Dissecting the ß-catenin-dependent and -independent functions of BCL9 and BCL9-2 in intestinal tumorigenesis

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Table of Contents

Zusammenfassung

Der hochkonservierte Wnt/ß-Catenin-Signaltransduktionsweg spielt eine wichtige Rolle während der Embryonalentwicklung, der Homöostase und der Tumorgenese in Adulten. Die BCL9 Proteine wurden zunächst als Kofaktoren dieses Signalweges identifiziert. Entsprechend agiert BCL9/*Legless* als essentieller Wnt/ß-Catenin-Kofaktor in *Drosophila*. Jedoch scheint die Rolle von BCL9 und BCL9-2, der Orthologe von Legless, in Vertebraten komplexer zu sein. Des Weiteren wurden die genauen Funktionen der BCL9 Proteine während der intestinalen Homöostase und Tumorgenese bislang wenig untersucht. Es konnte jedoch bereits gezeigt werden, dass BCL9-2 in intestinalen und Mammakarzinomen verstärkt expremiert wird.

Diese Arbeit beschreibt erstmalig den Einfluss und die Funktion von BCL9-2 während der intestinalen Tumorgenese. BCL9-2 beinflusst die Tumorprogression positiv durch Verstärkung des Wnt/ß-Catenin-Signaltransduktionsweg und der Expression von Zielgenen, die Tumorwachstum und -invasion vermitteln. Zudem aktiviert BCL9-2 die Transkription von ß-Catenin-unabhängigen Genen durch einen neuartigen Mechanismus.

Im Gegensatz zu BCL9, welches in allen humanen und murinen intestinalen Zelltypen expremiert wurde, beschränkte sich die BCL9-2 Expression auf die Zotten des Darmes. Die Wnt/ß-Catenin-positiven Krypten hingegen zeigten keinerlei BCL9-2-Expression auf, was darauf hinweist, dass BCL9-2 für den Wnt/ß-Catenin-Signalweg bei der intestinalen Homöostase entbehrlich ist. Während jedoch BCL9 Proteinlevel in Kolontumoren, im Vergleich zum normalen Epithel, unverändert blieben, wurde BCL9-2 bereits in frühen Stadien der Tumorgenese und in 90% aller Kolonkarzinome stark expremiert. Darüber hinaus führte transgene Überexpression von BCL9-2 im Darm von K19-BCL9-2;$APC^{Min/+}$ Mäusen zu einer verstärkten Formation von Adenomen, deren Invasion und einem verringerten Überleben der Versuchstiere.

Wie anhand von TOP/FOP Luciferase Reportergen-Versuchen gezeigt werden konnte, korrelierte die Stärke der BCL9-2-Proteinexpression mit der Aktivität des Wnt/ß-Catenin-Signalweges in Kolonkarzinomzellen. Zudem regulierte BCL9-2 die Transkription einiger ß-Catenin-abhängiger und darüber hinaus ß-Catenin-unabhängiger Zielgene, die bei der Tumorentstehung eine wichtige Rolle spielen. Des Weiteren zeigt diese Arbeit, dass in Kolonkarzinomzellen die BCL9-2 abhängige Transkription von CDX1 und CDX2 durch SP1-bindende Elemente über deren proximale Promotoren vermittelt wurde. Mittels Immunpräzipitation konnte zudem eine Interaktion zwischen BCL9-2 und SP1 in Kolonkarzinomzellen bestätigt werden.

Zusammenfassend zeigt diese Arbeit, dass BCL9-2-Überexpression in frühen Phasen der intestinalen Tumorgenese die Progression von benignen Tumoren in invasive Karzinome

fördert. Diese Eigenschaft wird durch verschiedene Mechanismen vermittelt: Zum einen verstärkt BCL9-2 die Expression einiger Wnt/ß-Catenin-abhängiger Zielgene; zum anderen reguliert BCL9-2 ß-Catenin-unabhängige Gene, die für die Tumorgenese eine wichtige Rolle spielen. Diese Funktion wird vermutlich durch die Bindung an SP1 Transkriptionsfaktoren und damit an die Promotoren von BCL9-2 Zielgenen vermittelt, was zu der verstärkten Expression von Genen führt, die die Tumorprogression und Invasion fördern.

Abstract

The Wnt/ß-catenin signaling pathway is highly conserved and plays an important role during embryonic development, in adult homeostasis and tumorigenesis. The members of the novel BCL9 family were characterized as co-activators of canonical Wnt-signaling. BCL9/*Legless* was found to be absolutely essential for Wnt/ß-catenin signaling in *Drosophila*. However, the role of BCL9 and the second homolog, BCL9-2, seems to be more complex in vertebrates. BCL9-2 was shown to be overexpressed in colon and breast cancers. However, detailed analyses of the BCL9 proteins and their function in normal intestines and during the multistep model of carcinogenesis were missing. Here, we show that BCL9-2 promotes intestinal tumor progression through the enhancement of Wnt/ß-catenin signaling and the regulation of target genes which trigger tumor growth and invasion. Moreover, we demonstrate that BCL9-2 activates the transcription of ß-catenin-independent genes by a novel mechanism.

Using Immunohistochemistry BCL9 was found to be expressed in all intestinal cell types and unchanged in colon cancer cells. In contrast, BCL9-2 protein expression was restricted to the villi in normal intestines, and absent in the crypts where Wnt-signaling is active, indicating that BCL9-2 is dispensable for Wnt/ß-catenin signaling in intestinal homeostasis. In addition, we found that BCL9-2 overexpression occurs early during intestinal tumorigenesis and is overexpressed in approximately 90% of human adenocarcinomas. Moreover, transgenic overexpression of BCL9-2 in the intestine of transgenic K19-BCL9-2;$APC^{Min/+}$ mice led to increased adenoma formation accompanied with local invasion which resulted in reduced survival.

Using TOP/FOP *Luciferase* reporter-gene assays, we found that BCL9-2 protein expression correlated with the level of Wnt/ß-catenin signaling activity in colon cancer cells. Moreover, BCL9-2 regulated the transcription of a subset of ß-catenin-target genes. In addition, we identified a set of BCL9-2 target genes which was apparently independent of ß-catenin signaling which have been implicated in tumorigenesis. Here, we demonstrate that the activation of CDX1 and CDX2 gene transcription by BCL9-2 was dependent on SP1-binding elements in their proximal promoters in colon cancer cell lines. This was corroborated by the novel finding that BCL9-2 associated with the specific protein 1 (SP1) in cancer cells.

In conclusion, this work demonstrates that BCL9-2 overexpression promotes early phases of intestinal tumorigenesis and contributes to the progression of tumors into invasive carcinomas. We showed that BCL9-2 enhances the activation of certain Wnt/ß-catenin target genes. Moreover, BCL9-2 regulates its own set of target genes apparently independent of ß-catenin. This function is mediated through binding to SP1 transcription factors and thereby to the promoters of BCL9-2 target genes, which results in aberrant expression of proteins that trigger the promotion of tumor progression and invasion.

List of Figures

List of Tables

Abbreviations

µl	micro
A/LysoPLA	A/lyso-phospholipase
aa	aminio acid
Ac	Acetylation
ACTB	ß-actin gene/mRNA
APC	adenomatous polyposis coli
bcatBD	ß-catenin binding domain
BCL9	B-cell CLL/lymphoma 9 protein
BCL9-2/BCL9L	B-cell CLL/lymphoma 9-like protein
BD	binding domain
BE	binding elements
BGH-polyA	bovine growth hormone polyadenylation
BMP	bone morphogenic protein
bp	base pairs
BrdU	Bromodeoxyuridine
BRE	TBIID recognition element
BRG1	brahma-related gene 1
CA1	carbonic anhydrase 1
CBP	CREB-binding protein
CCND1	CyclinD1 gene/mRNA
cDNA	complementary DNA
CDX	caudal type homeobox
C-HD	C-terminal homology domain
CIN	chromosomal instability
CIP	calf intestine phosphatase
CK1	casein kinase 1
CRC	colorectal carcinomas
CREB	cAMP responsive element binding protein 1
CTNNB1	ß-catenin gene/mRNA
CypA	Cyclopholin A
DAPI	4',6-diamidino-2-phenylindole
DKK1	Dickkopf-related protein 1
DMSO	Dimetylsulfoxide

DNA	Desoxyribonucleic acid
dNTP	Desoxyribonucleotide triphosphate
DPE	Downstream core promoter elements
DSH	Dishevelled
DSS	dextran sulfate sodium
DTT	DL-Dithiothreitol
E	embryonic day
EDTA	Ethylenediaminetetraacetate
EGTA	ethylene glycol tetraacetic acid
EMT	epithelial-mesenchymal-transition
EPH	Ephrin receptor
ephrin/EFNB	ephrin ligand
ER	Estrogen
ERα	estrogen receptor alpha
ESC	embryonic stem-cell
ESR1	Estrogen receptor alpha gene
EtBr	ethidium bromide
EtOH	Ethyl alcohol
EZH2	Enhancer of zeste (Drosophila) homolog 2
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FDR	fold discovery rate
FGF	fibroblast growth factor
for	(primer) forward
FZ	Frizzled
GREB1	Growth regulation by estrogen in breast cancer 1
GSK3ß	Glycogen synthase kinase 3ß
h	hours or human
H&E	Hematoxylin & Eosin
H2O2	Peroxygen
H3	histone 3
HAT	histone acetyltransferases
HCl	Hydrochloric acid
HD	homology domain
HDAC	histone deacetylases

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMT	histone methyltransferases
HRP	Horse radish peroxidase
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IHH	Indian Hedgehog
INR	initiator elements
ISC	intestinal stem-cells
IVT	in vitro transcription
K	lysine
K19	keratin 19
$KAlS_2O_2 \bullet 12H_2O$	Potassium aluminium sulfate
KCl	Potassium chloride
kDa	kilo Dalton
KLF	Krüppel-like Factor
KLF4	gut-enriched Krüppel-like factor 4
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LCT	lactase
LEF	Lymphoid enhancer factor
LEF	lymphoid enhancer factor
LGR5	Leucine-rich repeat-containing G-protein coupled receptor 5
lgs	*Legless*
LI-cadherin	liver-Intestine cadherin
LPP	Laemmli probe buffer
LRP	Low Density Lipoprotein Receptor-related Protein
LUC	*Luciferase*
m	Mouse
MaSC	mammary stem-cells
Me	Methylation
mg	Milligram
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
min	Minute
Min	multiple intestinal neoplasias
miRNA	micro RNA

mRNA	messenger RNA
MTT	Methyl Thiazolyl Diphenyl-tetrazolium Bromide
MUC2	mucin 2
Na_2SO_4	Sodium sulfate
Na_4PPi	Sodium pyrophosphate
NaCl	Sodium chloride
$NaHCO_3$	Sodium hydrogen carbonate
NaOH	Sodium hydroxide
NFκB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
N-HD	N-terminal homology domain
NLS	nuclear localization signal
nmol	nano mol
NP40	Nonidet-P40
OCT4	POU domain transcription factor 4
PBS	Phosphate Buffered Saline
PCR	polymerase chain reaction
PFA	Paraformaldehyde
PHD	Plant homeo domain
PIC	preinitiation complex
PPIA	Cyclopholin A gene/protein
PR	progesterone receptor
PROX	prospero-related homeobox 1
PyBD	Pygopus binding domain
PYGO	Pygopus
qPCR	quantitative real-time PCR
R	Arginine
RE	responsive element
rev	(primer) reverse
RNA	ribonucleic acid
rpm	rounds per minute
RT	reverse transcriptase or room temperature
S	Serine
s	Seconds
SAP	shrimp alkaline phosphatase
SAP	Shrimp Alkaline Phosphatase

SDNSF	stem-cell-derived neural stem/progenitor cell supporting factor
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SI	Sucrase isomaltase
siRNA	short interfering RNA
SMAD	sisters and mothers against decapentaplegic
SNAIL1	Snail
SNAIL2	Slug
SOX	SRY-related HMG-box gene
SOX2	SRY (sex determining region Y)-box 2
SP1/3	specific protein 1/3
ß-TRCP	ß-Transducing repeat containing protein
TA	transient amplifying cells
TAL	"Transkritomanalyselabor"
TBP	TATA-binding proteins
TBST	Tris-Buffered Saline Tween-20
TCF	T cell factor
TEMED	N,N,N',N'-Tetramethylethan-1,2-diamin
TF	transcription factor
TFIID	transcription factor II D
TG	Transgenic
TGFß	Transforming growth factor
TS	transcription start site
UTR	untranslated region
VEGF	Vascular endothelial growth factor
VIM	Vimentin
WRE	Wnt responsive elements
Y	Thyrosine
ZEB	zinc finger E-box binding homeobox
β-TRCP	beta-transducin repeat containing

1 Introduction

1.1 The Wnt/ß-catenin-signaling pathway in intestinal development, homeostasis and tumorigenesis

Wnts are a family of secreted proteins which activate several signaling pathways through binding to Frizzled and therefore, inducing the canonical (Wnt/ß-catenin)-, the non-canonical (planar-cell-polarity)- and the Wnt-Ca^{2+}- pathway (Habas and Dawid, 2005).

The Wnt/ß-catenin signal transduction pathway is highly conserved between all animals (Logan and Nusse, 2004;Nusse *et al.*, 1997;van and Clevers, 2002;Wodarz and Nusse, 1998). It plays an important role during embryonic development (Grigoryan *et al.*, 2008;Logan *et al.*, 2004;Niehrs, 2010) and adult tissue maintenance (Logan *et al.*, 2004;Nusse *et al.*, 2008;Polakis, 2012;van *et al.*, 2002) by modulating different cellular processes including proliferation, survival, cell migration, differentiation and patterning. In addition, canonical Wnt-signaling is implicated in various diseases, in particular in tumorigenesis (Bienz and Clevers, 2000;Logan *et al.*, 2004).

1.1.1 The role of ß-catenin in canonical Wnt-signaling

ß-catenin was first described as the segment polarity gene product, *Armadillo*, in *Drosophila* (Wieschaus and Riggleman, 1987). It harbors dual functions: as a component of adherence junctions it is necessary for regulating cell growth and adhesion (Brembeck *et al.*, 2006) and in addition, as a modulator of transcription as the key component of canonical Wnt-signaling. In the absence of extracellular Wnt signals, cytoplasmic ß-catenin is recruited to the destruction complex which consists of AXIN1/2, Adenomatous-polyposis-coli (APC), Glycogen synthase kinase 3ß (GSK3ß) and Casein kinase 1ε (CK1ε) (Golan *et al.*, 2004). ß-catenin is initially N-terminally phosphorylated by CK1ε at serine 45 (S45), followed by serine 33 (S33), serine 37 (S37) and tyrosine 41 (Y41) phosphorylation by GSK3β (Liu *et al.*, 2002a). This phosphorylation primes β-catenin for ß Transducing repeat containing protein (ß-TRCP) mediated ubiquitination and its subsequent degradation by the proteasome (He *et al.*, 2004a). Binding of extracellular Wnt ligands to the Frizzled (FZ) - lipoprotein receptor-related protein 5/6 (LRP5/6)-complex leads to activation of Dishevelled (DSH) and disassembly of the destruction complex.

Consequently, ß-catenin is stabilized and trans-locates to the nucleus where it binds to lymphoid enhancer factor/T cell factor (LEF/TCF) transcription factors (Behrens *et al.*, 1996). The function of β-catenin involves the recruitment of additional co-factors, including chromatin modifying and remodeling factors (Parker *et al.*, 2002), p300/ CREB-binding protein (CBP) (Hecht *et al.*, 2000), brahma-related gene 1 (BRG1) (Barker *et al.*, 2001) and TATA-binding proteins (TBP) (Bauer *et al.*, 1998;Hecht *et al.*, 1999) (see 1.4).

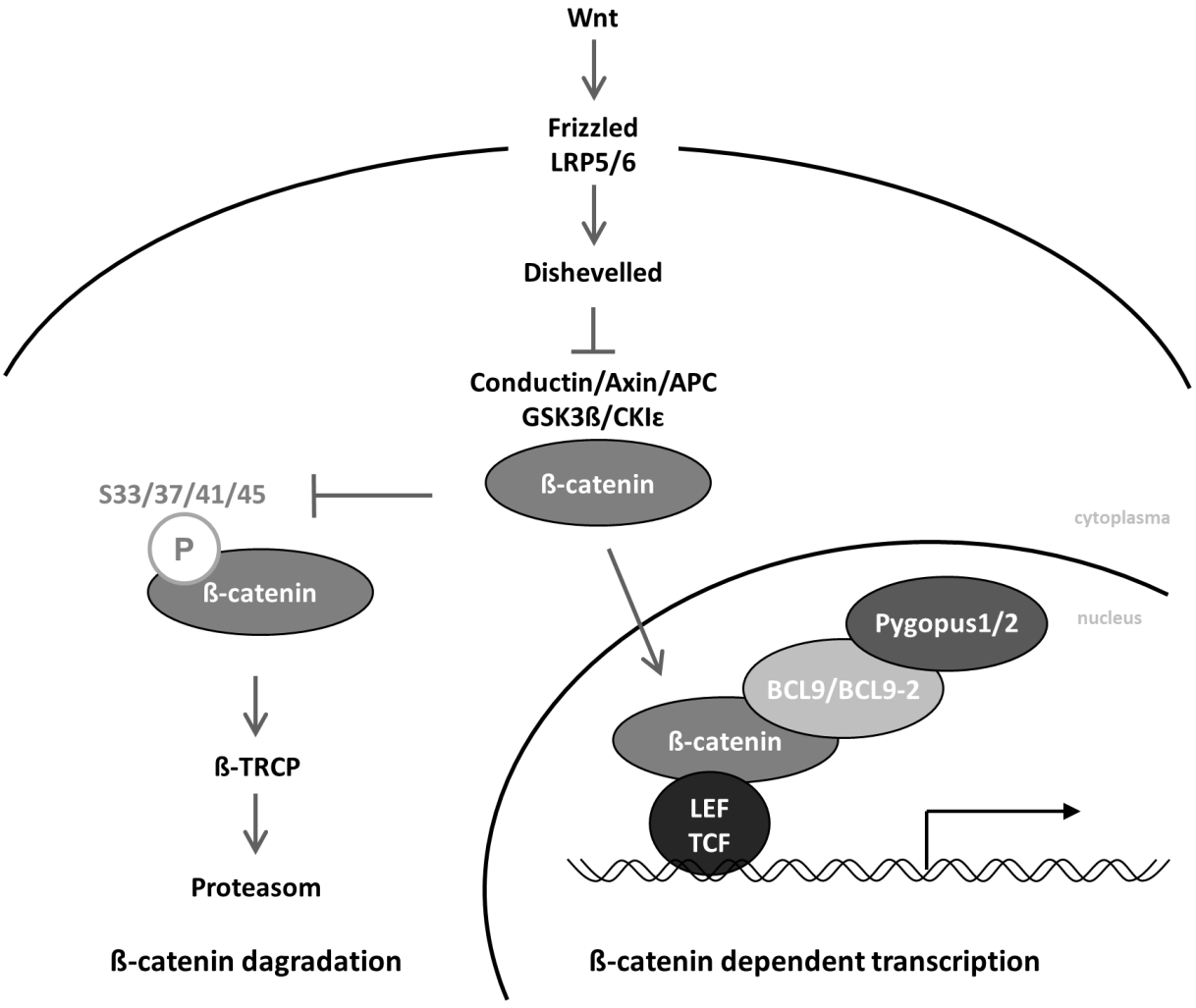

Figure 1: Schematic overview of the Wnt/ß-catenin-signaling pathway
Without extracellular stimuli, ß-catenin is phosphorylated by the destruction complex consisting of Axin1/2, APC, GSK3ß and CK1ε, ubiquitinylated by ß-TRCP and subsequently degraded by the proteasome. When Wnt signals bind to LRP/Fz receptors Dsh becomes activated which inhibits the destruction complex. Stabilized ß-catenin enters the nucleus and binds to LEF/TCF transcription factors and additional co-activators like the BCL9 and Pygopus proteins. This activates the transcription of ß-catenin-dependent target genes. (adapted from Brembeck *et al.,* 2006)

In addition, BCL9 proteins bind to ß-catenin and further recruit other co-activators like Pygopus1 and 2 which lead to enhanced ß-catenin target gene transcription (Belenkaya *et al.*, 2002;Brembeck *et al.*, 2004;Jessen *et al.*, 2008) (Figure 1) (see 1.4.2 and 1.4.3). In the absence of stabilized ß-catenin, TCF transcription factors are bound to Groucho as well as histone

deacetylases. This complex consequently acts as a transcriptional repressor of Wnt/ß-catenin target genes (Cavallo *et al.*, 1998;Chen and Struhl, 1999;Daniels and Weis, 2005)(see 1.4.2).

1.1.2 The Wnt/ß-catenin signaling pathway in intestinal development

Development of the intestine occurs early during embryogenesis. In mice, during E10 to E13, the endodermal germ layer gives rise to the primitive gut tube, whose inner endodermal core differentiates to the epithelium of the mature gastrointestinal tract. After this transition the different specific intestinal cell types develop (see 1.1.3.). In the post-natal period the small intestinal villi elongate and the crypts are formed. The development of the colon is delayed relative to the small intestine. In addition, no villis are formed in the colon, while the crypts merge into a surface epithelial layer (Gao *et al.*, 2009;Sherwood *et al.*, 2011;van der Flier and Clevers, 2009;van der Flier *et al.*, 2009).

These developmental processes require the expression of intestine-specific genes, which are regulated by the key-regulatory ParaHox genes. Those include the caudal related homeodomain proteins CDX1 and CDX2 (Gao *et al.*, 2009). These core-transcription factors are responsible for the activation of general intestine specific genes, such as Mucin 2 (MUC2), gut-enriched Krüppel-like factor 4 (KLF4) and Liver-Intestine cadherin (LI-cadherin), genes specific for the small intestine (e.g. sucrose isomaltase (SI), lactase (LCT) and A/lyso-phospholipase (A/LysoPLA)) and colonic epithelial genes (e.g. carbonic anhydrase 1 (CA1)) (Guo *et al.*, 2004) through evolutionarily conserved DNA promoter elements (Suh *et al.*, 1994). The activation of CDX1 and CDX2 target genes is highly dependent on the phosphorylation status of these proteins (Taylor *et al.*, 1997). The initial expression of CDX2 is induced by canonical Wnt-signaling between E7.5 and E8.5, while after E8.5 Wnt-signaling is no longer sufficient to activate CDX2 (Sherwood *et al.*, 2011). CDX2 protein expression in the posterior endoderm induces the expression of further intestinal Hox genes, including CDX1 and as a target gene of CDX2 and ß-catenin expression (Mutoh *et al.*, 2009; Lickert *et al.*, 2009). CDX1 protein expression appears after a short delay compared to CDX2 at E9.5 predominantly in the distal part of the developing intestine. CDX1 and CDX2 expression become restricted to the intestinal epithelium. As a consequence, a sharp anterior boundary is formed marking the transition from stomach to duodenum (Hu *et al.*, 1993;James *et al.*, 1994). Thus, the gradients of CDX2 and CDX1 define the three major parts of the intestine: the foregut which gives rise to the epithelia of esophagus, stomach, and duodenum, the midgut which becomes the small intestine and the hindgut, which develops into the cecum and colon. A gradient of CDX1 and

CDX2 forms the crypt-villus axis, with CDX1 expression primarily in the crypt and CDX2 in the villus region (Sherwood *et al.*, 2011).

In addition, Wnt-signaling contributes to the proper development of the intestine by modulating key-epithelial signals which define the epithelial integrity to the underlying mesenchyme by regulating the expression of e.g. Indian Hedgehog (IHH) (Buller *et al.*, 2012;Sherwood *et al.*, 2011). According to this, TCF1/TCF4 mutants display severe embryonic intestinal defects (Gregorieff *et al.*, 2004). In addition, ablation of ß-catenin in the node, notochord and anterior primitive streak abrogates definitive endoderm formation (Imai *et al.*, 2000;Lickert and Kemler, 2002). In summary, development of the gastrointestinal tract is governed by the cooperation of different core-factors including ß-catenin, which activates the expression of canonical Wnt-target genes, and the major specification transcription factor CDX2.

1.1.3 The Wnt/ß-catenin-signaling pathway and ß-catenin target genes in intestinal homeostasis and tumorigenesis

The intestine is covered by a single layer of epithelial cells surrounding the finger-like villi. These invade into the crypts of Lieberkühn consisting of non-differentiated, proliferating cells (Figure2). The crypts harbor the intestinal stem-cells, which give rise to transient-amplifying cells that divide 4-5 times before they differentiate into the absorptive enterocytes, mucous-secreting goblet cells, lysozyme producing Paneth cells and hormone-secreting enteroendocrine cells. The structural organization of the intestine leads to an extreme enlarged surface. In addition, the cellular organization conveys the major functions of the small intestine including digestion and absorption of nutrients and the formation of a barrier against luminal pathogens. In contrast, the colon lacks the constitution of villi. Consequently, the main function of the colon is the absorption of water and thus the compaction of stool mass. (Sancho *et al.,* 2003)

The bottom of the crypts consists of multipotent intestinal stem-cells (ISCs), which are bordered by single Paneth cells. The ISCs have a cell cycle period of 24h and reside in the lower part of the crypt. They give rise to the transient amplifying (TA) cells which reside in the crypts for up to six cell divisions with a cell cycle period of around 12h (Marshman *et al.*, 2002). When the TA-descendants reach the crypt–villus junction they undergo cell cycle arrest and differentiate into the four specific, epithelial intestinal cell types. While the intestinal cells differentiate they migrate along the crypt-villus axis. In mice the tip of the villus is reached by

the cells 3 days after their terminal differentiation, where they undergo apoptosis and are shed into the lumen (Hall *et al.*, 1994)(Figure 2). Paneth cells represent an exception. They move towards the bottom of the crypts upon differentiation and exhibit an enlarged life span of 20 days compared to the three other intestinal, epithelial, mature cell types (Garabedian *et al.*, 1997).

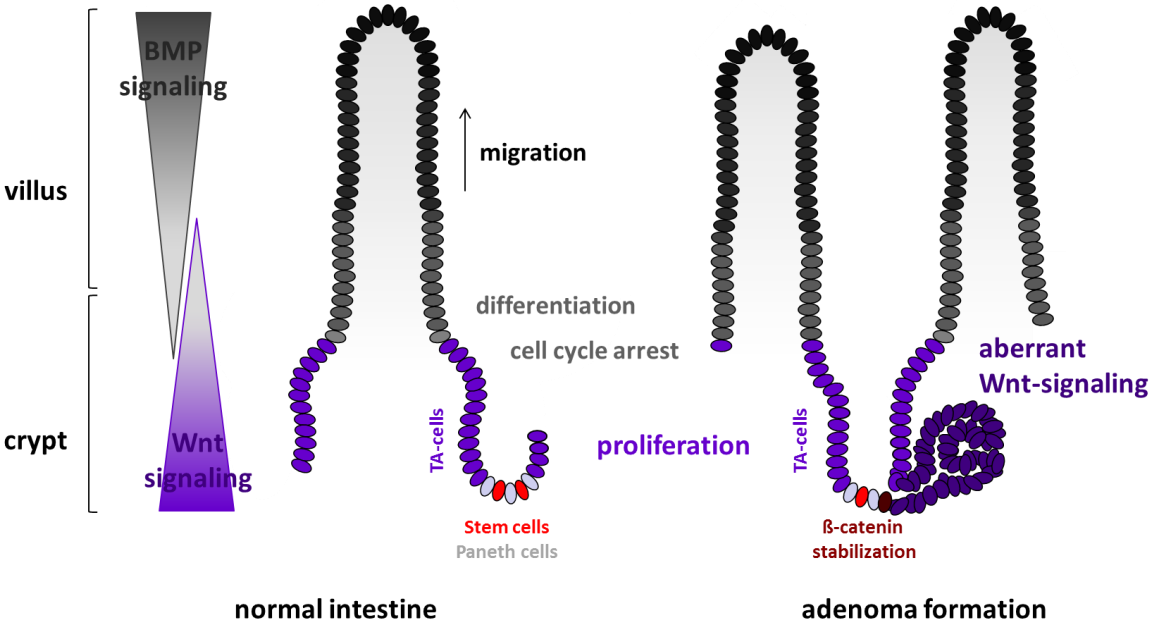

Figure 2: Intestinal homeostasis and adenoma formation
Overview of the organization of the crypt-villus organization of the small intestine and the activity of Wnt/ß-catenin-signaling in homeostasis (normal intestine) and tumorigenesis (adenoma formation). ß-catenin is transcriptional active in the crypts in normal intestine and controls the proliferation of intestinal stem-cells (ISCs) and transient amplifying cells (TA). Upon aberrant ß-catenin activation adenomas arise due to uncontrolled proliferation (Barker *et al.*, 2009).

For controlled homeostasis which includes the division of intestinal stem-cells and transient amplifying cells Wnt/ß-catenin signaling activity is indispensable. Accordingly, ablation of TCF4, ß-catenin, or ectopic expression of the inhibitor Dickkopf 1 (DKK1) lead to loss of proliferative epithelial cells in adult and embryonic intestine (Korinek *et al.*, 1998a;Korinek *et al.*, 1998b;Pinto *et al.*, 2003). In contrast, the initiation of cell cycle arrest at the crypt-villus border is of importance for the differentiation into mature epithelial, intestinal cell types. Consequently, a gradient of active Wnt-signaling is formed, with the highest activity in the bottom of the crypts accompanied with high nuclear ß-catenin, which gradually decreases to the interface of the villus. Thus, only membrane-bound ß-catenin is present in the villus resulting in the complete absence of Wnt-signaling in the tip of the villus (Figure 2, left) (Pinto *et al.*, 2003;van de Wetering *et al.*, 2002). In contrast, the Wnt-antagonizing bone

morphogenic protein (BMP) pathway is active in the mesenchyme of the villi and thereby inhibits canonical Wnt-signaling in the intestinal epithelium (Figure 2, left)(Crosnier *et al.*, 2006;Hartenstein *et al.*, 2010;He *et al.*, 2004b). In addition, canonical Wnt-signaling regulates the intestinal homeostasis by controlling the expression of other factors, including the ephrin ligands (*ephrin*) and receptors (*EPH*). A complex expression pattern of EPH/ephrin determines the position of differentiated cells along the crypt-villus-axis. EPHB2/3 receptors are strongly expressed in the crypts, ephrinB1 ligands in the villi. Consequently, a gradient of receptors and ligands is formed, which creates a unidirectional migration of differentiating cells from the crypt to the villus (Batlle *et al.*, 2002). Moreover, transforming growth factor ß (TGFß) and bone morphogenic protein (BMP) signaling in the villus inhibit Wnt/ß-catenin signaling and function as growth repressors in differentiated epithelial cells (Haramis *et al.*, 2004;Hardwick *et al.*, 2004;Sancho *et al.*, 2004) Thus, active and a well-defined dosage of Wnt/ß-catenin-signaling is indispensable in intestinal homeostasis.

To that effect, the majority of intestinal sporadic and hereditary adenomas derive from cells with aberrant stabilization of ß-catenin due to truncating mutations in Adenomatous-polyposis-coli (APC) (80%) (Ichii *et al.*, 1993;Nagase and Nakamura, 1993). Less colorectal carcinomas (CRC) occur upon stabilization of ß-catenin by mutations in Axin2 or ß-catenin itself, which abolish the phosphorylation and subsequent degradation of ß-catenin (Ilyas *et al.*, 1997;Liu *et al.*, 2000). Taken together, APC and ß-catenin mutations are the initial events in more than 90% of intestinal tumors (Fearon and Vogelstein, 1990). Increased ß-catenin activation leads to uncontrolled proliferation which results in a well-known sequence of histopathological changes. Dysplastic/hyperplastic crypts grow consequently out into microadenomas, which give rise to adenomas, carcinomas and subsequently invasive tumors (Fearon *et al.*, 1990;Fodde *et al.*, 2001)(Figure3).

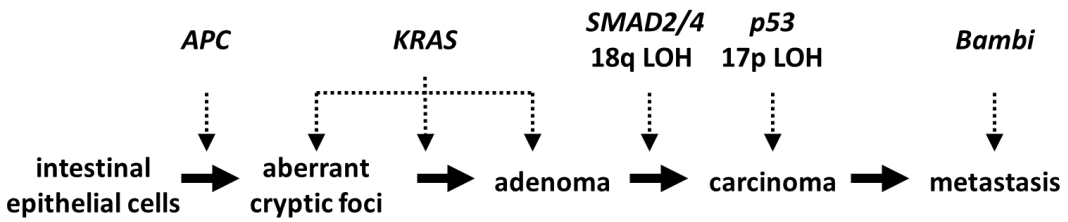

Figure 3: Gene mutations during the adenoma-carcinoma sequence
Development of intestinal adenomas occur following ß-catenin stabilization due to APC truncations or mutations of ß-catenin itself and mutations of *KRAS*. Additional mutations resulting in loss-of-function of tumor suppressors, like *SMAD2/4, p35* and *Bambi* induce the transformation into aggressive carcinomas. (adapted from Vogelstein and Kinzler, 1993)

It has been shown, that deletion of APC in Leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5) positive stem-cells leads to transformation of these cells to growing microadenomas that develop into macroscopic adenomas within three to five weeks (Barker *et al.*, 2009).

However, stabilized ß-catenin is not sufficient to induce the progression of adenoma into carcinoma. Additional mutations resulting in loss-of-function of tumor suppressors (e.g. sisters and mothers against decapentaplegic (SMAD), p35 or Bambi or activation of V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) are necessary to drive the progression of adenomas into aggressive carcinomas (Figure3) (Fearon *et al.*, 1990;Fodde *et al.*, 2001;Vogelstein and Kinzler, 1993;Vogelstein and Kinzler, 2004;Wang *et al.*, 2004).

Despite its fundamental role during canonical Wnt-signaling, ß-catenin acts as in processes of intracellular adhesion (Polakis, 2000). For cell-cell adhesion, cytosolic ß-catenin is recruited to the plasma membrane where it associates with E-cadherin. Consequently, adherence junctions are formed and stabilized which promotes a functional tissue architecture and morphogenesis (Gumbiner, 2000;Jamora and Fuchs, 2002). In tumorigenesis both functions of ß-catenin are often deregulated, which leads to aberrant ß-catenin accumulation, increases target gene expression and to loss of cell-cell adhesion (Brembeck *et al.*, 2006). These transformations promote enhanced migration and proliferation leading to invasion and metastasis (Perl *et al.*, 1998).

1.2 The BCL9 proteins

1.2.1 BCL9 and BCL9-2 encode the vertebrate orthologue of *Drosophila Legless*

In 1998 B-cell CLL/lymphoma 9 protein (BCL9) was found to be overexpressed in B-cell lymphomas caused by a t(1;14)(q21;q32) trans-location of the *B-cell lymphoma* gene locus (Willis *et al.*, 1998). More than 10 years later the orthologue of BCL9, *Legless* (*lgs*), was discovered in Drosophila in a genetic screen for dominant suppressors of the classical Wnt-phenotype. Rescue experiments in $lgs^{-/-}$ mutants revealed that the function of the segment polarity gene *lgs*/BCL9 is that of an absolutely required co-factor in Wnt/ß-catenin signaling in *Drosophila*. Maternal replacement of BCL9 in $lgs^{-/-}$ mutants was sufficient to achieve a complete rescue (Kramps *et al.*, 2002). BCL9-2, the vertebrate orthologue of BCL9 was

discovered as a ß-catenin related protein from a yeast-two-hybrid screen of a mouse cDNA library using a ß-catenin armadillo repeat domain as bait (Brembeck *et al.*, 2004;Kramps *et al.*, 2002). Seven domains of the BCL9 proteins are highly conserved with 90% amino acid sequence identity (Brembeck *et al.*, 2004;Kramps *et al.*, 2002)(Figure 4). Whereas vertebrate BCL9 proteins share only 35%, vertebrate BCL9-2 proteins share 60% overall amino acid sequence identity (Brembeck *et al.*, 2006).

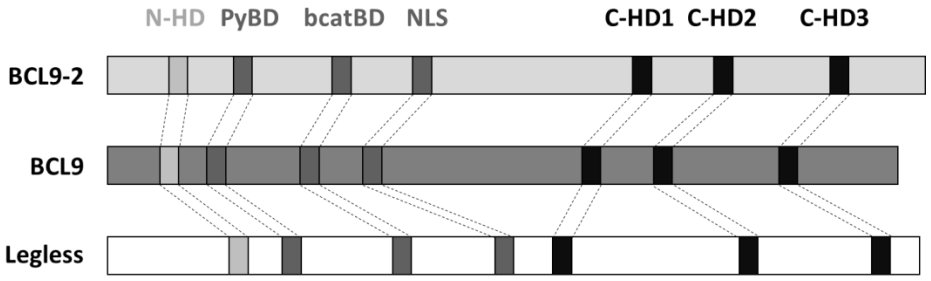

Figure 4: Schematic overview of the BCL9 proteins including seven evolutionary conserved domains
The vertebrate BCL9, BCL9-2 proteins and the *Drosophila* homolog *Legless* contain seven domains, which share up to 90% amino acid sequence homology: a N-terminal homology domain (N-HD), a Pygopus binding domain (PyBD), a ß-catenin binding domain (bcatBD), a classical nuclear localization signal (NLS) and three C-terminal homology domains (C-HD1, C-HD2 and C-HD3). (Brembeck *et al.*, 2006)

The N-terminus harbors an N-terminal homology domain (N-HD), which contains a putative sumoylation motif (K*K*KXE/D) (Melchior, 2000). All BCL9 family members also harbor a ß-catenin binding domain (bcatBD), a Pygopus binding domain (PyBD), a domain containing an un-functional classical nuclear localization signal (NLS, KRRK) and three C-terminal homology domains (Figure 4). In addition, the N-HD domain of BCL9-2 contains a functional nuclear localization signal (PRSKRRC). Consequently, deletion of the N-HD of BCL9-2 and therefore the NLS leads to cytoplasmatic de-localization (Adachi *et al.*, 2004;Brembeck *et al.*, 2004;Hicks and Raikhel, 1995). In contrast, BCL9 and *Legless* are no nuclear proteins. Both require Pygopus2 to be trans-located into the nucleus (Adachi *et al.*, 2004;Brembeck *et al.*, 2004;Townsley *et al.*, 2004).

1.2.2 The BCL9/*Legless* proteins are co-factors of the Wnt/ß-catenin signaling pathway

Legless and BCL9 are essential for canonical Wnt-signaling in *Drosophila*. They recruit Pygopus (PYGO) to the ß-catenin/TCF complex to transactivate ß-catenin-dependent target genes. The Pygopus protein family consists of the two homologs Pygo1 and Pygo2 in vertebrates which contain two highly conserved domains: an N-terminal homology domain (N-HD) interacting

with BCL9/*Legless* proteins and a C-terminal PHD (plant homeo-domain) zinc finger motif (Belenkaya *et al.*, 2002;Kramps *et al.*, 2002) which was shown to mediate trans-activatory properties. Moreover, Pygopus contributes to the activation of gene expression by recruiting chromatin remodeling factors and by modulating Histone modifications (see 1.4.3)(Belenkaya *et al.*, 2002;Kessler *et al.*, 2009;Kramps *et al.*, 2002;Nakamura *et al.*, 2007;Stadeli and Basler, 2005).

In vertebrates Pygopus binding to BCL9-2 is not absolutely required to co-activate gene transcription of Wnt/ß-catenin target genes (Adachi *et al.*, 2004). In addition, BCL9-2 binding promotes the trans-location of ß-catenin into the nucleus and therefore the expression of target genes. The switch between the adhesive and transcriptional function of ß-catenin is modulated by phosphorylation of thyrosine142 (Y142) of ß-catenin, which leads to preferred binding of the protein to BCL9-2 instead of α-catenin (Brembeck *et al.*, 2004).

BCL9-2 enhances Wnt/ß-catenin activity. For this function the C-terminus harboring the three C-HD is indispensable. In addition, BCL9 transcriptional co-activity requires the C-terminal region as well, although the mechanism is still unknown (Adachi *et al.*, 2004;Sustmann *et al.*, 2008).

1.2.3 BCL9 proteins in development, regeneration and tumorigenesis

Wnt/ß-catenin signaling is crucial for the dorsal-ventral mesoderm patterning in early, and organ specification in late embryonic development of *Danio rerio* (Huelsken and Birchmeier, 2001;Moon *et al.*, 2002). For Wnt8/ß-catenin signaling mediated mesoderm patterning in *Danio rerio* BCL9-2, but not BCL9/*Legless* is indispensable (Brembeck *et al.*, 2004). Moreover, other Wnt/ß-catenin-dependent developmental processes, like the posteriorization of anterior neuroectoderm (Lekven *et al.*, 2001) are BCL9-2 independent (Brembeck *et al.*, 2004). In contrast, BCL9 and BCL9-2 are required for the activation of Wnt/β-catenin signaling in adult mammalian myogenic progenitors during skeletal muscle regeneration. Under physiological conditions, Wnt1/3A/ß-catenin signaling promotes differentiation of proliferating myogenic progenitors in mice (Brack *et al.*, 2008;Rochat *et al.*, 2004). Reduction of BCL9/BCL9-2 abrogates myogenic differentiation (Brack *et al.*, 2009). Thus, the BCL9 proteins function as activators of Wnt/ß-catenin signaling in a temporal and cellular context dependent manner in vivo.

