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Investigation to determine the role of the TCF/TLE family of transcription factors in stem cell self-renewal and differentiation in myeloid malignancies.

Bachelor thesis from

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Wisdom is not the result of formal education, but rather the attempt throughout life, to acquire it.

Albert Einstein

I like to thank Dr Helen Wheadon for her inspiration and permanent support.

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Finally, I want to give thanks to my family and boyfriend who never lost the trust in me.

Leukaemia is a very bad disease and I am very happy and proud that I could help in the fight against it and I hope that my research will help to understand this disease a little bit better.

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Abbreviations

1x	one time
A	accelerated phase in CML
ADP ribose	Adenosine diphosphate ribose
AJs	adherens junctions
ALL	acute lymphoblastic leukaemia
AML	acute myeloid leukaemia
AML1	acute myeloid leukaemia 1, Runt-Related Transcription Factor 1 (*RUNX1*)
APC	adenomatosis polyposis coli
APS	Ammonium persulfate
B	blast phase or blast crisis in CML
B-cell	type of lymphocyte
b2m	beta-2 microglobulin
bcat	β-catenin, CTNNB1, Catenin (Cadherin-Associated Protein), Beta 1
BCR-ABL	Philadelphia chromosome
BIO	GSK-3 Inhibitor IX
BSA	Bovine serum albumin
C	chronic phase in CML
CD3E	CD3e Molecule, Epsilon (CD3-TCR Complex)
cDNA	complementary deoxyribonucleic acid
CHO cells	Chinese Hamster Ovary cells
CK1α	casein kinase 1α
CLL	chronic lymphocytic leukaemia
CLP	common lymphoid progenitors
CML	chronic myeloid leukaemia
CMML	chronic myelomonocytic leukaemia
CMP	common myeloid progenitors
CO_2	carbon dioxide, carbonic acid
CTNNB1	Catenin (Cadherin-Associated Protein), Beta 1
CTNNBIP1	Catenin, Beta Interacting Protein 1
DAPI	4',6-diamidino-2-phenylindole
ddH_2O	double demineralised water
dH_2O	demineralised water
DMEM	cell culture medium by Gibco®
DNA	deoxyribonucleic acid

dNTP	2'-deoxynucleoside 5'-triphosphates (dATP, dCTP, dGTP, dTTP)
ENOX2	Ecto-Nox-Disulfide-Thiol Exchanger
ES / ESC	embryonic stem cell
FBS	fetal bovine serum
G2NBL	guanine nucleotide binding protein (G protein)
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor 2
GMP	Granulocytes/Macrophage progenitor
GSK3β	glycogen synthase kinase 3 β
gusb	glucuronidase, beta
Ham's F12	cell culture medium by Gibco® for CHO cells
HCL	hairy cell leukaemia
HCl	Hydrogen chloride
HSC	haematopoietic stem cell
ICAT	CTNNBIP1, Catenin, Beta Interacting Protein 1
IF	immunofluorescence
IL-3	interleukin 3
kDa	kilo Dalton
LCK	Lymphocyte-Specific Protein Tyrosine Kinase
LEF1	Lymphoid Enhancer-Binding Factor 1
LIF	leukaemia inhibitor factor
LRP	lipoprotein receptor-related protein
LSC	leukemic stem cell
MDS	myelodysplastic syndromes
ME	Mercaptoethanol
MEM NEAA	MEM Non-Essential Amino Acids
MEP	Megakaryocyte/ Erythrocyte pro-genitor
mESC	murine embryonic stem cell
mRNA	messenger RNA
$MgCl_2$	Magnesium chloride
MPN	myeloproliferative neoplasms
MPP	multipotent haematopoietic progenitors
N	normal
ND1000	Nanodrop 1000
NDC	no drug control
NK cell	natural killer cell
OCT4	POU Class 5 Homeobox 1
P	Phosphor

PBS	Phosphate-buffered saline
PCR	polymerase-chain reaction
PDGfb	Platelet-Derived Growth Factor Beta Polypeptide
PEBP2α	α subunit of polyomavirus enhancer binding protein 2
Pen Strep	Penicillin & Streptomycin
PLL	prolymphocytic leukaemia
PMSF	phenylmethanesulfonylfluoride
Pre-amp	pre-amplification
qPCR	quantitative polymerase-chain reaction
PP2A	protein phosphatase 2A
RNA	ribonucleic acid
RPMI	cell culture medium by Gibco®
RT	reverse transcriptase
RUNX1	Runt-Related Transcription Factor 1, acute myeloid leukaemia 1 (*AML1*)
SDS page	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SHPTP2	Protein Tyrosine Phosphatase, Non-Receptor Type 11
T-cell	type of lymphocyte
TBE	Tris/Borate/EDTA
tbp	TATA box binding protein
TBS	Tris-buffered saline
TBSN	Tris-buffered saline containing Nonidet P40
TCF3	Transcription Factor 3
TCF4	Transcription Factor 4
TCF7	Transcription Factor 7
TCF7L1	Transcription Factor 7-Like 1
TCF7L2	Transcription Factor 7-Like 2
TE buffer	Tris-EDTA buffer
TEMED	Tetramethylethylenediamine
tet	tetracycline
TKI	tyrosine kinase inhibitor
TLE1/2/3/4	Transducin-Like Enhancer of Split 1/2/3/4
TNKS1/2	Tankyrase, TRF1-Interacting Ankyrin-Related ADP-Ribose Polymerase 1/2
TP	Tel/PDGFRβ; Ets Variant 6 and Platelet-Derived Growth Factor Receptor β
TYW1	Synthesizing Protein 1 Homolog
t(8,21)(qx,qy)	translocation between chromosome 8 and 21 at the area (qx, qy)
UBE2D2	Ubiquitin-Conjugating Enzyme E2D 2
UDG	Uracil-DNA Glycosylase

wg	wingless
Wnt	Wingless Int-1
Wnt3a	Wnt ligand, secreted glycol-protein
XAV	Tankyrase 1/2 Inhibitor III
XTT	2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

Abstract

Wnt signalling is very important in the maintenance of HSCs and abnormal Wnt signalling could be a reason for developing cancer. Wnt signalling activates the transcription of target genes, such as *TCF/LEF*, *TLE*, *ICAT* and *RUNX1*. However, it is not well known how altering the balance of these transcription factors increases self-renewal and suppresses differentiation and how in progressive CML-blast crisis and CMML the progenitors gain self-renewal potential. This study investigated the expression patterns of *TCF/LEF*, *TLE*, *ICAT* and *RUNX1* genes in CML and CMML and determined which family members are important regulators of the canonical Wnt signalling pathway. Therefore, a blast crisis CML cell line (K562), which express the tyrosine kinase fusion protein BCR-ABL, and a murine ES cell line (E14tg2a) with inducible expression of the leukemic oncogene Tel/PDGFRβ (TP) were used. The basal levels of *TCF/LEF*, *TLE*, *ICAT* and *RUNX1* genes were analysed in K562 and it was determined which family members of these genes are important regulators of the Wnt signalling through an activation and inhibition of the Wnt signalling pathway. E14tg2a were used to determine the alterations of these downstream genes when the oncogene TP was expressed because the oncogene caused haematopoietic differentiation and the preservation of self-renewal in this cell line. The findings indicate that *TCF7*, *RUNX1*, *TCF4*, *TLE2* and *TLE4* are important regulators of the Wnt signalling pathway and that the transcription factors *TCF7*, *LEF1* and *RUNX1* are highly expressed in K562 with and without an activation of the pathway. TCF7 and LEF1 were also highly expressed in E12tg2a cells; even the oncogene TP could not decrease the expression pattern. TCF7 and AML1 are important for self-renewal and pluripotency which confirm that myeloid progenitors in blast crisis CML and CMML gain self-renewal potential.

1. Introduction

1.1 Foundation and key terms

Myeloid malignancies are diseases concerning the blood, precisely “myeloid” cells. The myeloid cells consist of monocyte/macrophage, granulocyte, megakaryocyte and erythroid lineages (Hoffbrand et al., 2003).

Myeloid malignancies comprise a group of diseases involving the myeloid lineage like, myelodysplastic syndromes (MDS), myeloproliferative neoplasms (MPN), chronic myelomonocytic leukaemia (CMML) and acute myeloid leukaemia (AML). These diseases are clonal illnesses, which arise due to a genetic mutation/chromosomal translocation in a hematopoietic stem or progenitor cells (Murati et al., 2012).

This project focused on two myeloproliferative neoplasms (MPN), chronic myeloid leukaemia (CML) and CMML which arise due to chromosomal translocations which lead to the expression of constitutively active tyrosine kinase (TK) fusion proteins. In particular the emphasise of the project was to investigate how these TK, BCR-ABL in CML and Tel/PDGFβ in CMML altered the expression of the gene families of *TCF* (transcription factors) and *TLE* (transducin-like enhancers of split) which are key downstream regulators of the canonical Wnt signalling pathway. This pathway is known to be deregulated in MPNs.

Leukaemia is a type of cancer which affects the blood, the bone marrow and/or the lymphatic system and is therefore also known as blood cancer. There are different types of leukaemia. They can be distinguished as acute or chronic leukaemia depending on how fast the disease progresses and which cell type is affected (myeloid and lymphatic). The most common types of leukaemia are acute myeloid leukaemia (AML), chronic myeloid leukaemia (CML), acute lymphoblastic leukaemia (ALL) and chronic lymphocytic leukaemia (CLL). Acute leukaemias show an unrestrained proliferation of mostly undifferentiated cells, known as blast cells and if untreated are typically lethal within weeks. In chronic leukaemias the predominant cell type is more mature and the patients survive longer than those suffering from acute leukaemias (Pallister, 2005; Hoffbrand et al., 2003). In 2010, 6.5 % of 100,000 men and 4 % of 100,000 women died of blood cancer (Kaatsch et al., 2014). Furthermore, ALL is responsible for “approximately 30% of childhood cancers” (Pallister, 2005, P 145), whereas adults over an age of 30 years are more likely to develop chronic leukaemias (Pallister, 2005).

Haemopoiesis is a hierarchical system, normal hemopoietic stem cells differentiate into multipotent hemopoietic progenitor cells which then differentiate into common myeloid or lymphoid progenitors and further on to form all mature blood cells. Mature blood cells have a limited life span from years (B- and T-cells), up to three months (red blood cells) and to only a

few days (granulocytes). Because of this, new cells need to be constantly produced to replace old and damaged cells (Nemeth and Bodine, 2007). In leukaemia, cells gain a genetic alteration which gives them a proliferative or survival advantage leading to their expansion. In acute leukaemias this often results in a differentiation block, so leukaemic progenitor cells cannot differentiate into mature blood cells. Clonal expansions of malignant cells have a harmful effect on the function and differentiation of the normal haematopoietic cells as well (Nemeth and Bodine, 2007). Overtime this results in the leukaemic cells taking over the bone marrow resulting in defective normal haemopoiesis.

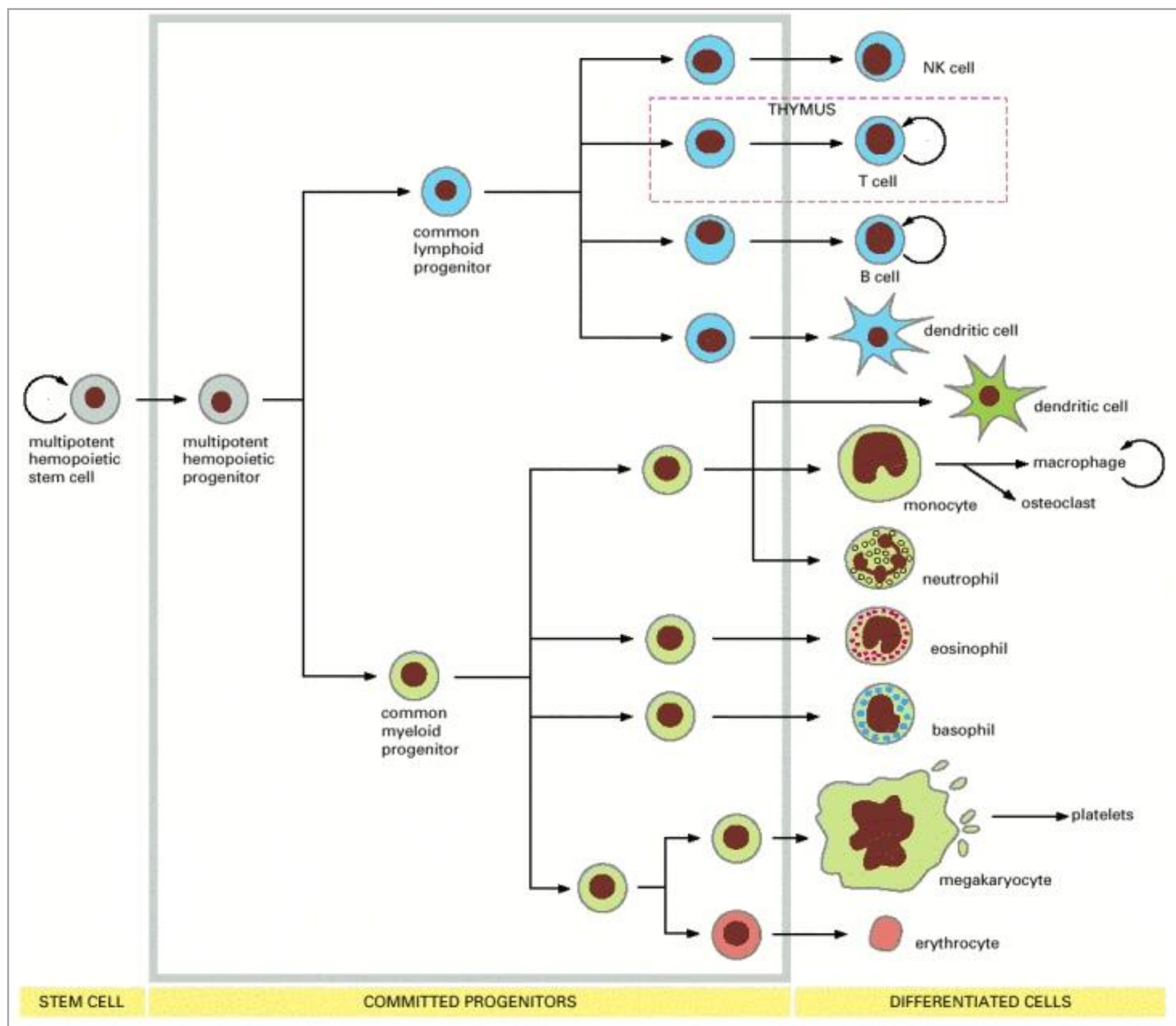


Figure 1: Schematic representation of normal haemopoiesis

Schematic diagram 1 shows the hierarchical haemopoiesis. Haemopoietic stem cells (HSC), are multipotent and able to self-renew and to differentiate into multipotent haematopoietic progenitors (MPP). MPP can then differentiate into common lymphoid progenitors (CLP) and common myeloid progenitors (CMP) which can further differentiate into all mature blood cells (Figure from Alberts et al., 2002).

1.1.1 Acute leukaemias

One characteristic of acute leukaemia is the presence of more than 30 % blast cells in the bone marrow (Pallister, 2005; Hoffbrand et al., 2003). The disease is also identified by proliferation of immature cells which are unable to differentiate properly into mature cells. The immature cells proliferate in the bone marrow and supersede normal haemopoietic cells which cause anaemia (Bain, 2004). The differentiation in AML and ALL depends on which blasts, myeloblasts or lymphoblasts, accumulate in the bone marrow (Hoffbrand et al., 2003). A two hit process has been postulated for acute leukaemia development, the first is the initiating genetic change which gives a self-renewal/proliferation advantage and the second causes a differentiation block. Patients therefore have complex cytogenetic and genetic changes making this group of diseases difficult to treat and manage (Shih et al., 2012; Kelly & Gilliland, 2002). In acute leukaemia there are three general mechanisms that cause morbidity and mortality: a lack in number and function of normal blood cells, infiltration of organs with damage of function, and metabolic imbalance due to systemic disruption. The consequences are infections, haemorrhage, anaemia and loss of function of vital organs (Pallister, 2005; Hoffbrand et al., 2003).

1.1.2 Chronic leukaemias

In chronic leukaemias the genetic change occurs in the HSC or progenitor population and leads to the expansion and differentiation of one particular lineage, characterised by an uncontrolled clonal proliferation of nearly well-differentiated blood cells. The differentiation in chronic myeloid leukaemia (CML) and chronic lymphoid leukaemia (CLL) depends on which lineage the malignant cell belongs to myeloid and lymphoid respectively. Each type can be subdivided. Chronic lymphoid leukaemia can be divided into three main subtypes: chronic lymphocytic leukaemia (CLL), prolymphocytic leukaemia (PLL) and hairy cell leukaemia (HCL) (Pallister, 2005). In chronic lymphoid leukaemia relatively mature B- and T-cell-lymphocytes propagate and the disease occurs mostly between the age of 60 and 80 (Hoffbrand et al., 2003).

There are six main subtypes of chronic myeloid leukaemia with the most common form being CML. This form of leukaemia is characterised by a chromosomal translocation giving rise to the Philadelphia chromosome t(9;22)(q34;q11). The abnormal chromosome contains the *BCR-ABL*-fusion gene which encodes a noval protein with a size of 210 kDa. This protein has constitutive tyrosine kinase activity unlike the normal protein, leading to the high proliferation rate observed in CML (Pallister, 2005; Hoffbrand et al., 2003).

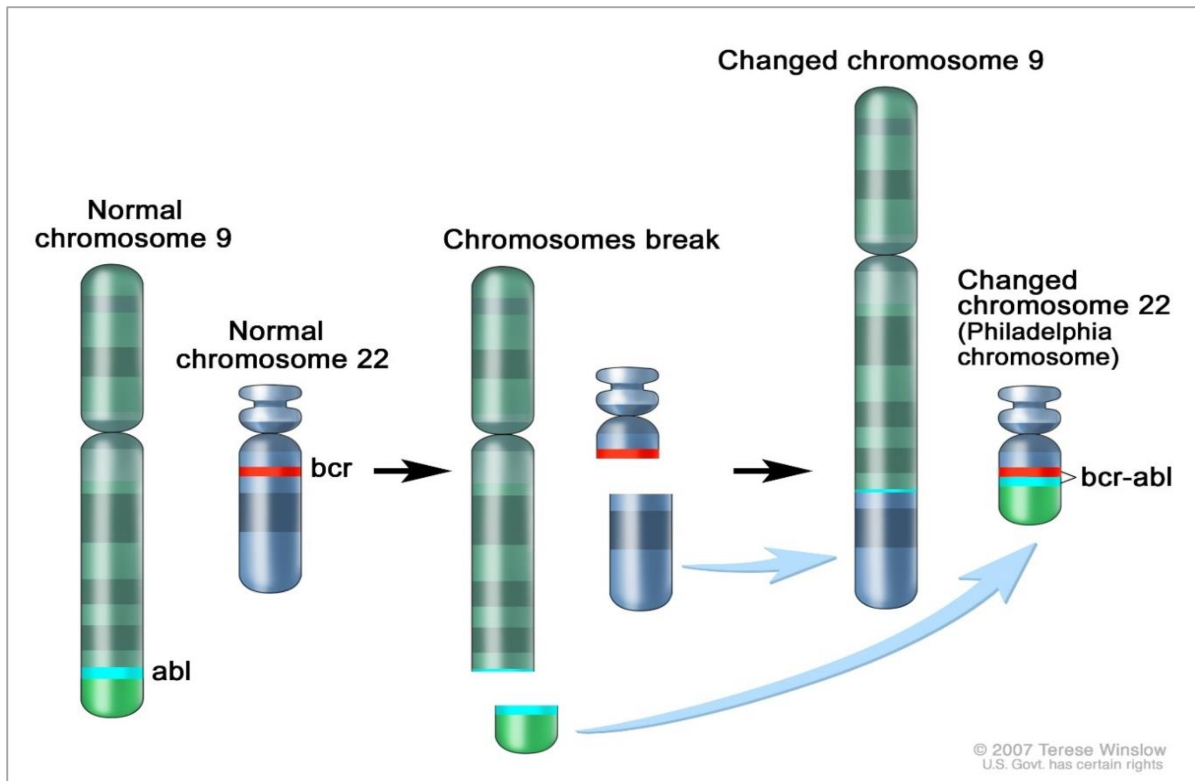


Figure 2: Development of the Philadelphia chromosome *BCR-ABL*

Schematic diagram 2 illustrates the development of the *BCR-ABL* fusion gene. A translocation between chromosome 9 and 22 leads to the Philadelphia chromosome (Figure from teresewinslow.com).

The Philadelphia chromosome is found in 95 % of CML patients. This translocation concurs to the clonal expansion of malignant haematopoietic progenitors (Konopka et al., 1985) but more events are necessary for disease progression (Sawyers, 1999; Goldman and Melo, 2003). CML can occur at any age and consists of three phases; the first one is the chronic phase, followed by the accelerated phase and a terminal blast phase, also called blast crisis. Normally only the abnormal chromosome is present during the chronic phase. Additional chromosomal abnormalities like an extra Philadelphia chromosome and p53 mutations occur in the accelerated phase. The progression to the blast phase is caused by an accumulation of proto-oncogene mutations. The blast phase is comparable with an acute leukaemia however, the treatment is not as successful as in acute leukaemia. The patients die mostly of infections (Pallister, 2005).

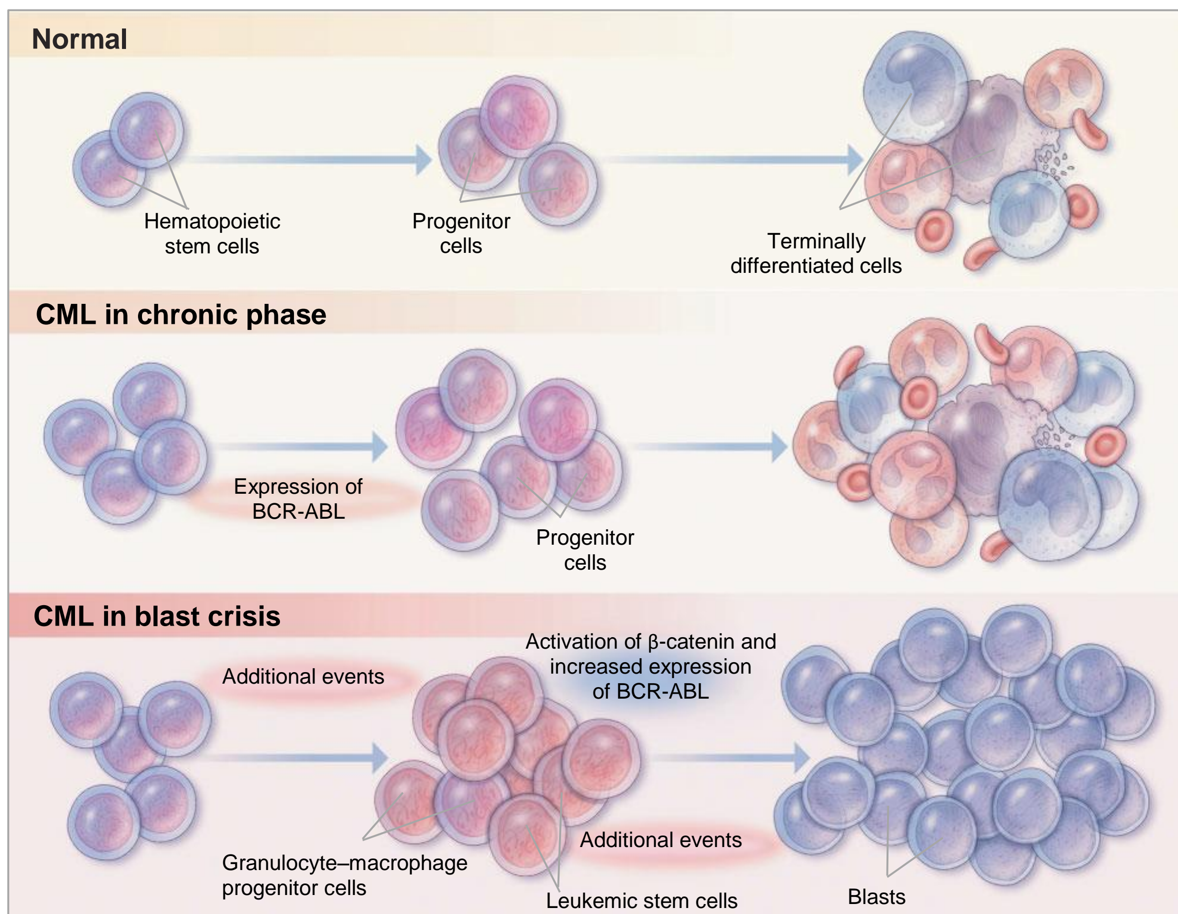


Figure 3: Model of the role of activated β–catenin in the progression of CML

Schematic diagram 3: In the chronic phase CML, cells within the progenitor pool have an enhanced proliferation ability compared to a healthy progenitor pool, because they mostly express the *BCR-ABL* fusion gene. A myeloproliferative syndrome is the consequence, however the pathways involved in differentiation and cell death are not affected. Additional events lead to the progression to blast crisis. These additional events include the activation of β-catenin in the granulocyte/macrophage progenitor (GMP) population. The proliferation and self-renewal capability increase and probably lead to the development of a leukemic stem cell. Further events are necessary for CML to progress, such as; prevention of cell death, inhibition of differentiation and circumvention of the immune response (Figure from Jamieson et al., 2004).

Previous studies have shown a connection between BCR-ABL and canonical Wnt signalling. Wild-type BCR protein can directly interact with β-catenin which leads to a negative regulation of the Wnt pathway (Ress and Moelling, 2005; Ress and Moelling, 2006). However the fusion between BCR and ABL averts this interaction leading to activation (Nemeth and Bodine, 2007). In addition GSK3β mutations and altered Axin levels, (key components of the β–catenin destruction complex) have been observed in CML, leading to activation of the Wnt pathway (Jamieson et al., 2004). In addition Jamieson et al. (2004) revealed that patients with CML in the accelerated or blast phase, displayed a larger progenitor pool in bone marrow compared to healthy people. Higher numbers of megakaryocyte-erythroid progenitors were found in

chronic phase CML samples, a rise in myeloid progenitors in samples from patients with accelerated phase and an increase of GMPs in blast crisis samples. In addition, BCR-ABL transcripts increased in myeloid progenitors in blast crisis compared to HSCs. Analysis revealed that the GMP population had gained self-renewal potential through active β –catenin signalling (Jamieson et. al., 2004). Recent evidence indicates that CML stem cells rely on the Wnt signalling pathway for survival following TKI therapy (Zhang et al., 2013).

1.1.3 Canonical Wnt Pathway

The canonical Wnt (wingless Int-1) signalling pathway forms part of the signal transduction network which enables cells to respond by extrinsic signalling. This pathway is important for the development of animal cells and is mostly activated during the embryonic stage. The name is derived from its ligand Wnt, which is a secreted signal protein. The *wingless* (*wg*) gene was discovered in *Drosophila* and it is responsible for the development of wings among other things. The *Int-1* gene was found in mice and it is known for promoting breast tumours (Alberts et al., 2002; Walter et al., 2012).

Components of this pathway play an important role in cell proliferation and differentiation. Mutations in genes involved in this pathway have been linked to developing cancer (Munk et al., 2008). The pathway is activated during the embryonic development and malfunction causes pronounced foetal abnormalities. However, in adult cells it is mostly inactive, apart from in stem cell niches such as the bone marrow and intestinal crypts (Komiya and Habas, 2008).

In addition, Wnt signalling plays an important role in HSC self-renewal (Reya et. al., 2003). However recent studies indicate that there needs to be a balance of activation as continuous Wnt signalling through constitutively active β-catenin can lead to the depletion of the HSC stem cell pool, through an inhibition of HSC self-renewal and an increase in multilineage differentiation. These findings indicate that increased activity of Wnt signalling may have a harmful effect on HSC function (Kirstteter et. al., 2006 and Scheller et. al., 2006). Moreover, activation of the Wnt pathway has been observed in AML (Simon et. al., 2005 and Ysebaert et. al., 2006) and in blast crisis CML patients (Muijtjens et. al., 2004).

If the pathway is inactive, a complex consisting of Axin, adenomatosis polyposis coli (APC), protein phosphatase 2A (PP2A), casein kinase 1α (CK1α) and glycogen synthase kinase 3 β (GSK3β) phosphorylates β-catenin. This targets β-catenin for ubiquitination and subsequent destruction. When the pathway is activated, the complex that phosphorylates β-catenin, cannot form and β-catenin accumulates in the cytoplasm. It translocates into the nucleus and binds to the *LEF/TCF* DNA-binding transcription factors where it activates the transcription of

target genes (Komiya and Habas, 2008; Munk et al., 2008) including *TCF7* (*TCF1*) (Roose et al., 1999) and *LEF1* (Hovanes et al., 2001; Filali et al., 2002).

