Roskilde University

Master Thesis

Sandra Dalby Johansen

Modulation of CDX2's gene regulatory activity through posttranscriptional modifications

Supervisor:

Professor Jesper T. Troelsen

Professor in Medical Biology, Ph.D, DMsc

Department of Science and Environment / Institut for Naturvidenskab og Miljø

1 PREFACE AND ACKNOWLEDGMENT

This master thesis was conducted at the Department of Science and environment at Roskilde university with Troelsen research unit. The research took place from 1 of August 2020 to the 1 of July 2021 and is equal to 60 ECTS point. The thesis was supervised by internal professor Jesper T. Troelsen.

I would like first and foremost to thank Jesper T. Troelsen for his infinite patience as well guidance with daily problems and pointing me in the right direction. I would like to thank the three Ph.D students Stine Bull Jensen, Josephine Bjergbæk Olsson and Janni Jørgensen for always being helpful in the laboratory and providing me with helpful tips for the project, when experiments when south and a thanks to Marianne Lauridsen for always helping with the technical parts of the experiments and never being tired of being asked the same questions repeatedly.

A special thanks to the two master students Susanna Selvarajah and Rikke Nørgaard Nielsen, for making it fun conducting experiments in the lab and for always helping during the long days in the laboratory.

Finally, I owe a huge thanks to my family and my boyfriend for their unyielding support and encouragement during the last year and for whom it would have been impossible for me to focus entirely on finishing my master's degree.

Sandra Dalby Johansen

01.07.2021

2 ABSTRACT

Patients with high-risk stage II colorectal cancer lack Caudal homeobox 2 (CDX2) expression and have appeared to benefit from adjuvant chemotherapy or surgery. CDX2 is a key regulator in maintaining the intestinal homeostasis and in important for the differentiation of epithelial cells. CDX2 needs to be tightly regulated to maintain homeostasis of the colon and the differentiation of the epithelial cells otherwise it can lead to cancerous consequences.

This thesis investigates modulations of CDX2's gene regulatory activity through posttranscriptional modifications. This is done by using the new cell line LS174T7D9, in which two system can regulate the CDX2 driven promoter activity of pGL4.10-IL-33-promoter-enhancer and Wnt driven promoter activity of TOPflash through induction with doxycycline (DOX). Screening of 196 kinase inhibitor aids in identifying a regulatory effect on the CDX2 and last identify CDX'2 regulatory effect on target genes *ABCB1, IL-33* and *MYC* expression.

From the 196 kinases inhibitor, 4 KIs was found to have a regulatory effect on CDX2 and showed significant difference. These were SB 525334, PD0332991, Everolimus and XL765, all kinase inhibitors that regulates cell proliferation, cell survival, cell growth, cell cycle and cell differentiation.

LS174T7D9 is a promising new model organism for researching modulations of CDX2's gene regulatory activity through posttranscriptional modifications and it provided 4 new pathways in which CDX2 is regulated on a posttranslational level.

3 TABLE OF CONTENTS

1 Preface and acknowledgment i
2 Abstract ii
3 Table of contents iii
4 Introduction 1
5 Background 3
5.1 CDX2 and its role in regulating and maintaining the colon epithelia 3
5.1.1 Normal homeostasis of colon epithelial 3
5.1.2 The canonical Wnt signalling pathway 4
5.1.3 CDX2's role in differentiation of the colon epithelial 7
5.1.4 How is the intestinal homeostasis regulated by CDX2? 7
5.1.5 Regulation of CDX2 9
5.2 Colorectal cancer development 10
5.2.1 CDX2's role in tumourigenesis and metastasis 12
5.2.2 Wnt signalling and tumourigenesis of CRC 15
5.2.3 CDX2 and Wnt signalling cross-talk 16
5.3 Cytokine alarmin IL-33's physiological function and regulation 17
5.3.1 Production, release, and processing of IL-33 18
5.3.2 IL-33 as a guardian of barriers and conductor of local inflammation 19
5.3.3 Regulation of intracellular IL-33 21
5.3.4 IL-33 and tumourigenesis of CRC 22
6 theoretical methods section 25
6.1 Tet-On/Tetracycline-response element, CMV-Lucia system, and IL-33/TOPflash constructs 25
7 Methods and materials 28
7.1 Cultivation of cell lines 28
7.2 Plasmid constructs 28
7.2.1 pGL.4.10 with IL-33 promoter and enhancer for analysis of CDX2 regulation 28
7.3 Transfection, Luciferase (firefly) promoter assay and Measurement of Lucia luciferase Quanti-Luc measuring of reporter activity 28
7.3.1 Induction with doxycycline 28
7.3.2 Transfection 29
7.3.3 Luciferase promoter assay 30

7.3.4 Quanti-Luc 30
7.4 Screening of kinase inhibitor library 30
7.4.1 Kinase inhibitor screening library 30
7.5 Kinase inhibitors regulatory effect on CDX2 target genes 31
7.5.1 RT-qPCR 31
7.6 Statistical analysis 32
8 Results 33
8.1 Creating the optimal protocol for investigating the expression of CDX2 through Reporter luciferase assays 33
8.1.1 Optimal promoter system 33
8.1.2 Validation of pGL4.10-IL-33-promoter-enhancer activity compared to other promoters 34
8.1.3 Optimal induction time and DOX concentration 35
8.2 Screening of kinase inhibitor library on LS174T-7D9 cells with TetOn and lucia system 36
8.3 Screening of kinase inhibitor library 37
8.3.1 CDX2 driven promoter activity of pGL4.10-IL-33-promoter-enhancer when stimulated with the selected 19 KIs 40
8.3.1 Wnt driven promoter activity of pGL4.10-IL-33-promoter-enhancer when stimulated with the selected 19 KIs 45
8.4 RT-qPCR of ABCB1, IL-33 and MYC 50
9 Discussion 53
9.1 LS174T7D9 an model organism to investigate regulatory mechanisms behind CDX2 53
9.2 a possible reestablishment of CDX2 in CRC cells and decrease in further progression 56
9.3 CDX2's possible involvement in chemoresistance, crosstalk with Wnt and local inflammation 60
9.3.1 ABCB1 transporter 60
9.3.2 Wnt pathway's target gene MYC 61
9.3.3 IL-33 a guardian of barriers and conductor of local inflammation 62
10 Conclusion 64
11 Future research 65
12 References 66
13 Appendix 1
13.1 Overview of knockout of CDX2 by TetOn and integration into the AAVS1 locus by ZFN (Pinto et al., 2017) 1

13.2 pSelect-zeocin-lucia promoter construct for recombinant insertion into AAVS1 locus site 1
13.3 In-Fusion cloning of pGL4.10-IL-33+promoter+enhancer (Larsen et al., 2021) 2
13.4 TOPflash promoter construct 2
13.5 Overview of Selleck Chemicals Kinase inhibitor screening library 3
13.6 Concentrations of purified RNA and total concentration in 2 6
13.7 Rt-qPCR program 6
13.8 Placement of primer and the different variant of the target genes used for RT-qPCR. 7
13.9 Data from kinase inhibitor screen with LS174T7D9 8
13.10 qPCR amplification plot and melting curve ananlysis on first set of data 15

4 INTRODUCTION

On average, from 2012-2016, 1.721 males and 1.658 females were diagnosed with colorectal cancer (CRC) each year in Denmark. The survival rate has been increasing over the last couple of years, with 84% males and 85% of females surviving after 1 year. After 5 years the survival rate is 63% for males and 65% for females (Nordcan & Kræftens bekæmpelse - visited 17/06/2021). Patients with CRC often undergoes treatment either by chemotherapy or surgery or a combination of both. However, 30% of these patient experience relapse after surgery. It has been found that patients with a high-risk stage II CRC lack Caudal homeobox 2 (CDX2) expression and have appeared to benefit from adjuvant chemotherapy. Therefore, it has been concluded that CDX2 expression is a possible prognostic biomarker for both stage II and stage III of colon cancer (Dalerba et al., 2016). However, the current knowledge of how the mechanism that is controlling the expression of the CDX2 gene/protein is deficient. CDX2 is a key regulator in the intestinal epithelium and the genes that are regulated by CDX2 are important for differentiation and homeostasis of the intestinal epithelia cells. Further studies have indicated that knock-out of the CDX2 gene removes the normal state of the epithelia function and thereby increases the risk of the intestinal cells to transform into cancer like cells. This indicated that the CDX2 function as a tumor suppressor (Hryniuk et la., 2014). However, non-intestinal tissue with aberrant CDX2 expression act as an oncogenic driver (Barros et al., 2012; Rawat et al., 2012; Tamagawa et al., 2012). Therefore, tight regulation of the CDX2 activity for normal differentiation and homeostasis in the different tissues are important, if not regulated properly it can lead to cancerous consequences. Moreover, Larsen et al. 2018 investigated the transcriptional regulation of *interleukin 33* (*IL-33*) and alarmin and found that CDX2 is an important transcription factor for IL-33 promoter activity and have a key role in the high expression levels in the intestinal epithelial cells. Currently, the mechanisms behind the transcription and regulation of IL-33 inside the nucleus remains elusive, however its function outside the cell is comprehensive. Furthermore, there have been indications of cross talk between CDX2 and the stem cell niche regulator Wingless-type (Wnt) signalling pathway, which controls the maintenance of the stem cell niche, cell proliferation, cell migration and cell fate all key components for maintaining the progression of CRC development. Based on these previous finding, this thesis will investigate the following hypothesis and aim:

Hypothesis:

CDX2 has a regulatory role in the development of colorectal cancer.

Aim:

To investigate possible modulations of CDX2's gene regulatory activity through posttranscriptional modifications.

More specifically, the goals were:

- To use the newly modified cell line LS174T7D9 for the induction of CDX2 driven promoter activity of pGL4.10-IL-33-promoter-enhancer construct and Wnt driven promoter activity of TOPflash construct
- To identify kinase inhibitors that have a regulatory effect on the promoter activity by screening a kinase inhibitor library
- To identify CDX2's regulatory effect on target genes *ABCB1*, *IL/33* and *MYC* expression, when stimulated with top five kinase inhibitors selected from the screening of the kinase inhibitor library

5 BACKGROUND

5.1 CDX2 AND ITS ROLE IN REGULATING AND MAINTAINING THE COLON EPITHELIA

5.1.1 Normal homeostasis of colon epithelial

The human intestinal epithelial are rapidly regenerated and cell proliferation and differentiation of the epithelial in the small intestines and colon are tightly regulated by various genes and transcriptions factors and through this homeostasis are maintained. The structure of the colon epithelial are lined with tubular glands called crypts, which turn inwards. The crypts are alle divided in to three parts and each part have a different function. At the bottom is the stem cell niche which divide and then produce the proliferating cells also called transient amplifying cells, which then moves up to reside in the middle of the crypt. The transient proliferating cells migrate further to the top of the crypt and the cells become more differentiated. Different types of colonocytes produced from the stem cells and transient amplifying cells are goblet cells, which secret mucous, Paneth cells, which is a part of the innate immune system, and enteroendocrine cells, which produce/release hormones. The differentiated cells remain at the luminal surface until they undergo apoptosis or phagocytosis and are thereby shredded into the lumen. This occurs approx. 5-7 days after the stem cells starts producing the cells at the bottom of the crypts.

At the bottom of the crypt is the stem cell niche, which is maintained by epidermal growth factors (EGF), Notch inhibitors of bone morphogenetics protein (BMP) and wingless-type (Wnt) (Spit et al., 2018) As shown in Figure 1, the expression of the different signalling components variants depending on where in the crypts you look. All four signalling components has highly expressed at the bottom of the crypt. When moving along the transient amplifying zone Wnt, EGF and Notch is still expressed, however, BMP inhibitors decreases and the level of BMP increases moving up to the differentiated cells and toward the luminal surface. When the cells are differentiated the expression levels of Wnt, EGF and Notch are decreased.

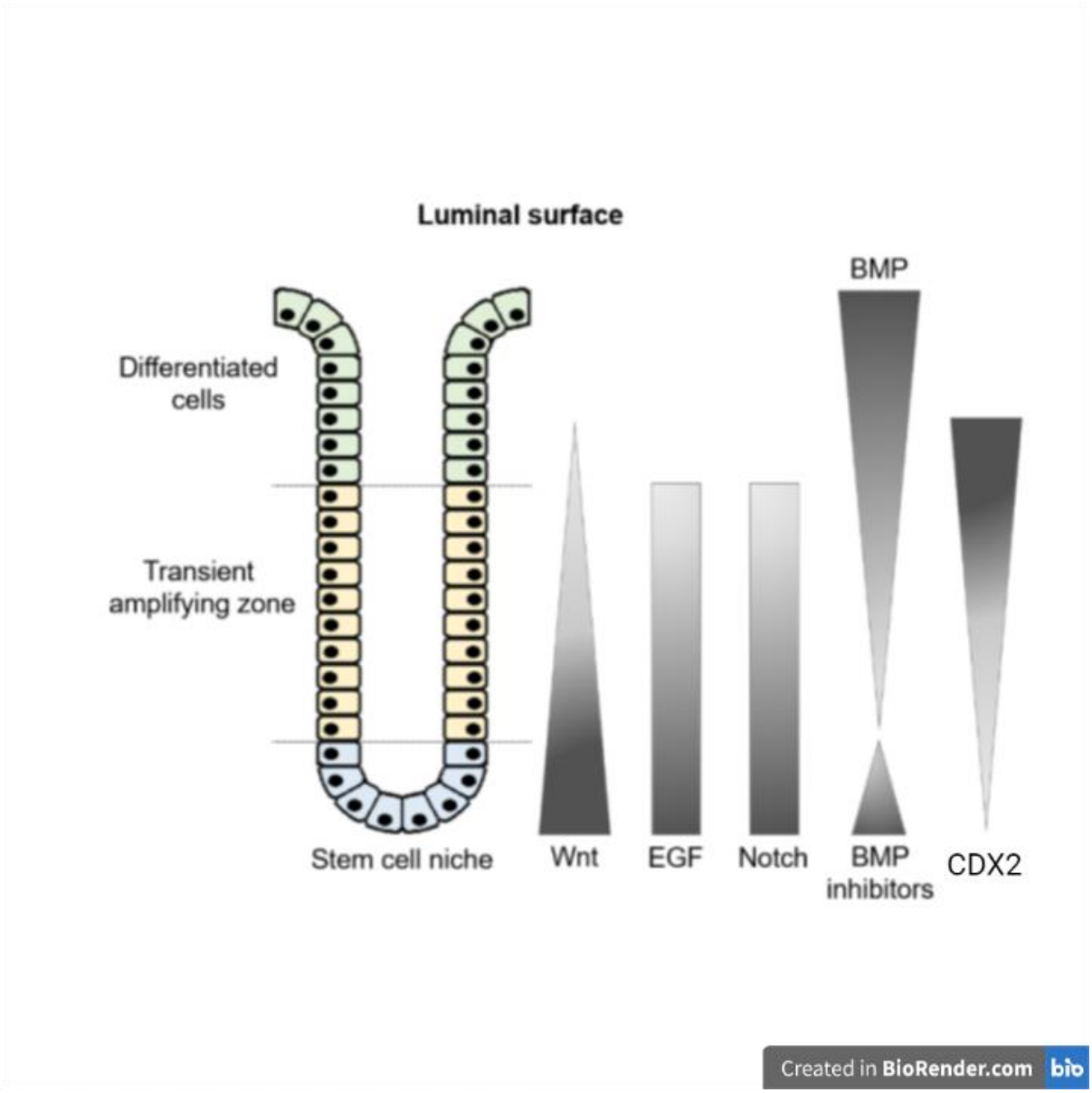


Figure 1 Signalling components in the stem cell niche, proliferation and differentiation of the colon epithelial. The figure shows that during the stem cell niche and proliferation the Wnt expression is high. EGF and Notch remain constant during the stem cell niche and almost throughout the cell proliferating state. BMP inhibitors are expressed during the stem cell niche but decreases before cell proliferation and then slowing start to increase as the cells goes from a proliferating state to differentiating state. CDX2's expression remains decrease during the stem cell niche but increase along cell proliferation and is high during differentiation. When Wnt decrease during cell proliferation, CDX2 increase in expression during cell proliferation into differentiation (modified version from Davidsen et al.,2018).

5.1.2 The canonical Wnt signalling pathway

Wnt transduction signalling happens through the binding of Wnt ligands from the extracellular matrix (ECM) to its receptor. Moreover, the activation of the Wnt pathway is also dependent on the presence of R-spondins, which is a soluble protein that binds to LGR family transmembrane receptor (Yan et al., 2017). If R-spondins are not present, this will lead to the degradation of the receptors Frizzled (FDZD) and lipoprotein receptor-related protein (LRP) through ubiquitination (Hao et al., 2012), which both are part of Wnt's receptor. The

signalling pathway can operate in two different ways, either through non-canonical or canonical. Non-canonical Wnt pathway regulates cell polarization during epithelial development and intracellular Ca2+ influx (Hoppler & Kavanagh, 2007), whereas the activation of the canonical Wnt pathway regulates targets genes and stem cell renewal, cell proliferation, cell migration and cell survival. (Komiya & Habas, 2008; Fagotto et al., 1998; Cong & Varmus, 2004; Schwarz-Romond et al., 2007).