Conditional ablation of BCL9/BCL9-2 in mouse intestinal epithelium leads to decreased expression of intestinal stem-cell markers like SRY-related HMG-box gene (*SOX6*) and *LGR5*. Moreover, knockout of the BCL9 proteins impaired the regeneration capacity of ulcerated colonic epithelia. Induction of dysplastic adenomas by dimethylhydrazine and DSS led to increased Wnt/ß-catenin signaling activity associated with nuclear β-catenin accumulation in wild-type and BCL9/BCL9-2 ablated tumors. Here a subset of the Wnt/ß-catenin target genes was reduced in knockout mice compared to wild-type tumors including *Axin2*, prospero-related homeobox 1 (*Prox1*), *Lef1* and *Tcf1* (Deka *et al.*, 2010) . Moreover, carcinomas occurred with similar incidence, although the size of mutant tumors was generally smaller. Comparative transcriptional expression profiles of wild-type and mutant carcinomas further revealed a role of BCL9/BCL9-2 in the control of epithelial-mesenchymal-transition (EMT) indicated by reduction of fifteen EMT-marker genes coding for proteins like Slug (*Snail2*) , Snail (*Snail1*), Vimentin (*Vim*), *Zeb1* and *Zeb2*. Moreover, fifty stem-cell related genes like *Lgr5* and *Sox6* were decreased in the gene set of BCL9/BCL9-2 conditional knockout mice indicating a role of the BCL9 proteins in modulating stem-cell-like traits in colon cancers (Deka *et al.*, 2010). Thus, Deka *et al.* pointed out that the function of BCL9/BCL9-2 function in intestinal regeneration and further, in colonic tumorigenesis by controlling a subset of Wnt/ß-catenin target genes that are implicated in EMT and stem-cell traits. However, the mouse model used by Deka *et al.* did not provide any information about the particular role of each of the BCL9 proteins. Thus, the observed effects on regeneration and tumorigenesis could have been mediated by BCL9, BCL9-2 or both. In contrast, Matsuura *et al.* investigated the particular function of BCL9-2. Complete knockout of BCL9-2 lead to growth arrest due to placental defect at around E10 resulting in embryonic lethality at E10.5 to E11.0, whereas heterozygous $BCL9^{+/-}$ mice were healthy and fertile. Those placental phenotypes are reminiscent of those resulting from cell fusion pathway defects (Matsuura *et al.*, 2011). Moreover, intestinal stem-cells with high Wnt/ß-catenin signaling are fusion partners of transplanted bone marrow-derived cells in tissue regeneration in the intestine. Transformed intestinal cells of $APC^{MIN/+}$ mice exhibit an increased frequency of fusion with bone marrow-derived cells (Lu and Kang, 2009).

The BCL9 proteins were described to play a role during tumorigenesis in different tumor entities. BCL9 and BCL9-2 overexpression was found in colorectal cancers tumors (Adachi *et al.*, 2004;Brembeck *et al.*, 2011;de la Roche *et al.*, 2008). Also, BCL9-2 mRNA expression correlated with the progressive grades of colorectal neoplasias (Sakamoto *et al.*, 2007). In this context BCL9 proteins induce canonical Wnt-signaling and thereby increase the ß-catenin mediated tumorigenic potential of tumor cells by contributing to stem-cell straits and EMT

(Matsuura *et al.*, 2011). Disruption of the BCL9 and ß-catenin interaction leads to decreased oncogenic Wnt-signaling activity and is therefore believed to be a promising therapeutic approach for treating Wnt-dependent cancers (de la Roche *et al.*, 2012a;Takada *et al.*, 2012).

BCL9-2 overexpression was found in breast cancers (Zatula and Brembeck, unpublished)(Mani *et al.*, 2009;Toya *et al.*, 2007). Human breast cancer tissue arrays revealed a correlation between high BCL9-2 and the expression of the hormone receptors of Estrogen (ER), Progesterone (PR), the human epidermal growth factor receptor 2 (HER2) (Zatula and Brembeck, unpublished) and of the proto-oncogene c-myc and p53 (Toya *et al.*, 2007). Moreover, high BCL9-2 expression was found during the ß-catenin/Wnt-dependent ductal outgrowth and during pregnancy in mouse mammary glands (Boras-Granic and Wysolmerski, 2008) suggesting the contribution of BCL9-2 to ß-catenin/Wnt activity dependent proliferation during these stages of development (Zatula and Brembeck, unpublished).

Investigations regarding the BCL9 proteins mainly focused on their ß-catenin associated function as activators of canonical Wnt-signaling (Adachi *et al.*, 2004;de la Roche *et al.*, 2008;Kramps *et al.*, 2002;Sakamoto *et al.*, 2007;Sustmann *et al.*, 2008). BCL9-2 plays an important role in Wnt/β-catenin pathway-mediated cell fusion, during embryonic development, tissue regeneration and cancer development but appears to be dispensable in intestinal homeostasis (Adachi *et al.*, 2004;Brembeck *et al.*, 2011;de la Roche *et al.*, 2008;Deka *et al.*, 2010). The gene locus of *BCL9* was shown to be trans-located in B-cell lymphomas leading to aberrant BCL9 protein expression, but nothing is known about the mechanisms which lead to overexpression of BCL9-2 in cancers so far. However, the particular function of BCL9 and BCL9-2 in tumorigenesis is not well understood. Until now, only the impact of the BCL9 proteins regarding their Wnt/ß-catenin-dependent function had been investigated in detail (Adachi *et al.*, 2004;Brack *et al.*, 2009;Brembeck *et al.*, 2006;Brembeck *et al.*, 2004;Kramps *et al.*, 2002). Moreover, a mechanism which explains the molecular ß-catenin-dependent or/and independent capacity of BCL9 and BCL9-2 is still to be found.

1.3. K19 dependent BCL9-2 overexpression *in vivo*

Since BCL9-2 is overexpressed in many human and mouse tumors we established a BCL9-2 overexpression mouse model to further investigate the function of this protein (Brembeck *et al.*, 2011)(Zatula and Brembeck, unpublished). In our mouse model ectopic expression of flag-tagged BCL9-2 was induced by the promoter of the epithelial specific Keratin 19 gene (K19)(for

detailed information see Materials and Methods 3.9.1). Flag-tagged BCL9-2 is consequently expressed in all simple epithelia including the stomach, intestine, liver, kidney, pancreas and mammary glands (Brembeck *et al.*, 2001;Brembeck and Rustgi, 2000).

Aged transgenic mice (more than 15 months of age) developed macroscopic tumors in the pancreas, the intestine and in mammary glands. In contrast to undifferentiated pancreatic and intestinal tumors, mammary gland tumors were well differentiated and composed of ductal-like and myoepithelial-like structures. Interestingly, mammary gland tumors were positive for nuclear Estrogen- (ERα) and Progesterone- (PR) receptors. Accordingly, primary cell cultures of K19-BCL9-2 tumors were dependent on Estrogen. Knockdown of BCL9-2 in MCF7 breast cancer cells lines led to a transient reduction of ERα (*Esr1*) and ERα target gene expression, indicating a regulatory role of BCL9-2 in Estrogen receptor signaling. Interestingly, MCF7 cells contain no transcriptionally active ß-catenin although BCL9-2 is highly expressed. This suggests a novel ß-catenin-independent mechanism (Zatula and Brembeck, unpublished).

1.4. Transcriptional regulatory mechanisms

The complex regulation of gene expression is accomplished by the collective activity of diverse transcriptional regulatory proteins. Transcription factors (TF) modulate the action of RNA polymerases through binding to specific cis-regulatory sequences within the promoter of a gene (Matsui *et al.*, 1980;Segall *et al.*, 1980).

1.4.1 Basal transcriptional regulatory mechanisms

1.4.1.1 Basal transcriptional activation

To initiate gene transcription RNA polymerase II requires the binding of specific transcription factors to attach to core promoter regions. The pre-initiation complex (PIC), consisting of general transcription factors (GTFs), transcription factor II D (TFIID) and polymerase II subsequently initiates transcription supported by variable co-factors (for example, see 1.4.2 and 1.4.3)(Blackwood and Kadonaga, 1998). Commonly, eukaryotic genes such as CDX1 harbor a transcription initiation site located in a region of approximately -30bp from the transcription start (TS) site, called canonical TATA box that binds the pre-initiation complex PIC trough transcription factor II D (Burley and Roeder, 1996;Nikolov *et al.*, 1996;Suzuki *et al.*, 2001).

However, approximately 76% of all human promoters transcribed by RNA polymerase lack TATA-like elements (TATA-less promoters) and even initiator elements (Weis and Reinberg, 1992). Initiator elements, downstream core promoter elements (DPE) and TBIID recognition element (BRE) comprising the transcription start site and are able to facilitate the binding of TBP in TATA-less promoters with or without the participation of additional cis-acting elements (Bucher, 1990;Javahery *et al.*, 1994;Smale and Baltimore, 1989) (Figure 5). Among others, motifs corresponding to specificity protein 1 (SP1) binding sites can be present instead of TATA or initiator elements and are therefore enriched in TATA-less promoters.

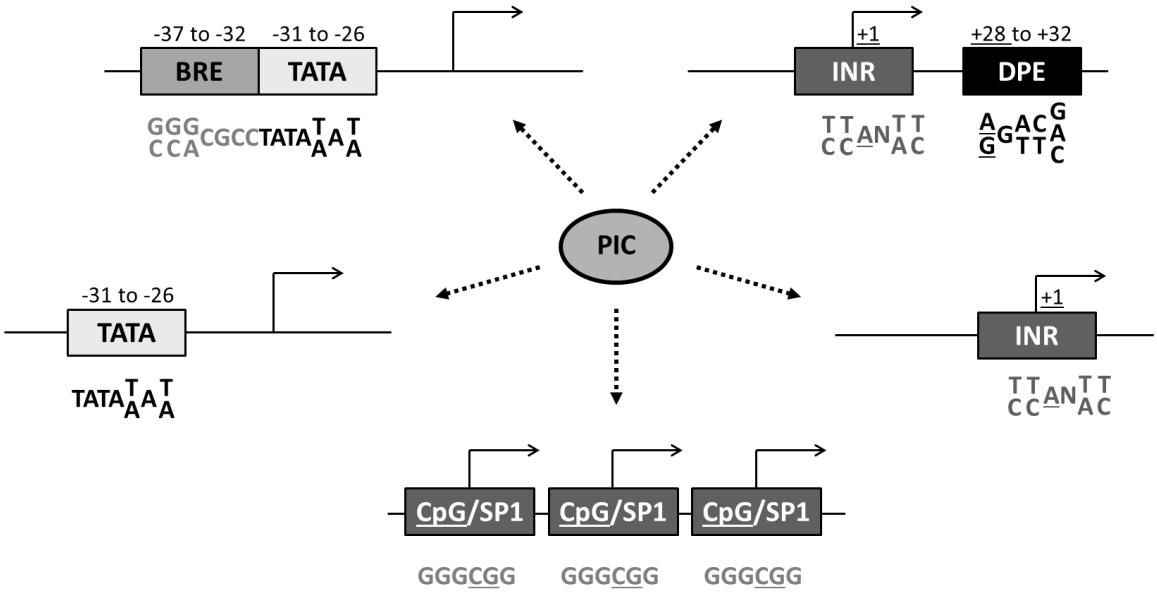

Figure 5: Transcriptional enhancers cooperating with the pre-initiation complex and initiating gene transcription through a variety of core promoters
The pre-initiation complex (PIC) and additional transcriptional enhancers participate in RNA polymerase II mediated transcription by binding to core promoter elements. Each of the regulatory elements is present in a subset of core promoters in different distributions, as indicated. (adapted from Blackwood *et al.*, 1998)

1.4.1.2 Transcriptional activation through Specific protein (SP) transcription factors in normal homeostasis and tumorigenesis

SP proteins belong to the family of SP/KLF transcription factors and can either enhance or repress the transcription of target genes. The family of SP/KLF factors includes the SP proteins (SP1 to SP9) and the krüppel-like factors (KLF1 to KLF16) (Simmen *et al.*, 2010; Black *et al.*, 2001; Suske, 1999). The different family members have been shown to preferentially, but not exclusively bind to different sequence motifs. Accordingly, KLFs prefer CACCC-boxes (Shields and Yang, 1998), whereas SPs preferentially bind to GC-boxes (Suske, 1999). SP motifs are frequently present in CpG islands (see 4.2.1) which are often un-methylated, GC-rich

sequences (Adachi and Lieber, 2002). CpG islands are commonly overrepresented in cis transcription factors (Brandeis *et al.*, 1994). Moreover, binding of SP1 to GC-rich elements can activate gene transcription without or in combination with initiator elements (INR) in absence of any TATA or downstream core promoter elements (Smale *et al.*, 1989;Smale *et al.*, 1990) (Figure 5).

SP1 was one of the first identified basal mammalian transcription factor (Kadonaga *et al.*, 1987). SP1-knockout mice are early embryonic lethal at E10.5. In contrast, knockout of its closest relative, SP3, causes postnatal death (Kruger *et al.*, 2007). SP1 and SP3 share over 90% DNA sequence homology in their DNA binding domain which consists of three adjacent Cys_2His_2-type zinc fingers (Suske *et al.*, 2005). SP1 and SP3 have different isoforms, which are generated through alternative splicing of SP1 and alternative translational initiation sites in SP3 (Li *et al.*, 2004;Sapetschnig *et al.*, 2004). SP1 and the longest SP3 variant contain two trans-activation domains (AD1 and AD2) and a domain, which is used for synergistic activation (SAD). In contrast to SP1, SP3 contains an additionally inhibitory domain (ID) (Figure 6). In addition, both transcription factors are post-translationally modified. SP1/3 can be sumoylated - which represses SP1 activity - or acetylated and phosphorylated, which results in increased transcriptional activity (Li and Davie, 2010;Li *et al.*, 2004).

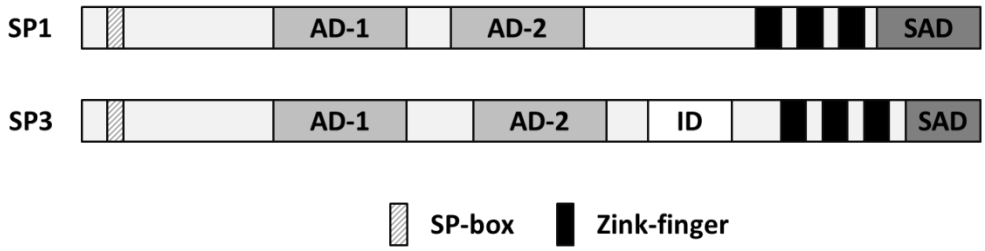

Figure 6: Schematic overview about the protein structure and modification motifs of SP1 and SP3
SP1 and SP3 contain an SP-box, a highly conserved DNA-binding domain consisting of three Zink-fingers two trans-activation domains (AD) and a C-terminal synergistic activation-domain (S-AD). In addition, SP1 contains an inhibitory-domain. (adapted from Bouwman *et al.*, 2002)

SP1 and SP3 can either induce or inhibit gene transcription, in dependency on their protein variant and post-translational modification. To this end, SPs directly interact with proteins in the pre-initiation complex and other nuclear cofactors which comprise the basal transcription factors and consequently initiate transcription. SP1-binding sites are commonly present in the promoters of TATA-less house-keeping genes such as acetyl-CoA synthetase 1 (Ikeda *et al.*, 2001;Lin *et al.*, 1996;Zhu *et al.*, 2008). SP1 and SP3 target gene products are involved in processes such as differentiation and cell cycle progression (Davie *et al.*, 2008). Moreover, overexpression of SP1 proteins in colon-, breast-, pancreatic- and gastric- and lung -cancers (Li *et al.*, 2010) results in increased expression of tumorigenesis-related genes. SP1 was shown to

interact with several transcription factors such as SMAD2/3/4 (Feng *et al.*, 2000), wild-type or mutated p53 (Schavinsky-Khrapunsky *et al.*, 2003) and OCT1 (Strom *et al.*, 1996), respectively. For example, in breast cancer cells SP3 plays a role for the ERα dependent trans-activation of the promoter of the vascular endothelial growth factor receptor 2 (VEGFR2) (Higgins *et al.*, 2006a;Higgins *et al.*, 2006b). In addition, transcription of VEGF is regulated by SP1 in pancreatic tumors (Safe and Abdelrahim, 2005). Moreover, it has been shown that mithramycinA mediated inhibition of SP1 expression is correlated with anti-angiogenic effects in human pancreatic cancers, suggesting that SP1 might be a useful therapeutic target (Jia *et al.*, 2007;Yuan *et al.*, 2007).

1.4.2 Mechanisms of ß-catenin dependent transcriptional regulation of Wnt/ß-catenin target genes

Upon Wnt-stimulation, ß-catenin trans-locates to the nucleus (see 1.1.1) and binds to LEF/TCF transcription factors (Behrens *et al.*, 1996). LEF/TCF transcription factors require specific consensus sequences in the promoters of target genes, also known as Wnt responsive elements [(a/t)(a/t)CAA(a/t)G] (Roose and Clevers, 1999). The LEF/TCF transcription factor family consists of LEF1, TCF1 (TCF7), TCF3 (TCF7L1) and TCF4 (TCF7L2). Of note, phenotypic rescue experiments revealed that the function of LEF/TCFs is not redundant (Mao and Byers, 2011). Moreover, also opposite effects of the transcription factors had been determined; TCF3 mainly, but not exclusively, acts as a repressor, while LEF1 primary exerts transcriptional activatory functions (Yi *et al.*, 2011). In addition, LEF/TCFs are expressed in diverse isoforms. Dependent on the experimental system and isoform which is used, TCF1 and TCF4 exert dual functions (Mao *et al.*, 2011).

Binding of ß-catenin displaces repressors like Groucho/TLE which subsequently promotes the interaction of ß-catenin with the TATA-binding protein (TBP) and the basal transcription machinery (see section 1.4.1.1)(Bauer *et al.*, 1998;Daniels *et al.*, 2005). The activation of ß-catenin-dependent gene transcription additionally requires the de-condensation of chromatin (Figure 7). Therefore, chromatin remodeling factors have been identified which bind to ß-catenin (Novak and Dedhar, 1999), such as the histone acteylase CBP/p300 (CREB-binding protein)(Greaves *et al.*, 1999), histone methyltransferase MLL2 (mixed-lineage-leukemia 2)(Willert and Jones, 2006) and BRG-1 (brahma related gene 1) which is a component of a nucleosome remodeling complex (Barker *et al.*, 2001). In addition, ß-catenin binds to DNA helicasess such as Pontin52 (Pon) via its armadillo repeats (Bauer *et al.*, 1998), which further

supports the transcriptional activation of ß-catenin target genes (Figure 7). Co-factors, such as BCL9 and Pygopus, additionally promote the transcriptional activity (see 1.4.4).

Without Wnt-stimulation, ß-catenin is degraded and the expression of target genes is transcriptionally inhibited by different mechanisms (Figure 7). TCF transcription factors reside in the nucleus, bound to the promoters of target genes. Thus, in the absence of ß-catenin, TCFs act as transcriptional inhibitors (Gregorieff and Clevers, 2005;Gregorieff *et al.*, 2004). Transcriptionally inactive TCFs bind to repressive nuclear factors such as Groucho/TLE (Cavallo *et al.*, 1998;Levanon *et al.*, 1998), which then recruit histone deacetylases such as HDAC1 (Chen *et al.*, 1999). De-acetylation of histones subsequently results in condensation of the chromatin which represses transcription.

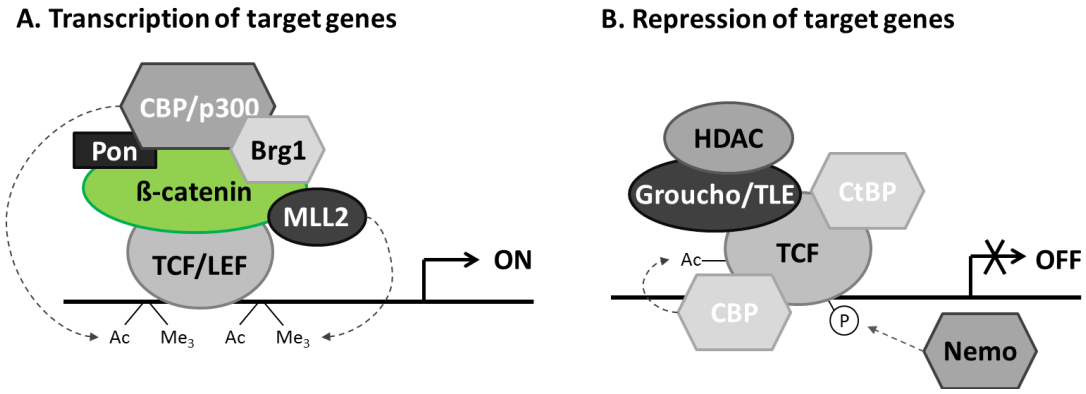

Figure 7: Transcriptional regulation of Wnt/ß-catenin target genes
(A) Transcriptionally active ß-catenin binds to LEF/TCF and recruits the helicase Pontin52 (Pon) and different chromatin remodeling factors like BRG-1 or CBP and MLL2 (MLL) which acetylate (Ac) and methylate (Me_3) histones, respectively. (B) TCF represses gene transcription in the absence of ß-catenin through the interaction with different co-repressors such as Groucho/TLE and CtBP which recruit chromatin remodeling factors like histone deacetylases (HDAC). Additionally, TCF is modified by CBP acetylases and Nemo phosphatases which inhibit binding to the DNA and ß-catenin, respectively. (adapted from Parker *et al.*, 2007)

Binding of CtBP to TCF leads to epigenetic chromatin modifications including histones de-methylation through the interaction with additional co-repressors (C-terminal binding protein) (Arce *et al.*, 2006;Brannon *et al.*, 1999;Chinnadurai, 2002). In addition, modifications of TCF promote the repression of target genes. NLK/Nemo kinases inhibit the DNA-binding affinity of the transcription factor through phosphorylation of TCF (Ishitani et al., 2003). Moreover, CBP interacts with the TCF-DNA-binding domain (HMG-box) and acetylates TCF at its N-terminal ß-catenin binding domain which prevents binding to ß-catenin and consequently the activation of transcription (Figure 7)(Waltzer and Bienz, 1998).

1.4.3. Crosstalk of LEF/TCF and SP1 in the transcriptional control of target gene expression

A crosstalk between LEF/TCF transcription factors and the function of SP transcription factors has been described (Clements *et al.*, 1996;Rossi *et al.*, 2006;Sheridan *et al.*, 1995;Thorpe *et al.*, 2005;Weidinger *et al.*, 2005;Yamaguchi *et al.*, 1999).

During central nervous system development Wnt/ß-catenin signaling activates the expression of SP5, which appeared to repress the transcriptional activation of SP1 target genes (Fujimura *et al.*, 2007).

Moreover, SP5 and the homologue SP5like mediate the function of Wnt8 in neuroectoderm and mesoderm patterning during zebrafish gastrulation (Thorpe *et al.*, 2005;Weidinger *et al.*, 2005). In mouse primitive steak and tailbud development Wnt responsive elements act in cooperation with SP1 binding sites in the promoter of T (Brachyury) to activate the expression of this gene (Clements *et al.*, 1996;Yamaguchi *et al.*, 1999). In addition, *in vitro* analyses suggest that LEF1 dependent transcription of the HIV-1 promoter requires the participation of SP1 in Jurkat T cells (Sheridan *et al.*, 1995). However, in human astrocytic cells TCF4 decreases the SP1 mediated transcription of the HIV-1 promoter through the prevention of SP1-DNA association. In this regard, interaction of TCF4 and SP1 leads to de-phosphorylation of SP1, which results in inhibited transcriptional activity (Rossi *et al.*, 2006).

The interaction of SP1 and LEF/TCF transcription factors and their modulation of each other's transcriptional functions had been described in many different systems (Clements *et al.*, 1996;Pesce *et al.*, 1999;Rossi *et al.*, 2006;Sheridan *et al.*, 1995;Thorpe *et al.*, 2005;Weidinger *et al.*, 2005;Yamaguchi *et al.*, 1999). The influence of SP1 on the function of TCF and LEF transcription factors differs in dependence of the genetic background. However, the authors pointed, that the specific functions of these interactions are highly context dependent (Rossi *et al.*, 2006;Sheridan *et al.*, 1995). Thus, the consequence of an interaction of SP and LEF/TCF could differ in dependence of the molecular background.

1.4.4 Transcriptional regulatory mechanism of Wnt/ß-catenin target genes by the BCL9 and Pygopus co-factors

In addition to basal transcriptional regulators and chromatin remodeling factors, the transcription of Wnt/ß-catenin target genes requires the co-activation by BCL9 and Pygopus proteins.

Nuclear ß-catenin, bound to LEF/TCF, interacts with BCL9/BC9-2, which further recruits Pygopus1/2 proteins (Figure 8)(Belenkaya *et al.*, 2002;Kramps *et al.*, 2002). Studies in Drosophila suggested that BCL9/*Legless* functions as an adaptor protein which mediates the recruitment of Pygopus to the basal transcriptional machinery. However, many studies demonstrated, that BCL9 and BCL9-2 themselves act as transcriptional co-activators in vertebrates (Adachi *et al.*, 2004;Brembeck *et al.*, 2011;Deka *et al.*, 2010;Sustmann *et al.*, 2008). In this regard, the C-terminus of the BCL9 proteins was shown to be essential for their co-activatory function, although the underlying mechanism is still unknown (Adachi *et al.*, 2004;Sustmann *et al.*, 2008). In contrast, Pygopus mediated transcriptional activity has been studied in more detail:

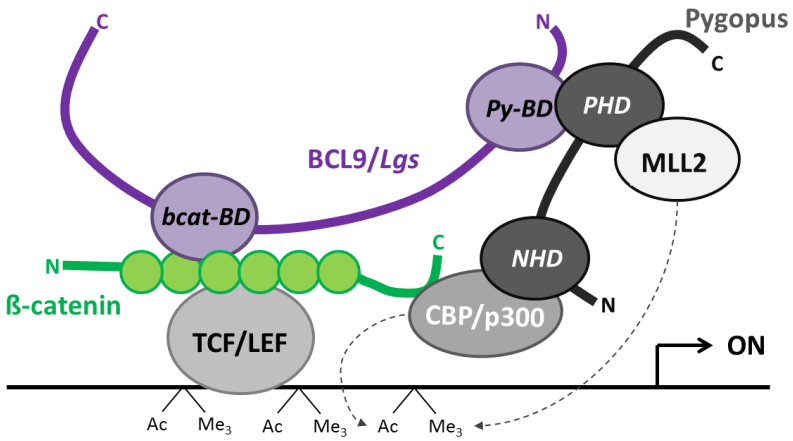

Figure 8: BCL9/Pygopus dependent transcriptional activation of Wnt/ß-catenin target genes
LEF/TCF transcription factors bind to ß-catenin, which is recruited by BCL9/*Legless* through its ß-catenin binding domain (bcat-BD). The Pygopus binding domain (Py-BD) of BCL9/*Legless* recruits Pygopus which is bound to the MLL2 methyltransferase through its PHD domain (PHD). Pygopus' NHD domain binds to CBP/p300 acetyltransferase which additionally interferes with ß-catenin which consequently activates gene transcription. (adapted from Andrews *et al.*, 2009)

Pygopus proteins modulate transcription through their C-terminal PHD plant homeo-domain (PHD) zinc finger motif and through their N-terminal homology domain (NHD). The PHD interferes with MML2 histone methyltransferases (HMT) which promotes transcription through trimethylation of histone 3 lysine residues ($H3K_4Me_3$) (Chen *et al.*, 2010;Gu *et al.*, 2009). In addition, Pygopus 2 had been shown to bind to the histone acetyltransferase

CBP/p300 (CREB binding protein). This interaction might promote the acetylation of histone 3 which subsequently results in de-condensation of chromatin and therefore transcriptional activation of Wnt/ß-catenin target genes (Figure 8)(Andrews *et al.*, 2009).

Interestingly, previous studies from our group indicated, that not all BCL9-2 dependent Wnt/ß-catenin target genes require the participation of Pygopus proteins. Consequently, BCL9-2 possibly co-activates the expression of Wnt/ß-catenin target genes independently of Pygopus interaction.

2 Aim of this work

The role of BCL9/*Legless* and Pygopus in *Drosophila* has been well described. However, little is known about the role of the BCL9 and Pygopus proteins in vertebrates. Our group has previously identified BCL9-2, the orthologue of BCL9/*Legless*, which also acts as a co-activator in the canonical Wnt/ß-catenin pathway. This study was carried out in regard to the function of BCL9 and Pygopus proteins 2 proteins during intestinal carcinogenesis. This work presented here will focus on the function of the BCL9 proteins

The aim of this study was to investigate the role of BCL9 and Pygopus proteins in intestinal homeostasis and during intestinal carcinogenesis. De-regulation of BCL9-2 in tumors might be further associated with de-regulation of other genes, which may contribute to tumor development and progression. We hypothesize that BCL9-2 in vertebrates acts not as a general enhancer of Wnt/ß-catenin-dependent transcription, but rather regulates a specific subset of target genes, possibly also independent of Wnt/ß-catenin signaling by a ß-catenin independent mechanism.

To address the role of BCL9 proteins during the multistep-model of intestinal tumorigenesis, the expression of BCL9/BCL9-2 was examined in normal, intestinal epithelia and during different stages of carcinogenesis using immunohistochemistry. In addition, adenoma formation of K19-BCL9-2/APC$^{MIN/+}$ compound mutant mice was followed to investigate the influence of BCL9-2 overexpression on tumor development.

We further investigated the specific gene signature that is induced by the BCL9 and BCL9-2 co-activators in comparison to known Wnt/ß-catenin target genes. To this end, qPCR, *Luciferase* reporter gene assays and microarray analyses were performed following knock down of the proteins.

In addition, this study addressed the regulatory mechanism concerning BCL9-2 mediated transcriptional regulation. A model system was used to dissect the ß-catenin-dependent and -independent functions of BCL9-2. For this purpose, two *Luciferase*-reporters containing the proximal promoter of a BCL9-2/ß-catenin-dependent- and a ß-catenin independent- BCL9-2 target gene were studied in detail. Finally, transcription factor binding elements which were present in both promoters were mutated. The activity of mutated *Luciferase*-reporters was analyzed in colon cancer cell lines following knockdown of BCL9-2. To identify a transcription factor that acts as putative BCL9-2 interaction partner from mediating a ß-catenin-independent co-activatory function of BCL9-2 co-immunoprecipitations were performed.

3 Materials and Methods

Unless stated otherwise, standard protocols were followed (Michael R.Green and Joseph Sambrook, 2012). Likewise, standard buffers and solutions were prepared and used as described in Sambrook, 2012.

3.1 General materials and chemicals

Disposables and basic material (Table 1), instruments and equipment (Table 2), chemicals and reagents (Table 3), commercial kits (Table 4), enzymes (Table 5) as well as antibodies (Table 6, Table 7 and Table 8) were obtained from the manufactures listed below.

Table 1: Disposables and basic material

Materials	**manufacturer**
Cryovials	Nunc
Culture plates (50 mm and 100 mm ∅)	Nunc
Cuvettes	Roth
Filters for solutions (0.2 µm and 0.45 µm)	Sartorius
Flasks for cell culture (75 cm^2 and 175 cm^2)	Sarstedt
Gloves (nitrile, latex)	Sempermed
Hybond-P PVDF membrane	GE Healthcare
Hypodermic needle (23 G)	Braun
Parafilm	Pechiney Plastic Packaging
Pasteur pipettes	Peske OHG
Petri dishes	Falcon
Pipette filter tips (10, 200 and 1000 µl)	Biozym
Pipette tips (10, 200 and 1000 µl)	MbP
Pipettes (2, 5, 10 and 25 ml)	Eppendorf
Plates for cell culture (6-well, 24-well and 96-well)	TPP, Nunc

Scalpels	Technic cut
Tubes for cell culture (polypropylene, 15 ml and 50 ml)	Falcon
Tubes for cell culture (polystyrene, 15 and 50 ml)	Falcon, Sarstedt
Tubes for molecular biology, Safelock (1.5 ml and 2 ml)	Eppendorf, Sarstedt
Whatman paper	Whatman
freezing boxes for cell cultures	Nalgene

Table 2: Instruments and equipment

Instrument	**manufacturer**
Camera DC 300 FX/Camera DFC 290	Leica
Cell counting chamber Neubauer	Brand
Cell culture incubator BBD 6220	Heraeus
Cell culture sterile bench LaminAir HB 2448	Heraeus
Refrigerated Microcentrifuge	Eppendorf
Microcentrifuge	Eppendorf
Refrigerated Bench-Top Hood Centrifuge	Eppendorf
Microcentrifuge MCF 2360	MS Co. LTD
Controlled-freezing box	Nalgene
Electrophoresis chambers for agarose gels	Peqlab
Electrophoresis chambers for SDS-PAGE	BioRAD
ELISA Reader SUNRISE A-5082	TECAN
Freezer (-150 °C) Ultra low temperature freezer MDF-C2156VAN	Sanyo
Freezer (-20 °C) PremiumNoFrost	Liebherr
Freezer (-80 °C) Ultra low temperature freezer U725	New Brunswick Scientific GmbH
Fridge (+4 °C) Electrolux SANTO	AEG
Gel documentation BioDocAnalyze	Biometra
Heating block - Thermostat plus	Eppendorf

Ice machine ZBE 70-35	Ziegra
Incubator	Memmert
Multimode Reader TriStar LB 946	Berthold
Micropipettes (2, 10, 100, 200, 1000 µl)	Eppendorf
Microscope DM 500	Leica
Microscope inverted DM IRB	Leica
Microwave oven	Powerwave
PCR cycler T3 Thermocycler	Biometra
Pipetting assistant MATRIX	Thermo Scientific
Power supplier EV231	Peqlab
Printer	Mitsubishi
Pump VDE0530	Adam.Baumüller GmbH
7900HT Fast Real-Time PCR System	Applied Biosystems
Rotator	GLW
IKA- Shaker MTS4	W.Krannich GmbH+Co.KG
Sonifier	Dr. Hielscher GmbH
Spectrophotometer ND-1000	NanoDrop
Stereomicroscope MZ FL III	Leica
Transilluminator UV Star	Biometra
UV lamp EBQ100 isolated	Leica
IKA ® Vortex	IKA
Water bath GFL 1003	W.Krannich GmbH+Co.KG
Water purification system	Millipore
Western Blot Documentation LAS-4000	Fujifilm
Wet Transfer Apparatus	Biorad
Shaker for bacteria	Sartorius
NanoDrop-Spektrophotometer	Thermo Scientific

Inkubator BBD 6220	Thermo Scientific
Rotator RM5	Assistant 348

Table 3: Chemicals and reagents

chemical/reagent	manufacturer
Acetic acid	Roth
Agarose	Invitrogen
APS (Ammonium persulfate)	Roth
benzyl-coelenterazine	SYNCHEM
Bradford reagent	BioRAD
BrdU (Bromdesoxyuridin)	Roche
Bromphenol Blue	Roth
Chloroform	Roth
Collagen	Sigma
Collagenase/Hyaluronidase Cocktail	Stemcell
D(+)-trehalose dihydrat	Roth
DAPI (4',6-diamidino-2-phenylindole)	Sigma
DEPC (diethyl pyrocarbonate)	Roth
Dexamethasone	Sigma
Dispase	Sigma
D-Luciferin potassium salt, 99%	Synchem
DMEM GlutaMAX™	Invitrogen
DMSO (dimethyl sulfoxide)	Roth
DTT (DL-Dithiothreitol)	Sigma
EDTA (ethylenediaminetetraacetate)	Roth
EGTA (ethylene glycol tetraacetic acid)	Roth
EtBr (ethidium bromide)	Roth

Ethanol	Chemie Vertrieb Hannover
FBS (Fetal bovine serum)	Invitrogen, Sigma
GeneRuler™ 1 kb DNA Ladder	Fermentas
Glycerol	Sigma
Glycine	Roth
H_2O_2 (Peroxygen)	Roth
HCl (Hydrochloric acid)	Roth
HEPES	Roth
Immu-Mount	ThermoScientific
Isopropanol	J.T.Backer
$KAlS_2O_2 \bullet 12H_2O$ (Potassium aluminium sulfate)	Sigma
KCl (potassium chloride)	Sigma
L-glutamine	Invitrogen
Lipofectamin 2000	Invitrogen
Luminol	Sigma
Methanol	J.T.Backer, Merck
$MgCl_2$ (Magnesium chloride)	Roche
$MgSO_4$ (Magnesium sulfate)	Roth
Na_2SO_4 (Sodium sulfate)	Roth
Na4PPi (Sodium pyrophosphate)	Roth
NaCl (Sodium chloride)	Roth
$NaHCO_3$ (Sodium hydrogen carbonate)	Merck
NaOH (Sodium hydroxide)	Sigma
Nicotinamid	Sigma
Nonidet-P40 (NP40)	Sigma
Opti-MEM®	Invitrogen
PageRuler™ Prestained Protein Ladder	Invitrogen

p-Coumaric acid	Sigma
Penicillin / streptomycin	Invitrogen
PFA (Paraformaldehyde)	Merck
phenyl-benzothiazole	City Chemicals
PMSF (Phenylmethanesulfonylfluoride)	Sigma
Polyacrylamide (30% Acrylamide / Bis)	Roth
Powdered milk	Roth
Protease Inhibitor cocktail tablets, EDTA free	Roche
restriction buffer O	Fermentas
restriction buffer R	Fermentas
restriction buffer XXX	Fermentas
RNA sample buffer	Fermentas
RNase Inhibitor	Fermentas
Roti®-Histokitt	Roth
Roti®-Phenol/Chloroform/Isoamylalkohol	Roth
SDS (Sodium dodecyl sulfate)	Sigma
Streptavidin-biotinylated HRP	GE Healthcare
SYBR GREEN I	Sigma Aldrich
TEMED (N,N,N',N'-Tetramethylethan-1,2-diamin)	Roth
Tris	Roth
Triton X-100	Sigma
Trypan blue	Sigma
Tween 20	Sigma
Xylene Cyanol	Roth
Xylol	Roth
β-Mercaptoethanol	Roth
Dako EnVision Kit	DAKO

Glycerin	Roth
Ampicillin	Roth
Bacto yeast extract	Roth
$CaCl_2$ (Calcium chloride)	Sigma
Bacto tryptone	Becton Dickinson
Agar-agar	Roth
5 x Passive lysis buffer	Promega
A-Sepharose	Sigma-Aldrich
Flag-M2 Agarose from mouse	Sigma-Aldrich

Table 4: Commercial kits

System	**manufacturer**
CloneJET™ PCR Cloning Kit	Fermentas
Fast Start Taq DNA Polymerase (dNTPs pack)	Roche
GeneJET™ Gel Extraction Kit	Fermentas
Plasmid Plus Midi Kit	Qiagen
Rapid DNA Ligation Kit	Fermentas
TRI Reagent RNA Isolation Kit	Ambion
GeneJET Plasmid Miniprep Kit	Thermo Scientific
SYBR Green	Sigma-Aldrich
Dako EnVision+ System HRP labeled	Dako EnVision+ System HRP labeled
Human 4 × 44 K design array 026652	Agilent Technologies
RNA Spike-In Kit for One color	Agilent Technologies
Low RNA Input linear Amplification Kit Plus, One Color	Agilent Technologies

Table 5: Enzymes

Enzyme	manufacturer
coenzyme A	AppliChem
DNase I, RNase-free	Fermentas
*Hin*dIII	Fermentas
HOT FIREPol DNA Polymerase	Solis BioDyne
Luciferin	SYNCHEM
PstI	Fermentas
EcoRI	Fermentas
Phusion High-Fidelity DNA Polymerases	Thermo Scientific
Proteinase K	Roche
Reverse transcriptase, MMLV-RT	Fermentas
RNase A	Roche
Trypsin / EDTA	Invitrogen
*Xho*I	Fermentas
Shrimp Alkaline Phosphatase (SAP)	Fermentas
BigDye® Terminator v1.1 Cycle Sequencing Kit	Applied Biosystems

Table 6: Antibodies for Western Blots

antibody	origin	dilution	product no.	source
anti-Flag-HRP	mouse	1:500	A8592	Sigma
anti-BCL9-2	rabbit	1:100	self-made	self-made (Brembeck *et al.*, 2011)
anti-β-catenin	rabbit	1:1000	self-made	self-made (Brembeck *et al.*, 2011)
anti-CypA	rabbit	1:1000	2175S	Cell Signaling
anti-α-tubulin	mouse	1:10000	T9026	Sigma

anti-β-actin	mouse	1:10000	A3853	Sigma
anti-laminB1	goat	1:100	sc-6216	Santa Cruz
anti-CDX2	rabbit	1:300	IHC-00126	Bethyl
anti-rabbit IgG HRP	goat	1:5000	111-035-144	Jackson Immunoresearch
anti-mouse IgG HRP	goat	1:5000	115-035-062	Jackson Immunoresearch

Table 7: Antibodies for immunohistochemistry (IHC)

antibody	**origin**	**dilution**	**product no.**	**manufacturer**
anti-bromodeoxyuridine	rat	1:100	ab6326	Abcam
anti-Flag	rabbit	1:250	F7425	Sigma
anti-ß-catenin	rabbit	1:2000	self-made	self-made (Brembeck *et al.*, 2011)
anti-EPHB2	goat	1:300	AF467	R&D Systems
anti-EPHB3	goat	1:300	AF432	R&D Systems
anti-EPHB4	goat	1:300	AF446	R&D Systems
anti-Bambi	rabbit	1:250	self-made	self-made (Brembeck *et al.*, 2011)
anti-mouse IgG Cy2	donkey	1:500	715-226-150	Jackson Immunoresearch
anti-rabbit IgG Cy3	donkey	1:1000	711-166-152	Jackson Immunoresearch
anti-panCK	mouse	1:300	C2562	Sigma
anti-αSMA	rabbit	1:1500	A2547	Sigma
anti-Cleaved caspase 3	rabbit	1:50	9661L	Cell Signaling
anti-rat Biotin IgG	goat	1:100	B7139	Sigma

Table 8: Antibodies for Immunoprecipitation (IP)

antibody	origin	dilution	product no.	manufacturer
anti-BCL9L (BCL9-2)	rabbit		A303-152A	Biomol/Bethyl
anti-SP1*	rabbit	1:1000	07-645	millipore
anti-PYGO2*	rabbit	1:1000	self-made	self-made (Brembeck *et al.*, 2011)

***** also used for Western Blot

3.2 Oligonucleotides and siRNA

Oligonucleotides were synthesized by ©IBA, Göttingen (Table 9 to Table 12). Restriction site sequences were introduced close to the 5' end of primers used for cloning applications to flank the resulting product with restriction sites, if required. Oligonucleotides for quantitative real-time PCR were designed using PrimerQuest^{SM} (Integrated DNA Technologies, Inc) or NCBI/Primer-BLAST, respectively. Primers were designed to span exon junctions when possible and to target all isoforms of one gene, if necessary and possible. The primer specificity was verified by nucleotide BLAST® from NCBI. Self-dimerization, hetero-dimerization and hairpin structures of the oligos were analyzed using OligoAnalyzer 3.1 (Integrated DNA Technologies, Inc).

