. For instance, this may be achieved by global gene expression analysis in tumors or in human colorectal cancer cell lines in the presence and absence of Pygo2 expression.

We also investigated the role of Pygo2 in the context of intestinal stem cells driven epithelial tissue regeneration following DSS treatment (Perše *et al.,* 2012; Puneet *et al.,* 2014). However, we did not find any change between Pygo2 knockout and control mice in their regeneration capacity after DSS induced epithelial tissue damage. Initially we used 5% (w/v) of DSS in drinking water for five days of the treatment, which we found to be lethal for mice as all of them were dead within a week due to probably severe toxic effects of DSS. Therefore, later we optimized DSS concentration to 2.5% (w/v) for 5 days of treatment to induce intestinal colitis and sacrificed mice on day 14 and day 28. Though we did not

examine, previous examinations proved that DSS induced mucin depletion, epithelial degeneration, and necrosis lead to disappearance of epithelial cells within 7 days after DSS treatment (Perše *et al.,* 2012). We noticed complete epithelial regeneration as early as 14 days after starting treatment. In addition, no change after 28 days suggested that intestinal epithelium necrosis had not progressed to be chronic.
Mainly, proliferation was restricted to only crypt region in both Pygo2 knockout and control mice on day 14 and day 28, which illustrated the absence of any obvious cellular and molecular aberrations including in Wnt signaling pathway. This was in line with previous reports in which they studied DSS induced colitis in C57BL/6JOlaHsd mice and had observed epithelial regeneration on day 7 or beyond after 5 days of treatment (Perše *et al.,* 2012). Of note, Pygo2 knockout and control mice in present study were on a pure C57BL/6 background. This study suggested that Pygo2 may not be required for the intestinal epithelial regeneration following DSS induced epithelial tissue damage. This was in fact in line with Pygo2 functional redundancy in normal intestinal epithelial homeostasis as well since Pygo2 loss did not affect epithelium regeneration (Brembeck and Schelp, unpublished). Overall, these data emphasized that Pygo2 was not necessary for epithelial regeneration after DSS induced tissue damage.

5.2). Pygo2 conditional deletion rescues intestinal hyperproliferation induced by stabilized ß-catenin, but not by APC truncation

Although Wnt co-factors including Pygo2, BCL9 and BCL9-2 were apparently redundant for the normal homeostasis (Deka *et al.,* 2010; Schelp and Brembeck unpublished), we and other groups have demonstrated that knockdown of these proteins in colon cancer cells reduced Wnt reporter activity as well as the expression of a subset of canonical Wnt target genes (Brembeck *et al.,* 2011; Thompson *et al.,* 2002). Furthermore, BCL9/BCL9-2 proteins have been implicated in intestinal tumorigenesis (Brembeck *et al.,* 2011, Deka *et al.,* 2010; Thompson *et al.,* 2002), indicating that although nuclear co-factors of the BCL,9 and Pygo families may be redundant for physiological levels of Wnt/β-catenin signaling in the normal intestinal epithelium, they might contribute to aberrant pathway activation in cancer. However, an in vivo evidence to demonstrate the role of Pygo2 during the intestinal tumorigenesis in appropriate genetic mouse models was lacking. From our examinations presented here, we found that conditional deletion of Pygo2, in mice with stabilized ß-catenin (GOF) due to conditional genetic disruption of a single ß-catenin allele, was sufficient to completely rescue acute hyperproliferation in intestinal epithelium by re-establishing the normal crypt homeostasis and tightly regulated stem cell differentiation. On the contrary, Pygo2 deletion did not rescue the intestinal hyperproliferation of APC homozygous or heterozygous mutants (LOF).

Throughout our study all ß-catenin GOF (Harada et al., 1999) and APC LOF (Robanus-Maandag *et al.,* 2010) mice were crossed with VillinCreERT2 mice (Marjou *et al.,* 2004) for the spatiotemporal somatic conditional genetic recombination. With this we achieved recombination of genes in almost all epithelial cells throughout small and large intestine since Villin has been shown to be expressed only in intestinal epithelial cells (Robine *et al.,* 1997). This induced the genetic recombination of APC, ß-catenin and Pygo2 in all epithelial cells, resulting in the induction of Wnt signaling pathway. In all colon cancer mouse mutants the type of Cre-expressing mice has been proved to play a crucial role that decided location of tumors as well as overall intestinal phenotype. For instance, FabplCre; APC (15lox/+) mice developed tumors primarily in large intestine due to the different source of Cre enzyme (Robanus-Maandag *et al.,* 2010), which was expressed under control of the fatty acid binding protein gene promoter (Saam JR *et al.* 1999). Another Cre source was AhCre, here Cre expression was under the control of P-450 cytochrome, and it was inducible with ß-naphthoflavone (Ireland *et al.,* 2004). Another Lgr5-EGFP-CreERT2 model was engineered by Hans Clevers group through which lineage tracing experiments, and stem cell specific genetic ablation became possible (Barker *et al.,* 2007). In addition, in case of germ line or inducible mutations, the type of mutation in APC gene has also resulted in different intestinal phenotypes. Embryonic lethality in all homozygous mice suggested the importance of APC wild type protein during the embryonic development (Ishikawa *et al.,* 2003; Oshima *et al.,* 1995). $APC^{Min/+}$ mice developed approximately 30 adenomas predominantly in small intestine, however, homozygosity in Min mice caused embryonic lethality (Moser *et al.,* 1990; Moser *et al.,* 1995). Other well studied APC models were $Apc^{\Delta 716/+}$ and the $Apc^{1638N/+}$ mice that showed on average 300 small intestinal tumors per mouse, and 10 intestinal adenomas throughout the intestine, respectively (Fodde *et al.,* 1994; Oshima *et al.,* 1995).

In our current experiments after induction of Wnt signaling pathway, tissue sections were first analyzed by hematoxylin and eosin staining. This had illustrated a massively dysplastic enlarged crypt-villus axes in APC LOF homozygous mice. In contrast enlargement of crypt-villus axes in ß-catenin GOF mice was less pronounced. Whereas BrdU positive cells were only in crypts of controls, they were expressed by all hyperproliferative cells in both APC LOF and ß-catenin GOF mice. Overall acute hyperproliferation was identically reflected in survival periods of APC LOF and ß-catenin GOF mice: APC LOF mice lived shorter due to relatively high degree of Wnt signaling induced highly acute hyperproliferation whereas ß-catenin GOF mice longer. Indeed, previous experiments from Sansom OJ et al reported that AhCre; $Apc^{580s/580s}$ mice lives only until 5 days maximum after treatment with β-naphthoflavone (Sansom *et al.,* 2004). These mice had severe intestinal phenotype due to the hyper-activation of Wnt signaling pathway as

illustrated by BrdU, ß-catenin stainings, which confirmed the indispensable role of APC as tumor suppressor in epithelium of the gut. In line with this, in our current study deletion of two functional alleles in APC LOF mice also caused a severe intestinal hyperproliferation with overexpressed ß-catenin and concomitant BrdU positive cells. Consequently, all mice were terminally ill and dead within 8 days. These experiments evidently demonstrated indispensability of APC tumor suppressor function to tightly control Wnt signaling pathway in gut homeostasis. Simultaneously, induction of Wnt signaling by oncogenic mutations in ß-catenin GOF mice resulted in intestinal hyperproliferation, although it was less pronounced compared to APC LOF. In accordance with previous reports, we also observed stabilized and nuclear ß-catenin in ß-catenin GOF mutants, an indicative of active Wnt signaling (Harada *et al.,* 1999). In fact, previous studies also reported intestinal hyperproliferation induced by ß-catenin gain of function mutations (Harada *et al*., 1999; Romagnolo *et al.,* 1999).

The aim of current study was to investigate in vivo role of Pygo2 in both mouse mutants. Therefore, both tumor models were analyzed with Pygo2 heterozygous and homozygous knockout background. Most importantly, Pygo2 deletion was able to completely reverse hyperproliferation in the presence of ß-catenin gain of function mutations in mice intestine. To the best our knowledge, these results showed for the first time an in vivo evidence for the pivotal function of Pygo2 in controlling intestinal hyperproliferation induced by ß-catenin mutations. Afterwards, we analyzed Pygo2 role during intestinal adenoma formation induced by APC single allele conditional truncation, which showed APCMin like phenotype (Robanus-Maandag *et al.,* 2010). Although we did not examine, these mice were predicted to exhibit also loss of remaining functional allele, loss of heterozygosity (LOH), similar to APC Min mice and FAP patients in long term (Luongo *et al*., 1994; Ichii *et al.,* 1992). In fact, germ line induced $Apc^{\Delta 15/+}$ truncated engineered mouse mutant revealed a Min- like phenotype, which were also used in our study (Robanus-Maandag *et al.,* 2010). In agreement with these investigations, $APC^{15lox/+}$ heterozygous mice also showed multiple adenomas similar to Min like phenotype, which had upregulated nuclear and cytoplasmatic ß-catenin with overexpression of nuclear Pygo2. In addition, we also found BCL9-2 overexpression in all adenomatous tissue. Most importantly, deletion of Pygo2 did not rescue the adenoma formation. Consistent with APC homozygous mice, this indicated that APC loss of function due to even single allele truncation deletion resulted in deregulated Wnt signaling and unlike in ß-catenin GOF mutants Pygo2 deletion was not sufficient to re-establish normal levels of Wnt signaling thereby failed to reconstitute normal crypt-villus structure.