Figure 2 illustrates the following: If Wnt is not present, LRP and FDZD maintain their position in the cell membrane. The degradation complex containing scaffold protein Axin, Casein kinase 1 α (CK1α), Glycogen synthase kinase 3 (GSK3), Adenomatosis Polyposis Coli (APC) and β-catenin. β-catenin is phosphorylated by CK1 α and then degraded through ubiquitin-mediated proteolysis, which leads to no transcriptional activation of target genes in the nucleus. In the absence of β-catenin , transcription factor 7-like 2 (TCF7L2) forms a complex with the co-repressor Groucho, making TCF7L2 a transcriptional repressor. When Wnt is present in the ECM it binds to the FDZD/LRP complex and induces membrane translocation. Dishevelled (Dsh) inhibits the activity of GSK-3β enzyme in the degradation complex, which leads to the accumulation and stabilization of β-catenin in the cytoplasm. The translocation of β-catenin to the nucleus remains elusive. It is proposed that it is transported together with other factors, Axin being one of the possible factors. In the nucleus, β-catenin replaces the co-repressor Groucho, thereby turning TCF7L2 into a transcriptional activator (Kriegl et al., 2010; Pinto et al., 2003), which makes β-catenin a co-activator. This complex then activates the transcription of downstream target genes such as *c-myc* and *cycline D1* (Fevr et al., 2007; Yu et al., 2019)

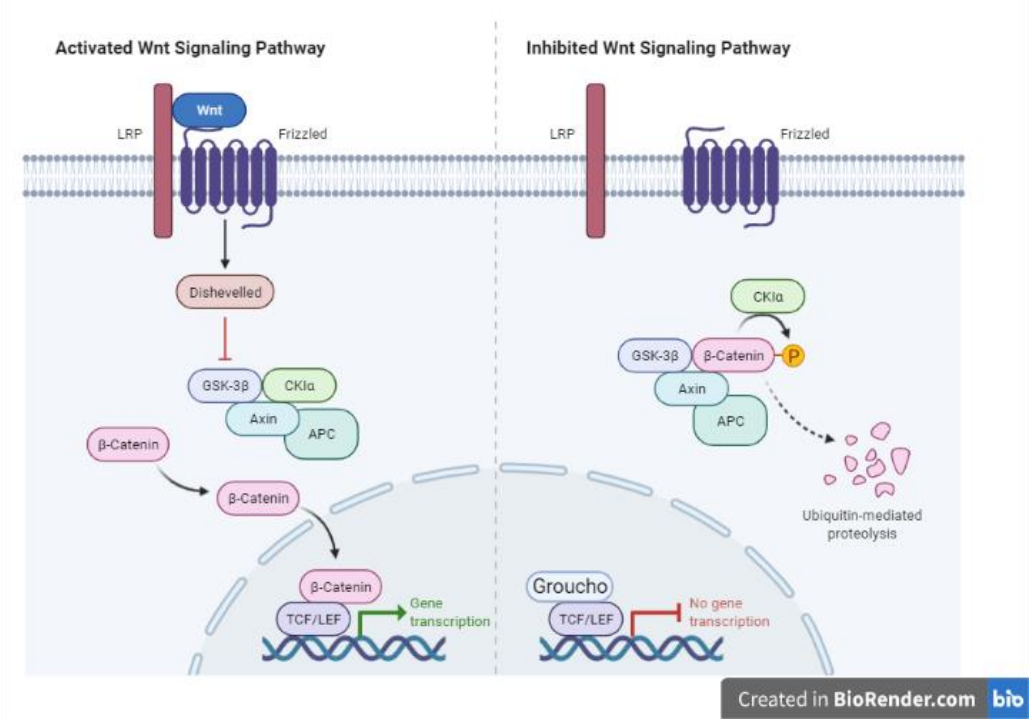


Figure 2 Wnt signalling pathway. Left: When Wnt is not present, beta-catenin is degraded by the degradation complex, which consists of GSK3beta, CKIalfa, APC and AXIN. Beta-catenin remain in the intracellular space and transcription of target genes is inhibited by Groucho binding to the TCFL" transcription factor. Right: When Wnt ligands are present and bind to the Wnt receptor (LRP/FZD), beta-catenin will accumulate inside the intracellular space and cross the nucleus membrane. Here it binds to TCF7L2 and initiate transcription of target gene (Created with biorender template for Wnt signalling pathway).

Studies of Wnt signalling have, as already stated, an important role in maintaining the stem cell niche, while other studies have investigated its role maintaining the transient amplifying zone of the intestinal crypts (Kuhnert et al., 2005; Pinto et al., 2003). A study using genetically modified mice, which lacked TCF7L2, showed the important role of Wnt signalling, in relation to the lack of proliferative ability of the intestinal crypts (Korinek et la., 1998). When deleting beta-catenin and TCF7L2 together with overexpression of Dickkopfl, an inhibitor of Wnt, resulted in a ceased proliferation of the crypts as well (Fevr et al., 2007; Pinto et al., 2003; van Es et al., 2012). Under normal homeostasis, Wnt decreases when the cell stops to proliferate and start to differentiate, this has been found to be correlated with TCF7L2 trigger the shift in the two states (Kuhnert et la., 2004; Mariadason et al., 2001; Pinto et al., 2003).

5.1.3 CDX2's role in differentiation of the colon epithelial

A key factor involved in the regulation of proliferation and differentiation of the epithelial is Caudal homeobox 2 (CDX2). CDX2 is a homeodomain transcription factor and is important for the development and homeostasis of the colon. CDX2 binds to gene enhancers and promoter regions on DNA and functions mainly as an activator (Pinto et al., 2017) but sometimes also a repressor (Drummond et al., 1997). Previous articles have shown that the differentiation of colon epithelial is downregulated in the absence of CDX2 and that CDX2 is important for the differentiation of the colon epithelium. Further results showed that murine experiments with knock-down of CDX2 revealed severe diarrhoea and rapid defecation, but continuation of proliferation and lack of differentiation of the colon epithelium (Natoli et al., 2013; Hryniuk et al., 2012). One study using intestinal organoids, which did not have CDX2 expression present in the cells, showed that the differentiation of the cells turns into a gastric like lineage, rather than the differentiation into the colon epithelium (Simmini et al., 2014). CDX2 further modulates and regulates cell adhesion, migration, and tumorigenesis. If the controlled expression of CDX2 is lost, it can lead to various intestinal diseases and developmental disorders such as inflammatory bowel disease, Crohn's disease, and colorectal cancer (CRC). The last is the focus of this thesis.

5.1.4 How is the intestinal homeostasis regulated by CDX2?

Many kinds of genes are involved in the functioning and structure of the colon. These genes have been shown to be transcriptionally regulated by CDX2. CDX2 regulates different processes taking place in the colon such as nutrients breakdown and uptake, cell-cell adhesion and tight junctions, regulation of transcription factors, the intestinal barrier and cell migration (**Error! Reference source not found.**). Transport of nutrients such as iron have been shown to be regulated by CDX2. Expression of the iron-transport protein *hephaestin* (*HEPH*), which transports iron from processed food, is regulated by CDX2 (Boyd et al., 2010; Coskun et al., 2012; Hinoi et al., 2005). Furthermore, the *enzyme Meprin 1 A* (*MEP1A*) and lactase is also regulated by CDX2. MEP1A is a membrane associated protein, that hydrolysis various peptides and protein substrates, whereas lactase is responsible for hydrolysis of lactose. These findings highlight CDX2's role in breakdown and transport of nutrients. To maintain and uphold the integrity of the colon, CDX2 has been found to regulate different factors which is important for cell adhesion and tight junctions. CDX2 has been linked to the cadherins family, which mediates cell-cell adhesion, and has been coupled to transcription of *glycoprotein A33* (*GPA33*) (Hinio et al., 2002; Pinto et al., 2017). GPA33 has been implicated in the cell-cell adhesion and maintenance of the intestinal barrier

function (Johnstone et al., 2000; Ackerman et al., 2008; Williams et al., 2015). CDX2 has further been linked to claudins, which is membrane proteins that act as a critical component in creating tight junctions between epithelial cells (Bhat et al., 2012; Sakaguchi et al., 2002; Satake et al., 2008). Tight junction further upholds the permeability of the epithelial barrier, and it has been shown that CDX2 regulates the transcription of *ST14*, which encodes transmembrane serine protease matripase a component of maintaining the permeability. On a post-translational level, matripase is regulated by two inhibitors *SPINT1* and *SPINT2*. *SPNT1* transcription is further regulated by CDX2 as well (Danielsen et al., 2018). Together with permeability, the surface of the gastrointestinal tract must be protected against acid, enzymes, food, and bacteria and one way to protect the surface is the production of mucins (Krishn er al., 2016). Mucins are secreted by goblet cells, and they give a protective layer of lubrication on the surface. A study has shown that a number of mucins are transcriptionally regulated by CDX2, these mucins being *MUC5AC*, *MUC5B* and *MUC6* (Pinto et al., 2017). In relation to cell migration taking place in the colon, two genes have been seen to be transcriptionally regulated by CDX2. The first being laminin subunit γ 2 (LAMC2) an extracellular matrix (ECM) protein important for cell migration and the second being *ELMO* 3 which encodes a scaffold protein that stimulate cell migration. CDX2 is further involved in transcriptional regulation of other transcription factors, which is important for e.g., proliferation, migration, adhesion, ECM organization, crypt formation, cell-cell adhesion, and goblet cell maturation. *Yes-associated protein 1* (YAP1) is a co-transcription factor and it have been shown that genes important for proliferation, adhesion, migration and ECM organization is regulated by YAP1. CDX2 regulates YAP1 by binding to its enhancer region and thereby regulates the before mentioned processes (Larsen et al., 2019). Another transcription factor regulated by CDX2 is *hepatocyte nuclear factor 4 α* (*HNF-α*) gene (Boyd et al., 2010), this transcription factor plays a vital role in crypt formation, cell-cell adhesion, and goblet cell maturation (Cattin et al., 2009; Garrison et al., 2006). Studies have indicated at link between CDX2 expression and Wnt-signalling. The studies found a correlation in expression between CDX2, APC and beta-catenin. Loss of CDX2 expression showed an inversely correlation with beta-catenin. Furthermore, experiments with colon cancer cells lines indicated that CDX2 bound to the promoter region of *APC* and *AXIN2*, lead to the activation of transcription (Olsen et al., 2014). Thereby increasing the degradation of beta-catenin and thus stopping cell proliferation, cell fate and cell migration. Taken together, these examples give an insight to how CDX2 cooperates with other broadly expressed transcription factors thereby regulating different aspects of the intestinal homeostasis.

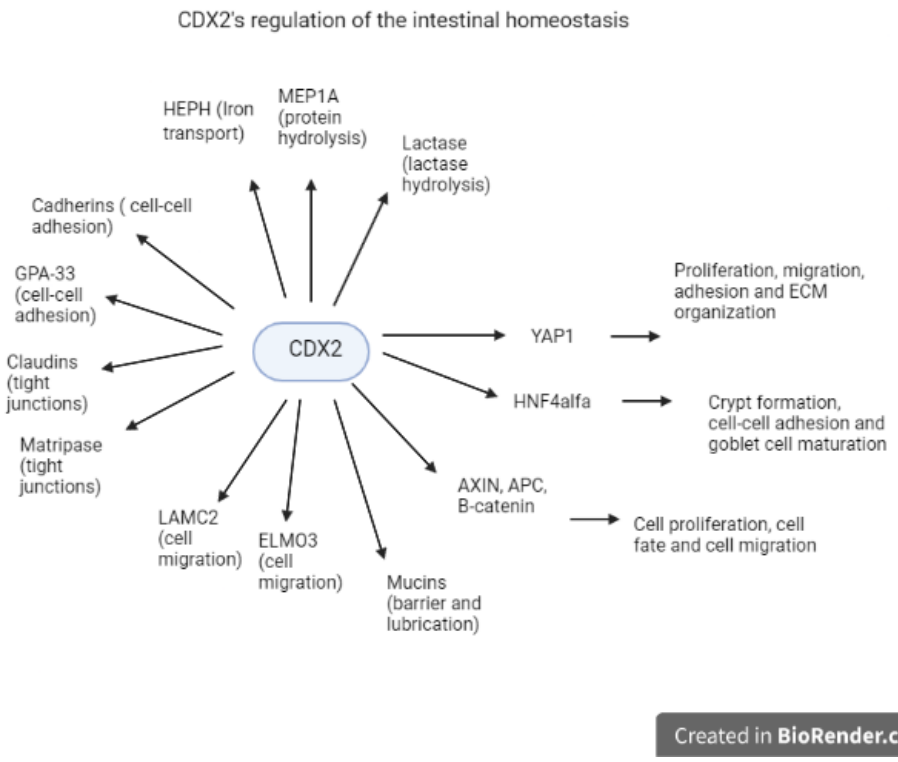


Figure 3 CDX2's role in regulation of the intestinal homeostasis. The illustration shows the CDX2 regulate nutrient uptake through HEPH, MEP1A and lactase. It regulates transcription factors such as YAP1 and HNF4alfa, intestinal barriers such as Mucins, cell migration through ELMO3 and LAMC2, Cell-cell adhesion and tight junction through cadherins, GPA-33, Claudins and Matriptase (Own creation through BioRender).

5.1.5 Regulation of CDX2

Expression of CDX2 is regulated by many different factors (Figure 4). First off, the expression of CDX2, can autoregulate itself by activating transcription from its own promoter (Barros et al., 2011; Boyd et al., 2010). Regulation of CDX2 can happen at both transcriptional and post-translational levels. Transcriptional regulation can happen with a combination of different factors, such as TCF7L2, HNF-alfa, GATA6, NF-KB and promoter methylation, whereas the latter inhibits the expression of CDX2 (Benahmed et al., 2008; Boyd et al., 2009; Kim et al., 2002; Graule et al., 2018). Post-translational regulation of the expression of CDX2 can happen through MAPK pathway, which can control/stimulates cell proliferation. When phosphorylating serine 60 via the MAPK pathway the transactivation capacity decrease of CDX2. This decrease was found by adding the kinase inhibitor U0126, which targets MEK1/2 in the MAPK pathway (Rings et al., 2001). Furthermore, when phosphorylated by p38, which is a member of the MAPK family, the transactivation of CDX2 increased. Kinase inhibitor SB203580 was used to inhibit p38 and thereby showing the increase of the transactivation (Houde et al., 2001). The inflammatory cytokine tumour necrosis factor-alfa (TNF-α) has been showed to influence the expression of CDX2 at the

invasive front and in tumour buddings in CRC. Moreover, oxygen supply might be able changes the expression of CDX2 through hypoxia-inducible factor-1α (HIF-1α). Research showed that *CDX2* mRNA levels decreased when hypoxia was induced in colon cancer cell lines (Zheng et al., 2014). Degradation of CDX2 has shown to be targeted by Cdk2 after its phosphorylation of CDX2 and thereby regulating CDX2 activity in coordination with the cell cycle process (Boulanger et al., 2005). Further regulatory factors of CDX2 are spares and need to be elucidated, which will be one of the main focuses of this thesis.

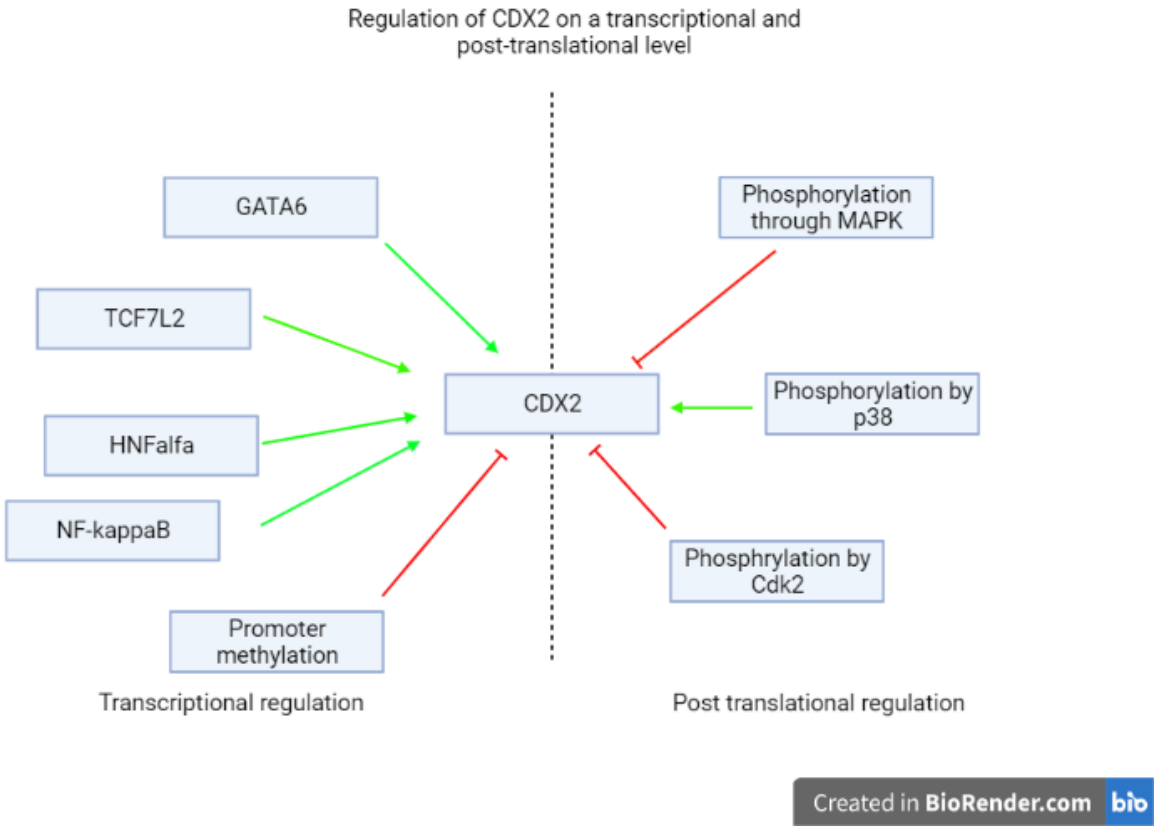


Figure 4 Transcriptional and post-translational regulation of CDX2. The figure illustrates the regulation of CDX2 on a transcriptional level and a post-translational level. CDX2 is regulated by GATA6, TCF7L2, HNFα, NF-κB and by promoter methylation at a transcriptional level. CDX2 is regulated by phosphorylation though MAPK, p38 and Cdk2 on a post-translational level (own creation through BioRender).

5.2 COLORECTAL CANCER DEVELOPMENT

Most colon cancers are adenocarcinomas, which originates from adenomatous precursor lesions/polyps and over time they will grow and degree of dysplasia, this resulting in tumours (Grady & Markowitz, 2016). As mentioned in the introduction the median age when diagnosed with CRC is approximately 70 (Brenner et al., 2014). Risk factors includes, age, which has been the most dominant factor, but also inflammatory bowel disease (Jess et al., 2012) smoking (Liang et al., 2009), obesity (Ma et al., 2013) excessive alcohol consumption

(Fedirko et al., 2011), diabetes (Jiang et al., 2011) high consumption of red and processed meats (Chan et al., 2011) and family history of colon cancer (Taylor et al., 2010). There are increasing incidents of CRC with young adults under 50 and this could be the result of difference in diet, sedentary lifestyle and rise of obesity (Cai et la., 2019; Loomans-Kropp & Umar, 2019). Some of the molecular risk factors for developing CRC includes genetic event and genomic instability (Hannahan & Weinberg, 2011). Genomic and epigenomic instability in colon cancer includes chromosomal instability (CIN), microsatellite stable (MSS), microsatellite instability (MSI) and CpG island methylator phenotype (CIMP). CIN is observed in 70-85% of sporadic cancer and typically comes as duplications or deletions of the either the whole chromosome or parts of the chromosome (Grady & Markowitz, 2016). For MSS and MSI, MSS is often associated with APC loss, which leads to adenomas, whereas MSI is associated with DNA mismatch repair (MMR) defects and these defects results in increased mutation rate of the entire genome (Novellasdemut et al., 2015). When CpG-island is hypermethylated it results in gene silencing through the CIMP pathway. Based on these different pathways a tumour can be the result or exhibit features from multiple pathways a once (Grady & Markowitz, 2016).

The degrees of colon cancer can be determined by describing the primary tumour (T), the involvement of regional lymph node (N) and metastasis to distant sites (M). TNM id further classified into staging groups I-IV, where stages I describe the tumour to be in the submucosa or muscular layer of the colon and stage IV describes the tumour with distant metastasis (Piñeros et al., 2019). A study has shown that 5-year survival with patients have stage I and II is 90% and patients with stages II and III is 71%. This indicate that the mortality rate is mostly dependent on stage of the disease at the time the patients is diagnosed (Mattiuzzi et al., 2019). When diagnosed with CRC the primary treatment is surgical resection of the tumour and dependents on the stage adjuvant chemotherapy is added. Adjuvant chemotherapy is often used for patients with stage III and IV diagnoses, however, some subsets of patients with stage II have shown also to benefit from adjuvant chemotherapy, even though 80% of these patients are cured slowly by surgery (Kannarkatt et al., 2017). In Denmark, the following adjuvant chemotherapy drug is used after surgery, those being 5-Flouroracil (5-FU), Capecitabin and Oxaliplatin (Kræftens bekæmpelse - Visited 17/06/2021). When patients undergo surgery, the surgery can induce a surgical stress response, which can lead to reduced anti-tumoural defense and can increase in inflammatory factors (Neeman & Ben-Eliyahu, 2013), which leads to an increased risk of postoperative

recurrence. Furthermore, van der Bij et al., 2009 found that after curative resection, the risk of relapse was up to 30% with patients. Patients with a high-risk stage II colon cancer lack CDX2 expression and have appeared to benefit from adjuvant chemotherapy. In the following section CDX2's role in colorectal cancer will be elaborated.