ON-TARGETplus siRNAs were obtained from Dharmacon, Bonn (Table 13).

Table 9: Oligonucleotides for mutagenesis PCR

oligonucleotide/target	forward primer sequence (5'-3')
-386bp hCDX1 XhoI for	ACTCGAGGTGAAGTTGGCCTAGAATCCCC
+73bp hCDX1 HindIII rev	GATAAGCTTGCTGGGCCCTGGAGC
-386bp hCDX1 XhoI -367bp **BCL9** mt for	TAGCTCGAGGTGAAGTTGGC**AGT**GAATCCC
hCDX1 -112bp **NFκB** mt for	CGACGGGT**AGA**CCCCTTTGATTCG
hCDX1 -112bp **NFκB** mt rev	CGAATCAAAGGGG**TCT**ACCCGTCG
hCDX1 -105bp **TCF4** mt for	GTTTCCCC**AGC**TGATTCGCGG

hCDX1 -105bp **TCF4** mt rev	CCGCGAATCA**GCT**GGGGAAAC
hCDX1 -66bp **OCT4** mt for	CCGCTTTGAAAT**AGC**AACCCGCCTC
hCDX1 -66bp **OCT4** mt rev	GAGGCGGGTT**GCT**ATTTCAAAGCGG
hCDX1 -74bp **TCF4** mt for	CTTCCCCCCG**AGC**TGAAATGC
hCDX1 -74bp **TCF4** mt rev	GCATTTCA**GCT**CGGGGGGAAG
hCDX1 -8bp **SP1**-B for	TGGGTGGGGC**AA**GCGCGGC
hCDX1 -8bp **SP1**-B rev	GCCGCGC**TT**GCCCCACCCA
hCDX1 -137bp **SP1**-A for	CTTTTGAACCCC**TT**GCCCCCGAC
hCDX1 -137bp **SP1**-A rev	GTCGGGGGC**AA**GGGGTTCAAAAG
-456bp hCDX2 XhoI for	TAGCTCGAGGCCCGTTTCCAAACCCAGCTTC
+142bp hCDX2 HindIII rev	GTAGGAGATCTTCTAGAAAGATAAGCTTGCTGC
hCDX2 -367bp **LEF** mt for	TGATTTCATT**AGC**TGAACCTGTGATTGG
hCDX2 -367bp **LEF** mt rev	CCAATCACAGGTTCA**GCT**AATGAAATCA
hCDX2 -234bp **OCT4** mt for	TCCCCTTTATCTTTTAAAAT**TAG**AATTATGTTTCGAG
hCDX2 -234bp **OCT4** mt rev	CTCGAAACATAATT**CTA**ATTTTAAAAGATAAAGGGGA
hCDX2 -219bp **BCL6** mt for	TTAAAATGCAAATTATG**AG**C**AGC**GGGTTGTGCGTA
hCDX2 -219bp **BCL6** mt rev	TACGCACAACCCG**CTG**C**TC**ATAATTTGCATTTTAA
hCDX2 -170bp **SMAD** mt for	ACGTCTCCAACCATTGGTG**AGC**GTGTCATTACTAATA
hCDX2 -170bp **SMAD** mt rev	TATTAGTAATGACAC**GCT**CACCAATGGTTGGAGACGT
hCDX2 -258bp **NFκB** mt for	CCTCTCACGAGCC**AGA**CTCCCCTTTATCTT
hCDX2 -258bp **NFκB** mt rev	AAGATAAAGGGGAG**TCT**GGCTCGTGAGAGG
hCDX2 +95bp **SMAD4** mt for	TGGGGCGCA**TGA**ACCCGCCGCT
hCDX2 +95bp **SMAD4** mt rev	AGCGGCGGGT**TCA**TGCGCCCCA
hCDX2 -133bp **CDX2** mt for	TCTTGTAAACACTCGTT**CC**TCACGGAAGG
hCDX2 -133bp **CDX2** mt rev	CCTTCCGTGA**GG**AACGAGTGTTTACAAGA
hCDX2 -30bp **SP1** mt for	GAAGAAAGGGA**TT**GAGGGAGGAGGCAG
hCDX2 -30bp **SP1** mt rev	CTGCCTCCTCCCTC**AA**TCCCTTTCTTC

hCDX2 -266bp **SP1** mt for	CTCTCACGAGCCT**AA**CTCCCCTTTATC
hCDX2 -266bp **SP1** mt rev	GATAAAGGGGAG**TT**AGGCTCGTGAGAG
hCDX2 -89bp **SP1** mt for	CCTGTGGC**TT**GTCTTCCAAGCCTCTGCAG
hCDX2 -89bp **SP1** mt rev	CTGCAGAGGCTTGGAAGAC**AA**GCCACAGG
hCDX2 -121bp **SP1** mt for	GTTAATCACGGAAGG**CC**GAAGGCCTGG
hCDX2 -121bp **SP1** mt rev	CCAGGCCTTC**GG**CCTTCCGTGATTAAC
hCDX2 +65bp **SP1** mt for	GCAGCCAC**AA**GCCGCTCCTCGAGATCT
hCDX2 +65bp **SP1** mt rev	AGATCTCGAGGAGCGGC**TT**GTGGCTGC

Table 10: Oligonucleotides for sequencing reactions of *Luciferase* constructs

oligonucleotide/target	primer sequence (5'-3')	reference/source
pGL2Basic *LUC* intern rev	TGTAAAAGCAATTGTTCCAGGAACCAG	self-made
pGL3Basic rev	CTTTATGTTTTTGGCGTCTTCCA	Promega
pGL2Basic for	TGTATCTTAAGGTACTGTAACTG	Promega
pJET for	CGACTCACTATAGGGAGAGCGGC	Fermentas
pJET rev	AAGAACATCGATTTTCCATGGCAG	Fermentas

Table 11: Oligonucleotides for qPCR

oligonucleotide/ target	forward and reverse primer sequences (5'-3')	reference
h*AXIN2*	TCAAGTGCAAACTTTCGCCAACCG	self-made
	TGGTGCAAAGACATAGCCAGAACC	
h*BAMBI*	ATTCGATGCTACTGTGATGCTGCC	self-made
	ATTCCAATGTGGGTATGGTGGTGC	
h*BMP4*	TCTATGTGGACTTCAGCGATGTGG	self-made
	AATTGACCAGGGTCTGCACAATGG	

h*CD44*	ATCCCTCGGGTGTGCTATGGATGG	self-made
	CCTCAGTGGAAAGCAATGCCCAGG	
h*CDX1*	TAAGACTCGGACCAAGGACAAGTACC	self-made
	CTTGTTCACTTTGCGCTCCTTTGC	
h*CDX2*	TGGAGAAGGAGTTTCACTACAGTCGC	self-made
	TCTGGGACACTTCTCAGAGGACC	
h*MYC*	TCTCCACACATCAGCACAACTACG	self-made
	TGTGTTCGCCTCTTGACATTCTCC	
h*EFNB3*	GGAGGTGGATGGTTCTTATTCTGTGG	self-made
	ACATGGCAGTCATCTTAGCTGTCC	
h*EFNB2*	TTTGCAGGGATTGCTTCAGGATGC	self-made
	TTAGCGGGATGATAATGTCACTGGGC	
h*EFNB1*	TGATGATGAGCAGGAAGATGACGC	self-made
	ATGGCAAGCATGAGACTGTGAACC	
h*EPHB2*	CGCCATCTATGTCTTCCAGGTGCG	self-made
	CTCCGAGTCAGCACGCTCAAACC	
h*EPHB3*	TGGTGCTGTCATAAAAGGGCAGGC	self-made
	CCTTTATTCTGCTGTCGGCCCAGC	
h*EPHB4*	GCCAGTGTCCAGCACATGAAGTCC	self-made
	AAATTGCCAACTCCTCACCCCACG	
h*LEF1*	ACCTCAGGTCAAACAGGAACATCC	self-made
	AGTACACTCAGCAACGACATTCGC	
h*TCF1*	GAACATTTCAACAGCCCACATCCC	self-made
	ACCAGAACCTAGCATCAAGGATGG	
m*Bcl9-2*	AATCATGGCAAGACAGGGAATGGC	self-made
	TCTTCAGACTTGAGTTGCTAGGCG	

h*CTNNB1* (ß-catenin)	TTCGAAATCTTGCCCTTTGTCCCG	self-made
	AATTCGGTTGTGAACATCCCGAGC	
h*ACTB*/h*Acbt* (ß-actin)	ATAGCACAGCCTGGATAGCAACGTAC	(West *et al.*, 2004)
	CACCTTCTACAATGAGCTGCGTGTG	
h*BCL9*	ACCACATAAAGTCCCAGGATTCCC	self-made
	AAACACGTACACCACTTTGGCTGG	
h*MSX2*	AAATCTGGTTCCAGAACCGAAGGG	(Diecke *et al.*, 2008)
	ATGGGAAGCACAGGTCTATGGAACG	
h*SOX9*	CAGCGAACGCACATCAAGACGGA	self-made
	GCTGGAGTTCTGGTGGTCGGTGTA	
h*SOX6*	GCAACTACCACACCATCGCCTC	self-made
	TAGGCTTCCGCCATCTGTCTTC	
h*SOX4*	CCCGGACTACTGCACGCC	self-made
	CGAGAACGCGGGCGAACGA	
h*OCT4*	GAGAACCGAGTGAGAGGCAACC	(Willems *et al.*, 2006)
	CATAGTCGCTGCTTGATCGCTTG	
h*CYPA*	CCCACCGTGTTCTTCGACAT	(Murphy *et al.*, 2003)
	CCAGTGCTCAGAGCACGAAA	
h*SDNSF*	TCAACAAACCAGAGGCGGAGATGT	self-made
	TTAGTGGTGCCTGTTCACTCCCTT	
h*LGR5*	ACACATTGCCCTGTTGCTCTTCAC	self-made
	TTTCTCAGGCTCACCAGATCCTCC	
h*BCL9-2*	ATCCCTCCAAACCATAAAGCACCC	self-made
	TGGAACTGGGCATTGCAAACTTGG	
h*CCND1* (CyclinD1)	TGGTGAACAAGCTCAAGTGGAACC	self-made
	ATGGAGGGCGGATTGGAAATGAAC	

h*PROX1*	AGAGCCTCCCATTACTCAGACCCG	self-made
	TGGTCAGGCATCACTGGACGGT	

Table 12: Oligonucleotides for conventional PCR

oligonucleotide/target	primer sequence (5'-3')	
BCL9-2 intron-TG for	CTGGTCATCATCCTGCCTTT	self-made
BCL9-2 exon1 for	TCCTGGCTAACAAGACAAGG	self-made
BCL9-2 exon2 rev	CTCTGAATCGAGGGATGGAG	self-made
MIN-wt for	GCCATCCCTTCACGTTAG	self-made
MIN-wt rev	TTCCACTTTGGCATAAGGC	self-made
MIN-mt for	TTCTGAGAAAGACAGAAGTT**A**	self-made
ß-actin for	TGGCACCACACCTTCTACAATGAGC	self-made
ß-actin rev	GCACAGCTTCTCCTTAATGTCACGC	self-made

Table 13: ON-TARGETplus siRNA (Dharacon)

siRNA/target	ID-No.	sequence (5'-3')
ON-TARGETplus Non-targeting Pool		not provided
Human *CTNNB1* (ß-catenin)	003482-09	GAUCCUAGCUAUCGUUCUU
	003482-10	UUAAUGAGGACCUAUACUUA
	003482-11	GCGUUUGGCUGAACCAUCA
	003482-12	GGUACGAGCUGCUAUGUUC
Human *BCL9L* (BCL9-2)	010858-05	GCUCAUGCCUUCACAGUUU
	010858-06	GAAAGCCUCCCUCGCAGUU
	010858-07	CACCCACAAUUGUAAUGUA
	010858-08	AACCAGAUCUCGCCUAGCA

Human *BCL9*	007268-05	CUACUGAGUAGGCCAAUAA
	007268-06	CCAAAGUGGUGUACGUGUU
	007268-07	GCGGAAGCCCUUGGAUAUA
	007268-08	UUUGAUCUAUCCCGCAUUA

3.3 Cell biology

3.3.1 Bacterial strains

For cloning procedures and plasmid preparation the chemo-competent *E. coli* strain One Shot® Top10F` (Invitrogen, Darmstadt) with the following genotype was used: *F´{lacIq Tn10 (TetR) mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL endA1 nupG.*

3.3.2 Cell lines and mouse strains

Cell lines were obtained from ATCC and LGC (LGC Standards GmbH, Wesel) and listed in Table 14. Utilized mouse strains are listed in Table 15.

Table 14: Cell lines

cell line	Description
SW480	Colon cancer cell line established from a primary adenocarcinoma of the colon of a 50 years old Caucasian male (Leibovitz *et al.*, 1976).
SW620	Colorectal adenocarcinoma isolated from the tissue (lymph node metastasis) of a 51 years old Caucasian male as was SW480 (Leibovitz *et al.*, 1976).
CACO2	Colorectal adenocarcinoma cell line derived from a 72 years old Caucasian male (Fogh *et al.*, 1977b).
CT26	N-nitroso-N-methylurethane-(NNMU), undifferentiated induced colon carcinoma cell line derived from BALB/c mice (Wang *et al.*, 1995).
HT29	Epithelial colorectal adenocarcinoma cell line from a 44 years old Caucasian woman (Fogh *et al.*, 1977a).

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).
WiDr	Colon adenocarcinoma line from a 78 year old female, derivative of HT-29 (Chen *et al.*, 1987;Noguchi *et al.*, 1979).
SW48	Colon cancer cell line established from a primary adenocarcinoma of the colon of a 82 years old Caucasian female (Leibovitz *et al.*, 1976).
Lovo	initiated in 1971 from a fragment of a metastatic tumor nodule in the left supraclavicular region of a 56 years old Caucasian male patient with a adenocarcinoma of the colon (Drewinko *et al.*, 1976).
Ls174T	variant of LS 180, which is a colorectal adenocarcinoma cell line from a 58 years old Caucasian woman (Tom *et al.*, 1976).
HEK293	Human embryonic kidney cell line (Graham *et al.*, 1977).
MCF7	Breast cancer cell line isolated in 1970 from a 69 years old Caucasian woman (Soule *et al.*, 1973).

Table 15: Utilized mouse strains

mouse strain	Description
$APC^{Min/+}$	Derived from an *N*-ethyl-*N*-nitrosourea (ENU) treated founder C57BL/6J mice, that induced a point mutation (T to A transversion of nucleotide 2549) in the APC gene. Already young mice are highly susceptible to spontaneous intestinal adenoma formation (Su *et al.*, 1992). (Jackson Laboratories)
K19-BCL9-2	Transgenic mice express BCL9-2 under control of a K19 promoter in simple epithelia, including the intestine, mammary glands and pancreas (Brembeck *et al.*, 2011).

3.4 Plasmids

Table 16: *Luciferase*-reporter plasmids

Plasmid	source /reference
ptkRenilla	Promega
TOPflash/FOPflash	R.T Moon, Seattle (Korinek *et al.*, 1997)
pGL2Basic	Promega
pGL3Basic	Promega
pGL3Basic -5,6kb Axin2	F. Constantini, New York (Jho *et al.*, 2002)
pA3 *LUC* Cyclin D1	R. Pestell, Philadelphia (Shtutman *et al.*, 1999)
pGL2Basic -3189bp CDX1	J. Lynch, Philadelphia (Suh *et al.*, 2002)
pGL2Basic -386bp CDX1	J. Lynch, Philadelphia (Suh *et al.*, 2002)
pGL2Basic -1434bp CDX2	J. Lynch, Philadelphia (unpublished)
pGL2Basic -456bp CDX2	J. Lynch, Philadelphia (unpublished)
pGL2Basic -281bp CDX2	J. Lynch, Philadelphia (unpublished)
pGL2Basic -386bp CDX1 -377BCL6-BS mt	this work
pGL2Basic -386bp CDX1 -122NFκB-BS mt	this work
pGL2Basic -386bp CDX1 -113LEF-BS mt	this work
pGL2Basic -386bp CDX1 -81OCT4-BS mt	this work
pGL2Basic -386bp CDX1 -82LEF-BS mt	this work
pGL2Basic -386bp CDX1 -82LEF-TF /-113LEF-BS mt	this work
pGL2Basic -386bp CDX1 –8SP1-BS mt	this work

pGL2Basic -386bp CDX1 –137SP1-BS mt	this work
pGL2Basic -386bp CDX1 –8SP1-BS mt/-137SP1-BS	this work
pGL2Basic -456bp CDX2 -229BCL6-BS mt	this work
pGL2Basic -456bp CDX2 -373LEF-BS mt	this work
pGL2Basic -456bp CDX2 -240OCT4-BS mt	this work
pGL2Basic -456bp CDX2 -264NFκB-BS mt	this work
pGL2Basic -456bp CDX2 -175Smad-BS mt	this work
pGL2Basic -456bp CDX2 +62Smad-BS mt	this work
pGL2Basic -456bp CDX2 -132CDX2-BS mt	this work
pGL2Basic -456bp CDX2 -30bp SP1-BS mt	this work
pGL2Basic -456bp CDX2 -266bp SP1-BS mt	this work
pGL2Basic -456bp CDX2 -89bp SP1-BS mt	this work
pGL2Basic -456bp CDX2 -121bp SP1-BS mt	this work
pGL2Basic -456bp CDX2 +65bp SP1-BS mt	this work
pGL2Basic -456bp CDX2 -30bp SP1-BS mt/-266 SP1-BS mt	this work

Table 17: Eukaryotic overexpression plasmids

Plasmid	**source/reference**
pcDNA-flag	Self-made (Brembeck *et al.*, 2004)
pcDNA-HA hOCT4	S. Monecke / R. Dressel, Göttingen (unpublished)

3.5 Buffers and solutions

Standard buffers and solutions were prepared as described in (Michael R.Green *et al.*, 2012). The compositions of additional buffers and solutions are listed in the corresponding method sections. For sterilization, solutions were autoclaved (120°C for 60 min, 2 bar) or filtrated with a 0.2 µm sterile filter**.**

3.6 Microbiology

3.6.1 Cultivation and storage of *E. coli*

For glycerin stocks and low scale plasmid preparations which required small volumes of *E. coli* bacterial suspensions 2 ml, for large scale plasmid preparations 250 ml LB medium was supplemented with Ampicillin antibiotics. *E. coli* bacterial suspensions were cultured o/n at 37°C in an incubator shaker at 220 rpm.

For long-term storage 700 µl *E. coli* suspension was vigorously mixed with 300 µl 100 % glycerin and stored at -80 °C.

3.6.2 Generation of chemo-competent *E. coli*

200 ml LB-medium was inoculated with 5 ml of an o/n *E. coli* suspension. The culture was incubated until an OD_{600} of 0.5 to 0.6 was reached. The bacterial suspension was incubated on ice for 10 min and afterwards centrifuged for 10 min at 4°C with 4600 rpm. The resulting pellets were resolved in 80 ml 100mM $CaCl_2$ and incubated on ice for further 10 min. Following an additional centrifugation step *E. coli* pellets were resolved in 8 ml 100 mM $CaCl_2$, supplemented with 600 µl glycerin and incubated on ice for 15 min. Aliquots of 100 µl competent bacterial suspensions were frozen in liquid nitrogen and stored at -80°C.

3.6.2 Transformation of *E. coli*

Plasmid DNA was introduced in *E. coli* bacteria using heat shock. Therefore, 10 µl ligation reaction was added to 50 µl of chemo-competent *E. coli* bacterial solution. Following 45 min of incubation on ice the transformation mix was set to 42°C for 60 s. After the solution was cooled down on ice 500 µl SOC medium were added and incubated at 37°C and 330 rpm for 60 min. The mixture was then plated on LB agar plates and cultivated o/n at 37°C.

3.7 Molecular biology

3.7.1 Isolation of nucleic acids

3.7.1.1 Isolation of genomic DNA from tails of transgenic mice

Genomic DNA from mouse tails was used for genotyping transgenic mice. 2 mm of a severed mouse tail tip was lysed by adding 50 µl Lysis buffer followed by o/n incubation at 65°C and 500 rpm. The mixture was diluted to a final volume of 500 µl and centrifuged with maximum speed. 1 to 2 µl supernatant was used for genotyping PCR (2.7.3.1).

Lysis buffer	100 mM Tris-HCl pH 8.5, 5 mM EDTA pH 8.0, 200 mM NaCl, 0.2 % (w/v) SDS, 200 µg/ml Proteinase K

3.7.1.2 Isolation of total RNA

Cells were seeded in 6-well plates (according to Table 18) for RNA isolation from cell cultures and transfected and incubated as described in 3.9.3.2. Cells were lysed by adding 1 ml TRI Reagent (Ambion, Darmstadt). For isolation of RNA from mouse tissues TRI Reagent was added to fresh tissues according to manufacturer's instructions. Cells and tissues were frozen in liquid nitrogen for storage at -80°C or homogenized, respectively. Total RNA was isolated using the TRI Reagent RNA Isolation Kit according to manufacturer's instructions. To avoid contamination of RNA with genomic DNA, RNA isolated from mouse tissues was digested with DNaseI (Fermentas, St. Leon-Rot) for 1.5 h according to manufacturer's instruction. RNA was then purificated according to 3.2.2.2. The quality and quantity of RNA was determined using NanoDrop and Agarose gel electrophoresis.

3.7.1.3 Isolation of plasmid DNA from *E. coli*

Plasmid DNA was isolated from o/n *E.coli* bacterial suspensions (2.6.1). The GeneJET Plasmid Miniprep Kit (Fermentas, St. Leon-Rot) was used for cloning procedures, the Plasmid plus Midi Kit (Qiagen, Hilden) for isolation of large amount of plasmid DNA according to manufacturer's instructions. DNA was dissolved in dH2O and stored at -20 °C.

3.7.2 Purification and extraction of nucleic acids

3.7.2.1 Extraction of nucleic acids from agarose gels

DNA fragments were extracted and purified from agarose gels using the GeneJET™ Gel Extraction Kit (Fermentas, St. Leon-Rot) according to the manufacturer's instructions.

3.7.2.2 Phenol chloroform extraction

After DNase digestion (3.7.1.2) RNA solutions were purified from proteins and other contaminants using phenol chloroform extraction. Therefore 1 volume of RNA solution was consecutively mixed thoroughly with 1 volume phenol chloroform isoamylalcohol solution. The solution was centrifuged at 4°C with 10500 rpm and the aqueous phase was transferred into a new reaction tube. Phenol remains were removed by adding ammonium acetate to a final concentration of 2.5 M and precipitated with 100% Ethanol (70% final) and a subsequent washing step with 1.5 ml 70 % Ethanol.

2.7.3 Amplification of nucleic acids

2.7.3.1 Detection of genomic DNA and transcripts by Polymerase chain reaction (PCR)

Genotypes of transgenic mice and the expression of transgenic BCL9-2 and ß-actin were examined by PCR using specific primers (Table 12) and the following cycling conditions

for genotyping of K19-BCL9-2 mice,
expression of TG-BCL9-2 and ß-actin mRNA

95°C	10 min	
95°C	30 s	⎡
60°C	30 s	⎢ 35 x
72°C	150 s	⎣
72°C	10 min	

for genotyping of $APC^{MIN/+}$ mice

95°C	10 min	
95°C	30 s	⎡
45°C	30 s	⎢ 40 x
72°C	150 s	⎣
72°C	10 min	

For analytical proposes a reaction was set up as follows:

1x Taq buffer, 2.5 mM $MgCl_2$, 0.2 µM primer each, 0.2 mM dNTPs each, 0.6 units FastTaq DNA Polymerase and in a final volume of 15 µl per reaction. 1 µl of genomic mouse tail DNA (see 3.2.1.1) or 10 ng (for ß-actin) to 500 ng (transgenic BCL9-2) cDNA from mouse tissues (see

3.2.3.2.) were used as templates. Instead, addition of H_2O was served as negative control. The resulting products were analyzed in 1 % agarose gels.

3.7.3.2 Mutagenesis Polymerase chain reaction (mt-PCR)

Single mutations were introduced in the promoters of *Luciferase*-reporters by mutagenesis PCR. Therefore forward and reverse oligonucleotides were used, which carry the final mutation. In a first step 2 PCR reactions were performed to introduce the mutated transcription factor binding site. To this end, forward mutagenesis primers and reverse primers targeting the 5' end of the promoter were used. The second PCR reaction was performed with forward primers targeting the 3' end of the promoter and reverse mutagenesis primers (Figure 9A). In a second step the amplified products of step 1 were used as templates for PCR. In addition, forward primers targeting the 3' - and reverse primers targeting the 5' end of the promoter were added to amplify the final product (Figure 9B).

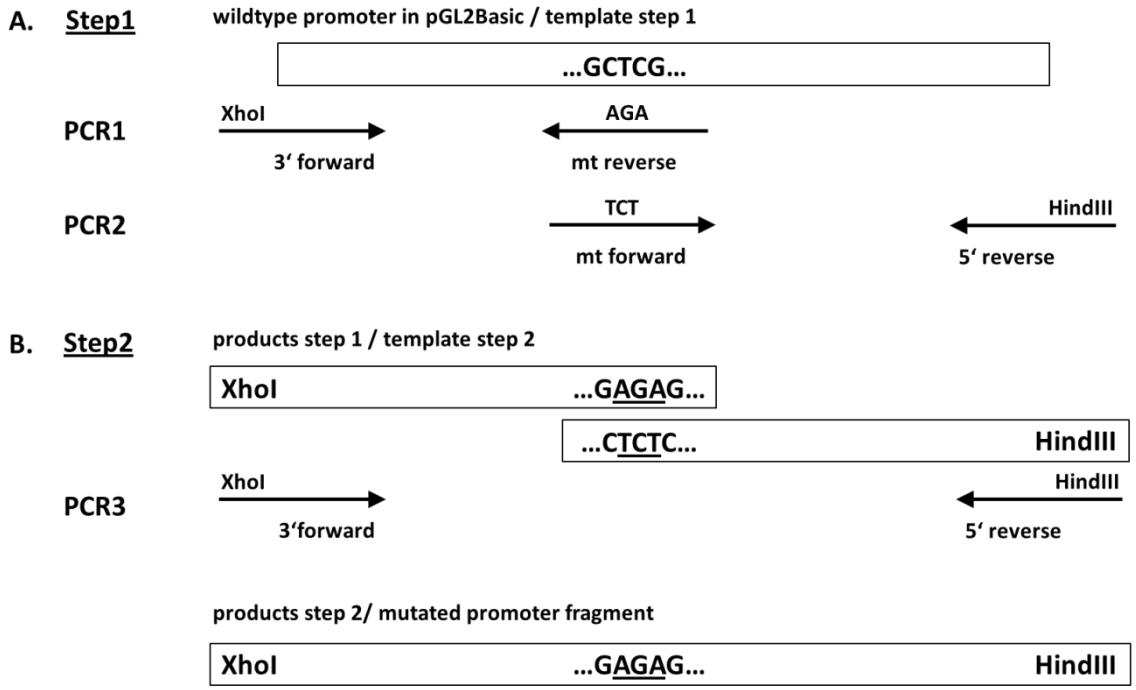

Figure 9: Scheme of mutagenesis PCR to indroduce mutations in the promoters of CDX1 and CDX2.

For preparative proposes Phusion polymerase was used, since this enzyme exhibits proof reading activity. Phusion driven reactions were set up as follows:

1 x Phusion PCR buffer, 0.8 mM dNTP-Mix, 10 pmol primers each, 2 U Phusion polymerase and 100 ng template in a final volume of 50 µl.

Cycle conditions for mutagenesis PCR were set as shown in the following. Thereby the melting temperature T_M of the used oligonucleotides (Table 9) was calculated as described in 2.2

95°C	10 min	
92°C	15 s	5 x
50 °C	15 s	
72°C	30 s	
95°C	15 s	35 x
60 °C	15 s	
72°C	30 s	
72°C	10 min	

2.7.3.3 Synthesis of copy DNA (cDNA)

RNA samples were transcribed into cDNA for qPCR and PCR. To this end, the Reverse transcriptase (RT), MMLV-RT system (Fermentas, St. Leon-Rot) was used and adapted as described.

For cDNA synthesis 2.5 µg RNA derived from cell cultures or 7.5 µg RNA isolated from mouse tissues were added to 3 pmol random hexamer primer in a total volume of 31 µl. 30 µl were used as cDNA sample, 1 µl was saved as –RT control. After an initial incubation of the samples at 65°C for 5 min the following master mix was added to a final volume of 60 µl: 1x MMLV RTase buffer, 20 mM DTT, 60 U RNase Out, 10 mM dNTPs, 150 U MMLV reverse transcriptase. The samples were subsequently incubated for 10 min at 25°C, followed by 60 min at 37°C and 10 min at 70°C. The resulting cDNA was stored at -20°C.

3.7.3.4 Quantitative real-time PCR (qPCR)

Depending on the expression level of the transcript of interest 15 ng to 30 ng of cDNA was used per sample. The cDNA was added to 3 pmol oligonucleotides and 7,5 µl SYBRGreen PCR Master Mix to a final volume of 10 µl. To investigate the transcription of specific mRNAs oligonucleotides with and efficiency of 2 ± 0.08 and an annealing temperature of 60°C ± 1°C were used, which resulted in the following cycle conditions: 12 min 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 60°C, 30 s at 72°C and subsequently 10 min at 72°C. A melting curve was added consisting of an initial 95°C denaturation step for 15 s, followed by 60°C for 30 s and a gradually heating step to 95°C.

SYBR Green PCR Master Mix	75 mM Tris-HCl pH 8.8, 20 mM (NH4)2SO4, 0.01% Tween-20, 3 mM MgCl2, 0.2 mM dNTP's, 20 U/ml HOT FIREPol DNA Polymerase, 0.25% TritonX-100, 500 mM D(+)-Trehalose Dihydrat, 1:80000 (v/v) Cybr

Green

Finally, the relative expression of target genes was calculated by $2^{-\Delta\Delta Ct}$ (Fleige *et al.*, 2006). The composition of the SYBR Green PCR Master Mix was kindly provided by Prof. Dr. Steven Johnsen, Dept. of Molecular Oncology, Georg August University Göttingen, Germany.

3.7.4 Molecular cloning

The purified *CDX1* and *CDX2* promoter fragments generated by mutagenesis PCR (3.7.3.2) were digested using the added restriction sites *Xho*I and *Hin*dIII (3.7.4.1) and subsequently subcloned into pJET2.1 (Fermentas, St. Leon-Rot) (3.7.4.3). Finally, the promoter fragments were cloned from pJET2.1 into pGL2Basic. For *CDX1* promoters, *Xho*I/*Hin*dIII were used, while for *CDX2* promoter fragments *Xho*I was used. In parallel, pGL2Basic vectors were restricted with respect to their prospective insert and dephosphorylated. The linear fragments were separated from the vector backbone or non-digested vector remains, respectively, by gel-electrophoresis and purified from the gels (3.7.2.1). After ligation (3.7.4.3) the successful insertion of the mutated *CDX1* and *CDX2* promoter fragments in pGL2Basic was finally checked by sequencing using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, California) according to manufacturer's instructions using specific primers listed in Table 10 and test restriction (3.7.4.1).

3.7.4.1 Restriction of nucleic acids with restriction endonucleases

DNA-fragments and plasmids were restricted according to DoubleDigest™ standard protocols available from the Fermentas web page (http://www.thermoscientificbio.com/webtools/doubledigest/). *CDX1* promoter containing pGL2Basic plasmids were resticted in R-buffer with *EcoRI* and *XhoI*, *CDX2* promoter containing pGL2Basic vectors were restricted in O-buffer with *EcoRI* and *XhoI* (Fermentas, St. Leon-Rot).

3.7.4.2 Dephosphorylation of nucleic acids

Phosphate residues were removed from pGL2Basic vectors using calf intestine phosphatase (CIP). To this end, 10 U of Shrimp Alkaline Phosphatase (SAP) was added to 10 µg restriction digest and incubated for 60 min at 37 °C.

3.7.4.3 Ligation of DNA fragments

For sub-cloning of mutated *CDX1* and *CDX2* promoter fragments into pJET2.1 the CloneJET™ PCR Cloning Kit (Fermentas, St. Leon-Rot) was used according to manufacturer's instructions.

Digested and purified CDX1/CDX2 promoter fragments and pGL2Basic vectors were ligated using the Rapid DNA Ligation Kit (Fermentas, St. Leon-Rot). 100 ng vector DNA and 200 ng promoter fragments were incubated with 1 µl T4 Ligase in 1 x Rapid ligation buffer for 10 min at 22°C.

The resulting ligation reaction was used for transformation of competent *E. coli* (3.6.2).

3.7.5 Microarray analysis

Biological triplicates of HCT116, SW480 and MCF7 cells were transfected with 2 specific siRNAs for ß-catenin, BCL9 and BCL9-2 knockdown and 4 unspecific non-targeting siRNAs as controls, with a final concentration of 50 nmol for 48h according to standard protocols. Extraction of RNA was performed using TRI Reagent (Ambion, Darmstadt) according to manufacturer's instructions (3.7.1.2).

cDNA synthesis, *in vitro* transcription, hybridization and initial analyses of expression data were performed in the "Transkritomanalyselabor" (TAL), Göttingen. cDNA synthesis and *in vitro* transcription (IVT) were performed according to the manufacturer's recommendation using "Low RNA Input linear Amplification Kit Plus, One Color" protocol (Agilent Technologies, Böblingen) and the RNA Spike-In Kit for One color (Agilent Technologies, Böblingen). Global gene expression analysis was done using the Human 4 × 44 K design array 026652 (Agilent Technologies, Böblingen). Hybridizations were performed for 17 hours at 10 rpm and 65°C in the Hybridization Oven (Agilent Technologies, Böblingen). Cy3 intensities were detected by one-color scanning using an Agilent DNA microarray scanner at 5 micron resolution. Intensity data were extracted using Agilent's Feature Extraction (FE) software (version 10.7.3.1) including a quality control based on internal controls using Agilent's protocol GE1_107_Sep09. All chips passed the quality control and were analyzed using the Limma package (1) of Bioconductor (2).

3.8 Biochemistry

3.8.1 Isolation of proteins from cell cultures

3.8.1.1 Isolation of whole cell protein lysates for SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Whole cell protein lysates were obtained from confluent cell cultures grown in 10 cm culture dishes. After 2 washing steps with ice-cold 1 x PBS, cells were lysed with 1 x RIPA buffer supplemented with protease inhibitors, harvested with a cell scraper and transferred into a new reaction tube. Lysates were centrifuged with maximum speed at 4°C after incubation on ice for 10 min. The supernatant was transferred into a new reaction tube and stored at -80°C or immediately used for SDS-PAGE.

Protein concentrations were determined according to Bradford standard protocol (Bradford, 1976). To the appropriate amount of protein, 4 x Laemmli probe puffer (LPP) was added. It was thenboiled for 5 min at 95°C and stored at -20°C.

3.8.1.2 Isolation of nuclear and cytoplasmatic proteins for SDS-PAGE

To separate nuclear and cytoplasmatic protein fractions, confluent cell cultures grown in 6 well culture plates were immediately lysed in 100µl of buffer A supplemented with protease inhibitors, scabbed off and transferred into a new reaction tube. After 15 min incubation on ice 5µl of 10% NP40 were added. The samples were vortexed for 20 s and centrifuged for 2 min at 3000 rpm at 4°C. An appropriate amount of 4 x LPP was added to the supernatant containing the cytoplasmatic fraction. The remaining nuclear pellet was resuspended in 60 µl ice-cold buffer C (supplemented with protease inhibitors) and incubated for 15 min at 4°C while rocking. After centrifugation for 5 min at 3000 rpm 4°C the supernatant was supplemented with an appropriate amount of 4 x LPP. All samples were boiled for 5 min at 95°C and stored at -20°C.

3.8.1.3 Isolation of protein lysates for immunoprecipitation

Proteins for immunoprecipitations were isolated according to 2.8.1.2 and 2.8.1.3. For generation of whole cell lysates, in contrast, MS buffer was used to lyse the cells. For generation of nuclear lysates 10 cm culture dishes containing confluent cells were lysed with 500 µl buffer A. Finally, solubilisation of nuclear pellets was achieved with 100 µl buffer A. For immunoprecipitation 400 µl MS buffer was added to the nuclear protein lysate.

75 µl of cell lysates served as INPUT-control. To these, 25 µl 4 x Laemmli probe buffer (LPP) was added. They were stored at -20°C.

MS buffer	20 mM Tris pH7.4, 150 mM NaCl, 1% NP-40, 0.5% Sodium Deoxychelate, 1mM EDTA, 0.1% SDS , 20 mM NaF, Protease Inhibitor Cocktail (Roche)
MSW buffer	50 mM Tris pH7.4, 150 mM NaCl, 20 mM NaF
Buffer A	10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA
Buffer C	20 mM Hepes, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA

3.8.1.4 Isolation of proteins for *Luciferase* assays

Whole cell lysates for *Luciferase* assays were obtained from cells previously transfected with *Luciferase*-reporters as described in 3.9.3.2.

For isolation of *Luciferase* proteins the medium was removed and 50 µl of 1 x passive lysis buffer (Qiagen, Hilden) was added to the confluent cells. The reaction was incubated for 60 min at RT. Cell lysates were transferred into reaction tubes and centrifuged for 2 min with maximum speed. Measurement of *Luciferase* activity was performed as described in 3.8.4.

3.8.2 SDS-PAGE and Western Blot

Protein samples from whole cell lysates or nuclear/cytoplasmatic fractions were used for SDS-PAGE and subsequent Western blotting to determine the expression of specific proteins in cell cultures per se or after treatment with siRNAs. The molecular weight of the separated proteins was determined by comparison with a pre-stained protein ladder.

Proteins were concentrated in the stacking gel at 100 V, followed by separation at 150 to 200 V. Subsequently, proteins were transferred onto methanol-activated Hybond-P PVDF membranes at 4°C using a wet blot system. Depending on the size of proteins 0.5 mA were applied for 1.25 h to 1.5 h. For immunostaining the membranes were previously blocked with blocking solution to avoid unspecific binding of the primary antibody (Table 6) which was incubated o/n at 4°C with dilution according to the instructions of the manufacturer. After the membranes were washed 3 times with TBST, the horseradish peroxidase (HRP) coupled secondary antibody diluted in TBST with 5 % powdered milk was added. The membranes were then incubated for 45 min at RT. The membranes were again washed 3 times with TBST.