Given that ß-catenin GOF driven hyper activation of Wnt signaling was Pygo2 dependent mice, we hypothesized that Pygo2 might be able to downregulate the expression of Wnt target genes in stabilized ß-catenin mutants thereby restructuring the disturbed crypt-villus morphology.

5.3). Pygo2 is redundant for the maintenance of normal intestinal epithelial homeostasis

Our previous immunohistochemical analysis suggested that Pygo2 was functionally redundant for normal intestinal epithelial homeostasis (Schelp and Brembeck unpublished data) since intestinal epithelium was able to regenerate after Pygo2 loss as well. Therefore, in the current study we further analyzed the effect of Pygo2 conditional deletion on the Wnt transcriptome level. In fact, we found that the complete loss of Pygo2 did not affect the conventional Wnt target genes in the normal mouse intestine, including the expression of stem cell markers like Lgr5 and Ascl2 (Barker *et al.,* 2007; van der Flier *et al.,* 2009), suggesting that intestinal stem and progenitor cells were totally unaffected by ablation of Pygo2. Given the relevance of Wnt signaling in the epithelial regeneration of intestine, this might be the reason why the epithelial homeostasis in Pygo2 deficient mice was unaffected. This was in accordance with a previous report that ablation of Pygo2 expression in mice caused defects in brain, eyes, hair follicles, and lung but no abnormality was observed in embryonic intestine (Li *et al.,* 2007). In addition, conditional deletion of both BCL/BCL9-2 co-factors was also shown to be redundant in mice intestine (Deka *et al.,* 2010).

5.4). Expression of Pygo2 and BCL9 co-factors during intestinal tumorigenesis in vivo and human colon cancer cells

Despite their redundancy in normal epithelial cells maintenance in the intestine, Pygo2 and BCL9 co-factors were implicated in mice intestinal tumorigenesis and colon cancer cell lines in our previous examinations (Brembeck *et al.,* 2011).

Therefore, in our current investigation the expression of these co-factors were also studied in the genetic ß-catenin GOF and APC LOF tumorigenesis models and chemically induced tumor mouse models. In fact, we found strong Pygo2 up-regulation in the tumor cells of chemically induced tumors and conditional mouse mutants suggesting a potential role in tumor formation. Nevertheless, Pygo2 RNA was mostly unaltered, indicating the role of stabilization of Pygo2 protein in deregulated Wnt signaling conditions.

Future studies addressing multifactorial transcription and protein stabilization mechanistic role that might contribute to Pygo2 up-regulation could reveal new insights.

BCL9 protein was not induced in chemically induced tumor and in conditional mutants. However, BCL9-2 was differentially expressed across these three models for the intestinal tumor induction: BCL9-2 was only slightly induced in chemically induced tumors and unaffected by Pygo2 loss whereas it was not present in the hyperproliferative regions of the conditional mutants. We have reported in our recent studies that Pygo2, BCL9 and BCL9-2 are not Wnt target genes (Brembeck *et al.,* 2011). This was however in contrast to findings of the Bienz group, in which they stated that BCL9 and BCL9-2 may represent Wnt inducible genes in HEK293 cells and colon cancer cell lines (de, la, Worm, J. & Bienz, M *et al.,* 2008). In disagreement with Bienz group, we found in our current study again that BCL9-2 was strongly negatively regulated by active Wnt signaling in hyperproliferative regions of intestinal epithelium in both conditional models. In addition, BCL9-2 was only expressed in villi in normal epithelium of intestine. This might be due to the suppression of BCL9-2 expression by active ß-catenin signaling in crypt region. We in fact showed in SW480 colon cancer cells, which harbor APC mutations, that ß-catenin negatively regulated the expression of BCL9-2 (Brembeck *et al.,* 2011). In the current study we also analyzed the ß-catenin dependent regulation of BCL9-2 in another human colon cancer cell lines, DLD1, harboring APC mutations. Again, in this cell line too ß-catenin negatively regulated the expression of BCL9-2. Therefore, we speculated that the absence of BCL9-2 in early hyperproliferative regions in both conditional mutants might be due to the active ß-catenin signaling in the genetically recombined cells in hyperproliferative region (Brembeck *et al.,* 2011). Active Wnt/ß-catenin signaling in normal crypts and in transformed epithelial cells in conditional mutants might trigger BCL9-2 promoter methylation thereby silencing BCL9-2 expression. Future investigations in this direction probably reveal further details. In contrast, in advance stage tumors of chemically induced mouse models and in adenomatous tissue of APC heterozygous mice, BCL9-2 was highly overexpressed. This was supported by previous reports in which higher BCL9-2 expression was found in high-grade neoplasia and invasive carcinomas (Brembeck *et al.,* 2011; Adachi *et al.,* 2004; Sakamoto *et al.,* 2006). Even in noninvasive breast carcinomas (Ductal carcinoma in situ) BC9-2 expression was associated with the tumor grade, HER-2, c-myc and p53 and interestingly BCL9-2 expression was not related to ß-catenin expression pattern (Toya *et al.,* 2007). Chromosomal rearrangements might have induced translocation of BCL9-2 gene and oncogenic overexpression of BCL9-2, identical to BCL9 in hematopoietic malignancies, (Willis *et al*., 1998). We indeed showed, when $APC^{Min/+}$ crossed with BCL9-2 overexpressing mice (K-19-BCL9-2), that BCL9-2 promoted the intestinal cancer progression (Brembeck *et al.,* 2011), suggesting that overexpression of

BCL9-2 synergizes tumor development with accompanying Wnt deregulating mutations. However, in the future further examinations are required to analyze underlying molecular mechanism for the overexpression of Pygo2 and BCL9 co-factors in colorectal cancer.

5.5). Pygo2 knockout alters the degree of Wnt signaling output in chemically induced tumors, APC LOF and ß-catenin GOF mice

In order to obtain further insight to support our hypothesis that Pygo2 deletion directed the downregulation of overexpressed Wnt signaling target genes and of other known tumor driver genes in ß-catenin GOF mice in contrast to APC LOF mice, we analyzed mRNA and protein expression in chemically induced tumors and in our two genetic tumor models.

Axin2, which interacts with β-catenin and GSK3β (Behrens *et al.,* 1998), is a negative feedback regulator of Wnt signaling and found to be transcriptionally activated by ß-catenin-Lef1/Tcf4 complex (Leung *et al.,* 2002; Jho *et al.,* 2002). Axin2 is in fact considered to be the best candidate gene that represents Wnt signaling activity in vertebrates (Jho *et al.,* 2002; Weidinger *et al.,* 2005). Therefore, we investigated the expression of Axin2 in all mutants. Axin2 transcription was remarkably increased in all conditional and chemically induced mutants. In fact, previous studies also reported elevated levels of Axin2 in colorectal cancer patients (Yan *et al*., 2001; Lustig *et al.,* 2002). In another study of a large cohort of colon cancer patients, Axin2 was upregulated in tumor cells with concomitant overexpression of ß-catenin, although it did not have any prognostic value (Schaal *et al.,* 2013). Hence, Axin2 overexpression may also have contributed to hyperproliferation in conditional mutants, tumor initiation and progression in chemically induced mice. However, in our study, the complete downregulation in ß-catenin GOF mutants and partial downregulation in APC LOF mice and chemically induced tumors indicated the dependency of Axin2 transcription on Pygo2. Notably, Axin2 was sensitive even to the deletion of a single Pygo2 allele in our conditional mutants. This may be linked to the Wnt signaling dosage effect controlled totally or partially by Pygo2, which in turn affected Axin2 expression.

Next, expression of Lef1, Tcf1 andTcf4 in our tumor models was analyzed. DNA binding Lef/Tcf transcription factors complex in the nucleus with ß-catenin by replacing Groucho/Tcf3 (Daniels *et al.,* 2005; Solberg *et al.,* 2012), and initiates Wnt target gene expression (Behrens *et al.,* 1996; Molenaar *et al.,* 1996). Even for the normal development of intestine, these transcription factors were important

because Tcf4 mutations resulted in depleted crypt compartments in intestinal epithelium, suggesting Tcf4 mediated Wnt signaling was needed for the crypt homeostasis (Korinek *et al.,* 1998). In contrast Lef1 is not expressed by epithelial cells in normal intestine (Lau, de & Clevers, 2001; van Genderen *et al.,* 1994). This might suggest the redundancy of Lef1 expression for the continual renewal of normal intestinal epithelium and thus ß-catenin-Tcf4 transcriptional complex may be sufficient. In contrast to normal intestinal epithelium, Lef1 and Tcf4 were implicated in colon carcinogenesis, suggesting their role during the tumorigenesis (Hovanes *et al.,* 2001; Xie *et al.,* 2012). In another experiment, knockdown of Lef1 showed tumor inhibitory effects in vitro and in tumor xenograft models (Wang *et al.,* 2013).