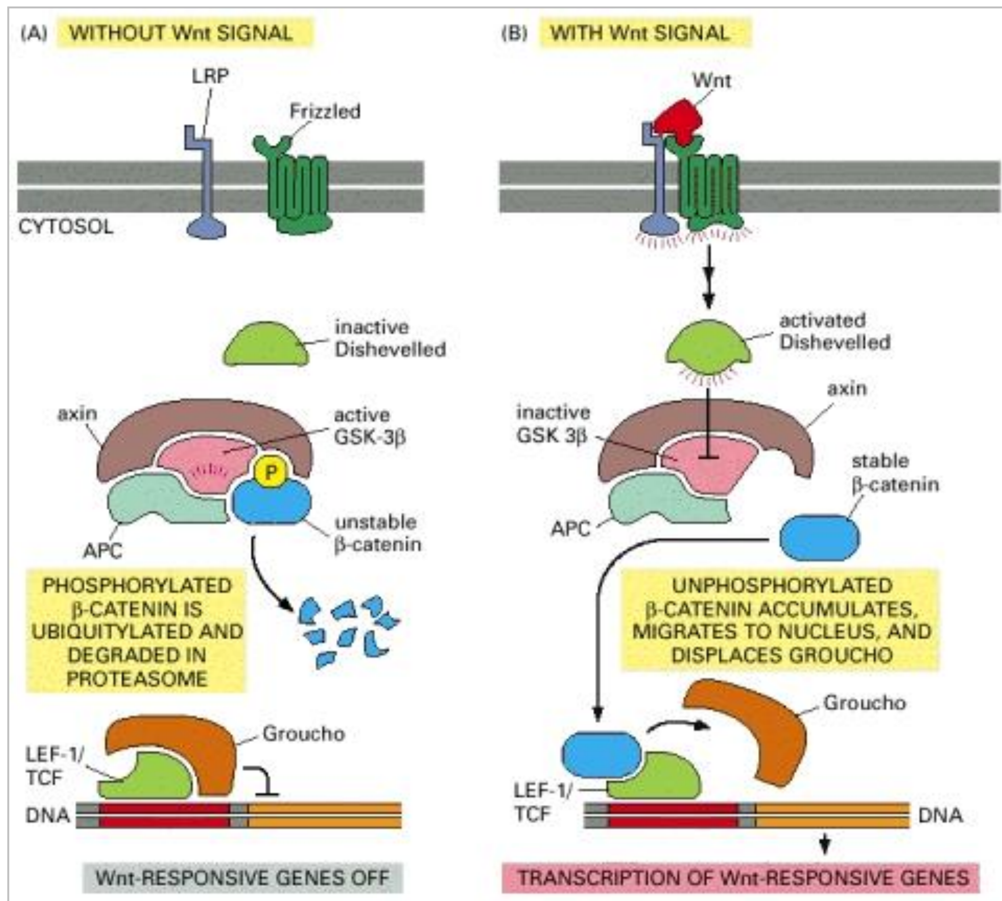


Figure 4: The canonical Wnt signalling pathway in the inactivated and active state

Schematic diagram 4 of the Wnt signalling pathway. The inactivated pathway is depicted in (A). Without a signal, dishevelled is inactive and the complex containing Axin, active GSK3β and APC can phosphorylate β-catenin, which is then degraded. In (B) the pathway is activated. A ligand binds to the receptor and the signalling protein deactivates the complex, which can no longer phosphorylate β-catenin. Now β-catenin accumulates in the cytoplasm and enters the core, where it binds to the transcriptional regulator LEF-1/TCF and activates the transcription of target genes (Figure from Alberts et al., 2002).

1.1.4 Genes of interest

Wnt signalling plays an important role in the maintenance of stem cell pluripotency, developmental haemopoiesis and adult tissue homeostasis. Deregulation of the pathway is linked to many cancers especially colon cancer and leukaemia. However it is a complex pathway regulated by a network of transcriptional activators and repressors. The exact role of individual activators and repressors in stem cell self-renewal and differentiation is still not clearly defined. It is known that Wnt signalling leads to the activation of multiple genes, this is mediated through the TCF family of transcription factors.

The *TCF* gene family comprises *TCF3 (TCF7L1), TCF4 (TCF7L2), LEF1 (TCF7L3)* and *TCF7.* The *TCF* genes are members of the T-cell factor family of transcription factors, which can bind to DNA and are able to activate or inhibit transcription (Barker et al., 2000).

TCF7 is a family member of HMG box containing transcription factors. These factors associate with β-catenin (bcat) and mediate Wnt signalling (Barker et al., 2000; Clevers and van de Wetering, 1997; Eastman and Grosschedl, 1999). But in the absence of bcat, *TCF7* operates as a transcriptional repressor (Barker et al., 2000; Clevers and van de Wetering, 1997). Furthermore, *TCF7* plays a role in B- and T-cell development (Staatl and Clevers, 2005).

TCF3 is a member of the T-cell factor family and an important component of the regulatory network that decides between self-renewal and differentiation (Cole et al., 2008). In addition, *TCF3* acts as a repressor of the *Nanog* gene in ES cells, which is important for maintaining pluripotency and self-renewal (Pereira et al., 2006).

The protein encoded by *TCF4* is involved in Wnt signalling and plays an important role in several developmental processes and carcinogenesis (Clevers & van de Wetering, 1997). During Wnt signalling, β-catenin binds to *TCF4.* This complex activates the transcription of target genes (Cuilliere-Dartigues et al., 2006).

LEF1 belongs to the *TCF/LEF* family of HMG-domain containing transcription factors as well as *TCF7* (Klaus and Rudolf, 1993; He et al., 1998; Shtutman et al., 1999). *LEF1* is an activator of the OCT4 promotor and it works synergistically with β-catenin. Furthermore, it is suggested that *LEF1* works with *Nanog* to promote self-renewal in ESCs (Huang and Qin, 2010).

Other genes of interest involved in the pathway are: *Catenin, Beta Interacting Protein* (*ICAT*) and *Acute Myeloid Leukemia 1 Protein* (*AML1 or RUNX1*). The *AML1* gene encodes a transcription factor (Ito, 2004; Speck and Gilliland, 2002) and contains a Runt domain which is homolog with the *Drosophila* runt protein and an α subunit of polyomavirus enhancer binding protein 2 (PEBP2α) (Erickson et al., 1992; Daga et al., 1992; Ogawa et al., 1993). *AML1* can bind to DNA through the Runt domain (Meyers et al., 1993; Kagoshima et al., 1993) and is very important for haematopoiesis (Dzierzak and Speck, 2008) but not necessary for the preservation of adult HSCs (Tsuzuki and Seto, 2012, Ichikawa et al., 2004; Growney et al., 2005). The *AML1* gene is disrupted in acute myeloid leukaemia (AML) by the t(8;21) translocation (Miyoshi et al., 1991) and in blast crisis CML by the t(3;21) translocation which leads to a fusion gene (Nucifora et al., 1993; Mitani et al., 1994; Nucifora et al., 1993; Nucifora et al., 1994). Additionally, the *AML1* gene generates three alternative spliced variants. These variants were named *AML1a*, *AML1b* and *AML1c*. *AML1a* is the short and *AML1b* and *c* are the long transcript variants *(Miyoshi et al., 1995). AML1b* and *AML1c* proposed to function as

transcriptional activators (Bae et al., 1994; Tanaka et al., 1995; *Miyoshi et al., 1995*) which reduce HSC repopulation (Tsuzuki et al., 2007) and promote differentiation (Tsuzuki et al., 2007). *AML1a* seems to be a negative regulator because it suppresses transcriptional activation by *AML1b* and it has a higher affinity to DNA than *AML1b* (Tanaka et al., 1995; Miyoshi et al., 1995). In addition, it fosters Maintenance and proliferation (Tsuzuki et al., 2007).

Wu et al. discovered that *TCF7* and *RUNX1* mediate the transcription of genes which are up-regulated in stem cells. They also showed that *TCF7* and *RUNX1* bind to their own promotors and to each other's promotors and may co-regulate each other in a feed-back loop. Furthermore, both genes bind and regulate an overlapping bunch of target genes which may be transcription factors. The protein of the short isoform of *RUNX1* disappeared if *TCF7* was knocked down, but there was no change in the protein level of the long isoform of *RUNX1* (Wu et al., 2012).

The pathway is negatively regulated by another gene family called *TLE, which* covers *TLE1, TLE2, TLE3 and TLE4.* The *TLE* genes are transducing-like enhancers of split and act as transcriptional co-repressors (Stifani et al., 1992; Hartley et al., 1988). They are widely expressed and involved in several signalling pathways during animal development, like haematopoiesis (Gasperowicz and Otto, 2005; Jennings and Ish-Horowicz, 2008). *TLE* encoded proteins interact with the regulatory region of target genes but it is not yet known how *TLE* switches off transcription (Jennings and Ish-Horowicz, 2008).

ICAT acts as a negative regulator of the canonical Wnt signalling pathway through an interference of the β-catenin-TCF4 complex. But *ICAT* levels did not change significantly when the Wnt pathway was activated. Moreover, during mouse embryonic development *ICAT* mRNA was expressed almost constantly (Tago et. al., 2000).

1.1.5 Drugs

GSK-3 Inhibitor IX (BIO), Tankyrase 1/2 Inhibitor III (XAV) and a Wnt3a ligand were used to treat cells.

The drug BIO and the ligand Wnt3a activate the Wnt pathway. The Wnt3a ligand is one of 19 secreted glycol-proteins which are crucial for normal development (Logan and Nusse, 2004). Wnt3a binds to the Frizzled and LRP receptor at the cell surface and activates the Wnt signalling (Figure 4). In addition, ESC proliferation is stimulated by Wnt3α (Singla et al., 2006).

BIO is an ATP competitive inhibitor which inactivates GSK3β, by binding to the Leu132 residue of GSK3β, preventing that the complex that phosphorylates β-catenin forming. This causes an increase of β-catenin in the cytoplasm (Meijer et al., 2003). GSK3 inhibitors were identified in the late 1970s but they were first used in the mid-1990s for type 2 diabetes and Alzheimer's

disease. GSK3 phosphorylates proteins and generates multisite phosphorylation domains. This phosphorylation can affect the biological activity of the substrate (Cohen and Goedert, 2004). GSK3 can prepare some substrates, like ß-catenin, for "ubiquitylation and subsequent destruction by the proteasome" (Cohen and Goedert, 2004: Page 479). The substrates contain transcription factors and enzymes which are involved in regulating metabolism. GSK3 can also be inhibited in the Wnt-signalling during the embryonic development which leads to an accumulation of β-catenin and translocation to the nucleus, where it stimulates the transcription of certain genes. In several cancer cells many components of the Wnt signalling are overexpressed or mutated (Cohen and Goedert, 2004).

XAV stabilises Axin which is the concentration limiting component of the destruction complex (Salic et al., 2000) which phosphorylates β-catenin and thus causes its destruction. The main components of the destruction complex are APC, Axin and GSK3. Axin is regulated by the Wnt-signalling pathway itself via the TNKS1/2 proteins. XAV binds to TNKS1 and TNKS2 and inhibits these proteins. Therefore TNKS1/2 can no longer alter Axin through PARsylation (the addition of several ADP-ribose units), leading to its destruction. Importantly, the accumulation of Axin takes several hours. In cancer cells, the protein level of Axin was strongly increased after XAV treatment, however, this was not observed at the mRNA level (Huang et al., 2009).

1.1.6 The oncogene *Tel/PDGFRβ*

Tel/PDGFRβ (TP) is a fusion protein which consists of the Platelet-Derived Growth Factor Receptor β (PDGFRβ) and a noval *ets*-like gene, tel. A cell surface tyrosine kinase receptor is encoded by *PDGFRβ* which is localised on chromosome 5. This receptor plays an important role in the regulation of embryonic development, survival, cell proliferation, chemotaxis, migration and differentiation (National Center for Biotechnology Information, 2014; Golub et al., 1994). *Tel*, also called ets variant 6, encodes a transcription factor from the ets family which can bind to DNA (National Center for Biotechnology Information, 2014; Pruitt et al., 2007).

TP, a chimeric oncogene, can be found in a subgroup of chronic myelomonocytic leukaemia (CMML) patients and accrues from the t(5;12)(q33;p13) translocation (Golub et al., 1994). A constitutive activation of the PDGFRβ tyrosine kinase and a stimulation of the PDGFRβ signalling pathway are the results of the fusion protein (Carroll et al., 1996; Jousset et al., 1997).

The expression of TP in the ES cell model E14tg2a leads to haematopoietic differentiation and the preservation of self-renewal. Furthermore, it increases myelopoiesis and represses erythropoiesis (Dobbin et al., 2008).

1.2 Objectives

The first objective of this project was to measure the basal level of the *TCF/LEF*, *TLE*, *ICAT* and *RUNX1* genes in CML cells to discover which family members are expressed in this disease. We then went on to activate and repress the pathway to determine which family members are important regulators of Wnt signalling in CML.

The second objective was to investigate which of these downstream genes were altered when the oncogene TP was inducible expressed in stem cells as a previous study showed that TP suppresses Wnt signalling leading to decreased pluripotency and increased haemopoietic differentiation of mES cells.

2. Materials and Methods

2.1 Materials for Cell Culture

2.1.1 Cell-lines:

K562

K562 is an immortalised myelogenous leukaemia cell line which was derived from a 53 year old female CML patient in blast crisis. This cell line was first cultured by a team from the Laboratories of Cytogenetics and Spleen Pathophysiology, University of Tennessee Memorial Research Center and Hospital in 1974. It stably expresses the *BCR-ABL* fusion protein even after serial subcultivations (Lozzio and Lozzio, 1975). This cell line was kindly provided by the Paul O'Gorman Leukaemia Research Centre, Glasgow.

Chinese hamster ovary cells (CHO)

CHO cells are immortalised cells derived from the ovary of the Chinese hamster. They were first isolated by Theodore T. Puck in 1957. CHO cells are the most common cell lines in biotechnology, cell biology and molecular biology because they grow very fast and produce a high yield of protein. CHO cells only have 22 chromosomes which is interesting for various cell culture studies. They are the most frequently used expression systems for biopharmaceuticals. The cell line is used in many experiments because of the genetic polymorphism which means that there are several gen variants and CHO cells can be cultured in suspension and adherent. Furthermore, the post-translational glycosylation of the resulting proteins when used for expression studies is very important because it determines the effect of the biopharmaceutics. CHO cells synthesise a broadly similar glycan pattern to humans, enabling any differences in species specific protein formation being diminished through molecular biology interventions (Thüte, 2012; Klausing, 2013).

In this project CHO LIF (Leukaemia inhibitor factor) cells and CHO Wnt3a cells were cultured to receive the proteins LIF and Wnt3α. The cells contain a plasmid which has the genetic information for each protein attached to an immunoglobulin signal peptide to ensure the protein is secreted into the media.

These cell lines were kindly provided by the Paul O'Gorman Leukaemia Research Centre, Glasgow.

E14tg2a

E14tg2a cells are mouse embryonic stem cells. The cell line was developed in 1987 by M. Hooper to be feeder-independent (Smith and Hooper, 1987).

The parental E14tg2a cell line (R63), which was used, expresses the tetracycline (tet)-sensitive transactivator. Furthermore, three cell lines were developed from R63. The first one expresses the oncogene TP (TPI), the second one expresses constitutively active bcat (Clone II) and the third one expresses both, the oncogene TP and constitutively active bcat (TP-βC), under the control of the tetracycline inducible expression system (Tet-off system) (Dobbin et al., 2008).

These cell lines were kindly provided by the Paul O'Gorman Leukaemia Research Centre, Glasgow.

2.1.2 Media, reagents and drugs

The following reagents, media and drugs were used in the cell culture lab:

DMSO (#D2650)	Sigma-Aldrich
DPBS (#14200083)	Gibco®, Life Technologies
FBS (#10500-064)	Gibco®, Life Technologies
FBS for mESC (#FB-1001S/500ml)	Bio-Sera
GSK-3 Inhibitor IX (BIO) (#667463-62-9)	Calbiochem
HAM's F-12 Nutrient mix (#11765-054)	Gibco®, Life Technologies
KnockOut™ DMEM (#10829-018)	Gibco®, Life Technologies
KnockOut™ serum replacement (#10828-028)	Gibco®, Life Technologies
L-Glutamine (#25030-024)	Gibco®, Life Technologies
Leukaemia inhibitor factor protein (LIF) (#LIF2010)	Millipore
MEM NEAA (100x) (#11140-035)	Gibco®, Life Technologies
PBS (#10010-056)	Gibco®, Life Technologies
Penicillin and Streptomycin (Pen Strep) (#15140-122)	Gibco®, Life Technologies
RPMI 1640 Media (#31870-074)	Gibco®, Life Technologies
Tankyrase ½ Inhibitor III (XAV) (#575545-10MG)	Calbiochem
Tetracyclin (#T7660)	Sigma-Aldrich
Trypan blue (#302643-100g)	Sigma-Aldrich
Trypsin-EDTA (#T4049-500ml)	Sigma-Aldrich
Trypsin-EDTA for mESC (#25200-072)	Gibco®, Life Technologies

2.2 Materials for molecular biology lab

2.2.1 Kits

The following kits were used in the molecular biology lab.

- RNeasy Plus Mini Kit (50), cat# 74134, QIAGEN
- Super Script™ III Reverse Transcriptase (RT), cat# 18080-093, Invitrogen
- QIAGEN Multiplex PCR Master Mix, 2x, cat# 206143, QIAGEN
- TaqMan® Gene Expression Master Mix, cat# 4369016, AB by Life Technologies
- Fast SYBR® Green Master Mix, cat# 4385612, AB by Life Technologies
- SensiFAST™ SYBR Hi-ROX Kit, cat# BIO-92005, BIOLINE

2.2.2 Software

The following software's were used in the molecular biology lab.

- Real Time PCR: SDS 2.3.lnk by Novell
- Analyse data from Real Time PCR: RQ Manager 1.2.lnk by Novell
- Nano-Drop: ND 1000 V3.3.0
- Plate reader: SoftMax Pro V5.2
- Fluorescence microscope: Axio vision Rel.

2.2.3 Genes/Primer

TCF3 and *TCF4* are genes which encode a member of the E protein family of helix-loop-helix transcription factors. E proteins activate the transcription by binding to regulated E-box sequences on target genes as heterodimers or homodimers. They also play a crucial role in lymphopoiesis. The encoded protein is needed for B- and T-lymphocyte development (National Center for Biotechnology Information, 2014; Pruitt et al., 2007).

The *TCF7* gene encodes a protein, which is a transcriptional activator and plays an important role in lymphocyte differentiation. This protein is predominantly expressed in T-cells and can bind to an enhancer element, which activates the cluster of differentiation 3 epsilon (*CD3E)* gene. The *CD3E* gene is important for the T-cell development. Through a feedback mechanism it may also repress the *bcat* and *TCF7L2* (*TCF4*) genes (National Center for Biotechnology Information, 2014; Pruitt et al., 2007).

TLE1, *TLE2*, *TLE3* and *TLE4* code for proteins which are transcriptional corepressors. Transcriptional activation mediated by the *TCF* family following Wnt signalling is inhibited by the TLE family of proteins (National Center for Biotechnology Information, 2014; Pruitt et al., 2007).

The protein encoded by the *ICAT* gene (also called *CTNNBIP1)* binds β-catenin and averts interaction between the TCF family members and β-catenin. Therefore, ICAT is a negative regulator of the Wnt signalling pathway (National Center for Biotechnology Information, 2014; Pruitt et al., 2007).

The *RUNX* (*AML1,* Runt-Related Transcription Factor) gene encodes a protein which binds to several enhancers and promoters, including murine leukaemia virus, polyma virus enhancer, T-cell receptor enhancer, lymphocyte specific protein tyrosine kinase (LCK), interleukin-3 (IL-3) and granulocyte macrophage colony stimulating factor (GM-CSF) promoters. The protein binds DNA and it seems to play a role in the development of normal haematopoiesis (National Center for Biotechnology Information, 2014; Pruitt et al., 2007).

LEF1 (Lymphoid Enhancer-Binding Factor one) is a protein-coding gene, which encodes a transcriptional factor belonging to a family of proteins that shares homology with the high mobility group protein 1. The encoded protein can bind to a functionally important site in the T-cell receptor-alpha enhancer and enables maximal enhancer activity. The members of the *TLE* family repress the transactivation mediated by *LEF1* (National Center for Biotechnology Information, 2014; Pruitt et al., 2007).

β-catenin (*CTNNB1*, Catenin (Cadherin-Associated Protein), *Beta 1*) encodes a protein which is part of a complex that constitutes adherens junctions (AJs). Furthermore, the protein anchors the actin cytoskeleton and possibly acts to repress dividing when the epithelial sheet is complete, through transmitting a contact inhibition signal. The protein is encoded by three transcript variants (National Center for Biotechnology Information, 2014; Pruitt et al., 2007).

Guanine nucleotide binding protein *(G2NBL)*, Ecto-Nox-Disulfide-Thiol Exchanger (*ENOX2)*, Synthesizing Protein 1 Homolog *(TYW1)* and Ubiquitin-Conjugating Enzyme E2D 2 (*UBE2D2)* are human house keeping genes. These genes are expressed in all cells and can be used as control for different analysis (National Center for Biotechnology Information, 2014; Pruitt et al., 2007).

TATA box binding protein (*tbp*), beta-2 microglobulin (*b2m*) and glucuronidase, beta (*gusb*) are mouse house keeping genes. These genes are expressed in all cells and can be used as control for different analysis (The Jackson Laboratory, 2015).

2.2.4 Media and reagents

Due to the high number of media and reagents, they are listed in the annex.

2.3 Methods used for cell culture

2.3.1 Cell culture

All work was done in a laminar flow hood and all solutions and equipment were sterile.

K562 cells were routinely cultured in tissue culture flasks with RPMI 1640 media supplemented with 10 % foetal bovine serum (FBS), 1 % L-glutamine and 1 % Pen Strep, and kept constantly

in a humidified incubator at 37˚C with 5 % CO_2. The cells were passaged every two or three days after reaching 70-80 % confluence.

CHO cells were cultured in cell culture flasks with Ham's F12 Media which was supplemented with 10 % FBS and 1 % Pen Strep, and were kept constantly in a humidified incubator (37°C with 5 % CO_2). The cells were passaged when they reached 80 % confluence.

Murine ESCs were cultured in cell culture dishes coated for 30 minutes with 1 % gelatine prior to culture. The KnockOut™ DMEM Media was supplemented with 15 % KnockOut™ serum replacement, 1 % FBS for mESC, 0.1 mM 2– Mercaptoethanol, 2 mM L-glutamine, 0.1 mM MEM NEAA and 10 % LIF CM. 0.1 % recombinant LIF was directly added to the culture dish. The cell lines TPI, TPβC and Clone II, expressing dominant positive β-catenin (dp-bcat), were treated with 1 µg ml^{-1} Tet to prevent the expression of TP and bcat. To induce expression cells were washed x3 in PBS and cultured for 48 h without Tet, to induce the expression of the oncogene (TP) and constitutively active bcat (dp-bcat). The cells were passaged when they reached a high confluence but the media was changed every two days.

2.3.2 Drug treatment

K562

The drug treatment of K562 was performed with concentrations of 1 µM and 5 µM BIO, 1 µM and 5 µM XAV or 20 % and 50 % Wnt3a in Ham's F12 Media for 8 h and 24 h.

The 8 h treatment K562 cells were plated at 1 x 10^6 cells per ml, and for the 24 h treatment 1 to 2 x 10^5 cells per ml.

mESC

Murine ESC were treated with 5 µM BIO and with 1 µg ml^{-1} Tet to inhibit the expression of the oncogene and bcat.

The ES cells were washed 3 times with PBS and plated at different concentrations. TPI were plated from 6 x 10^5 to 1 x 10^6 cells per dish without the addition of Tet and 5 x 10^5 cells per dish with Tet. TPβC were plated at 1 x 10^6 cells per dish for all variants. R63 were plated at 6 x 10^5 cells per dish with Tet and 5 x 10^5 cells per dish without Tet. Clone II (dp-bcat) at 5 x 10^5 cells per dish with Tet and 6 x 10^5 cells without Tet.

2.4 Methods used for molecular biology

2.4.1 Preparation of RNA and cDNA

Total RNA was extracted from cells using the RNeasy Plus Mini Kit from Qiagen. To receive a good yield, the cell sample was homogenised with a small syringe in lysis buffers as per the manufacturer's instruction.

After preparation, the concentration of RNA was measured using the Nanodrop 1000 (ND1000) at a wavelength of 260 nm in a volume of 1.5 µl. If the concentration of RNA was too high, the RNA was diluted and the measurement repeated. A dilution of 1:10 was usual.

First-strand cDNA was synthesised from 500 ng of total RNA using the Super Script™ III Reverse Transcriptase (RT) kit as per the manufacturer's instruction, Invitrogen.

2.4.2 Preparation of protein

Protein was extracted from a minimum of 2 x 10^6 cells. The lysis buffer (solubilisation buffer) was prepared in advance without PMSF, this was added shortly before using the buffer. For the recipe see 6.2.12 protein solubilisation buffer, page 89. Cells were counted and an aliquot was taken and centrifuged (5 min, 400 x *g*). The pellet was resuspended and washed 2 times in PBS. After the second wash the cells were centrifuged at full speed for 5 minutes at 4°C in a microcentrifuge. The supernatant was aspirated and 30 µl lysis buffer per 1 x 10^6 cells was added. Subsequently, the lysates were incubated on ice for 30 minutes to solubilize the proteins. Afterwards the solution was centrifuged at 4°C for 5 minutes at full speed in a microfuge to pellet the debris and the supernatant containing the protein was transferred to a clean tube and stored at -80°C prior to further use. To quantify the protein concentration a Bradford assay (Bio-Rad) were performed and samples measured against a BSA standard curve.

2.4.3 Immunofluorescence

Slides were prepared with 30 µl of 5 µg ml^{-1} Poly-L-Lysine in Tetraborate buffer per well and incubated at room temperature for 2 h to overnight. Afterwards, the wells were washed three times with 1x PBS.

A concentration of 6 x 10^4 cells per well was used and the following antibodies were tested: LEF1, TCF7, AML1, TLE1/2/3/4, active bcat and bcat. A control was conducted for all drugs and concentrations. Following adherence of cells for 45 min at 37°C, cells were fixed with 4 % Paraformaldehyde for 10 min and then permeablised with 0.5 % Triton-X100 in PBS for 15 min at RT. Cells were then blocked for 1 h with blocking solution for immunofluorescence (6.2.12). The primary antibodies were incubated overnight. The secondary antibody was prepared 1:200 in IF Blocking solution and cells incubated for 2 h. DAPI mounting solution was used to visualise the cells. Cells were washed x5 between each stage of the protocol using PBS.

2.4.4 XTT-Assay

A XTT Assay was previously conducted with BIO and XAV to determine the IC50 of these drugs for the cells used in this study.

2.4.5 Pre-Amplification (pre-amp) and Exonuclease I digest

Pre-amplification using unbiased Taq polymerase to increase the copy number of the genes analysed.

The first step was to prepare primer master mix using 500 nM forward and reverse primer from up to 6 genes of interest and 2 house keeping genes per 0.5 ml micro test tube. Each primer master mix was made up to 200 µl with TE buffer. This procedure was repeated for additional primer sets as required but with different endogenous controls.

Before the pre-amp master mix was set up, the number of samples had been calculated. The master mix for 1 primer set contained the following reagents and amounts:

Reagent	Amount in µl per cDNA sample
2x Qiagen Pre-amp Master Mix	2.5
double demineralised water	0.5
primer set x	0.75
Total volume	3.75

3.75 µl of the pre-amp master mix was dispensed into a PCR tube and 1.25 µl of the cDNA sample was added. Subsequently, the suspension was mixed and centrifuged. The amplification was carried out in a thermocycler with the following conditions:

	temperature in ˚C	time in min	number of cycles
Stage 1	95	15	1
Stage2	94	0.5	14
	60	1.5	
	72	1.5	
Stage 3	72	10	1

The needed amount of Exonuclease I master mix was prepared on ice. The master mix contained the following reagents and amounts:

Reagent	Per 1 x 5 µl pre-amp-sample
double demineralised water	1.4 µl
Exo I Buffer	0.2 µl
Exo I enzyme (20 units/µl)	0.4 µl

The master mix was vortexed and centrifuged afterwards. 2µl of this master mix was dispensed into each pre-amp sample and each sample was vortexed and centrifuged again. The incubation in the thermocycler was conducted under the following conditions:

1) 37˚C for 30 minutes
2) 80˚C for 15 minutes

After this treatment, 18 µl of TE buffer was added to each tube and each sample was transferred into a 0.5 ml micro test tube. The total volume was 25 µl and the sample was stored at -20˚C prior to further use.

To estimate the concentration of the amplified cDNA a polymerase chain reaction (PCR) with house keeping genes was performed. The amplified cDNA was diluted 1:2 if the concentration was high enough.

2.4.6 Polymerase chain reaction (PCR)

The concentration of the pre-amplified cDNA was tested by PCR and agarose gel electrophoresis. Therefore the following reagents were added together in a PCR tube and mixed gently.

Reagent	Concentration per 25 µl tube
5x Green GoTaq® Flexi Buffer	1x
$MgCl_2$ Solution (25 mM)	3 mM
PCR Nucleotide Mix (10 mM)	0.2 mM each dNTP
Sense Primer (25 mM)	0.2 mM
Antisense Primer (25 mM)	0.2 mM
GoTaq DNA polymerase (5 u $µl^{-1}$)	1 u
Template DNA (diluted)	2 µl
Double demineralised water to	25 µl

Afterwards, the solution was heated to 94˚C for 1 minute to denature the cDNA. Subsequently, 30 cycles which consisted of denaturing at 94˚C for 30 s, annealing at 60˚C for 30 s and extension at 72˚C for 40 s, were performed. At the end the solution was heated to 72˚C for 5 min and then cooled down to 4˚C before being removed. All these steps were performed in a thermocycler. The samples were run on a 2 % (w/v) agarose gel at 100 V for approximately 40 min in TBE buffer.

2.4.7 Quantitative PCR (qPCR)

At first, a 25 µM primer stock needed to be prepared for each primer that was used for the quantitative PCR. Therefore, 10 µl of forward and reverse primer from a 100 µM stock were dispensed in a 0.5 ml micro test tube. Another 20 µl TE buffer was added to receive a final volume of 40 µl.