Proteins bound to antibody-complexes were visualized after incubation with 1:1 (v/v) ECL solution A and B for 60 s. The resulting chemo-luminescent signals were detected with LAS-4000 imaging system. Finally, the membranes were washed with TBST to remove ECL solution residues and air-dried for storage at 4°C.

SDS transfer buffer	2.5 mM Tris, 19.2 mM Glycine, 0.01% (w/v) SDS
Stacking gel	12 % Acrylamid/Bis (10%), 25 % (v/v) Stacking buffer, 0,01 % (v/v) TEMED, 0,1 % (v/v) APS
Separating gel (10 %)	33 % Acrylamid/Bis (30 %), 25 % Separating buffer, 0.01 % (v/v) TEMED, 0.1 % (v/v) APS (10 %)
Separating gel (10 %)	10% Acrylamide/Bis, 2.5 ml Separating Buffer (4 x), 5 ml water, 100 µl APS (10%), 10 µl TEMED
TBST	50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.05% (v/v) Tween 20
Blocking Solution	5% powdered milk in TBST
ECL solution A	2.5 mM Luminol, 0.4 mM p-coumaric acid, 0.1 M Tris-HCl (pH 8.5)
ECL solution B	0.05% (v/v), 35% H_2O_2, 0.1 M Tris-HCl (pH 8.5)
Stacking buffer (pH 6.8)	0.5 M Tris, 0.4% (w/v) SDS
Separating buffer (pH 8.8)	1.5 M Tris, 0.4% (w/v) SDS

3.8.3 Immunoprecipitation

Protein lysates used to identify specific protein-protein-complexes were obtained according to 3.8.1.3.

To couple specific proteins to sepharose beads 2 µg of respective antibodies were added to the cell lysates and incubated for 30 min at 4°C followed by addition of 100 µl pre-swollen A-sepharose beads (50% slurry) in MS buffer. The protein-antibody-bead-complexes were generated during incubation of the sample o/n at 4°C rotating. Loaded beads were pelleted and centrifuged with 2000 rpm at 4°C. After washing the complexes 6 times with MS wash buffer the pellets were resuspended in 40 µl MS buffer and directly used for mass-spectrometric analyses. For Western Blot analyses 25 µl 4 x Laemmli probe buffer (LPP) was added. Samples were stored at -20°C.

3.8.4 *Luciferase* assays

Luciferase assays were performed in duplicates according to the protocols published by Hampf (Hampf and Gossen, 2006).

40 µl of the supernatant of lysed cells (as described in 2.8.1.4) was transferred into 96-well luminometer plates. Luminescence intensity was measured with the Berthold Multimode Reader TriStar LB 946. Injectors were washed before and after usage with 20 cycles of H_2O, 20 cycles of ethanol, 20 cycles of H_2O and 20 cycles of air.

Firefly *Luciferase* activity was determined by injecting 100 µl P/Fluc-A solution, followed by 0.5 s delay and 10 s measurement of luminescence. Accordingly, Renilla *Luciferase* activity was quantified by injecting 75 µl P/Rluc-B solution followed by 0.5 s delay and 10 s measurement of luminescence. Firefly *Luciferase* activity was normalized to the respective Renilla *Luciferase* activity. In addition, *Luciferase* (*LUC*)-promoter-reporter activities were relativized to their empty control *LUC*-reporters. Mean values of at least 3 independent experiments and their standard deviations were calculated and displayed graphically using Microsoft Excel. Statistical significances were based on conventional students t-tests.

P/Fluc-A solution (pH 8.0)	15mM $MgSO_4$, 0.1mM EDTA, 25mM DTT, 1mM ATP, 200 µM coenzyme A, 200 µM luciferin, 200 mM Tris-HCl
P/Rluc-B solution (pH 5.0)	0.05% (v/v), 35% H_2O_2, 0.1 M Tris-HCl (pH 8.5) 10mM NaAc, 15mM EDTA, 500mM NaCl, 50 µM phenyl-benzothiazole, 4 µM benzyl-coelenterazine, 500mM Na_2SO_4, 25mM $Na4PP_i$

3.9 *In vivo* model systems

3.9.1 Mouse model systems

To investigate the role of BCL9-2 *in vivo* a mouse model has been established (Brembeck *et al.*, 2011). To this end the 2.1-kilobase mouse keratin 19 promoter (Brembeck *et al.*, 2001) was linked to a rabbit ß-globin intron and the flag tagged, full-length cDNA of mouse BCL9-2 and a BGH-polyA. Genotypes of K19-BCL9-2 transgenic offspring were determined by conventional PCR (as described in 2.7.3.1) using specific oligonucleotides targeting the BCL9-2 transgene (Table 12).

Transgenic K19-BCL9-2 mice were bred with $APC^{Min/+}$ mice (Table 15) to investigate the influence of transgenic BCL9-2 expression on tumor formation. $APC^{Min/+}$;K19-BCL9-2 mice were followed up to 5.5 month of age or until mice showed severe tumor-related diseases before they were dissected. Macroscopic tumors of the intestine (≥0.5 mm^2) were identified with a dissection lens. The number and size of adenomas was determined in the small intestine and the colon. To secure accurate analyses counted numbers of adenomas were regularly re-checked by additional counting and measuring by a second person. Box Plot analyses were evaluated and graphically visualized by SPSS 19.0. Significances were calculated using the Whitney-U test. The significance for survival analysis was defined according to P log-rank test.

To label proliferating cells in the intestine 0.1 mg/g of bodyweight bromo-deoxyuridine in 1 x PBS (Table 3) was injected intraperitoneally 2h before the mice were dissected. Mice were raised by the ENI animal facility, Göttingen with the approval by governmental authorities.

3.9.2 Tissue processing for immunohistochemistry and Immunofluorescence

Tissue of transgenic and control mice were fixed in 4% paraformaldehyde in 1 x PBS at 4°C overnight and washed with cold 1x PBS. For long-term storage, tissue samples were transferred into 70% ethanol or dehydrated according to standard protocols (75% Ethanol, 80% Ethanol, 90% Ethanol, 96% Ethanol and 2 times 100% Ethanol for 1.5 h each). For 3 µm serial sections samples were incubation in xylol 2 times 1.5 h and paraffin for 1.5 h to 12 h and stored at RT or processed according to 3.3.5.

3.9.3 Immunostaining of tissues

For Hematoxylin and Eosin (H&E) staining and immunostaining 3 µm serial tissue sections (3.9.2) from transgenic and control mice were rehydrated. Tissue sections were incubated 3 times for 5 min in Xylol, for 3 min in 100 %, 96 %, 80 % and 70 % ethanol each and finally washed in dH_2O.

3.9.3.1 Hematoxylin and Eosin staining (H&E)

De-waxed and rehydrated tissue sections were incubated in Hematoxylin for 2 min and rinsed with dH_2O for 5-10 min followed by 2 min incubation in Eosin. Hematoxylin stained sections were dehydrated in a rising ethanol-series (70 % for 10 sec, 80 % for 10 sec, 96 % for 3 min and

twice in 100 % for 3 min), followed by 3 times incubation in xylol for 3 min. They were finally mounted with Roti®-Histokitt (Roth, Karlsruhe).

3.9.3.2 Immunohistochemistry

The expression of proteins was examined by staining of de-waxed and rehydrated tissue sections with the corresponding specific antibodies (Table 7). To this end, samples were boiled in pre-heated Antigene retrieval buffer for 20 min and cooled down to RT in a water bath. The sections were incubated with 1 % H_2O_2 for 10 min at RT and then washed once in dH_2O for 5 min and twice in 1 x PBS for 5 min each. Endogenous peroxidases were blocked by incubation in IHC blocking solution for 30 min at RT. Tissue sections were incubated o/n at 4°C with the specific primary antibody diluted in IHC blocking solution in a humidified atmosphere. After washing for 3 times 5 min in 1 x PBS the samples were incubated for 30 to 45 min at RT with the secondary HRP-conjugated antibody (Dako EnVision Kit, Dako, Hamburg) and then washed 3 times in 1 x PBS for 5 min. Antibody complexes were detected according to manufacturer`s instructions. For counter-staining, sections were incubated for 10 sec with Hematoxylin and 5 to 10 min rinsed in dH_2O. For imaging, stained sections were dehydrated and mounted with Roti®-Histokitt (Roth, Karlsruhe).

Antigene retrieval buffer	10 mM Tris, 1mM EDTA, pH 9.0
IHC Blocking Buffer	10% rabbit serum, 1% BSA in 1x PBS

3.9.3.3 Immunofluorescence staining on paraffin embedded tissues

Protein expression, corresponding specific antibodies (Table 10) and the indirect fluorescent staining were used. The paraffin sections were rehydrated by incubation in xylol (3 x 5 min), ethanol (100%, 96%, 80% and 70% for 3 min each step) and then washed in dH_2O. Following rehydration, the antigene retrieval was performed by boiling the samples in preheated Antigene retrieval buffer (Table 5) for 20 min. The samples were cooled down to RT in a cold water bath. After 30 min (RT) blocking in AB buffer (Table 5), the sections were incubated over-night (4°C) with the specific primary antibody (diluted in blocking solution) in a humidified atmosphere. After washing (3 x 20 min in PBST) the samples were incubated for 30 to 45 min (RT, humidified atmosphere) with the secondary fluorophore-conjugated antibody (diluted in AB buffer) and then washed again in PBST (3 x 20 min).The counter-staining with DAPI was performed in the penultimate washing step by diluting DAPI stock solution to 0.1 µg/ml in

PBST. For microscopy the sections were mounted in Immu-Mount (ThermoScientific, Bonn) and stored at 4°C in the dark.

AB buffer	0.1% Tween, 10 % horse serum in 1x PBS
PBST	1 x PBS supplemented with 0.05% (v/v)Tween
Blocking Solution	1 x PBS supplemented with 0.5% (v/v) Triton X-100, 0.5% (w/v) BSA

3.9.4 Scoring of human tissue microarray

Colorectal cancer tissues were obtained from the Tumor Bank of the Department of Surgical Oncology, Charité Medical School Berlin. All patients underwent surgery followed up for a minimum of three years. Specimens were transferred to a paraffin array block and 1 µm tissue sections subjected to immunohistochemistry. ß-catenin, BCL9 and BCL9-2 stainings were scored by three independent investigators using the expression score according to (Sinicrope *et al.*, 1995). The immunoreactive score (0 to 9) was calculated as the product of the staining intensity and the percentage of positive epithelial cells. The staining intensity was defined as 0 (negative), 1 (low), 2 (moderate) and 3 (strong).The percentage of positive epithelial cells was scored as 0 (0%), 1 (<30%), 2 (30%-60%) and 3 (>60%). Statistics were evaluated using the Mann-Whitney-U test.

3.9.5 Cell culture model systems

3.9.5.1 Cultivation and storage of cell cultures

Cell cultures were stored in 1 x culture/trasnfection medium supplemented with 10% DMSO in cryo tubes. To this end, cells were slowly frozen o/n at -80°C in freezing boxes followed by long-time storage at -150°C. For cultivation, cells were thawed in a water bath at 37 °C and transferred into 10 ml 1 x PBS. After centrifugation at 1000 rpm for 3 min, cell-pellets were resuspended in culture medium, supplemented with 10% FCS as recommended by the suppliers and transferred into a 75 cm^2 or 175 cm^2 flasks and cultivated at 37°C in a 5% CO_2 / 95 % humidity atmosphere. Depending on their confluence cells were split 1:5 to 1:10 every 3 to 5 days. Therefore, cells were detached with 1ml trypsin after washing with 1 x PBS. Detached cells were resuspended in culture medium and spread into new culture flasks.

3.9.5.2 Transfection

Cell lines were transfected with siRNA (Table 13) or endotoxin-free, highly pure plasmid DNA (Table 16) using Lipofectamine2000 (Invitrogen, Darmstadt) according to manufacturer's instructions. 100 ng TOP/FOP-, -136 bp CyclinD1- and -5600 bp Axin2-reporters or 200 ng of *CDX1* and *CDX2* promoter *LUC*-reporters were used for transfection. In addition, 25 ng Renilla *Luciferase* was added to normalize *Luciferase*-reporters activities.

Cells were seeded 12 h - 16 h prior to transfection. The used cell numbers of different cell lines were summarized in Table 18.

Table 18: Cells / ml used for transfection with siRNA and with overexpression-plasmids (oe)

cell line	cells/ml (siRNA) in 24 well plates	cells/ml (siRNA) in 6 well plates	cells/ml (oe) in 10 cm dishes
HEK293			1.25×10^5
SW480	1.875×10^5	1.25×10^5	
DLD1	1.875×10^5	1.25×10^5	
HCT116	1.25×10^5	1.67×10^5	
SW48	1.875×10^5		

For over-expression of proteins *in vivo* in addition to *Luciferase*-reporters (*LUC*-reporters) or for protein isolation, cells were transfected with transfection mixes according to Table 19. Transfection mix A including OptiMEM and siRNA/plasmids and Transfection mix B including OptiMEM and Lipofectamine2000 were incubated for 5 min before they were mixed and further incubated for 20 min at RT. Consequently, the mix was added to the cells. In analytical approaches, the plates were centrifuged for 10 min at 300 rpm to increase the transfection efficiency.

After siRNA transfection cells were incubated for 48h followed by transfection of *LUC*-reporters and further incubation of 36h. For overexpression with and without *LUC*-reporter transfection cells were incubated for 48 h. Cells were harvested according to 3.8.4.

Table 19: Components of Lipofectamine based transfection mixes

detergents	in 24 well plates	in 6 well plates	in 10 cm dishes
OptiMEM Mix A and B	50 µl each/well	250 µl each/well	2,5 ml each/well
Lipofectamine2000	0.5 µl	X	15 µl
Overexpression and *LUC*-Firefly plasmids	100 – 200 ng	X	15 - 20 µg
LUC-Renilla plasmid	25 ng	X	
Lipofectamine2000	1 µl	5 µl	X
siRNA	1 µl (25 pmol each)	5 µl (25 pmol each)	x

3.10 Software

Table 20: Software

software	developer/manufacturer
Microsoft Office 2010	Microsoft
GENtle	University of Cologne, Germany
SPSS 19.0	SPSS Inc.
Transfac® (MATCH/PATCH)	Transfac Professional 2008.4 (Kel *et al.*, 2003;Matys *et al.*, 2003)
T-COFFEE®	T-Coffee Home Page (Di *et al.*, 2011;Notredame *et al.*, 2000)
Scaffold3	Proteome Software, Inc.
Leica Application Suite	Leica

3.11 Statistics

Statistical significances of qPCR and *Luciferase*-reporters gene assays were calculated by the conventional two sided t-test using Microsoft Excel. Significances of adenoma development were calculated by P log-rank test, significances of nuclear expression scores by the Mann-Whitney U Test using SPSS 19.0 software.

4 Results

4.1 BCL9 and BCL9-2 expression in normal intestine and during intestinal tumorigenesis

So far, the expression of the Wnt/ß-catenin co-factors BCL9 and BCL9-2 normal intestinal epithelia and tumors of mice and humans were not characterized. Recent studies described that BCL9 proteins are deregulated in different tumor entities (Brembeck *et al.*, 2004;Sakamoto *et al.*, 2007;Toya *et al.*, 2007). However, detailed investigations were missing.

4.1.1 BCL9-2 expression is restricted to the villi of the normal intestine and is up-regulated in early stages of intestinal tumorigenesis

To get an insight into the distribution and expression of the BCL9 proteins, intestinal tissue sections from late embryonic and adult mice were subjected to immunohistochemistry (Figure 10A). Antibodies were generated that specifically recognize BCL9 and BCL9-2 and ß-catenin (Brembeck *et al.*, 2011). In addition, the expression of the proteins in adenomas from $APC^{MIN/+}$ mutant mice was determined (Figure 10). Immunohistochemical stainings of tissue microarrays from human normal mucosa and adenomas were scored for the nuclear expression of the three proteins (Figure 10C).

In the intestines of embryonic and adult mice, nuclear BCL9 was strongly expressed in all mesenchymal and epithelial cells in the crypts and villi the intestine (Figure 10A, left panel). In contrast, BCL9-2 expression was restricted to the villi of late embryonic and adult intestines (Figure 10A, right panel, black arrowheads) but was absent in the crypts of the small intestine and in the colon (Figure 10A, right panel, white arrowhead), where ß-catenin/Wnt-signaling is active. Adenomas of $APC^{MIN/+}$ mice were highly proliferative as indicated by BrdU staining. Increased nuclear ß-catenin expression was determined suggesting actived Wnt/ß-catenin signaling in adenomas (Figure 10B and C). Interestingly, adenomas of $APC^{MIN/+}$ mice and human specimen also exhibited increased BCL9-2 expression (Figure 10B, black arrow) compared to adjacent crypts of the normal mucosa (Figure 10C). In contrast, BCL9 protein expression remained unchanged in adenomas of $APC^{MIN/+}$ mice and human specimen (Figure 10B and C).

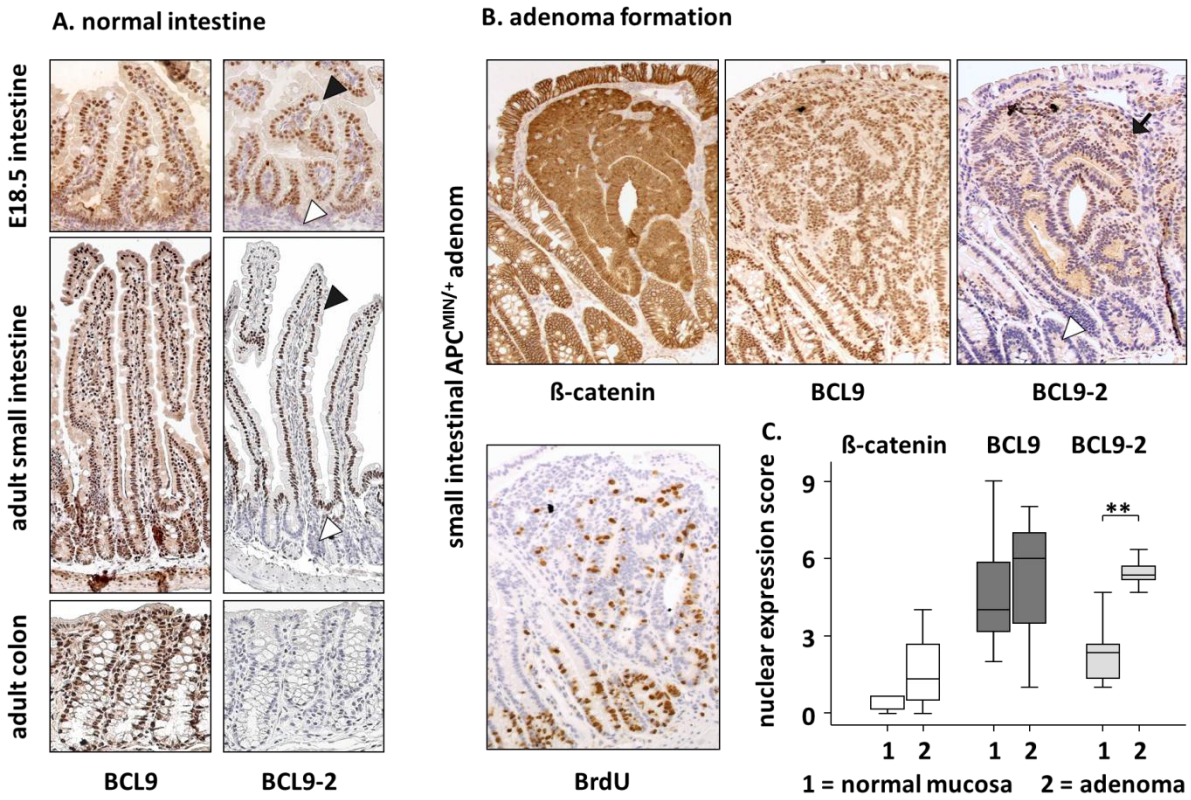

Figure 10: Expression of BCL9 and BCL9-2 in normal embryonic and adult intestines and in adenomas
Immunohistochemistry on serial sections of (A) normal intestines of late embryonic and adult mice and (B) a representative adenoma of an $APC^{MIN/+}$ mouse. Specific antibodies targeting the indicated proteins were used. (C) nuclear expression score of ß-catenin, BCL9 and BCL9-2 obtained from human normal mucosa and adenomas (*p≤0.05). (Brembeck, Wiese *et al.*, 2011)

4.1.2 BCL9-2, but not BCL9, is highly expressed in human colon cancers

The distribution and expression of ß-catenin, BCL9 and BCL9-2 was further investigated in different stages during colon carcinogenesis. Therefore a human tissue microarray of paired samples from 105 patients was established containing normal mucosa, adjacent transitional mucosa and colorectal cancers without (stage I, n=58) or with metastases (stage III/IV, n=47). The nuclear expression score was defined as the product of the presence of immunopositive cells and intensity of the staining.

Highly significant increased expression of BCL9-2 was found in more than 90% of human colon tumors. Comparison of the nuclear expression score of stage I (without metastasis) and stage II/IV (with metastasis) cancers revealed that BCL9-2 was strongly expressed in all cancers, independently of the presence of metastasis (Figure 11A and B). Strong ß-catenin expression was only found in approximately 60% of the tumors (Figure 11A). In contrast, no significant changes were detected for the BCL9 protein (Figure 11B).

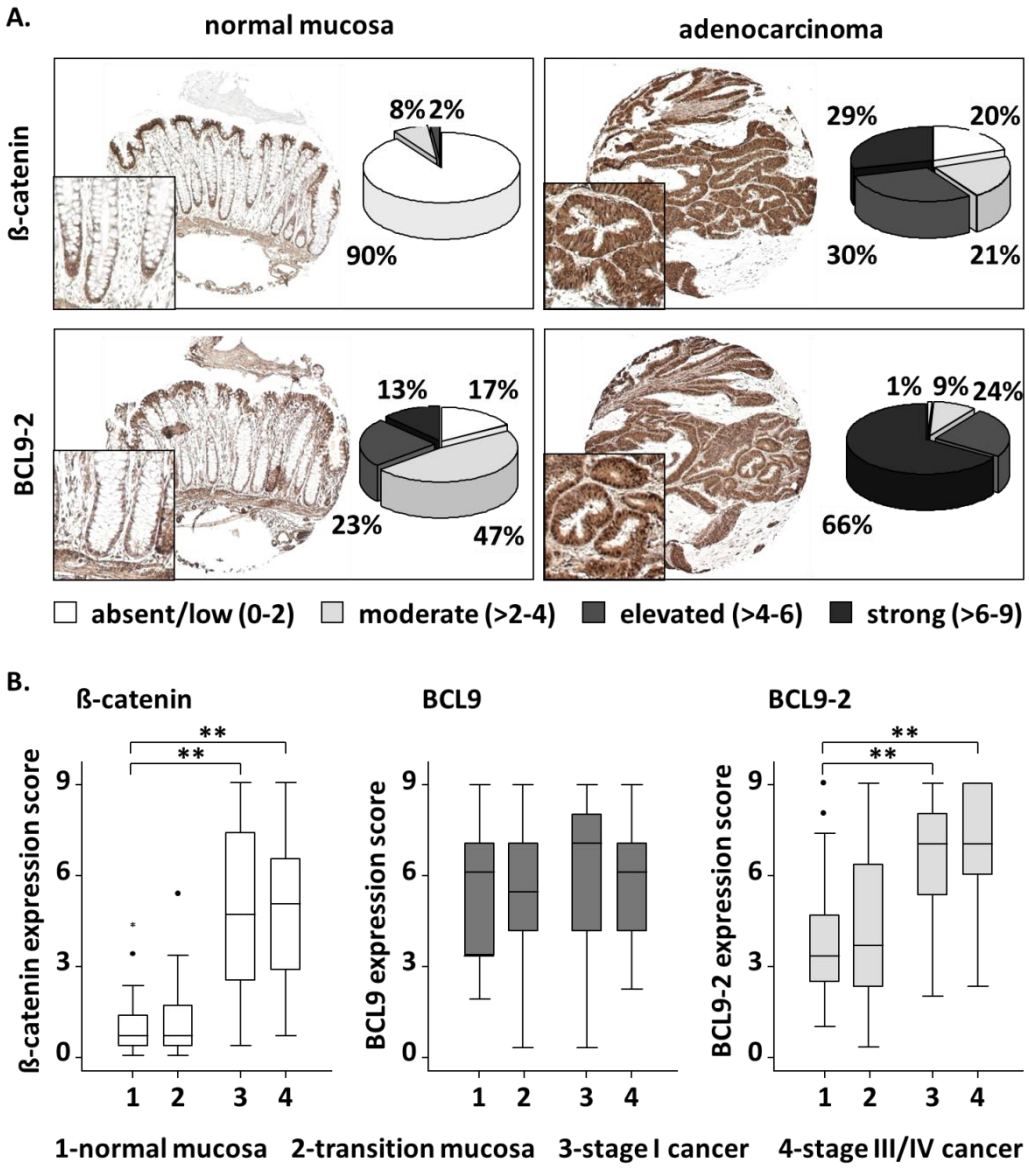

Figure 11: BCL9-2 is up-regulated in human adenocarcinomas
Immunohistochemistry and scoring of human tissue microarrays of matched specimens from patients with stage I, III and IV colon cancer (A) Immunostainings depict the protein expression of ß-catenin and BCL9-2 on matched, representative tumor samples. Pie charts show the relative frequency of the immunoreactive score for BCL9-2 and ß-catenin expression in adenocarcinomas and normal mucosa, (B) Boxplot analyses of the nuclear expression score of ß-catenin, BCL9 and BCL9-2 in normal and transient mucosa, non-metastatic(stage I) and metastatic cancer (stage II/IV) ($p<0.01$). (Brembeck, Wiese *et al.*, 2011)

Thus, intestinal BCL9-2 expression is restricted to the villi and not present in the crypts, where Wnt-signaling is active. Moreover, up-regulation of BCL9-2 protein expression occurs early during intestinal tumorigenesis, culminating in 90% of highly BCL9-2 positive human adenocarcinomas. In contrast, BCL9 expression is not changed during early and even late stages of intestinal tumorigenesis compared to normal mucosa. These data indicate that BCL9-

2 might contribute to tumor progression already in early stages of intestinal tumorigenesis. Further, BCL9-2 might be a useful marker, which indicates tumor progression.

4.2 BCL9-2 overexpression promotes intestinal tumor development

4.2.1 BCL9-2 expression in compound APC$^{Min/+}$; K19-BCL9-2 transgenic mice

To investigate the tumorigenic potential of BCL9-2 in vivo, a transgenic mouse model was analyzed (Brembeck *et al.*, 2011). In this model, BCL9-2 overexpression was induced by a K19 promoter which is active in simple epithelia, including the intestine (Brembeck *et al.*, 2000). Ectopic expression of BCL9-2 on a pure C57BL/6 background led to infrequent, undifferentiated intestinal tumor formation in aged mice (>15 month)(Brembeck *et al.*, 2011). To induce tumorigenesis K19-BCL9-2 transgenic mice were further crossed with C57BL/6-APC$^{Min/+}$- mice, which displayed spontaneous development of adenomas in the small intestine and colon (Su *et al.*, 1992). The specific expression of ectopic BCL9-2 in compound transgenic mice was verified RT-PCR (Figure 12A) and immunohistochemistry (Figure 12B).

Endogenous BCL9-2 protein was expressed in adenomas of APC$^{Min/+}$ control mice (Figure 12B, left) and strongly increased in adenomas of compound APC$^{Min/+}$; K19-BCL9-2 mice (Figure 12B, right). Moreover, transgenic expression of flag tagged BCL9-2 was found in intestinal tissue sections of adenomas of compound APC$^{Min/+}$; K19-BCL9-2 mice (Figure 12A+B).

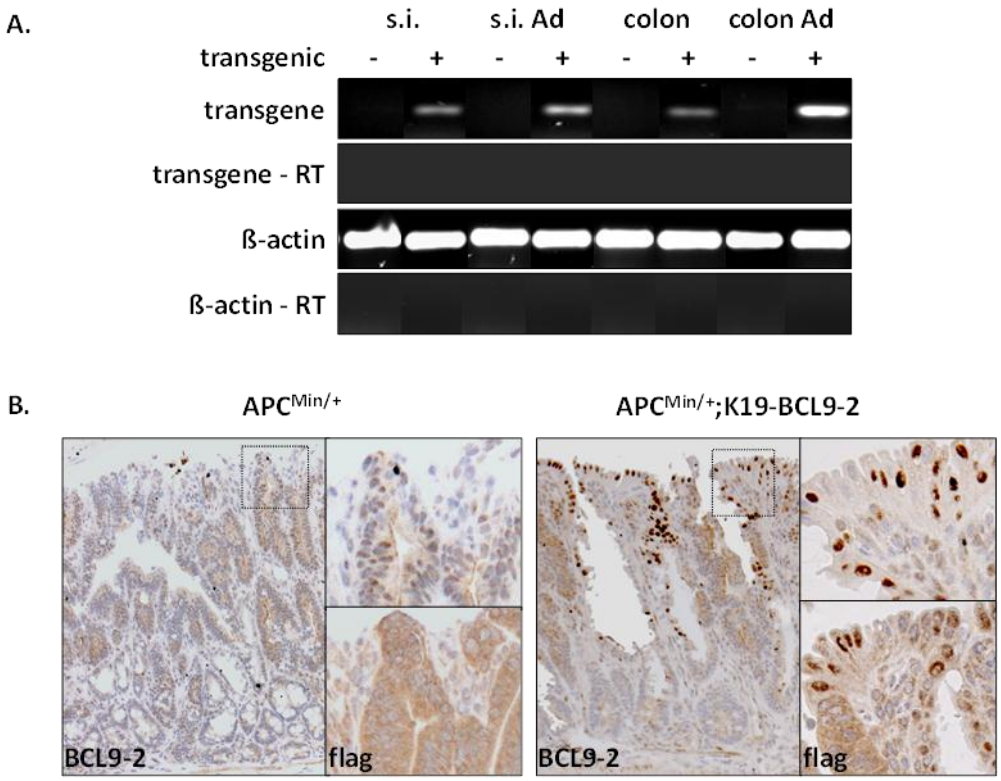

Figure 12: Transgenic BCL9-2 expression in compound APC$^{Min/+}$; K19-BCL9-2 intestinal adenomas and adjacent normal tissues compared to non-transgenic APC$^{Min/+}$ littermate controls
(A) Transgenic RNA expression in adenomas (Ad), normal tissues of the small intestine (s.i.) and colon was analyzed by RT-PCR. Specific primers were used to target the 3' flag tagged BCL9-2 transgene and ß-actin as internal control. Templates without reverse transcription were used as controls (-RT). (B) Transgenic and endogenous BCL9-2 protein expression was visualized by Immunohistochemistry on representative adenomas of the indicated genotypes using specific antibodies directed against BCL9-2 and the flag-tagged transgenic protein. (Brembeck, Wiese *et al.*, 2011)

4.2.2 BCL9-2 overexpression promotes tumor formation and local invasion

K19-BCL9-2 transgenic mice were crossed to C57BL/6-APC$^{Min/+}$ mice to investigate the impact of BCL9-2 on tumor development and progression. Overexpression of BCL9-2 resulted in a significant decrease of survival of compound APC$^{Min/+}$; K19-BCL9-2 mice compared to non-transgenic APC$^{Min/+}$ littermates (Figure 13). To follow the development of intestinal tumors due to aberrant BCL9-2 expression adenoma formation in the small intestine and colon of 5.5 month old compound APC$^{Min/+}$; K19-BCL9-2 mice and non-transgenic APC$^{Min/+}$ littermates were investigated in detail. H&E staining and box plot analysis showed that transgenic mice exhibited significant increase both in number and in size of adenomas in the small intestine compared to age matched non-transgenic APC$^{Min/+}$ littermates (Figure 14A and B). A similar trend of tumor progression was observed in the colon, although APC$^{Min/+}$ mice do not frequently develop adenomas in the colon: APC$^{Min/+}$; K19-BCL9-2 formed a slight, but not significantly higher number of larger adenomas than APC$^{Min/+}$ mice (Figure 14B).

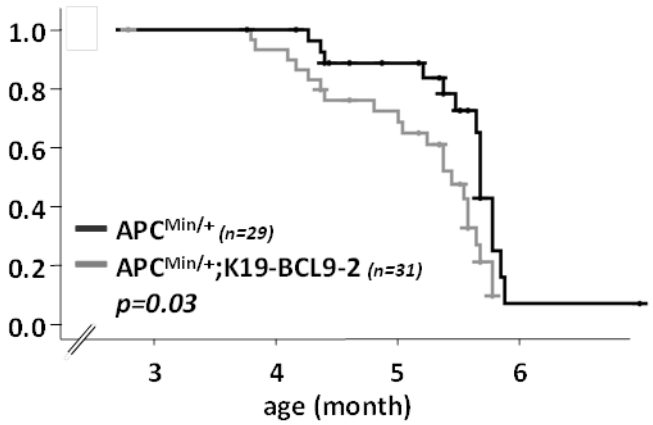

Figure 13: BCL9-2 overexpression leads to impaired survival of compound APC$^{Min/+}$; K19-BCL9-2 mice
Kaplan–Meier survival analysis of compound mutant (n=31) and of littermate APC$^{Min/+}$ control mice (n=29). The survival was defined as the time point of death or the time point at which mice were sacrificed due to severe tumor-related disease. Statistics were evaluated by the P log-rank test. (Brembeck, Wiese *et al.*, 2011)

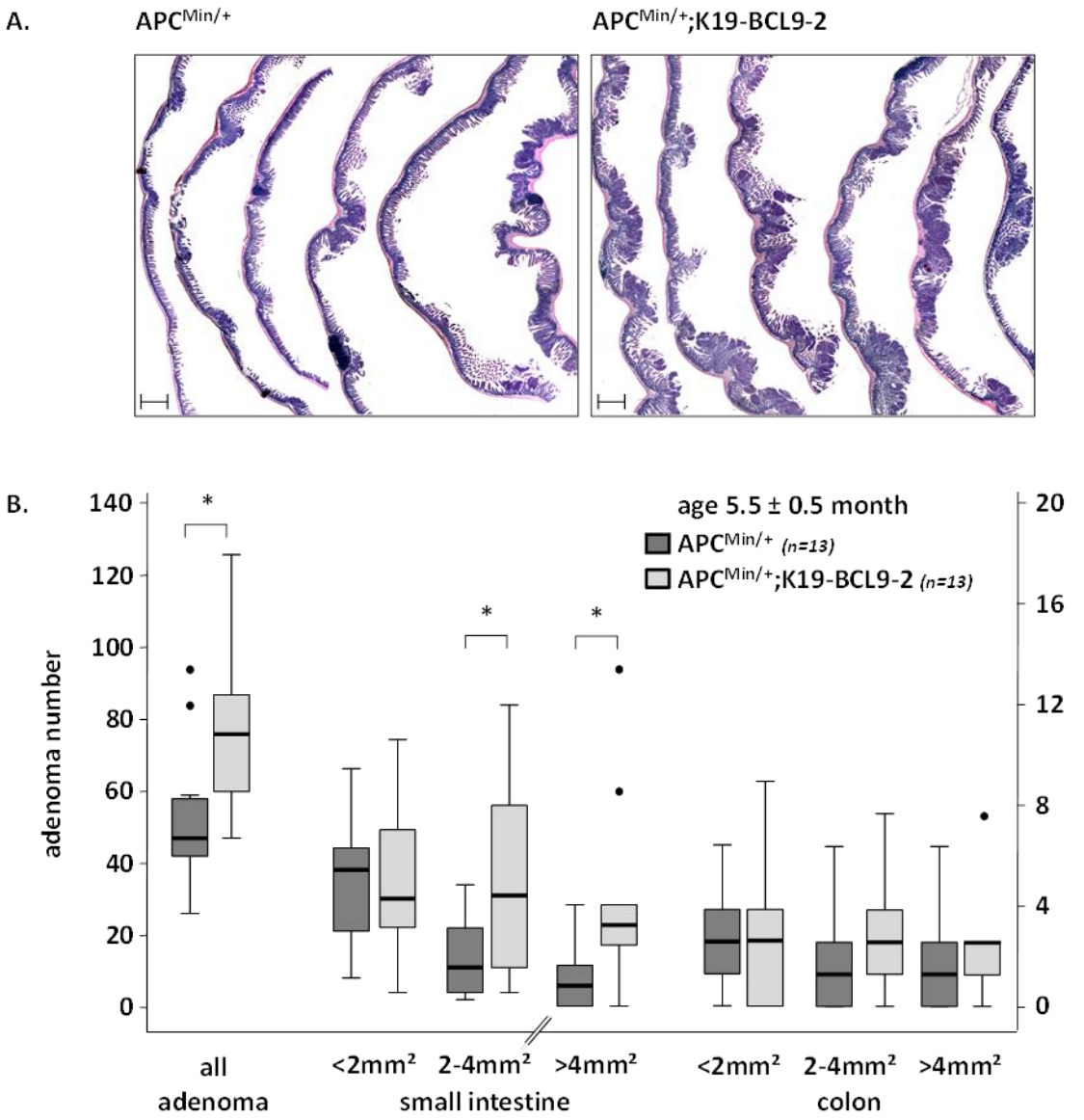

Figure 14: BCL9-2 overexpression promotes adenoma formation in number and size in compound APCMin/+; K19-BCL9-2 mice
(A) H&E staining of representative small intestines of APCMin/+; K19-BCL9-2 and non-transgenic controls show an overview of the severe formation of adenomas in transgenics, (bar – 1mm). (B) Box blot analyses showing the total number of adenomas (left) and adenoma formation for the indicated sizes in the small intestine and colon (right) of 5,5 ± 0,5 month old transgenic (n=13) and non-transgenic littermates (n=13). For calculation of statistical significances the Whitney-U test test was used (*p≤0.05). (Brembeck, Wiese *et al.*, 2011)

$APC^{Min/+}$ mice are known to develop benign tumors with well-formed boundaries (Moser *et al.*, 1990). In contrast, adenomas from compound K19-BCL9-2 mutant mice already showed local invasion with an incidence of 14%, which was never observed in control mice. Co-Immunofluorescence of $APC^{Min/+}$; K19-BCL9-2 intestinal sections with pan-cytokeratin and smooth muscle actin monitored invasive tumors that disrupted the basal membrane. Invasive tumors grew beyond the mucosa into the submucosa (Figure 15A, middle) or even into the

muscularis (Figure 15A, right). In contrast, adenomas of $Apc^{Min/+}$ mice were restricted to the mucosa with an intact basal membrane (Figure 15A, left). Immunohistochemical analyses on sections of invasive tumors of compound $APC^{Min/+}$; K19-BCL9-2 revealed high expression of BCL9-2 and of nuclear ß-catenin at the invasive front (Figure 15B).

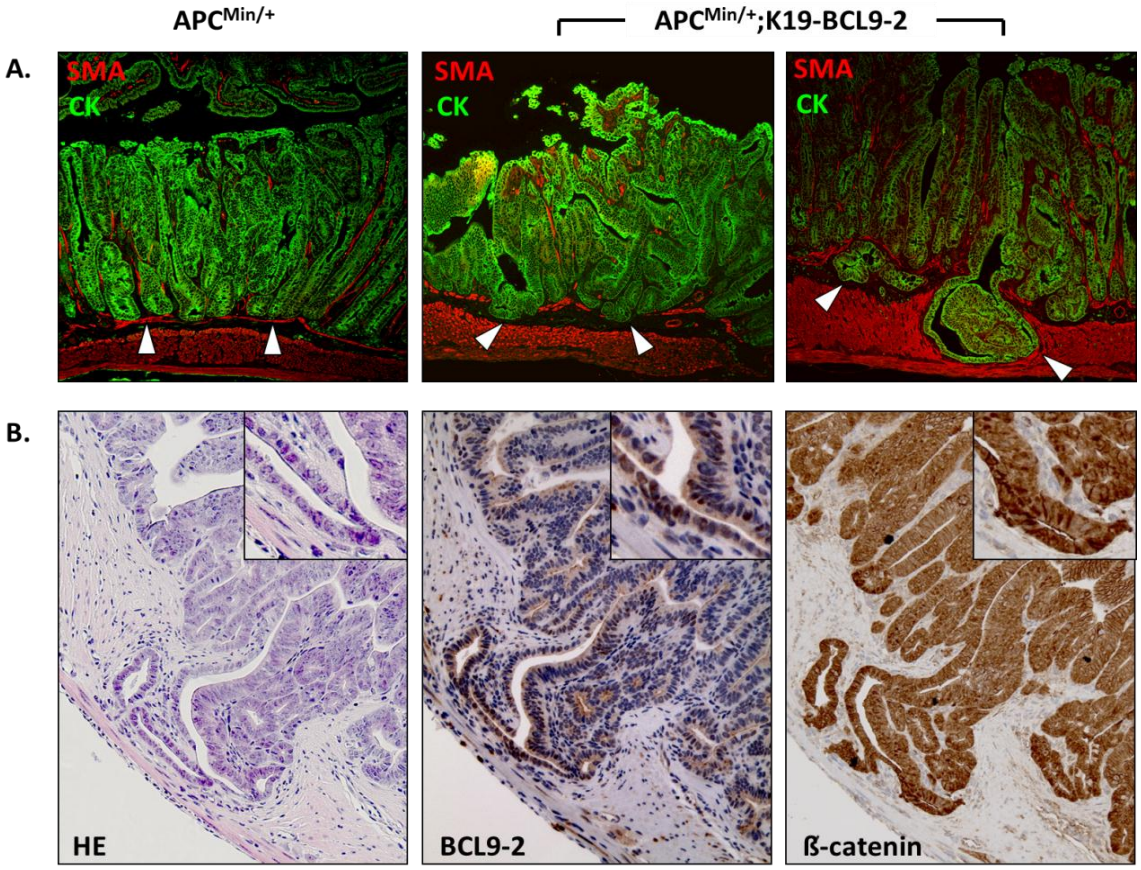

Figure 15: BCL9-2 overexpression induced invasion in compound $APC^{Min/+}$; K19-BCL9-2 mice
(A) Co-immunofluorescence staining of smooth muscle actin (SMA, red) and pan-cytokeratin (CK, green) on representative adenomas of an APCMin/+ control mice (left) and of compound APCMin/+; K19-BCL9-2 mice (middle, right). Arrowheads indicate the invasion into the basal membrane (200 x magnification). (B) H&E (left) staining and immunohistochemistry (middle and right) on an invasive adenoma of compound APCMin/+; K19-BCL9-2 mice of BCL9-2 and ß-catenin, as indicated (400 x magnification). (Brembeck, Wiese *et al.*, 2011)

These data demonstrate that overexpression of BCL9-2 *in vivo* promotes tumor progression in the intestine.