Therefore, in our current study Lef1 and Tcf4 were also evaluated across all conditional mutants and chemically induced tumor models. Indeed, Lef1 and Tcf4 were highly upregulated in all mutants. Previous reports from Angus-Hill suggested that Tcf4 was expressed in crypts and villi of intestines with higher expression in villi (Angus-Hill *et al*., 2012), as we found in control mice. However, in transformed cells Tcf4 was relatively highly expressed throughout crypt-villus axes and in tumor cells of chemically induced mice. Lef1 was not expressed in the normal intestinal epithelium in agreement with previous reports although it was highly overexpressed in hyperproliferative regions of conditional mutants and chemically induced tumor models. These observations indicated elevated amounts of ß-catenin-Tcf1/Lef4 nuclear complex formation in genetically recombined cells of conditional mutants and tumor cells of chemically induced mice and might be important for the induction of Wnt target genes.

Remarkably, Tcf4 was downregulated even to lower levels compared to controls in all Pygo2 knockout mice. A similar phenomenon was also observed in single Pygo2 conditional knockout mice. However, Tcf4 moderate downregulation by Pygo2 loss may be of no relevance since it did not result in any intestinal phenotype that affected epithelial homeostasis or regeneration. In conclusion, Tcf4 normal expression in general might be dependent on Pygo2 expression alone. Therefore, we cannot also rule out the possibility of Pygo2 mediated Tcf4 transcriptional activation. Hence, studies in this direction may provide new insights regarding the regulation of Tcf4 by Pygo2.

In contrast to Lef1 and Tcf4 expression in our examinations, immunohistochemical analysis from colon cancer patients suggested highly heterogeneous pattern of Tcf4 and Lef1 expression, and reported different prognostic values for Lef1 and Tcf4 with only Lef1 being a positive prognostic factor (Kriegl *et al*., 2010). In an array of total 214 colon cancer patient specimens, they found LEF-1 was expressed in 56 (26%) and TCF4 in 99 (46%) of colon carcinomas (Kriegl *et al*., 2010). In another study Lef1

overexpression was also found to correlate with poor survival of colon cancer patients (Wang *et al*., 2013). These data suggested a correlation between tumor pathology in human patients and tumor mouse models. Nevertheless, this might indicate a limitation of mouse models in recapitulating intestinal tumorigenesis in humans. In human patients advanced tumors acquired genetic heterogeneity and invasiveness due to multiple concurrent genetic aberrations. In contrast, single gene mutations in mouse models such as APC or ß-catenin recapitulate one specific step of tumorigenesis.

Next another Wnt target gene, Tcf1, was also analyzed. Tcf1 was described to be activated by Wnt signaling pathway using Tcf1 knockout mice and found it as a possible feedback repressor of Wnt target genes (Roose *et al*., 1999). In addition, they showed that Tcf1 mutant mice also developed adenomas in gut and mammary glands. Even though Tcf1 was vastly expressed in T-lymphocytes in adult cells (Van de Wetering *et al*., 1996), they were also shown to be expressed by colorectal cancer cell. In addition, Tcf1 overexpression was in relation to metastatic nature of cells (Mayer *et al*., 1997). Whereas Tcf4 (Full length) expressed only one isoform that can bind to ß-catenin and can induce Wnt signaling (Najdi *et al*., 2009), Tcf1 gene expressed different isoforms with alternative promoters. The first promoter region encodes full length Tcf1 with ß-catenin binding domain. A second promoter encodes for a truncated version that does not contain ß-catenin binding domain and is reported to be a suppressor of Wnt signaling (Van de Wetering *et al*., 1996). Further, in a study to reveal the role of Tcf1 isoforms showed that in fact dominant negative forms of Tcf1 in colon cancer cells was corrupted, compared to normal colon cells: whereas dominant negative Tcf1 was equally distributed between cytoplasm and nucleus in normal cells, it was downregulated in nucleus of colon cancer cells with concurrent nuclear overexpression of full length Tcf1 (Najdi *et al*., 2009). Therefore, in our current study we also intended to investigate total levels of truncated and full length forms of Tcf1 following Wnt signaling activation, compared to controls. However, we were not able to successfully evaluate dominant negative TCf1 by measuring total endogenous levels compared to full length Tcf1 since we did not observe any differences using multiple different primers in our qRT analyses. Despite this we observed elevated levels of Tcf1 full length protein and mRNA expression in all conditional and chemically induced mouse mutants, and this was indeed highly nuclear in proliferating cells with active Wnt signaling (Najdi *et al*., 2009). Since Tcf1 full length protein with its ß-catenin binding domain interacts with ß-catenin to promote Wnt signaling (Archbold *et al*., 2012; Arce *et al*., 2006; Chatterjee *et al*., 2015), overexpression of Tcf1 full length protein indicated that Tcf1 might have further enhanced expression target genes together with Tcf4 and Lef1. Interestingly, RNA levels of full length Tcf1 was still upregulated in Pygo2 knockout conditional mutants, whereas TCF1 protein was totally suppressed. This may suggest possible alterations in

posttranslational protein stabilization after deleting Pygo2 resulting in downregulation of Tcf1. Thus Pygo2 may have a direct or indirect role in protein stabilization. However, examinations are required to confirm the role of Pygo2 on regulation of Tcf1 or other protein stabilization.

Further intestinal stem cell markers were examined including Lgr5 and Ascl2. Lgr5 was first characterized as a Wnt target gene that marks adult stem cells in many organs, which also included intestinal epithelium (Barker *et al*., 2009, Barker *et al*., 2010 and Jaks *et al*., 2008). In addition, upregulation of Lgr5 was reported in many cancers including hepatocellular carcinoma (Fukuma et al., 2013; Yamamoto et al., 2003), colon and ovarian cancers (Uchida *et al*., 2010; McClanahan *et al*., 2006), basal cell carcinomas (Tanese *et al*., 2008) and gastric cancer (Yamanoi et al., 2013), indicating its possible role during tumor growth.

Similarly, in our mouse models Lgr5 was also upregulated in all conditional mutants and chemically induced tumors, which confirmed it as a Wnt target. This may indicate an increased number of Lgr5 expressing stem like cells as a result of aberrant Wnt signaling activation. In a recent study Lgr5 was shown to be highly overexpressed in colon cancer patient tissues and associated with poor patient survival as well as Lgr5 knockdown in colon cancer cell lines (HT-29) induced growth arrest (Chen X *et al*., 2014; Ann-Marie Baker *et al*., 2014; Liu *et al*., 2014). These findings suggested that Lgr5, apart from being a stem cell marker, was also indispensable for tumor growth. However, how Lgr5 alone could drive tumor multiplicity is unknown or entire stemness and existence of stem cells may be dependent on Lgr5 expression. Lgr5 was demonstrated to be a receptor for soluble R-spondins, through which it potentiates Wnt signaling (de Lau *et al*., 2011; Carmon *et al*., 2011; Ruffner *et al*., 2012). Hence, Lgr5 overexpression in our models might have also implied in R-spondins mediated Wnt signaling potentiation. Remarkably, Lgr5 was totally downregulated to control levels in ß-catenin GOF mice after Pygo2 conditional deletion. Conversely, Pygo2 loss only partially suppressed in APC LOF and chemically induced tumors. These data indicated a possible dependency of Lgr5 expression on Pygo2 either entirely or partially. Thus, Pygo2 may in part control tumor cell regeneration in conditional mutants and chemically induced tumors by suppressing Lgr5 expression. In contrast, only Pygo2 conditional deletion in intestine did not affected Lgr5 expression. This may suggest that Pygo2 may have gained the function of controlling Wnt target genes only in the context of deregulated Wnt signaling. In conclusion, our comprehensive study was first to reveal the role for Pygo2 in strictly governing the stem like cell population in intestinal tumorigenesis, in particular conjunction with mutated ß-catenin. These data are supported also by recent other investigations in which Pygo2 was implicated in activation of stem cells in hair follicle and skin

hyperplasia reference however after Pygo2 deletion deregulated Wnt/ß-catenin signaling did not appropriately induce stem cell activation and hair follicle regeneration (Peng Sun *et al*., 2014). In another study in U251 human glioblastomas Pygo2 knockdown inhibited the expression of the neural stem cell marker genes CD133, Nestin, and Sox-2(Li *et al*., 2015).