5.2.1 CDX2's role in tumorigenesis and metastasis

Loss of CDX2 expression is a common event in tumorigenesis labelling CDX2 a tumour suppressor (Mallo et al., 1997). However, the gene *CDX2* is rarely mutated, but epigenetic changes, e.g hypermutation of the *CDX2's* promoter, are thought to be the leading course of downregulation or loss of expression of CDX2 (Xia et al., 2009; Dawson et al., 2014; Graule et al., 2019). Complete deletion of CDX2 in mice embryos resulted in death before birth and heterozygote *CDX2* mice developed multiple adenomatous polyps (Chawengsakophak et al., 1997). Further studies with mice also showed that when a mosaic deletion of *CDX2* was present the mice a gastric-like lesion occurred but did not spontaneously evolve into cancer. But when paired with mice containing mutant *Apc* there was a significant increase in tumour formation (Balbinot er al., 2018; Hrynuik et al., 2014). It was further indicated that CDX2 depleted cells do not become tumorigenic, but the depleted cells stimulate tumorigenesis in adjacent CDX2 intact *Apc* mutated cells (Balbinot et al., 2018) As mentioned before, CDX2 can function as a tumour suppressor, but studies have found that CDX2 possess oncogenic properties. CDX2 has been considered an oncogene in cancers such as leukaemia, gastric intestinal and esophagus cancers (Barros et al., 2012; Rawat et al., 2012; Tamagawa et al., 2012). Human colon cancer cells, which exhibits chromosomal instability, by having amplified the CDX2 locus, which plays a crucial role in colon cancer cell survival (Subtil et al., 2007). In vitro and in vivo experiments have seen that knock down of CDX2 in colon cancer cells promoters cell proliferation and accelerates tumour formation, respectively. However, overexpression of CDX2 suppresses cell proliferation and viability, which could indicate an inhibition of proliferation of colon cancer cells through CDX2 (Yu et al., 2019). As mentioned earlier, CDX2 regulates the transcription of many genes, that when dysregulated can become tumorigenic. Hereunder is the YAP1 gene, which mentioned earlier is a key regulator in proliferation, adhesion, migration and ECM organization, but it has also been linked to cancer progression in relation with CDX2 (Liang et al., 2014; Steinhardt et al., 2008; Tschaharganeh et al., 2013). Furthermore, YAP1 expression has been associated with poor prognosis in colon cancer and other cancer types as well this being in relation to nuclear localization and overexpression (Xia et al., 2018). On the surface of mucosal epithelial cells

there can be found mucins. Mucins are highly glycosylated, and their biological functions are to form mucus/lubrication on the cell surface, and they are involved in cell signalling. Association between colon cancers and mucins has been found when there occurs alteration in the expression, the glycosylation and organization of the mucins (Krishn et al., 2016). One mucin protein, MUC5AC, is not expressed in healthy colon epithelium and is rendered a target of CDX2 (Pinto et all., 2017) It has been observed that expression is upregulated in early stages of colon cancers, but when dysplasia increases expression of MUC5AC decreases. It has also been estimated that patients which is MUC5AC negative have a worse overall survival and more aggressive diseases (Imai et al., 2013; Wang et al., 2017). Lack of mucin expression through dysregulation of CDX2 may possibly drive to colon cancer development. Some colon cancers can in later stages release cells from the adenocarcinoma and create metastasis. Cancer progression and the development of metastasis have been linked to CDX2. Cells which are released from the primary adenocarcinoma are called tumour budding cells and these cells have reduced CDX2 expression, despite the tumour being CDX2 positive, (Graule et al., 2018; Hansen et al., 2018) this being related to downregulation of CDX2 with metastatic process. The cells further ability to adherer to other cells can be affected by loss of CDX2, this implicating CDX2 in the downregulation of adhesion in tumour budding cells. Furthermore, cancer cells have been found to downregulation cell-cell adhesion through modifications of cadherin-catenin complex (Aamodt et al., 2010) and downregulation of claudins it thought to reduce the integrity of tight junctions (Tabariès and Siegel, 2017). Studies have shown that CDX2 mediate E-selectin ligand expression and hereby being involved in cancer cell attachment at distant sites of metastasis and therefore making it possible for the tumour budding cells to attach themselves to metastatic sites (Sakuma et al., 2012)

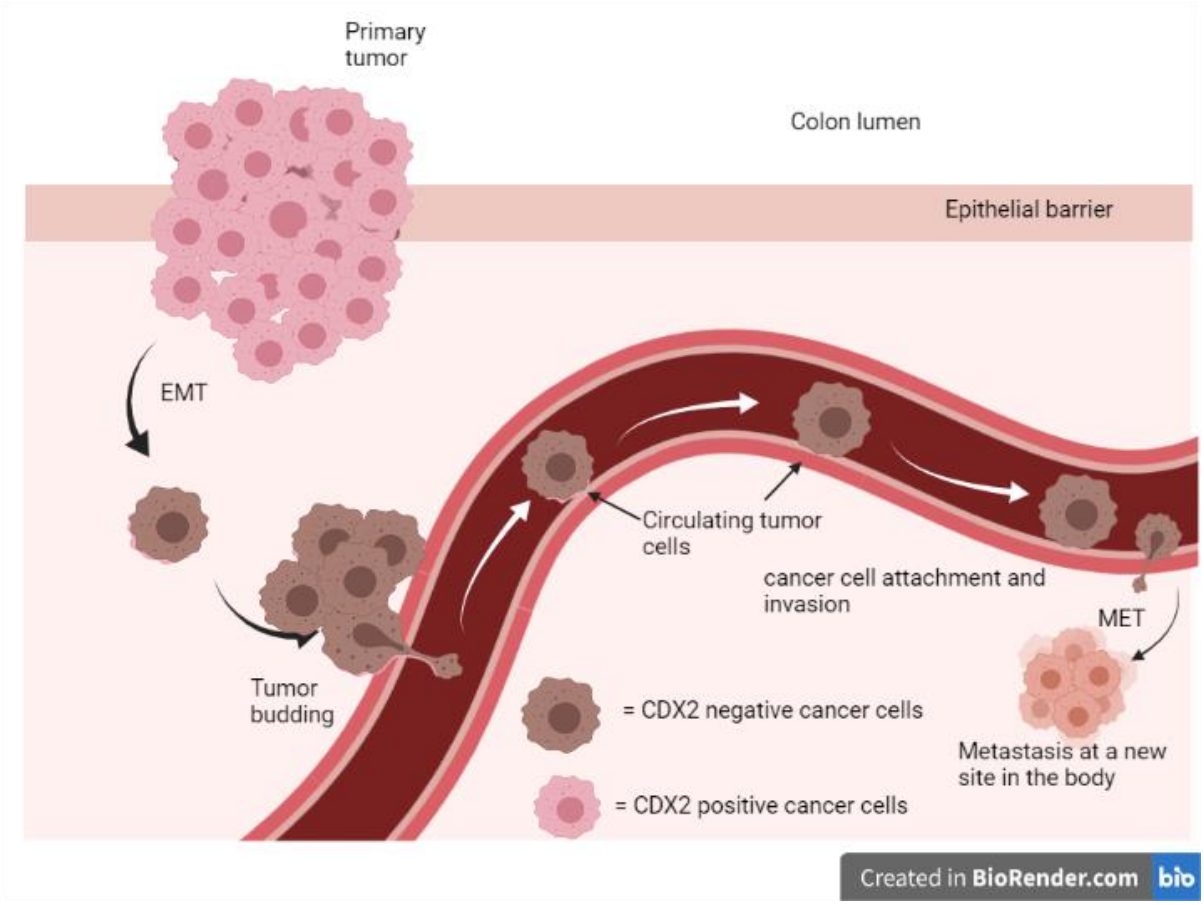


Figure 5 Downregulation of CDX2 possible role in transition of epithelial to mesenchymal cells in metastatic colon cancer cells. The figure illustrates that CDX2 positive cancer cells can transition in to CDX2 negative cells due to downregulation of CDX2. The transition from epithelial to mesenchymal state is initiated and the CDX2 negative cancer cells buds out into the blood vessels and disperse to a new site in the body. The CDX2 negative cancer cells starts to attach and invade at the new site and transition from mesenchymal to epithelial cells again and then progress into a new tumor. (Created with BioRender - own creation - with inspiration from Brablets et al., 2004; Zhang et al., 2015;Gross et al., 2008; Zheng et al., 2011).

Downregulation of CDX2 in a primary tumour has been proposed to be a precursor to epithelial to mesenchymal transition (EMT) in metastatic colon cancer cells, has been indicated in Figure 5. When the circulating tumour budding cells travels along the blood vessel to at metastatic site and establish metastasis, the expression of CDX2 is then re-established and the cancer cells then convert from mesenchymal to epithelial transition (MET) again (Brablets et al., 2004; Zhang et al., 2015). It has further been observed that decrease in mobility and dissemination of cancer cells happens when overexpression of CDX2 in colon cancer cell lines occur, this just indicating CDX2's further implication in the metastatic process (Gross et al., 2008; Zheng et al., 2011).

Most colon cancer cells show that CDX2 is aberrant and therefore have been thought of as a potential prognostic marker. Studies have further implied that the expression of CDX2 can be applied as a tissue marker, which can be used to determine the origin of primary tumours.

However, the potential to be a prognostic marker is controversial because studies have found that epigenetic changes e.g., hypermethylation of CDX2 promoter led to transcriptional downregulation or loss of expression of CDX2 (Dawson et al., 2014; Graule et al., 2018). This indicating that the loss of CDX2 may be related to other changes in the colon cancers cells, besides the signature feature that CDX2 expression is lost in undifferentiated colon cancer cells and poorly differentiated cancers, which have been correlated with a higher risk of relapse (Merlos-Suárez et al., 2011). A review of studies investigating the applicability of CDX2 as a prognostic marker, showed that there is a relatively larger amount of publication which imply that CDX2 is a possible prognostic marker, however there where many factors in the studies compromising the true value of CDX2.

5.2.2 Wnt signalling and tumorigenesis of CRC

Studies have indicated that there is more than one way of the Wnt signalling pathway to indue tumorigenesis. This is interesting due to Wnt signalling pathway crucial role in maintaining stemness of the colon, cell proliferation, cell fate decision, cell polarity (Saito-Diaz et al, 2013; MacDonald et al, 2009) and furthermore, control the transient amplifying zone of the intestinal crypts. This is done by activating the transduction signalling pathway and then initiating transcription of target genes necessary from the before mentioned processes. Table 1 gives a quick overview of which genes are mutated in/by the Wnt signalling pathway and how this could possibly lead to the development of CRC. However, further studies have shown that not only does these mutations play a role in the development of CRC, but further mutations within these regions can progress the CRC to malignancy and invasiveness (Bienz & Clevers, 2000)

Table 1 Mutations in Wnt signalling pathway as possible factors for CRC. The table indicates what genes are affected and how they are affected by mutations. The table is modified from Saito-Diaz et al., 2016.

Gene	Type of change	Reference
AXIN 1/2	Truncation/loss of function	Salahshor & Wodgett, 2005 Liu et al., 2000 Lammi et al., 2004
APC	Truncation/loss of function	Bienz & Clevers, 2000 Novellasdemunt et al., 2015 Zhan et al., 2017 Nishisho et al., 1991

CTNNB1 (β-catenin)	Missense/in-frame deletions/gain of function	Korinek et al., 1998 Bienz & Clever et al., 2000 Liu et la., 2002 Morin et al., 2015
TCF7L2 (TCF4)	Missense/deletion/truncation/gain of function	Cilliere-Dartigues et al., 2006 Duval et al., 1999 Novellasdemunt et al., 2015

As shown in the table, these mutations often lead to loss of function, which can in *AXIN 1/2* and *APC* lead to an accumulation of β-catenin and an uncontrolled transcription initiation of target genes. Mutation in *CTNNB1/β-catenin* is also referred to as activating mutations. These activating mutations can affect cell-cell adhesion (REF), the regulatory N-terminal serine/threonine residues, which can affect the binding of β-catenin to TCF7L2 inside the nucleus. Mutations in *TCF7L2* are still under investigation and the Wnt activating potential of the mutation is not fully characterized. However, experiments testing the loss-of-function of TCF4 showed that the mouse intestines had crypt degeneration (Novellasdemunt et al., 2015). Moreover, TCF7L2 has been found to have a proliferating-stimulation activity in CRC, but at the same time is acts as an invasion suppressor, which has been shown through loss-of-function assays. A large fraction of CRC tumors has been found with mutation in the *TCF7L2* gene (Wenzel et al., 2020).

5.2.3 CDX2 and Wnt signalling cross-talk

As mentioned in Wnt signalling and tumorigenesis, there exists many Wnt regulators and in addition to these CDX2 also acts as a Wnt regulator. Through TOP/FOP-flash reporter system (explained in theoretical method section) the effect of CDX2 on Wnt signalling have been investigated (Veeman et al., 2003). A study indicated that CDX2 is inversely related to B-catenin expression, together with the expression of cyclin D1 and c-myc, which are target genes of Wnt signalling (Yu et al., 2019). Furthermore, another study found that CDX2 inhibits the binding of B-catenin to the transcription factor TCF7L2 by binding itself to B-catenin (Guo et al., 2010). CDX2 have been linked to the cadherin family, however, an indirect effect of CDX2 on β-catenin activity has been found, and this effect happens through CDX2s regulation of protocadherin Mucdh1 transcription, which then interacts and inhibits the transcriptionally activity of B-catenin (Hinkel et al., 2013). A study found that in

differentiated colon cancer cells, a reduction of CDX2 showed an increase of transcription factor TCF7L2 and moreover CDX2 was found to bind and activate the transcription of HBP1, which is a transcriptional repressor that inhibits TCF transcription factors and supress Wnt signalling (Boyd et al., 2010). The degradation complex's different proteins APC, AXIN2 and GSK3-beta have through many studies have indicated an involvement in Wnt signalling with cross talk through CDX2. In relation to APC, knock out and overexpression of CDX2 did not show any changes in the expression pattern of APC (Yu et al., 2019), however contradictory with this another study did found that APC var transcriptionally regulated by CDX2 (Olsen et al., 2013). Furthermore, Nuclear levels of APC was associated with the loss of CDX2, but in relation to the cytoplasmic expression of APC no association was found in colon cancer liver metastasis (Tòth et al., 2018). Regarding AXIN2 research have indicated that knock out and overexpression of CDX2 had opposite effect on AXIN2 expression. When CDX2 was knocked out, he expression of AXIN2 decreases whereas when CDX2 was overexpressed the mRNA level of AXIN increased. (Yu et al., 2019). ChIP-seq data showed that when cells differentiate compared to proliferating cells there is an increase of CDX2's binding to the promoter region of AXIN2. This could possibly indicate that CDX2 upregulate the expression of AXIN2 during differentiation in colon cancer cells (Boyd et al., 2010) and AXIN2 has also been proven to transcriptionally be regulated by CDX2 also in colon cancer cells (Olsen et al., 2013). GSK3-beta expression decreased in colon cancer cells having knocked out CDX2, while overexpression of CDX2 led to an increase of GSK3-beta expression at mRNA level (Yu et al., 2019). It was further found that CDX2 binds to the promoter region of GSK3-beta, however, is does no activate transcription of GSK3-beta (Olsen et al., 2013). TNF-alfa was found to induces the expression of Wnt signalling components, when CDX2 expression was downregulated (Coskun et al., 2014). These findings indicate a possible cross talk between CDX2 and Wnt signalling both as an inhibitor and activator.

5.3 CYTOKINE ALARMIN IL-33'S PHYSIOLOGICAL FUNCTION AND REGULATION

The following section will include a short introduction of IL-33 as an "alarmin" and how it is regulated through CDX2 and current knowledge of how it can promote tumorigenesis. IL-33 was first identified as a member if the IL-1 family of cytokines and *IL-33* is located on chromosome 9. Common properties of IL-1 family are that all possesses a core tetrahedron-like structure, which is composed of 12 β-strands these being indispensable for their biological activity. The cytokines bind as monomers to the IL-1 receptor subfamily of Toll-

interleukin 1 receptor (TIR) domain-containing receptors. Heterodimeric transmembrane receptors complexes are formed, which initiate signalling mechanisms. Most of IL-1 family are proinflammatory cytokines, with pleiotropic processing. They can exert the acute inflammatory processes locally, but in severe cases, they can exert them systematically as well. In addition, the IL-1 cytokines assist in shaping the adaptive immunity by skewing the differentiation of naïve helper T lymphocytes and by directly influence the functions of subsets of T and B lymphocytes. IL-33 has been found to effect different target genes in many organs. Studies has implied that IL-33 is set to 'favour' immune response in T helper type 2 (T_H2) lymphocytes, due to the fact that the ligand-binding chain of IL-33 receptor complex IL-1R4/ST2 was found expressed on T_H2 lymphocytes (Martin & Martin, 2016) (Important: IL-1R4/ST2 have two forms, where soluble ST2 (sST2) function as a decoy receptor for IL-33 and stop possible initiation of signalling mechanisms in T_H2 cells; ST2/ST2L, which is the membrane bound ST2 IL-33 binds to. The receptor will from hereafter be referred to as ST2 or sST2 depending on which function is described. The major production and release of IL-33 in human are in epithelial barrier tissues. These tissues include the lungs, skin, stomach, and large intestines. IL-33 is also expressed in lymphoid organs such as the lymph nodes and spleen and has been found in the endothelial of blood vessels (Caryol and Girard, 2014). Several diseases such as allergic asthma, chronic inflammation of the gut, arthritis, disorders of the central nervous system and cardiovascular diseases has paved the way for how IL-33 function as a proinflammatory cytokine. However, IL-33 has been found to be beneficial or harmful, depending on the development of the given disease (Martin & Martin, 2016).

5.3.1 Production, release, and processing of IL-33

The initiation of transcription of IL-33 remains to this day sparse and need to be elucidated more. However, what is known is that IL-33 is constitutively produced in different epithelial cells such as the keratinocytes, lung and gut epithelial, fibroblast and fibrocytes and smooth muscle cells. As illustrated on Figure 6 Release and processing of IL-33 in human epithelial cells. Left: after the cells are injured or necrosis occur the full-length bioactive IL-33 is released. IL-33 is then modified by Calpain, esterases, Cathepsin G or Elastase, which gives to products, one being 10-30X more active that the full-length IL-33. When IL-33 has worked put it role it is inactivated outside the cells by chymases and other proteases or by caspase 3 or 7 or by forming disulphide bridges through oxidation. Right: After apoptosis, IL-33 is release from the nucleus and then inactivated by caspase 3 or 7 and then it becomes biologically inactive. (Martin & Martin, 2016)full-length IL-33 resides inside the cell's nucleus as a biologically active protein. The

full-length protein is 270 amino acids. Upon injury or necrosis of epithelial cells the full-length IL-33 is processed by different proteases such as Calpain, Esterase, Cathepsin G and Elastase outside the cell. It has been shown that different sizes of IL-33 give different biological activity compared to the full-length. When the injury or necrosis is under control, IL-33 is inactivated outside the cell as well by either chymases, capsase 3 or 7 or it can happen the formation of disulphide bridges. This leads to an alteration in the tetrahedron core structure and thereby bioactivity of IL-33 is lost. In the case of apoptosis, the inactivation of IL-33 happens through cleavage by caspase-3 or caspase 7, inactivating it as a cytokine or alarmin.