4.3 BCL9 and BCL9-2 protein expression in colon cancer cell lines

The majority of human colorectal carcinomas exhibited BCL9-2 overexpression (Figure 11) (Brembeck *et al.*, 2011). To investigate the molecular function of BCL9 proteins for colorectal

carcinogenesis, different colon carcinoma cell lines were analyzed for endogenous expression of BCL9-2 and the homologue BCL9. Therefore, our newly developed antibodies were analyze endogenous BCL9 and BCL9-2 protein expression. Using Western Blot analysis the proteins were detected at approximately 180kDa. Highest protein expression of BCL9-2 was observed in cell lines with mutant APC (Rowan *et al.*, 2000), e.g. in human SW480, CaCo2, HT29, DLD1 cells or the murine CT26 cell line. Absence or lower BCL9-2 protein expression was detected in cell lines with ß-catenin stabilizing mutation (Kim *et al.*, 2003), e.g. HCT116, SW48 and the cell line LS174T with mutated E-cadherin (Efstathiou *et al.*, 1999) (Figure 16, upper panel). However, BCL9 was ubiquitously expressed at approximately equal levels in all analyzed cell lines (Figure 16, middle panel).

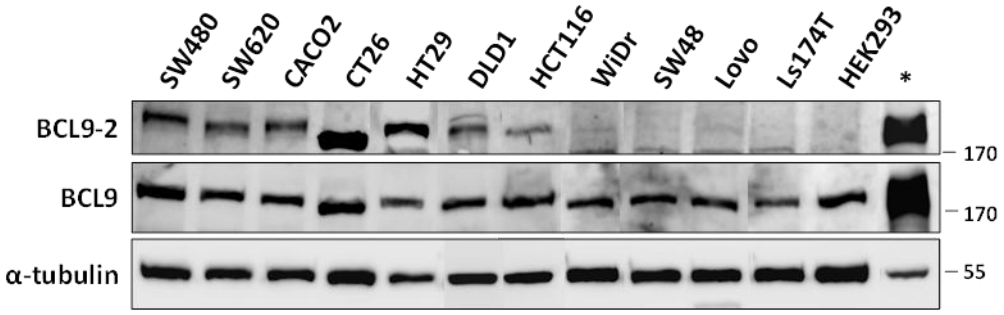

Figure 16: Expression of BCL9 proteins in different colon cancer cell lines
Western Blot analyses were performed with 60 µg whole cell lysate obtained from the indicated cell lines, using specific antibodies for endogenous BCL9 and BCL9-2, respectively. As loading control α-tubulin was used. Whole cell lysates obtained from transiently transfected HEK293 cells with BCL9 or BCL9-2 cDNA served as positive controls (indicated by an asterisk). (Brembeck, Wiese *et al.*, 2011)

4.4 Analysis of the dependency of the transcriptional control of ß-catenin, BCL9 and BCL9-2 on RNA Interference

Colon cancer cell lines revealed high levels of ß-catenin caused by a mutation in APC and high levels of BCL9-2 (Figure 16). To investigate the transcriptional function of BCL9 proteins compared to the function of ß-catenin, RNA interference was used. A pool of 4 different non-targeting *si*RNAs was used as control. To cause minimal, unspecific effects 50 pmol of modified Dharmacon® ON-TARGET plus™ *si*RNAs were used which were modified to reduce off-targeting caused by either sense or antisense strands. Further, off-target effects were minimized by the usage of a pool of two different *si*RNAs resulting in a minimal concentration of 25 pmol per *si*RNA. The potency of 4 single and pooled *si*RNAs to reduce the expression of BCL9, BCL9-2 and ß-catenin transcripts in SW480 cells was investigated by qPCR.

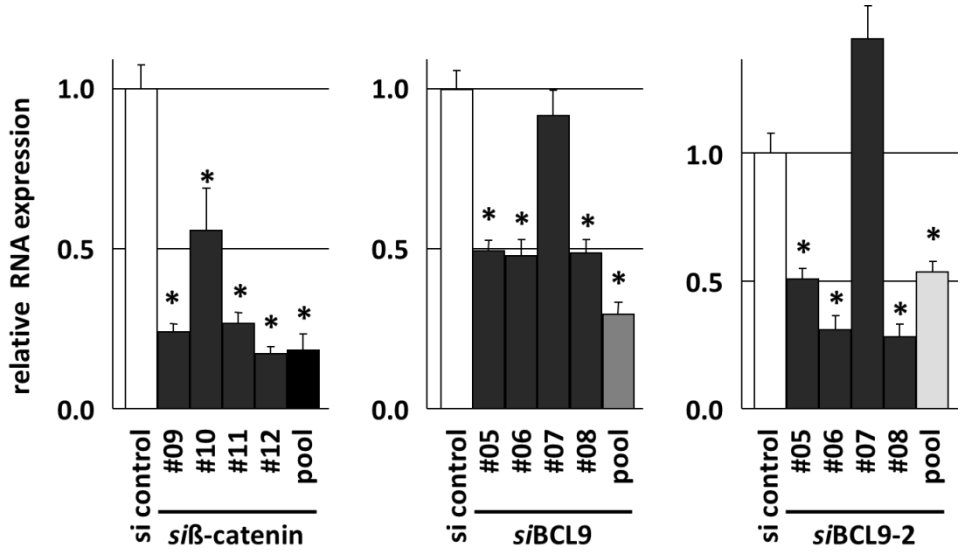

Figure 17: Knockdown efficiency of 4 single and pooled siRNAs targeting BCL9, BCL9-2 and ß-catenin in SW480 cells
Quantitative real time PCR of SW480 showing the mean of least 3 independent experiments and their standard deviation after 48h *si*RNA treatment. The relative expression of the indicated transcripts is shown as % of control *si*RNA-treated cells (*p≤0.05).

Pools containing four single *si*RNAs reduced the mRNA expression of ß-catenin to 20 %, of BCL9 to 30 % and of BCL9-2 to 50 % compared to control *si*RNA treated cells. RNA interference using te single *si*RNAs targeting ß-catenin, BCL9 and BCL9-2, revealed that only three of the four single *si*RNAs efficiently reduced the respective transcript (Figure 17). To increase the knockdown efficiencies, a pool of the two most efficient single *si*RNAs was used for further investigations (#09 and #12 for ß-catenin, #06 and #08 for BCL9 and #06 and #08 for BCL9-2 knockdown). The knockdown efficiency induced by these 2 pooled *si*RNAs targeting each transcript was further investigated on the mRNA and protein level (Figure 18). Treatment of SW480 cells with 2 specific *si*RNAs strongly reduced the RNA- and protein-levels of the respective targets (Figure 18A and B) to at least 25% compared to treatment of the cells with a pool of all 4 *si*RNAs.

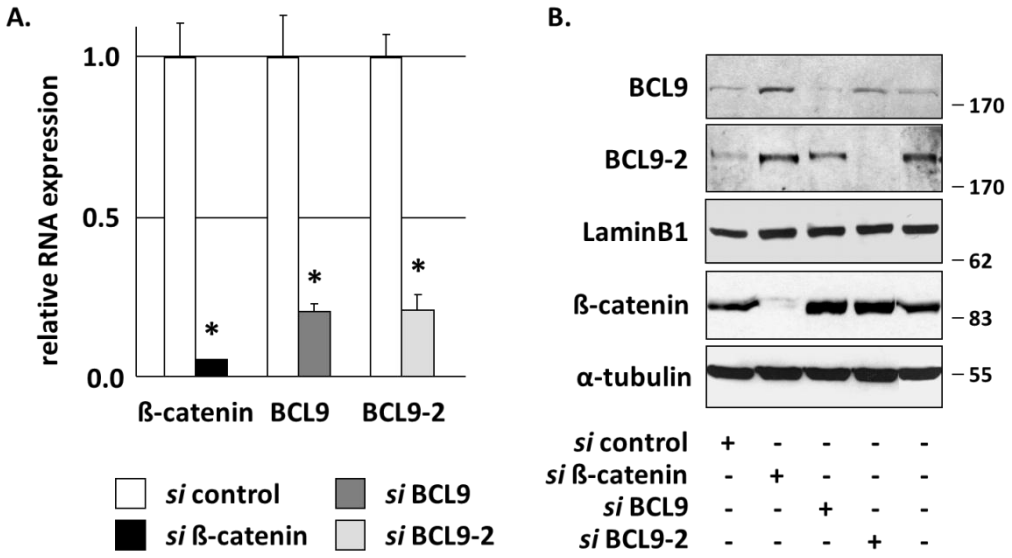

Figure 18: BCL9, BCL9-2 and ß-catenin knockdown in SW480 cells
(A) Quantitative real time PCR of SW480 showing the mean of least 3 independent experiments and their standard deviation after 48h *si*RNA treatment. The relative expression of the indicated transcripts is shown as % of control *si*RNA-treated cells (*p≤0.05). (B) Western Blot analysis of SW480 cells followed by 72h RNA interference. Nuclear extracts were used for BCL9, BCL9-2, whole cell lysates for ß-catenin detection. LaminB1 and α-tubulin served as internal controls. (Brembeck, Wiese *et al.*, 2011)

Further investigations with these pools of specific *si*RNAs were carried out using different colon cancer cell lines such as SW480, DLD1, HCT116 and SW48. In addition, the breast cancer cell line MCF7 was used, which contains high levels of BCL9-2 protein (Zatula and Brembeck, unpublished).

4.5 BCL9 and BCL9-2 knockdown revert the mesenchymal malignant phenotype of cancer cell lines

The investigated cancer cell lines showed a characteristic change of the morphology after treatment with *si*RNA targeting BCL9 and BCL9-2 or ß-catenin after 72h. In comparison to the more mesenchymal-like morphology of control *si*RNA treated cells (Figure 19, upper panel), cells in which BCL9-2 was knocked down showed a more epithelial-like phenotype characterized by a flattened appearance and tight cell-cell contacts. This phenotype was also observed for MCF7 breast cancer cells, which are highly positive for BCL9-2 (Zatula and Brembeck, unpublished). Treatment with BCL9 *si*RNA led to similar, but less pronounced morphological changes like BCL9-2 knockdown (Figure 19, middle panels). In contrast, knockdown of ß-catenin enhanced the mesenchymal-like, spindle-shaped morphology of the cells that did not form tight cell-contacts (Figure 19, bottom panel).

These data indicate that BCL9-2 overexpression enhances the mesenchymal phenotype in colon and breast cancers possibly mediated by the promotion of epithelial-mesenchymal transition.

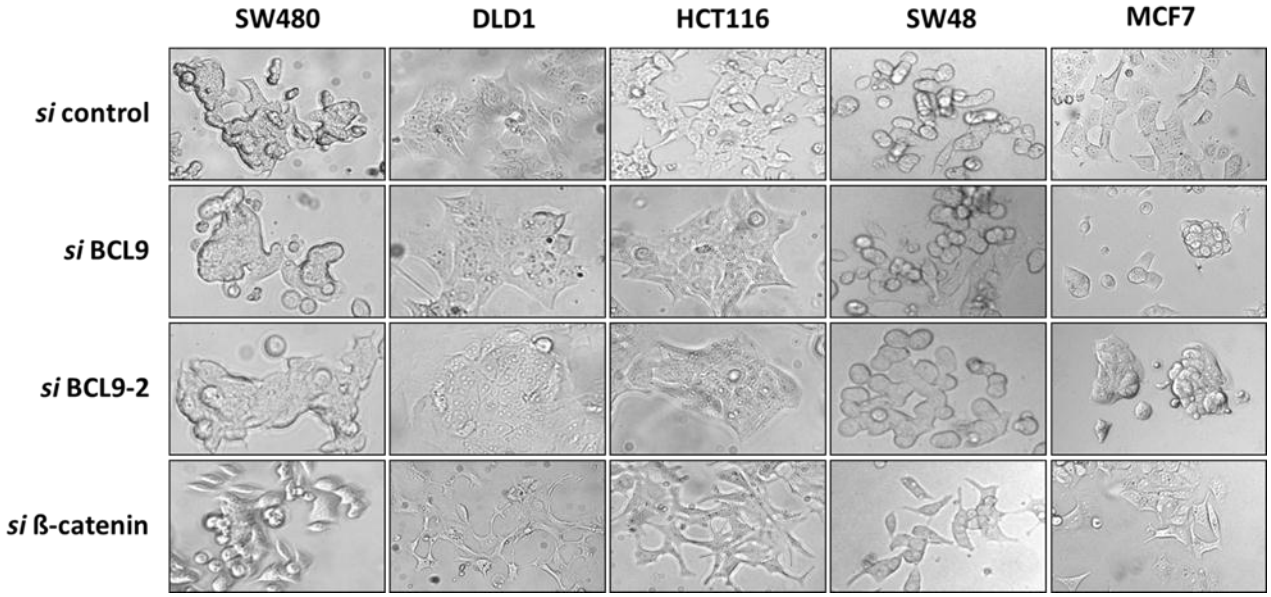

Figure 19: Knockdown of BCL9 and BCL9-2 induces characteristic morphological changes
Bright field images of human colon (SW480, DLD1, HCT116, and SW48) and breast (MCF7) cancer cells that were treated with a pool of 2 specific siRNAs against β-catenin, BCL9, BCL9-2 for 72 hours and non-targeting control siRNA (200x magnification).

4.6 BCL9 and BCL9-2 are not target genes of ß-catenin

To investigate, if BCL9 and BCL9-2 gene expression is regulated by Wnt/ß-catenin signaling, SW480 were treated with the respective *si*RNAs for 48h. The target gene transcription was determined by quantitative real-time PCR.

ß-catenin knockdown did not reduce BCL9 or BCL9-2 expression (Figure 20A) and neither, BCL9 nor BCL9-2 had an influence on each other's transcription. Interestingly, BCL9-2 expression was significantly induced after knockdown of ß-catenin (Figure 20A).

These results show that BCL9 and BCL9-2 are not target genes of ß-catenin signaling in colon cancer cells. Moreover, ß-catenin appears to negatively regulate BCL9-2 expression.

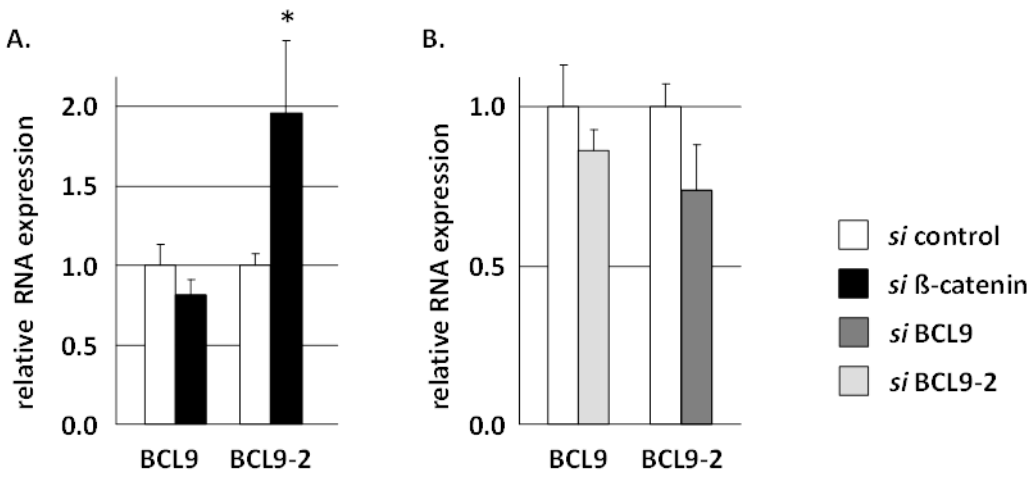

Figure 20: BCL9 and BCL9-2 are not target genes of ß-catenin
Expression of BCL9 and BCL9-2 was assessed as % of control *si*RNA-treated cells following knockdown of (A) ß-catenin, (B) BCL9 and BCL9-2 for 48h. Quantitative real time PCRs show the mean of at least 3 independent experiments and their respective standard deviation (*p≤0.05). (Brembeck, Wiese *et al.*, 2011)

4.7 ß-catenin/Wnt-signaling activity correlates with the expression level of BCL9-2 in human colon cancer cells

BCL9-2 co-activates the Wnt/ß-catenin signaling pathway in HEK293 cells (Brembeck *et al.*, 2004). For that reason, *Luciferase* assays with different ß-catenin-dependent reporters were performed in colorectal cancer cell lines to examine the dependency of the ß-catenin/Wnt-signaling activity on the BCL9 proteins in colon cancer cells.

Colorectal cancer cell lines with different levels of BCL9-2 were examined (Figure 16): SW480 cells with high, DLD1 cells with moderate, HCT116 cells with low and SW48 cells with no BCL9-2 expression. BCL9 was equally expressed in the cell lines (Figure 16). The cell lines were treated for 48 h with *si*RNAs targeting ß-catenin, BCL9 and BCL9-2, respectively, before they were transfected with 3 different *Luciferase*-reporters for further 36h. Since the promoters of the synthetic TOP/FOP reporter harbors three optimal, functional (TOP) or mutated (FOP) LEF/TCF binding sites (Molenaar *et al.*, 1996) that serve as a direct readout for canonical Wnt-signaling. The regulation of well-known Wnt/ß-catenin signaling target genes was determined using a *LUC*-plasmid containing 5600 bp of the Axin2- (Jho *et al.*, 2002) or -136pb of the CyclinD1- (Shtutman *et al.*, 1999) promoter that consequently control the *Luciferase*-reporters gene expression.

SW480 cells expressing highest levels of BCL9-2 showed also highest Wnt/ß-catenin activity, as determined by the TOP/FOP *Luciferase*-reporters. The level of TOP/FOP and Axin2 reporter activity was significantly decreased after knockdown of BCL9 and BCL9-2. Interestingly, the CyclinD1 reporter was only dependent on ß-catenin, but not on the BCL9 proteins (Figure 21, left panel).

In DLD1 cells which contain moderate levels of BCL9-2 the TOP/FOP *Luciferase*-reporters was expressed with moderate levels and only knockdown of BCL9-2 and not BCL9 inhibited the Axin2 and TOP/FOP *Luciferase*-reporters.

In contrast, HCT116 with low and SW48 cells with absent BCL9-2 expression showed the lowest Wnt-activity. Accordingly, knockdown of neither, BCL9 or BCL9-2 had any influences on Axin2 and TOP/FOP reporter gene expression.

In conclusion, BCL9-2 expression levels correlate with ß-catenin/Wnt signaling activity in colon cancer cell lines. In addition, these data indicate that promoters with core Wnt-responsive elements such as the synthetic TOP/FOP and the Axin2 promoter are dependent on BCL9-2, but not the CyclinD1 promoter.

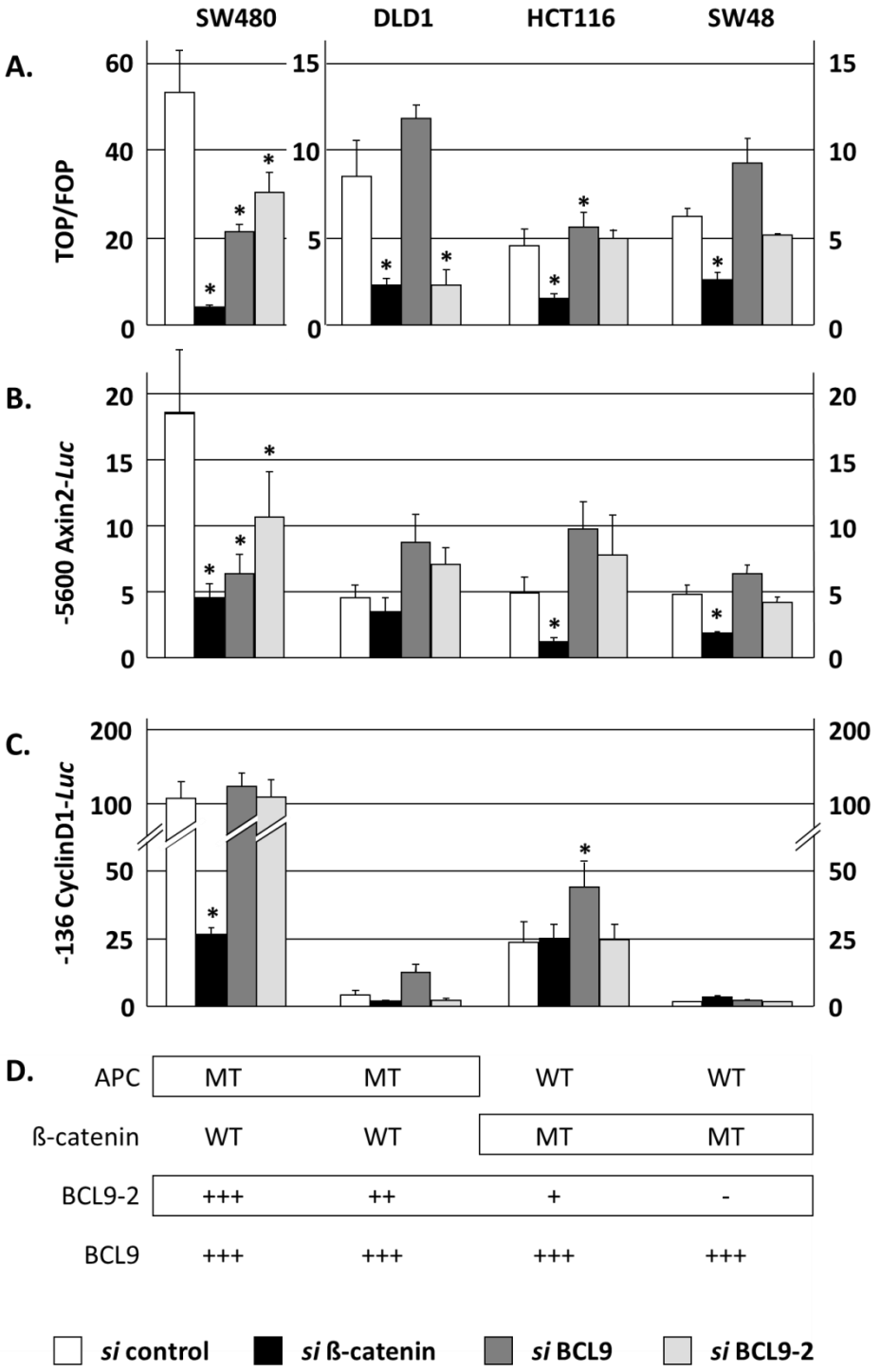

Figure 21: BCL9-2 expression levels correlates with canonical Wnt signaling activity and reporter gene expression in different colon cancer cell lines

(A-C) For *Luciferase* assays the indicated cell lines were pretreated with siRNA targeting ß-catenin, BCL9 or BCL9-2 for 48h, followed by 36h transfection with *LUC*-reporters: (A) TOP/FOP, (B) -5600 bp Axin2-*Luc* and (C) 136 bp CyclinD1-*Luc*. (D) The cell lines harbor different mutations and BCL9 protein levels, as indicated (MT-mutated, WT-wild-type). Graphs show the fold change of empty control plasmid of the mean of at least four independent experiments with the respective standard deviation (*p≤0.05).(Brembeck, Wiese *et al.*, 2011)

4.8 BCL9-2 regulates ß-catenin-dependent and -independent target genes

To further understand the role of BCL9-2 and its homolog BCL9 in Wnt/ß-catenin signaling during tumorigenesis it is crucial to characterize the dependency of known Wnt/ß-catenin target genes on the BCL9 proteins. (Canonical Wnt-target genes are listed on the Wnt Homepage: http://www.stanford.edu)).

4.8.1 BCL9-2 is not required for the expression of all canonical Wnt target genes and regulates ß-catenin independent genes in colon cancer cells

To study the impact of the BCL9 proteins in colon cancer in detail, endogenous BCL9, BCL9-2 and ß-catenin expression was silenced in SW480 colon cancer cells for 48h by RNA interference. The dependency of known Wnt/ß-catenin target genes and the ephrin B ligands on ß-catenin and the co-factors was determined by qPCR. The ephrin B receptors (EPHB2/B3 and B4) are known to be expressed in intestinal crypts as Wnt-target genes in normal intestinal homeostasis. The ephrin B ligands (ephrinB1/B2 and B3) are no target genes of ß-catenin and expressed in normal intestinal villi where BCL9-2 protein is expressed. Therefore we tested whether the ephrin B ligands and receptors are target genes of ß-catenin and BCL9-2.

Different subsets of target genes of BCL9-2 and ß-catenin were identified (Figure 22):

The first set of genes was strongly down-regulated after knockdown of ß-catenin and BCL9-2 (Figure 19A), e.g. Axin2, EPHB2, CD44, TCF1, c-myc, BMP4 and Bambi. BCL9 knockdown also reduced the mRNA expression of this gene set with the exception of BMP4, while Bambi was reduced only slightly, but still significant.

The second set of Wnt/ß-catenin target genes was not regulated by BCL9 or BCL9-2 (Figure 19B). ß-catenin knockdown led to strongly reduced mRNA levels of LEF1, MSX2 and CyclinD1. Knockdown of BCL9-2 and BCL9 had no effect on the expression of the ß-catenin target genes LEF1, MSX2 and CyclinD1. This further supports our previous using a minimal *Luciferase*-reporter - CyclinD1. Interestingly, LGR5 mRNA was slightly, but significantly reduced after BCL9, but not BCL9-2 knockdown.

The third set of genes consists of genes of the ephrin/EPHB family which was differentially regulated by ß-catenin and BCL9-2 (Figure 19C). Knockdown of BCL9-2 resulted in reduced

expression of the analyzed genes, while ß-catenin knockdown had no influence or even increased the mRNA expression of the ephrin receptors EPHB3/B4 and the ephrin ligands B1, B2 and B3. In contrast, RNA Interference against BCL9 only led to a reduced mRNA expression of EPHB2, B3 and B4.

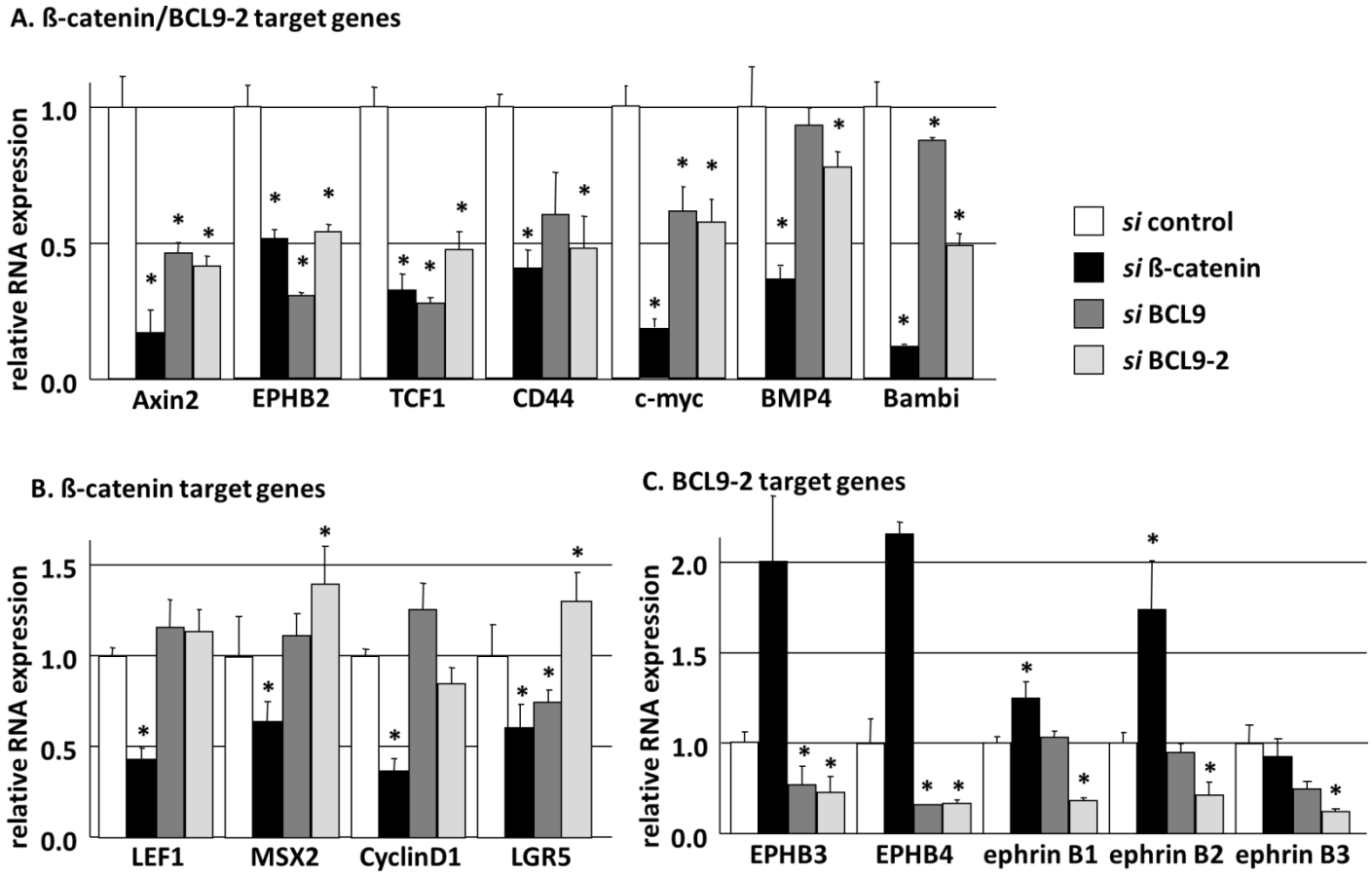

Figure 22: BCL9-2 regulated its own subset of target genes, apparently independent of ß-catenin
Expression of the indicated genes was determined following knockdown of ß-catenin, BCL9 and BCL9-2 for 48h relative to control *si*RNA-treated cells. Quantitative real time PCRs show the mean of least 3 independent experiments and their respective standard deviation (* $p<0.05$). (A) Target genes of BCL9-2 and ß-catenin. (B) BCL9-2 independent ß-catenin target genes. (C) BCL9-2 and ß-catenin differentially regulated genes (*$p \leq 0.05$). (Brembeck, Wiese *et al.*, 2011)

In summary, BCL9 and BCL9-2 regulate only a subset of Wnt/ß-catenin target genes. Moreover, these data demonstrate that BCL9-2 also regulates ß-catenin independent target genes.

4.8.2 ß-catenin-dependent and -independent BCL9-2 target genes are expressed at the invasive front of adenomas of compound K19-BCL9-2; $APC^{Min/+}$ mice

BCL9-2 contributes to adenoma formation from compound K19-BCL9-2; $APC^{Min/+}$ mice and induces locally invasive tumors which invade beyond the basal membrane into the submucosa

or even into the muscularis (Figure 14 and Figure 15). Therefore, accumulation of ß-catenin in $APC^{Min/+}$ mice alone appears not to be sufficient to induce local invasions. However, BCL9-2 activates its own gene cluster independently from ß-catenin (Figure 19). Thereby some of these genes might be responsible for the development of invasive tumors in compound K19-BCL9-2; $APC^{Min/+}$ mutant mice. To investigate whether BCL9-2 targets are expressed in the invasive areas of BCL9-2; $APC^{Min/+}$ derived adenomas immunohistochemistry was performed using specific antibodies which detect the ß-catenin/BCL9-2 target genes Bambi and EPHB2 and the BCL9-2 target genes EPHB3 and B4. The invasive areas were previously stained positive for nuclear ß-catenin and BCL9-2 (Figure 15B) indicating high transcriptional activity.

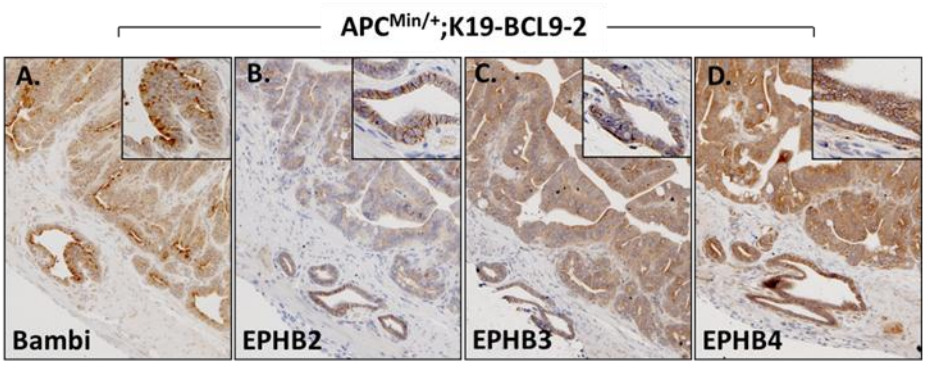

Figure 23: Expression of BCL9-2 targets in invasive areas of compound K19-BCL9-2; $APC^{Min/+}$ mice
(A-D) Immunohistochemistry of (A) Bambi, the ephrin receptors (B) EPHB2, (C) EPHB3 and (D) EPHB4 on an invasive adenoma of compound $APC^{Min/+}$; K19-BCL9-2 mice (400x magnification).

Strong protein expression of the ß-catenin/BCL9-2 target genes Bambi and EPHB2 was observed in the invasive area compared to the central tumor area (Figure 23A and B). In addition, the ß-catenin independent BCL9-2 targets EPHB3 and EPHB4 (Figure 23C and D) were expressed in the invasive areas and in the central tumor.

In summary, BCL9-2 contributes to tumor formation and invasion probably by the regulation of an own set of target genes, which is partially independent of ß-catenin.

4.9 Whole genome microarray analyses demonstrate that BCL9, BCL9-2 and ß-catenin activate different gene expression profiles

BCL9-2 is up-regulated in the majority of colon cancers (Figure 11 and Figure 16) and contributes to cancer progression by promoting tumor growth (Figure 14) and invasion (Figure 15 and Figure 19). In addition, BCL9 and BCL9-2 regulate the expression of target genes

independently from ß-catenin (Figure 22). To understand the impact of BCL9-2 and its homolog BCL9 in comparison to ß-catenin during these processes, it is important to analyze the gene cluster which is affected by these proteins in comparison to known ß-catenin target genes. (A list of described canonical Wnt-target genes is available from the Wnt Homepage http://www.stanford.edu)

To explore the gene signature of BCL9 proteins and ß-catenin in tumorigenesis human whole genome microarrays were analyzed. Colon cancer cell lines with different levels of BCL9-2 were examined. Expression data from previous unpublished human whole genome microarrays of SW480 cells, which exhibit the highest BCL9-2 protein level, were already available (Brembeck, unpublished). These data were completed for DLD1 colon cancer cells with moderate and HCT116 cells with low BCL9-2 protein levels. In addition, our current studies MCF7 breast cancer cells to express also very high levels of BCL9-2 protein (Zatula and Brembeck, unpublished). Since, MCF7 cells did not show measureable active canonical Wnt signaling, as determined by TOP/FOP reporter gene assays, these breast cancer cells served as an additional model to investigate ß-catenin independent functions of BCL9-2.

The gene expression profile of the described cell lines was determined in triplicates after knockdown of endogenous BCL9, BCL9-2 and ß-catenin for 48h using a pool of two different *si*RNAs, respectively, and normalized to cells treated with a pool of four non-targeting control *si*RNAs. The efficacy of all *si*RNAs was previously tested in all used cell lines by qPCR (data not shown). The gene signatures in the identified whole genome micro arrays resulting from knock down of ß-catenin, BCL9 and BCL9-2 in the different cell lines, were subsequently analyzed. Each data set consisted of biological triplicates. The data sets for each treatment (*si*ß-catenin, *si*BCL9 and *si*BCL9-2) were relativized to the data set originated from *si*control treated cells. For further analyses only genes were used, which were expressed with fold discovery rate (FDR ≙ adjusted p-value) ≤ 0.05. In addition, only genes which were expressed with a minimum of a moderate expression level of 7 from total 15 were further analyzed.

4.9.1 ß-catenin and BCL9/BCL9-2 induce different gene sets in cancer cells

To investigate, whether BCL9 and BCL9-2 regulate specific sets of target genes independently of ß-catenin, Venn diagram analyses were performed. These illustrated the overlap of all genes that were significantly reduced ≥1.5 fold following knockdown of ß-catenin, BCL9 and BCL9-2 in SW480, DLD1 and MCF7 cells (Figure 24). In addition, heat map analyses compared all genes by a color coded expression score. To this end all genes were included, that showed ≥2 fold

down-regulation following knockdown of ß-catenin and of BCL9-2 in SW480 and MCF7 cells (Figure 25).

The microarray analyses confirmed the distinct transcriptional roles of ß-catenin and of BCL9/BCL9-2. The induced gene sets of BCL9 and BCL9-2 were only in part overlapping with the gene cluster of ß-catenin (Figure 24 and Figure 25). Only 4% of BCL9-2 and 6% of BCL9 dependent genes were present in the gene set regulated by ß-catenin in SW480. In DLD1 cells 13% of BCL9-2 and 14% of BCL9 target genes were regulated by ß-catenin. In addition, BCL9 and BCL9-2 itself shared almost 10% of their target genes in SW480 and DLD1 (Figure 24, left and middle).

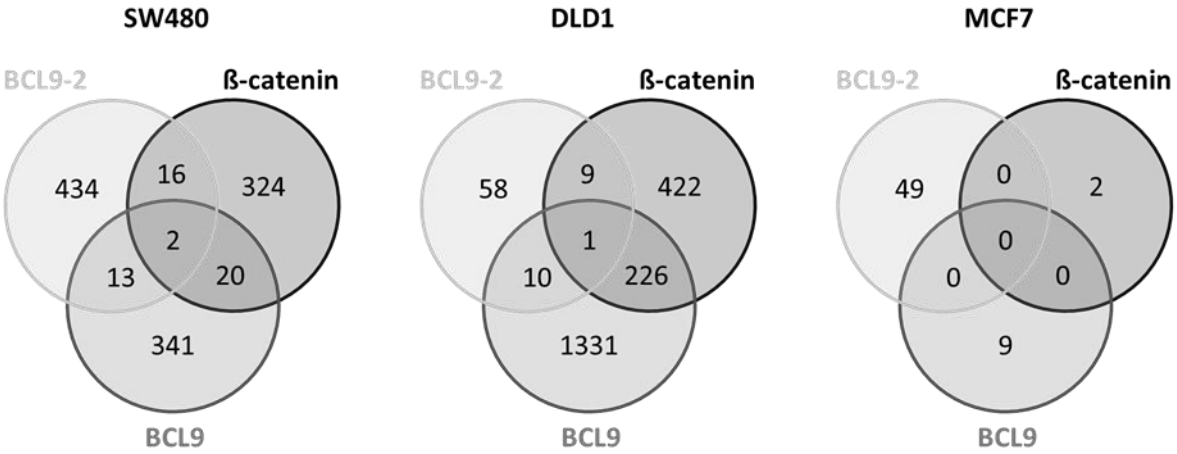

Figure 24: ß-catenin and BCL9/BCL9-2 induce different gene sets in cancer cell lines
Venn diagrams illustrating the overlap of ≥1.5 fold down-regulated genes in SW480, DLD1 and MCF7 cells following knockdown of the proteins for 48h, as indicated (FDR≤0.05).