Next we analyzed the transcription factor Ascl2 (Achaete-scute like 2). Ascl2 was reported as Wnt target gene that was exclusively expressed in Lgr5 positive intestinal stem cells and Ascl2 expression was indispensable for the fate of Lgr5 positive cells (van der Flier *et al*., 2009). In addition, Ascl2 is also overexpressed in intestinal neoplasia (A M Jubb *et al*., 2006). Ascl2 overexpression was also associated with squamous cell lung carcinoma, and shown to be a potential prognostic marker (Hu *et al*., 2015). In addition, silencing of Ascl2 in HT-29 and LS174T colon cancer cell lines increased mesenchymal to epithelial cell conversion by inhibiting ZEB1 expression (Tian *et al*., 2014). In accordance with these studies, we also found Ascl2 overexpression on the RNA level in all mouse mutants and chemically induced tumors. However complete deletion of Pygo2 did not entirely downregulate Ascl2 to control levels. This was apparent in both APC LOF and ß-catenin GOF mice. We did not perform Ascl2 protein expression analysis on mutant neoplastic tissues and colon tumors to find a correlation to mRNA since our immunohistochemical study was limited by the lack of specific antibodies. However, Ascl2 mRNA elevation may suggest increased amount of Ascl2 protein. A study by the group of Hans Clevers showed that Ascl2 directly binds to Lgr5 promoter region to activate Lgr5 gene expression. This illustrated that Lgr5 directed “stemness” may rely on Ascl2 expression (van der Flier *et al*., 2009). Given Ascl2 indispensability for Lgr5 positive stem cells, it may be also implicated in other malignancies with activated Wnt signaling. In our current study we also found elevated Ascl2 in all genetically recombined cells in conditional mutants and colon tumors may suggest that may have attained stem cell like features. Whether Ascl2, being transcription factor, is able to activate any genes within stem cells that required for stem cells behavior is unknown. However, Ascl2 downregulation after Pygo2 loss suggested that although Ascl2 transcription was induced by ß-catenin Lef/Tcf nuclear complex, Pygo2 was necessary for Ascl2 expression. We speculate that Pygo2 loss may have reversed hyperproliferation in ß-catenin GOF mice by affecting Ascl2 transcripts, which in turn reduced Lgr5 expressing stem like cells.

Aberration in stem cell differentiation into distinct cell lineages following dysregulated Wnt pathway in our study was assessed by analyses of Lysozyme and Dclk1. Lysozyme is a paneth cell secreting anti-microbial agent (Peeters & Vantrappen, 1975; Bevins *et al*., 2011), which is also deregulated in tumor cells (Rubio *et al*., 2009; Rubio *et al*., 2004; Rubio *et al*., 1996; Reitamo *et al*., 1981; Fahlgren *et al*., 2003; Donghai Wang *et al*., 2010). Similarly, we also found aberrant expression of Lysozyme in

hyperproliferative regions in all mouse mutants. This suggested differentiation defects of intestinal stem cells in mutant mice due to over activated Wnt signaling. In fact, it was shown that Paneth cells express EGF, TGF-α, Wnt3 and Notch ligand Dll4 which are important for stem-cell maintenance, suggesting that that Paneth cells represent the niche cells for Lgr5 stem cells (Sato, T *et al*., 2010). Pygo2 conditional deletion in our current study severely restricted lysozyme expressing cells to crypts in ß-catenin GOF mice. This suggested that Pygo2 loss may also inhibit paneth cells constituting into a niche of Lgr5 stem cells. Next, we analyzed the expression of Dclk1, which is expressed in tuft cells of normal intestine, and a putative cancer stem cell marker (Nakanishi *et al*., 2012). In our study, Dclk1 was completely lost in APC homozygous mutants and partially in chemically induced colon tumors, whereas it was unchanged in ß-catenin GOF mice. The reason for the complete downregulation of Dclk1 in APC homozygous mutant mice is unknown. This might reflect the severe differentiation defects due to acute APC loss. In a recent study, Dclk1 expression was shown to be downregulated due to hyper-methylation of the promoter in colorectal cancer patients, compared to normal intestinal mucosa (Vedeld *et al*., 2014). Similarly, hyper-methylation might have caused Dclk1 expression acute loss in APC LOF mice, which also indicated a possible epigenetic alteration during early acute hyperproliferation. Although Wnt pathway was activated in ß-catenin GOF mutants, the dosage of Wnt signaling gene output may be not sufficient to transform Dclk1 expression. Therefore, further studies are required to analyze if Dclk1 expression is dependent on dosage of Wnt signaling or is linked to APC loss of function independently. In contrast to our findings, Dclk1 complete ablation induced regression of adenomas in APC^{Min} mice and was suggested as a reliable marker for cancer stem cells (Nakanishi *et al*., 2012). Hence, further extensive analysis of Dclk1 expression in cancer cell lines in vitro and in human cancer patients is required to re-affirm Dclk1 as a cancer stem cell marker or its function in retaining stem cell characteristics. In conclusion, our present study suggested that Dclk1 deregulation may be dependent on Wnt signaling dosage and Pygo2 expression may not influence Dclk1 expression.

In addition, E-Cadherin expression was also tested. Previous studies from our lab indicated that Pygo2 knockdown in human cancer cell lines can modulate expression of E-cadherin and induces epithelial-mesenchymal transitions (Wiese and Brembeck, unpublished data). In fact, several studies indicated that ß-catenin nuclear translocation during Wnt signaling activation disturbed the E-Cadherin-ß-catenin complex that resulted in epithelial to mesenchymal transition (Vermeulen *et al*., 2010; Lamouille *et al*., 2014; Gonzalez *et al*., 2014). In addition, loss of E-cadherin was also associated with tumor growth and metastasis in colorectal cancer patients (Kim *et al*., 2016). However, we found no change of E-cadherin transcription and protein expression in all conditional mutants and colon tumors. Moreover, Pygo2

deletion did not change E-cadherin Pygo2 expression in vivo. This might suggest that ß-catenin nuclear translocation in all conditional mutants and chemically induced tumors may not have triggered E-cadherin deregulation. Thus, additional genetic aberrations likely are required for deregulated E-cadherin expression. Numerous studies revealed E-cadherin to be repressed by transcriptional silencing through many transcription factors including Snail and Slug, SIP1 and ZEB1, by directly binding to E-cadherin promoter (Peinado *et al.,* 2004; Cano *et al.,* 2000; Bolós *et al.,* 2003; Gregory *et al.,* 2008; Moreno-Bueno *et al.,* 2008). In addition, E-cadherin downregulation has also been linked to Hepatocyte growth factor (HGF) and Receptor tyrosine kinase (RTK) activation (Andl *et al.,* 2005; Han *et al.,* 2005).

Next we estimated levels of Cyclin D1. Cyclin D1, a known Wnt target gene, is crucial for cell proliferation during G1 phase of cell cycle (Sherr *et al*., 1996; Arber *et al*., 1997). Lef1 was described to directly bind to CyclinD1 promoter region and ß-catenin overexpression induced Cyclin D1 expression (Shtutman *et al*., 1999). ß-catenin was also shown to regulate Cyclin D1 expression in colon cancer cell lines (Osamu Tetsu *et al*., 1999; Brembeck *et al*., 2011). Moreover, Cyclin D1 inhibition also arrested tumorigenicity in colon cancer cells (Arber *et al*., 1997). Not surprisingly, CyclinD1 was also overexpressed in human colon cancer patients and proposed even as a prognostic marker (Bahnassy *et al*., 2004; Handa *et al*., 1999; Ioachim E *et al*., 2008; Nosho K *et al*., 2008). In accordance with these findings, in our conditional and chemically induced colon cancer models Cyclin D1 was also highly overexpressed. Especially, in APC LOF mutants Cyclin D1 overexpression was predominantly localized in the hyperproliferative region, and was more pronounced compared to ß-catenin GOF mutants. This may be linked to higher degree of Wnt signaling in APC LOF mice. In fact, previously also Cyclin D1 was shown to be deregulated in other colon cancer mouse mutants such as in APC 1638N and APC^{Min} mice (Shinozaki *et al*., 2003; Zhang T *et al*., 1997), which was also linked to increased cell proliferation activity. Over all, Cyclin D1 expression might be reflecting Wnt signaling dosage and negatively regulating survival of conditional mutants. In our study, Cyclin D1 overexpression was also seen in tumors sections from Pygo2 control and deficient chemically induced mice. In fact Cyclin D1 was previously also shown to be overexpressed in AOM treated mice (Wang *et al*., 1998). Most importantly, Cyclin D1 expression was totally independent of Pygo2 expression since no significant difference was observed between Pygo2 wild type, heterozygous and homozygous mice in APC LOF and ß-catenin GOF mutants as well as in Pygo2 control and deficient chemically induced tumors. On contrary to APC LOF mice, Cyclin D1 transcription was not induced in ß-catenin GOF mutant mice although Cyclin D1 protein was seen in hyperproliferative regions. This may be attributed to protein stabilization of Cyclin D1 in ß-catenin GOF mice by an unknown, yet to be characterized mechanism. Notably, Cyclin D1 was also highly nuclear in all genetically recombined cells in hyperproliferative regions

of conditional mutants, and in tumor cells of chemically induced mice. Numerous investigations indicated nuclear Cyclin D1 as a clear indicator and promoter of cancerous growth (Kim *et al*., 2009; Aggarwal *et al*., 2010; Baldin *et al*., 1993; Andrew B. Gladden *et al*., 2006). Altogether, Cyclin D1 elevation was indisputably an indicator of active Wnt signaling across all models examined. Importantly Pygo2 loss did not affect the expression of Cyclin D1 in mutant models, suggesting that Cyclin D1 may be a prominent Wnt target gene involved in cell proliferation that is independent of Pygo2.