Subsequently, the assay master mix had to be set up. The number of samples which should be analysed was calculated. Each sample was analysed in triplicate. Preferably, a plate layout with all samples and genes was drawn. For Example (96-well-plate):

	Gen	1	2	3	4	5	6	7	8	9	10	11	12
A	K562 a	TCF 7	TCF 7	TCF 7	LEF 1	LEF 1	LEF 1	ICA T	ICA T	ICA T	TLE 2	TLE 2	TLE 2
B	K562 a	ENO X	ENO X	ENO X									
C	K562 b	TCF 7	TCF 7	TCF 7	LEF 1	LEF 1	LEF 1	ICA T	ICA T	ICA T	TLE 2	TLE 2	TLE 2
D	K562 b	TYW	TYW	TYW									
E	NTC	TCF 7	LEF 1	ICAT	TLE 2	ENO X	TY W						
F													
G													
H													

For each primer dilution the following reagents were dispensed in a 1.5 ml micro test tube.

TaqMan® Gene Expression Master Mix	Volume per 10 µl reaction in µl
double demineralised water	2.3
AB Gene expression MM (2x)	5
Primer dilution	0.2
Eva green (SYBR) (20x)	0.5
Total volume	8

or

Fast SYBR® Green Master Mix	Volume for per 10 µl reaction in µl
Fast SYBR® Green MM (2x)	5
Primer dilution	0.2
double demineralised water	2.8
Total volume	8

or

SensiFAST™ SYBR Hi-ROX Kit	Volume for per 10 µl reaction in µl
2x SensiFAST SYBR Hi-ROX Mix	6
Primer dilution	0.2
double demineralised water	1.8
Total volume	8

The qPCR for K562 1, 8 h samples was carried out with the TaqMan® Gene Expression Master Mix and the qPCRs for K562 2, 8 h, 1 and 2 24 h were carried out with the Fast SYBR® Green Master Mix.

The qPCR with mESC samples were performed with the SensiFAST™ SYBR Hi-ROX Kit, cat# BIO-92005, BIOLINE.

The master mix was placed on ice while 2 µl of the diluted cDNA (sample) was added into a 96- or 384-well-plate well.

Secondly, 8 µl of the well mixed master mix was added to the wells. Afterwards, an optically clear plate was added to seal the plate and it was centrifuged at 500 x *g* for 1 min in the Sorvall

Legend T centrifuge (Thermo Scientific). Further, the amplification was performed using the following conditions:

TaqMan® Gene Expression Master Mix	Temp in ˚C	Duration	Cycles
UDG Incubation	50	2 min	Hold
AmpliTaq Gold®, UP Enzyme Activation	95	10 min	Hold
Denature	95	15 s	40
Anneal/Extend	60	1 min	

or

Fast SYBR® Green Master Mix (Instrument: 7900HT Fast)	Temp in ˚C	Duration	Cycles
AmpliTaq® Fast DNA Polymerase, UP Activation	95	20 s	Hold
Denature	95	1 s	40
Anneal/Extend	60	20 s	

or

SensiFAST™ SYBR Hi-ROX Kit	Temp in ˚C	Duration	Cycles
Polymerase activation	95	2 min	Hold
Denaturation	95	5 s	40
Annealing/extension	60	20 s	

At the end a melting curve analysis was implemented.

2.4.8 Western blotting

Gels with different percentages were prepared to ensure maximum separation of the proteins under investigation. 7.5 % gels were prepared for TLE1/2/3/4, TCF3, bcat and active bcat. 10 % gels were prepared for TCF7 and TCF4 and 12 % gels were prepared for LEF1 and AML1.

40 µg protein were fractionated by SDS-PAGE in 1x Running Buffer at 80 V for 15 minutes and 180 V for 30 to 45 minutes. Afterwards, semi-dry blot was conducted to transfer the protein on nitrocellulose membranes (Whatman™ Protran BA 85). For this purpose, the gel were placed on top of the membrane and sandwiched between Whatman paper strips which were soaked in 1x Transfer Buffer. The conditions for the blotting were 40 mA per Gel for 60 minutes. Subsequently, the successful transfer was proofed by staining with Ponceau S Solution and stain removed with 1x TBS. Except for AML1, all membranes were blocked with 5x BSA blocking solution for over one hour on a shaker. Membranes which were tested for AML1, were blocked with 5 % milk blocking solution. The primary antibodies were incubated overnight and the secondary antibodies were incubated for 1-2 h. After the incubation the signal was detected by chemiluminescence on a CL-XPosure™ Film. Afterwards, the membrane was stripped using 1x stripping buffer and a different antibody or a control antibody was tested.

3. Results

3.1 Micro Array Data

The following data was extracted from an extensive Affimetrix microarray data set performed by Professor Copland on normal, CML chronic phase, CML accelerated stage and CML blast crisis patients. Cells were sorted into HSC and progenitor populations. Array data provided by the Paul O'Gorman Leukaemia Research Centre in Glasgow. The microarray data shows the gene expression of certain genes of interest during cell differentiation and in healthy cell as well as in the chronic, accelerated and blast phase of CML.

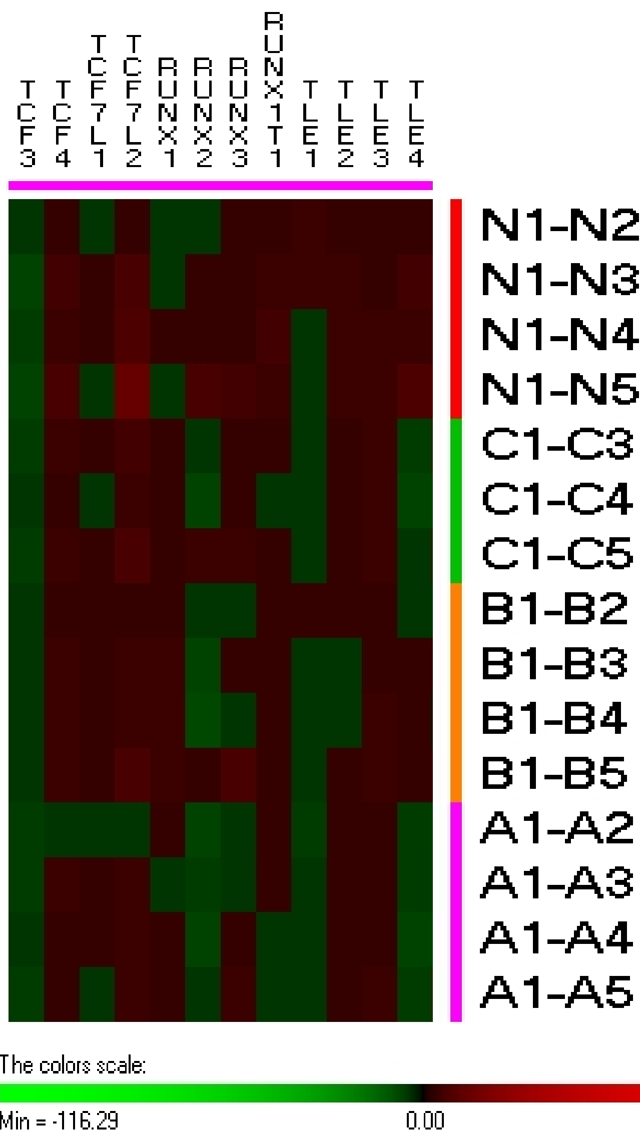
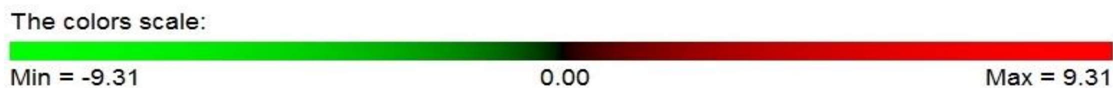


Figure 5 displays the gene regulation of certain genes in normal cells (N) and cells from CML in chronic phase (C), accelerated phase (A) and blast crisis (B) in comparison to more differentiated progeny (1 - HSC, 2 – MPP, 3 – CMP, 4 – GMP, 5 – MEP).
The expression of *TCF3* was down-regulated in all progenitor populations compared to HSC. Whereas *TLE3* was up-regulated with no difference in expression pattern seen in CML compared to the normal pattern.
RUNX1, *RUNX2*, *TLE1* and *TLE4* showed the biggest differences in expression with *RUNX1* being highly expressed in all phases of CML in both HSC and progenitor populations whereas *RUNX2* was down-regulated. Interestingly *TLE1* was down-regulated in all phases of CML whereas *TLE4* was down-regulated in CP and AP but upregulated in BC. This is in sharp contrast to *TLE2* which showed the opposite pattern.

Figure 5: Gene expression of certain genes in normal and CML cells during differentiation.

Microarray data analyses, displayed in Figure 5, revealed a change in the gene expression of *RUNX1*, *TLE1*, *TLE2* and *TLE4* during differentiation in the chronic, accelerated and blast

phase of CML compared to the more immature cell type. *RUNX1* was down-regulated during differentiation in normal cells, but highly up-regulated during disease progression. *TLE1* was down-regulated in all phases of CML. *TLE4,* which was down-regulated in chronic and accelerated phase, displayed an up-regulation during cell differentiation in normal cells and blast crisis. By contrast *TLE2* was up-regulated in all phases except from blast phase CML. These might indicate that the Wnt pathway be defective in its negative regulatory mechanisms in CML.



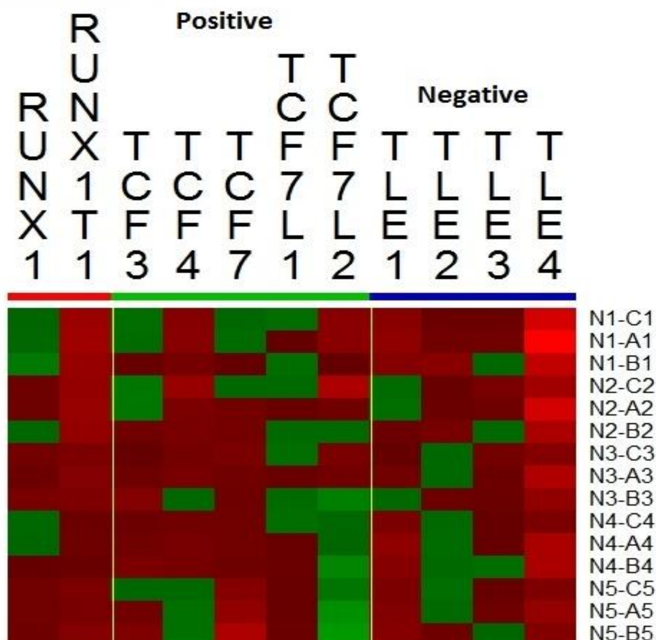


Figure 6: Gene expression of Wnt genes during differentiation in normal cells compared to CML cells.

Figure 6 shows the gene expression of key Wnt regulatory genes in normal cells (N) during differentiation (1 - HSC, 2 – MPP, 3 – CMP, 4 – GMP, 5 – MEP) in comparison to cells from chronic phase (C), accelerated phase (A) and blast crisis (B) CML. *RUNX1* was up-regulated in CML HSC, BC MPPs and CP/AP GMPs. *RUNX1T1* and *TLE4* were highly down-regulated in CML. *TCF3* and *TCF7* were up-regulated in more immature CML cells. By contrast, *TCF4* was only up-regulated in CML MEPs. *TLE1* and *TLE3* were down-regulated, whereas *TLE2* was up-regulated in CML CMPs, GMPs and MEPs.

In Figure 6 further genes of interest were depicted. *RUNX1* showed an increase in gene expression in HSCs, BC MPPs and CP/AP GMPs and a down-regulation in CP/AC MPPs,

CMPs and MEPs. *TCF7* was up-regulated in HSCs, however *TCF7* was down-regulated in all progenitors. The expression of *TCF3* and *TCF4* were quite different from each other. The *TCF3* expression was up-regulated in HSC and MPPs. *TCF4* on the other hand was only up-regulated in MEPs. The *TLE1, TLE3* and *TLE4* expression were down-regulated in all differentiation states. *TLE2* expression was only down-regulated in HSCs and MPPs.

3.2 Treatment of K562, CML cell line

K562 cells were cultured and treated with Wnt3a, BIO and XAV for 8 h and 24 h. The samples were analysed via Western blot, quantitative PCR and immunofluorescence.

3.2.1 Results Western blot

Western blotting was performed to analyse the cytoplasm protein level of bcat, TCF7, LEF1 and TLE family in K562 cells with and without 8 h and a 24 h treatment with Wnt3a, BIO, and XAV.

Wnt3a and BIO activate the Wnt-signalling pathway. In contrast XAV stabilises Axin which is a main component and a limited factor in the maintenance of the destruction complex.

Treatment with Wnt3α

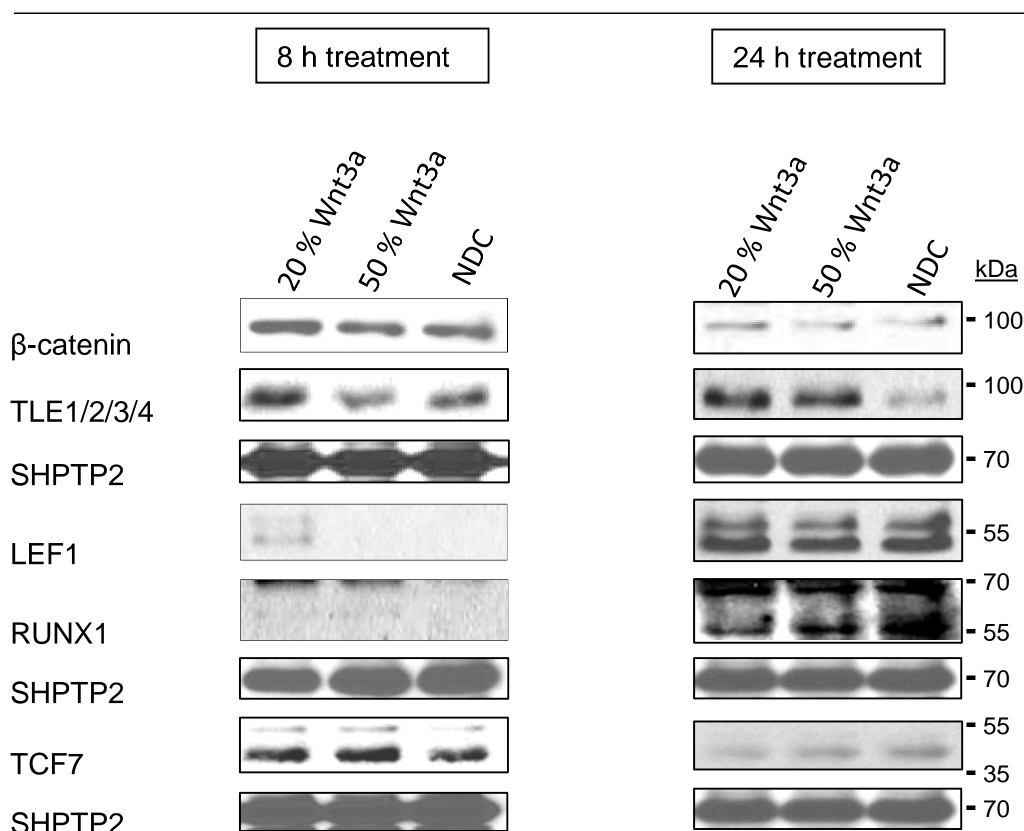


Figure 7: Western blotting results of K562 treated cells with Wnt3a after an incubation of 8 h and 24 h.

Figure 7: Western blot results of K562 whole cell lysates treated with and without Wnt3a. On the left side are the results of the 8 h treatment and on the right side from the 24 h treatment. SHPTP2 was used as a control protein.

After the 8 h treatment with 20 % and 50 % Wnt3α β-catenin, TLE1/2/3/4, LEF1 and TCF7 showed little difference in the protein level in comparison to the no drug control (NDC). The bcat level in the cytoplasm was slightly increased after the Wnt3a treatment. Therefore, a

significant change in the protein levels of the other proteins was not assumed. But the expression of TLE1/2/3/4 decreased after an 8 h treatment with 50 % Wnt3a and faintly increased after the 8 h treatment with 20% Wnt3a and much stronger after a 24 h treatment. Also LEF1 and TCF7 showed an increase after an 8 h incubation. After 24 h, the TCF7 level decreased with 20 % Wnt3a and RUNX1 expression decreased with 20% and 50% Wnt3a treatment.

Treatment with BIO

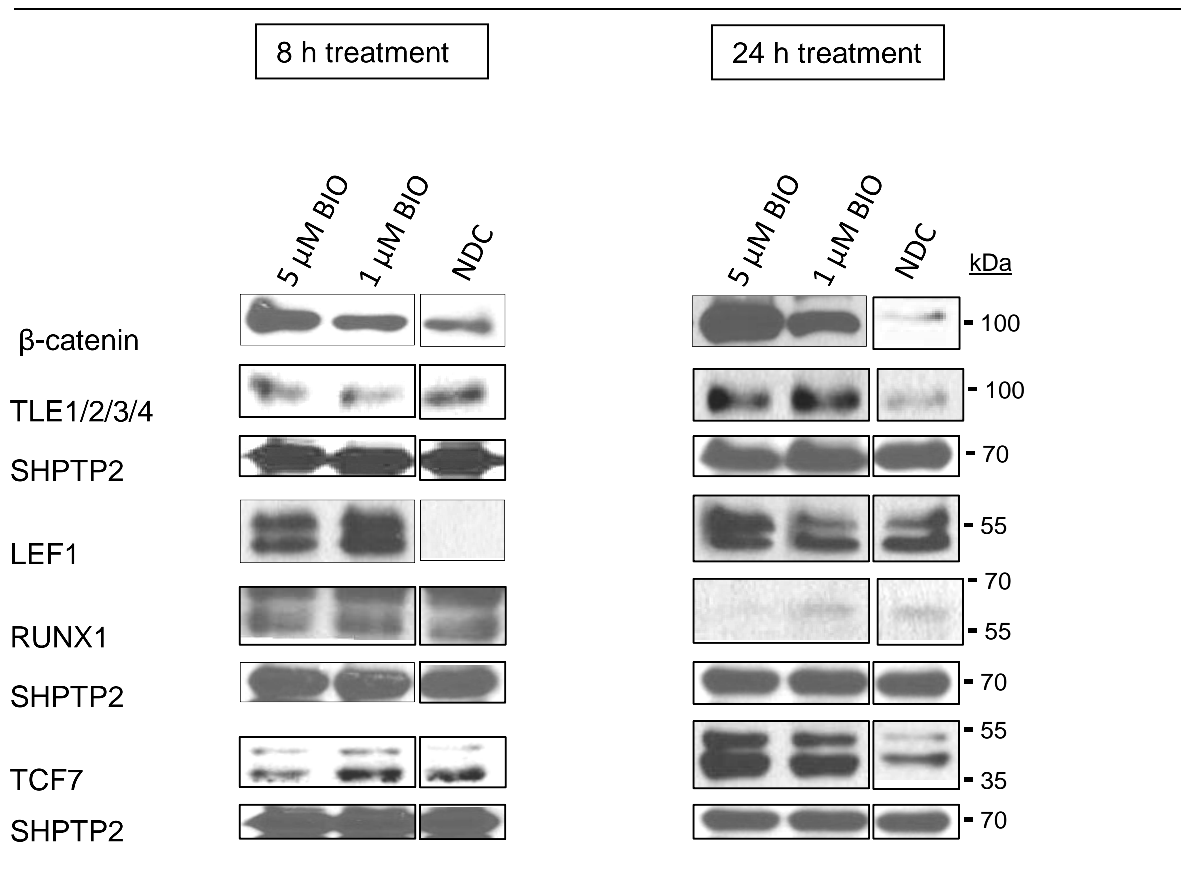


Figure 8: Western blotting results of K562 treated cells with BIO after an incubation of 8 h and 24 h.

Figure 8: Western blot results of K562 whole cell lysates treated with and without BIO. On the left side are the results of the 8 h treatment and on the right side from the 24 h treatment. SHPTP2 was used as a control protein.

The bcat protein level increased strongly after a treatment with 5 µM BIO after 8 h and 24 h and slightly with 1 µM BIO. This result shows that BIO has a higher impact on K562 cells. TLE1/2/3/4 slightly decreased with both, 1 µM and 5 µM BIO, after 8 h and increased after 24 h treatment. The protein level of LEF1 strongly increased after 8 h and 1 µM BIO as well as with 5 µM BIO. After 24 h, there was only an increase with 5 µM BIO. Furthermore, LEF1 showed two bands and with 5 µM BIO the upper band was much stronger than the upper bands with 1 µM BIO and the NDC after 24 h. It was even stronger than the lower band. With

1 µM BIO and the NDC the lower band was bigger after 24 h. TCF7 showed two bands as well. The upper band was less strong when compared to the lower band in all blots. After 8 h TCF7 level showed little change but after 24 h the level strongly increased especially with 5 µM BIO. The RUNX1 level did not change after an 8 h treatment.

Treatment with XAV

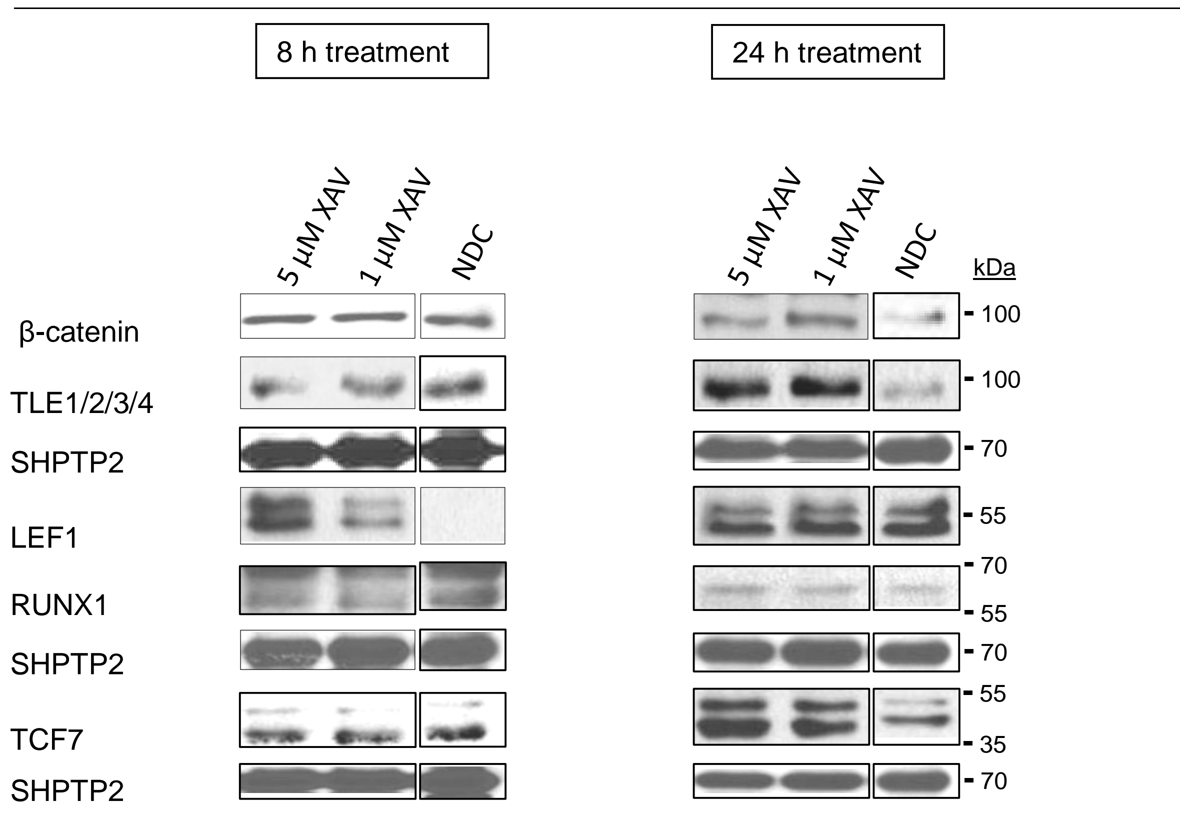


Figure 9: Western blotting results of K562 treated cells with XAV after an incubation of 8 h and 24 h.

Figure 9: Western blot results of K562 whole cell lysates treated with and without XAV. On the left side are the results of the 8 h treatment and on the right side from the 24 h treatment. SHPTP2 was used as a control protein.

After 8 h, the protein level of bcat did not decline, it even seemed to increase after 24 h. This leads to the assumption, that XAV cannot decrease the bcat protein level. But the treatment with XAV showed a decrease after 8 h and an increase after 24 h of TLE1/2/3/4. LEF1 rose after 8 h and dropped after 24 h. RUNX1 did not show any change in protein expression. The TCF7 protein level intensely increased after a 24 h treatment with 1 µM XAV and even more with 5 µM XAV.

3.2.2 Quantitative PCR

Quantitative PCR was carried out to analyse the fold changes of expression levels of K562 treated cells.

K562 were treated with 20 % and 50 % Wnt3a conditioned media. The treatment with BIO and XAV was conducted with 1 µM and 5 µM.

Following treatment, RNA was extracted from treated cells and cDNA was prepared (see 2.4.1 Preparation of RNA and cDNA, page 21). Subsequently, the cDNA was amplified (see 2.4.5 Pre-Amplification (pre-amp) and Exonuclease I digest, page 23), and tested (see 2.4.6 Polymerase chain reaction (PCR), page 24). Afterwards quantitative PCR was conducted (see 2.4.7 Quantitative PCR (qPCR), page 24). All treatments were done in duplicate for 8 h and 24 h.

For illustration purposes only the results of the 20 % Wnt3a treatment and 5 µM BIO and XAV treatment were depicted in Figure 10, Figure 11 and Figure 12.

Treatment with Wnt3a

The diagrams below show the results from K562 treated cells with 20 % Wnt3a against the no drug control.

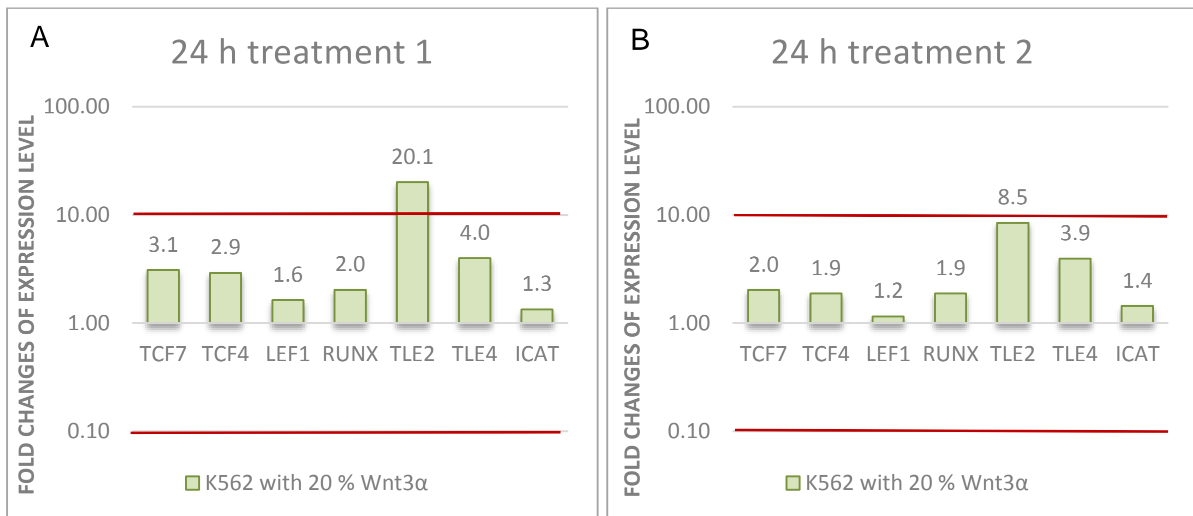


Figure 10: Fold changes of expression levels of K562 treated cells with 20 % Wnt3a after 24 h.

Figure 10: Displayed are the expression patterns of genes activated by canonical Wnt signaling in K562 treated with Wnt3α compared with untreated K562. A: Results of the first 24 h treatment. B: Results of the second 24 h treatment.

Following 8 h Wnt3a treatment the levels of the genes showed no significant change compared to NDC (data not shown). However by 24 h treatment there was an up-regulation of *TCF7*, *TCF4*, *RUNX1* and *TLE 2* & *4* indicating strong activation of the downstream targets of Wnt signalling.

Treatment with BIO

The diagrams below show the results from K562 treated cells with 5 µM BIO against the no drug control.

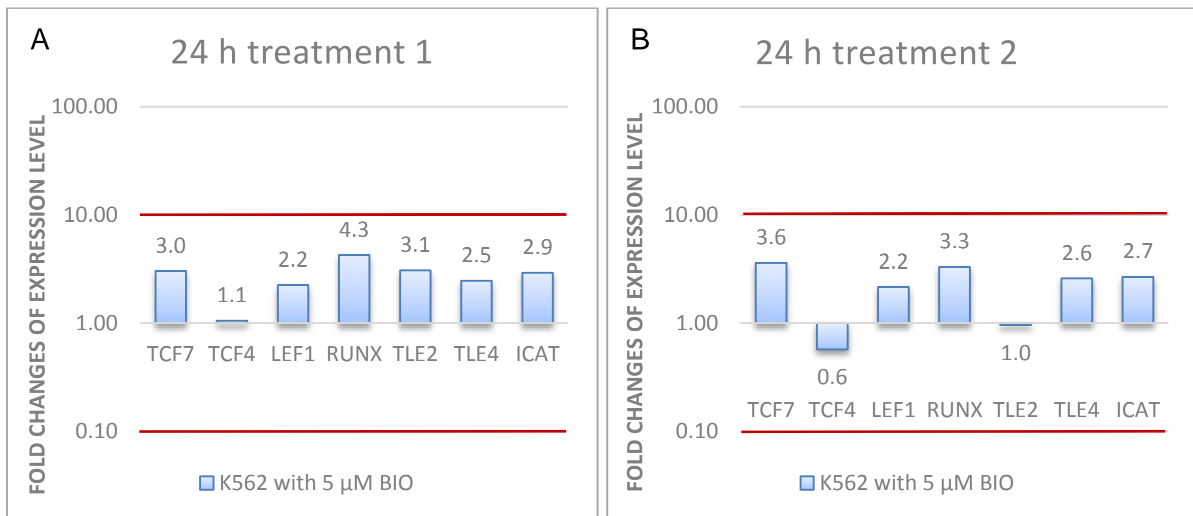


Figure 11: Fold changes of expression levels of K562 treated cells with 5 µM BIO after 24 h.