Moreover, knockdown of ß-catenin in MCF7 led to reduction only one gene covered by two oligonucleotides and which represented ß-catenin itself. In addition, BCL9 knockdown in MCF7 appeared to be less effective in transcriptional modulation compared to BCL9-2, since only nine genes were regulated by BCL9 compared to 49 genes which appeared in the BCL9-2 gene set (Figure 24 and Figure 25, right). In contrast, knockdown of BCL9 in DLD1 cells resulted in more than 1500 differentially regulated genes, while BCL9-2 knockdown led to reduction of only 78 genes. Since DLD1 cells contain moderate levels of BCL9-2, BCL9 appeared to have a stronger impact on gene transcription than BCL9-2 (Figure 24, middle).

The heat map analyses for all highly significant regulated genes revealed that BCL9-2 indeed shares many genes with the cluster of ß-catenin in SW480 colon cancer cells. However, BCL9-2 significantly regulates several additional target genes independently of ß-catenin (Figure 25, left).

Moreover, heat map analyses showed that there is almost no detectable transcriptional function of ß-catenin in MCF7 cells. In contrast, BCL9-2 appeared to be a potent transcription factor for the regulation of target genes in breast cancer cells (Figure 25, right).

These results demonstrate that BCL9-2 dependent transcriptional regulation in different cancer cells does not rely completely on ß-catenin and that BCL9 and BCL9-2 are able to transactivate a gene set independently of ß-catenin/Wnt signaling.

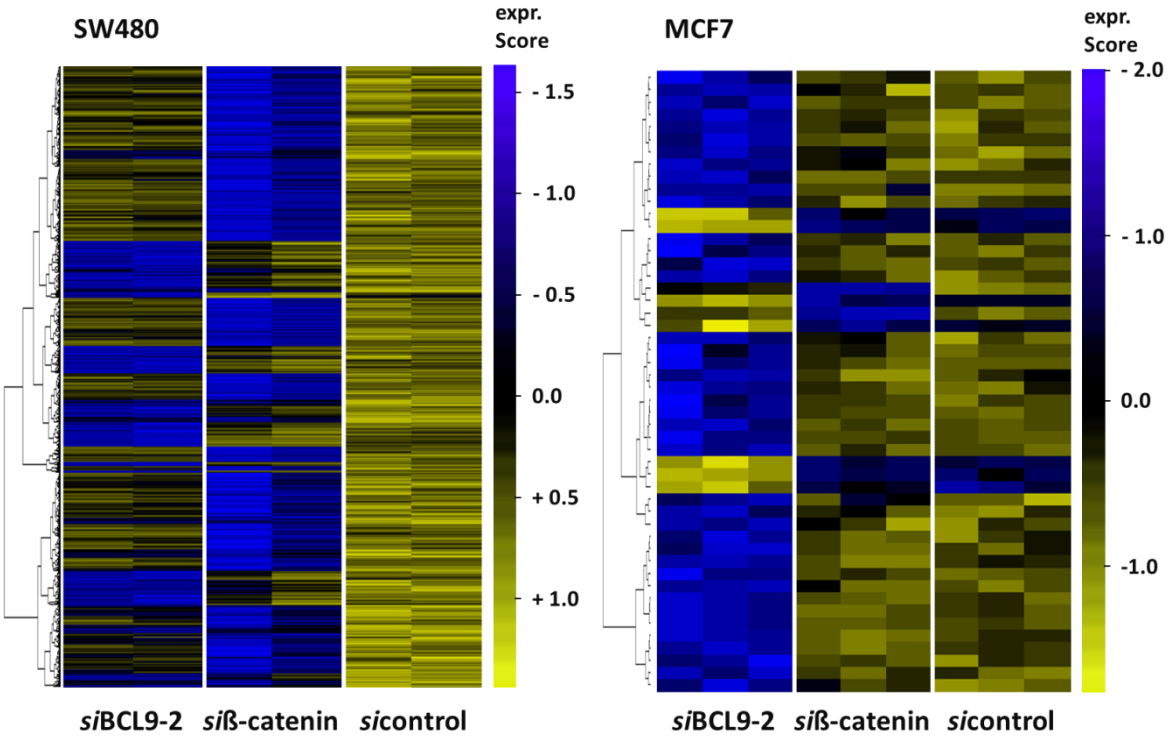

Figure 25: BCL9-2 regulates target genes independently of ß-catenin.
Heat maps show all ≥2 fold down-regulated genes in SW480 and MCF7 cells after knock down of ß-catenin and BCL9-2 (FDR≤0.05). The expression of genes is shown in comparison to non-targeting siRNA treated samples and depicted by a color coded expression score.

In summary, the induced gene signature resulting from knockdown of BCL9 and BCL9-2 in different cancer cells are only in part overlapping with the gene cluster of regulated by ß-catenin. In addition, the number of BCL9-2 regulated genes correlated positively with the expression level of BCL9-2 in the different cancer cell lines.

4.10 Novel BCL9-2 target genes in cancer cells identified by microarray analyses

4.10.1 Comparison of the gene expression profile of cancer cell lines and of tumors from the intestine specific double knock out of VilCre;BCL9$^{-/-}$/ BCL9-2$^{-/-}$ mice

Deka and colleagues developed a intestine specific BCL9$^{-/-}$/BCL9-2$^{-/-}$ knockout mouse model in which they chemically induced dysplastic colon adenomas (Deka *et al.*, 2010). Tumors deriving from wild-type and BCL9$^{-/-}$/BCL9-2$^{-/-}$ mice were analyzed by comparative gene expression profiling and the regulated genes were publicly available for our further analyses. This model reflects the *in vivo* situation which is mimicked by *si*BCL9 and *si*BCL9-2 treatment of our colon cancer cell lines. We compared the gene expression profile of BCL9$^{-/-}$/BCL9-2$^{-/-}$ derived tumors and *si*BCL9, *si*BCL9-2 and *si*ß-catenin treated SW480 cells to investigate the regulatory functions of BCL9 and BCL9-2 in comparison to ß-catenin in colon cancer.

We compared target genes of BCL9 and BCL9-2, which were significantly ≥1.5 fold down-regulated in SW480 cells and target genes, which displayed ≥2 fold decreased transcription, originating from the expression profile of BCL9$^{-/-}$/BCL9-2$^{-/-}$ mice derived tumors. The graphs show the fold change of the respective genes compared to SW480 cells treated with non-targeting siRNA treated or tumors derived from wild-type mice, respectively (Figure 26).

From 1200 genes, which appeared in the expression profile of BCL9$^{-/-}$/BCL9-2$^{-/-}$ mice derived tumors, 28 were also present in the gene sets of siBCL9/siBCL9-2-treated SW480 cells. Out of these 28 genes, only knockdown of BCL9 resulted in reduction of 5 specific genes, like the gene of ras-related protein 2b (RAP2B) (Figure 26C), whereas only knockdown of BCL9-2 led to the reduction of 19 genes such as the gene of transforming growth factor beta 3 (TGFB3) (Figure 26D). Interestingly, only 4 genes appeared in the gene set of *si*ß-catenin-treated SW480 cells, such as Axin2 (Figure 26A and B).

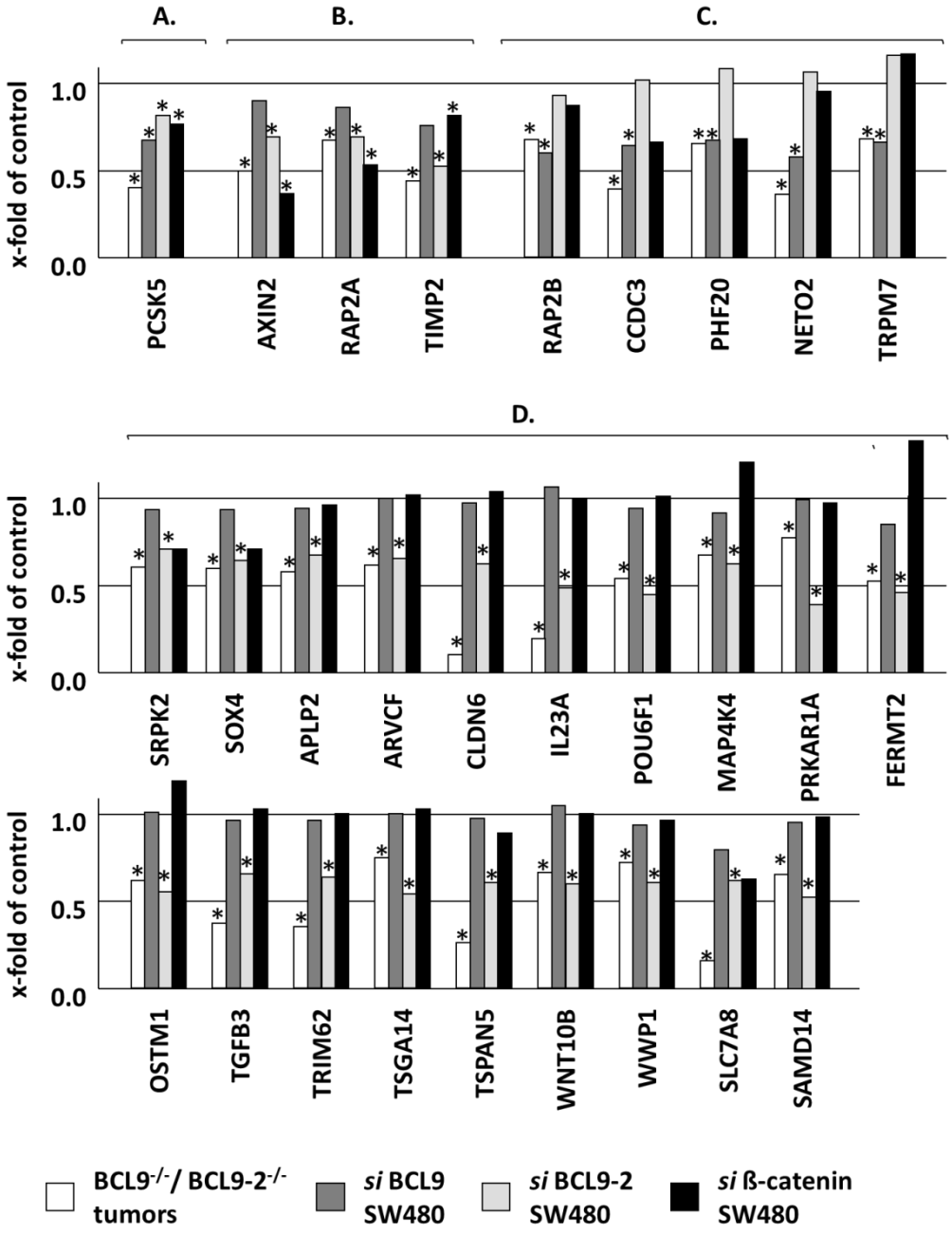

Figure 26: Comparative gene expression profiles from VilCre;BCL9$^{-/-}$/ BCL9-2$^{-/-}$ and microarrays from *si*RNA treated SW480 cells identified new target genes of the BCL9 proteins
Comparative gene expression profiles from tumors of VilCre;BCL9$^{-/-}$/ BCL9-2$^{-/-}$ and SW480 cells treated with *si*RNA targeting ß-catenin, BCL9 and BCL9-2, as indicated. The graphs show the log fold change (logFC) of target genes of (A) BCL9, BCL9-2 and ß-catenin, (B) BCL9-2 and ß-catenin, (C) BCL9 and (D) BCL9-2 as % of control *si*RNA-treated cells and tumors of non-transgenic mice, respectively. Asterisks indicate a fold discovery rate (FDR<0.05).

In summary, based on the expression profiles obtained from our microarray analyses in SW480 cells, the impact of BCL9-2 knockout in BCL9$^{-/-}$/BCL9-2$^{-/-}$ mice appeared to be more severe in comparison to BCL9 knockout. Moreover, ablation of BCL9-2 had a dramatic influence on gene expression in the induced tumors. Remarkably, this influence was not observed after

knockdown of ß-catenin in SW480 cells. This indicates a partially ß-catenin-independent influence of BCL9-2 on gene transcription in colon cancer.

Comparison of the differentially regulated genes from our microarray analyses in SW40 cells to those from the tumors derived from BCL9/BCL9-2 knockout mice, we found the HOX gene SOX4 which appeared in both analyses. Moreover, Deka et al. identified SOX6 and PROX1 as putative target genes of the BCL9 protein *in vivo*. An additional HOX gene, SOX9, was present in the BCL9-2 mediated gene set in SW480 cells.

However, determination of differentially regulated genes in BCL9$^{-/-}$/BCL9-2$^{-/-}$ mice does not dissect the dependency on BCL9 or BCL9-2, since the arrays were performed from double mutant animals. To analyze the dependency of the HOX genes in colon cancer cells on BCL9-2 or/and BCL9 in comparison to ß-catenin, qPCRs were performed following knockdown of the proteins in SW480 cells (Figure 27A and B).

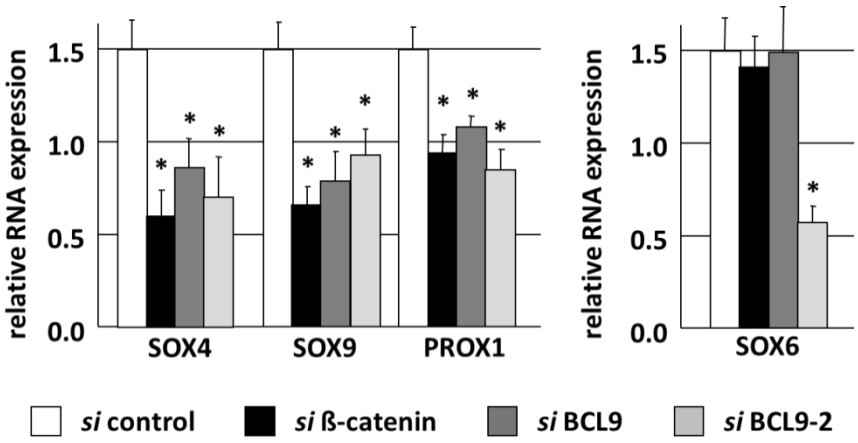

Figure 27: BCL9-2 regulates the expression of HOX genes
Expression of the indicated genes was determined following knockdown of ß-catenin, BCL9 and BCL9-2 for 48h relative to control *si*RNA-treated SW480 cells. Quantitative real time PCRs show the mean of least 3 independent experiments and their respective standard deviation (*p≤0.05).

The mRNA expression of the HOX genes SOX4, SOX9 and PROX1 in SW480 cells was strongly down-regulated after knockdown of all three proteins. In contrast, SOX6 expression was only regulated by BCL9-2, but not by BCL9 or ß-catenin.

In summary, BCL9-2 transcriptionally regulates different genes of the HOX gene family, which are implicated in cancer progression (Petrova *et al.*, 2002), and which might further trigger the oncogenic potential of BCL9-2 during intestinal tumorigenesis.

4.10.2 Cyclopholin A (CypA) and the stem-cell-derived neural stem/progenitor cell supporting factor (SDNSF) are newly identified BCL9-2 core target genes in cancer cell lines

Venn diagrams were used to compare all genes that were significantly down-regulated ≥1.5 fold in MCF7, SW480, DLD1 and HCT116 cancer cells. The number of BCL9-2 regulated genes correlated well with the expression level of BCL9-2 in the cell lines (Figure 16 and Figure 28). BCL9-2 knockdown in SW480 showed the strongest effects with 605 affected genes. In contrast, BCL9-2 knockdown was less effective with 18 transcriptional reduced genes in HCT116 cells, with low BCL9-2 protein levels (Figure 28). Moreover, these analyses identified two novel core target genes which were only regulated by BCL9-2 in all analyzed cancer cell lines (Figure 28): Cyclophilin A (CypA) and stem-cell-derived neural stem/progenitor cell supporting factor (SDNSF).

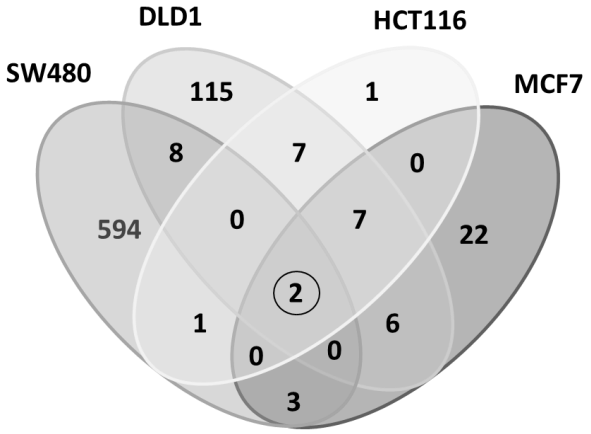

Figure 28: Microarray analysis identified 2 genes as new BCL9-2 core target genes in cancer cell lines
Venn diagram showing the overlap of 1.5 fold down-regulated genes upon BCL9-2 knock-down in various cancer cell lines, as indicated (p≤0.05).

Quantitative real time PCRs and Western Blot analyses were performed, to validate the dependency of CypA and SDNSF on BCL9-2. Only knockdown of BCL9-2 resulted in strong reduction of mRNA levels of the new core target genes in all tested cell lines (Figure 29A, B and C). Accordingly, protein levels of CypA were reduced after treatment of SW480 and HCT116 cells for 72h with *si*RNA targeting BCL9-2 (Figure 30A, upper panel). ß-catenin and BCL9 knockdown had no effect on CypA and SDNSF expression (Figure 29A).

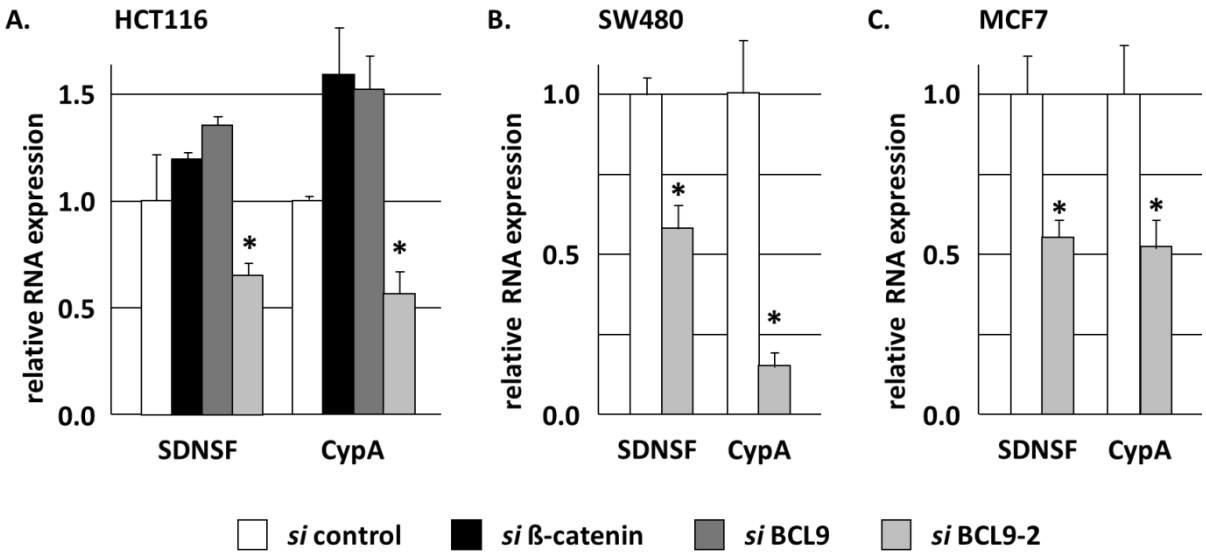

Figure 29: BCL9-2 but not BCL9 and ß-catenin regulates the mRNA and protein expression of Cyclophilin A (CypA) and stem-cell-derived neural stem/progenitor cell supporting factor (SDNSF) in cancer cell lines

(A, B and C) qRT-PCR determined mRNA levels of SDNSF and CypA following knockdown of ß-catenin, BCL9 and BCL9-2 for 48h in different cancer cell lines, as indicated. Graphs show the mRNA expression as % of control *si*RNA treated cells (*p≤0.05).

In summary, BCL9-2 regulates a subset of core target genes in colon cancer cells which might trigger the oncogenic role of BCL9-2 independent of ß-catenin. First, Cyclophilin A, which has already been shown to play a role in different tumor entities, might also mediate the oncogenic role of BCL9-2 (Obchoei *et al.*, 2009). Second, SDNSF, which regulates cell-survival (Toda *et al.*, 2003) could also be implicated in BCL9-2 induced tumorigenesis.

4.11 Dissecting the ß-catenin-dependent and -independent functions of BCL9-2

Dissection of the ß-catenin-dependent and -independent function of BCL9-2 is important to characterize the molecular mechanisms for the role of this co-factor for the control of specific target genes. BCL9-2 itself does not contain a classical DNA-binding domain, but might act a co-factor for several transcription factors besides ß-catenin/LEF/TCFs. Therefore, we analyzed the promoters of BCL9-2 regulated genes to identify putative BCL9-2 responsive elements and to characterize a nuclear binding partner of BCL9-2. In this regard, we analyzed the transcriptional regulation of the promoters of the ß-catenin/BCL9-2 target gene CDX1 and of the BCL9-2 target gene CDX2.

4.11.1 BCL9-2 regulates the endogenous expression of caudal type homeobox 1 and 2 (CDX1/2)

The homeodomain transcription factor caudal type homeobox 1 and 2 proteins (CDX1 and CDX2) have been previously identified as Wnt/ß-catenin target genes during embryonic intestinal development in mice (Hryniuk *et al.*, 2012;Lickert *et al.*, 2000;Lickert *et al.*, 2002). However, it was shown, that CDX2 expression is not activated by canonical Wnt-signaling after E10.0 in mice (Gao *et al.*, 2009). Moreover, CDX1, but not CDX2, is a well characterized Wnt/ß-catenin target gene in the adult intestine (Hryniuk *et al.*, 2012).

We have analyzed whether BCL9, BCL9-2 and ß-catenin regulate the expression of these two homeodomain transcription factors, and performed qPCRs were performed after treatment of SW480 cells with *si*RNA for 48h targeting BCL9, BCL9-2 and ß-catenin. BCL9 knockdown had no influence on CDX1 and CDX2 expression. In contrast, CDX1 mRNA was reduced after knockdown of ß-catenin and BCL9-2 in SW480 cells. Remarkably, CDX2 was only affected by BCL9-2, but not by ß-catenin knockdown (Figure 30B). Accordingly, BCL9-2 knockdown for 72h reduced the CDX2 protein level in HCT116 and SW480 cells as analyzes by Western Blot analysis (Figure 30A).

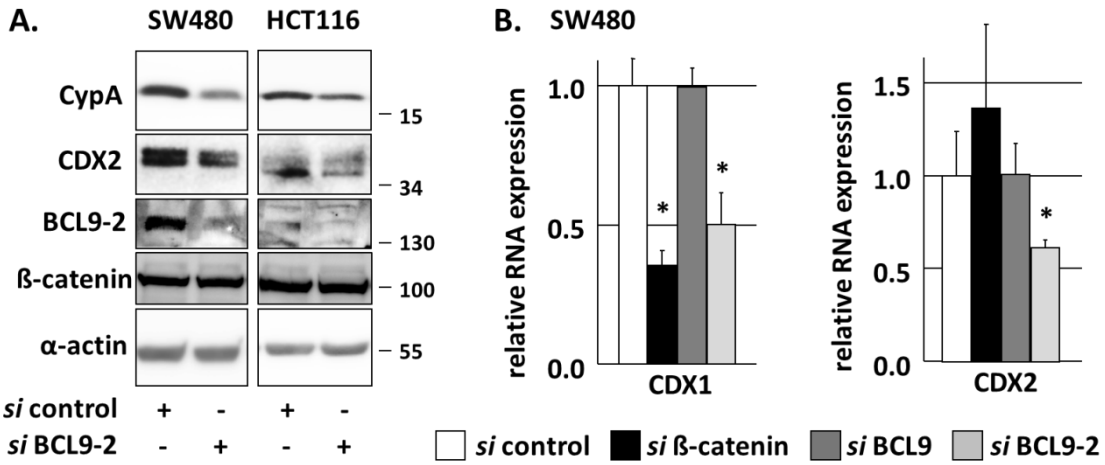

Figure 30: The Wnt/ß-catenin target gene CDX1 and the ß-catenin-independent gene CDX2 are regulated by BCL9-2 in colon cancer cell lines
(A) 30µg of nuclear protein lysates were used in Western Blot analysis to determine CDX2 and CypA protein expression following knockdown of BCL9-2 for 72h (second panel) in SW480 and HCT116 cells. β-actin (bottom panel) and ß-catenin (third panel) served as controls for equal protein loading. (B) qPCR of CDX1 and CDX2 following knockdown of ß-catenin, BCL9 and BCL9-2 in cancer cell lines, as indicated. Graphs show the mRNA expression relative to control *si*RNA treated cells (*p≤0.05).

In summary, BCL9-2 regulates the endogenous expression of CDX1 and CDX2, which had been shown to be up-regulated during early stages of tumor development (Bakaris *et al.*, 2008;Phillips *et al.*, 2003). Remarkably, only BCL9-2, but not ß-catenin or BCL9, regulates the mRNA and protein expression of CDX2 in colon cancer cell lines. In contrast, endogenous expression of CDX1 is dependent on ß-catenin and BCL9-2.

5.11.2 Transcriptional activation by the proximal promoters of the homeodomain transcription factors CDX1 and CDX2 requires BCL9-2, but not ß-catenin in colon cancer cell lines

To identify a promoter region, which is responsible for BCL9-2 mediated transcriptional activation, *Luciferase* assays were performed in SW480 and HCT116 colon cancer cell lines following knockdown of ß-catenin and BCL9-2. To this end, *Luciferase*-reporter constructs, containing different length of the proximal promoter of the human CDX1 (Suh *et al.*, 2002) and CDX2 were used. All *Luciferase* constructs contained different fragments of the upstream CDX1 and CDX2 promoter regions and +73bp of the CDX1- and +76bp of the CDX2-3'UTR, respectively, relative to the transcription start site (Figure 31and Figure 32).

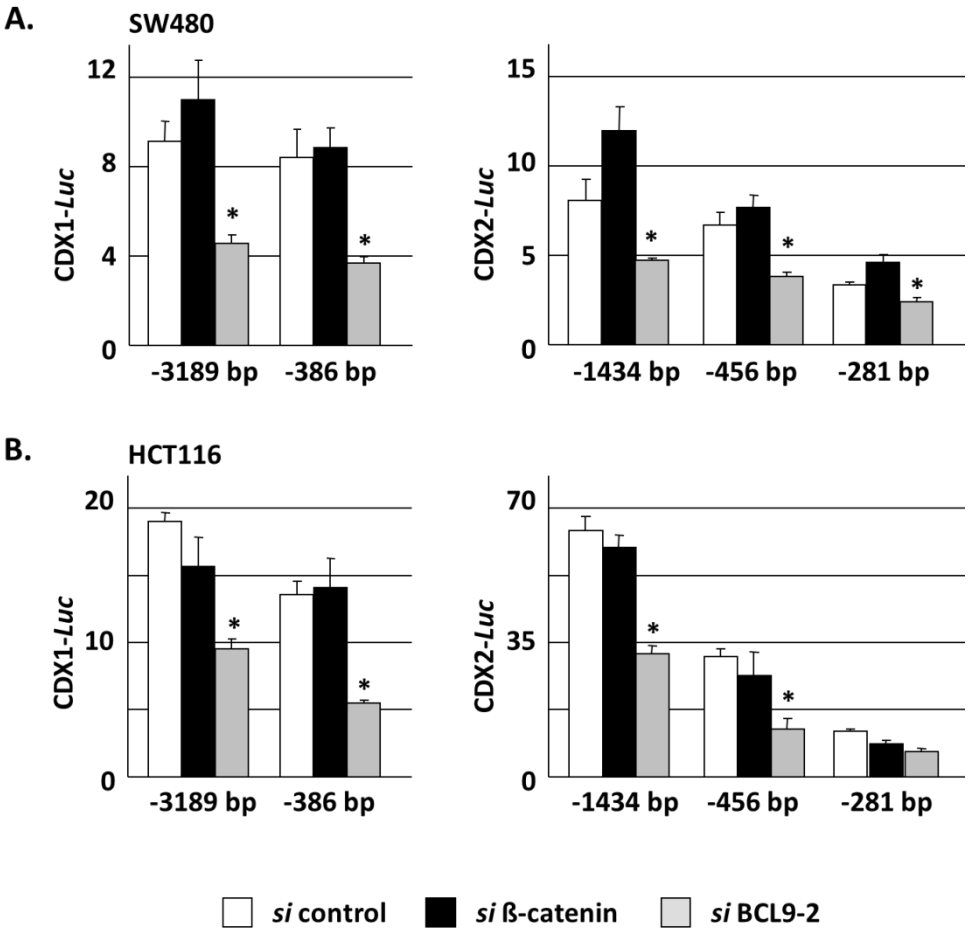

Figure 31: BCL9-2, but not ß-catenin knockdown reduces the activity of CDX1 and CDX2 Luciferase-reporters
For *Luciferase* assays (A) SW480 and (B) HCT116 cells were pretreated with *si*RNA for 48h targeting ß-catenin, or BCL9-2 and non-targeting *si*RNA, followed by 36h transfection of CDX1 and CDX2 *Luciferase*-reporters containing different length of each of their proximal promoters, as indicated. The graphs show the fold change of empty control plasmids with their respective standard deviation (*p≤0.05).

CDX1 and CDX2 promoter driven *Luciferase* expression was independent of ß-catenin in both colon cancer cell lines, although all CDX1 reporters and the -1434bp and -456bp CDX2 reporters contain LEF/TCF responsive elements. Compared to the control, knockdown of BCL9-2 reduced the activity of the -3189bp and the smaller -386bp CDX1 *Luciferase*-reporter to 50% (Figure 31A and B, left). Moreover, knockdown of ß-catenin even slightly induced all CDX2 *Luciferase*-reporter in SW480 (Figure 31A, right). This tendency was already observed on CDX2 mRNA level after knockdown of ß-catenin in qPCR (Figure 30A and B). BCL9-2 knockdown significantly inhibited the activities of the -1434bp and the -456bp CDX2 reporters in SW480 and HCT116. The -281bp proximal promoter fragment of CDX2 still responded to BCL9-2 knockdown in both cell lines, but to a lesser extent compared to the larger -456bp CDX2 reporters (Figure 31A and B, right).

In conclusion, CDX2 and the Wnt/ß-catenin target gene CDX1 is regulated by BCL9-2 through a responsive element in the proximal promoter of -386/+73bp CDX1 and of -456/+76bp CDX2. Moreover, both CDX reporters were independent of ß-catenin indicating that the LEF/TCF binding elements (Wnt responsive elements - WRE) in the proximal promoters of the CDX genes are not responsive to ß-catenin in colon cancer cell lines.

4.11.3 Identification of putative transcription factor binding sites in the proximal promoters of CDX1 and CDX2

The promoters of CDX1 and CDX2 were further analyzed in more detail, to evaluate the BCL9-2 mediated transcriptional regulation of the ß-catenin/BCL9-2 target gene CDX1 compared to the BCL9-2 target gene CDX2. TRANSFAC® MATCH/PATCH (Transfac Professional 2008.4 (Kel *et al.*, 2003;Matys *et al.*, 2003)) was used to identify putative transcription factor binding elements (BE), which were present in the -386/+73bp CDX1 and the -456/+76bp CDX2 promoter. TRANSFAC® MATCH identifies predicted transcription factor binding elements by using a library of positional weight matrices, while TRANSFAC® PATCH identifies pattern-based transcription factors. Both applications use a set of binding sites provided from TRANSFAC® Public 6.0 (http://www.gene-regulation.com/pub/databases.html#transfac).

TRANSFAC® MATCH analyses identified various putative binding elements (BEs) in both proximal promoters: Binding elements for LEF/TCF, OCT4 (octamer-binding transcription factor 4), NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells), BCL6 (B-Cell Lymphoma 6 protein) and also for CDX2 were present in both promoters (Figure 32A and B).

Of note, CDX2 itself was shown to bind to the TATA box of the CDX1 promoter and thereby activate CDX1 expression (Mutoh *et al.*, 2009) . The two LEF/TCF binding sites in the CDX1 promoter were previously described as functional elements in early mouse embryonic intestines (Lickert *et al.*, 2000;Lickert *et al.*, 2002). In addition, the CDX2 promoter contains a previously identified SMAD-binding element, which is activated by BMP2/4 in intestinal metaplasia (Barros *et al.*, 2008). An second SMAD-binding element was identified in the proximal promoter of the CDX2 gene close to the transcription start site by TRANSFAC® MATCH (Figure 32B).

Moreover, both promoters harbor multiple SP1-binding elements (Specificity Protein 1), which are very common in GC-rich promoters. TRANSFAC® MATCH identified two SP1-binding elements in the proximal promoter of the CDX1 gene. In the TATA-less promoter of CDX2 SP1

binding elements (BEs) were randomly distributed as identified by TRANSFAC® PATCH. In addition we found three suboptimal SP1-BEs the CDX2 proximal promoter (Figure 32A and B).

To investigate whether the identified TF-binding elements are conserved between *Mus musculus* and *Homo sapiens*, T-COFFEE® was used (Di *et al.*, 2011;Notredame *et al.*, 2000). This tool allows the combination of multiple sequence alignment including the determination of the degree of conservation between sequences. To this end, the proximal promoter sequences of human and mouse CDX1 and CDX2 were analyzed (Figure 32A and B). The proximal promoter of the CDX1 gene showed overall less conservation compared to that of CDX2. Previously identified transcription factors binding sites for LEF, OCT4 and CDX2 in the -386bp CDX1 and -456bp CDX2 promoter were highly conserved in humans and mice. Both SMAD-binding elements were highly conserved in mouse and human CDX2 promoters and a conserved NFκB-binding element was present in the proximal promoter of CDX1 while only human species harbor this element in the CDX2 promoter. BCL6-binding elements were found in both CDX promoters of *Homo sapiens*, but not in the proximal promoter of rodent CDX1, suggesting a less important role of BCL6. However, SP1-binding elements were present in all promoters of the CDX genes, but only the very proximal SP1-elements located very close to the transcription start were conserved in the promoters of both species. In contrast, the more distal SP1-binding elements were only present in the human CDX1 and CDX2 promoters (Figure 32B).

Thus, multiple similar binding elements were present in the proximal promoters of CDX1 and CDX2 which are highly conserved between human and mouse including the binding sites for BCL6, NFκB, OCT4, CDX2, LEF/TCF and SP1 transcription factors.

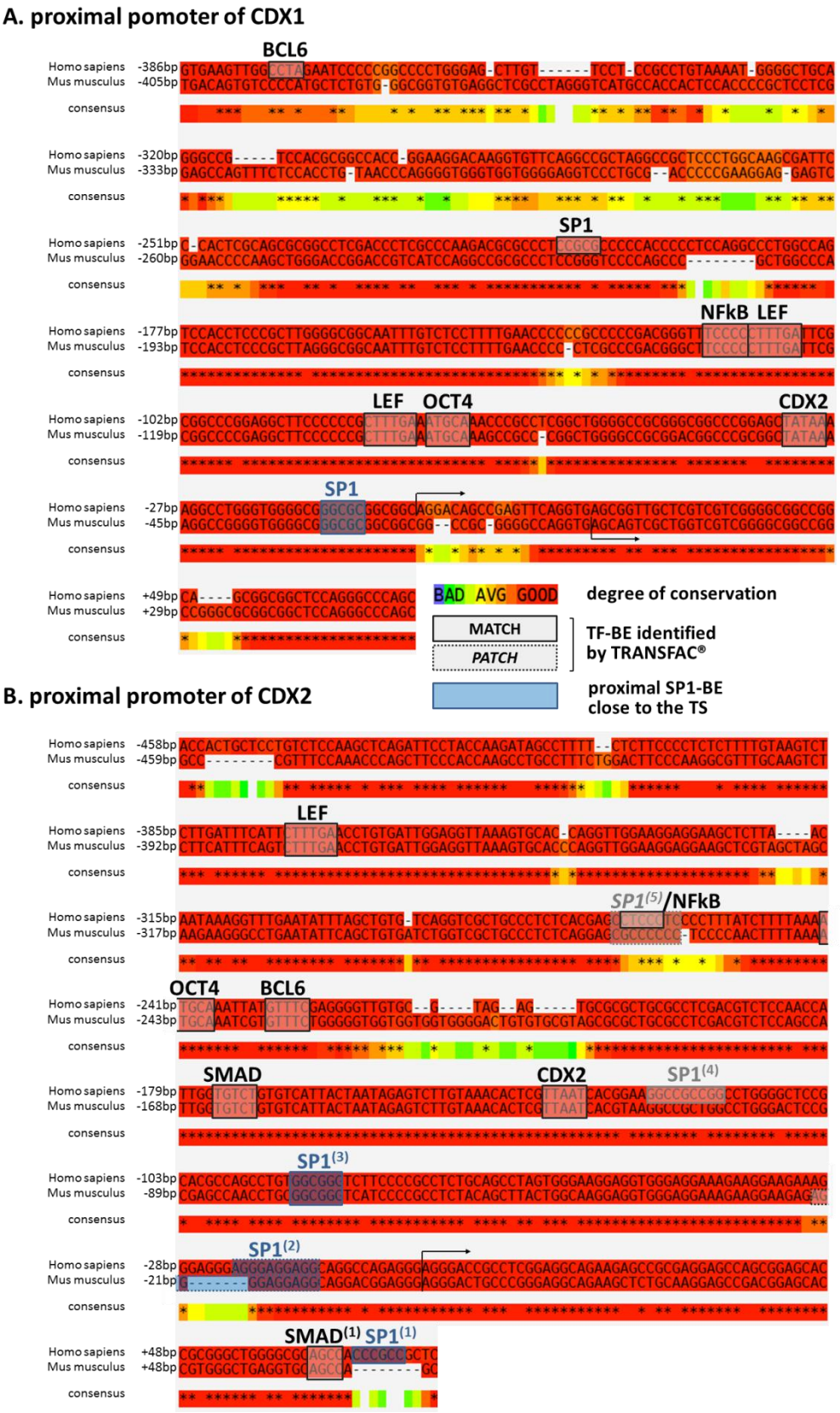

Figure 32: Transcription factor binding elements in the CDX1 and CDX2 proximal promoter
Proximal promoters from *Homo sapiens*, *Mus musculus* and *Rattus norwegicus* **(A)** of CDX1 and **(B)** CDX2 including trough TRANSFAC® identified putative transcription factor binding elements (TF-BE) on the indicated position from the transcription start (TS). The degree of conservation between the organisms is indicated by a color code.

4.11.4 BCL9-2 modulates CDX1 and CDX2 reporter activity by Specificity Protein 1 (SP1)-binding elements in their proximal promoter in colon cancer cell lines

To identify a binding site which is responsible for BCL9-2 transcriptional regulation, all binding elements as identified above (see 4.11.3) were mutated using mutagenesis PCR to disrupt the binding of the respective transcription factors. The activities of the mutated Luciferase-reporters were analyzed in SW480 and HCT116 which were pretreated for 48h with *si*RNA targeting BCL9-2 and compared to control *si*RNA treated cells.

A) Transcriptional regulation of CDX1 (Figure 33)

In general, mutation of all TF-binding elements in the promoter of CDX1 only slightly reduced the reporter activities in SW480 cells. In HCT116 mutation of the binding motif for LEF, NFκB, OCT4 and SP1 led to a reduction of reporter activities of approximately 45-60%. However, each of the mutated reporters was still expressed with well detectable levels (Figure 33).

Remarkably, single and double LEF mutated CDX1 *Luciferase*-reporters still responded to knockdown of BCL9-2 in SW480 and HCT116 cells (Figure 33), indicating that BCL9-2 regulated their expression independently of these LEF-binding elements.

Also the mutations of the BCL6-, NFκB- or OCT4-binding elements still showed a reduced CDX1 reporter activity following BCL9-2 knockdown (Figure 33).

Mutation of the CDX2-binding element in the proximal CDX1 promoter (Figure 32A) was not investigated since this binding site is located close to the transcription start site and mutation will lead to a complete loss of reporter activity (Mutoh *et al.*, 2009).

The distal SP1-binding element mutant still responded to BCL9-2 knockdown. However, mutation of the very proximal SP1-binding element lost the response to knock down of BCL9-2. Similarly, CDX1 reporter containing SP1-binding element-double mutations also did not respond to BCL9-2 knock down (Figure 32A and Figure 33).

These data demonstrate that BCL9-2 regulates CDX1 gene transcription through a newly identified SP1 binding element in the proximal promoter region close to the transcription start site.