The transcription factor Prox1, the orthologue of Drosophila Prospero, was characterized as an oncogene that was also a target of Wnt signaling (Ragusa *et al*., 2014). Prox1 overexpression in APC^{Min} mice enhanced tumor formation, while its loss inhibited tumor progression, indicating its key role in tumor promotion (Petrova *et al*., 2008). Hence, in our present examinations Prox1 was also investigated. We found that Prox1 was highly elevated in APC LOF and ß-catenin GOF mutants, and in chemically induced models. In contrast, Prox1 was not expressed in normal intestinal epithelial cells suggesting that it may not be implicated in the maintenance of normal intestinal epithelium. Thus, only an oncogenic Wnt signaling might be able to activate Prox1 expression. Prox1 was relatively highly overexpressed in ß-catenin mutants compared to APC LOF mutants. Surprisingly, Prox1 was totally downregulated in both conditional mutants following Pygo2 complete deletion, suggesting that h Prox1 expression was completely dependent on Pygo2 during oncogenic Wnt signaling activation. This is to our knowledge the first time to demonstrate Pygo2 importance for Prox1 expression in early intestinal hyperproliferation. Strikingly, APC LOF intestinal phenotype was not affected by Prox1 loss in Pygo2 knockout mice demonstrating that Prox1 might not have any functional importance during early intestinal hyperproliferation induced either by APC LOF or ß-catenin GOF. Therefore, Prox1 overexpression probably represents an effect of overactive Wnt signaling, but apparently had no functional role for early intestinal hyperproliferation. In contrast, Prox1 was highly overexpressed in Pygo2 control and deficient tumors of chemically induced mice however Pygo2 loss had not resulted in the downregulation of Prox1 in these tumors. This was a major difference we found between conditional mouse mutants and chemically induced tumors regarding Prox1. One explanation for this difference may be that our conditional APC LOF and ß-catenin GOF mouse models represent models for early proliferation, but chemically induced tumors present advanced tumor stages of colon cancer. Thus, Prox1 might have preferentially gained a specific role during tumor progression as it was shown in previous experiments (Petrova *et al*., 2008). Including Wnt signaling, any unknown mechanism for the upregulation of Prox1 in advanced tumors can also not be ruled out. Numerous studies proved that Prox1 expression correlated with advanced stages of metastatic colon cancer and was important for epithelial to mesenchymal

transition (Ragusa *et al*., 2014; Lu *et al*., 2012; Skog *et al*., 2011). Prox1 might play a key role for the local invasion into the submucosa as we observed in some tumors of chemically induced mouse models.

In summary, Prox1 was overexpressed by deregulated Wnt signaling pathway during early intestinal hyperproliferation and tumor development. Pygo2 differentially controlled the expression of Prox1 in different stages of cancer development. Importantly, Pygo2 completely controlled Prox1 expression during early hyperproliferation, but did not change Prox1 expression in advanced tumors.

After this, Sox9, another prominent Wnt regulated transcription factor, was studied. Sox9 is selectively expressed in highly proliferating crypts of intestinal epithelium (Blache *et al*., 2004). We also observed Sox9 only in crypts of normal intestinal epithelium. In addition, Sox9 also plays a key role in cancer development. Recent studies from several groups illustrated the importance of Sox9 expression in malignancies including colon cancer (Matheu *et al*., 2012; Huang *et al*., 2013; Lü *et al*., 2008). In agreement with these examinations, we also found highly overexpressed Sox9 throughout hyperproliferative regions of conditional mutants and in chemically induced tumors. Notably, in APC LOF conditional mutants Pygo2 loss completely failed to influence the transcription and protein levels of Sox9, indicating that Pygo2 expression might be redundant for Sox9 expression. Thus, Sox9 overexpression might have contributed to consistent intestinal phenotype in APC LOF mice. In ß-catenin GOF mice also Sox9 transcription was not affected by Pygo2 knockout. However, Sox9 protein was partially downregulated after Pygo2 loss but not completely to the control levels despite of the complete rescue of the intestinal hyperproliferation. Altogether, these data suggested that Sox9 regulation by Pygo2 may be dependent on Wnt dosage effect since Sox9 has been partially suppressed after Pygo2 loss only in ß-catenin GOF mice. In addition, Sox9 analysis on chemically induced tumors also suggested that Pygo2 loss did not affect the expression of Sox9 in colon tumors although Sox9 transcription was marginally downregulated in a subgroup of Pygo2 deficient chemically induced tumors. In conclusion, Sox9 overexpression illustrated its known role during early hyperproliferation in conditional mutants and tumor formation in chemically induced mice. However, Pygo2 might not be required for the expression of Sox9 during oncogenic Wnt signaling.

Finally, c-myc, a global gene amplifier, a reported essential Wnt target gene for the persistent maintenance of colorectal carcinoma was analyzed in our study (Dang *et al*., 2006; He *et al*., 1998). Numerous studies re-affirmed the overexpression of c-myc in many malignancies, including colorectal cancer (Rochlitz *et al*., 1996; Sikora *et al*., 1987; Erisman *et al*., 1988; Erisma *et al*., 1989; Stewart *et al*.,

1986). Indeed, in our experiments also c-myc was highly overexpressed in all conditional and chemically induced mutants. Remarkably, Pygo2 loss failed to influence c-myc overexpression in APC LOF and chemically induced tumors. In contrast, Pygo2 loss in ß-catenin GOF mutant mice completely downregulated c-myc that was indeed achieved by deletion of one single Pygo2 allele. This supported the crucial role of relatively higher degree of Wnt signaling due to complete dysfunction of APC in APC LOF mice that might have dictated Pygo2 limited ability in controlling c-myc. In fact, previous studies already demonstrated APC mutations in AOM treated mice (Maltzman *et al*., 1997; Møllersen *et al*., 2004). Hence, the failure of Pygo2 loss to downregulate c-myc in APC LOF mice and chemically induced mice may be attributed to APC mutations induced oncogenic Wnt signaling and possibly other Wnt independent functions of mutated APC (Aoki *et al*., 2007). The crucial role of c-myc in tumor initiation and promotion was demonstrated by c-myc deletion in APC truncated mutant mice in which c-myc loss completely rescued the intestinal phenotype (Sansom *et al*., 2007). In addition, c-myc deletion in $APC^{Min/+}$ mice also showed inhibition of intestinal polyposis by reducing angiogenesis (Yekkala *et al*., 2007; Ignatenko *et al*., 2006). In another study, c-myc, as global gene activator, was shown to bind to promoter regions of genes and induce transcription, which resulted in elevated transcription of each gene within the cell specific expression (Lin, C. Y. *et al.*, 2012). Similarly, c-myc expression might have induced intestinal epithelial specific transcription of numerous genes including those tested in our study, which are implicated in hyperproliferation. Since c-myc is crucial for intestinal tumorigenesis, the degree of c-myc overexpression may also influence the overall degree of target gene expression and therefore the severity of hyperproliferation. This was in fact apparent in our model since c-myc was relatively higher in APC LOF compared to ß-catenin GOF mice. This may in turn probably influence the severity of the intestinal phenotype and survival of mice after hyperproliferation induction.

In summary, c-myc, a highly crucial single regulator of intestinal tumorigenesis, was overexpressed by active Wnt signaling in our mouse models, which was independent of Pygo2 expression in the context of APC mutations. In contrast, in the context of mutated ß-catenin in ß-catenin GOF mice, Pygo2 loss is sufficient to downregulate c-myc expression which is presumably linked also to the rescue of the phenotype. Thus, c-myc expression under a certain Wnt signaling dosage might be reversed by Pygo2 loss.

These data along with previous findings suggested that c-myc is a key player for the induction and maintenance of colorectal carcinoma, which can be regulated by Pygo2 to a specific level. Hence, Pygo2

as regulator of c-myc is undoubtedly an attractive therapeutic target for cancer therapy in the context of mutated ß-catenin.