Figure 11: Displayed are the expression patterns of genes activated by canonical Wnt signalling in K562 treated with BIO compared with untreated K562. A: Results of the first 24 h treatment. B: Results of the second 24 h treatment.

After 8 h treatment with BIO there was no significant change in gene expression (data not shown) however 24 h BIO treatment resulted in up-regulation of *TCF7*, *LEF1*, *RUNX1*, *TLE4* and *ICAT* indicative of strong activation of the Wnt signalling pathway.

Treatment with XAV

The diagrams below show the results from K562 treated cells with 5 µM XAV against the no drug control.

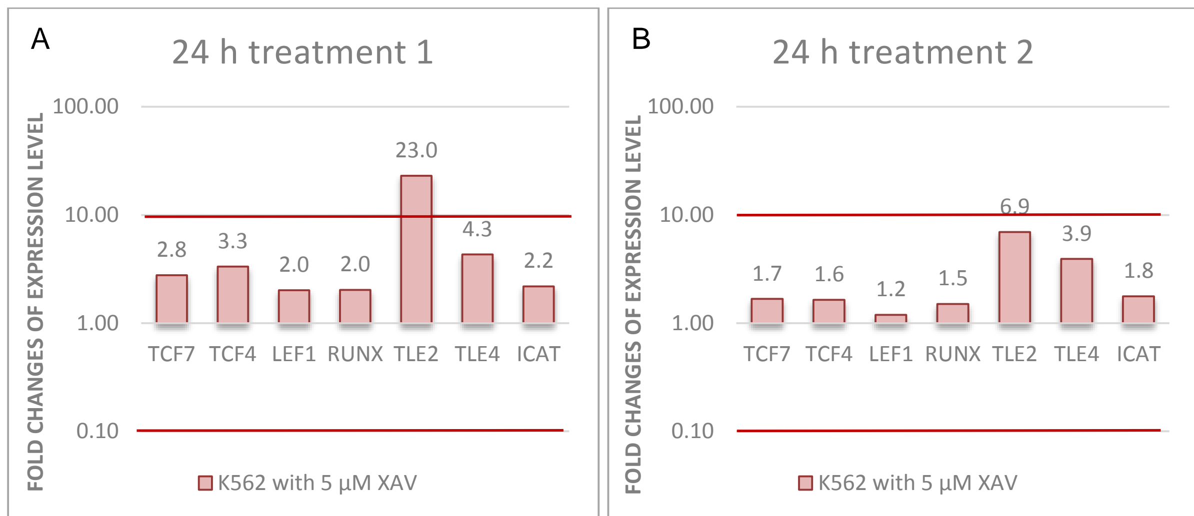


Figure 12: Fold changes of expression levels of K562 treated cells with 5 µM XAV after 24 h.

Figure 12: Displayed are the expression patterns of genes activated by canonical Wnt signalling in K562 treated with XAV compared with untreated K562. A: Results of the first 24 h treatment. B: Results of the second 24 h treatment.

Treatment with XAV lead to no significant change in gene expression after 8 h (data not shown), however by 24 h treatment there was a strong induction of TLE2 expression, and up-regulation of TLE4 and ICAT expression. Surprisingly modulation of the pathway both positively and negatively resulted in similar alterations in gene expression highlighting the complex interplay between these key transcription factors.

3.2.3 Immunofluorescence (IF)

Immunofluorescence was conducted to analyse the protein expression of K562 treated cells with Wnt3α, BIO and XAV.

The first row displays the staining of the protein of interest, the second row shows the DAPI staining, the third row contains the overlay of the protein and DAPI staining and the last row displays the control with protein and DAPI staining. The control was performed in the same way but without adding the primary antibody.

β-catenin

The figures below show the change in the protein expression of β-catenin in K562 treated cells.

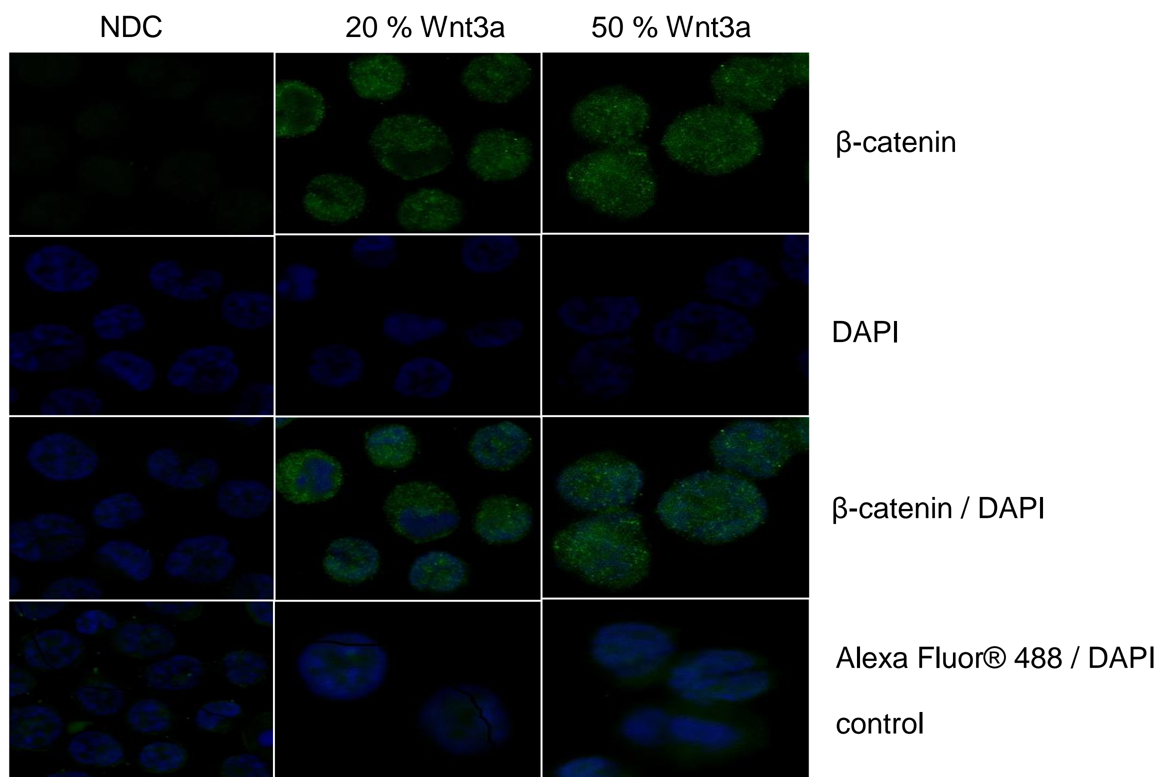


Figure 13: IF results of bcat protein level after 24 h treatment with Wnt3a, 400x enlargement

Figure 13: Depicted are the bcat/Alexa Fluor® 488 (first row) and DAPI stained (second row) K562 cells after a 24 h treatment with 20 % and 50 % Wnt3a and without any drug (NDC). The overlay of bcat/Alexa Fluor® 488 and DAPI staining is shown in row 3. The last row displays the control staining.

Total bcat staining showed undetectable expression in K562 untreated cells, this could potentially be the sensitivity level of the anti- β-catenin antibody. However 24 h stimulation with Wnt3a led to a strong expression of this protein.

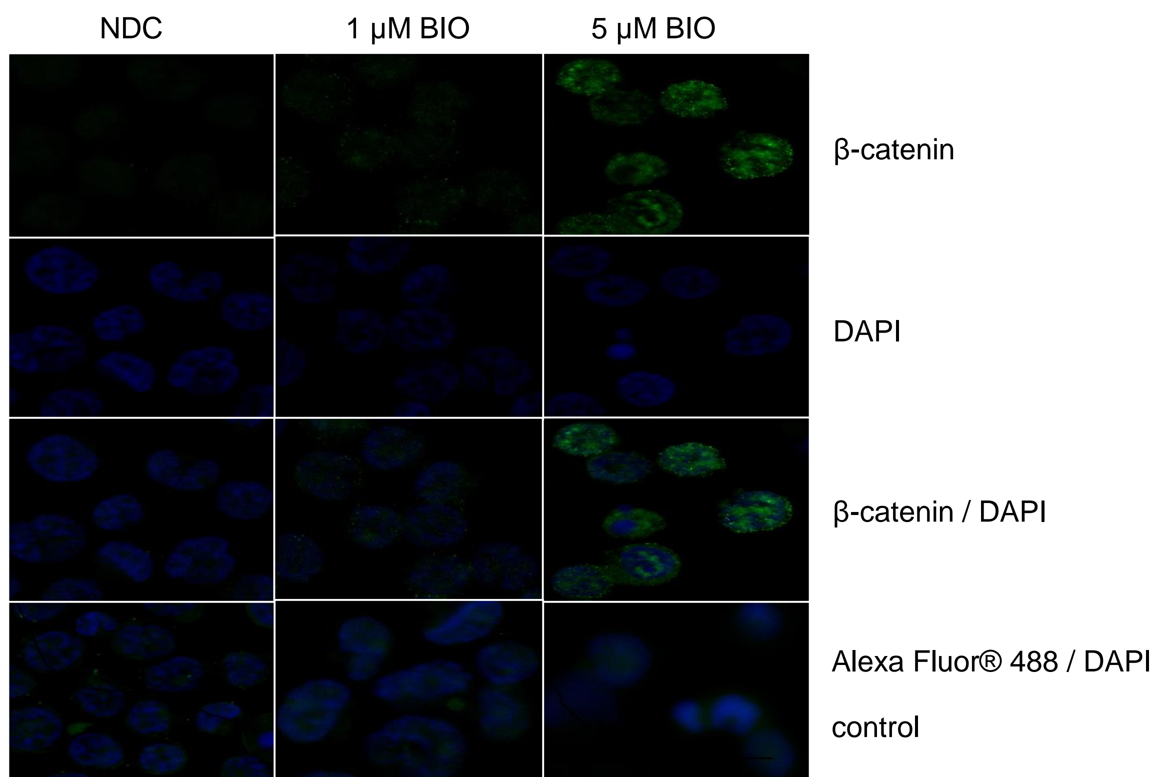


Figure 14: IF results of bcat protein level after 24 h treatment with BIO, 400x enlargement

Figure 14: Depicted are the bcat/Alexa Fluor® 488 (first row) and DAPI stained (second row) K562 cells after a 24 h treatment with 1 µM and 5 µM BIO and without any drug (NDC). The overlay bcat/Alexa Fluor® 488 and DAPI staining is shown in row 3. The last row displays the control

Treatment with BIO, also resulted in up regulation of β-catenin expression especially with 5 µM BIO, after 24 h.

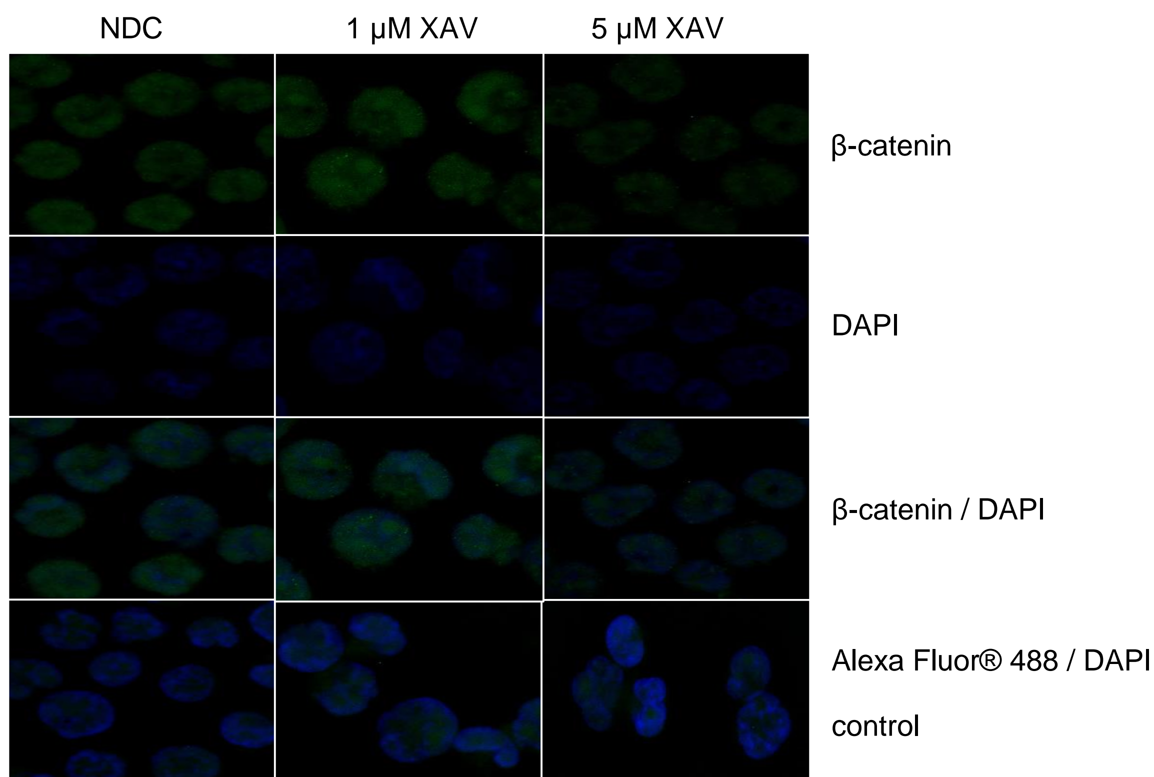


Figure 15: IF results of bcat protein level after 8 h treatment with XAV, 400x enlargement

Figure 15: Depicted are the bcat/Alexa Fluor® 488 (first row) and DAPI stained (second row) K562 cells after a 8 h treatment with 1 µM and 5 µM XAV and without any drug (NDC). The overlay of bcat/Alexa Fluor® 488 and DAPI staining is shown in row 3. The last row displays the control staining.

XAV led to no change in overall β-catenin levels.

Active β-catenin

The images below show the change in the protein expression of active bcat in K562 treated cells.

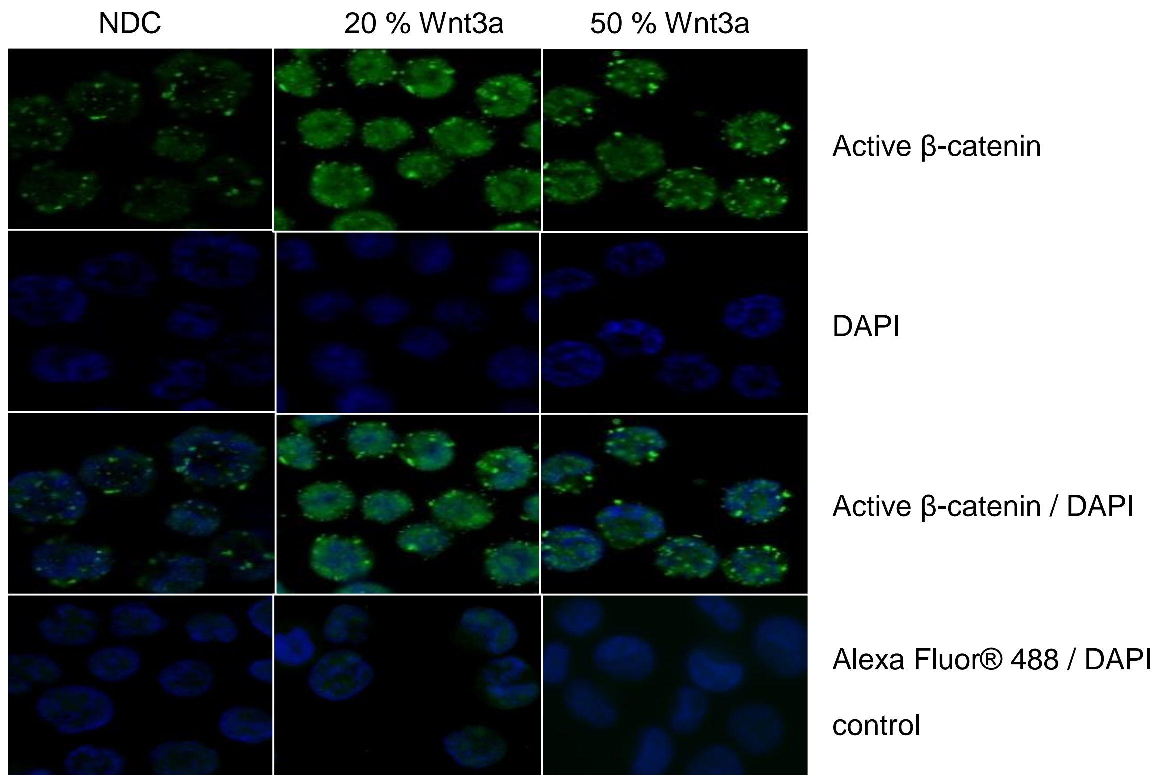


Figure 16: IF results of active bcat protein level after 8 h treatment with Wnt3a, 400x enlargement

Figure 16: Depicted are the active bcat/Alexa Fluor® 488 (first row) and DAPI stained (second row) K562 cells after an 8 h treatment with 20 % and 50 % Wnt3α and without any drug (NDC). The overlay of active bcat/Alexa Fluor® 488 and DAPI staining is shown in row 3. The last row displays the control staining.

Active bcat is marginally expressed in untreated K562 cells. Wnt3a stimulation increased the expression of active bcat after 8 h, indicating good activation of the canonical Wnt signalling pathway.

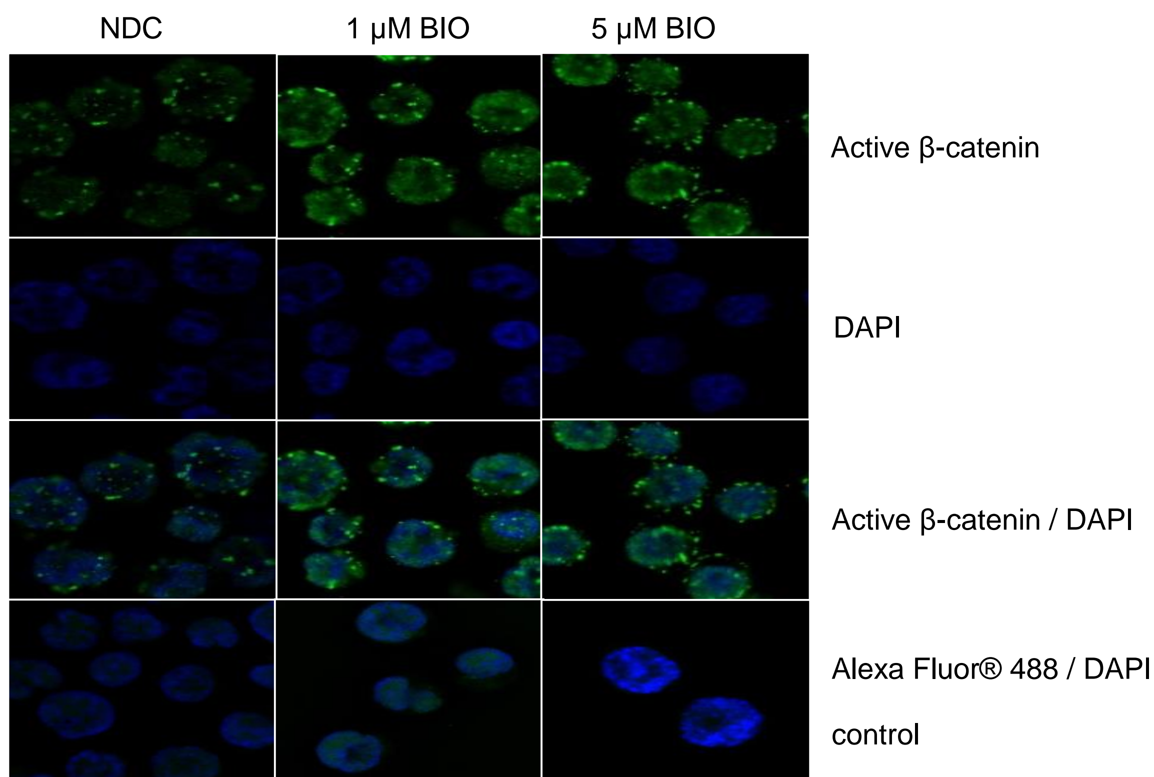


Figure 17: IF results of active bcat protein level after 8 h treatment with BIO, 400x enlargement

Figure 17: Depicted are the active bcat/Alexa Fluor® 488 (first row) and DAPI stained (second row) K562 cells after an 8 h treatment with 1 µM and 5 µM BIO and without any drug (NDC). The overlay of active bcat/Alexa Fluor® 488 and DAPI staining is shown in row 3. The last row displays the control staining.

Treatment with GSK3β inhibitor BIO caused a rise in the expression of active bcat in K562, indicating good activation of the canonical Wnt signalling pathway.

TCF7

The images below show the change in the protein expression of TCF7 in K562 treated cells.

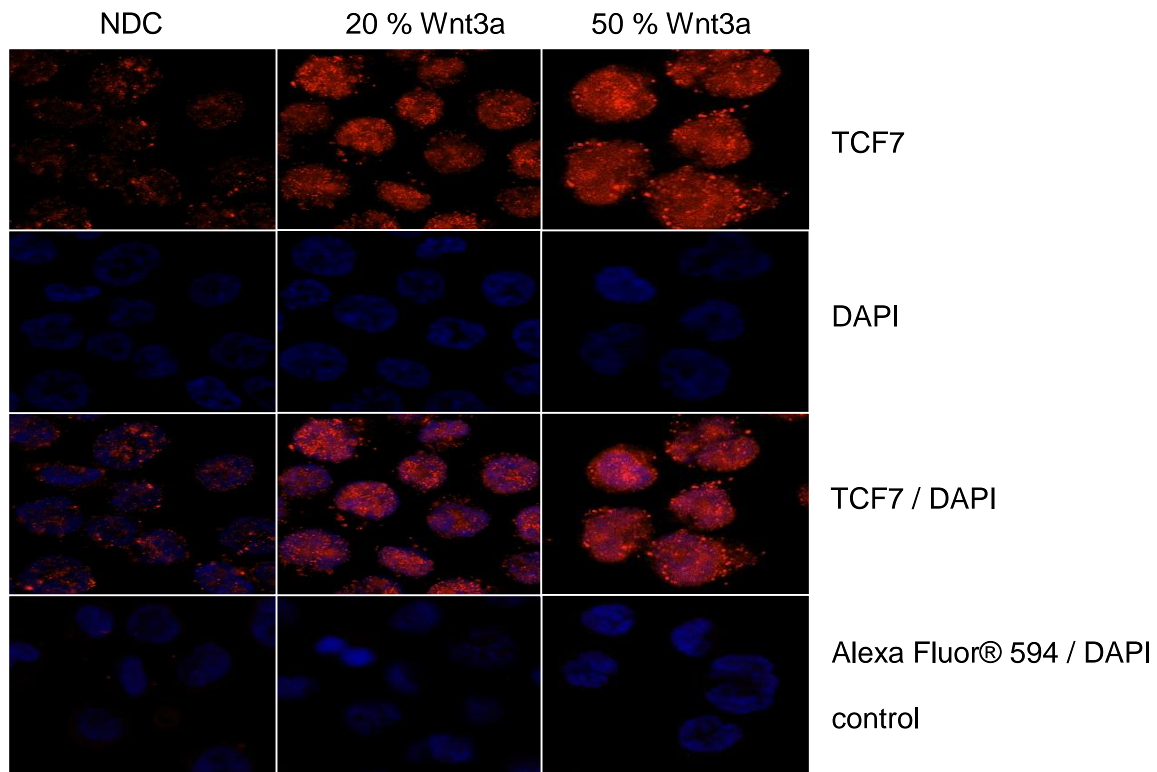


Figure 18: IF results of TCF7 protein level after 24 h treatment with Wnt3α, 400x enlargement

Figure 18: Depicted are the TCF7/Alexa Fluor® 594 (first row) and DAPI stained (second row) K562 cells after a 24 h treatment with 20 % and 50 % Wnt3α and without any drug (NDC). The overlay of TCF7 and DAPI staining is shown in row 3. The last row displays the control staining.

These images indicate a significant increase in the protein level of TCF7 after a 24 h treatment with Wnt3a. The untreated cell line expressed TCF7 in a small amount. Whereas stimulation with WNT3a led to a more intense staining of TCF7 in the cytoplasm and in the nucleus, indicating higher expression of the protein.

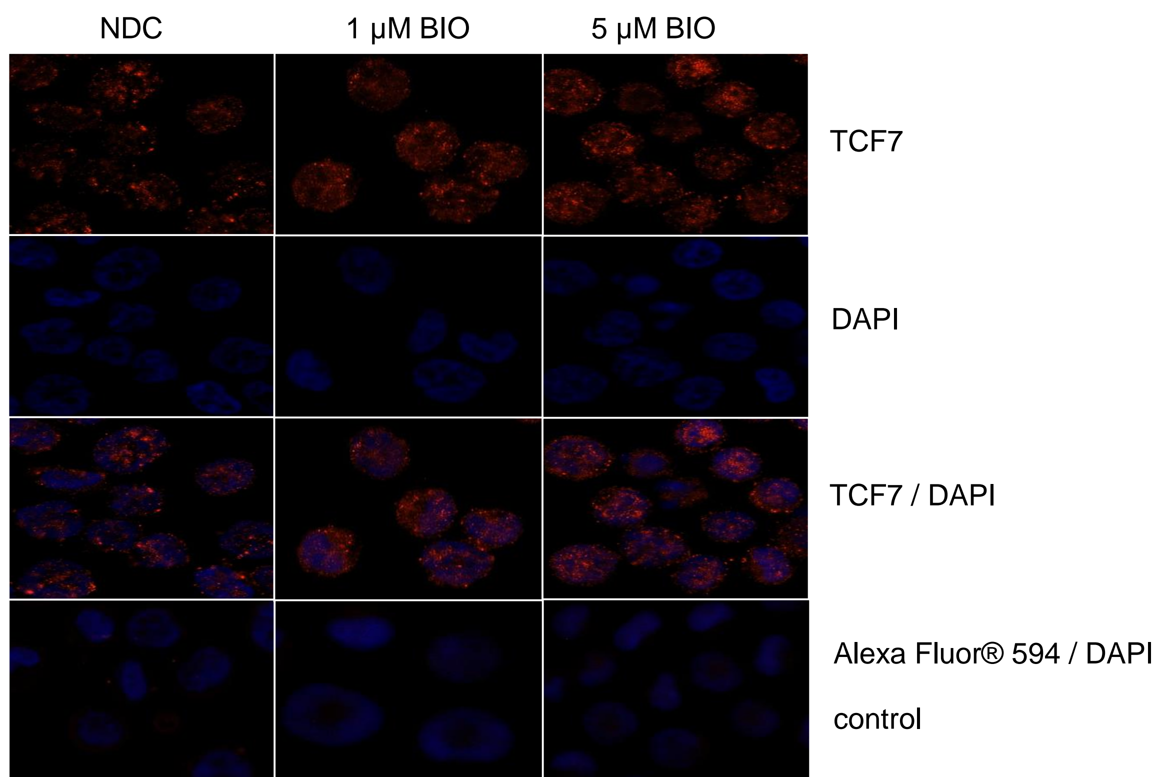


Figure 19: IF results of TCF7 protein level after 24 h treatment with BIO, 400x enlargement

Figure 19: Depicted are the TCF7/Alexa Fluor® 594 (first row) and DAPI stained (second row) K562 cells after a 24 h treatment with 1 µM and 5 µM BIO and without any drug (NDC). The overlay of TCF7/Alexa Fluor® and DAPI staining is shown in row 3. The last row displays the control staining.

The 24 h treatment with BIO led to a modest increase in the protein level of TCF7.