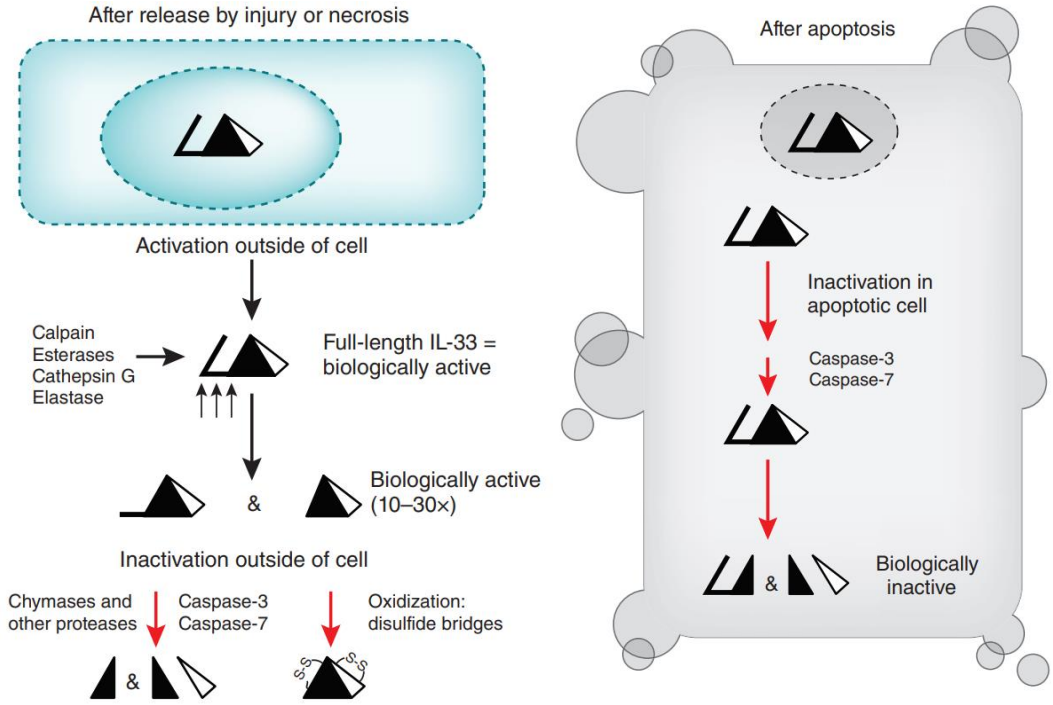


Figure 6 Release and processing of IL-33 in human epithelial cells. Left: after the cells are injured or necrosis occur the full-length bioactive IL-33 is released. IL-33 is then modified by Calpain, esterases, Cathepsin G or Elastase, which gives to products, one being 10-30X more active that the full-length IL-33. When IL-33 has worked put it role it is inactivated outside the cells by chymases and other proteases or by caspase 3 or 7 or by forming disulphide bridges through oxidation. Right: After apoptosis, IL-33 is release from the nucleus and then inactivated by caspase 3 or 7 and then it becomes biologically inactive. (Martin & Martin, 2016)

5.3.2 IL-33 as a guardian of barriers and conductor of local inflammation

The following will depict a working model of how IL-33 function as an alarmin during local inflammation and how it proceeds to induce an immune response and recruit immune response cells to the site of inflammation. However, the working model were made based/taking on/from different contexts and should be considered as preliminary working model. Upon breach of the epithelial barrier by an allergen/antigen (this example a parasitic

worm (Helminth)) IL-33 is released from the cells as full-length bioactive IL-33 alarmin then binds to cells expressing ST2 receptors. If it is the first time an allergen/antigen invade (naïve state) cells expressing ST2 could be mast cells, dendritic cells, macrophages and group 2 innate lymphoid cells (ILC2). If it is a recall situation such as allergens, cells could be effector and memory T and B lymphocytes, which includes regulatory T cells Figure 7 A. When IL-33 is bound to mast cells, the mast cell can release proinflammatory cytokines, such as IL-4 and IL-5, this leading to the recruitment of eosinophiles and basophiles to the site of acute inflammation. The activated mast cells release proteases and as explained earlier these proteases can in fact increase the specific biological activity of IL-33 by cleavage in the N-terminus (Figure 7 B). Mast cells start to produce cytokine IL-33 and other cytokines. Eosinophiles start to attack the antigen from the Helminth. IL-33 activates ILC2, macrophages and B1 lymphocytes, which then release mediators. Fibroblast are recruited to initiate wound healing, which then seals the breach where the antigen entered (Figure 7 C). When the acute inflammation is under control, macrophages synthesize anti-inflammatory cytokines, IL-33 stimulates B1 lymphocytes to produce IL-10, which leads to a dampening of the inflammation. IL-33 is then degraded by capsase 3 or 7 or by the formation of the disulphide bridges (Figure 7 D).

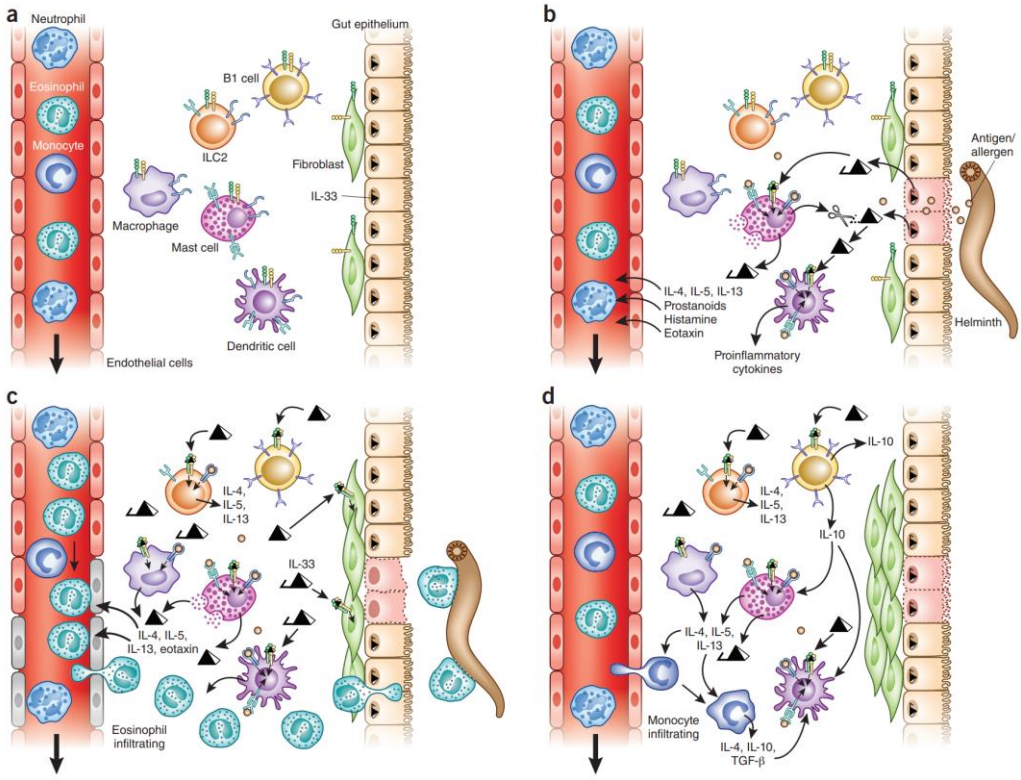


Figure 7 Working model of how IL-33 interact as an alarmin during local inflammation. A) the bioactive IL-33 reside inside the nucleus of the epithelial cells. B) upon attack with the parasite Helminth, IL-33 is release from the cells and binds to the receptor ST2 found on immune cells. IL-33's binding to different immune cells (mast cells, dendritic cells, macrophages and group 2 innate lymphoid cells (ILC2)) can release cytokines. C) Mast can produce further IL-33, eosinophiles starts attacking the parasite and ILC2, macrophages and B-lymphocytes release mediators. Fibroblasts are recruited to the site, where it starts wound healing. D) IL-10 is release from B1 lymphocytes, which dampen the inflammation. IL-33 is degraded by caspase 3 or 7, by formation of disulphide bridges (Martin & Martin, 2016).

5.3.3 Regulation of intracellular IL-33

The understanding of the regulation of IL-33 expression remains to this day insufficient at a genetic level. As mentioned, IL-33 is positioned on the short part of chromosome 9 on seven exons, where in mice is encoded on chromosome 19 and contain 11 introns. In mice, studies have indicated that two alternative promoters create transcripts *il33a* and *il33b*, which is produces by macrophages, embryonal fibroblasts, and different tissues in a cell type-specific manner, which both encodes full-length IL-33 on eight exons. Furthermore, the expression of *il33* is controlled by transcription factors IRF-3 and CREB and yields two mRNA species. Several studies have also proven that IL-33 mRNA is not stabile and is degraded within hours after synthesis (Martin & Martin), which could indicate that the process of converting it to an alarmin happens fast to ensure the protective properties during injury or breach by antigens.

A newly published study by Larsen et al., 2021, found that in the human colon cancer cell line LS174T that IL-33 proved to be regulated by CDX2. CDX2 was found to be an important transcription factor for the promoter activity of IL-33, and it was important for the high expression levels in the intestinal epithelial cells. The mRNA from IL-33 was

significantly upregulated by CDX2. The study further indicated that CDX2 recognizes and binds to the enhancer of IL-33 thereby upregulating the mRNA expression of IL-33 in intestinal epithelial cells (Figure 8).

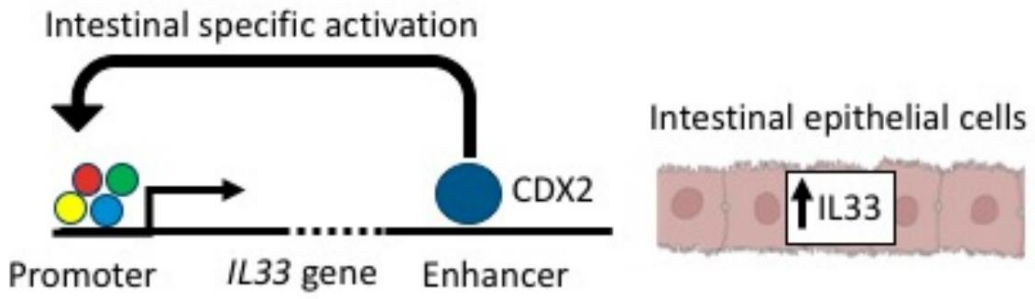


Figure 8 Regulation of IL-33 gene. CDX2 recognize and binds to the enhancer region of IL-22 thereby enhancing the transcription of IL-33, this leading to an up regulation of IL-33 in the cells (Larsen et al., 2021).

Based on this and the limited knowledge regarding IL-33 and its regulation inside the epithelial cells, IL-33 will be the main promoter applied for the measurements of CDX2 expression in LS174T cells.

5.3.4 IL-33 and tumorigenesis of CRC

IL-33 expression has been proven to be increased in CRC patient tissue compared to non-cancer adjacent tissue). However, the IL-33/ST2 signalling axis has been an elucidating part in the cancer pathology as well. Not only does the axis play an important part in inflammatory response, tissue injury and tissue repair, but its role in the development of CRC has been found to be both pro-and anti-tumour tumour microenvironment (TME). Studies have created an overview of how the pro- and anti-tumour TME properties of IL-33/ST2, which can be seen in Figure 9. The figure illustrates that in a pro-tumour TME situation, IL-33 recruits pro-inflammatory cells, such as mast cells, myeloid derived suppressor cells (MDSC), neutrophiles, macrophages and T regulator cells (T_{reg}). The immune cells start to secret cytokines, inflammatory molecules, and growth factors, which promote angiogenesis, invasion, migration, tumour growth and metastasis. The secreted molecules work in paracrine and autocrine manners, which creates a tumour promoting loop, that could lead to a further enhancement of the growth and metastasis of the tumour. In an anti-tumour TME situation, IL-33 recruits natural killer (NK) cells and cytotoxic T cells, to the TME, where they start to secret INF-γ and cytokines which leads to tumour regression and rejection. The pro-tumorigenic effect on tumours has been found in both mouse models and patient tissue and

cells lines in CRC, whereas anti-tumorigenic effect on tumours has only been seen in mouse model and cell lines (Table 2) (Larsen et al., 2018.

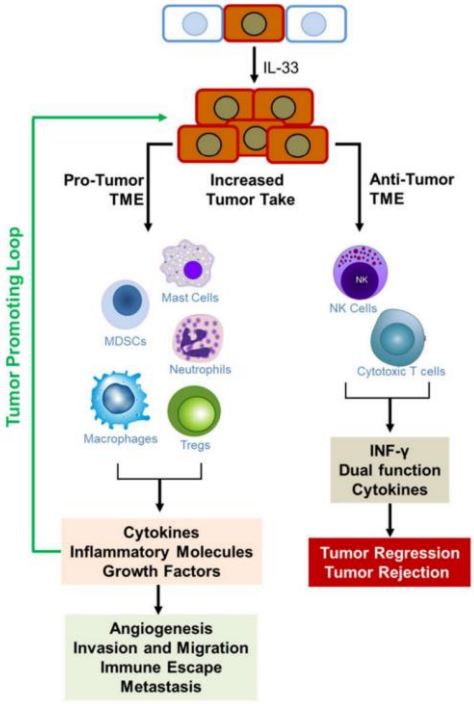


Figure 9 IL-33s pro- and anti-tumorigenic effect on tumours. The figure shows that IL-33 can either function as a pro-tumour for the tumor-microenvironment (TME) or as an Anti-Tumour for the TME. The black arrows indicate the effect IL-33 expression on immune cell recruitment, production of cytokines and pro- and anti-inflammatory cells. Pro-tumour TME will recruit Mast cells, myeloid dendritic cells (MDSCs), Neutrophiles, macrophages and T regulatory cells (t reg), which leads to an increase in cytokines, inflammatory molecules, growth factors. These three parameters lead to an tumour promoting loop, that will further increase the tumour take. The cytokines, inflammatory molecules and growth factors will lead to angiogenesis, invasion and migration, immune escape, and metastasis. Anti-tumour TME shows that IL-33 will lead to the recruitment of Natural killer (NK) cells and cytotoxic T cells. these leads to interferon gamma (INF-γ), a dual function and cytokines. These three parameters lead to tumour regression and rejection (Larsen et al., 2018)

Table 2 Summary of data indicating the effect of IL-33/ST2 axis on the development in CRC. (Larsen et al., 2018- modified version) . the table show type of cancer and where pro- or anti-tumorigenic effect has been found.

Type of cancer	**Pro- or anti-tumorigenic effect on tumor**	**Model used**	**References**
CRC	Pro- tumorigenic	Mouse model Patient tissue and cell lines	Maywald et al., 2015 Mertz et al., 2016 Zhang et al., 2017 Akitomo et al., 2016 (both mouse and patient samples)

			Fang et al., 2017 He et al., 2017 (both mouse and patient) Cui et al., 2015 Liu et al., 2014
	Anti-tumorigenic	Mouse model and cell lines	O'Donnell et al,. 2016 Eissmann et al., 2018

Other cancer types, such as breast cancer, gastric cancer, hepatocellular cancer, hepatobiliary cancer, pancreatic cancer, lung cancer and prostate/kidney cancer, have also shown that IL-33/ST2 axis have either pro- and/or anti-tumor TME properties in them, however they will not be address further in this thesis (Larsen et al., 2018).

6 THEORETICAL METHODS SECTION

6.1 TET-ON/TETRACYCLINE-RESPONSE ELEMENT, CMV-LUCIA SYSTEM, AND IL-33/TOPFLASH CONSTRUCTS

The thesis will apply a newly develop cell line LS174T7D9, which contains a variety of different components for a tightly controlled regulation of CDX2 expression through DOX stimulation. The following section will give an insight of how the individual components are made, how they work and working models of how they interact inside the cells.

In the LS174T7D9 two different genes have been inserted on the two alleles in the AAVS1 locus, which is a safe harbour locus used for integrating new genetic materials. Pinto et al., 2017 inserted on the first allele Tetracycline Response Element (TRE) and CDX2 in LS174T6D6 cells. TRE is a part of tetracycline controlled TetOff and TetOn (also referred to as Tet3G, however TetOn will be used in this thesis) gene expression system, which is activated by the effector doxycycline (DOX). DOX is a tetracycline antibiotic and is used as a research reagent both in vitro and in vivo. The TetOn was inserted, using Zinc Finger Nucleases (ZFN), in the CDX2 gene thereby knocking out CDX2 expression and at the same time introducing a constitutive expression of TetOn. CDX2and TRE was inserted in the AAVS1 locus also by ZFN (appendix 13.1). LS174T7D9 is induced with DOX, which binds to the TetOn protein in the cytoplasm and forms as complex with the protein. The complex of TetOn and DOX enters the nucleus where it binds to TRE, which initiates the transcription of CDX2 (Figure 10 top half of the cell).

LS174T6D6 was later modified by PhD student Stine Bull Jensen (data not published) on the second allele in the AAVS1 locus. By using homolog recombination and CRISPR-Cas9, it was possible to incorporate the *Lucia luciferase* gene and its promoter hEF1 (Appendix 13.2). *Lucia luciferase* is expressed by a synthetic gene designed on the naturally secreted luciferases from marine copepods, whereas luciferase is a natural bioluminescent found in fireflies and click beetles. Another difference is that firefly luciferase catalyses the oxidation of firefly luciferin and acquires ATP to execute the reaction, whereas Lucia luciferase uses coelenterazine as a substrate to emit an intense flash instead of a light/glow and do not use ATP to get activity. When Lucia luciferase is translated into protein, it will be exported out of the cell by vesicles where it will remain in the media until further assessment (Figure 10 bottom half of the cell).