5.5.1). Downregulation of specific Wnt target genes in tumors from a sub-group of Pygo2 deficient mice: Pygo2 requirement for Wnt target gene expression may depend on tumor progression

After the confirmation of dysregulated Wnt signaling in colon tumors, we further investigated the transcription of prominent Wnt target genes in tumor cells (Lef1, Tcf4, c-myc, Cyclin D1, Prox1, Sox9, Lgr5, Ascl2 and Tcf1). In tumor sections of control and Pygo2 deficient mice all these proteins were highly upregulated suggesting Pygo2 knockout was not sufficient to completely downregulate these target genes to control level. RNA expression analyses confirmed upregulation of all these genes mentioned. Strikingly, downregulation of transcripts of Wnt target genes in tumors from a subgroup of Pygo2 deficient animals was observed. This suggested that during intestinal tumorigenesis induced by AOM treatment, Pygo2 complete deletion was potently able to suppress the upregulation of Wnt target genes in a sub group of Pygo2 deficient tumors, which in turn delayed overall tumor growth in these mice. Thus, Pygo2 deficiency might have contributed to possible delayed tumor progression. Overall, these data suggested that knockout of Pygo2 apparently cannot prevent tumor development, but may delay tumor progression by suppressing the transcription of specific Wnt target genes.

5.5.2). Tri/di methylation of H3K4 (Lysine4 of Histone3) is independent of Pygo2 expression, which is also not induced during intestinal tumorigenesis

Pygo2 was previously shown to be involved in chromatin remodeling as it bound to histone H3 tail methylated at lysine 4 (Gu *et al*., 2009). Therefore, we have also studied any possible relevance of Pygo2 association with tri/di methylated histone 3 at lysine4 during intestinal hyperproliferation by immunohistochemistry. Of note, previously Pygo2-tri/di-methylated lysine4 of histone3 interaction has been demonstrated by co-immunoprecipitation (Co-IP) studies (Gu *et al*., 2009; Cantù *et al*., 2013) therefore we assumed that present study may be insufficient to bring any firm conclusion because immunohistochemical examinations recognized only methylated lysine4 of histone3 and we did not directly show any interaction between Pygo2 and methylated histone3. Therefore, further Co-IP studies may be needed to re-affirm our examinations.

In fact, we observed the expression of tri/di methylated lysine4 of histone3 in all intestinal epithelial cells of control mice. However, it was not induced in intestinal hyperproliferative cells in APC LOF and ß-catenin GOF mice. Importantly, Pygo2 complete knockout in APC LOF and ß-catenin GOF mutants did not affect the expression of tri/di methylated lysine4 of histone3. In addition, it was also not induced in tumor sections of AOM treated mice regardless of Pygo2 expression. However, this was in contrast to previous findings where Pygo2 was stated to induce mammary progenitor cell expression via interacting with histone H3K4 methylation (Gu *et al*., 2009). In another study, Pygo2 was demonstrated to regulate histone gene expression and histone3 (K56) Lysine56 in mammary epithelial cells (Gu *et al*., 2012). This showed that Pygo2 may have different tissue specific functions. In agreement with present study, Pygo2 and H3K4me2/3 interaction was shown to be dispensable for mouse development and Wnt signaling mediated transcription (Cantù *et al*., 2013). On contrary to mouse experiments, Drosophila experiments showed that Pygo interaction with H3K4me via its PHD Zinc finger domain was important for wing development since defects in wings were found Pygo was knocked out (Fiedler *et al*., 2008). This might indicate Pygo/Pygo2 requirement for development and Wnt signaling has evolutionarily been selected. Together, these data indicated that methylation of histone3 at lysine4 was not induced during intestinal tumorigenesis, and it was not dependent on Pygo2 expression.

5.6). Regulation of target gene expression by Pygo2 in human colon cancer cell lines

In order to perform a comparative analysis with our in vivo results Wnt dependent target gene transcription was also investigated in human colon cancer cell lines. Here, DLD1 cells harboring APC mutations and HCT116 cells with ß-catenin mutation were analyzed after knockdown of Pygo2, compared to ß-catenin.

ß-catenin knockdown induced downregulation of several Wnt target genes including Sox9, c-myc and Axin2 suggesting these are Wnt inducible genes as we reported earlier in SW480 cells harboring APC mutation (Brembeck *et al*., 2011). However, Tcf4 was not regulated by ß-catenin knockdown in these two cell lines. This was in line with previous report that Tcf4 was shown to be heterogeneously upregulated in colorectal cancers but had no correlation with ß-catenin expression (Kriegl *et al*., 2010). This was intriguing since we found slightly elevated levels of Tcf4 transcription only in ß-catenin GOF mice but not in APC LOF mice although both had activated Wnt signaling. Conversely, we found highly nuclear Tcf4 protein in both mouse models. In addition, similar to in vivo models Tcf4 was regulated by Pygo2 knockdown also in both cancer cell lines tested. Differential Tcf4 regulation in human patients

compared to in vitro or in vivo models may imply Wnt independent regulation of Tcf4 expression. How Pygo2 may influence Tcf4 expression can possibly be linked to its chromatin modulating capacity (Valerie Horsley *et al*., 2009).

Cyclin D1 was another gene that was upregulated in current in vivo models. However, it was not regulated by ß-catenin knockdown in both cancer cell lines. In fact, Cyclin D1 expression and role was controversially discussed in previous studies since it was shown not to be a target of ß-catenin in APC LOF mice (Sansom et al., 2005). In contrast, Cyclin D1 heterozygous expression in APC^{Min} mice reduced tumorigenicity (Hulit *et al*., 2004). Hence, Cyclin D1 may be required for enhancing tumorigenesis. However, the different regulation of Cyclin D1 in current in vivo and in vitro studies suggested that translating results of in vivo examinations to human patients should be done with caution. Finally, whether Cyclin D1 is Wnt dependent or independent or maintained by various mechanisms is yet to be disclosed.

Axin2 regulation was also analyzed (Lustig *et al*., 2002). In agreement with our previous results, and present in vivo studies Axin2 was dependent on ß-catenin and Pygo2 expression in both colon cancer cells (Brembeck *et al*., 2011), indicating Axin2 was a prominent Wnt target gene. In contrast, Sox9 significant downregulation only in DLD1 after ß-catenin knockdown was unknown (Blache *et al*., 2004). However, Pygo2 knockdown downregulated Sox9 in both cell lines similar to that in in vivo models. Altogether, Pygo2 may be regulating Sox9 in colon cancer cell lines.

C-myc is a known Wnt target gene and can be downregulated by ß-catenin knockdown in cell lines (He TC *et al*., 1998; Brembeck *et al*., 2011). In accordance with this, we also found c-myc downregulation after ß-catenin knockdown in both cell lines tested here, more significantly in DLD1 cell lines. However, this was in contrast to in vivo c-myc regulation by Pygo2 because in APC LOF mice Pygo2 loss failed to downregulate c-myc whereas in cell lines, DLD1, with APC mutations pygo2 partially downregulated it. This different regulation may be attributed to c-myc expression and stability because c-myc deregulation can happen in multiple ways in in vivo during tumorigenesis, whereas in present in vivo models it was achieved only by Wnt activation during early hyperproliferation (Meyer *et al*., 2008).

In addition, previously a comprehensive analysis of colon cancer cell lines indicated variable growth in nude mice, suggesting that different colon cancer cell line has unique characteristics (Flatmark *et al*., 2004). In another study, different epigenetical and genetic changes were reported in different colon cancer cells (Ahmed *et al*., 2013). Therefore, unlike current in vivo models which have activated Wnt

signaling, in colon cancer cells several concurrent deregulated genetic and epigenetical events may cross talk and control each other. These complex anomalies in human cancer may contribute to differential Pygo2 regulation of Wnt target genes. Hence, animal models can be ideal to dissect genetic events mostly in a single pathway to examine colorectal cancer events.

5.7). Wnt signaling target gene output in ß-catenin GOF mutant mice may be just at the "right threshold" to be repressed by Pygo2

Given the total suppression of intestinal hyperproliferation in ß-catenin GOF mutant mice following Pygo2 conditional loss, the reason for Pygo2 inability to exert similar effects in APC LOF mice and chemically induced tumors was elusive.

Overall, the intestinal phenotype of APC LOF mice may be linked to two distinctive mechanisms. One was deregulation Wnt independent functions of APC in APC LOF homozygous and heterozygous mice; second was possible differential dosage of Wnt signaling gene output by APC LOF and ß-catenin GOF mutations.