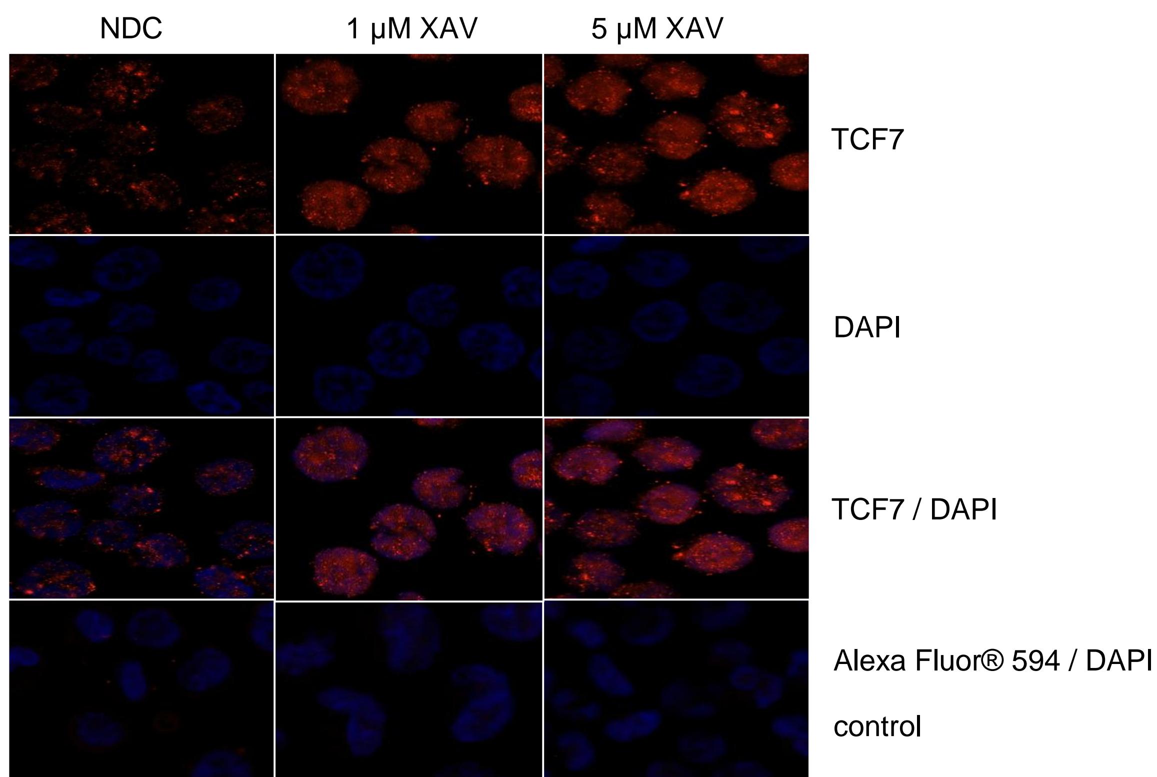


Figure 20: IF results of TCF7 protein level after 24 h treatment with XAV, 400x enlargement

Figure 20: Depicted are the TCF7/Alexa Fluor® 594 (first row) and DAPI stained (second row) K562 cells after a 24 h treatment with 1 µM and 5 µM XAV and without any drug (NDC). The overlay of TCF7/Alexa Fluor® and DAPI staining is shown in row 3. The last row displays the control staining.

Surprisingly, XAV treatment also caused a rise in the protein level of TCF7 similar to the increased observed with Wnt3a stimulation.

LEF1

The figures below show the change in the protein expression of LEF1 in K562 treated cells.

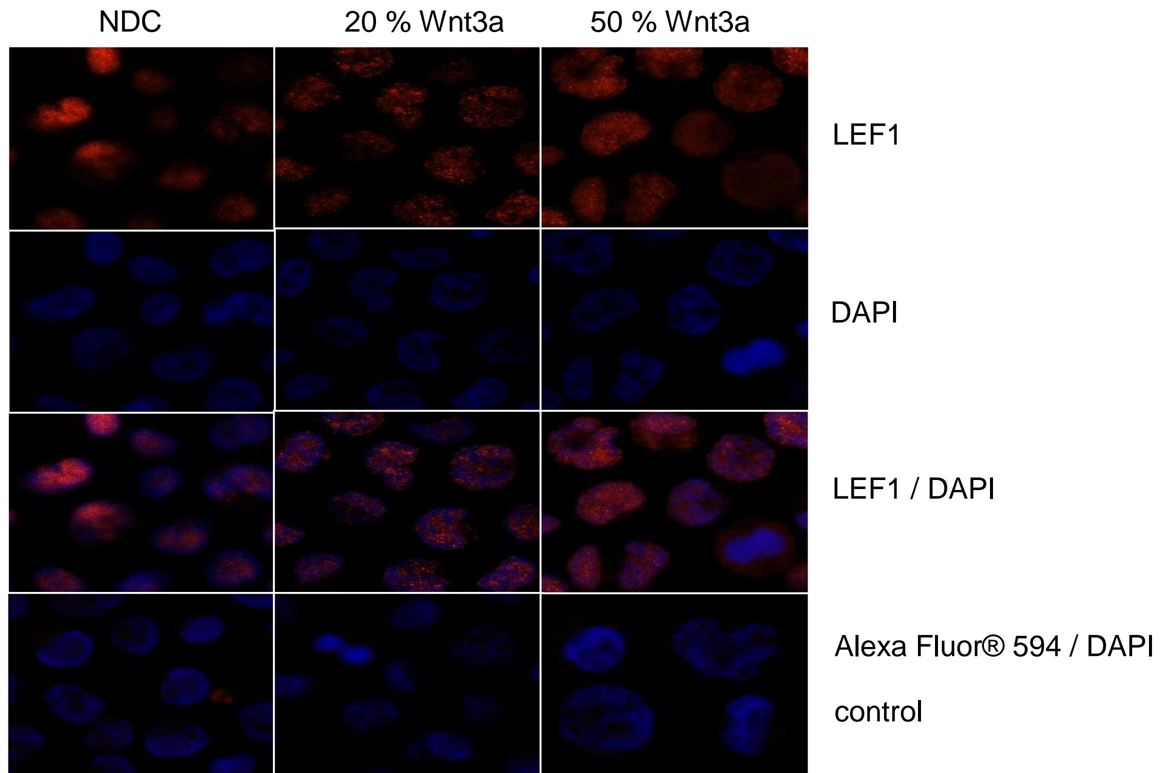


Figure 21: IF results of LEF1 protein level after 24 h treatment with Wnt3a, 400x enlargement

Figure 21: Depicted are the LEF1/Alexa Fluor® 594 (first row) and DAPI stained (second row) K562 cells after a 24 h treatment with 20 % and 50 % Wnt3a and without any drug (NDC). The overlay of LEF1/Alexa Fluor® and DAPI staining is shown in row 3. The last row displays the control staining.

The LEF1 protein level was already high in untreated K562 cells. Nevertheless, Wnt3a stimulation led to a small increase in the protein level of LEF1.

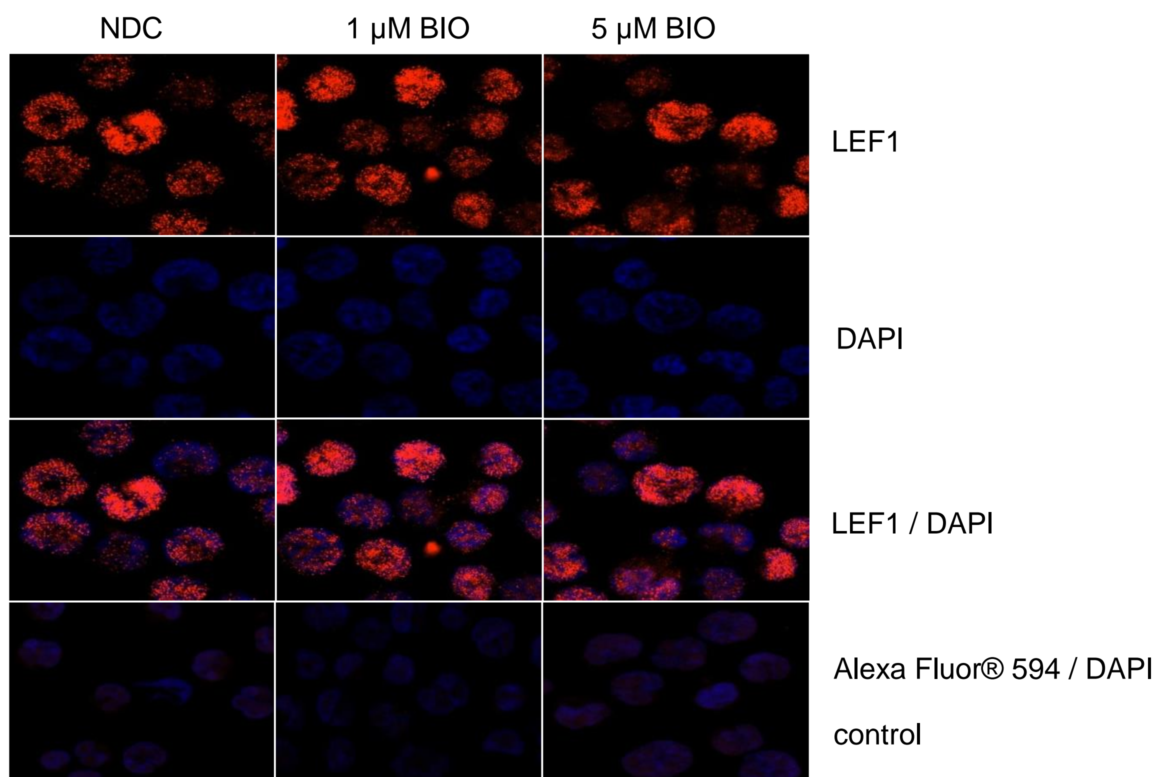


Figure 22: IF results of LEF1 protein level after 8 h treatment with BIO, 400x enlargement

Figure 22: Depicted are the LEF1/Alexa Fluor® 594 (first row) and DAPI stained (second row) K562 cells after an 8 h treatment with 1 µM and 5 µM BIO and without any drug (NDC). The overlay of LEF1/Alexa Fluor® and DAPI staining is shown in row 3. The last row displays the control staining.

An 8 h treatment with BIO (1 µM and 5 µM) caused no detectable change in the amount of LEF1 in K562, however the cells already express high levels of the protein making any subtle changes difficult to detect.

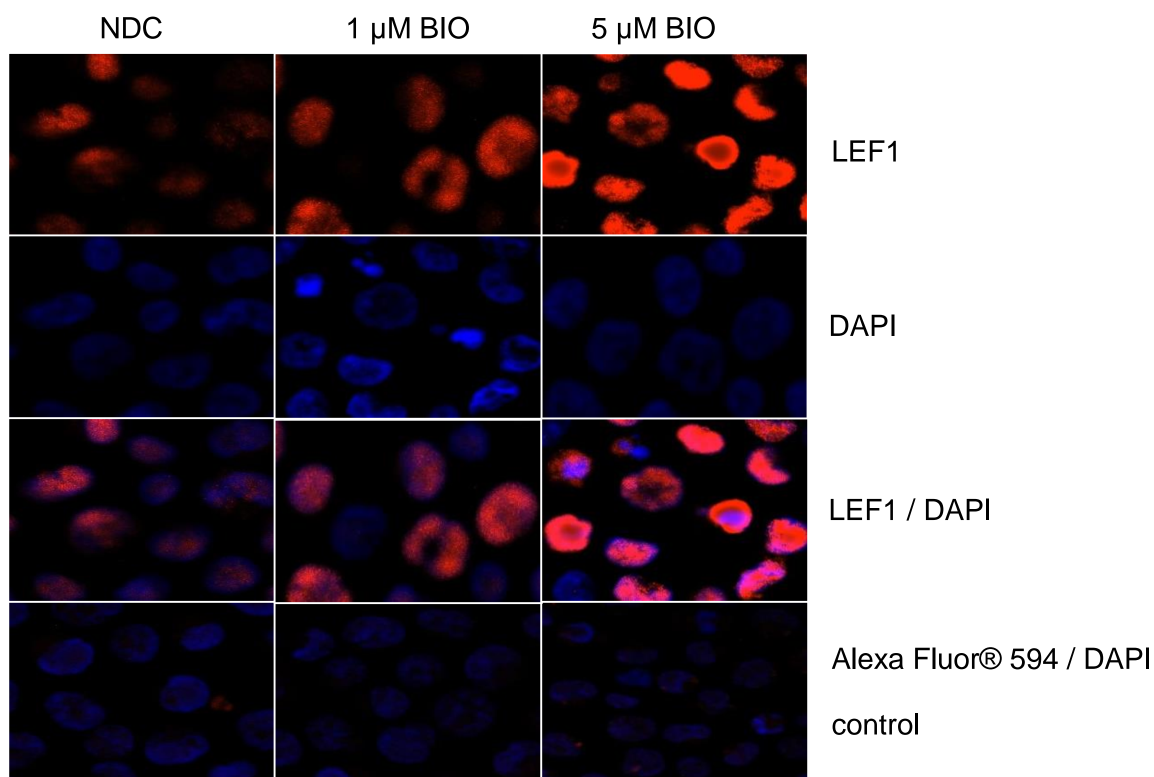


Figure 23: IF results of LEF1 protein level after 24 h treatment with BIO, 400x enlargement

Figure 23: Depicted are the LEF1/Alexa Fluor® 594 (first row) and DAPI stained (second row) K562 cells after a 24 h treatment with 1 µM and 5 µM BIO and without any drug (NDC). The overlay of LEF1/Alexa Fluor® and DAPI staining are shown in row 3. The last row display the control staining.

However, a 24 h treatment with BIO resulted in an increase in the protein level of LEF1.

AML1

The images below show the change in the protein expression of AML1 in K562 treated cells.

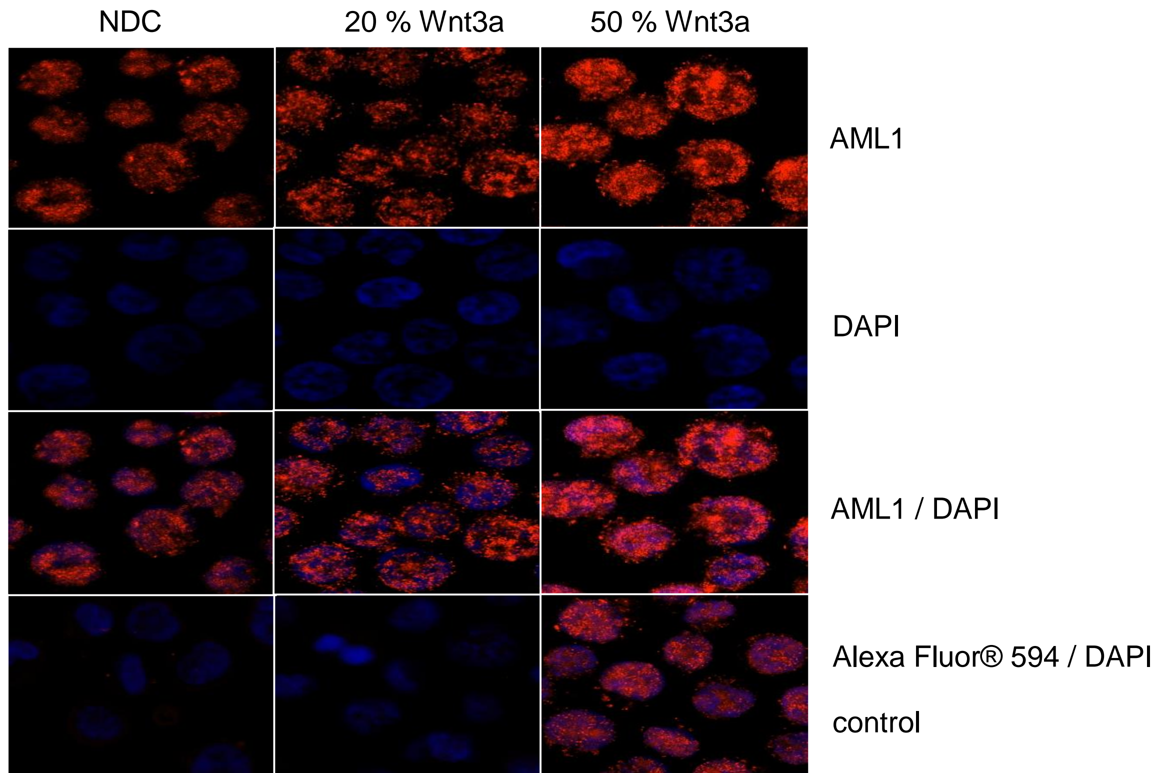


Figure 24: IF results of AML1 protein level after 24 h treatment with Wnt3a, 400x enlargement

> Figure 24: Depicted are the AML1/Alexa Fluor® 594 (first row) and DAPI stained (second row) K562 cells after a 24 h treatment with 20 % and 50 % Wnt3a and without any drug (NDC). The overlay of AML1/Alexa Fluor® and DAPI staining is shown in row 3. The last row displays the control staining.

AML1 is reported to be a downstream target of the *TCF* family of transcription factors. However, AML1 was already strongly expressed in K562 cells making it difficult to determine whether Wnt3a stimulation led to a further rise in expression.

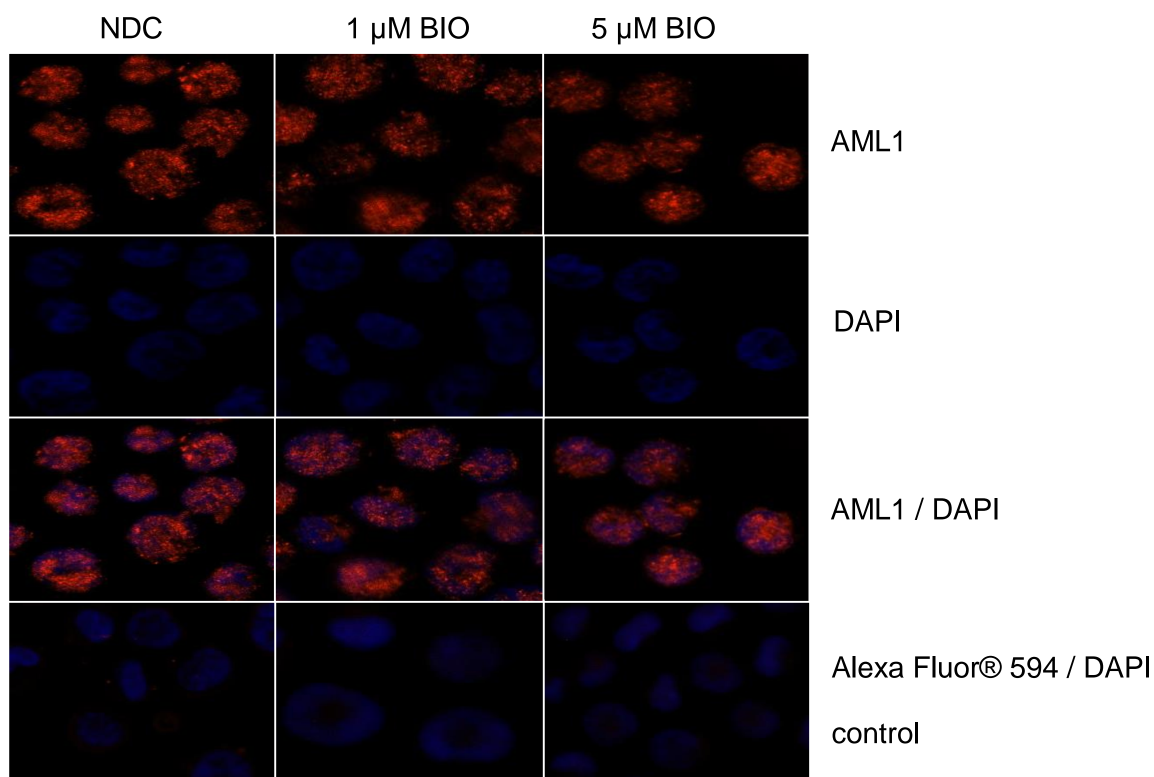


Figure 25: IF results of AML1 protein level after 24 h treatment with BIO, 400x enlargement

Figure 25: Depicted are the AML1/Alexa Fluor® 594 (first row) and DAPI stained (second row) K562 cells after a 24 h treatment with 1 µM and 5 µM BIO and without any drug (NDC). The overlay of AML1/Alexa Fluor® and DAPI staining is shown in row 3. The last row displays the control staining.

The treatment with BIO led to no change in the expression of AML1 in K562.

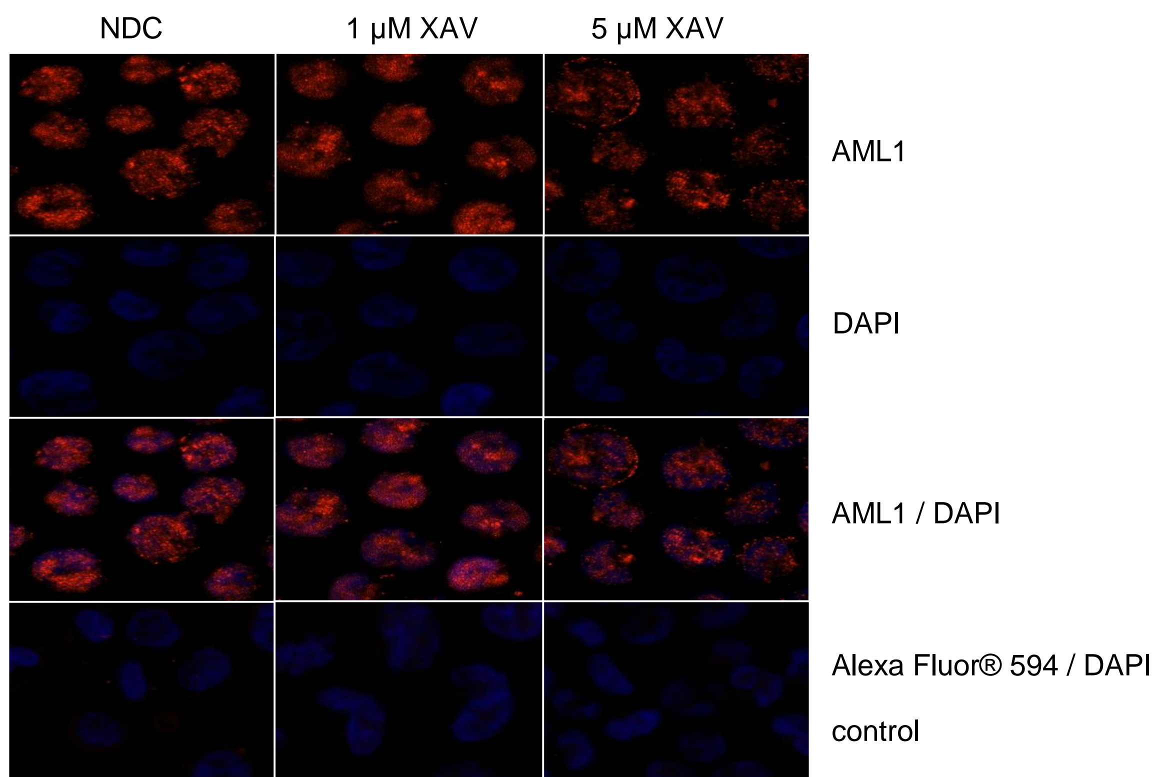


Figure 26: IF results of AML1 protein level after 24 h treatment with XAV, 400x enlargement

Figure 26: Depicted are the AML1/Alexa Fluor® 594 (first row) and DAPI stained (second row) K562 cells after a 24 h treatment with 1 µM and 5 µM XAV and without any drug (NDC). The overlay of AML1/Alexa Fluor® and DAPI staining is shown in row 3. The last row displays the control staining.

XAV caused a modest decrease in the expression of AML1 after 24 h.

TLE1/2/3/4

The figures below show the change in the protein expression of TLE1/2/3/4 in K562 treated cells.

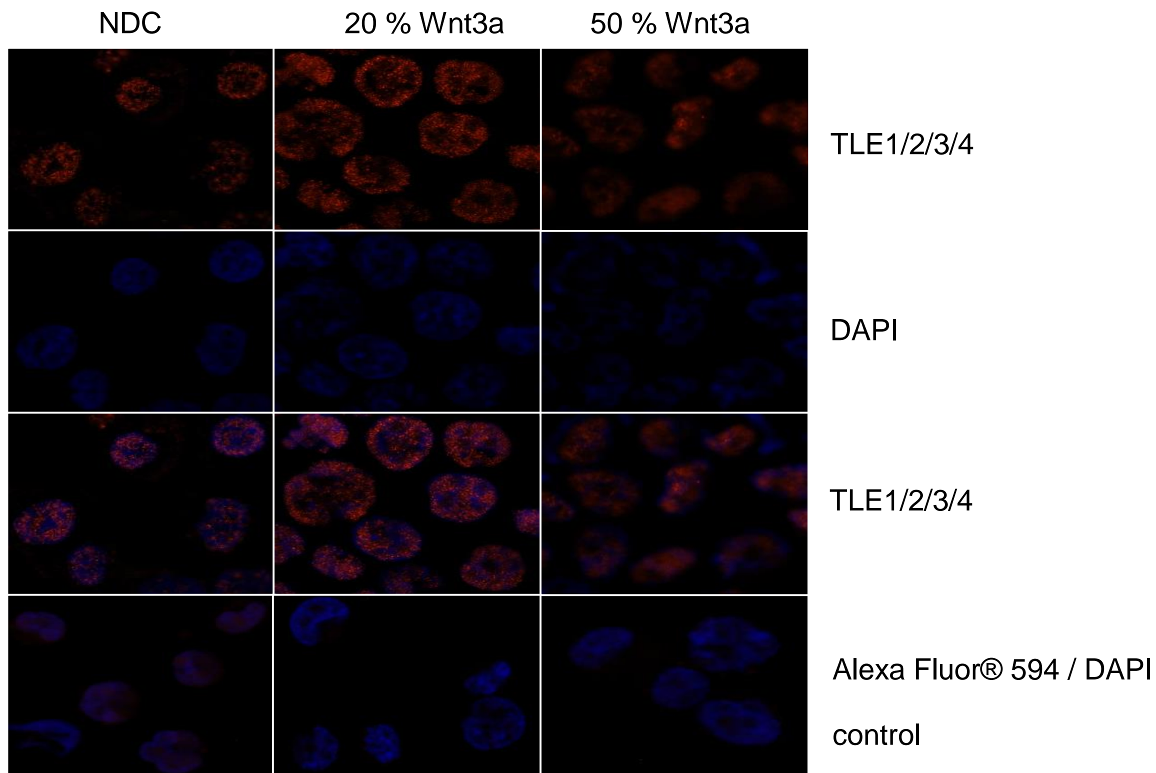


Figure 27: IF results of TLE1/2/3/4 protein level after 8 h treatment with Wnt3a, 400x enlargement

Figure 27: Depicted are the TLE1/2/3/4/Alexa Fluor® 594 (first row) and DAPI stained (second row) K562 cells after an 8 h treatment with 20 % and 50 % Wnt3a and without any drug (NDC). The overlay of TLE1/2/3/4/Alexa Fluor® and DAPI staining is shown in row 3. The last row displays the control staining.

TLE1/2/3/4 was marginally expressed in untreated K562. Wnt3a caused a rise in the protein level of TLE1/2/3/4, the negative feedback loop involved in switching off Wnt signalling.

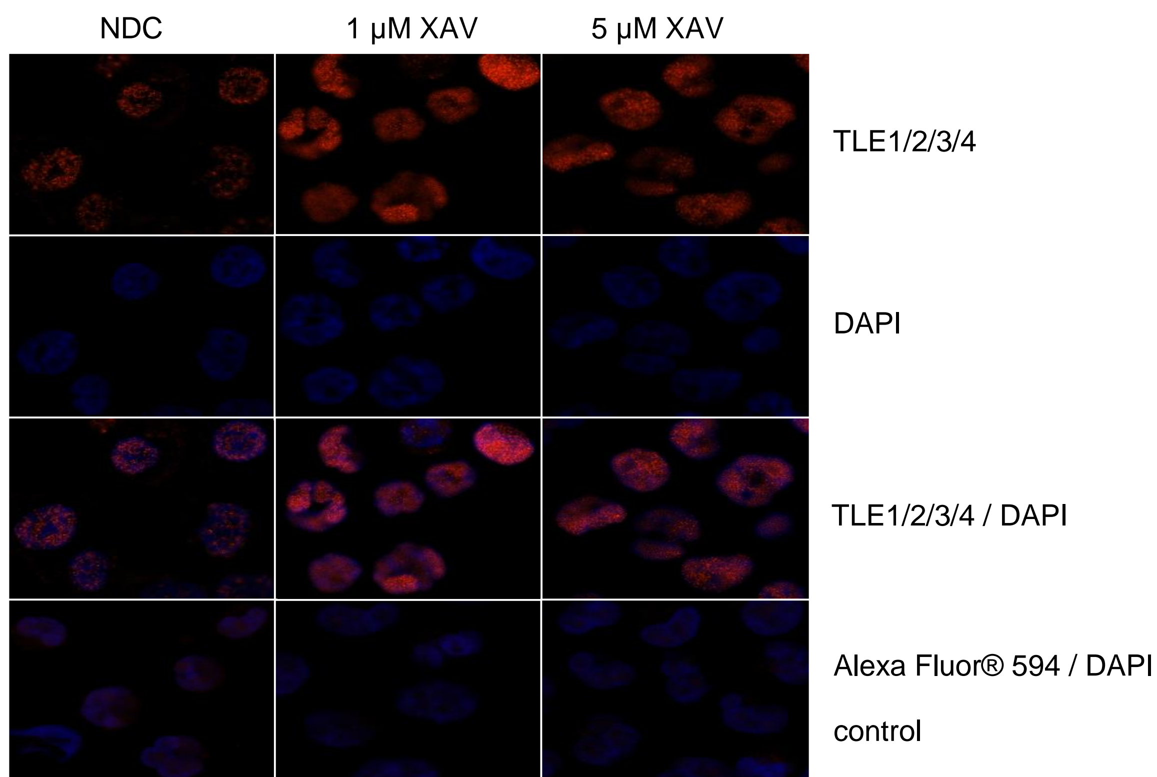


Figure 28: IF results of TLE1/2/3/4 protein level after 8 h treatment with XAV, 400x enlargement

Figure 28: Depicted are the TLE1/2/3/4/Alexa Fluor® 594 (first row) and DAPI stained (second row) K562 cells after an 8 h treatment with 1 µM and 5 µM XAV and without any drug (NDC). The overlay of TLE1/2/3/4/Alexa Fluor® and DAPI staining is shown in row 3. The last row displays the control staining.