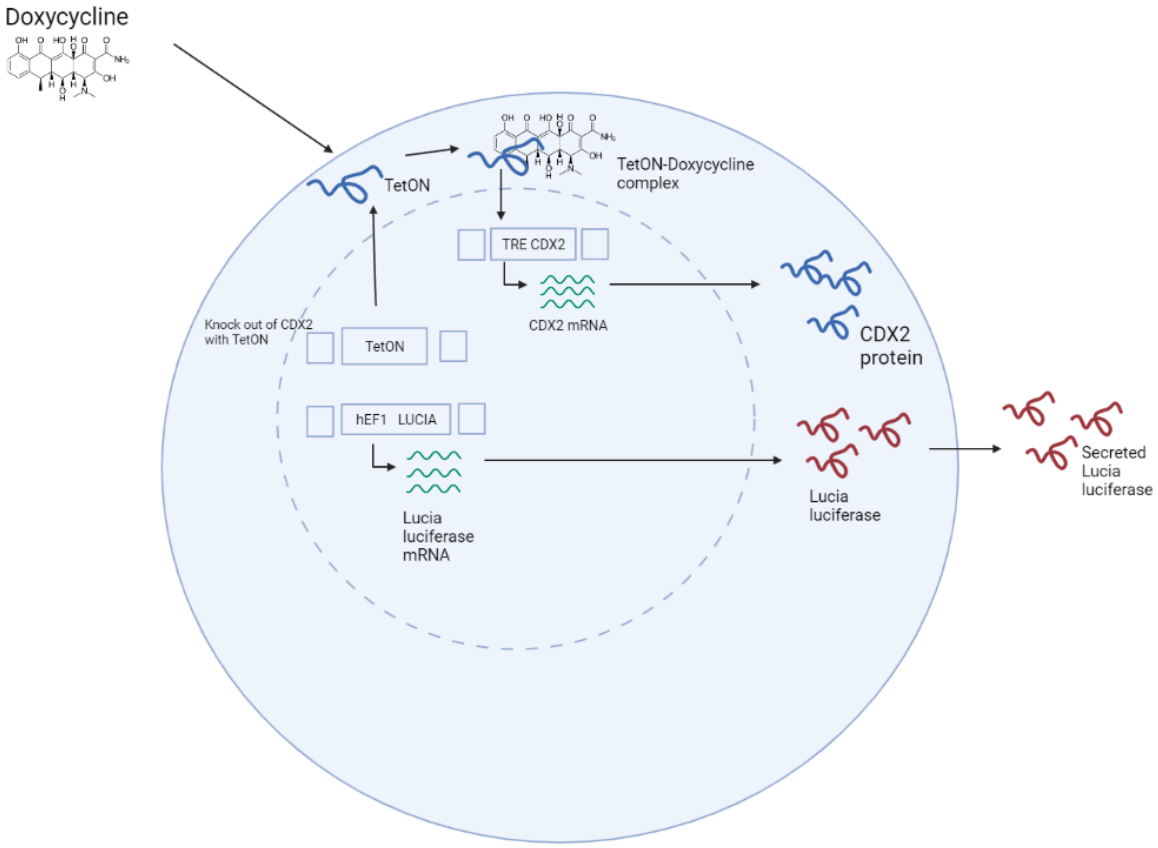


Figure 10 Modified DOX inducible LS174T7D9 cell line with Lucia luciferase. Dox binds to TetOn protein in the cytoplasm, where it creates a complex that will find and bind to the tetra response element (TRE). TRE will initiate the transcription of CDX2 and thus CDX2 protein will reside in the cytoplasm. For the expression of lucia luciferase hEF1 promoter will initiate the transcription of Lucia. Lucia is translated and after entering the cytoplasm vesicles will export the protein out of the cell into the media (Create with BioRender).

LS174T7D9 is then transfected with either pGL4.10-IL-33-promoter-enhancer (appendix 13.3) or TOPflash constructs (appendix 13.4). pGL4.10-IL-33-promoter-enhancer resides inside the nucleus. DOX induced CDX2 from the TetOn system re-enters the nucleus where is recognize and binds to a specific sequence in the enhancer region of pGL4.10-IL-33. By binding to the enhancer, transcription of *luciferase* is initiated and is then translated into protein. Luciferase proteins reside inside the cytoplasm of the cell, where is will be until harvest (Figure 11 - left). If LS174T7D9 is transfected with TOPflash, the transcription of luciferase will be initiated by Wnt ligands. TOPflash contains 7 TCF binding sites placed before *luciferase* gene and thereby controls the expression of *luciferase*. Wnt present in the cell media will activate the accumulation of β-catenin inside the cell. β-catenin translocate to the nucleus and recognize the TCF region in the construct, forms a complex, and initiates the transcription of *luciferase*. The expression of Wnt will be proportional with amount of luciferase in the transfected LS174T7D9 (Figure 11 - right).

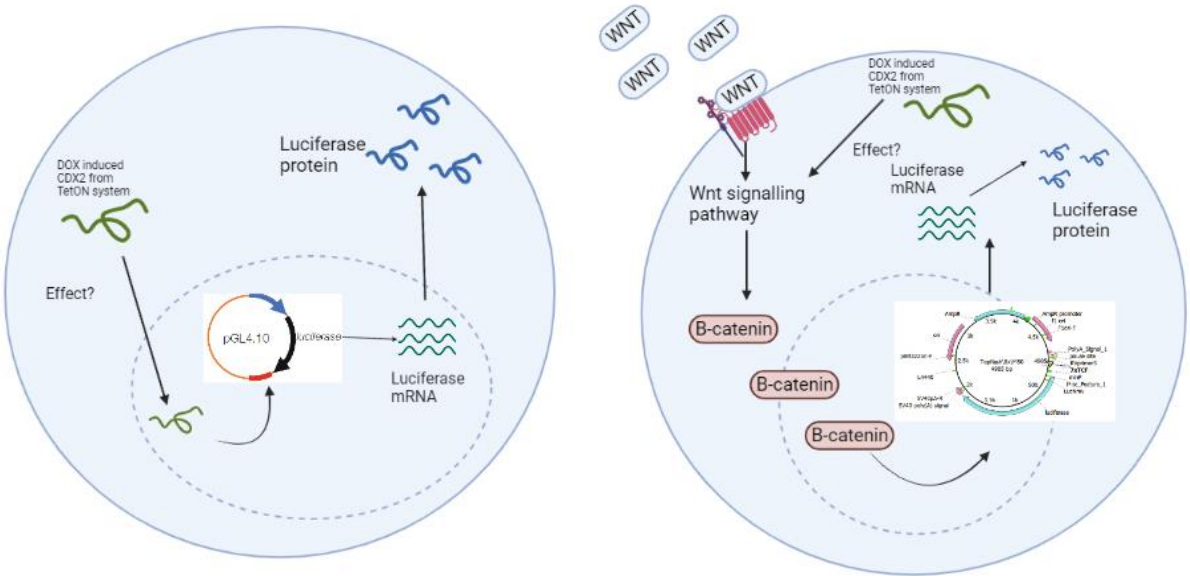


Figure 11 CDX2 driven promoter activity of pGL4.10-IL-33-promoter-enhancer (left) and Wnt driven promoter activity of TOPflash (right). Left: LS174T7D9 is transfected with pGL4.10-IL-33-promoter-enhancer construct The DOX induced CDX2 from the TetOn system will enter the nucleus and bind to the enhancer region of pGL4.10-IL-33-promoter-enhancer where is initiates the transcription of luciferase. The luciferase protein is translated and resided in the cytoplasm until harvest. LS174T7D9 is transfected with TOPflash construct and here Wnt will activate the Wnt pathway by binding to its receptor, which leads to an accumulation of β-catenin inside the cell. β-catenin translocate to the nucleus and binds to the TFC region in the TOPflash construct, this leading to the transcription of luciferase. Luciferase will reside inside the cell until harvest (create with BioRender).

7 METHODS AND MATERIALS

7.1 CULTIVATION OF CELL LINES

Human colon cancer cell line LS174T7D9 was grown and cultivated in Dulbeco's modified Eagle medium (DMEM) with 10% fetal bovine serum and 1% Penicillin-Streptomycin (Pen-strep) antibiotic. The cell line was maintained at 37℃ and humidified atmosphere of 5% CO_2 and was sub-cultivated once to twice a week, depending on assay line ups. Cells were grown in T75 culture flasks and if necessary zeocin was added to further protect the cells from contamination. During sub-cultivation, the cells were washed in phosphate-buffered saline (PBS) modified without Ca^{2+} and Mg^{2+} and then trypsinized with PBS containing 2% trypsin and 0.02% EDTA.

7.2 PLASMID CONSTRUCTS

7.2.1 pGL.4.10 with IL-33 promoter and enhancer for analysis of CDX2 regulation

pGL4.10-IL-33 promoter and pGL4.10-IL-33 promoter-enhancer constructs were used in transfections assays and pGL4.10-IL-33 promoter-enhancer was later used in the screening of the kinase inhibitor library. The pGL4.10-IL-33-promoter-enhancer construct was created through In-Fusion cloning (Larsen et al., 2021) and was used to measure CDX2 driven promoter activity of pGL4.10-IL-33 promoter-enhancer via induction with DOX through the TetOn expression system.

TOPflash promoter construct was used for the measurement of the Wnt driven promoter activity of TOPflash. The construct was only used after the selection and validation of the 19 KIs and for the top five KIs validation experiments.

7.3 TRANSFECTION, LUCIFERASE (FIREFLY) PROMOTER ASSAY AND MEASUREMENT OF LUCIA LUCIFERASE QUANTI-LUC MEASURING OF REPORTER ACTIVITY

7.3.1 Induction with doxycycline

To control and assess the expression of CDX2, LS174T7D9 is induced with DOX. DOX concentrations were assayed at 0-32 ng/ml to find the optimal DOX concentration. 16 ng/ml was found to be the optimal concentration and is therefore used in the screening protocol. Cells were treated with DOX for approx. 72 hours before measuring CDX2 driven luciferase

activity using pGL4.10-IL-33-promoter-enhancer construct and the Wnt driven promoter activity of TOPflash.

7.3.2 Transfection

For transfection assay, 50 ng of plasmids (12.5 ng of promoter plasmids, 8.5 ng of CMV-lacZ and 29 ng of empty plasmid pBSK+II) were incubated for 1 hour at room temperature with 2μM of polyethylenimine (PEI). PEI was mixed with the plasmids and then added to a 24 well plate containing 50000 cell/well. Seeding of the 50000 cells were done 72 hours before harvest and the plasmid/PEI mixture was added 48 hours before harvest. After seeding the plates media was changed when plasmid/PEI was added and left for 4 hours of incubation with the plasmid/PEI mixture. After incubation media was changes again and then re-stimulated with DOX.

Beside pGL4.10-IL-33-promoter-enhancer construct, HEPH, YAP1 and GPA-33 promoter were tested under the same conditions as mentioned before, however, instead of a 24 well plate a 96 well plate was used instead and only 8500 cell were seeded 72 hours before.

Before assessing the kinase inhibitor library, two KIs were tested on LS174T7D9. Previous studies have identified a regulatory effect on CDX2 expression by stimulating with U0126 (Rings et al., 2001) and SB203580 (Houde et al., 2001). The transfection assay had the same condition as the before mentioned assays, however, to find the optimal time for stimulation 1 μM of kinase inhibitor (KI) was added 24 hours, 48 hours, and 2 hours before harvest.

After finding the optimal condition for the screening kinase library, the screening was conducted with 50 ng of plasmid, where 12.5 ng of either pGL4.10-IL-33-promoter-enhancer or TOPflash were added. The cells were then induced with 0 ng/ml and 16 ng/ml DOX 72 hours before harvest and stimulated with 1 μM of KIs also 72 hours before. The total screening of the 196 kinases were done in duplicates in a 96 well plate seeded with 8500 cells/well. Validation experiments hereafter were done in triplicates first in 96 well plates and then in 24 well plates, seeding 8500 and 50.000 cells/well, respectively. Media was changes after the 4 hours of incubation with plasmid/PEI mix.

All transfection assays were carried out in a LAF bench and placed at 37°C and 5% CO_2 atmosphere, incubation happened in a total of 72 hours and the plates were only taken out when the cells were added with plasmid/PEI mix 24 hours after seeding the plates.

7.3.3 Luciferase promoter assay

Glomax® 96 Microplate Luminometer was applied to measure the luciferase activity produced during the transfection assays. After the total 72 hours of incubation, plates were taken out and placed at a work bench in 20℃ environment. The cells were washed twice with PBS modified without Ca^{2+} and Mg^{2+} and then lysed with lysis solution and Dithiothreitol (DTT) from Tropix® Dual-Light® luminescent reporter gene assay kit (ThermoFisher – Dual light™). Lysis happened during 5 min of shaking the plate and here released luciferase from LS174T7D9. For the detections of luciferase (produced by pGL4.10-IL-33-promoter-enhancer or TOPflash) and β-galactosidase (produced through the CMV-lacZ plasmid added during transfection), two buffers were made: Buffer A and buffer B. To buffer B Galacton-plus substrate was added. The Glomax® 96 Microplate Luminometer first measures luciferase and then β-galactosidase. The assay must incubate for approx. 30-45 min after measurement of luciferase, before measuring β-galactosidase. This is done to make sure that luciferase decays before measuring β-galactosidase. β-galactosidase is measured at a different pH and therefore Accelerator-II is added, by the machine, after the 30-45 min of incubation.

7.3.4 Quanti-Luc

From the transfection assays, after 72H of DOX induction and stimulation with KIs, samples of cell culture supernatant are taken out before lysing the cells. 20 µl of the samples are places in a white 96 well plate and measured on SpectraMax instrument. The samples are analysed for secreted lucia luciferase. Experiments performed by Ph. D student Stine Bull Jessen (not published) have demonstrated that the amount of Lucia luciferase secreted to the media parallels the number of cells in the well and can be used as a proxy for cell viability.

7.4 SCREENING OF KINASE INHIBITOR LIBRARY

7.4.1 Kinase inhibitor screening library

The kinase inhibitor library contained 196 different KIs was purchased from Selleck Chemicals (Appendix 13.6). The library consisted of 3 96 well plates where the KIs where distributed. KI library was diluted from 1 mM to 1 µm. The screening was done as describe in 7.3.2 transfection and measured as done in 7.3.4 luciferase promoter assay.

7.5 KINASE INHIBITORS REGULATORY EFFECT ON CDX2 TARGET GENES

7.5.1 RT-qPCR

7.5.1.1 RNA extraction and cDNA synthesis

50.000 LS174T7D9 cells were seeded in a 24 well plate and induced with 0 ng/ml or 16 ng/ml DOX and stimulated with 1 μm of either SB525334, PD0332991, Neratinib, Everolimus and XL765. RNeasy® Mini Kit (QIAGEN) kit was used in accordance with the manufacturer's protocol. Cells were collected using at pipette tip, homogenized in a centrifuge, and then extracted. Sample concentrations were measured on NanoDrop, and total RNA was calculated (appendix 13.5).

cDNA was synthesized from 250 ng RNA using First Strand cDNA synthesis Kit (Thermo Fishers) applying the manufacturer's protocol. Three target genes were investigated: *ABCB1, IL-33 and MYC* and the primers applied to the cDNA for the RT-qPCR is shown in Table 3. The genes were normalized to the reference gene ribosomal protein large subunit P0 (RPLP0). Furthermore, GAPDH was used as a positive control (provided by First strand cDNA synthesis Kit) and 100 ng of RNA was used for cDNA synthesis.

Melting curve analysis was performed during the RT-qPCR run and assessed postamplification.

Table 3 Primer overview for RT-qPCR of the three possible target genes ABCB1, IL-33 and MYC

Gene	Forward primer 5'-3'	Reverse primer 5'-3'	Size (bp)/Tm of primers
ABCB1 (Borchert et al, 2020)	GGAACTCAGCTCTCTGGTGG	CAGGGCTTCTTGGACAACCT	**138 / 65°C**
IL-33 (Larsen et al., 2020)	AAGAACACAGCAAGCAAAGCC	AGTGAAGGCCTTTTGGTGGT	**158 / 65°C**
MYC (Coskun et al., 2014)	CCACAGCAAACCTCCTCACAG	GCAGGATAGTCCTTCCGAGTG	**105 /64.9°C**
RPLP02	GCAATGTTGCCAGTGTCTG	GCCTTGACCTTTTCAGCAA	**141 /61.7°C**

7.5.1.2 Quantitative PCR

RT-qPCR was conducted in technical duplicates, since the sample is the same only the primers is the variant in these experiments. The program for the RT-qPCR is shown in appendix 13.6). The RT-PCR was done on MX3005P and in the program was set to measure the amplification during the third step of amplification. The primer annealing temperature was set to 58. To measure the amount of amplified DNA, QuantiTect® SYBR® Green PCR Kit (200) was used.

7.6 STATISTICAL ANALYSIS

Statistical analysis included Two-tailed Student T-test and was done when it was relevant to determine the difference in promoter activity driven by CDX2 or Wnt between the control without KI stimulation and samples stimulated with KIs. For quantitative experiments standard deviation (STD), were calculated based on the triplicates. Optimally, for the screening of the kinase inhibitor library, triplicates for the initial testing of all 196 would have been applied, however, variance coefficient (CV%) was applied. The CV% give an indication of the dispersion of the different data around the mean and further give a ratio between the standard deviation and the mean. The significance level was set to 0.05, therefore p-values equal to or below 0.05 were considered significant (p-value < 0.05*, p-vlaue < 0.01** and p-value < 0.001***).

8 RESULTS

8.1 CREATING THE OPTIMAL PROTOCOL FOR INVESTIGATING THE EXPRESSION OF CDX2 THROUGH REPORTER LUCIFERASE ASSAYS

The first part of the results is the establishment of an optimal protocol for measurement of promoter activity driven by CDX2, which is done in modified LS147T7D9. For the optimal protocol, the following parameters were tested: 1) Optimal DOX concentration for the induction of CDX2, 2) Testing pGL4.10-IL-33-promoter-enhancer construct and enhancer activity against already published promoters in LS174T7D9 and 3) Optimal induction time for DOX.

8.1.1 Optimal promoter system

pGL4.10-IL-33-promoter's enhancer is recognised at the enhancer regions by CDX2 at a specific sequence. Therefore, the first part of the results will focus on the two constructs pGL4.10-IL-33 promoter and pGL4.10-IL-33-promoter-enhancer, to see if there was a difference in CDX2 driven promoter activity when adding the enhancer to the construct. LS174T7D9 was transfected with the two constructs and stimulated with a range of DOX concentration spanning from 0-32 ng/ml (Figure 12). The figure shows that when LS174T7D9 is transfected with pGL4.10-IL-33-promoter, the promoter activity is low when inducing with 0-32 ng/ml DOX. However, when transfecting the cells with pGL4.10-IL-33-promoter-enhancer, there is an increase in the promoter activity, when inducing the LS174T7D9 with 0-32 ng/ml DOX. Therefore, when CDX2 is induced with 0-32 ng/ml DOX and LS174T7D9 is transfected with pGL4.10-IL-33-promoter-enhancer, the promoter activity increase with approx. 3x at 0 ng/ml, 9x at 8 ng/ml, 7x at 16 ng/ml and 11x at 32 ng/ml compared to the pGL4.10.IL-33-promoter construct.