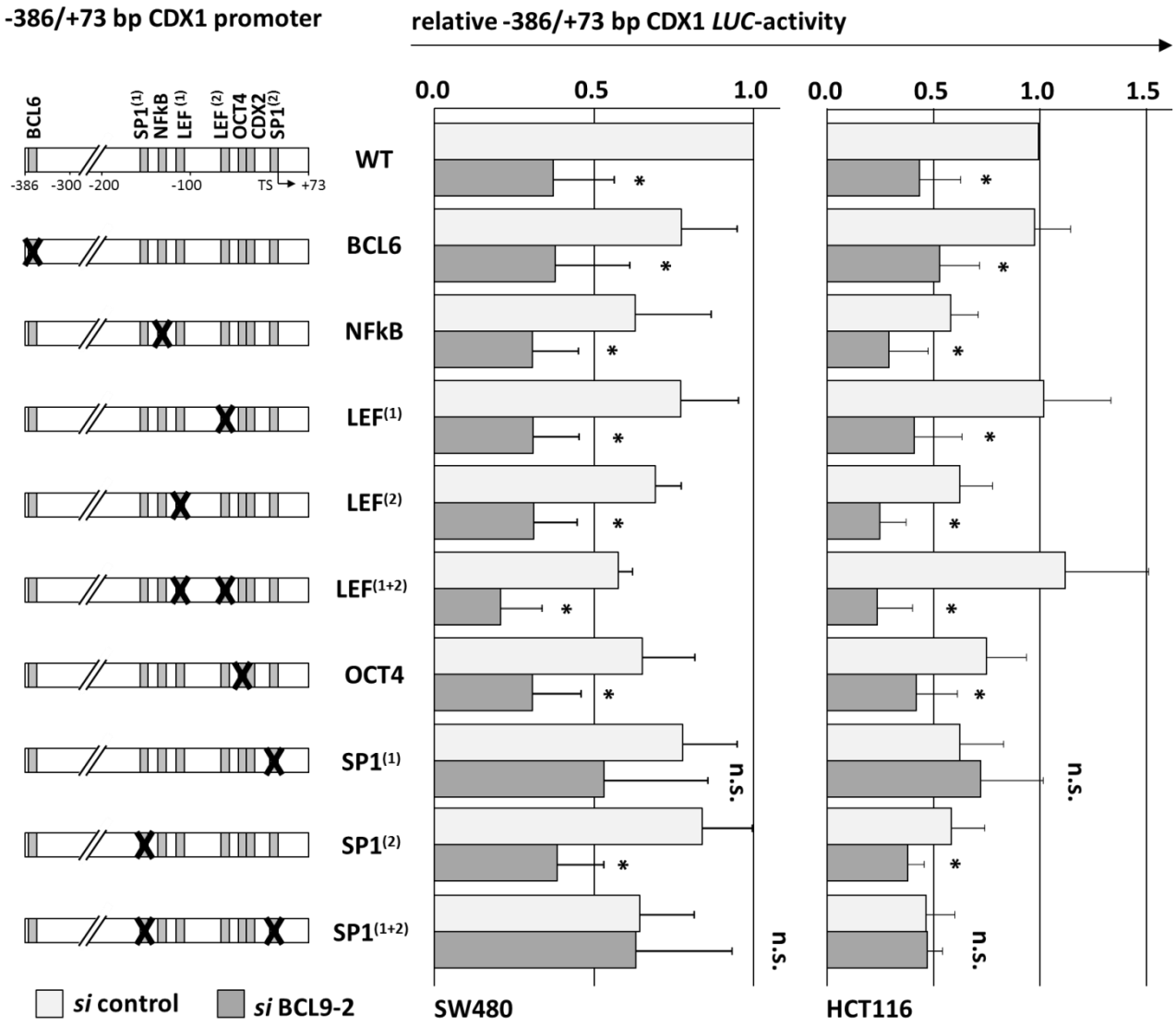

Figure 33: BCL9-2 regulates CDX1 reporter gene expression by an SP1 responsive element in the proximal promoter
For *Luciferase* assays SW480 and HCT116 cells were pretreated with *si*RNA targeting BCL9-2 and non-targeting *si*RNA for 48h, followed by 36h transfection of -386bp/+73bp CDX1 *Luciferase*-reporters containing different mutations of TF-responsive elements, as indicated (left). The graphs show the relative *Luciferase*-reporter activity to the CDX1-*Luciferase* wild-type (WT) activity and their standard deviation. Significances were calculated to the respective control *si*RNA treated cells (*p≤0.05).

B) Transcriptional regulation of CDX2 (Figure 34)

Mutation of LEF-, BCL6- and CDX2-binding elements had just minor effects on the -456bp CDX2 Luciferase-reporter activity in HCT116 and SW480 cells Figure 34). In SW480 cells, only mutation of the SMAD- and NFκB-binding elements decreased the activity to a greater extent to approximately 50-60%. In HCT116 cells, mutation of NFκB- and the distal SMAD-binding elements reduced the -456bp CDX2 Luciferase-reporter activity to approximately 40-70%. However, each of the mutated -456bp CDX2 Luciferase-reporters was still expressed with well detectable levels in SW480 and HCT116 cells (Figure 34).

Similar to our results for the CDX1 promoter, mutation of the LEF-binding element in the CDX2 reporter also had no influence on the repressive function of BCL9-2 knockdown. In addition, also mutations of the BCL6-, NFκB- or CDX2-binding elements did not change the capability of BCL9-2 knockdown to further reduce these CDX2 reporter activities (Figure 34).

Interestingly, one of each mutant for the two SMAD-binding elements was potent to inhibit the BCL9-2 effect in either, HCT116 or in SW480 cells, respectively (Figure 34). This indicates that the SMAD pathway is efficient to modulate CDX2 reporter activity in these cells.

Like many TATA-less genes, the CDX2 promoter harbors multiple putative SP1-binding elements. SP1-MT(2) and -MT(5) are mutated optimal SP1 sites, while SP1-MT(1), -MT(3) and -MT(4) are mutations of suboptimal SP1 binding sites (Figure 32B).

In HCT116, the basal activity of the CDX2 reporters containing the mutation of the four proximal SP1-binding elements (MT1-4) was reduced to approximately 60- 40 % of the unmutated promoter, while in SW480 mutation of these SP1-binding elements (MT1-4) had no influence on the reporter activities. In addition, the most distal SP1-binding element (MT5) did not affect the basal CDX2 reporter activity in both cell lines. However, double mutation (dMT) of the two optimal SP1-binding elements (dMT2+5) strongly reduced the reporter activity in HCT116 and SW480 cells (Figure 34).

In SW480 cells, single mutations of the distal SP1-binding elements (MT4 and 5),) and the proximal SP1-binding element (MT2) still showed significantly reduced reporter activity following BCL9-2 knockdown. In contrast, mutation of two SP1 sites in the proximal promoter (MT1 and 3), completely lost the inhibition of promoter activity after knockdown of BCL9-2 (Figure 34).

Remarkably, all mutated SP1-binding elements (MT1-5) of the CDX2 promoter did not further respond to BCL9-2 knockdown in HCT116 cells. In both cell lines, double mutations of SP1 sites (MT2+5) also did not respond to BCL9-2 knockdown (Figure 34).

These results clearly demonstrate that the BCL9-2 mediated transcriptional regulation of the CDX2 promoter requires the presence of several functional SP1 binding elements in both cell lines.

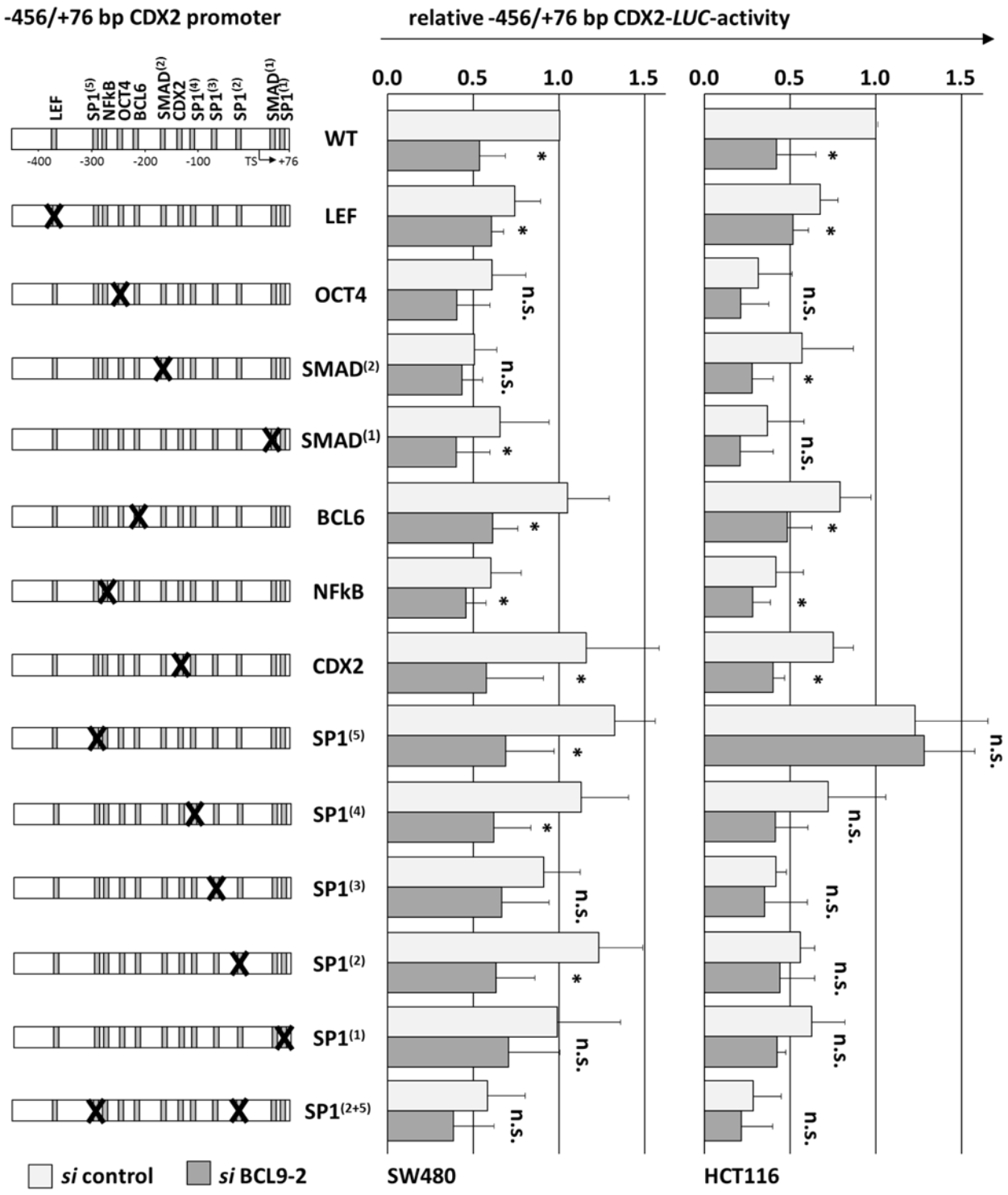

Figure 34: SP1 binding elements mediate the BCL9-2 dependent regulation of the CDX2 promoter
For *Luciferase* assays SW480 and HCT116 cells were pretreated with siRNA targeting BCL9-2 and non-targeting siRNA for 48h, followed by 36h transfection of -456bp/+76pb CDX2 *LUC*-reporters containing different mutations of TF-BSs, as indicated. The graphs show the relative *LUC*-reporter activity to the CDX2-*LUC* wild-type (WT) activity and their standard deviation. Significances were calculated to the respective control siRNA treated cells (*p≤0.05).

In addition, the CDX2 reporter activity was strongly reduced after mutation of the OCT4-binding element, indicating the importance of this element for the transcription of CDX2 in

colon cancer cell lines. Moreover, BCL9-2 knockdown did not further significantly reduce the OCT4 mutated reporter in HCT116 and SW480 cells (Figure 34).

Because CDX1 and CDX2 reporter activities were dependent on the presence of an OCT4 binding element, OCT4 was overexpressed in SW480 cells to determine whether the reporters are inducible by OCT4 or if the OCT4 binding element generally exhibits some activating function independently from OCT4 protein expression (Figure 35).

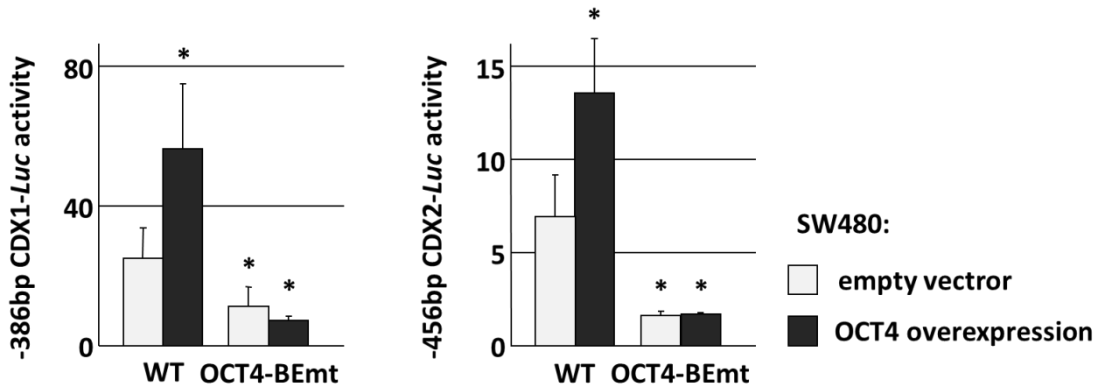

Figure 35: OCT4 induces the CDX1 and CDX2 reporter activities through a newly identified OCT4 binding element
For *Luciferase* assays SW480 were transfected with 200ng OCT4 overexpression plasmid and -456bp/+76 pb CDX2 or -386bp/+75bp CDX2 *Luciferase*-wild-type (WT) or OCT4-binding element mutated (OCT4-BEmt) reporters, as indicated. The graphs show the absolute *Luciferase*-reporter activities and their standard deviation. Significances were calculated to the activity of the respective WT reporters without OCT4 overexpression (*p<0.05).

Interestingly, overexpression of OCT4 resulted in significant induced CDX1 and CDX2 activity of wild-type (WT) reporters in SW480 cells. As shown previously, mutation of the OCT4-binding elements in both promoters led to a significant inhibition of reporter activities. Accordingly, OCT4 overexpression showed no longer any activating effects (Figure 35).

In summary, LEF-binding elements in the proximal promoter of CDX1 and CDX2 are dispensable for BCL9-2 mediated gene transcription, indicating that BCL9-2 acts independently from ß-catenin in the regulation of these target genes. However, the proximal promoter of CDX1 and CDX2 contain novel OCT4 responsive elements which mediate the transcriptional activation of the CDX1 and CDX2 reporters.

The most important results of these studies reveal that the cis-regulatory elements of both, the CDX1 and CDX2 gene, harbor multiple SP1-binding elements, which are essential for the activation of the proximal promoters in dependence of BCL9-2. Interestingly, the proximal SP1 binding elements close to the transcription start site are most important for the BCL9-2 mediated CDX1- and CDX2-gene transcription.

4.12 BCL9-2 interacts with the transcription factor SP1 in colon cancer cell lines

BCL9-2 regulated the expression of the CDX1- and CDX2-*Luciferase* reporters through SP1-binding elements in their proximal promoters. To determine whether BCL9-2 regulates gene expression by binding to SP1 and thereby probably to the promoter of target genes, co-immunoprecipitations were performed in HCT116 and SW480 colon cancer cell lines. Specific antibodies (Table 8) were used to precipitate BCL9-2 and SP1, respectively. In addition, Pygopus 2 was precipitated as known interaction partner of BCL9-2, rabbit serum (IgG) served as negative control.

The specific antibody recognizing BCL9-2 only precipitated its targeted protein, but not BCL9 (Figure 36, bottom panel). Moreover, binding to Pygo2 was confirmed by precipitation of BCL9-2 and vice versa (Figure 36, third panel).

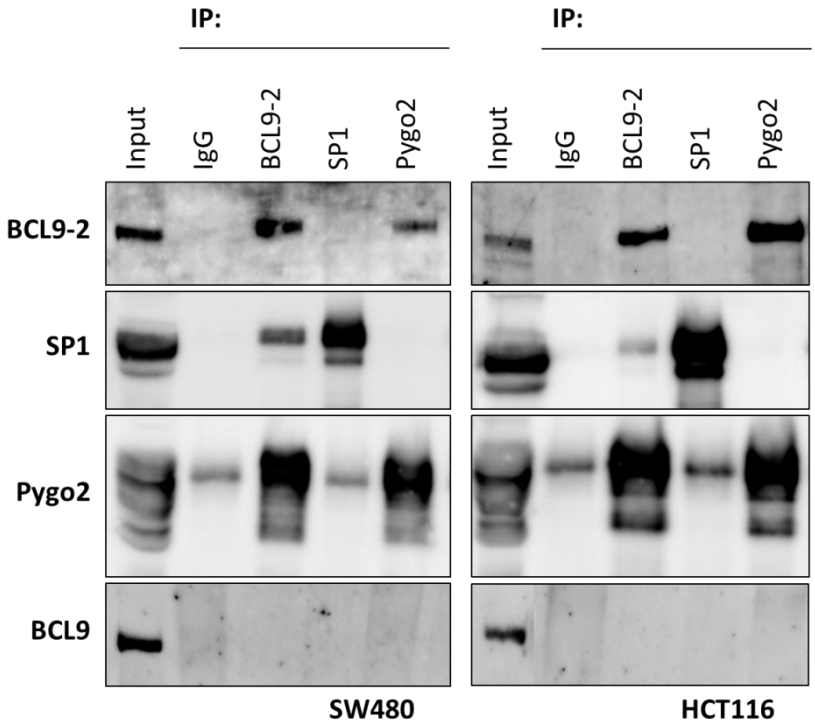

Figure 36: Co-Immunoprecipitation identified SP1 as a new interaction partner of BCL9-2
Co-Immunoprecipitations (Co-IPs) were performed from SW480 and HCT116 using specific antibodies, as indicated (IP). IgG rabbit serum served as negative control. 10% of IPs and 5% of the respective inputs were used for SDS-PAGE in addition to specific antibodies detecting the indicated proteins/interaction partners (left).

Of particular note, co-Immunoprecipitation identified BCL9-2 as interaction partner of the transcription factor SP1 in SW480 and HCT116 cells. After immunoprecipitation of BCL9-2, SP1

was identified by Western blotting in the precipitated complexes in both cell lines. The reverse experiment using a SP1-anti*b*ody was not successful. HCT116 cells express lower levels of BCL9-2 compared to SW480 cells (Figure 16, upper panel and Figure 36, upper panel). According to this, less SP1 protein was detected in the BCL9-2-IP in HCT116 cells compared to SW480 cells.

In conclusion, the co-immunoprecipitation experiments show that BCL9-2 can be found in a complex not only with Pygopus 2, but also with SP1 in colon cancer cells.

These completely novel results uncover a mechanism which explains the transcriptional regulation of BCL9-2 target genes which are ß-catenin-dependent and -independent.

5 Discussion

The role of BCL9/*Legless* in Drosophila has been studied intensively (Kessler *et al.*, 2009;Kramps *et al.*, 2002;Townsley *et al.*, 2004). BCL9/*Legless* was shown to be essential for canonical Wnt signaling during embryogenesis in *Drosophila* (Kramps *et al.*, 2002). In this context BCL9/*Legless* mediates the recruitment of the segment polarity gene Pygopus which recruits and modulates components of the chromatin remodeling machinery (Chen *et al.*, 2010;Fiedler *et al.*, 2008;Kessler *et al.*, 2009;Nakamura *et al.*, 2007). The function of the vertebrate BCL9 proteins was shown to be primarily context-dependent (Brembeck *et al.*, 2006;Brembeck *et al.*, 2004;Sustmann *et al.*, 2008). However, the role of the vertebrate BCL9 and BCL9-2 seems to be more complex than in *Drosophila*. BCL9-2 double knockout mice are early embryonic lethal (Matsuura *et al.*, 2011) while conditional ablation of BCL9/BCL9-2 in the intestine caused no obvious phenotype (Deka *et al.*, 2010). This indicates that the BCL9 proteins are not necessary for normal homeostasis while at least BCL9-2 exerts important functions during embryonic development. BCL9-2 is overexpressed in cancers (Zatula and Brembeck, unpublished)(Adachi et al. 2004; Deka et al. 2010; Sakamoto et al. 2007; Toya et al. 2007b). These findings provide further evidences for BCL9-2 to act as a proto-oncogene in colon tumorigenesis.

However, a complete investigation of the BCL9 proteins and their function in normal intestines and during the multistep model of carcinogenesis was still missing. Here, their expression and transcriptional function in normal and tumorigenic epithelia in mice and humans was examined. Moreover, for the first time, this study provides evidence for a ß-catenin independent function of BCL9-2 and defines a novel mechanism how BCL9-2 might act as transcriptional co-activator independently of ß-catenin.

5.1 The role of BCL9 and BCL9-2 in intestinal homeostasis

The Wnt/ß-catenin pathway is crucial for intestinal homeostasis and diverse developmental processes such as specification of the mesoderm and neuroectoderm, body axis formation and intestinal specification during embryogenesis (Gadue *et al.*, 2006;Kemp *et al.*, 2005;Mohamed *et al.*, 2004;Nostro *et al.*, 2008;Sherwood *et al.*, 2011), as well as intestinal homeostasis in the adult organism (Pinto *et al.*, 2003). In addition, BCL9 and BCL9-2 have been shown to be essential for different ß-catenin-dependent developmental processes such as mesoderm

patterning in zebrafish, myogenic differentiation and for proper placenta development in mice (Brack *et al.*, 2009;Brembeck *et al.*, 2004;Matsuura *et al.*, 2011), while it appears, that other processes do not require the participation of the BCL9 proteins, like the formation of the dorsal organizer or the posteriorization of the anterior neuroectoderm (Brembeck *et al.*, 2004).

We addressed the question whether BCL9 and BCL9-2 participate in the Wnt/ß-catenin signaling-dependent maintenance of intestinal homeostasis. To this end, we characterized the expression of BCL9 and BCL9-2 in intestinal tissues of mice using specific antibodies (Brembeck *et al.*, 2011). BCL9 protein expression was found in all intestinal cell types including the crypts. Canonical Wnt signaling in the intestinal crypt compartment and the subsequent expression of ß-catenin target genes is required for the maintenance of the proliferative capacity of intestinal stem cells (Korinek *et al.*, 1998a). Our results showed that intestinal ß-catenin target genes such as TCF1, EPHB2 and PROX1 depend on BCL9 expression in colon cancer cells. Possibly, BCL9 might also play a role in the transcriptional regulation of these target genes in intestinal homeostasis.

In contrast, BCL9-2 expression was restricted to the mature differentiated epithelial cells, which reside in the villi. In the villi TGFß and BMP-signaling inhibit Wnt/ß-catenin signaling and function as growth repressors in differentiated epithelial cells (Haramis *et al.*, 2004;Hardwick *et al.*, 2004;Sancho *et al.*, 2004). This inhibition through paracrine factors like BMPs is necessary for the differentiation of the cells into mature specialized cells (Crosnier *et al.*, 2006;Hartenstein *et al.*, 2010;He *et al.*, 2004b). Interestingly, our studies in colon cancer cell lines showed that BMP4 gene expression is regulated by BCL9-2. Probably, BCL9-2 contributes to the expression of Wnt/ß-catenin signaling repressive genes. Expression of the paracrine factor BMP4 could therefore be activated in intestinal epithelial cells. BMP4 leads to activation of BMP signaling in the mesenchyme that consequently inhibits Wnt signaling in the villi (Haramis *et al.*, 2004;He *et al.*, 2004b). BCL9-2 is not expressed in the crypts, where Wnt/ß-catenin signaling is active. Thus, BCL9-2 appears to be dispensable for intestinal Wnt/ß-catenin signaling which is restricted to the proliferative crypt compartment, although it might function in a ß-catenin-independent mechanism in the mature epithelial cells residing in the villi.

In accordance to our findings, Deka *et al.* showed that conditional ablation of both BCL9 proteins in intestinal epithelia did not disturb the normal intestinal homeostasis (Deka *et al.*, 2010). The distribution and differentiation of intestine-specific cell lineages remained unaltered, although the expression of the stem-cell marker Leucine-rich repeat containing G protein-coupled Receptor 5 (LGR5) and other intestinal epithelial genes, e.g. SOX6, were

significantly reduced in intestinal epithelia of BCL9/BCL9-2 deficient animals (Deka *et al.*, 2010). However, these studies do not provide detailed information about the particular function of BCL9 or BCL9-2, since the changes in the gene expression pattern of conditional $BCL9^{-/-}$/$BCL9\text{-}2^{-/-}$ knockout mice may have been caused by both or just one of the proteins.

In our studies on colon cancer cells LGR5 mRNA expression was only dependent on ß-catenin and BCL9, while SOX6 expression was regulated by BCL9-2, suggesting diverse functions of BCL9 and BCL9-2 in malignant intestinal cells. Other intestinal canonical Wnt target genes such as SOX9 and PROX1 (Blache *et al.*, 2004;Karalay *et al.*, 2011;Petrova *et al.*, 2002;Petrova *et al.*, 2008) are dependent on ß-catenin and the BCL9 proteins. We found other differentially expressed genes in SW480 colon cancer cells after knockdown of ß-catenin and BCL9/BCL9-2. These genes are known to be important for intestinal homeostasis: the EPHB receptors and ephrin ligands were only partially dependent on ß-catenin in colon cancers (this work). Other studies have shown that EPHB receptors as Wnt/ß-catenin target genes are key-regulators of migration and proliferation in the intestinal stem-cell niche (Holmberg *et al.*, 2006). Further intestinal proteins like LGR5 (van der Flier *et al.*, 2007) and SOX9 (Blache *et al.*, 2004) have been shown to be expressed in the crypts in dependency of ß-catenin , while BCL9-2 regulated proteins reside in the villi, e.g. CDX2 (Guo *et al.*, 2004).

In summary, since previous investigations showed that knockout of BCL9 and BCL9-2 causes no obvious intestinal phenotype (Deka *et al.*, 2010), it is conceivable that they are dispensable for normal intestinal homeostasis. However, the gene expression pattern of $BCL9^{-/-}$/$BCL9^{-/-}$ mutant epithelia was changed indicating that BCL9 and BCL9-2 regulate the expression of intestine specific genes whose functions are particular important for tumorigenesis (see below)(Brembeck *et al.*, 2011;Deka *et al.*, 2010). BCL9-2 might therefore participate in the regulation of the ephrinB ligands and CDX2 in a ß-catenin independent manner outside of the crypts. Additionally, canonical Wnt-signaling exerts a role in normal crypts by activating the expression of the EPHB receptors, CDX1, SOX9 and LGR5 probably with or without the contribution of BCL9 (Figure 37)(Batlle *et al.*, 2002).

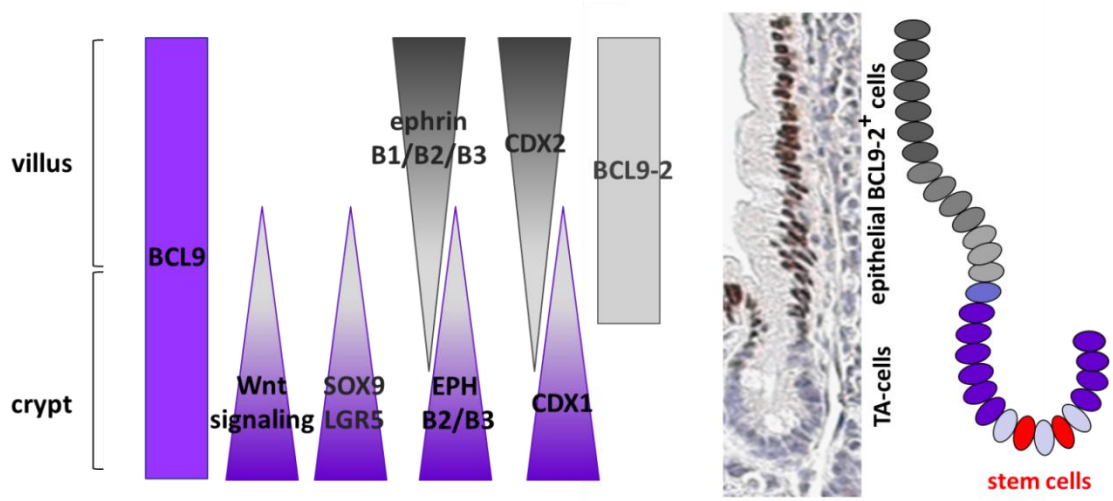

Figure 37: BCL9-2 expression and Wnt/ß-catenin signaling in the intestine
BCL9-2 is expressed in the villi of the intestine, apart from active Wnt/ß-catenin signaling in the crypts. Therefore BCL9-2 might contribute to the intestine specific gene expression pattern along the crypt-villus axis independently of ß-catenin. BCL9, in contrast, is expressed in all intestinal cells and has the spatial ability to contribute to ß-catenin-dependent gene transcription in the crypts.

5.2 BCL9-2 is up-regulated independently of Wnt/ß-catenin signaling in early stages of intestinal tumorigenesis

Previous analyses mainly focused on the overexpression of BCL9-2 in advanced tumors (Adachi *et al.*, 2004;Sakamoto *et al.*, 2007;Toya *et al.*, 2007) while comprehensive analyses regarding early stages of tumorigenesis were still lacking. We found that early stages of tumor development, represented by adenomas of $APC^{Min/+}$ mice, exhibited high nuclear BCL9-2 protein expression. In contrast, BCL9-2 protein was absent in adjacent crypts. As already shown in mice, human tissue microarrays revealed significantly increased nuclear BCL9-2 protein in human adenomas whereas BCL9 protein expression remained unchanged compared to normal mucosa. Moreover, 90% of human colon cancers contained elevated or high BCL9-2 protein while high and elevated nuclear ß-catenin was present in only 59% of the colon cancer samples. In addition, we found that colon cancer cell lines carrying APC-mutations (Rowan *et al.*, 2000) contain high levels of BCL9-2. In contrast, most cell lines with a ß-catenin stabilizing mutation (Kim *et al.*, 2003) exhibited low or absent BCL9-2 protein expression. As already observed in $APC^{Min/+}$ tumors, BCL9 was ubiquitously expressed in colon cancer cell lines at approximately equal levels compared to normal mucosa. Here, we show that BCL9-2 protein expression is significantly increased in early stages of tumorigenesis and highly expressed in tumors harboring an APC mutation while BCL9 expression is not altered.

Previous studies provide evidences for a dependency of BCL9-2 expression on Wnt/ß-catenin signaling (de la Roche *et al.*, 2008). De la Roche *et al*. found increased BCL9 and BCL9-2 mRNA expression following Wnt3a stimulation of HEK293 cells. However, we did not observe any reduction of BCL9/BCL9-2 mRNA or protein levels after knockdown of ß-catenin in SW480 colon cancer cells indicating that the expression of BCL9 and BCL9-2 in colon tumorigenesis does not depend on Wnt/ß-catenin signaling. Moreover, BCL9-2 mRNA and protein expression was even significantly induced in SW480 cells after knockdown of ß-catenin. These data suggest that BCL9 and BCL9-2 are not targets of ß-catenin and that Wnt/ß-catenin signaling might negatively regulate the expression of BCL9-2. It has been shown that LEF1 is activated through binding of ß-catenin that consequently results in suppression of E-cadherin gene transcription in keratinocytes (Jamora *et al.*, 2003). Possibly ß-catenin negatively regulates BCL9-2 expression in colon cancer cells through a similar mechanism. However, the precise undelaying mechanism remains to be discovered.

Further findings *in vivo* support our hypothesis that ß-catenin negatively regulates BCL9-2 expression. The crypt compartment in normal intestine lacks BCL9-2, which is probably due to the negative influence of ß-catenin on BCL9-2 transcription. In the villi, ß-catenin resides at the cell membrane and therefore exerts no transcriptional function. Thus, BCL9-2 expression is restricted to the villi.

The negative effect of ß-catenin on BCL9-2 expression might be overcome by other mechanisms, which lead to overexpression of BCL9-2. For instance, we found elevated levels of BCL9-2 protein in adenomas of $APC^{Min/+}$ mice (this study). Loss of function mutations in APC are associated with chromosomal instability (CIN) (Alberici and Fodde, 2006;Caldwell and Kaplan, 2009;Fodde *et al.*, 2001). CIN is a common event in tumorigenesis often resulting in the trans-location of genetic loci e.g. of oncogenes (Caldwell *et al.*, 2009;Dikovskaya *et al.*, 2007;Radulescu *et al.*, 2010). Also, BCL9 overexpression was linked to trans-location of its gene locus in B cell malignancies (Willis *et al.*, 1998). It is possible that the gene locus of BCL9-2 could be also rearranged likewise resulting in increased transcription levels. Indeed, the chr11q22 which harbors the gene locus of BCL9-2, was found to be altered in 64% of colon cancers (Knosel *et al.*, 2002). Accordingly,ilt might be possible that gene re-arrangement of the BCL9-2 gene locus occur due to chromosomal instability induced by APC mutations.

5.3 BCL9-2 promotes intestinal tumorigenesis

5.3.1 BCL9-2 expression enhances Wnt/ß-catenin signaling activity in intestinal tumorigenesis

The majority of colon cancers show high levels of canonical Wnt-signaling due to mutations causing ß-catenin stabilization or APC truncation, since these mutations lead to aberrant transcriptional activity of ß-catenin (Nagase *et al.*, 1993;Polakis, 2000;Reya and Clevers, 2005;van der Flier *et al.*, 2007). We analyzed the level of Wnt/ß-catenin signaling in different colon cancer cell lines that contain high, moderate or low levels of BCL9-2 and found that BCL9-2 protein levels correlated with the level of Wnt/ß-catenin signaling activity in these cell lines.

The dosage of Wnt/ß-catenin signaling is crucial for many different events, including normal developmental processes, such as the maintenance of pluripotency and intestinal specification (Gadue *et al.*, 2006;Kemp *et al.*, 2005;Mohamed *et al.*, 2004;Nostro *et al.*, 2008;Sherwood *et al.*, 2011). In addition, adult tissues require a well-defined dosage of canonical Wnt-signaling: intestinal homeostasis (Pinto *et al.*, 2003) and pathological events like the formation of intestinal tumors and breast cancers (Mohinta *et al.*, 2007;Reya *et al.*, 2005).

Since the level of Wnt/ß-catenin activity is critical for cell renewal and differentiation, many regulatory mechanisms exist which control the Wnt/ß-catenin-dependent activation of target genes. In embryonic development and homeostasis paracrine signaling molecules form concentration gradients. These lead to different signaling responses which subsequently define the resulting tissues. For example, in the adult intestine APC is inversely expressed to active ß-catenin along the crypt villus which results in a gradient of canonical Wnt-signaling activity (Gaspar and Fodde, 2004). In addition, nuclear repressors and activators modulate the function of ß-catenin: Groucho generally represses ß-catenin dependent transcription through the recruitment of repressive chromatin (Fisher and Caudy, 1998;Palaparti *et al.*, 1997), while other transcriptional co-regulators like CBP/p300 can either activate or repress canonical Wnt signaling in dependency of the recruited co-factors (Li *et al.*, 2007).

Additionally, BCL9-2 modulates the activity of canonical Wnt-signaling in different ways.

Our group previously showed that BCL9-2 promotes the trans-location of ß-catenin to the nucleus that enhances Wnt/ß-catenin-dependent target gene transcription (Brembeck *et al.*,

2006;Brembeck *et al.*, 2004). In addition, BCL9-2 modulates the switch between ß-catenin´s transcriptional and adhesive function due to the preferred binding of Y142 phosphorylated ß-catenin to BCL9-2 instead of α-catenin (Brembeck *et al.*, 2004). Moreover, several studies showed that BCL9 and BCL9-2 co-activate the transcription of ß-catenin target genes (Adachi *et al.*, 2004;Brembeck *et al.*, 2004;Brembeck *et al.*, 2011;Deka *et al.*, 2010;Sustmann *et al.*, 2008).

Because BCL9-2 appears to be redundant for normal intestinal homeostasis but exerts tumorigenic properties, some studies aimed to target the interaction between ß-catenin and BCL9/BCL9-2 to inhibit tumor growth. Takada and colleagues developed a stabilized α helix of BCL9 (SAH-BCL9), which interferes with its ß-catenin binding domain. This resultes in an impaired binding to ß-catenin. Remarkably, SAH-BCL9 led to the suppression of tumor growth, angiogenesis, invasion and metastasis formation in mouse xenografts. In addition, de la Roche *et al.* identified carnosic acid to inhibit β-catenin binding to the BCL9 co-factors (de la Roche *et al.*, 2012b). Their study demonstrated that binding to BCL9 proteins protect un-phosphorylated β-catenin against degradation. Thus, treatment of colon cancer cells with carnosic acid lead to destabilization of oncogenic β-catenin and consequently to transcriptional inactive oncogenic ß-catenin (de la Roche *et al.*, 2012b).

In summary, the function of BCL9-2 in canonical Wnt-signaling is most likely the enhancement of a critical signaling threshold which results in the activation of certain Wnt target genes in pathologic processes. The Wnt/β-catenin signaling pathway plays a crucial role for the balance of stemness and differentiation in the intestinal crypts (Fodde and Brabletz, 2007). However, Fodde and colleagues resumed that ß-catenin stabilizing APC mutations alone might not be sufficient to cause aberrant ß-catenin transcriptional activation. Additional factors appear to be necessary to induce a pathological transcription level, which leads to tumorigenesis (Gaspar *et al.*, 2004), for instance, BCL9-2, which enhances the ß-catenin signaling output. Our studies support this role, since Wnt/ß-catenin activity correlates with the protein level of BCL9-2 in colon cancer cell lines. Overexpression of BCL9-2 results in the transcriptional co-activation of Wnt/ß-catenin target genes. This might be promoted by BCL9-2 mediated nuclear retention of ß-catenin and the regulation of ß-catenin´s transcriptional function. Although BCL9/BCL9-2 appear to be dispensable for intestinal homeostasis, they play an important role in pathologic processes.

5.3.2 BCL9-2 is not required for the expression of all canonical Wnt target genes and regulates additional ß-catenin-independent genes implicated in tumorigenesis

BCL9-2 and BCL9 are potent co-activators of canonical Wnt-signaling. We addressed the question whether the proteins are essential or just partially required co-factors for Wnt/ß-catenin signaling or whether they regulate target genes independently of ß-catenin. We analyzed different Wnt/ß-catenin-dependent *Luciferase*-reporters and the endogenous expression of known ß-catenin-target genes and other genes implicated in colon cancer. To this end, the expression of the target genes was analyzed after knockdown of BCL9 and BCL9-2 in comparison to the knockdown of ß-catenin in colon cancer cells.

Knockdown of BCL9-2 and BCL9 resulted in strong reduction of Axin2 and TOP/FOP Wnt reporter activities in cell lines with high levels of BCL9-2. In addition, we found several endogenous Wnt target genes which were highly dependent on BCL9-2 and BCL9, e.g. Bambi, TCF1 and EPHB2. But not all ß-catenin target genes were regulated by the BCL9 proteins. The CyclinD1 reporter was not affected by BCL9 or BCL9-2 knock down. In line with these findings, CyclinD1 mRNA was not changed after knock down of the BCL9 proteins. In addition, genes such as LEF1 and MSX2 also not responded to BCL9 and BCL9-2. Thus, BCL9-2 promotes only a subset of Wnt/ß-catenin target genes (Figure 38).

Interestingly, Clarke and Clevers recently postulated that CyclinD1 is not a direct target gene of ß-catenin. They found that endogenous CyclinD1 expression was not altered following antagonism of the Wnt pathway *in vitro* or even was not induced by conditional loss of APC *in vivo* (Sansom *et al.*, 2005). In consequence it is possible that also BCL9-2, as co-factor of canonical Wnt-signaling, is not able to induce CyclinD1 expression. Thus, secondary effects appear to be required for CyclinD1 overexpression in cancers. For example, p53 mutations lead to activation of NFκB, which positively regulated CyclinD1 transcription (Rocha *et al.*, 2003). Therefore, it is likely that ß-catenin regulates the expression of CyclinD1 by a BCL9-2-independent activation of an inhibitor or by inhibition of a suppressor such as p53. However, the underlying mechanism is still unknown and needs to be investigated.

BCL9-2 independent ß-catenin target genes are co-regulated by other factors and obviously do not require the co-activation through the BCL9 proteins. For example, MSX2 was shown to be co-regulated by cooperative binding of SMAD4 and LEF1 in murine embryonic stem cells (Hussein *et al.*, 2003). Interestingly, Hussein and colleagues found, that Wnt/ß-catenin-dependent activation of MSX2 required the presence and functionality of SMAD binding

elements. Moreover, we found that the mRNA expression of Bambi was highly dependent on BCL9-2 in colon cancer cells. Expression levels of Bambi, an inhibitor of TGFß-signaling, were shown to correlate with the metastasis-free survival time of colon cancer patients. In addition, Fritzmann *et al.* found that overexpression of Bambi increases migration of colon cancer cells and subsequently metastasis formation (Fritzmann *et al.*, 2009).