The 8 h treatment with XAV led to an increase in the protein level of TLE1/2/3/4.

3.3 Treatment of mESC

Murine ESC were cultured with and without tetracycline and treated with BIO for 48 h. The samples were analysed via Western blotting and quantitative PCR.

3.3.1 Western blot results

Western blotting was performed to analyse the protein expression of LEF1 and TCF7 in mESC with and without the oncogene TP and constitutively active β-catenin.

The addition of tetracycline inhibited the expression of the oncogene and the constitutively active bcat. BIO should activate the Wnt-signalling pathway.

PDGfb showed, if the oncogene is expressed.

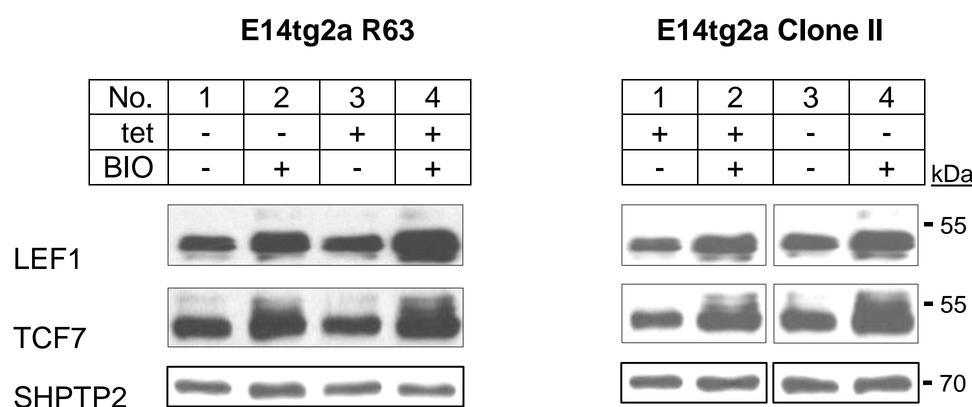


Figure 29: Western blotting results of E14tg2a treated cells with tet and BIO after an incubation of 48 h.

Figure 29: Western blot results of E14tg2a R63 and Clone II whole cell lysates. The treatment was carried out for 48 h and with or without the addition of tet and BIO. PDGfb showed the presence of the oncogene TP. SHPTP2 was used as a control protein. Tet 1 μg ml^{-1}, BIO 5 μM

The parental cell line R63 and Clone II did not contain the oncogene TP. Clone II contains constitutively active bcat (dp-bcat) which will be expressed, if tet was not added.

LEF1 and TCF7 were already expressed in this mESCs (lane 1). But an addition of BIO led to an increase in the expression of LEF1 and a strong increase in TCF7 levels (lane 2). If tet was not added to Clone II (lane 3), dp-bcat was expressed. This led to a slight rise in the expression of LEF1 and TCF7.

When tet and BIO were added to R63, an induction in the expression of LEF1 and TCF7 occurred. The effect of the dp-bcat with BIO in Clone II led to a further increase in TCF7 and LEF1 levels (compare lane 2 with lane 4 right hand blots).

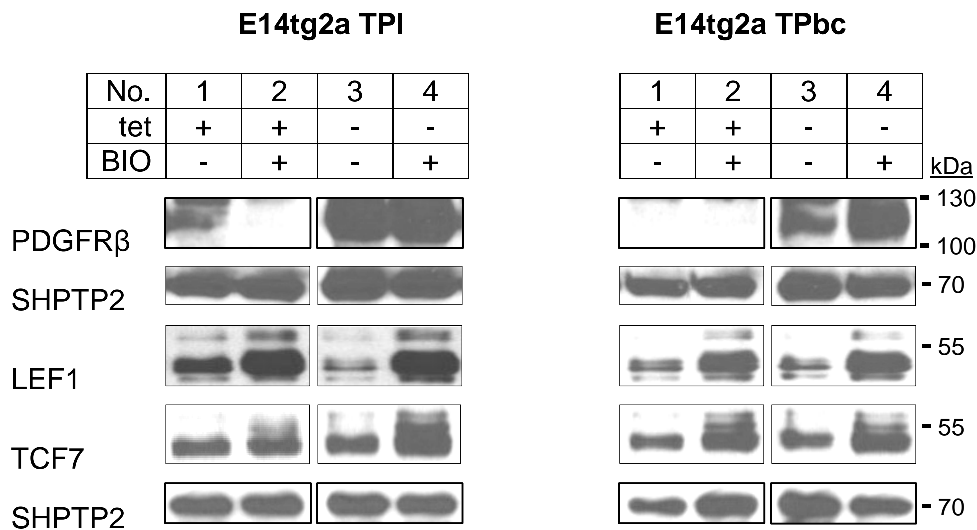


Figure 30: Western blotting results of E14tg2a treated cells with tet and BIO after an incubation of 48 h.

Figure 30: Western blot results of E14tg2a TPI and TPbc whole cell lysates. The treatment was carried out for 48 h and with or without the addition of tet and BIO. PDGfb shows the presence of the oncogene TP. SHPTP2 was used as a control protein. Tet 1 μg ml^{-1}, BIO 5 μM

TPI and TPbc both contained the oncogene TP, under the control of the tetracycline regulatory system, with TPbc also expressing dp-bcat when tet is removed from the culture. Following tet removal both TP1 and TPbc showed a PDGFRβ band by Western blotting which corresponded in size to the oncogene Tel/PDGFRβ. Therefore, the oncogene was expressed in these cell lines.

In both cell lines LEF1 and TCF7 were expressed without any treatment as well (lane 1), but TPI had a slightly higher protein level and TPbc a lower level of LEF1 and TCF7 compared to R63. The addition of BIO caused an increase in the expression of LEF1 in the TPI cell line and a small increase in TCF7. In BIO treated TPbc there was a rise in both protein levels (lane 2). The oncogene in TPI led to a decrease in LEF1 and seemed to have no influence on the expression of TCF7 (lane 3 left hand blots). The combination of the oncogene and BIO treatment caused a big increase in the expression of LEF1 and TCF7 in TPI cells (lane 4 left hand blots).

If tet was not added to the TPbc cell line, the oncogene and dp-bcat were expressed. This caused a small decrease in LEF1 and a small rise in TCF7 (lane 3 right hand blots). As well as in TPI, both the addition of BIO and the omission of tet, led to a strong increase of LEF1 and TCF7 (lane 4 right hand blots). These results indicate that inhibiting GSK3β causes strong activation of the Wnt pathway in embryonic stem cells, which can counteract the repression of the pathway observed by the oncogene Tel/PDGFRβ.

3.3.2 Real time PCR

Quantitative PCRs were carried out to analyse the fold changes of expression levels of mESC treated cells.

The cell lines Clone II, TPI and TPbc were cultured with and without tet whereas the parental cell line R63 was cultured without tet, for 48 h plus and minus 5 μM BIO treatment as indicated.

After the treatment, RNA was extracted from the cells and cDNA was prepared (see 2.4.1 Preparation of RNA and cDNA, page 21). Subsequently, the cDNA was amplified (see 2.4.5 Pre-Amplification (pre-amp) and Exonuclease I digest, page 23), and tested by PCR (see 2.4.6 Polymerase chain reaction (PCR), page 24). Afterwards a quantitative PCR was conducted (see 2.4.7 Quantitative PCR (qPCR), page 24) for the genes of interest.

The figures below show the comparison to the parental cell line R63. All cell lines, Clone II, TPI and TPbc, were expected to display the same gene expression as R63 in the presence of tet as this suppresses expression of TP and active β-catenin.

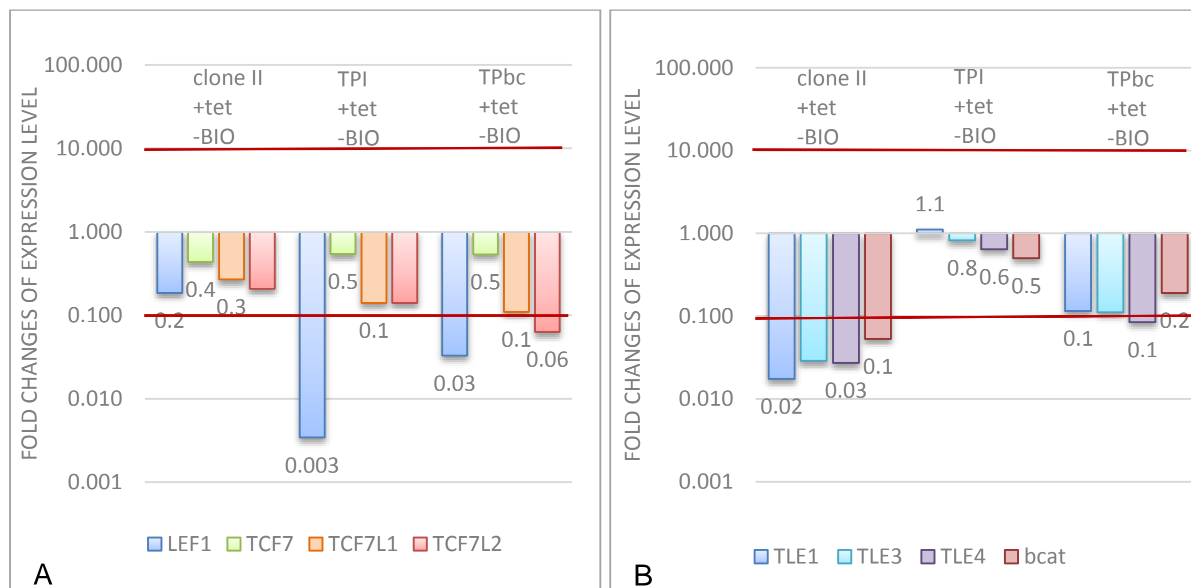


Figure 31: Fold changes of expression levels of genes of interest from Clone II, TPI and TPbc cell line against the parental cell line R63

Figure 31: Displayed are the expression patterns of genes activated by canonical Wnt signaling in E14tg2a Clone II, TPI and TPbc with the addition of tet compared to R63 (-tet). A: Change in the gene expression of the *TCF* family genes after a 48 h treatment. B: Change in the gene expression of the *TLE* family genes and *bcat* after a 48 h treatment. Tet 1 μg ml^{-1}

However, all cell lines gave a different expression of *LEF1*, *TCF7*, *TCF7L1 (TCF3)* and *TCF7L2 (TCF4)*. The expression of *TLE1*, *TLE 3*, *TLE 4* and *bcat* are similiar to R63 in the TPI cell line, but Clone II and TPbc gave a different expression, with all genes down-regulated. These findings indicate that either tet is affected the gene expression pattern or that the system is slightly leaky leading to low level expression of TP and active β-catenin.

Because of this, the next figures for each cell line were calibrated against the corresponding cells + tet and – BIO.

The figures below display the effect of BIO, constitutively active bcat and oncogene TP on the gene expression of *LEF1*, *TCF7*, *TCF3* and *TCF4*.

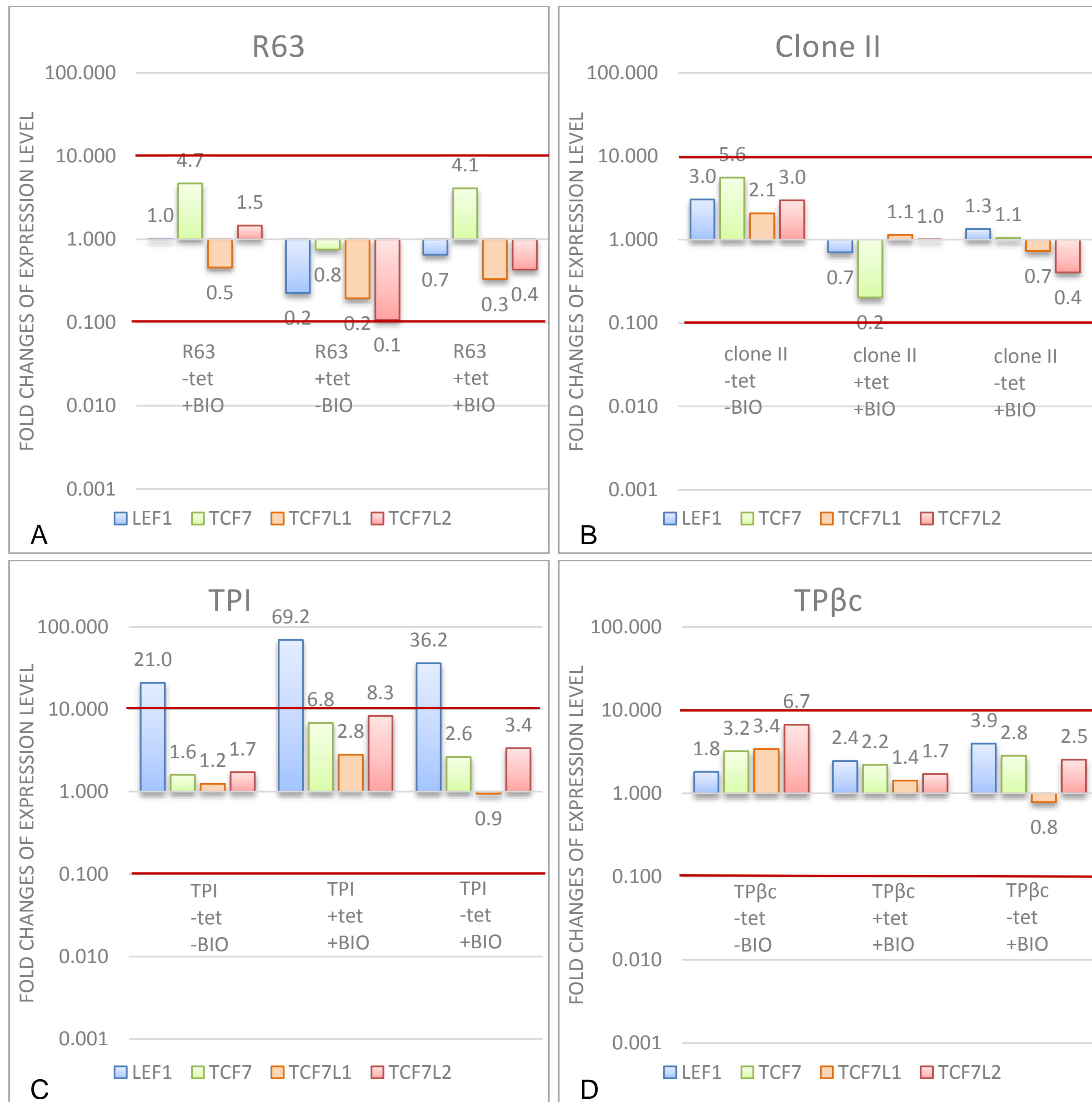


Figure 32: Fold changes of expression levels of genes of interest from R63, Clone II, TPI and TPbc cell line

Figure 32: Displayed are the expression patterns of *TCF* family genes in E14tg2a R63, Clone II, TPI and TPbc with and without the addition of tet and BIO compared to the normal expression pattern of each cell line. A 48 h treatment was conducted. A: Results of gene expression in R63. B: Results of gene expression in Clone II. C: Results of gene expression in TPI. D: Results of gene expression in TPbc. Tet 1 µg ml^{-1}, BIO 5 µM

The basis to R63 is R63 – tet and – BIO. R63 is the control cell line. The figure shows the effect of tet and BIO on a healthy cell. BIO caused an increase in the gene expression of *TCF7* and a marginal decrease of *TCF7L1*. Tet led to a decrease of *LEF1*, *TCF7L1* and *TCF7L2* and

both together, tet and BIO, caused an increase of *TCF7* and a decrease of *TCF7L1* and *TCF7L2*.

In Clone II all genes were up-regulated in the presence of constitutively active bcat (- tet). BIO only led to a diminished amount of *TCF7*. The omission of tet and the addition of BIO together caused only a small abate of *TCF7L2*. TPI cells up-regulated *LEF1* in the presence of TP, BIO and both together. Only BIO treatment caused a rise of *TCF7*, *TCF7L1* and *TCF7L2*. With BIO and TP all genes were up-regulated except for *TCF7L1*. TPbc displayed only a small change in the gene expression. The biggest change happens when tet was not added. Then all genes were up-regulated, especially *TCF7L2*. The treatment with BIO resulted in a small increase of the expression of all genes. The omission of tet and the addition of BIO led to the same result as TPI, with the exception that *LEF1* was not as highly increased.

The figures below display the effect of BIO, constitutively active bcat and oncogene TP on the gene expression of *TLE1, TLE3, TLE4* and *bcat.*

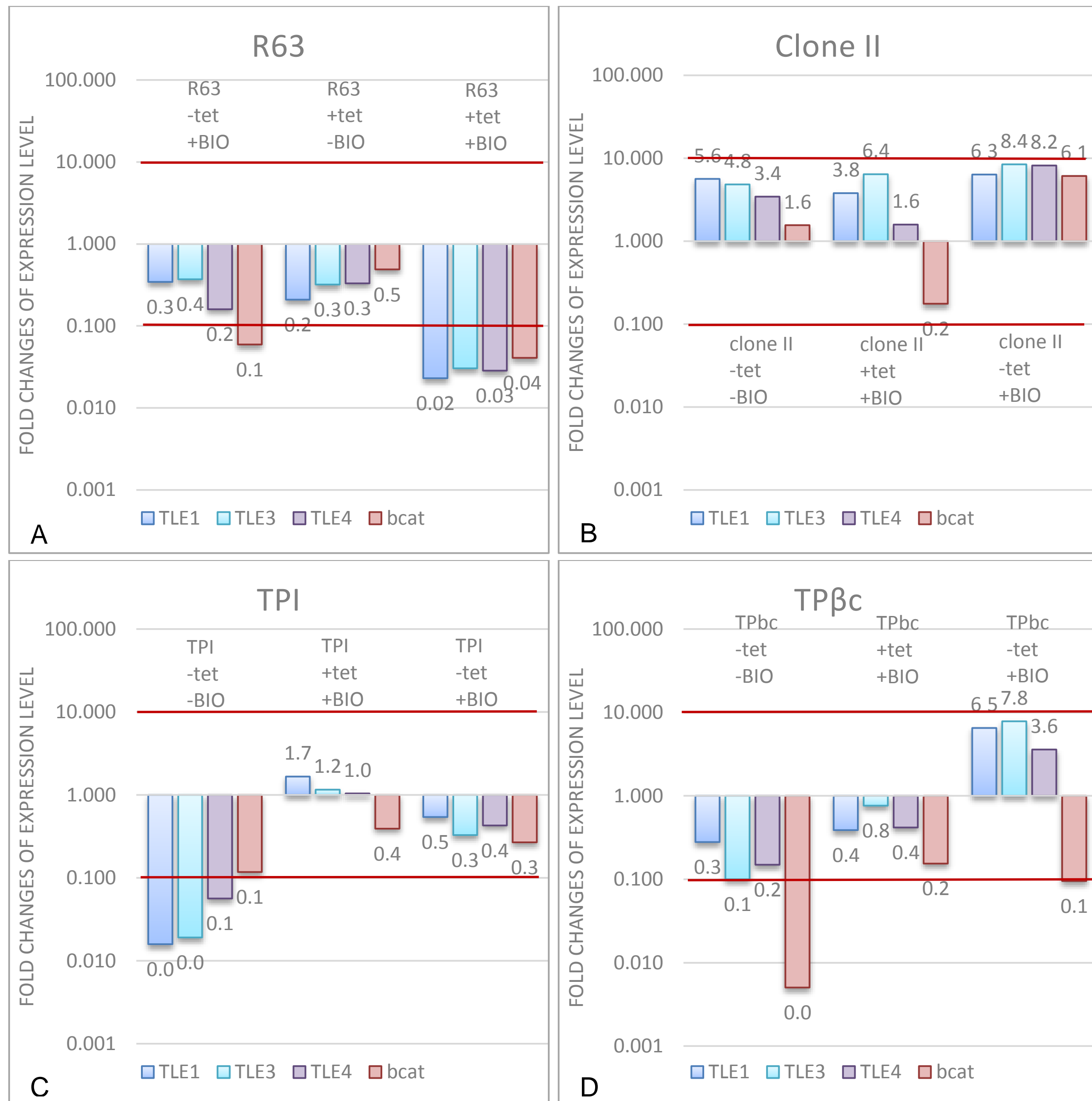


Figure 33: Fold changes of expression levels of genes of interest from R63, Clone II, TPI and TPbc cell line

Figure 33: Displayed are the expression patterns of TLE family genes and *bcat* in E14tg2a R63, Clone II, TPI and TPbc with and without the addition of tet and BIO compared to the normal of each cell line by itself. A 48 h treatment was conducted. A: Results of gene expression in R63. B: Results of gene expression in Clone II. C: Results of gene expression in TPI. D: Results of gene expression in TPbc. Tet 1 µg ml^{-1}, BIO 5 µM

R63 displayed again the effect of BIO and tet on a healthy ESC concerning to the genes *TLE1, TLE3, TLE4* and *bcat*. All variants cause a decrease in the gene expression of *TLE1, TLE3, TLE4* and *bcat.* Only with BIO *bca*t was strongly abated and the combination of BIO and tet led to a very strong drop in the gene expression.

The omission of tet in Clone II cultures caused a rise in the gene expression of all genes, but the strongest was with *TLE1* and *TLE3*. The treatment with BIO led to an up-regulation of *TLE1*, *TLE3* and *TLE4* and a fall in the expression of bcat. The combination - tet and + BIO led to an increase in all gene expressions.

In the cell line TPI the expression of the oncogene caused a strong drop in the expression of *TLE1*, *TLE3* and *TLE4* and a drop in *bca*t. The addition of BIO did not seemed to have a big effect on the expression, only *bcat* was slightly down-regulated. The third case, - tet and + BIO, led to a small decrease in all genes.

If TPbc was cultured without tet, all genes were down-regulated, especially *TLE3* and *bcat*. BIO caused only a small decrease in all expression. The highest drop was recognised with *bcat*. The addition of BIO and the omission of tet at TPbc led to an increase of the *TLE* genes and a strong drop in *bcat*.

4. Discussion

The canonical Wnt signalling is very important for embryonic development, but this pathway is mostly inactive in adult cells (Komiya and Habas, 2008). Previous studies have shown that the pathway is activated in CML during blast crisis (Luis et al., 2012). The genes: *TCF7, TCF3, TCF4, TLE1, TLE2, TLE3, TLE4, AML1 (RUNX1), LEF1* and *ICAT* are critical down-stream transcriptional factors involved in the regulation of the canonical Wnt signalling and transcription of its effector genes. Genes of the *TLE* family are transcriptional co-repressors and bind to the TCF/LEF transactivation domain (Jennings and Ish-Horowicz, 2008), *TCF7* and *LEF1* are transcribed if the pathway is active (Roose et al., 1999; Hovanes et al., 2001; Filali et al., 2002*). AML1* is activated by *TCF7 (*Wu et al., 2012*)* and *ICAT* interacts with β-catenin and inhibits the interaction with *TCF4* so β–catenin cannot bind to the *TCF/LEF* transactivation domain (Tago et al., 2000).

Microarray data revealed that the gene expression of Wnt activated genes changes during disease progression and cell differentiation. *RUNX1* was down-regulated during normal cell differentiation, but up-regulated in CML HSCs and MPPs. *TCF7* and *TCF3* were also up-regulated in more immature CML cells. In contrast, TCF4 was down-regulated in CML except for MEPs. The expression pattern of *TLE* family genes changed as well. Usually, *TLE4* expression increases during normal cell differentiation but during differentiation in chronic and accelerated phase CML *TLE4* is down-regulated as well as in MPPs in blast crisis. Noteworthy is that *TLE4* is highly down-regulated in more immature cells in CML as well as *TLE1* and *TLE3*. *TLE2* expression was decreased in more immature CML cells.

A reason for that could be the BCR-ABL translocation because BCR-ABL tyrosine kinase effects modifications in splicing of pre-mRNA in hematopoietic progenitor cells (Salesse et al., 2004; Perrotti & Neviani, 2007) and triggers tyrosine (Y) phosphorylation of bcat (Coluccia et al., 2007). This Y-phospho bcat cannot be bound by the Axin/GSK3β complex. So the BCR-ABL fusion protein causes bcat protein stabilisation and transcriptional activation by promoting *TCF4* binding (Coluccia et al., 2007). Usually, bcat is phosphorylated by GSK3β at the serine/threonine (S/T) specific N-terminal residues (Klymkowsky, 2005) which causes the degradation of bcat.

Today, CP CML is mostly treated with tyrosine kinase inhibitors (TKIs), like imatinib, dasatinib and nilotinib. The enzymatic activity of BCR-ABL tyrosine kinase is blocked by these inhibitors (Perrotti et al., 2010; Druker et al., 2006; Quintás-Cardama et al., 2009). Therefore, TKI treatment leads to a reduced level of S/T-nonphospho bcat and an increased cytosolic Axin/bcat binding. Furthermore, reduced levels of bcat enhanced the sensibility of CML to TKI (Coluccia et al., 2007; Zhang et al., 2013) and decreased self-renewal and leukaemia

development in leukemic stem cells (LSCs) (Zhao et al., 2007; Hu et al., 2009). Indicating that this pathway plays an important role in the disease phenotype.

Wnt signalling activated by Wnt ligands in CML cells has also been shown to result in an increased expression of bcat, even after TKI treatment. Exogenous Wnt signalling inhibits TKI mediated apoptosis and therefore protects CML stem/progenitor cells. These indicates that activated bcat through Wnt signalling plays an important role in reducing the effect of TKIs. Bcat protein level could be reduced due to TKI treatment but this did not change Wnt reporter activity and expression of Wnt target genes in CML stem/progenitor cells. For this reason, there must be an additional mechanism which deregulates bcat signalling (Zhang et al., 2013). Zhang et al. (2013) postulated that activation of the Wnt signalling by Wnt ligands is an additional mechanism, independent from BCR-ABL kinase activity. In addition myeloid BC GMP engraft more often than BC HSC but normal GMP did not engraft. These confirm that BC GMP achieved self-renewal capacity (Abrahamsson et al., 2009) and behave like LSCs if they overexpress BCR-ABL tyrosine kinase (Jamieson et al., 2004).

The up-regulation of genes which are important for self-renewal and the down-regulation of repressors like *TLE* family genes during cell differentiation in CML could potentially lead to more mature progenitor cells gaining the ability to self-renew. Furthermore, the increased levels of bcat in myeloid progenitors are evidence for activate Wnt signalling in more mature haematopoietic cells.

To analyse the gene expression of Wnt activated genes in blast crisis CML, a drug treatment of K562 cells was conducted. K562 cells are immortalised cells from a patient in blast crisis CML (Lozzio and Lozzio, 1975). First we determined whether the leukemic cells expressed all the key Wnt activated genes and if the expression level of these genes altered following extrinsic activation/inhibition using Wnt ligands and small molecule inhibitors to modulate the pathway.

Therefore, WNT3a, BIO and XAV treatments were used to investigate changes in the gene expression. The drugs BIO and Wnt3α activated the Wnt pathway. Wnt3a is a ligand that binds to the Frizzled and LRP receptor and activates the Wnt signalling from the outside. BIO deactivates GSK3β directly, causing the destruction complex that phosphorylates bcat for proteosomal degradation to no-longer be active (Meijer et al., 2003). Both mechanisms should causes an increase of bcat.

Abrahamsson et al. (2009) discovered that in BC CML progenitors GSK3β is already less expressed compared to normal progenitors, which causes an increase of bcat. Abrahamsson et al. (2009) also revealed that GSK3β was mis-spliced (m-GSK3β) in 4 of 7 myeloid BC CML

samples and lacked the FRAT and Axin binding domains. This m-GSK3β isoform cannot phosphorylate bcat. The m-GSK3β protein was more present in BC GMP then in BC HSC. Overexpression of BCR-ABL tyrosine kinase might cause the mis-splicing of GSK3β and could be a key event for the development of LSC (Abrahamsson et al., 2009). It was therefore hypothesised that the treatment with BIO and WNT3a would lead to a further increase in the expression of bcat and this probably lead to an up-regulation of *TCF7*, *RUNX1* and *LEF1* and a down-regulation of *TLE* family genes.

Abrahamsson et al. (2009) also explored a decreased expression of Axin 2, which is the concentration-limiting component of the destruction complex (Huang et al., 2009). The drug XAV stabilises Axin and therefore the destruction complex thus potentiating the degradation of bcat and down-regulates the expression of *TCF7*, *LEF1* and *RUNX1*.

An 8 h treatment was performed to study a short-term effect of the treatments on the cells. A 24 h treatment was also carried out to investigate the effects of a longer incubation time; to ensure the cells had time to change the protein expression. It is important to remember, that the Wnt signalling is already activated in K562 cell lines. This makes the response of the cells to the drugs much more interesting in terms of their potential for further activation. The results from the 8 h treatment showed little alteration in gene expression. However significant changes in gene expression were observed following 24 h.