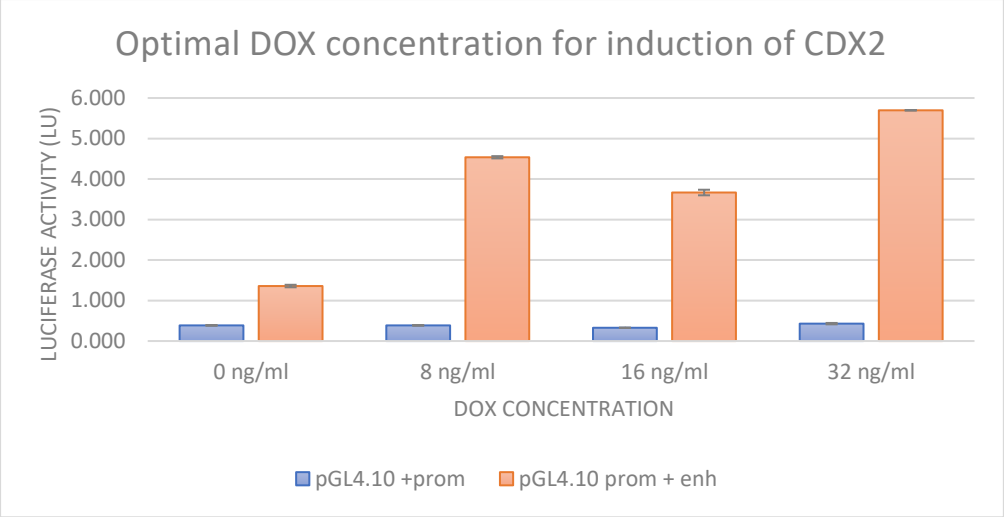


Figure 12 Optimal DOX concentration for induction of CDX2. The graph shows how the CDX2 driven promoter activity of IL-33 behaves when induced with 0, 8, 16 and 32 ng/ml DOX. LS174T7D9 was transfected with either a construct containing only IL-33's promoter (blue) or a construct containing both IL-33's promoter and enhancer (orange). The assay was done in triplicates. The data points are luciferase activity, which is measured in light units (LU), which is on the y-axis and the DOX concentration from 0-32 ng/ml is placed on the x-axis. Due to technical issues the luciferase activity was not corrected to the β-galactosidase activity.

8.1.2 Validation of pGL4.10-IL-33-promoter-enhancer activity compared to other promoters

Next, a test to see how pGL4.10-IL-33-promoter-enhancer promoter activity increase compared to previously published promoters, which had also been identified as to be driven by CDX2. The previous promoters were HEPH, YAP1 and GPA-33. A new range of DOX concentration were applied for the experiment ranging from 0-12 ng/ml. The figure shows that the CDX2 driven promoter activity for pGL4.10-IL-33-promoter-enhancer, HEPH, YAP1 and GPA-33 increased when induced 6 and 8 ng/ml DOX.

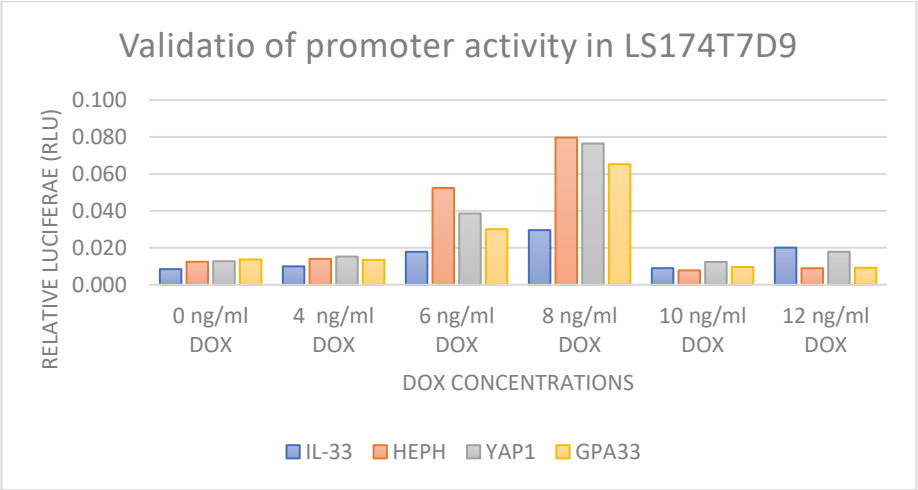


Figure 13 Validation of pGL4.10-IL-33-promoter-enhancer (Blue) activity compared with HEPH (orange), YAP1(Grey) and GPA-33 (yellow) promoters. The graph shows LS174T7D9 transfected with pGL4.10-IL-33-promoter-enhancer, HEPH, YAP1 and GPA-33 and induced with DOX concentration ranging from 0-12 ng/ml DOX. The assay was done in duplicates. The data points were adjusted to β-galactosidase numbers, which give the relative light units (RLU), which is on the y-axis and DOX concentrations on the x-axis.

8.1.3 Optimal induction time and DOX concentration

This next part investigated the optimal induction time, DOX concentration and when to stimulate the cells with KIs when using pGL4.10-IL-33-promoter-enhancer construct or TOPflash construct. Two kinases were chosen, SB203580 and U0126 with previous published CDX2 modulatory activity (Rings et al., 2001; Houde et al., 2001). U1026 targets MEK1 and MEK2, which is a part of the Ras/Raf/MEK/ERK pathway and SB203580 targets p38 mitogen, which is a part of the MAPK pathway.

Validation experiments showed that when LS174T7D9 were stimulated with U0126 and induced with 0 ng/ml and 16 ng/ml the CDX2 driven promoter activity of pGL4.10-IL-33-promoter-enhancer construct increased when induced with 16 ng/ml DOX at both 24 h and 72 H before harvest and measurement (Figure 14 A). When stimulating LS174T7D9 with SB203580 the promoter activity increases when induced with 16 ng/ml DOX as well, however only for 24 H (Figure 14 B).

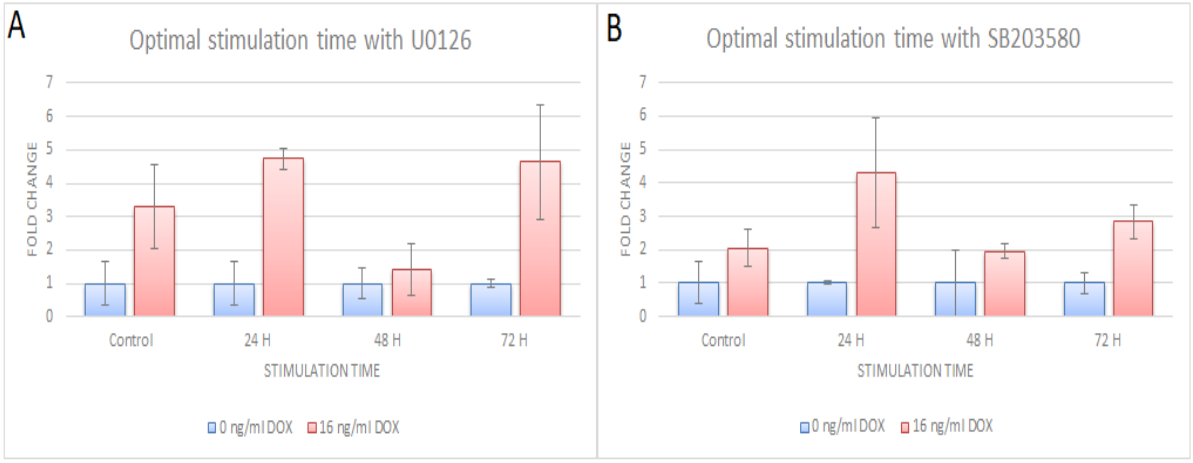


Figure 14 Optimal stimulation time with U0126 and SB203580 in LS174T7D9 transfected pGL4.10-IL-33-promoter-enhancer. A) LS174T7D9 were induced with 0 ng/ml and 16 ng/ml DOX. Cells were stimulated with 1 µM U0126 inhibitor 24 H, 48H and 24 H before harvest and measurement. B) LAS174T7D9 were induced with 0 ng/ml and 16 ng/ml DOX. Cells were stimulated with 1 µM SB230580 24 H, 48 H and 72 H before harvest and measurement. Controls were not stimulated with KIs but were induced with 0 ng/ml and 16 ng/ml as well. The y-axis shows the fold change in regulation and the x-axis the stimulation time. Assay was done in duplicates.

Next, LS174T7D9, transfected with the Wnt driven promoter construct TOPflash, was stimulated with U0126 24 h, 48 H and 72 H before harvest and measurement and induced with 0 ad 16 ng/ml DOX (Figure 15A). The figure shows that the control for both U0126 and SB203580, decreased in the Wnt driven promoter activity of TOPflash. When stimulated with U0126 the Wnt driven promoter activity of TOPflash decrease when induced with 16 ng/ml DOX and stimulated 24 H 48 H and 72 H before harvest and measurement. However, the promoter activity did not decrease further when comparing 24 H 48 H and 72 H with the

control. The stimulation at 48 H shows to have almost the same promoter activity as the control. When stimulating LS174T7D9 with SB203580, the Wnt driven promoter activity of TOPflash show a small decrease at 24 H, 48 H and 72 H (Figure 15 A). When comparing the three stimulation times with the control, the promoter activity does not decrease the promoter activity further when induced with 16 ng/ml DOX.

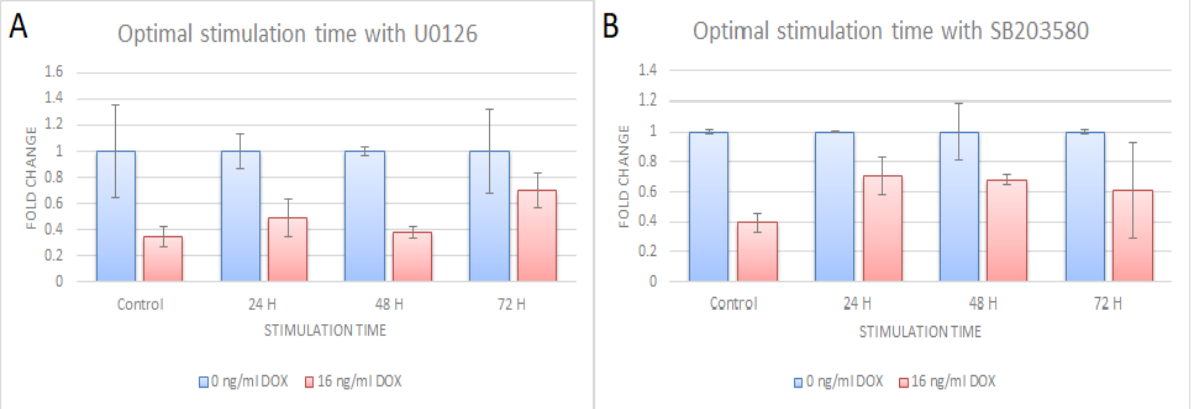


Figure 15 Optimal stimulation time with U0126 and SB203580 in LS174T7D9 transfected with TOPflash. A) LS174T7D9 were induced with 0 ng/ml and 16 ng/ml DOX. Cells were stimulated with 1 µM U0126 inhibitor 24 H, 48H and 24 H before harvest and measurement. B) LAS174T7D9 were induced with 0 ng/ml and 16 ng/ml DOX. Cells were stimulated with 1 µM SB230580 24 H, 48 H and 72 H before harvest and measurement. Controls were not stimulated with KIs but were induced with 0 ng/ml and 16 ng/ml as well. The y-axis shows the fold change in regulation and the x-axis the stimulation time. Assay was done in duplicates.

.

To summarize, when testing the optimal stimulation time for U0126 and SB203580 needed for LS174T7D9 transfected with pGL4.10-IL-33-promoter-enhancer construct the results show that at 24 H and 72 H there is a difference in promoter activity when stimulated with 0 ng/ml and 16 ng/ml DOX. However, when testing the optimal stimulation time for U0126 and SB203580 when using the Wnt driven promoter construct TOPflash, the optimal stimulation time was 72 H. Due to the limited time available in the thesis, a validation of the data was not possible. Therefore, stimulation time when assessing the screening of the kinase inhibitor library, will be 72 H for both pGL4.10-IL-33-promoter-enhancer and TOPflash constructs.

8.2 Screening of kinase inhibitor library on LS174T-7D9 cells with TetOn and Lucia system

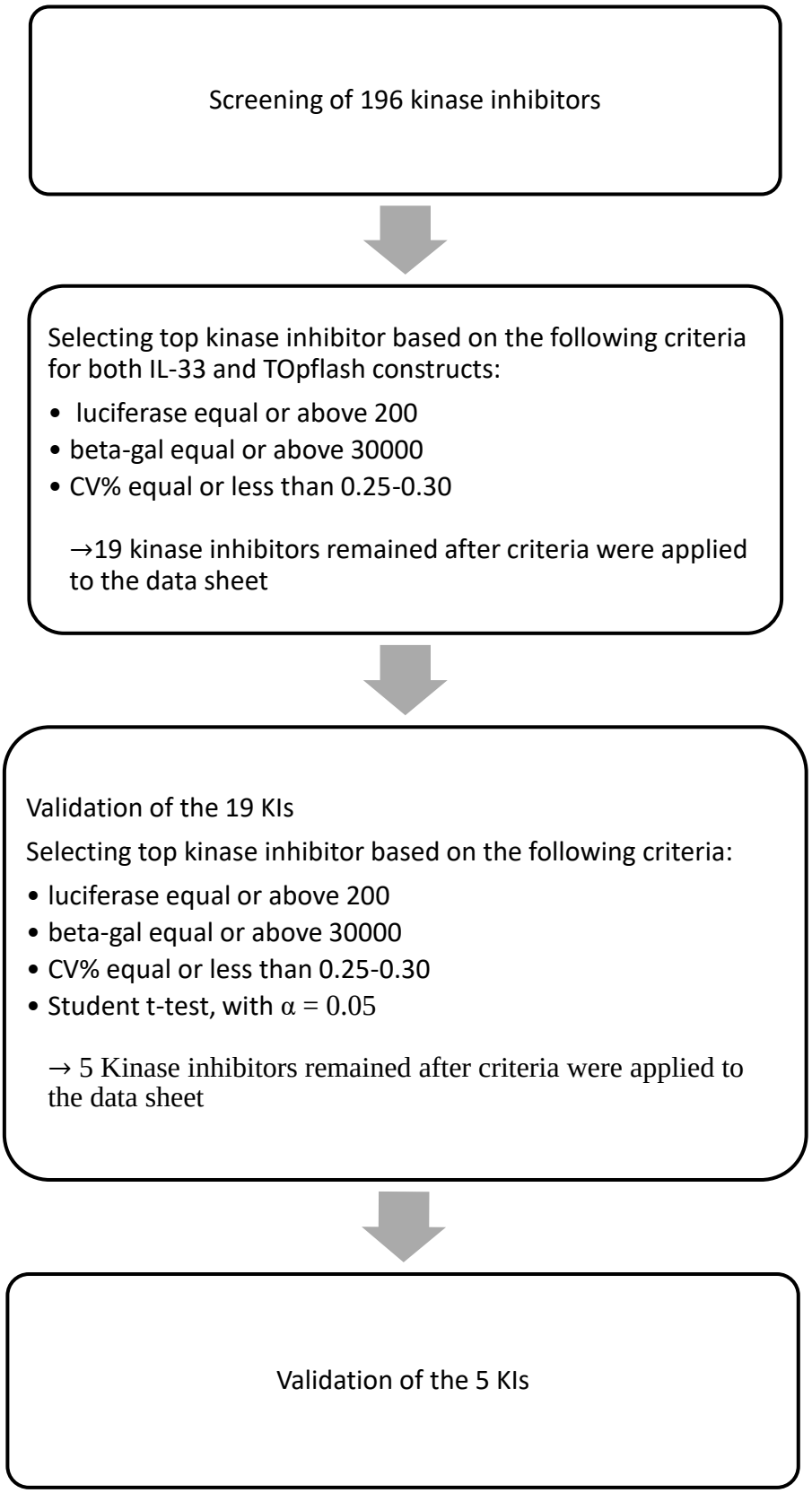
CDX2 is regulated both on a transcriptional and post translational level in the cell, this happening through different factors such as GATA6, TCF7L2, HNF-α, NF-κB, promoter methylation (transcriptional regulation), phosphorylation through MAPK and p38 and CDK2

(translational regulation). Here in the second part of the results, the same kinase inhibitor screening library will be screened on LS174T7D9 to see how the kinases affect the CDX2 driven promoter activity of either pGL4.10-IL-33-promoter-enhancer and TOPflash.

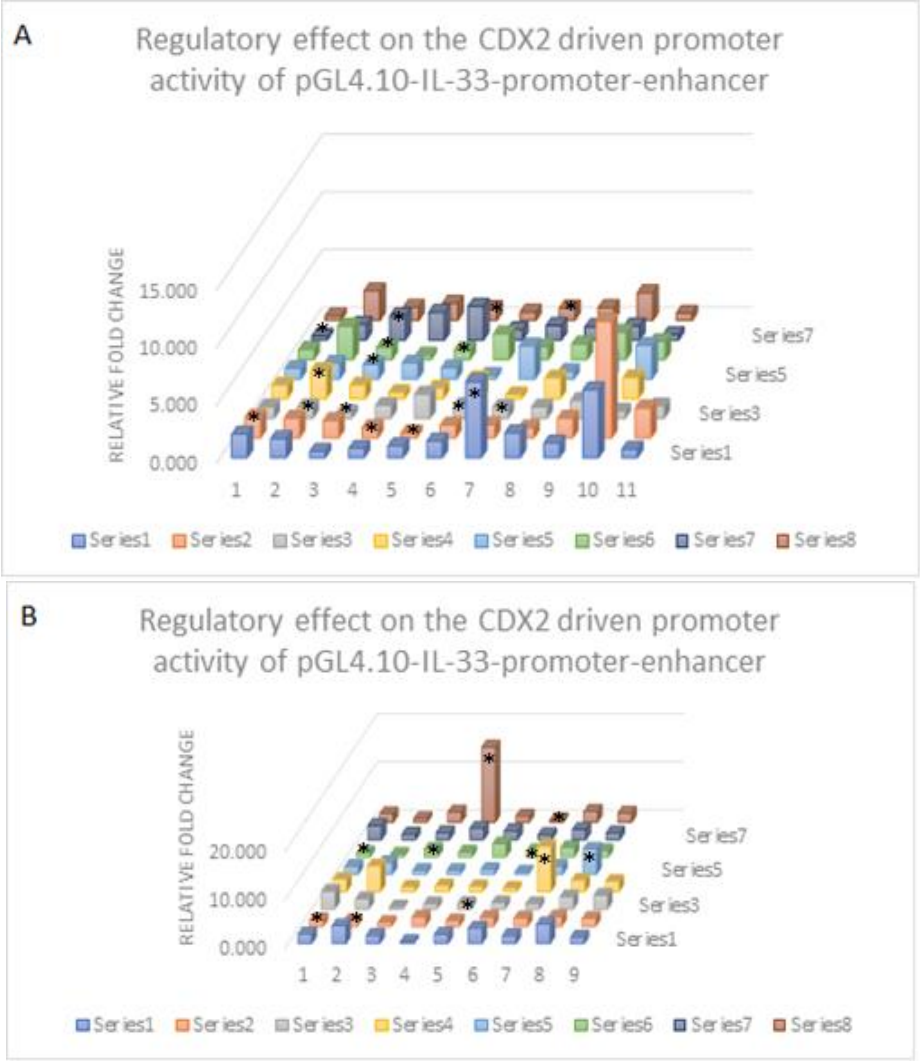
8.3 SCREENING OF KINASE INHIBITOR LIBRARY

The following section contains the data obtained from the screening of the kinase inhibitor library. The screening of the 196 KIs was designed to be done in duplicates. Whereas the validation of the selected KIs were done in triplicates, for the possibility of doing a 2 tailed paired student t-test and seeing if there is a difference in CDX2 driven and Wnt driven promoter activity in LS174T7D9. The following flow sheet gives an overview of the selection process of the KIs and what criteria were applied to the data sheet.

Table 4 Overview of screening and selection process. From Selleck's kinase inhibitor screening library 196 KIs were screened to see their possible regulatory effect on CDX2 through its driven promoter activity on pGL4.10-IL-33-promoter-enhancer and TOPflash constructs. Based on luciferase, β-galactosidase numbers and CV% criteria 19 KIs were selected for further assessment. After validation of the 19 KIs the same criteria for the 196 were applied to the data together with student t-test with a p-value of 0.05. After the selection 5 KIs were validated and analysed.