BCL9-2 and BCL9 are not required for all ß-catenin target genes, as our studies demonstrated. Microarray analyses and qPCR of different cancer cell lines supported these observations. Each of the BCL9 proteins mediated its own set of ß-catenin- target genes (Figure 38). Thus, genes like LGR5 were dependent on ß-catenin and BCL9 but not on BCL9-2 levels. In addition, CDX1 and BMP4 were co-regulated by ß-catenin and BCL9-2, and independent of BCL9 in colon cancer cells.

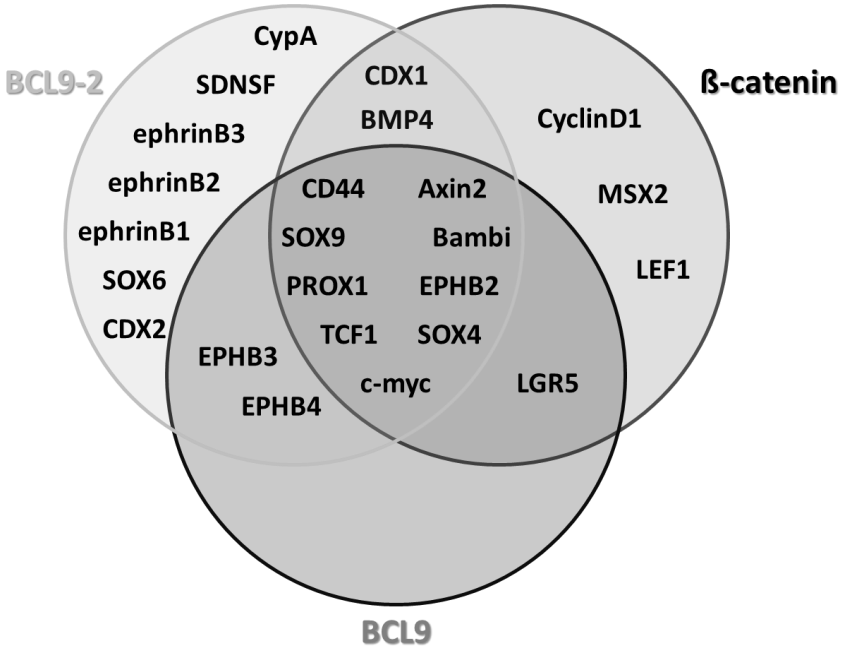

Figure 38: BCL9-2 and BCL9 regulate a subset of Wnt/ß-catenin target genes and regulate the expression of distinct genes independently of ß-catenin
Venn diagram showing the different genes which were identified in this study to be transcriptionally regulated by BCL9, BCL9-2 or ß-catenin, as indicated.

Interestingly, BCL9-2 induced an additional set of target genes which was apparently independent of ß-catenin indicating that BCL9-2 controls the expression of ß-catenin independent genes in colon and breast cancer. This gene set includes CDX2, the EPHB3/4 receptors and ephinB1/2/3 ligands (Figure 38).

These BCL9-2 target genes might further trigger intestinal tumorigenesis. As we have shown for BCL9-2, the ephrinB1, B2 and B3 ligands and EPHB3 and B4 (Hafner *et al.*, 2004;Lugli *et al.*, 2005) receptors, as well as CDX1 and CDX2 (Ee *et al.*, 1995;Ren *et al.*, 2000;Silberg *et al.*, 1997) have been described to be up-regulated in early phases of intestinal tumorigenesis.

Investigations regarding the function and expression of CDX1 and CDX2 are partially controversial. Both proteins have been described to exert tumor-promoting and -repressing functions. However, CDX1 and CDX2 are up-regulated in intestinal adenomas (Ee *et al.*, 1995;Ren *et al.*, 2000;Silberg *et al.*, 1997) and to some extent silenced in carcinomas. For example, CDX2 expression might be maintained in most colon cancers and lost in tumors with a high frequency of microsatellite instability (Hinoi *et al.*, 2001).

The underlying mechanisms which lead to the repression of the proteins are not completely understood yet. It has been suggested that hypermethylation of the CDX1 promoter causes transcriptional silencing (Suh *et al.*, 2002). In addition, CDX2 silencing was shown to be in part mediated by a dominant negative transcriptional repressor (Hinoi *et al.*, 2003). CDX1 was hypothesized to promote proliferation in different colon cancer cell lines (Di Guglielmo *et al.*, 2001;Soubeyran *et al.*, 2001), whereas other studies claimed that CDX1 inhibits Wnt/ß-catenin signaling and thereby exerts anti-proliferative functions (Lynch *et al.*, 2003).

However, our results show that CDX1 and CDX2 are expressed in different colon cancer cell lines in dependency of BCL9-2 expression. Some studies determined a tumor-suppressive function of CDX2 (Lorentz *et al.*, 1997;Mallo *et al.*, 1998). In contrast, others claimed that CDX2 exerts tumor promoting functions (Bonhomme *et al.*, 2003;Oshima *et al.*, 1995), which to some extent reflect the influences we observed after BCL9-2 overexpression in our transgenic mouse model. For example, Salari *et al.* demonstrated that CDX2 is de-regulated in colorectal cancers through chromosomal focal amplification and acts as a lineage-survival oncogene. Thus, cancer cells deriving from the CDX2-lineage require the presence of this oncogene for continued growth and survival. Moreover, the study of Salaris *et al*. claimed that CDX2 is implicated in Wnt/β-catenin signaling and therefore contributes to colorectal tumorigenesis (Salari *et al.*, 2012). In addition, it was recently shown that CDX2 regulates claudin1 expression (Bhat *et al.*, 2012),which has been linked to tumor progression and metastasis in association with EMT (Dhawan *et al.*, 2005). Thus, BCL9-2 dependent activation of CDX2 might further promote the epithelial-mesenchymal transition of cancer cells.

In conclusion, the function of CDX1 and CDX2 depends on their temporally and locally expression. It is likely that, depending on the genetic background, the proteins exert different

functions. Further investigation will provide more insights into the complex role of the CDX proteins by dissecting the tumor-promoting and -repressing functions in dependency on the cellular context.

In addition to CDX proteins, the expression of the EPHB receptors and ephrinB ligands were strongly dependent on BCL9-2. In intestinal homeostasis, the EPHB/ephrins have been shown to regulate cell migration and intestinal cell fates through contact-mediated cell repulsion (Sancho *et al.*, 2003;Sancho *et al.*, 2004). This function suggests a role of the proteins for tumor invasion. Many studies have shown that the EPHs and ephrins are overexpressed in early stages of tumorigenesis and silenced in advanced stages due to hypermethylations of their promoters (Hafner *et al.*, 2004;Lugli *et al.*, 2005). Although overexpression of the Wnt/ß-catenin target EPHB2 is mainly associated with loss of cancer progression, further studies provided evidence that aberrant expression of the ß-catenin independent BCL9-2 target gene products EPHB4 and ephrinB2 give rise to metastases (Liu *et al.*, 2002b;Liu *et al.*, 2004;Stephenson *et al.*, 2001). However, up-regulation in particular of the BCL9-2 regulated EPHB/ephrins was shown to promote tumor invasion which was also observed as a consequence of BCL9-2 overexpression.

Moreover, using microarray analyses we identified the new BCL9-2 core target genes Cyclophilin A (CypA) and the stem-cell-derived neural stem/progenitor cell supporting factor (SDNSF) which were independent of ß-catenin and BCL9 in cancer cell lines (Figure 38). SDNSF and CypA are novel candidates which could further trigger tumor progression by overexpression of BCL9-2. Both proteins had been previously shown to be implicated in tumorigenesis (Gashaw *et al.*, 2007;Mosca *et al.*, 2010;Obchoei *et al.*, 2009).

The physiological function of SDNSF is the transport of selected proteins as component of a receptor which is important for the endoplasmatic reticulum (ER) - Golgi apparatus (Zhang *et al.*, 2003). SDNSF supports the survival and multipotency of neuronal stem/progenitor cells without the addition of fibroblast growth factor (FGF) or epidermal growth factor (EGF) *in vitro*, which are indispensable for retention of the potential to self-renew under physiological conditions (Toda *et al.*, 2003). The EGF-signaling pathway was shown to regulate colon cancer stem-cell proliferation and apoptosis (Feng *et al.*, 2012). Possibly, SDNSF promotes the maintenance of pluripotency of cancer cells, which is a common event in tumorigenesis (Abdul Khalek *et al.*, 2010). In this regard SDNSF might contribute to the autonomy and independence of tumor cells on self-renewing promoting factors like FGF or EGF. However, detailed studies regarding the expression and role of SDNSF in tumorigenesis are still missing: Gashaw and

colleagues postulated SDNSF to be marker for testicular germ cell tumors (Gashaw *et al.*, 2007). Moreover, some studies provided evidences that SDNSF is up-regulated in breast cancers (Mosca *et al.*, 2010).

Cyclophilin A (CypA), the second newly identified BCL9-2 target gene is a peptidylprolyl cis-trans-isomerase (PPI) which accelerates the folding of proteins and is involved in intracellular protein trafficking (Andreeva et al., 1999. CypA is overexpressed in many tumor, e.g. in pancreas, breast and colon cancer (Obchoei *et al.*, 2009). Importantly, overexpression of CypA prevents hypoxia- and cisplatin-induced apoptosis in HCT116 colon cancer cells but has no influence on cell proliferation (Choi *et al.*, 2007). In addition, it has been shown that the new BCL9-2 target gene is a promising candidate for treatment and early diagnosis of diverse cancers like hepatocyte and endometrial tumors (Lee, 2010a;Lee, 2010b;Obchoei *et al.*, 2009).

However, detailed analyzes for the role of SDNSF and CypA in intestinal tumors are missing. Further investigations regarding their function and expression during intestinal tumorigenesis could further shed light on the role of BCL9-2 in colon cancer.

In summary, this study revealed that BCL9-2 plays a more prominent role than BCL9 for the co-activation of tumorigenesis-related genes. Interestingly, both co-factors mediate a gene signature which is apparently independent of ß-catenin in cancer cells. In addition, we identified CypA and SDNSF as new BCL9-2 target genes. SDNSF and CypA were both shown to be overexpressed and to some extent implicated in the maintenance of tumor-related properties. This supports our hypothesis that target gene products of BCL9-2 trigger the ß-catenin independent, oncogenic role of this protein.

5.3.3 BCL9-2 promotes tumor development and local invasion

BCL9-2 is up-regulated in the majority of colon cancers. It has been shown, that the phenotypes of aberrant expressed BCL9-2 target genes result in severe phenotypes (see above and the following text). We used a BCL9-2 transgenic mouse model, to investigate whether BCL9-2 overexpression is involved in tumor development and progression *in vivo*. In our model, BCL9-2 overexpression in mice was achieved by a keratin 19 (K19) promoter which leads to expression of the transgene in simple epithelia including the intestine (Brembeck *et al.*, 2011).

BCL9-2 overexpression alone induced the development of undifferentiated tumors in the small intestine in aged mice (>15 month) with a relatively low incidence of 20%. To analyze whether BCL9-2 overexpression contributes to tumor formation in combination with other genetic alterations *in vivo*, adenoma development was analyzed in compound $APC^{Min/+}$;K19-BCL9-2 mice.

Remarkably, overexpression of BCL9-2 in compound $APC^{Min/+}$;K19-BCL9-2 mice resulted in significantly increased adenoma formation in the small intestine with regard to number and size compared to $APC^{Min/+}$ non-transgenic littermates. The same tendency was observed in the colon, although the changes were not significant due to the rare development of colonic tumors in $APC^{Min/+}$ mice per se. The life span of APCMin/+ mice is generally shortened due to anemia resulting from the intestinal adenomas (Moser *et al.,* 1990).

Our results are in agreement with studies by Deka *et al.* for intestinal tumorigenesis in BCL9/BCL9-2 mutant animals: Induction of dysplastic adenomas by dimethylhydrazine in BCL9/BCL9-2 knockout mice led to tumor formation with similar incidences in knockout and wild type mice. However, the size of $BCL9^{-/-}$/$BCL9\text{-}2^{-/-}$ derived tumors was significantly decreased in comparison to wild type mice. Thus, loss of BCL9/BCL9-2 suppresses tumor growth. Accordingly, $APC^{Min/+}$;K19-BCL9-2 animals developed huge adenomas, indicating that BCL9-2 overexpression leads to tumor development under supra-pathological conditions. APC mutation resulting in ß-catenin stabilization alone were suggested to be not sufficient to cause aberrant ß-catenin transcriptional activation (Gaspar *et al.*, 2004). Thus, our studies reveal that BCL9-2 overexpression induces an increased supra-pathological transcription level of ß-catenin-dependent and -independent target genes, which further promote tumor progression.

Remarkably, adenomas of $APC^{Min/+}$;K19-BCL9-2 mice were locally invasive with tumor cells growing invading the submucosa and muscularis, which was never observed in $APC^{Min/+}$ control

mice. $APC^{Min/+}$ mice develop benign tumors with well-formed boundaries (Moser *et al.*, 1990). Thus, canonical Wnt-signaling requires the co-activation of genes by additional factors to induce invasion, as observed in our compound mutant mice (see 5.3.1).

This study identified the Wnt/ß-catenin target gene PROX1 to be highly dependent on BCL9-2. Indeed, it has been shown that ß-catenin alone is not sufficient to activate PROX1 gene expression in colon cancer cells (Petrova *et al.*, 2008). Petrova and colleagues demonstrated that PROX1 is a dose-dependent target gene of Wnt/ß-catenin signaling. Moreover, a yet unknown factor is required for transcriptional activation of PROX1 in addition to nuclear ß-catenin. Our study suggests that BCL9-2 might be the missing link to induce the specific activation of target genes like PROX1. Accordingly, high PROX1 expression was found in SW480 and HT29 (Petrova *et al.*, 2008) which contain high levels of BCL9-2 (this study). In contrast, DLD1, WiDr and HCT-116 cells with no or moderate BCL9-2 protein expression (this study) were negative for PROX1 (Petrova *et al.*, 2008). According to BCL9-2, overexpression of PROX1 promotes intestinal tumor progression and invasion (Petrova *et al.*, 2008). Overexpression of PROX1 correlates with poor prognosis in colon cancer patients (Skog *et al.*, 2011). Elyada *et al.* provided evidence, that PROX1 mediates invasion through the alteration of cell-polarity and -adhesion in a TP53-dependent manner (Elyada *et al.*, 2011). However, the detailed underlying mechanism has not been described so far. Future experiments are required to investigate the dependency of PROX1 on BCL9-2 in more detail.

We already showed, that invasive areas of $APC^{Min/+}$;K19-BCL9-2 mice derived tumors express the ß-catenin/BCL9-2 regulated EPHB2 and Bambi as well as the BCL9-2 dependent EPHB3 and B4 proteins, which were differentially regulated by ß-catenin. The expression of the ephrinB3 and EPHBB4 receptors was negatively regulated by ß-catenin in SW480 suggesting that ß-catenin overexpression might cause inhibition of these genes in colon tumors. Moreover, deregulation of EPHB4 and ephrinB2 was shown to correlate with metastases formation (Liu *et al.*, 2002b;Liu *et al.*, 2004;Stephenson *et al.*, 2001). This findings support the hypothesis that BCL9-2 is capable of regulating the transcription of target genes independently from ß-catenin which promote tumorigenesis. In addition, BCL9-2 might be essential for the expression of ß-catenin target genes like PROX1, which further contribute to invasion.

Invasion is a process which requires the activity of proteins that contribute to the disruption of the extracellular matrix and proteins which induce a motile, epithelial phenotype of the cancer cells (reviewed in (Kong *et al.*, 2011;Yilmaz and Christofori, 2009). Our studies provide evidences that BCL9-2 overexpression contributes to the malignant phenotype of cancer cells

through the promotion of epithelial-mesenchymal transition (EMT). Accordingly, we observed a transition of the mesenchymal-like phenotype of cancer cells to a more epithelial-like morphology after knock down of BCL9-2 and to a lesser extend after BCL9 knockdown. Earlier studies from our group showed that BCL9-2 negatively regulates the mRNA expression of the epithelial marker E-cadherin, which supports our hypothesis that BCL9-2 contributes to EMT. Also in zebra fish embryos, BCL9-2 was shown to trigger EMT (Brembeck *et al.*, 2004). In agreement with our findings, Deka and colleagues found that $BCL9^{-/-}$/$BCL9\text{-}2^{-/-}$ mutant tumors exhibited a reduced expression of target genes which had been associated with EMT (e.g., Branchury (T) and Vimentin (Vim)), intestinal stem-cell traits (e.g. LGR5) (Deka *et al.,* 2010). Moreover, in addition to Wnt/ß-catenin-dependent mesenchymal markers, further characteristic EMT-key player genes were reduced in the gene set of $BCL9^{-/-}$/$BCL9\text{-}2^{-/-}$ mutant tumors, e.g. snail homolog 2 (SLUG), TWIST1 and zinc finger E-box binding homeobox 1 and 2 (ZEB1/2). Until now it is not clear whether BCL9-2 itself participates in the transcriptional regulation of EMT key components like SLUG and SNAIL, or whether only an indirect process underlies the promotion of EMT like the activation of PROX1 or the inhibition of E-cadherin expression. Therefor future experiments need to examine the influence of BCL9-2 on the regulation of EMT marker genes in cancer cells.

Taken together, our findings indicate that BCL9-2 overexpression promotes invasion through the contribution to epithelial-mesenchymal transition (EMT) directly or indirectly through the activation of EMT-promoting factors. In line with these results, Deka et al. claimed that BCL9/BCL9-2 expression is associated with LGR5-positive intestinal multipotent stem-cells and consequently with cancer stem-cells that exert stem-cell like properties (Deka *et al.,* 2010).

In summary, BCL9-2 promotes intestinal tumorigenesis and local invasion. These features are obviously mediated by two different mechanisms: First, aberrant expression of BCL9-2 increases canonical Wnt signaling and many of the Wnt/ß-catenin/BCL9-2 target gene products have been described by to mediate tumor promoting properties like invasiveness (Han *et al.*, 2012). Second, BCL9-2 activates its own gene set and thereby triggers tumor development and invasion and that BCL9-2 further promotes epithelial-mesenchymal transitions.

5.4 A novel mechanism for BCL9-2 to regulate target gene transcription independently of ß-catenin

Here we identify a mechanism which is responsible for the ß-catenin independent activation of BCL9-2 target genes. We used a system which allows the comparison of the transcriptional regulation of a ß-catenin/BCL9-2-dependent- (CDX1) and a ß-catenin independent BCL9-2 target gene (CDX2) in colon cancer cell lines. Similar to BCL9-2, the caudal related homeobox genes CDX1 and CDX2 are up-regulated in early stages of intestinal carcinogenesis (Ee *et al.*, 1995;Ren *et al.*, 2000;Silberg *et al.*, 1997). We found that BCL9-2 regulates the expression of CDX1 and CDX2. Interestingly, only CDX1 was dependent on the expression of ß-catenin while CDX2 appeared to be negatively regulated.

5.5.1 BCL9-2 regulates the expression of CDX1 and CDX2 independently of ß-catenin in colon cancer cells

During mouse embryonic development, the initiation of CDX2 transcription is induced by canonical Wnt-signaling at around E8.0. However, CDX2 expression was suggested to be independent of ß-catenin in later stages and adults (Sherwood *et al.*, 2011). These observations are in line with our studies in colon cancer cells: ß-catenin knockdown had no influence on the CDX2 mRNA expression in colon cancer cells. Moreover, our studies suggest, that the LEF binding elements also known as Wnt responsive elements (WRE) in the proximal promoter of CDX2 are dispensable for BCL9-2 mediated reporter gene activity. This indicated that BCL9-2 acts independently of ß-catenin in the regulation of these target genes.

Further studies showed that the -3600bp proximal promoter of CDX1 harbors four Wnt responsive elements which are essential for embryonic development (Lickert *et al.*, 2000). However, the Wnt responsive elements in the -386bp promoter of CDX1 were shown to be not necessary for the activation of CDX1 expression in later stages than E8.5 (Lickert *et al.*, 2000). Our study confirmed that the Wnt responsive elements in the CDX1 proximal promoter are not functional colon cancer cells. In contrast, endogenous CDX1 expression was dependent on ß-catenin, obviously through more distal Wnt responsive elements. Lickert and colleagues showed that Wnt/ß-catenin signaling regulates CDX1 expression in intestinal tumors in mice (Lickert *et al.*, 2000). Our results support this observation.

In conclusion, only BCL9-2, but not ß-catenin, regulates the endogenous expression of CDX2 in colon cancer cells. Moreover, we found that BCL9-2 regulates the expression of CDX1 through the proximal promoter probably independent from ß-catenin.

However, since BCL9-2 mediates the transcriptional activation of diverse Wnt/ß-catenin target genes we cannot exclude that BCL9-2 acts also on more distal Wnt responsive elements in the promoter of target genes. Here we focused on the proximal promoters of CDX1/2 and suggest a ß-catenin independent function of BCL9-2 to regulate the basal transcription of these genes. (Figure 40).

5.5.2 Identification of a new OCT4 responsive, transcription factor binding element in the proximal promoters of CDX1 and CDX2

Here, we identified OCT4 as a novel transcriptional activator of CDX1 and CDX2 gene expression. OCT4 and CDX2 are key transcription factors together with NANOG, SRY (sex determining region Y)-box 2 (SOX2) and gut-enriched Krüppel-like factor (KLF4) in early embryonic development where they define the lineage decisions in the mouse blastocyst (Niwa *et al.*, 2000;Pesce and Scholer, 2001;Wei *et al.*, 2009;Zhang *et al.*, 2010). CDX2 expression leads to trophoblast lineage differentiation, whereas OCT4, NANOG, SOX2 and KLF4 expression are necessary for the self-renewing potential of pluripotent stem-cells (Niwa *et al.*, 2000). In this context both, OCT4 and NANOG are necessary to inhibit CDX2 expression through binding to cis-regulatory elements in the CDX2 promoter (Chen *et al.*, 2009). CDX1 is expressed in later stages during embryonic development and not correlated with OCT4 expression (Lickert *et al.*, 2002).

None of the already described OCT4-binding elements are present in the proximal promoter constructs used in our studies. Surprisingly, mutation of a newly identified OCT4 binding element in the proximal promoter of CDX2 led to strong reduction of reporter activity. Moreover, OCT4 overexpression activated CDX1 and CDX2 reporter activities through the novel OCT4 binding element in each of the promoters. This indicates that OCT4 is an essential transcription factor for CDX2 reporter activity. Thus, our analyses revealed that OCT4 exerts distinct functions in intestinal tumorigenesis and embryonic development.

We found that BCL9-2 driven CDX2 reporter activity requires the functionality of the novel OCT4 binding element in colon cancer cell lines. Further analyses are required to analyze the putative interaction of OCT4 and BCL9-2 (Figure 39A). Our preliminary studies showed that

overexpressed BCL9-2 did not bind to exogenous expressed OCT4 in HEK293 cells, suggesting an indirect binding of BCL9-2 to OCT4. In addition, BCL9-2 might modulate the expression of CDX2 by binding to the OCT4 responsive element through another DNA-binding protein such as OCT1 (Figure 39A) or OCT4 mediated transcription requires a co-factor which is dependent on BCL9-2 expression (Figure 39B). Thus, we will further investigate the BCL9-2 dependent function of OCT4 in colon cancer cell lines. Consequently, the expression of other OCT4 known target genes, e.g. NANOG and SOX2 (Jung *et al.*, 2010) will be analyzed. Furthermore, BCL9-2 might also modulate the expression of OCT4 itself or of another related transcription factor (Figure 39B), which mediates the transcriptional activation of CDX2 through the novel OCT4-binding element.

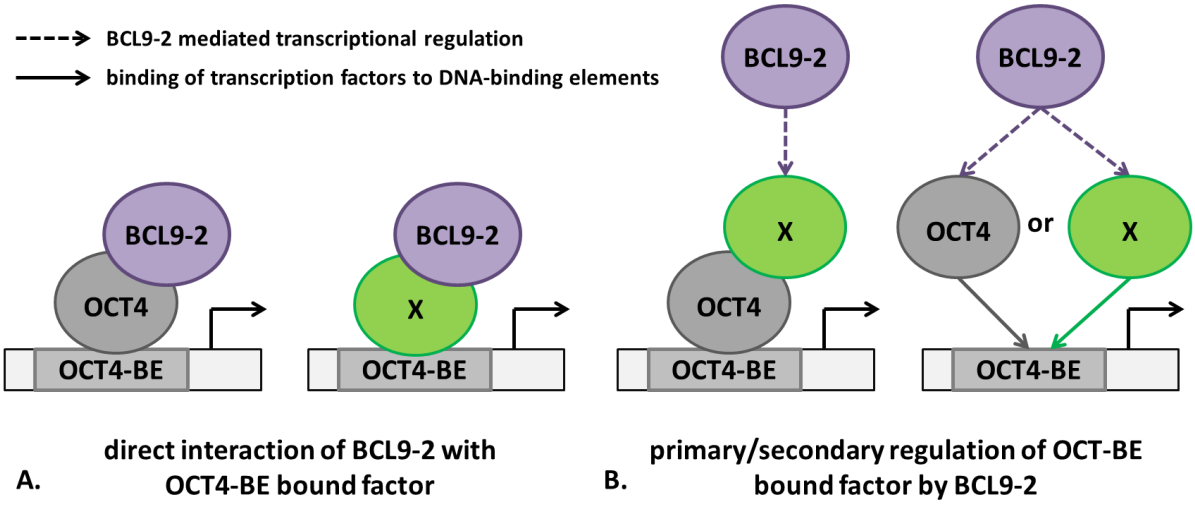

Figure 39: Overview about putative mechanisms for BCL9-2/OCT4 transcriptional activation of CDX2. BCL9-2 might either act through (A) direct interaction with OCT4 or an related transcription factor or (B) secondary, through the transcriptional regulation of OCT4, of an OCT4-required co-factor, or of a transcription factor, which binds to the OCT4-binding element (OCT4-BE).

OCT4 is only expressed in embryonic stem-cells and primary silenced in adults with rare exceptions of some adult stem-cells such as breast- and skin-stem-cells (Pesce *et al.*, 2001;Tai *et al.*, 2005). However, the function of OCT4 in cancer cells appears to be different to its function in embryonic stem cells (this study). Overexpression of CDX2 in mESCs induces trophoblast differentiation (Tolkunova *et al.*, 2006), while knockdown of OCT4 results in endoderm and trophoblast differentiation (Hay *et al.*, 2004). Interestingly, several publications reported that OCT4 is expressed in different breast cancer and colon cancer cell lines (Jin *et al.*, 1999;Steingart *et al.*, 2002;Wang *et al.*, 2003). Overexpression of OCT4 induces the reactivation of pluripotency associated factors which contribute to tumorigenesis (Hochedlinger *et al.*, 2005). Moreover, it was shown that OCT4 overexpression resulted in dedifferentiation of melanoma cells into “cancer stem-cells” (CSC). These cells acquired the

typical CSC characteristics such as the ability to form tumor spheroids and the ability to resist chemotherapeutics and hypoxia (Kumar *et al.*, 2012). Moreover, we found that OCT4 and moderate levels of BCL9-2 are co-expressed in mouse embryonic stem cells (mESCs) (data not shown). Probably, also in cancer cells, which to some extent recapitulate similar processes as during early embryogenesis, OCT4 and BCL9-2 may contribute to stem-cell like properties, either in cooperation or independently of each other. Future studies will therefore be extended to mouse embryonic stem-cells to analyze their function in stem cells. To this end, the expression of BCL9-2 in pluripotent and differentiating mESCs will be examined. Furthermore the phenotypes of mESCs following overexpression or downregulation of BCL9-2 and their pluripotency and differentiation potential will be analyzed. On the other hand, it is important to investigate whether OCT4 overexpression or downregulation in colon cancer cell lines with different levels of BCL9-2 changes their stem cell characteristics, motility and ability to proliferate.

5.5.3 CDX1 and CDX2 expression is regulated by BCL9-2 through newly identified SP1 binding elements in their proximal promoter

BCL9-2 does not contain a DNA-binding motif and is therefore not able to bind to cis-regulatory elements in the promoters of target genes itself (Brembeck *et al.*, 2004). Therefore the co-activator requires an adaptor protein, which links BCL9-2 to the transcription machinery where it exerts transcriptional activating functions. Here we describe a novel interaction for this factor. BCL9-2 binds to specificity protein 1 (SP1) transcription factors (TF) in colon cancer cell lines, while this interaction was not detected for Pygopus 2. Thus, Pygopus 2 plays a role in the BCL9-2 dependent activation of target genes but appears to be dispensable for BCL9-2/SP1 complex formation.

In addition, multiple SP1 binding elements in the proximal promoters of CDX1 and CDX2 were identified in this work. Moreover, expression of CDX2 was independent of the presence of a TATA box or even a TATA like element. Therefore it is likely that the initiation of transcription is mediated trough GC-rich elements located closely to the transcription start site, as shown for other TATA-less genes (Davie *et al.*, 2008;Lu and Archer, 2010;Singh *et al.*, 2012;Suske, 1999). Investigations regarding the transcriptional regulation of the CDX2 gene primarily focus general transcription factors, which activate the expression of CDX2 such as FGF signaling through SMADs (Barros *et al.*, 2008;Camilo *et al.*, 2012). So far, studies investigating the TATA-less transcriptional activation of the CDX2 gene were still missing. We did not found common

elements in TATA-less promoters such as downstream core promoter elements, TBIID recognition element or initiator elements in promoter of the CDX2 gene. However, our study revealed that the activity of CDX1 and CDX2 *Luciferase* reporters containing the proximal promoters of the genes were highly dependent on SP1-binding elements (BEs). Moreover, the very proximal SP1 binding elements close to the transcription start site were essential for the BCL9-2-dependent transcriptional regulation of both reporters. These results suggest that SP1 in dependence of BCL9-2 initiate the transcription of CDX1 and CDX2 genes.

Our results indicate that BCL9-2 exerts co-activatory functions through binding to SP1 and thereby to the DNA of target genes. Moreover, the transcriptional regulation of CDX1 and CDX2 by BCL9-2/SP1 appeared to be independent of ß-catenin, since knockdown of the protein had no effect on CDX reporter activity.

Further investigations will determine whether SP1/BCL9-2 complex formation is specific for BCL9-2 or if also its homologue BCL9 binds to SP1. To proof the SP1-mediated recruitment of BCL9-2 to specific cis-acting elements in the promoters of CDX1, CDX2 and other target genes, chromatin-immunoprecipitation (ChIP) and electrophoretic mobility shift assays (EMSA) will be performed. These studies will further compare known Wnt-responsive elements bound by ß-catenin (WRE) and putative BCL9-2 bound elements in the promoters of these target genes.

Interestingly, LEF/TCF transcription factors had been shown to interact with SP1/SP5 in different biological systems. For example, as previously shown for BCL9-2, SP5 is important for Wnt8-dependent transcriptional regulation of Wnt/ß-catenin target genes in zebrafish mesoderm specification. In this regard, SP5 dependent transcription might be dependent on BCL9-2. Possibly, SP1 binding elements are further important to mediate the BCL9-2 dependent transcriptional regulation of target genes in dependency of Wnt responsive elements, which are bound by LEF/TCF in intestinal carcinogenesis (Figure 40, left). Since the impact of SP/LEF/TCF complexes on gene transcription differs in dependence on the genetic and biological background, detailed analyses in colon cancers are necessary to determine the consequence of the putative SP1/BCL9-2/LEF/TCF complex formation.

To identify a BCL9-2 protein domain, which is responsible for the binding of SP1, binding of the SP transcription factors to different truncated versions of BCL9-2 will be analyzed. These analyses will provide information about the dependency of this complex formation on different domains in the BCL9 proteins, e.g. whether ß-catenin binding or the BCL9-2 C-terminus is required for an interaction with SP1. The C-terminus of BCL9 was previously described by Sustmann and colleagues to harbor a transactivation domain (Sustmann *et al.*,

2008). The three C-terminal domains of BCL9-2 and BCL9 show high similarities (Brembeck *et al.*, 2004;Kramps *et al.*, 2002) and are important for the co-activatory function of BCL9 and BCL9-2 in Wnt/ß-catenin signaling (Brembeck *et al.*, 2004;Sustmann *et al.*, 2008). In addition, the C-terminal domains might play a role in the SP1/BCL9-2 complex mediated transcriptional activation due to the facilitation of the SP1-BCL9-2 interaction. Moreover, until now it is unclear whether SP1/BCL9-2 complexes are a feature of cancer cells or a general mechanism in many cell types including untransformed, normal epithelial cells. Thus, future studies will be extended to other cell types such as additional breast cancer-, colon cancer- and untransformed- cell lines like the human embryonic kidney cell line HEK293.

SP1 transcription factors are ubiquitously expressed in all mammalian cells and belong to the highly conserved Protein/Krüppel-like Factor (SP/KLF) transcription factor family. SP/KLF factors contain a DNA binding domain and three Zink finger domains (Brandeis *et al.*, 1994;Davie *et al.*, 2008;Smale *et al.*, 1990). SP1 proteins contain two trans-activation domains and serine/threonine-rich subregions which can be post-translational modified which primary results in inhibited transcriptional activity (Yang *et al.*, 2001). The protein structure of SP1/KLF proteins is highly conserved. Thus, BCL9-2 might also bind to other SP1/KLF proteins. The closest structural relative of SP1 is SP3, which recognizes the same promoter binding elements. Like BCL9-2 knockout mice, SP1 knockout mice are embryonic lethal at E10.5, whereas SP3 knockout causes postnatal death suggesting a more prominent role of SP1 during embryonic development. In addition, BCL9-2 and SP1 knockout resulted in placental defects (Kruger *et al.*, 2007). Interestingly, similar to BCL9-2, SP1 and SP3 are overexpressed in breast, colon and other tumor entities (Li *et al.*, 2010).

Future studies will investigate whether BCL9 proteins interact with other SP related such as KLF4. Like OCT4, KLF4 is an important transcription factor in mouse embryonic stem-cells (ESCs). In addition, many studies claimed a dependency of SP1-TF and KLF4-TF on each other regarding their potential to activate transcription (Black *et al.,* 2001;Brembeck *et al.,* 2000;Shie *et al.,* 2000). For example, CyclinD1 gene regulation is mediated by KLF4 which is in part dependent by binding to SP1 in gastric cancer cells (Shie *et al.,* 2000). In addition, Brembeck *et al.* showed that the K19 promoter is active in gastrointestinal cancer cells through the transcriptional regulation by KLF4 and SP1. This study further claimed that the functional interaction of ubiquitous (SP1) and tissue-restricted (KLF4) transcription factors determines tissue- and neoplasm-specific patterns of gene expression (Brembeck *et al.,* 2000). In this regard, BCL9-2 might play a tissue specific role for the induction of SP/KLF target genes. Thus, BCL9-2 transcriptional activity is dependent on SP1 and probably on OCT4. Therefore, we will

address the question whether KLF4 plays a BCL9-2 dependent role in colon cancer and stem-cell maintenance.

Moreover, SP1, SP3 as well as KLF4 transcription factors have been shown to be implicated in the transcriptional regulation of pluripotency genes such as OCT4 and NANOG in mESCs (Pesce *et al.*, 1999;Wu and Yao, 2006). Possibly, BCL9-2 and SP proteins might further function in a complex in embryonic stem cells which activate pluripotency genes in ESCs (see section 5.4.2). BCL9-2 might additionally exert a function in a SP1/SP3 mediated co-regulation of OCT4 in cancer cells. BCL9-2 and SP transcriptional complexes in colon cancers might contribute to the expression of genes which are essential for the development of stem-cell like features. As it was already shown for many BCL9-2 target genes, SP1 target gene products are also implicated in tumorigenesis where they act in different cancer-related processes like cell cycle progression/arrest, proliferation, invasion, metastasis and angiogenesis (Li *et al.*, 2010). Subsequently, the SP1/BCL9-2 complex dependent transcription might activate the expression of cancer-related genes that trigger the oncogenic function of BCL9-2

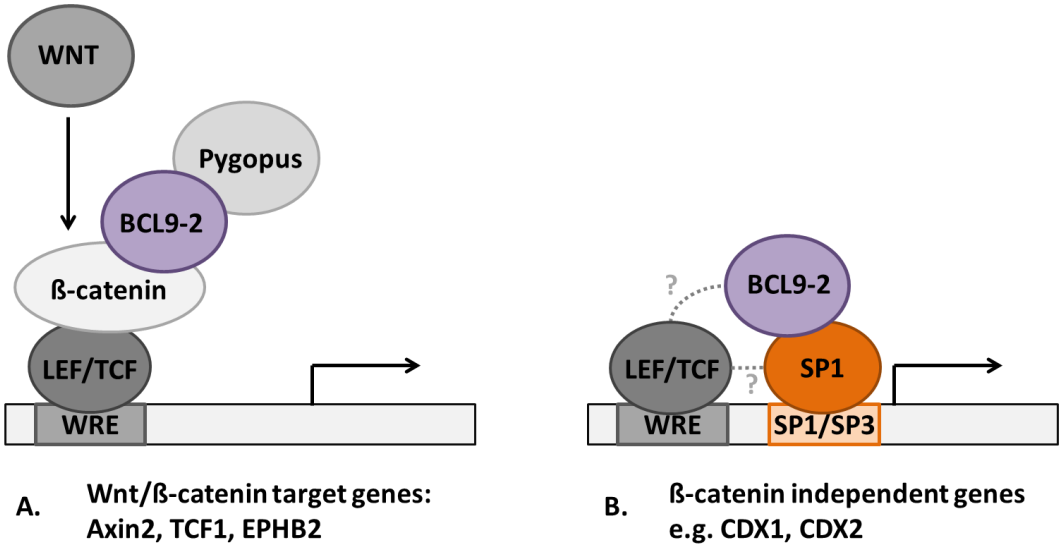

Figure 40: BCL9-2 regulates canonical Wnt-target genes and ß-catenin independent genes
BCL9-2 is important for the regulation of (A) Wnt/ß-catenin target gene and binds to LEF/TCF trough ß-catenin, which is activated by paracrine WNT signals. In this regard, BCL9-2 links Pygopus 2 to the complex. (B) BCL9-2 activates ß-catenin-independent target genes through complex formation with SP1 transcription factors. In addition BCL9-2 or/and SP1 might be recruited to LEF/TCF transcription factors and possibly exerts ß-catenin-independent function trough Wnt-responsive elements (WRE) in the promoters of target genes.

6 Summary and Conclusion

The function of B-cell CLL/lymphoma 9 protein (BCL9)/*Legless* in *Drosophila* has been investigated intensively (Kessler *et al.*, 2009;Kramps *et al.*, 2002;Townsley *et al.*, 2004). However, the role of the vertebrate orthologs in Wnt/ß-catenin signaling and in further processes seems to be more complex than in *Drosophila.* This work investigated the ß-catenin-dependent and -independent functions of BCL9 and BCL9-2 in normal intestine and tumorigenesis.

BCL9 was expressed in all intestinal cell types and unchanged in colon cancer cells. In contrast, BCL9-2 protein expression was restricted to the villi in untransformed, normal intestines, and absent in the crypts where Wnt-signaling is active, indicating that BCL9-2 is dispensable for Wnt/ß-catenin signaling in intestinal homeostasis. However, BCL9-2 overexpression enhanced ß-catenin mediated transcription of a subset of target genes and correlated with the level of Wnt/ß-catenin signaling activity in colon cancer cells. Moreover, BCL9-2, and partially BCL9, regulated the expression of ß-catenin independent genes which have been implicated in tumorigenesis. Moreover, BCL9-2 was already overexpressed in early stages of intestinal tumorigenesis and additionally elevated in approximately 90% of human adenocarcinomas independent of metastases formation. Ectopic overexpression of BCL9-2 in the intestine of transgenic K19-BCL9-2;$APC^{Min/+}$ mice led to increased adenoma formation accompanied with local invasion which resulted in reduced survival. In cancer cells BCL9-2 associates with specific protein 1 (SP1). Moreover, BCL9-2 mediated activation of CDX1 and CDX2 reporter gene transcription was dependent on SP1-binding elements in their proximal promoters in colon cancer cell lines.

In conclusion, this work demonstrates that BCL9-2 promotes early phases of intestinal tumor development and contributes to the progression of tumors into invasive carcinomas. These features are obviously triggered by the transcriptional regulation of partially ß-catenin-independent target genes. Moreover, BCL9-2 mediates its co-activatory function ß-catenin-independently through binding to SP1 transcription factors and thereby to the promoters of target genes which are implicated in early phases of tumor progression.

Future studies will also focus the implication of other SP/KLF transcription factors in the BCL9-2 mediated transcriptional regulation of target genes. In addition, the recruitment of BCL9-2 to ß-catenin-dependent and -independent cis-acting elements will be proofed in cancer- and untransformed- cell lines.

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8 Curriculum Vitae

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