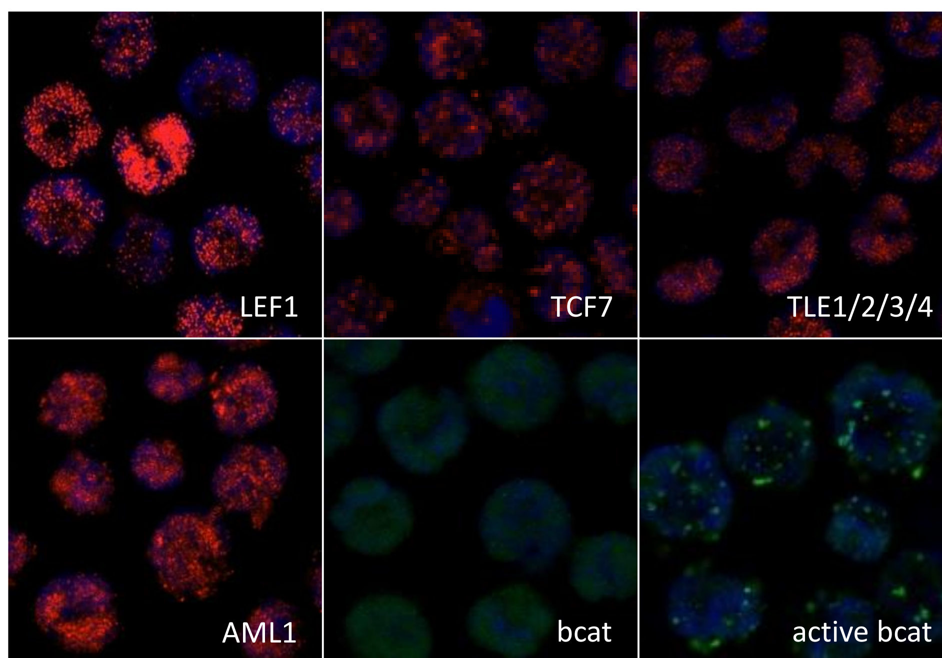


Figure 34: IF results of untreated K562, 400x enlargement

Figure 34 shows the protein level of LEF1, TCF7, TLE1/2/3/4, AML1, bcat and active bcat in untreated K562 cells. LEF1, TCF7, TLE1/2/3/4 and AML1 are stained with Alexa Fluor® 594 and DAPI. Bcat and active bcat are stained with Alexa Fluor® 488 and DAPI.

Figure 34 displays the protein levels of LEF1, TCF7, TLE1/2/3/4, AML1, bcat and active bcat in untreated K562 cells. The protein levels of LEF1 and RUNX1 were already very high in K562. In addition, TCF7 and active bcat protein were also present in the normal K562 cell line. It was also observed that the protein levels of bcat and TLE1/2/3/4 in untreated K562 cells were lower. The protein levels of LEF1, TCF7 and AML1 were an evidence for an active Wnt signalling pathway, but curious was the lower bcat level in untreated K562. It seems that the cells are actively trying to switch off Wnt signalling by degrading β-catenin to keep it at a low level to counteract the high levels of LEF1, AML1 and TCF7 in this cell line.

The following figures display the fold changes of expression levels of *TCF7*, *LEF1, AML1* and *TCF4* in K562 treated with BIO, Wnt3a and XAV in comparison to the 8 and 24 h treatments.

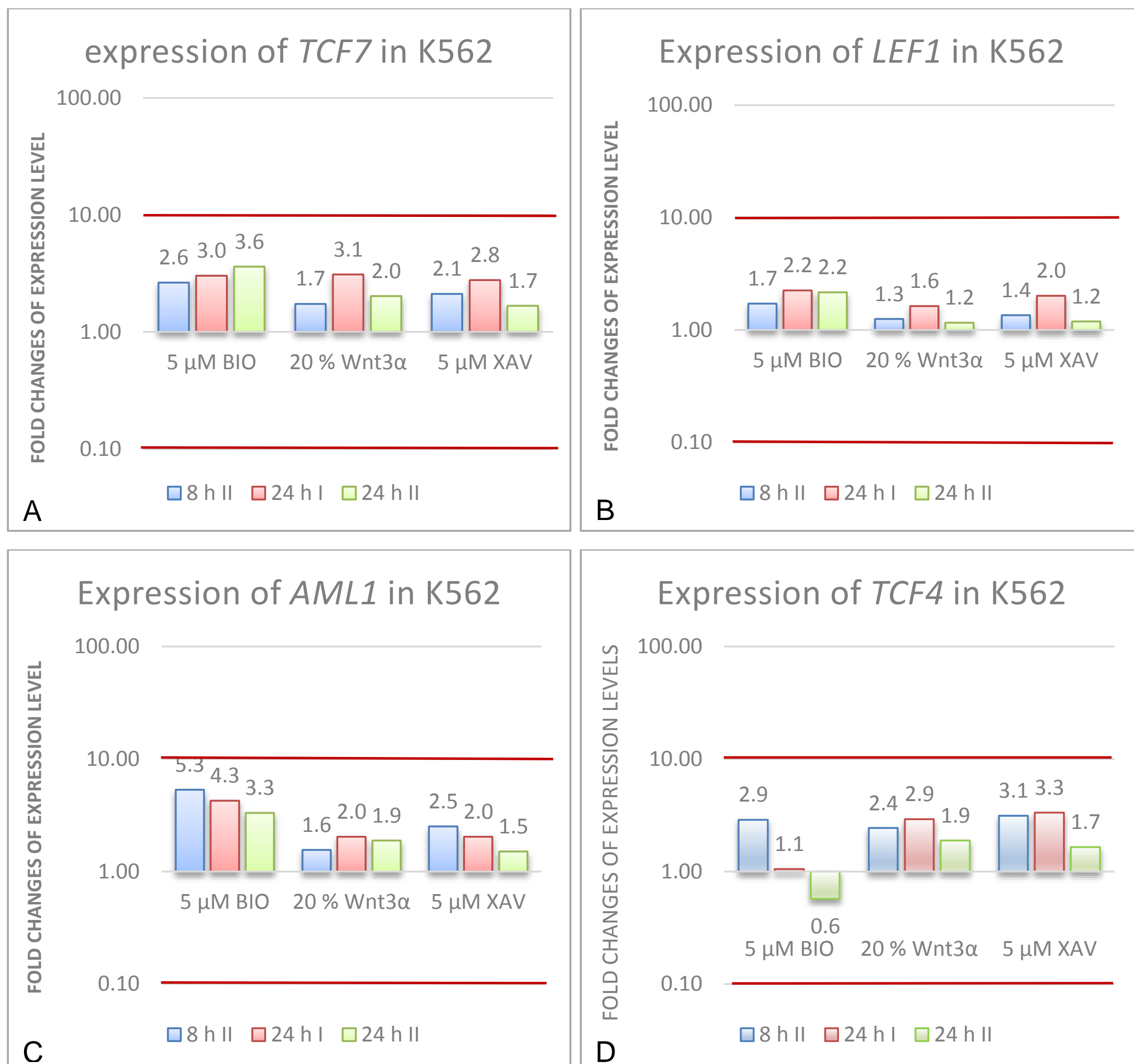


Figure 35: Fold changes of expression levels of *TCF7*, *LEF1, AML1* and *TCF4* in K562 treated cells after 8 h and 24 h

Figure 35: The expression pattern of *TCF7* (A), *LEF1* (B), *AML1* (C) and *TCF4* (D) in K562 treated with BIO, Wnt3α and XAV are displayed. The arrangement show the difference between the 8 h II, 24 h I and 24 h II treatments.

The Wnt3a treatment did not significantly increase the gene expression of *TCF7*, *LEF1* and *RUNX1* in K562, but the bcat and active bcat protein level increased. As mentioned before, the Wnt signalling was probably already active in K562 and a further activation through Wnt3a was ineffective. But Wnt3a treatment did result in a rise in the protein level of TLE1/2/3/4 and a strong increase in the gene expression of *TLE2* and *TLE4* after the 24 h treatment. This could be an indication for the response of the cell to the stimulation, because if the cell is undergoing chronic activation of the pathway, it will try and switch it off by up-regulating

negative regulators. The TLE family genes repress this effect because these genes inhibit the transcription of Wnt signalling target genes. Longer treatments with Wnt3a are necessary to analyse if the *TLE* genes can reduce the expression of *TCF7*, *LEF1* and *RUNX1*. The results of the Wnt3a treatment led to the assumption that *TCF7*, *RUNX1*, *TCF4*, *TLE2* and *TLE4* are important regulators of the Wnt signalling pathway in BC CML.

The drug BIO led to a strong increase in the protein level of bcat and active bcat. This results in a higher protein level of TCF7 and an up-regulation of *TCF7* (3.6 fold higher) as well as *RUNX1* (4.3 fold higher). But the protein level of RUNX1 did not increase during the 8 and 24 h treatment. Since *RUNX1* was activated by TCF7, the accumulation of RUNX1 seems to take more time than 24 h. High TCF7 and RUNX1 protein level were an evidence for immature cells. The protein level of LEF1 and the gene expression patern showed no increase. But the Western blot showed a change in the isoform of the protein. After the 24 h treatment with BIO the protein level of the bigger isoform increased and the level was higher for the smaller isoform. So BIO might lead to a different isoform expression of LEF1. This isoform may potentially have a different influence in the behaviour of the cell, the smaller isoform therefore warrants further investigation. The gene expression of *TLE4* was up-regulated after 8 h (3.2 fold higher) but dropped slightly by 24 h (2.5 fold higher). *TLE2* expression did not show a significant change, suggesting that inhibition of Wnt signalling by *TLE* was not occurring following BIO treatment. Further work is necessary to prove whether BIO treatment inhibits the expression of the *TLE* genes in some way. On the other hand, the treatment with BIO led to an increase in the gene expression of *ICAT* after 24 h. *ICAT*, like *TLE*, is a repressor of the Wnt signalling pathway. ICAT interact with bcat so that bcat cannot bind to the *TCF/LEF* transactivation domain anymore. But it was improbable that the level of ICAT was high enough to totally stop further activation of the Wnt signalling. These results suggest that *TCF7*, *LEF1*, *RUNX1*, *TLE4* and *ICAT* are important regulators of the Wnt signalling pathway in BC CML.

XAV caused a decreased protein level of bcat after 8 h treatment however the protein level of active bcat did not change indicating that the signalling pathway was still active. In contrast the protein level of TCF7 increased, as did the gene expression (2.8 fold higher). LEF1 protein level and gene expression did not significantly change. A decrease was seen in the protein level of AML1 but not in the gene expression. The co-repressors *TLE2 (23 fold higher)* and *TLE4 (4.3 fold higher)* were highly up-regulated similar to the Wnt3a treated cells. In addition *TCF4* gene expression was increased (3.3 fold higher). All these repressors will relieve the effect of the activated Wnt signalling. Although XAV could cause a promising inhibition of the canonical Wnt signalling pathway in other cancers, in CML there was very little effect on the chronic Wnt activation observed. The treatment suggested that *TCF7*, *RUNX1*, *TLE4*, *TLE2*, *TCF4* and *ICAT* are important regulators of the Wnt signalling pathway.

An activation through BIO led to a rise in the expression of *TCF7* and *AML1*, and a different isoform expression of LEF1. Wnt3a further activated the transcription of TLE co-repressors as did XAV.

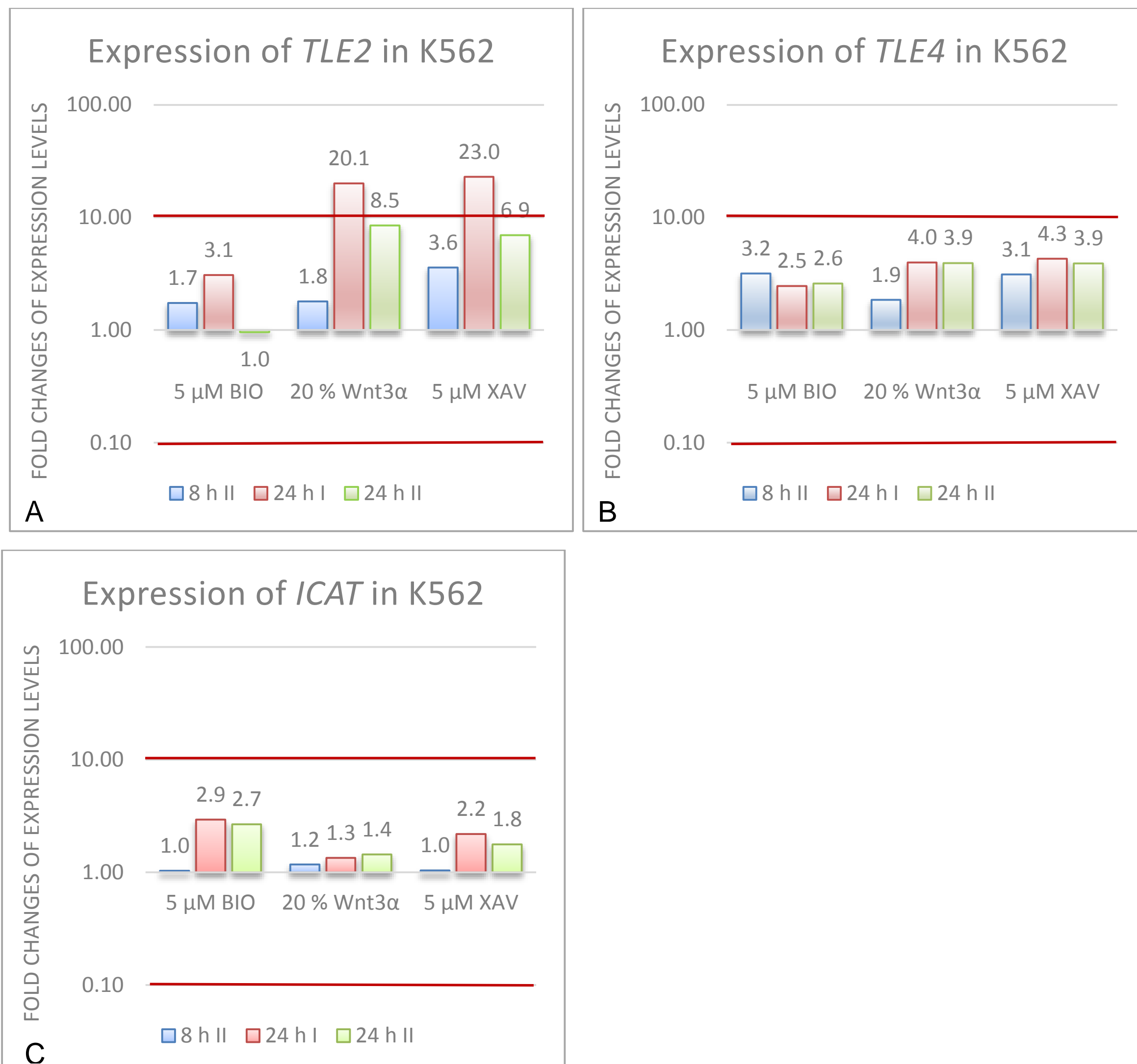


Figure 36: Fold changes of expression levels of *TLE2*, *TLE4* and *ICAT* in K562 treated cells after 8 h and 24 h

Figure 36: The expression pattern of *TLE2* (A), *TLE4* (B) and *ICAT* (C) in K562 treated with BIO, Wnt3α and XAV are displayed. The arrangement shows the difference between the 8 h II, 24 h I and 24 h II experiments.

Overall these data indicate that the gene and protein expression in blast crisis CML can be changed through the further activation or inhibition of the Wnt signalling pathway. The Wnt signalling pathway is very important in maintenance of HSCs (Reya et. al., 2003). Our data indicates that this pathway is already switched on in CML BC cells with further activation up-regulating *TCF7* and *AML1*, important mechanisms involved in stem cell maintenance and self-renewal. Further investigations are necessary to analyse the effect of these treatments of

CML self-renewal and differentiation alongside changes in expression of genes involved in the cell cycle in blast crisis CML.

The Wnt signalling is active in ESCs as well as in HSCs (Komiya and Habas, 2008). Therefore, the genes of the *TCF* and *TLE* family should also be expressed in these cells. The murine embryonic stem cell line E14tg2a was transfected with the oncogene TP (TPI) and with constitutively active bcat (Clone II) and with both, TP and constitutively active bcat (dp-bcat) (TPbc). The oncogene TP is found in a subgroup of CMML (Golub et al., 1994). TP expression in E14tg2a leads to differentiation and preservation of haemopoietic progenitor self-renewal. TP also repressed erythropoiesis and increased myelopoiesis (Dobbin et al., 2008). It has also been previously shown that TP inhibits Wnt signalling by GSK3β mediated degradation of β-catenin (personal communication Dr Wheadon). Through treatment with BIO, which activate Wnt signalling, we hypothesised that the effect of TP should be rescinded. In addition, previous studies revealed that pluripotency in human and mouse embryonic stem cells can be maintained by BIO (Sato et al., 2004). It was also suggested that dp-bcat reverses the effect of TP and possibly increases the expression of the *TCF* and *TLE* gene families.

The Western blot results (see 3.3.1 Western blot results, page 53) confirm the assumption in the case of *LEF1*, that TP inhibits Wnt activated expression. The cell line TPI showed a decrease in the protein level of LEF1 when the oncogene was expressed. However, the protein level of TCF7 did not change during the expression of TP. The dp-bcat in TPbc reduced the effect of TP, by restoring the LEF1 protein level nearly back to the levels observed in the control cells (without TP and constitutively active bcat). Again, there was no change in the protein level of TCF7. The control cell line R63 did not show a change in the protein level of TCF7 and LEF1 if tet was added to the cells. Thus tet has usually no influence on the protein levels of TCF7 and LEF1 in E14tg2a. The expression of dp-bcat led to a rise in the protein level of TCF7 and LEF1 in Clone II, which confirmed the assumption that dp-bcat possibly reduces the effect of TP.

The treatment with BIO led to an increase of the protein levels of TCF7 and LEF1 in all cell lines. In all cases apart from TPI, the protein level of TCF7 is more increased than LEF1 by BIO. As previously reported our data confirms that BIO led to an activation of the Wnt signalling pathway. The addition of BIO to cells expressing TP led to a strong increase in the protein level of TCF7 and LEF1 a similar effect was seen in cells expressing both TP and dp-bcat. Interestingly the levels of TCF7 and LEF1 are higher in TPI than in TPbc. TP expressing cells also had higher levels of TCF7 than Clone II. These results indicate that the oncogene is also modulating the down-stream transcription factors involved in this pathway independently of pathway activation.

The fact, Clone II, TPI and TPbc with the addition of tet did not have the same gene expression as R63 (- tet), was on interest. The gene expression in the control cell lines; Clone II, TPI and TPbc (+ tet, - BIO) compared with the parental cell line R63 showed down-regulation in nearly all cases. Only the gene expression of the *TLE* family and *bcat* in TPI was nearly the same as in R63. This is either due to the genetic manipulation of the cells or due to the addition of tet to the culture. Further investigations are necessary to clarify these finding.

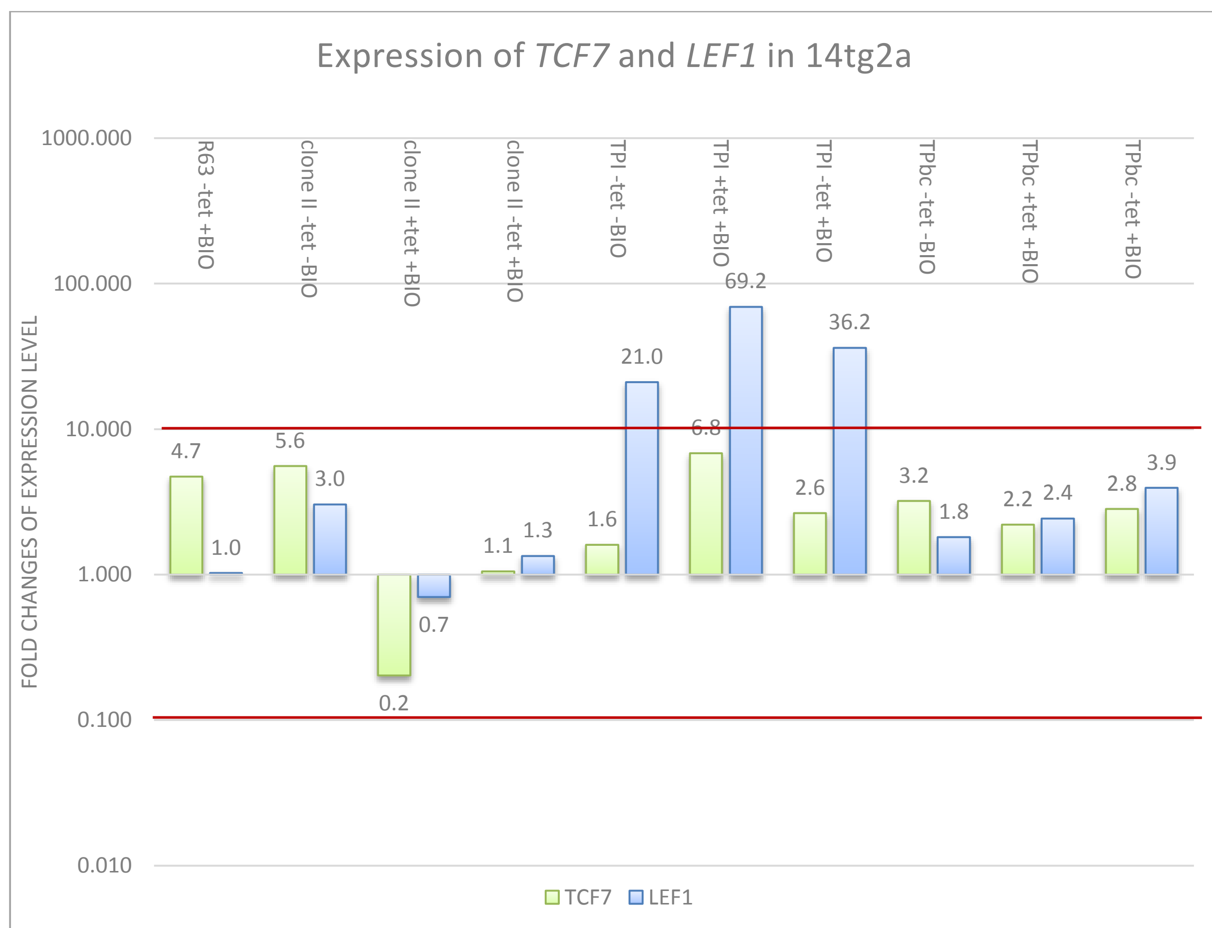


Figure 37: Fold changes of expression levels of *TCF7* and *LEF1* from R63, Clone II, TPI and TPbc cell line

Figure 37: The changes in the gene expression of *TCF7* and *LEF1* in E14tg2a cell lines treated for 48 h with and without tet and BIO are displayed. Tet 1 µg ml^{-1}, BIO 5 µM

The expression of dp-bcat in Clone II (-tet, -BIO) led to an increased expression of *TCF7* and *LEF1* as well as *TCF7L1*, *TCF7L2*, *TLE1, 3, 4* and *bcat* which was assumed. Surprisingly, TPI without the addition of tet showed a big increase in *LEF1* expression and a down-regulation of *TLE* family genes. It was assumed that TP led to a decreased of *TCF7*, *LEF1* and *bcat* but only *bcat* was down-regulated The expression of TP and dp-bcat in TPbc caused a small rise in the gene expression of *TCF7* and *LEF1*, these results led to the assumption that dp-bcat partly reverses the effect of TP. Additionally, the increase in dp-bcat possibly led to the strong

down-regulation of *bcat* in the presence of TP because there was no decrease in *bcat* expression in Clone II.

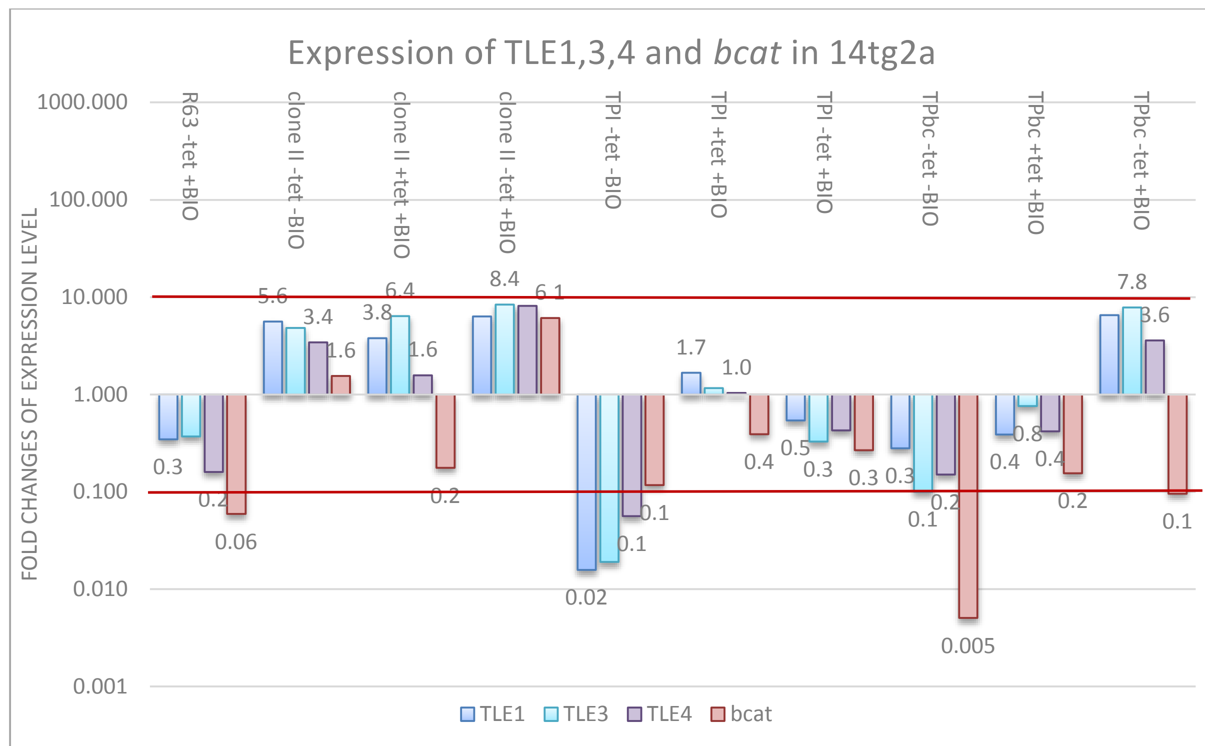


Figure 38: Fold changes of expression levels of *TLE1*, *TLE3*, *TLE4* and *bcat in* R63, Clone II, TPI and TPbc cell line

Figure 38: The changes in the gene expression of *TLE1*, *TLE3*, *TLE4* and *bcat* in E14tg2a cell lines treated for 48 h with and without tet and BIO are displayed. Tet 1 µg ml^{-1}, BIO 5 µM

The addition of BIO did not led to an up-regulation of *TCF7* and *LEF1* in all cell lines as expected. Clone II showed a down-regulated of *TCF7* and *LEF1* and R63 only an up-regulation in *TCF7.* Nevertheless, western blot results showed an increase in the protein levels. A reason for this was possibly a product inhibition because *TLE1* and *TLE3* were up-regulated under these conditions and maybe inhibited the expression of *TCF7* and *LEF1. TCF7* and *LEF1* were highly up-regulated in TPI and marginally in TPbc treated with BIO and tet. In these cases BIO activated the Wnt signalling pathway. The expression of *bcat* was down-regulated in TPI and TPbc treated with BIO. A reason for that could be a high protein level of bcat and this level represses the further expression of *bcat*.

As presumed, the influence of BIO on cells expressing the oncogene TP led to a high expression of *LEF1* but only to a small increase in *TCF7*. The addition of BIO to dp-bcat expressed Clone II did not show a further increase in the gene expression of *TCF7* and *LEF1* but did increase *TLE1,3,4* and *bcat.* In contrast, the protein level of TCF7 increased strongly. As TCF7 is able to regulate its own transcription, it could potentially be inhibiting its own

expression in this case. It seems that the additional expression of *dp-bcat* in TPbc treated with BIO led to a smaller increase in the expression of *TCF7* and *LEF1* when compared to TP expressing cells treated with BIO.

Due to the high concentration of dp-bcat and the accumulation of bcat in the Clone II cell line, the cell might tried to reduce the activation of the Wnt signalling by increasing the expression of *TLE* repressors. Furthermore, the accumulation of bcat protein in the cell maybe led to an inhibition of further expression of *bcat* as well.

The assumption that TP inhibits Wnt activated expression could not be confirmed by the gene expression results. TPI cells expressing the oncogene showed a strong increase in the expression of *LEF1*. In fact, if TP inhibits the Wnt activated expression, *LEF1* expression should be down-regulated. In addition, it seems that the dp-bcat reduced the effect of TP but also inhibits the effect of BIO. The mechanism of Wnt activated expression inhibition by TP, also needs to be investigated further to determine how modulation of these transcriptional activators and repressors are regulating self-renewal and differentiation in ESC.

TP expression alone led to an increased gene expression of *LEF1* and *TCF7* and a decreased expression of *TLE* family genes and *bcat*. By contrast, the protein level of TCF7 did not change whereas the protein level of LEF1 decreased. The treatment with BIO repressed the effect of TP. Dp-bcat enhanced the effect of BIO in case of *TLE* family genes and *bcat* expression but decreased the expression in case of *TCF7* and *LEF1*. However, dp-bcat also decreased the effect of the oncogene TP.

These results confirm the effect of the oncogene TP in CMML because the up-regulation of *TLE* co-repressors could support differentiation and the up-regulation of *TCF7* and *LEF1* might preserve the self-renewal ability in more mature haemopoietic cells.