First, all three plates containing the 196 were screened on LS174T7D9 induced with 0 ng/ml and 16 ng/ml DOX. The data showing the KIs inhibitory effect on the CDX2 driven promoter activity of pGL4.10-IL-33-promoter-enhancer together with the regulatory difference between the control (no KI is added) and samples is shown in appendix 13.9 Table 5. For a graphical overview of all 196 KIs regulatory effect on the promoter activity, the relative fold change is illustrated in Figure 16. The three graphs are noted as A, B and C. On the graph the selection of the 19 KIs for further validation are indicated by a black star.



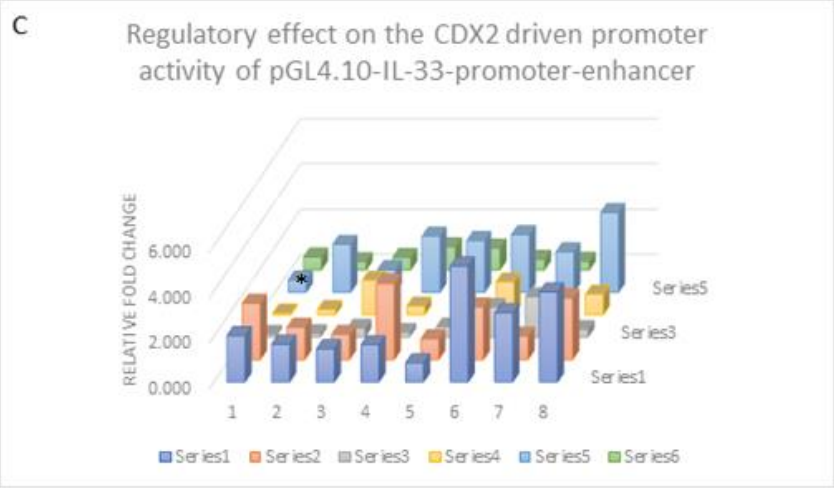


Figure 16 Overview of the regulatory effect on the CDX2 driven promoter activity of pGL4.10-IL-33-promoter-enhancer construct shown in relative fold change (y-axis). The graph illustrates the fold change in CDX2 driven promoter activity when the cells are induced with 0 ng/ml and 16 ng/ml DOX and stimulated with 1 µM. On alle three graphs (A, B, C), Series 1 # 1 represents the control (no KI is added), and the rest of the columns represents one KI pr. Column. A = first plate of KIs, B = second plate of KIS and C = third plate of KIs. Black stars indicate selected 19 KIs, for further validation.

8.3.1 CDX2 driven promoter activity of pGL4.10-IL-33-promoter-enhancer when stimulated with the selected 19 KIs

Based on the selection process 19 KIs met the applied criteria: Ki8751, Flavopiridol (Alvocidib), XL880 (GSK1363089), SB 525334, PD0332991, MGCD-265, PHA-793887, Neratinib, AZD0530, Dastinib, BI 2536, Vinorelbine (Navelbine), PI-103, Everolimus (RAD001), PD98059, WYE-345, XL 147, XL765 and DOC-2036. The purpose with this validation was to find KIs that increase the CDX2 promoter activity of pGL4.10-IL-33-promoter-enhancer and when compared to the control's 0 ng/ml had the same promoter activity at 0 ng/ml. The comparison with the control was done to find KIs that increase the promoter activity, when having the same starting points as the control, when no KI was added. Samples that decrease the promoter activity will not be assessed in this thesis. Statistical analysis showed a significant difference in the promoter activity when LS174T7D9 were stimulated with Flavopiridol(Alvocidib), XL880(GSK1363089), SB 525334, PD0332991, PI-103, Everolimus(RAD001), PD98059, WYE-354, XL147, XL 765 and DCC-2036. KIs that both increase the promoter activity and had the same promoter activity at 0 ng/ml as the control were XL880(GSK12603089), SB 525334, PD0332991, PI-103, Everolimus, PD98059, WYE-354, XL147 and XL 765. However, the control, Ki8751, MGCD-265, PHA-793887, Neratinib, AZD0530, Dastinib, BI 2536, Vinorelbine (Navelbine) and PD98059 did not showed to be significantly different when inducing with 16 ng/ml DOX. On the graph, the significance level is shown by stars for those KIs that had a difference in the promoter activity (Figure 17).

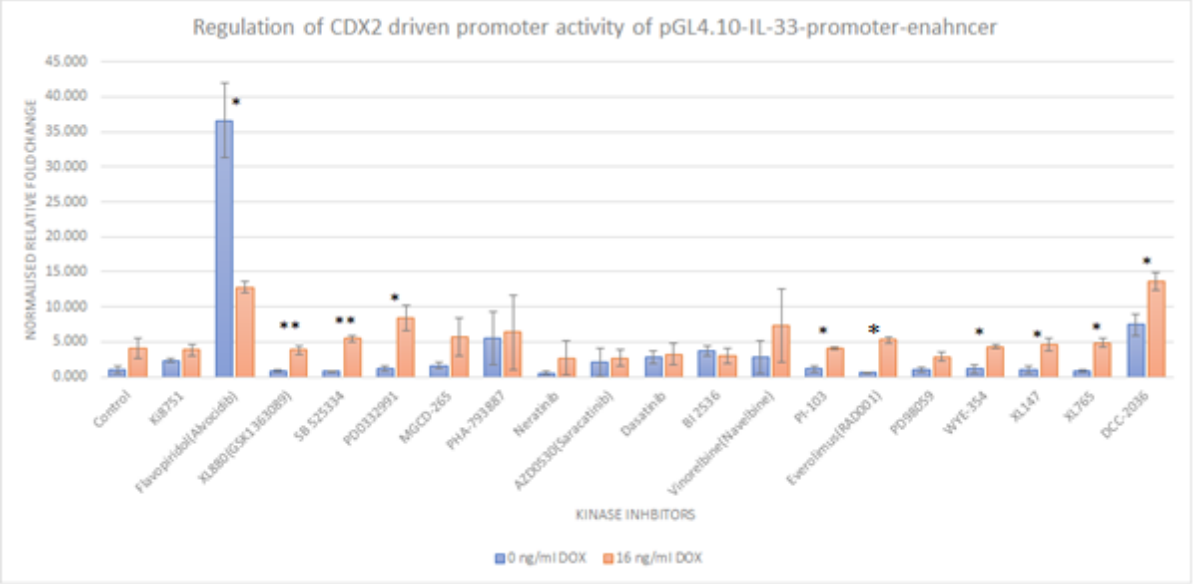


*Figure 17 Validation of the 19 KIs chosen after selected based on the predetermined criteria (see Flowchart). The graph shows how the CDX2 driven promoter activity of pGL4.10-IL-33-promoter-enhancer is regulated by Ki8751, Flavopiridol (Alvocidib), XL880 (GSK1363089), SB 525334, PD0332991, MGCD-265, PHA-793887, Neratinib, AZD0530, Dastinib, BI 2536, Vinorelbine (Navelbine), PI-103, Everolimus (RAD001), PD98059, WYE-345, XL 147, XL765 and DOC-2036, when normalised to the relative fold change in regulation. The x-axis shows the KIs. The control used in the assay had no KI added. The assay was done in triplicate. Statistical analysis was done with a Student T-test, where KIs showed a significant difference with a p-value of <0.05 in promoter activity when LS174T7D9 was induced with 16 ng/ml DOX is noted with *. p-value < 0.05*, p-value < 0.01** and p-value < 0.001***.*

Each KI was compared to the control to assess the change in the regulatory effect of the CDX2 driven promoter activity of pGL4.10-IL-33-promoter-enhancer construct (Figure 18). The focus here was to find KIs that further increased the promoter activity, which happened when LS174T7D9 was stimulated with XL880(GSK1363089), SB 525334, PD0332991, Neratinib, Everolimus and XL765. Above each KIs is given, in percentage, how much each KI compared to the control either increase or decrease the CDX2 driven promoter activity of pGL4.10-IL-33-promoter-enhancer setting the control to 100%. The graph shows the relative fold change in percentages of CDX2 driven promoter activity of pGL4.10-IL-33-promoter-enhancer, where XL880(GSK1363089) further increases the promoter activity with 9.5 %, SB 525334 with 74.3%, PD032991 with 72.6%, Neratinib with 23.9%, Everolimus with 96.2% and XL765 with 44.3% when compared with the control.

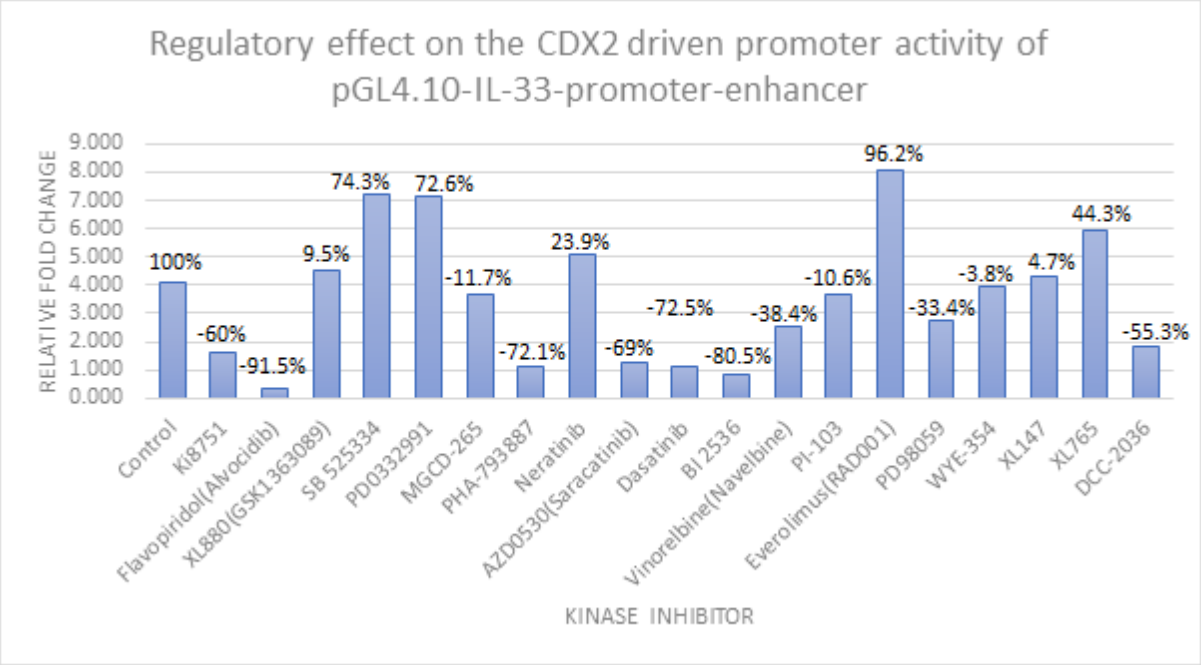


Figure 18 Regulatory effect on the CDX2 driven promoter activity of pGL4.10-IL-33-promoter-enhancer. The relative fold change (y-axis) was derived from the difference between 16 ng/ml DOX divided with 0 ng/ml DOX. Each KI was compared to the control, and the KIs that showed a further increase in the promoter activity was considered relevant. These were XL880(GSK1363089), SB 525334, PD0332991, Neratinib, Everolimus and XL765. The regulatory effect in percentage is shown above each KI and they are calculated based on the control being 100%.

To make sure that the KIs only exert their regulatory effect on LS174T7D9 and not killing the cells a cell viability check was done by assessing the secreted Lucia luciferase found in the media of LS174T7D9. The assays cell viability was checked before LS174T7D9 was harvested and measured for luciferase and β-galactosidase activity (Figure 19). The Graph shows that LS174T7D9 cell viability does not remain constant when KIs is added to the cells. KIs Flavopiridol, PHa-793887, Neratinib, AZD0530, Dastinib, BI 2536, Vinorelbine and Everolimus all show a decrease in secreted Lucia luciferase, because of a possible effect through the KI target.

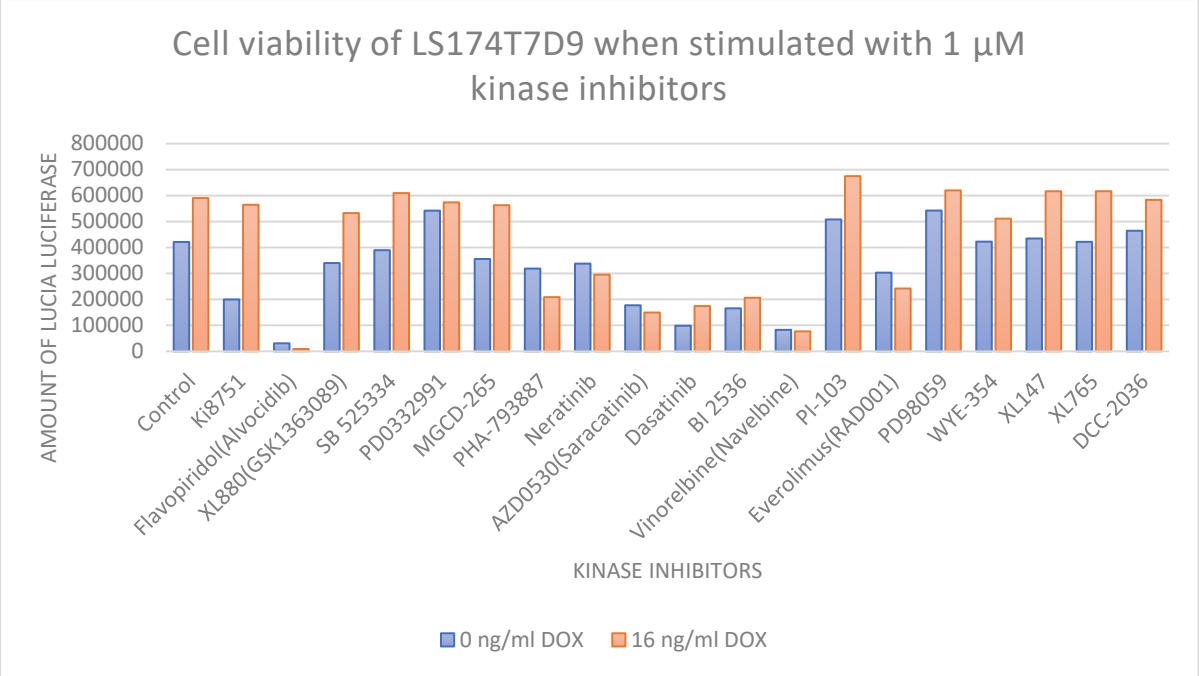


Figure 19 Secreted Lucia luciferase from LS174T7D9. The cells were stimulated with 1 µM KI, incubated for 72 H and induced with 0 ng/ml and 16 ng/ml DOX. The cell media was taken out before harvest and measurement, where the secreted Lucia luciferase is obtained. The y-axis is the amount of secreted Lucia luciferase measured and the x-axis is the kinase inhibitors.

In brief, based on the result outcome from the CDX2 driven promoter activity, where the promoter activity increase when CDX2 drives the promoter of pGL4.10-IL-33-promoter-enhancer that showed a significant difference between 0 ng/ml and 16 ng/ml DOX, five KIs were chosen further assessment. These five KIs regulatory effect showed a further increase promoter activity. These five KIs were SB 525334, PD0332991, Neratinib, Everolimus and XL765.

The following data shown here is not validated and therefore shows the same outcome as the 19 KIs data for the CDX2 driven promoter activity of pGL4.10-IL-33-promoter-enhancer construct. However, for a better overview, the data is displayed only for the top five KIs.

The top five KIs SB 525334, PD0332991, Neratinib Everolimus and XL765 all had the same promoter activity at 0 ng/ml DOX as the control, however when assessing which KIs had a further increase in CDX2 driven promoter activity SB 525334, PD0332991, Everolimus and XL765 showed an increase expect from Neratinib (Figure 20). Statistical analysis showed that SB 525334, PD0332991, Everolimus and XL765 were significant when inducing from 0 ng/ml to 16 ng/ml DOX. However, for the control and Neratinib did not show a significant difference when inducing from 0 ng/ml to 16 ng/ml DOX.

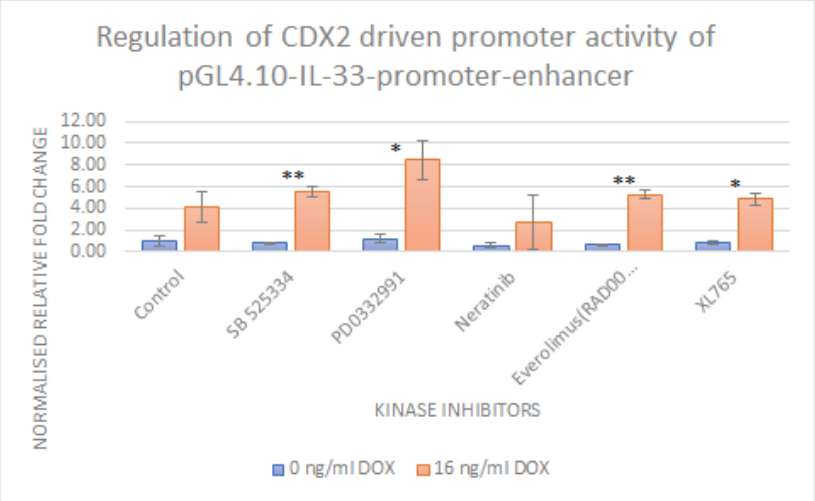


*Figure 20 Validation of the top five KIs regulatory effect on the CDX2 driven promoter activity of pGL4.10-IL-33-promoter-enhancer. The graph shows the normalised relative fold change of how the CDX2 driven promoter activity of pGL4.10-IL-33-promoter-enhancer is regulated by SB 525334, PD0332991, Everolimus (RAD001) and XL765. The control used in the assay had no KI added. The assay was done in triplicate Statistical analysis was done with a Student T-test, where KIs showed a significant difference with a p-value of <0.05 in promoter activity when LS174T7D9 was induced with 16 ng/ml DOX is noted with *. p-value < 0.05*, p-value < 0.01** and p-value < 0.001***.*

The regulatory effect of the five KIs on CDX2 driven promoter activity of pGL4.10-IL-33-promoter-enhancer construct showed that they all increase the promoter activity further when compared with the control. SB 525334 had 74.5 %. PD0332991 had 72.6 %, Neratinib had 23.9%, Everolimus had 96.2% and XL765 had 44.3% further increase of the promoter activity when setting the control to 100% (Figure 21).