Although ß-catenin gain of function or APC loss of function resulted in oncogenic Wnt signaling activation, ß-catenin and APC are different functionally. ß-catenin function is mainly linked to the role in cell-cell adhesion and in Wnt signaling (Valenta *et al*., 2012). In contrast, APC, apart from its tumor suppressor role, has diverse functions such as gene segregation, cell migration, apoptosis and differentiation (Kaplan *et al*., 2001; Näthke *et al*., 2004). Moreover, loss of APC drives the chromosomal instability as a result of mitotic and apoptotic defects (Fodde *et al*., 2001). Wild type APC assembles with Bub1 and BubR1 to direct the mitotic checkpoint machinery (Kaplan *et al*., 2001). In addition, APC is also involved in cytoskeleton regulation. Strictly controlled migration, division, and differentiation of intestinal epithelial cells are vastly dependent on full length APC to regulate cytoskeleton and limit the lifetime of intestinal epithelium. Therefore, APC loss of function in APC LOF mice might have derailed all these strictly controlled molecular and cellular events that rendered accumulation of epithelial cells (Hermiston *et al*., 1995). Consequently, in current APC LOF homozygous models loss of all these APC functions might have contributed to proportionately higher intestinal proliferation. Pygo2 ablation could not compensate these Wnt independent APC functions, which may be one reason for failed rescue of the APC LOF intestinal phenotype. On contrary, full length APC protein might have regulated cytoskeleton in intestinal epithelial cells of ß-catenin GOF mice that eventually influenced limited life time of epithelium. Thus,

APC wild type protein in the presence of ß-catenin mutations might have partly contributed to less severe intestinal phenotype induced by active Wnt signaling in ß-catenin GOF mice.

Indeed, a recent work had demonstrated the prominence of APC wild type protein, in which full length APC protein restoration in intestines of colorectal cancer murine models was shown to re-establish the normal crypt homeostasis by total regression of colon cancer even in the presence of K-ras and TP53 mutations (Dow *et al*., 2015). Despite no examinations were performed to investigate chromosomal instability and other APC Wnt independent functions in this study due to APC loss, it is conceivable that after APC wild type protein restoration all APC Wnt independent function might have also been restored.

Second and probably the most important was the effect of dosage of Wnt signaling gene output, which likely decided the potential of Pygo2 to suppress tumorigenicity. Because conditional mutations in APC and ß-catenin induced evidently differential levels of oncogenic Wnt signaling that was apparently represented in their intestinal phenotype and overall survival period. The main aim of this study was to examine Pygo2 potency to influence active Wnt signaling. We used two models to induce Wnt signaling: APC tumor suppressor conditional truncation and ß-catenin oncogenic activation mutation. In APC LOF mice, homozygous for APC truncation resulted in severe intestinal phenotype because of acute Wnt signaling induced by biallelic loss of 15 exon in APC gene, which expressed predominant portion of APC protein (Luis *et al*., 2011). Virtually with no APC functional protein expression in genetically transformed intestinal epithelial cells, ß-catenin was translocated to nucleus to increase Wnt signaling gene output (Robanus-Maandag EC *et al*., 2010). Concurrently, all additional Wnt independent functions of APC were also lost. Thus, dysregulation of all Wnt dependent and -independent functions of APC might have caused the massive intestinal proliferation. Hence, despite having an essential role during active Wnt signaling (Brembeck *et al*., 2011; Townsley *et al*., 2004), Pygo2 loss did not compensate the loss of APC protein and thereby hyper-activated Wnt signaling. This may result in upregulation of downstream Wnt target genes that cannot be influenced by Wnt co-factors such as Pygo2. In human colon cancers nonsense or frame shift mutations of APC mostly within the so called mutation cluster region lead to progressive intestinal tumorigenesis (Rowan *et al*., 2000). Hence, induction of rather severe loss of total APC was used in our study as an experimental model to analyze any possible role for Pygo2 in APC driven tumorigenesis. Biallelic loss of 15^{th} exon in our APC model resulted in severe APC truncation with no ß-catenin binding domains causing highly active Wnt signaling. In addition, epigenetic alterations such as promoter methylation might have also assisted to increase deregulation of prominent Wnt target genes

and even other unknown targets. Pygo2 loss was overall presumably not sufficient to rescue this severe intestinal hyperproliferation induced by acute loss of APC within few days.

In monoallelic loss of APC function induced adenoma formation in APC heterozygous mice that resemble APC^{Min}-like phenotype (Robanus-Maandag *et al*., 2010; Brembeck *et al*., 2011) and chemically induced tumors were not rescued by Pygo2 loss. In addition to active Wnt signaling, APC loss in APC heterozygous mice and chemically induced tumors most likely also caused chromosomal instability. Riccardo Fodde et al first examined in mouse embryonic stem cells harboring homozygous Min or APC^{1638T} alleles and proved that APC mutations indeed caused chromosomal instability (Fodde *et al*., 2001). Another study in human colon cancer patients also illustrated APC mutations induced chromosomal aberrations that may assisted in adenoma progression (Giaretti *et al*., 2004). In AOM/DSS treated mice a, over 81% of tumors were shown to be chromosomally instable and to be ideal to study chromosomal instability (CIN) (Gerling *et al*., 2011). Therefore, most likely adenomas of APC heterozygous mice and chemically induced tumors in current study may also exhibit chromosomal instabilities. Thus, in the context of APC heterozygous mice, Pygo2 loss may not have any impact on this dysregulation. In addition, in chemically induced tumors, Pygo2 loss may have suppressed tumor growth largely by reducing Wnt signaling output, but probably not by influencing chromosomal instabilities.

Besides aberrations in Wnt signaling pathway, overexpression of BCL9-2 likely was also a significant contributor for tumor progression in heterozygous APC LOF and chemically induced mice. Previous studies from our lab revealed high levels of BCL9-2 only in colon cancer cell lines that harbor APC mutations whereas low or absence of BCL9-2 was detected in colon cancer cells with ß-catenin mutations reference (Brembeck *et al*., 2011). In addition, we also demonstrated tumor invasiveness in APC^{Min} mice when BCL9-2 was overexpressed concurrently (Brembeck *et al*., 2011). Therefore, it is possible that BCL9-2 overexpression in adenomas of heterozygous APC LOF mice and chemically induced tumors may have further contributed in tumor progression, which cannot be compensated by Pygo2 loss.

In contrast, hyperproliferation in ß-catenin GOF mice was entirely rescued after Pygo2 ablation. This was owing to single allelic ß-catenin mutation induced Wnt signaling gene output that might be under a threshold to be suppressed by Pygo2 loss. Whereas APC biallelic loss in APC LOF mice induced severe Wnt signaling that exerted presumably above the Wnt signaling gene output threshold hence Pygo2 loss failed to reverse it. In fact, Wnt signaling dosage effects have been well described, especially in the

context of intestinal tumorigenesis, hematopoietic stem cell system and embryonic stem cell differentiation (Kielman *et al*., 2002; Luis *et al*., 2011; Gaspar *et al*., 2004). During embryonic stem cell differentiation introducing different APC ($APC^{1638N/1638N}$, $APC^{Min/Min}$) and ß-catenin ($ß\text{-}catenin^{exon3/+}$) mutations were shown to induce different dosages of Wnt signaling that caused diverse embryonic defects, in addition these Wnt signaling deregulation was also stated to be cell autonomous (Kielman *et al*., 2002). Similarly, different APC and ß-catenin mutations in hematopoietic system induced different levels of Wnt signaling gene output that affected hematopoietic stem cell differentiation (HSC) (Luis *et al*., 2011). However, a study more relevant to our model has illustrated that varying APC mutations differentially affected intestinal stem cell differentiation, susceptibility to tumorigenesis, that relied on Wnt signaling dosage exerted by APC mutations (Gaspar *et al*., 2004). Altogether, these examinations supported the current models in which APC and ß-catenin mutations activated different dosages of Wnt signaling that was reflected in intestinal phenotype and survival of APC LOF and ß-catenin GOF mice.

However, whether Pygo2 loss rescued hyperproliferation in ß-catenin GOF mice predominantly by inhibiting its nuclear translocation was elusive. In drosophila studies however Pygo was shown to be indispensable for ß-catenin nuclear expression (Townsley *et al*., 2004). Collectively, our present and previous investigations showed that Pygo2 loss in conditional mutants, chemically induced tumors, and in human colon cancer cells including DLD1, SW480 and HCT 116 cells induced downregulation of prominent Wnt target genes (Brembeck *et al*., 2011); nevertheless, whether this was because of reduction in nuclear ß-catenin has not been studied. Therefore, further studies are required to investigate in parallel the effect of Pygo2 loss in Wnt signaling output and plausible alterations in ß-catenin nuclear and cytoplasmic levels in murine models and in vitro in colon cancer cell lines. This can disclose new insights about Pygo2 governing effect on Wnt signaling pathway.

This investigation provided the first in vivo evidence to illustrate the prominent role of Pygo2 during intestinal tumor initiation and progression. Recent studies in vivo revealed Pygo2 role in the context of other tumor formations such as mammary tumor progression, hair follicle stem cell generation and skin hyperplasia (Watanabe *et al*., 2014; Sun *et al*., 2014). Pygo2 deletion in MMTV-Wnt1 mice delayed mammary tumor formation and also suppressed tumor initiating cells, surprisingly all this was largely Wnt signaling independent (Watanabe *et al*., 2014), indicating Pygo2 context dependent role in different tissues. In another study, skin hyperplasia induced by stabilized ß-catenin driven Wnt signaling in vivo was suppressed after Pygo2 deletion, which also reduced Wnt target genes such as Lef1 and Cyclin D1 (Sun *et al*., 2014). This particular study indicated that Pygo2 ability in suppressing stabilized ß-catenin

mediated Wnt signaling gene output may be cell autonomous, which could be the reason why Pygo2 exerted similar effect in present study in ß-catenin GOF mice. Of note, the requirement of Pygo2 for stabilized ß-catenin mutations induced Wnt signaling activation may not be linked to Pygo2 chromatin modulation functions since we did not find any apparent change in Pygo2 mediated epigenetical modifications. Future investigations about the Wnt dependent and independent functions of Pygo2 are required to unravel additional cellular and molecular modification regulated by Pygo2.