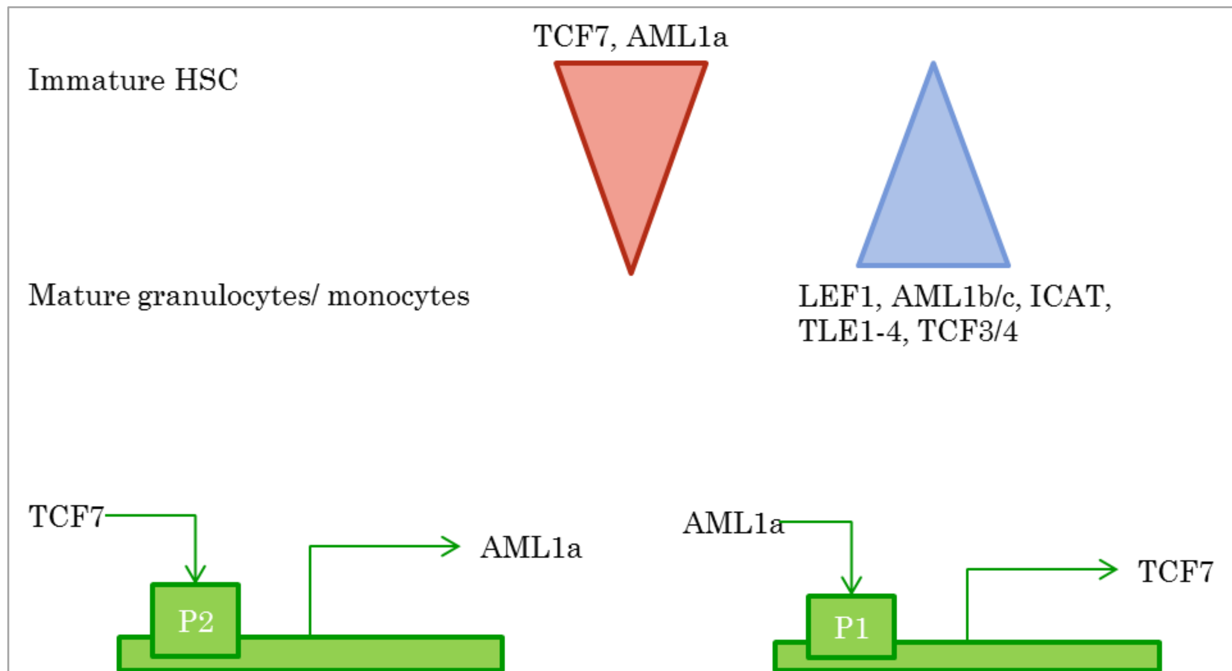


Figure 39: The protein levels of genes of interest in immature and mature hematopoietic cells and the relation between TCF7 and AML1

Figure 39: The protein level of TCF7 and AML1a are high in normal immature HSC and low in normal mature granulocytes/monocytes. By contrast, the protein levels of LEF1, AML1b/c, ICAT, TLE1/2/3/4 and TCF3/4 are low in immature HSC and high in mature granulocytes/monocytes. TCF7 binds to the promotor (P2) of AML1a and activates the transcription. AML1a itself binds to the promotor (P1) of TCF7 and activate the transcription of TCF7.

In this study we aimed to determine how the two active tyrosine kinase fusion proteins BCR-ABL in CML and Tel/PDGFRβ in CMML modulated the Wnt pathway through the key downstream transcriptional activators and repressors of the pathway. Our data clearly demonstrates that both oncogenes result in high expression of *TCF7* and *AML1* important regulators of stem cell self-renewal. As depicted in the schematic diagram above, these transcription factors are important for suppressing differentiation when highly expressed. Activation of the pathway by Wnt3a stimulation or GSK3β inhibition led to a further enhancement in the levels of *TCF7*. These findings have important implications for the management of these malignancies because they highlight the potential mechanism through which Wnt signalling alters the balance of transcription factors involved in HSC self-renewal and differentiation. By changing this balance, Wnt signalling favours self-renewal and LSC expansion, the cells which are less sensitive to TKI treatment and responsible for residual disease and progression in MPN.

5. Summary

Myeloid malignancies are clonal diseases of hematopoietic stem and progenitor cells. They develop through several genetic and epigenetic alterations. Self-renewal, proliferation and differentiation are disturbed through these alterations (Murati et al., 2012). Among other things, the canonical Wnt signalling pathway is important for self-renewal, proliferation and differentiation in HSCs (Reya et al., 2003). Several key transcription factors, including *TCF3*, *TCF4*, *TCF7* and *LEF1*, and transducing-like enhancers, like *TLE1*, *TLE2*, *TLE3* and *TLE4*, are regulated through this pathway (Komiya and Habas, 2008; Munk et al., 2008), but it is not well known, how these genes are regulated by Wnt signalling.

This study investigated the transcription levels of *TCF/LEF*, *TLE*, *ICAT* and *RUNX1* genes in CML and CMML. In both diseases a constitutively active tyrosine kinase (TK) fusion protein were expressed. It was also determined which family members are important regulators of the canonical Wnt signalling pathway in CML and which genes of the Wnt signalling were altered when TK was expressed in CMML.

The transcription factors *TCF7* and *LEF1* activate the transcription of key genes which are important for self-renewal and proliferation (Huang and Qin, 2010; Wu et al., 2012). For example, the transcription of a further transcription factor, *AML1*, is activated by *TCF7*. *AML1* is another key transcription factor involved in HSC self-renewal and myelopoiesis. AML1 itself also activates the transcription of *TCF7* which causes a higher level of both genes (Wu et al., 2012). *TCF4* is needed by β-catenin to activate the transcription of target genes (Cuilliere-Dartigues et al., 2006). Genes of the *TLE* family are transcriptional co-repressors (Hartley et al., 1988, Stifani et al., 1992) and inhibit transcription of Wnt signalling target genes (Jennings and Ish-Horowicz, 2008).

Results from a microarray which was provided by the Paul O'Gorman Leukaemia Research Centre in Glasgow, showed a change in the gene expression during progression on CML. *TCF7* and *RUNX1* are up-regulated in immature CML cells. Genes of the *TLE* family are down-regulated during disease progression.

The expression of the Wnt signalling activated genes were analysed in a blast crisis CML cell line (K562) and in mESC (E14tg2a) which were transfected with the oncogene TP and constitutively active β-catenin (dp-bcat). TP stands for Tel/PDGFRβ, which is a fusion protein and can be found in a subgroup of CMML (Golub et al., 1994). The oncogene causes a constitutive activation of a tyrosine kinase (Carroll et al., 1996; Jousset et al., 1997) which leads to haematopoietic differentiation (Dobbin et al., 2008). Four transfected variants were tested, the parental cell line R63, Clone II with dp-bcat expression, TPI with the oncogene TP expression and TPbc with dp-bcat and TP expression.

The experiments included a drug treatment with the drugs BIO and XAV and the Wnt3a ligand on K562 cells for 8 and 24 h. MESC were treated with and without tet and BIO for 48 h. BIO and Wnt3a activated the Wnt signalling and therefore the expression of the genes of interest (Meijer et al., 2003; Singla et al., 2006). XAV increased the phosphorylation of β-catenin by stabilising Axin (Salic et al., 2000). This should led to a decrease of β-catenin and a reduced transcription of target genes.

The protein levels in untreated K562 showed high levels of LEF1 and AML1. TCF7 and active β-catenin were also expressed. The level of β-catenin was surprisingly low in untreated K562. The levels of the transcription factors are probably an indication of active Wnt signalling, but contrary is the low level of β-catenin. If the pathway is activated, the β-catenin protein level should be higher, however chronic constitutive activation could potentially lead to down-regulation. The cause for the high levels of LEF1 and AML1 could be one or more mutations, which causes an expression of active β-catenin, because active β-catenin was detected in untreated K562. These results indicate that the transcription factors *LEF1*, *AML1* and *TCF7* were expressed in BC CML cells.

Samples of drug treated K562 were analysed by Western blot, immunofluorescence and qPCR. The Wnt3a treatment caused only a small increase in the gene expression and protein levels of LEF1, TCF4, TCF7 and AML1, but on the other hand a big increase in the protein levels of β-catenin, active β-catenin and TLE1/2/3/4. Even the gene expression of *TLE2* and *TLE4* increased heavily. The high β-catenin/ active β-catenin level possibly caused an activation of the *TLE* gene expression. Conceivably, Wnt3a did not further activate Wnt signalling but increased the expression of Wnt repressors. These results suggested that *TCF7*, *RUNX1*, *TCF4*, *TLE2* and *TLE4* are important regulators of the Wnt signalling pathway in BC CML.

The effect of BIO on K562 was quite pronounced. The results displayed a further activation of the Wnt signalling pathway. Protein levels of TCF7, AML1, LEF1, β-catenin and active β-catenin were increased as well as at the gene expression level. *TLE2* and *TLE4* did not increase substantially. But the gene expression of *ICAT* increased with BIO treatment. The expression might be activated through the high β-catenin and active β-catenin levels. These results indicate that although already active further activation of the Wnt signalling pathway was possible in blast crisis CML and that *TCF7*, *LEF1*, *RUNX1*, *TLE4* and *ICAT* were important regulators of the Wnt signalling pathway. Moreover, an activation of the signalling through BIO led to a different isoform expression of LEF1.

XAV treatment resulted in an increased expression of *TLE2* and *TLE4* and a decrease of the β-catenin protein level. Surprisingly, XAV led to an increase in *TCF7* and *RUNX1* expression.

These results support that that *TCF7*, *RUNX1*, *TLE4*, *TLE2*, *TCF4* and *ICAT* are important regulators of the Wnt signalling pathway in BC CML, but further studies are necessary to analyse if the cell cycle and proliferation behaviour are changed under the influence of these treatments.

The Wnt signalling pathway is active in E14tg2a cells, but the addition of oncogene TP to the cell line TPI has previously been shown to inhibit Wnt signalling. It was hypothesised that the treatment with BIO would counteract the effect of TP. Furthermore it was expected that dp-bcat would also reduce the effect of TP. The expression of TP in TPI cells did not reduce the gene expression of *TCF7* and *LEF1* but heavily down-regulated the expression of the *TLE* family genes and of *β-catenin*. These might be the reason that myeloid progenitors in CMML obtained self-renewal ability.

The addition of BIO led to an increase in the expression of *LEF1* and *TCF7* in TPI with and without expressed TP and BIO reduced the effect of TP on *TLE* family genes and *β-catenin* expression. The presence of dp-bcat led to a small increase in protein levels and gene expression patterns of *TCF7* and *LEF1* in TPbc compared to TPI cells. Interestingly, the protein level and the gene expression pattern were completely different if BIO was added. The protein level of LEF1 and TCF7 increased while the gene expression was down-regulated. The gene expression of *LEF1* and *TCF7* in TPbc did not increase to the same level as in TPI. But it can be confirmed that constitutively active β-catenin increases the expression of *TLE* genes and *β-catenin* in TP expressed cells. Western blot results confirm that the addition of BIO and the expression of constitutively active β-catenin reduces the effect of the oncogene TP in E14tg2a mouse embryonic stem cells.

Overall these findings highlight the complex interplay between the transcriptional activators and repressors involved in the Wnt pathway. Moreover it highlights how leukaemia oncogenes alone and in combination with active Wnt signalling can alter the balance to promote LSC expansion and suppress differentiation. Mechanisms involved in TKI resistance and disease progression in MPN.

6. Annex

6.1 List of Literature

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6.2 Materials

6.2.1 Machines

Machine	Company
Automatic flake-ice machine AF100	Scotsman
ChemiDoc™ XRS+ System (765/07825)	BIO RAD
Centrifuge 3-16 PK (Rotor 11180), 1-15 PK, 1-14, SciQuip (Rotor 12132-H)	Sigma
Centrifuge: Sorvall Legend T (75004367) Four-Place swinging Bucket Rotor (75006445) Inlay: Microplate Carrier (75006449)	Thermo Scientific – Sorvall Heraeus
Developer, SRX-101A	Konica Minolta
Fluorescence Microscope, M1 AX10	Zeiss Imager
Freezer (-80˚C)	Sanyo
Freezer (-20˚C)	Bio Cold
Fridge	Lab Cold
Haemocytometer	Neubauer
Hood VB 85	Gelaire
Hood Hera Safe	Thermo scientific
Hot plate PC-101	Corning
Incubator, Galaxy 170 s	New Brunswick
Nanodrop ND-1000	Labtech
Optical microscope, TMS No 300729	Nikon
pH meter 209	HANNA instruments
Pipettes	Gilson, Fisherbrand
Power Pac Basic and Power Pac-HC	BIO RAD
Real Time PCR 7900 HT	Science
Roller mixer SRT9	Stuart
Scale PF-602	Fisherbrand
Scale XS105 DualRange	Mettler Toledo
Shaker, gyro-rocker SSL3	Stuart
Spectra max M5	Molecular Devices
Trans-blot® SD, Semi-dry transfer cell	BIO RAD
Thermocycler TC-412	Techne
Vortex Genius 3	IKA®
Water bath, SUB28	Grant
Mini-PROTEAN Tetra cell	BIO RAD

6.2.2 Equipment

Equipment	Company
Cell Culture Flask	Cellstar
Cell Cultur Dishes	Cellstar
Centrifuge Tube (15 and 50 ml)	Scientific laboratory supplies
CL-XPosureTM Film (18 x 24 cm), #34089	Thermo Scientific
Cell culture Dish for ESC (6 and 10 cm) #3296	Corning
Cryos™	Greiner Bio-one
Deckgläser (24 x 60 mm)	Menzel
Filter Tips	Greiner Bio-one, Sorenson
Microseal B-Adhesive Seals MSB-1001	BIO RAD
Micro test tubes (0.5, 1.5, 2.0 ml)	Greiner Bio-one

Equipment	Company
Multispot microscope slides, PTFE and specialised coatings	Hendley-Essex
Needle 0.45 x 12 mm	Terumo
Pasteur pipette	Greiner Bio-one
PCR filter tips	Sorenson, Multiguard
Stripette (5 ml, 10 ml and 25 ml)	Costar
Syringe	BD Plastipak
Syringe-Filter (0.20 µm)	Sartorius Stedim
Tips	Greiner Bio-one
Tubes (0.5, 1.5, 2.0 ml)	Greiner Bio-one
Whatman™ Chromatography paper (cat#3030917)	GE Healthcare
Whatman™ Protran BA 85 (cat#10401196)	GE Healthcare

6.2.3 Reagents

Reagent	Company	cat
10x Exonuclease I Reaction Buffer	BioLabs.inc	B02935
10x TBE Buffer	Ambion	LIN 1303047
2-Mercaptoethanol (2 ME)	Sigma Aldrich	M7522
5x Green GoTaq® Flexi Buffer	Promega	M891A
6x Blue/Orange Loading Dye	Promega	G190A
Acrylamide 30%	Sigma	A3699
Agarose	Web scientific	AGR-500
Ammoniumpersulfate (APS)	Sigma	A9164-100g
Bradford Quick Start	BIO RAD	#500-0205
Bromophenol Blue	Sigma Aldrich	B0126
BSA	Roche	10735094001
DAPI	Vector Scientific	101098-044
dNTP Mix (10mM)	Invitrogen	18427-013
Ethanol	VWR chemicals	20821d.330
Ethidiumbromide	Sigma	1001571022
Evagreen	VWR	31000-T
Exonuclease I (20`000 Units/ml)	BioLabs.inc	M0293L
Gelatine	Sigma	G2500-500G
GeneRuler	Thermo Scientific	SM1331
Glycine	Sigma	G8898-1KG
Go Taq® Flexi DNA Polymerase	Promega	M8308
Hydrochlorid acid	Sigma	07102-2.52
Immobilon^{TM} Western Chemiluminescent HRP Substrate	Millipore	W3KLS0500
Magnesium chloride ($MgCl_2$)	Promega	A351H
Nonidet P40 Substitute	Fluka	74385
oligo(dT)$_{20}$ (50µM)	Invitrogen	18418-020
Ovalbumin	Sigma	A5378-25g
Paraformaldehyd	Aldrich	16005
Phenylmethanesulfonyl fluoride (PMSF)	Sigma	329-98-6

Reagent	Company	cat
Poly-L-Lysine	Sigma-Aldrich	P1274-100mg
Ponceau S Solution	Sigma	P7170-1L
Potassium chloride	Sigma	P9541
Potassium phosphate	Sigma	P9791-500g
QIAGEN Multiplex PCR Master Mix, 2x	Qiagen	206143
Restore Western Blot Stripping Buffer	Thermo Scientific	21059
Restore Plus Western Blot Stripping Buffer	Thermo Scientific	46430
RNase-free water	Qiagen	129112
RNaseOUT™ (40 units/µl)	Invitrogen	10777-019
RNeasy Plus Mini Kit (50)	QIAGEN	74134
Secondary AB Polyclonal Goat Anti-mouse	Dako Denmark A/S	P0447
Secondary AB Polyclonal Goat Anti-Rabbit	Dako Denmark A/S	P0448
Sigma 7-9 (Trisma Base)	Sigma Aldrich	T1378-1KG
Sodium azide	Sigma Aldrich	S8032
Sodium chloride	Sigma Aldrich	S7653-5KG
Sodium Dodecyl Sulphate (SDS)	Sigma Aldrich	L4390
Sodium phosphate	Sigma	S9763
Sodium Tetraborate	Sigma-Aldrich	221732
SuperScript™ III Reverse Transcriptase	Invitrogen	18080-093 18080-044 18080-085
TaqMan® Gene Expression MM	AB by life technologies	4369016
TE Buffer	Invitrogen	12090-015
Tetramethylethylenediamine (TEMED)	Sigma	T9281-25ml
Triton X-100 SigmaUltra	Sigma	T9284-500ml

6.2.4 Genes/Primer human

Gen	Company	Forward	Reverse	Size
TCF7	Eurofins mwg operon	TCAACCAGATCCTGGG TCGC	CCTTTCCTTGCGGGC CAG	83 bp
TCF4	Eurofins mwg operon	AATCAAAACAGCTCCTC CGATT	CCATCTTGCCTCTTG GCCG	113 bp
TLE2	Eurofins mwg operon	TATGAGATGTCGTACG GGC	CAGGAAGGGGATAAT CTGAGC	96 bp
TLE4	Eurofins mwg operon	GAAAACCACCAGGAGT TGACC	GTCAGCTCTCCGTTC ATTCC	118 bp
LEF1	Eurofins mwg operon	CACTGACAGTGACCTA ATGC	CAACGACATTCGCTC TCATT	134 bp
ICAT	Eurofins mwg operon	CATGCTGCGGAAGATG GGAT	GGAAAACGCCATCAC CACGT	145 bp
RUNX 1	Eurofins mwg operon	CACCTACCACAGAGCC ATCAA	CTCGGAAAAGGACAA GCTCC	109 bp
RUNX 1_A_4	IDT Integrated DNA Technologies	CAGAGTCAGATGCAGG AGG	CATAACGTGCATTCTG AGG	162 bp

Gen	Company	Forward	Reverse	Size
RUNX 1_BC	IDT Integrated DNA Technologies	TCAGTCCTACCAATACC TGG	TCGCTGAACGCTGTC AGG	150 bp
RUNX 1_C	IDT Integrated DNA Technologies	GAAGAGGGTGCATTTT CAGG	AAGGATTCATTCCAAG TATGC	110 bp

6.2.5 House-keeping genes human

Gen	Company	Forward	Reverse	Size
G2NB L	Eurofins mwg operon	TCCATACCTTGACCAGC TTG	GCAGATTGTCTCTGGA TCTC	179 bp
ENOX 2	Eurofins mwg operon	GAGCTGGAGGGAACCT GATTT	CACTGGCACTACCAAA CTGCA	123 bp
UBE2 D2	Eurofins mwg operon	CCATGGCTCTGAAGAGA ATCC	GATAGGGACTGTCATT TGGCC	138 bp
TYW1	Eurofins mwg operon	ATTGTCATCAAGACGCA GGGC	GTTGCGAATCCCTTCG CTGTT	168 bp

6.2.6 Gene/Primer mouse

Gen	Company	Forward	Reverse	Size
tcf7	Eurofins mwg operon	ATAGACAGCACTTCCCT GCC	ACCAGATCCCAGCATC AAGG	86 bp
tcfl1	Eurofins mwg operon	ACGAATCGGAGAATCAG AGC	TCACTTCGGCGAAATA GTCG	105 bp
tcfl2	Eurofins mwg operon	AGACAAACCCTCAAGGA TGC	GTTGCACCACCGGTAC TTTG	217 bp
tle1	Eurofins mwg operon	CGTCTGCTGAAGAAAGA TGC	ATGCAGGCTCACTTCT TTGG	93 bp
tle2	Eurofins mwg operon	CATTATGTGATGGCTGC ACC	CGTTTCACAATCTCAG CCTG	143 bp
tle3	Eurofins mwg operon	TGCAAGCTCAGTATCAC AGC	CAATCTCTGTCTGCTTG TGC	138 bp
tle4	Eurofins mwg operon	GATGTGTCCAATGAGGA TC	CTGGAAGATGCAACAG AAGC	131 bp
lef1	Eurofins mwg operon	AGAGAACACCCTGATGA AGG	ACGGGTCGCTGTTCAT ATTG	112 bp
βcat IP1	Eurofins mwg operon	GGAATTTCTGCGCACCT ATG	ATCTGGAAAACGCCAT CACC	107 bp

6.2.7 House-keeping genes mouse

Gen	Company	Forward	Reverse	Size
tbp	Eurofins mwg operon	GAGAGCTCTGGAATTGT ACC	GCTGCTAGTCTGGATT GTTC	233 bp
b2m	Eurofins mwg operon	GGCGTCAACAATGCTGC TTCT	CTTTCTGTGTTTCCCGC TCCC	278 bp
gusb	Eurofins mwg operon	CACGAGGATTCAGATAT CCG	GCCTCGTTGCCAAAAC TCTGT	215 bp

6.2.8 Primary antibodies

Antibody	Catalogue reference	Species	Protein size (kDa)	Blocking	Conc. for Western blot
Anti-AML1	Calbiochem, PC284	Rabbit	58	5% milk blocking soln	1:1,000 in 1x BSA Blocking soln
TLE1/2/3/4	Cell Signaling, #46815	Rabbit	78 – 90	5x BSA Blocking soln	1:2,000 in 1x BSA Blocking soln

Antibody	Catalogue reference	Species	Protein size (kDa)	Blocking	Conc. for Western blot
TCF1 (TCF7)	Cell Signaling, #2203	Rabbit	48, 50	5x BSA Blocking soln	1:1,000 in 1x BSA Blocking soln
LEF1	Cell Signaling, #2230	Rabbit	25 - 58	5x BSA Blocking soln	1:1,000 in 1x BSA Blocking soln
β-catenin	BD, #610154	Mouse	92	5x BSA Blocking soln	1:2,500 in 1x BSA Blocking soln
Active β-catenin	Millipore, #05-665	Mouse	92	5x BSA Blocking soln	1:500 in 1x BSA Blocking soln
SH-PTP2	Santa cruz, sc-280	Rabbit	70	5x BSA Blocking soln	1:1,000 in 1x BSA blocking soln
PDGfβ	Upstate, #06-498	Rabbit	92	5x BSA Blocking soln	1:2,000 in 1x BSA Blocking soln

6.2.9 Secondary antibodies

Antibody	Catalogue reference	Conc. for Western blot
Secondary AB Polyclonal Goat Anti-mouse	Dako Denmark A/S, P0447	1:10,000 in 1x TBSN
Secondary AB Polyclonal Goat Anti-Rabbit	Dako Denmark A/S, P0448	1:10,000 in 1x TBSN

6.2.10 IF antibodies

Antibody	Catalogue reference	Concentration
TCF7	see table “Primary antibodies”	1:100 in IF blocking soln
LEF1	see table “Primary antibodies”	1:100 in IF blocking soln
TLE1/2/3/4	see table “Primary antibodies”	1:100 in IF blocking soln
Anti-AML1	see table “Primary antibodies”	1:100 in IF blocking soln
β-catenin	see table “Primary antibodies”	1:100 in IF blocking soln
Active β-catenin	see table “Primary antibodies”	1:100 in IF blocking soln
Alexa Fluor® 488 Goat Anti-Rabbit	Thermo fisher scientific, A-11008	1:200 in IF blocking soln
Alexa Fluor® 594 Goat anti-Rabbit	Thermo fisher scientific, A-11072	1:200 in IF blocking soln
Alexa Fluor® 488 Goat anti-Mouse	Thermo fisher scientific, A-11001	1:200 in IF blocking soln
Alexa Fluor® 594 Goat anti-Mouse	Thermo fisher scientific	1:200 in IF blocking soln

6.2.11 Drugs

GSK-3 Inhibitor IX (BIO), cat# 361550, Merck Millipore (Calbiochem)

Tankyrase 1/2 Inhibitor III (XAV), cat# 575545, Merck Millipore (Calbiochem)

Wnt3α, from CHO cells extracted protein in Ham’s F12 Media. Kindly provided form Paul O’Gorman Leukaemia Research Centre, Glasgow.

6.2.12 Media

0.1 M Sodium Tetraborate in PBS, pH 8.3

0.1 % (w/v) Gelatine in ddH_2O

0.5% (w/v) Triton-X100 in PBS

1M Tris-HCl (ph 6.8, 7.5 and 8.8)

In a 500 ml bottle 60.6 g Trisma base was diluted in dH_2O and the pH was adjusted to 6.8, 7.5 or 8.8.

1x PBS – Phosphate Buffered Saline

137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4 was added to a 1l bottle and diluted with dH_2O. The pH was adjusted to 7.4 with HCl and filled up with dH_2O to 1 l.

1x TBSN – Tris Buffered Saline with NP 40

0.5 % (v/v) of 10 % NP 40 was diluted in 1000 ml of 1x TBS

4% (v/v) Paraformaldehyde in PBS

5x Sample Buffer

10 % (w/v) SDS was diluted in ddH_2O, 200 mM Tris-HCl pH 6.8, 50 % (w/v) glycerol, Bromophenol blue and 5 % (v/v) 2-mercaptoethanol (50 µl ml^{-1}) were added.

10 % APS

0.1 g APS was diluted in 1 ml dH_2O.

10 % NP 40

25 mL Nonidet P40 was combined with 225 ml of dH_2O.

10 % SDS

10 g SDS was diluted in 91 ml dH_2O.

10x SDS-page running buffer

In a 1 L bottle 149.2 g Glycine, 30 g Trisma base and 10 g SDS were dissolved in 1 ml dH_2O. For 1x SDS-page running buffer 10 % Stock was diluted in dH_2O.

10x Semi-dry transfer buffer

29.3 g Glycine, 58.1 g Trisma base and 3.75 g SDS were added to a 1 L bottle and diluted with dH_2O to a total volume of 1 L. For 1x Semi-dry transfer buffer 200 ml Methanol, 100 ml 10x stock and 700 ml dH_2O were combined.

10x TBS – Tris Buffered Saline (pH 7.5)

In a 1l bottle 87.6 g NaCl and 24.2 g Trisma base were mixed in 900 ml dH_2O, the pH was adjusted to 7.5 with HCl and filled up to 1 L. For 1x TBS 100 ml of 10x TBS was combined with 900 ml of dH_2O.

Blocking solutions for Immunofluorescence

5 % (w/v) BSA and 1 % (w/v) Ovalbumin in TBS with 0.2 % Triton-X100

Blocking solutions for Western Blot

5x BSA blocking solution:

25 g BSA, 5 g Ovalbumin and a pinch of sodium azide were dissolved in 500 ml 1x TBS.

1x BSA blocking solution:

20 % (v/v) of 5x BSA blocking solution was diluted in 1x TBS.

5 % milk blocking solution:

1.5 g Marvel dry milk was dissolved in 30 ml 1x TBS.

Gels for Western blotting

Running gel for Western Blot

Reagent	7.5% gel	10% gel	12% gel
ddH_2O	36.55 %	28.39 %	21.87 %
1M Tris-HCl, pH 8.8	36.55 %	36.55 %	36.55 %
10% SDS	1.63 %	1.63 %	1.63 %
30% Acrylamide	24.48 %	32.64 %	39.16 %
10% APS	0.65 %	0.65 %	0.65 %
Temed	0.13 %	0.13 %	0.13 %

5% Stacking gel for Western Blot

Reagent	5% gel
ddH_2O	65.65 %
1M Tris-HCl, pH 6.8	13.68 %
10% SDS	1.64 %
30% Acrylamide	18.27 %
10% APS	0.55 %
Temed	0.22 %

Poly-L-Lysine stock

10 mg ml^{-1} Poly-L-Lysine was dissolved in 0.1 M Sodium Tetraborate in PBS.

Protein Solubilisation Buffer

This buffer was used to lyses and store protein samples. If PMSF was added to the buffer, it could be used up to one week, without PMSF the buffer could be stored for up to three month.

First, the Solubilisation Buffer was prepared: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 % Nonidet P40, 10 % Glycerol and 5 mM EDTA pH 8.0.

Afterwards, the Phosphatase and Protease Inhibitors were added: 1 mM Sodium Orthovanadate, 1 mM Sodium Molybdate, 1 mM Sodium Fluoride, 40µg ml^{-1} Phenylmethylsulphonyl Fluoride, 0.7µg ml^{-1} Pepstatin A, 10 µg ml^{-1} Aprotinin, 10 µg ml^{-1} Leupeptin, 10 µg ml^{-1} Soybean trypsin inhibitor.

Stripping buffer for Western Blot

Mild stripping buffer:

15 g Glycine, 1 g SDS and 10 ml Tween 20 were added to a 1 L bottle, the pH was adjusted to 2.2 and filled up to 1 L with ddH_2O.

Strong stripping buffer, 5x:

10 g SDS was dissolved in 31.25 ml Tris-HCl pH 7.5 and filled up with dH_2O to 100 ml.

Strong stripping buffer, 1x:

5x stripping buffer was diluted to 1x stripping buffer with ddH_2O and 660 ml 2-ME per 100 ml 1x stripping buffer was added.

Working stock Poly-L-Lysine, 5 µg ml^{-1}

The Stock was diluted in 0.1 M Sodium Tetraborate in PBS.

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