5.8). Targeting Pygo2 might be a promising therapeutic strategy, particularly in malignancies with ß-catenin mutations.

Our in vivo examinations showed that Pygo2 deletion rescued early intestinal hyperproliferation induced by ß-catenin gain of function mutations. Thus, targeting Pygo2 can be an attractive therapeutic strategy to control or even total suppresses cancer with ß-catenin mutations including colorectal cancer in human patients. Only a minority (5%) of colon cancers are initiated due to gain of function mutations in ß-catenin (Johnson *et al*., 2005; Morin *et al*., 1997) however gain of function mutations of ß-catenin are more frequently found in other malignancies such as 40% of hepatocellular carcinomas (HCC) (Huang *et al*., 1999), 85% of sporadic desmoid tumors (Lazar *et al*., 2008), 90% of solid-pseudopapillary tumors of pancreas (Abraham *et al*., 2002) and almost all ovarian solid pseudopapillary tumors (Kominami *et al*., 2014). In addition, ß-catenin mutations were also reported in anaplastic thyroid carcinoma (Garcia-Rostan *et al*., 1999), human skin tumors (Chan *et al*., 1999), ovarian endometrioid tumors (Wright *et al*., 1999), hepatoblastomas (Wei *et al*., 2000; Koch *et al*., 1999) and intestinal-type gastric cancer (Park *et al*., 1999).

Hence, future studies should focus on thesis malignancies to analyze the expression and role of Pygo2. So far, in vitro examinations concluded that expression of Pygo2 partially or predominantly controlled many cancer cell lines, which were Wnt dependent or independent (Thompson *et al*., 2002; Andrews *et al*., 2007; Popadiuk *et al*., 2006; Fang *et al*., 2003; Wang *et al*., 2010; Brembeck *et al*., 2011). To the best of our knowledge, the current study has provided the very first in vivo evidence for the prominence of Pygo2 during intestinal tumorigenesis. Therefore, further studies should concentrate to reveal the importance of Pygo2 in other cancers ideally in suitable in vivo tumor models to evaluate whether targeting Pygo2 can be an ideal option.

Because of the undisputable role of Wnt signaling pathway during colon cancer development it has been a target for therapeutic intervention (Kahn *et al*., 2014; Voronkov *et al*., 2012; Sawa *et al*., 2015). Many small molecule inhibitors were shown to modulate Wnt signaling cascade. These include porcupine inhibitors (Chen *et al*., 2009; Dodge *et al*., 2012; Wang *et al*., 2013; Proffitt *et al*., 2013), Wnt inhibitors (Morell *et al*., 2008), Frizzled inhibitor (Chen *et al*., 2009), Axin inhibitor (Chen *et al*., 2009; Kulak *et al*., 2015), CK1 inhibitor (Thorne *et al*., 2010), GSK3inhibitor (Coghlan *et al.,* 2000; Sato *et al*., 2004), TCF/ß-catenin inhibitor (Chen *et al*., 2009; Lepourcelet *et al*., 2004), tankyrase inhibitors (de la Roche M *et al*., 2014). Carnosic acid, an extract from rosemary plant, in colorectal cancer cells was proved to decrease ß-catenin transcriptional activity by interfering binding between BCL9 and ß-catenin (de la Roche M *et al*., 2012; Schwarz & Ternes, 1992). However, further crucial in vivo examinations may be necessary to confirm the potency of this compound.

However, so far no effective therapeutic agent is available to target Pygo2. One exception is Pyrvinium pamoate, an anti-helminth drug, which was shown to be effective to degrade Pygo2 endogenous levels including modulating CK1α and ß-catenin in colon cancer cells lines with APC and ß-catenin mutations (Thorne *et al*., 2010). Oral administration of Pyrvinium was shown to inhibit the Wnt signaling and polyposis in $APC^{Min/+}$ mouse models by activating CK1α and degrading Pygo2 simultaneously (Li *et al*., 2014). In fact, in colon cancer cell lines c-myc was observed to be downregulated following treatment with Pyrvinium pamoate indicating the suppression of Wnt signaling (Wiegering *et al*., 2014). Nevertheless, until now Pyrvinium pamoate effectiveness was restricted to only in vitro examinations and murine models. Therefore, future studies should focus to test its ability to inhibit the cancer growth probably primarily in organoid cultures, and eventually in human patients, while not ruling out the likeliness of side effects. Recently small molecules that can block affinity of Pygopus PHD binding domain have also been discovered. These molecules, benzimidazoles, could interfere Pygopus coupling with BCL9 and H3k4me thereby modulating the PHD zinc finger functions in histone methylation and Wnt signaling (Miller *et al*., 2014). However, Wnt modulating efficacy of these specific molecules is yet to be examined. Theoretically although Pygo2 can be an ideal target for inhibiting the intestinal tumor harboring stabilized ß-catenin, manipulating adenomatous polyposis coli (APC) gene by restoring wild type APC expression was demonstrated to promote cellular differentiation and re-establishment of normal crypt homeostasis in colorectal cancer (Dow *et al*., 2015). In another trail APC full length protein restoration enhanced cell adhesion in colon cancer cell lines (Faux *et al*., 2003).

Altogether, these data suggest that Pygo2, despite being dispensable for the normal intestinal homeostasis, appears to be required for the initiation and progression of intestinal tumors induced by stabilized ß-catenin. Thus, Pygo2 conditional deletion may arrest the tumor growth harboring ß-catenin proto-oncogenic mutations, indicating that targeting Pygo2 may be a novel attractive therapeutic strategy. Currently it is impossible to directly manipulate any particular gene in a tissue specific manner in human patients. However, small molecules that aimed at modulating or even blocking Pygo2 expression might achieve identical effects. Latest CRISP-Cas9 is an impressive and revolutionary novel gene editing method for targeted gene recombination in humans in near future, with the efficiency of its delivery to the target cells of any particular disease yet to be optimized (Savić & Schwank, 2015; Sachdeva *et al*., 2015; O'Geen *et al*., 2015; Beaudet & Meng, 2015).

Summary

Canonical Wnt signaling pathway is an essential regulator of embryonic development as well homeostasis of adult tissues and stem cell proliferation. Familial or sporadic mutations of genes of Wnt signaling components are reported to aberrantly activate the pathway, which is frequently found in human cancers, including colon cancer. Pygopus, as a nuclear co-factor of ß-catenin, demonstrated to be indispensable for Wnt signaling in Drosophila. On the contrary, Pygopus homologues in mammalians have rather a context dependent role, and they are expressed differentially especially in human and mice. Pygopus2 in particular, is expressed more frequently including in human intestinal epithelium, where genetic mutations within Wnt signaling primarily initiate early intestinal hyperproliferation and induce colon cancer. Nonetheless, until now a specific role for Pygo2, as a co-factor of Wnt signaling, during intestinal tumorigenesis has not been studied.

Our data confirmed, that conditional deletion of Pygo2 did not affect the Wnt target gene cascade in normal intestinal epithelium, supporting that Pygo2 is dispensable for Wnt-signaling in the normal intestine. In addition, we also found that Pygo2 is redundant for the epithelial regeneration following the intestinal inflammation.

In contrast, we found that Pygo2 is required for the early intestinal hyperproliferation induced by stabilized ß-catenin since Pygo2 knockout blocked specific upregulated Wnt target genes thereby rescued the intestinal hyperproliferation in ß-catenin mutant mice. In contrast, Pygo2 deletion cannot rescue the hyperproliferation in APC mutants, which might be due to the deregulation of additional APC functions. Moreover, Pygo2 constitutive knockout reduced the colon tumors size and number in chemically induced cancer models and downregulated Wnt target gene expression in a subgroup of these tumors. Importantly, we found c-myc overexpressed in both conditional mutants, and chemically induced tumors.

Pygo2 knockout completely downregulated c-myc only in ß-catenin mutants whereas it failed to act on c-myc similarly in APC mutants and chemically induced tumor models which might be the key player for promoting intestinal tumorigenesis in the context of hyperactivated Wnt signaling.

Together, these data revealed an important role of Pygo2 during early intestinal hyperproliferation and during tumor progression. Thus, targeting Pygo2 in cancers with ß-catenin mutation can be an attractive therapeutic intervention to inhibit the tumor growth.

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