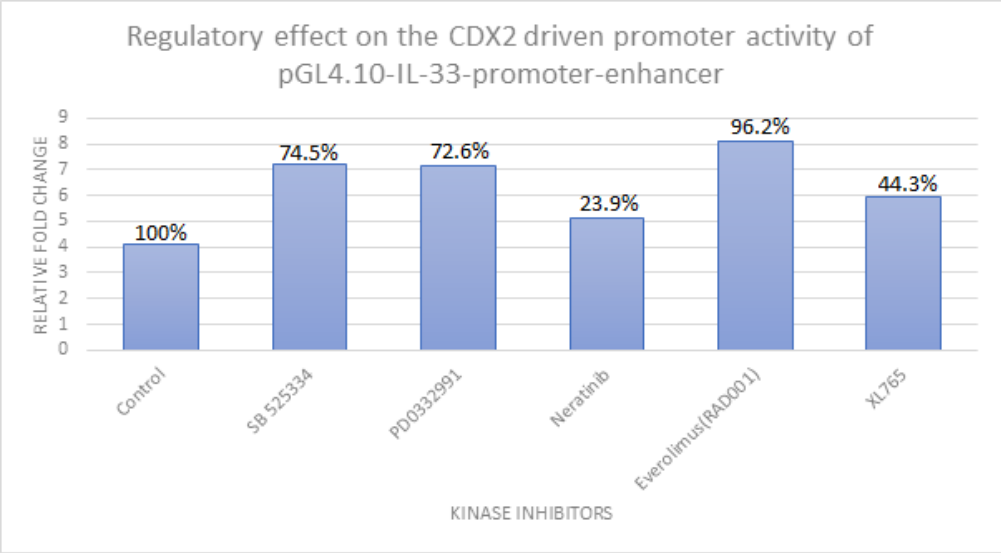


Figure 21 Regulatory effect on the CDX2 driven promoter activity of pGL4.10-IL-33-promoter-enhancer. The relative fold change (y-axis) was derived from the difference between 16 ng/ml DOX divided with 0 ng/ml DOX. Each KI was compared to the control, and the KIs that showed a further increase in the promoter activity was considered relevant. These were SB 525334, PD0332991, Neratinib, Everolimus and XL765. The regulatory effect in percentage is shown above each KI and they are calculated based on the control being 100%.

8.3.1 Wnt driven promoter activity of pGL4.10-IL-33-promoter-enhancer when stimulated with the selected 19 KIs

Based on the selection process 19 KIs met the applied criteria Ki8751, Flavopiridol (Alvocidib), XL880 (GSK1363089), SB 525334, PD0332991, MGCD-265, PHA-793887, Neratinib, AZD0530, Dastinib, BI 2536, Vinorelbine (Navelbine), PI-103, Everolimus (RAD001), PD98059, WYE-345, XL 147, XL765 and DOC-2036. The purpose with this validation was to find KIs that decrease the Wnt promoter activity of TOPflash and when compared to the control's 0 ng/ml and that have the same promoter activity at 0 ng/ml. Here samples that increases the promoter activity will not be assessed in this thesis. Statistical analysis showed a significant difference in the promoter activity when LS174T7D9 were stimulated with PD0332991, MGCD-265, PI-103, XL765 and DCC-2036. KIs that both decrease the promoter activity and has the same promoter activity at 0 ng/ml as the control were XL880(GSK1363089), Dasatinib, PI-103, PD98059, XL174 and XL765. On the graph, the significance level is shown by stars for those KIs that had a difference in the promoter activity (Figure 22).

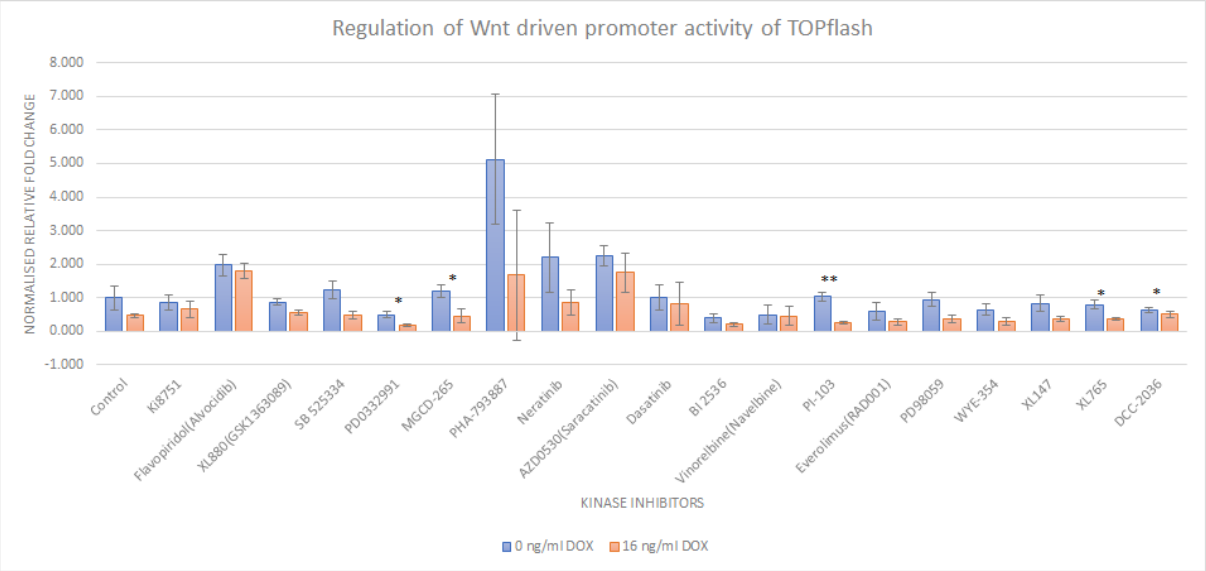


*Figure 22 Validation of the 19 KIs chosen after selected based on the predetermined criteria (see Flowchart). The graph shows how the Wnt driven promoter activity of TOPflash is regulated by Ki8751, Flavopiridol (Alvocidib), XL880 (GSK1363089), SB 525334, PD0332991, MGCD-265, PHA-793887, Neratinib, AZD0530, Dastinib, BI 2536, Vinorelbine (Navelbine), PI-103, Everolimus (RAD001), PD98059, WYE-345, XL 147, XL765 and DOC-2036 when normalised to the relative fold change in regulation. The x-axis shows the KIs. The control used in the assay had no KI added. The assay was done in triplicate. Statistical analysis was done with a Student T-test, where KIs showed a significant difference with a p-value of <0.05 in promoter activity when LS174T7D9 was induced with 16 ng/ml DOX is noted with *. p-value < 0.05*, p-value < 0.01** and p-value < 0.001***.*

Each KI was compared to the control (no KI was added) to assess the change in the regulatory effect of the Wnt driven promoter activity of TOPflash construct (Figure 23

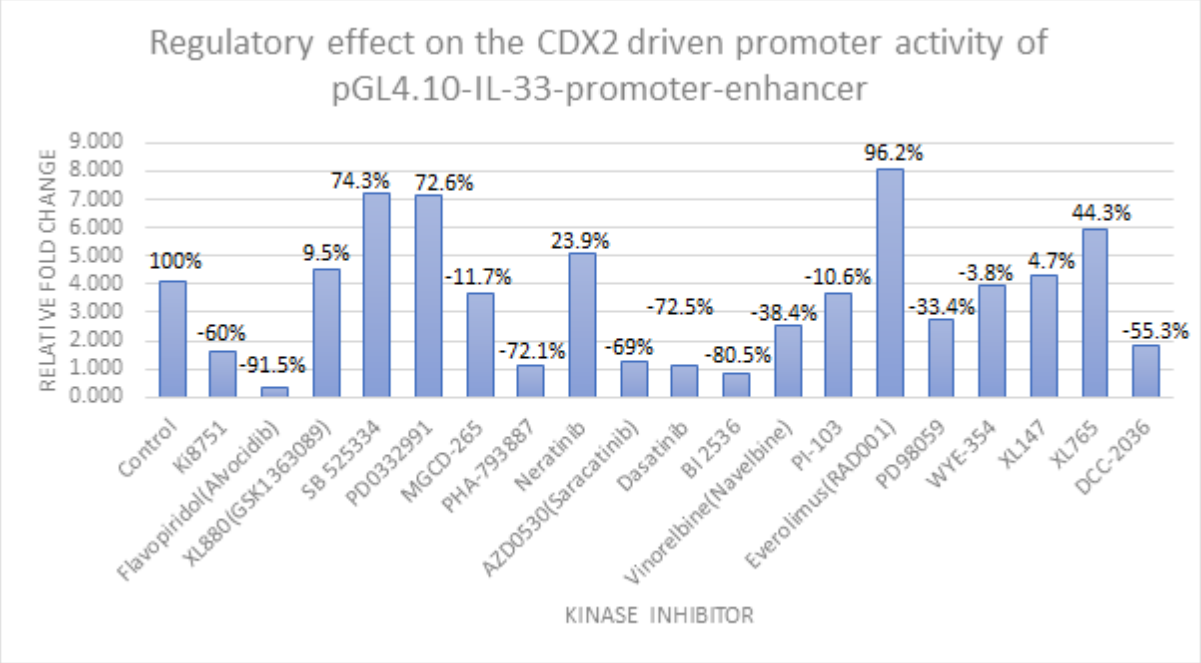


Figure 18 Regulatory). The focus here was to find KIs that further decreased the promoter activity, which happened when LS174T7D9 was stimulated with SB 525334, PD0332991, MGCD-265, PHA-793887, Neratinib, PI-103, Everolimus(RAD001), PD98059, WYE-354, XL147 and XL 765. Above each KIs is given, in percentage, how much each KI compared to the control either increase or decrease the Wnt driven promoter activity of TOPflash setting the control to 100%. The graph shows that relative fold change in percentage of the Wnt driven promoter activity of TOPflash, where SB 525334 further decreases the promoter activity with 25.9%, PD0332991 with 39.2%, MGCD-265 with 29.9%, PHA-793887 with 51.5%, Neratinib with 26%, PI-103 with 91.1%, Everolimus(RAD001) with 4%, PD98059 with 34.9%, WYE-354 with 3.9%, XL147 with 11.5% and XL 765 with 6.6% when compared with the control.

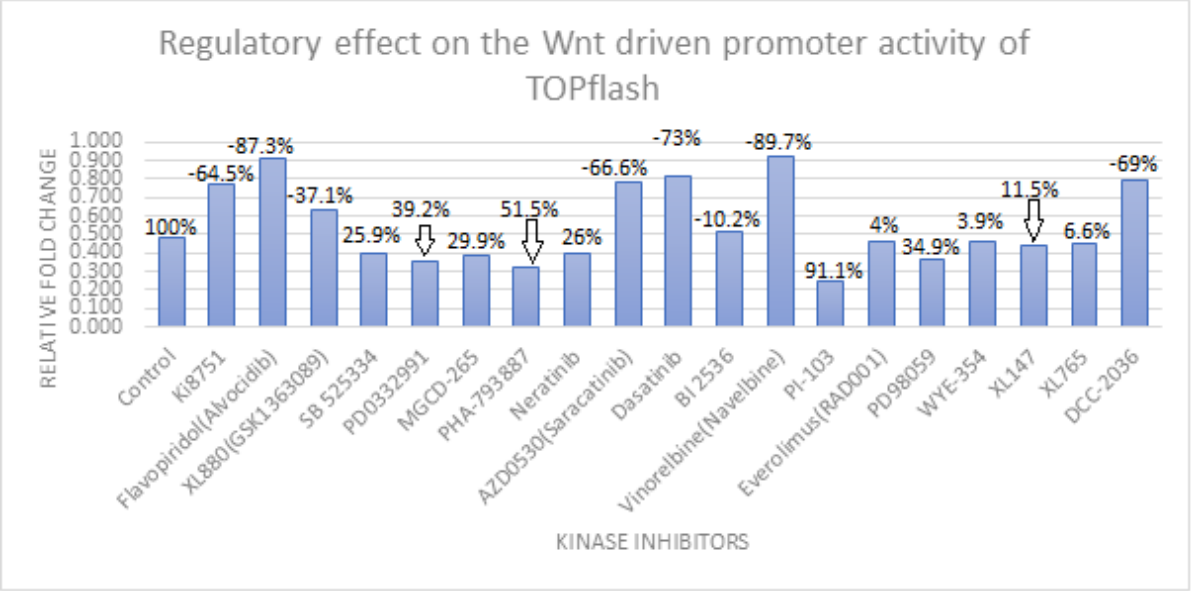


Figure 23 Regulatory effect on the Wnt driven promoter activity of TOPflash. The relative fold change (y-axis) was derived from the difference between 16 ng/ml DOX divided with 0 ng/ml DOX. Each KI was compared to the control, and the KIs that showed a further decrease in the promoter activity was considered relevant. These were SB 525334, PD0332991, MGCD-265, PHA-793887, Neratinib, BI-2536, PI-103, Everolimus(RAD001), PD98059, WYE-354, XL147 and XL765.The regulatory effect in percentage is shown above each KI and they are calculated based on the control being 100%.

To make sure that the KIs only exert their regulatory effect on LS174T7D9 and not killing the cells a cell viability check was done by assessing the secreted Lucia luciferase found in the media of LS174T7D9. The assays cell viability was checked before LS174T7D9 was harvested and measured for luciferase and β-galactosidase (Figure 24). The graph shows that LS174T7D9 cell viability does not remain constant when KIs is added to the cells. KIs Flavopiridol, PHA-793887, Neratinib, AZD0530, Dasatinib, BI-2536, Vinorelbine and Everolimus all show a decrease in secreted lucia luciferase, because of a possible effect through the KIs target.

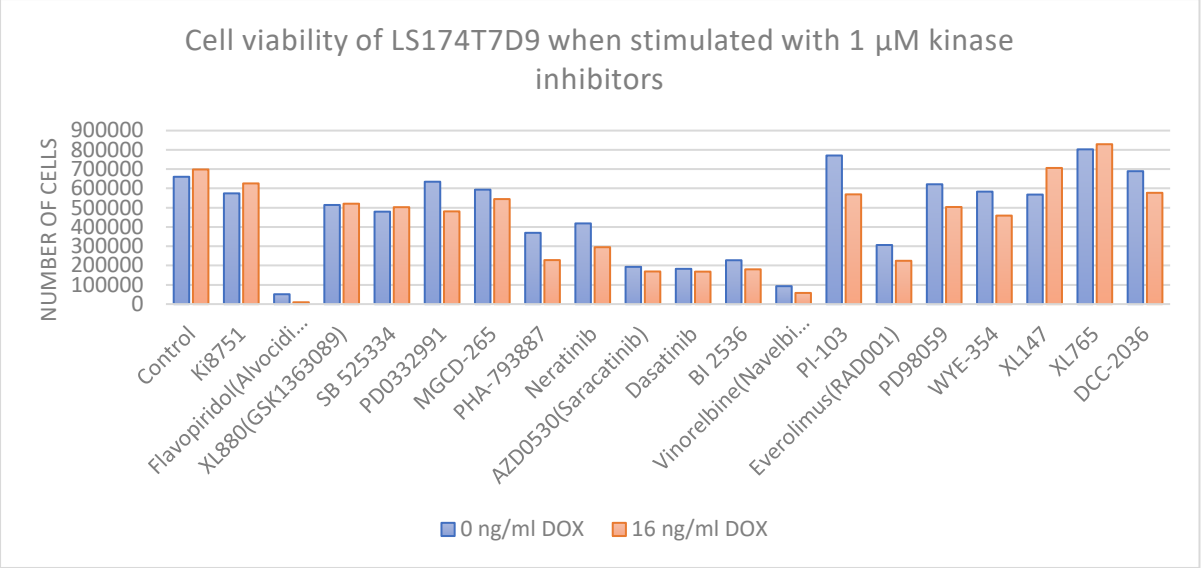


Figure 24 Secreted Lucia luciferase from LS174T7D9. The cells were stimulated with 1 µM KI, incubated for 72 H and induced with 0 ng/ml and 16 ng/ml DOX. The cell media was taken out before harvest and measurement, where the secreted Lucia luciferase is obtained. The y-axis is the amount of secreted Lucia luciferase measured and the x-axis is the kinase inhibitors.

To summarize, based on the result outcome of the Wnt driven promoter activity of TOPflash, a further decrease in the promoter activity and KIs that showed to have a significant difference between 0 g/ml and 16 ng/ml DOX, only two KIs showed both effects. The KIs were PD 525334 and PI-103. However, the Wnt promoter activity of TOPflash were assessed again but with the five KIs chosen from the CDX2 driven promoter activity.

The Wnt driven promoter activity of TOPflash was assessed with the top five KIs (Figure 25). The graph showed that SB525334, PD0332991, Everolimus and XL765 did not have the same starting point at 0 ng/ml DOX as the control, only Neratinib showed the same starting point. However, statistical analysis showed that the control, SB 525334, PD0332991 and Everolimus had a significant difference in the promoter activity, whereas Neratinib and XL765 did not.

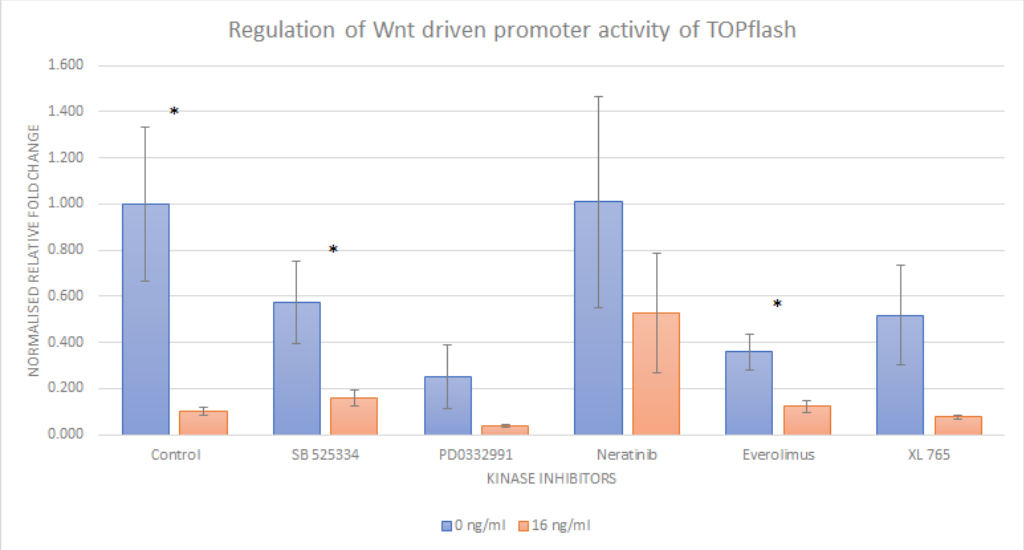


*Figure 25 Validation of the top five KIs regulatory effect on the Wnt driven promoter activity of TOPflash. The graph shows the normalised relative fold change of how the WNT driven promoter activity of TOPflash is regulated by SB 525334, PD0332991, Everolimus (RAD001) and XL765. The control used in the assay had no KI added. The assay was done in triplicate Statistical analysis was done with a Student T-test, where KIs showed a significant difference with a p-value of <0.05 in promoter activity when LS174T7D9 was induced with 16 ng/ml DOX is noted with *. p-value < 0.05*, p-value < 0.01** and p-value < 0.001***.*

The regulatory effect on Wnt driven promoter activity of TOPflash with the 5 KIs showed that none of them decreased the promoter activity additionally when compared to the control. For a better overview of a possible decrease in promoter activity the regulation between 0 ng/ml and 16 ng/ml were found by taking the logarithm to the numbers, showing the normalized relative fold change in log with a base of 2. SB525334 had a decrease of the promoter activity with -44.02%, PD0332991 with -15.9%, Neratinib with -71.6%, Everolimus with -53.5% and XL765 with -16.4% when compared with control of 100% (Figure 25). The graph shows that none of the KIs showed a further decrease in Wnt driven promoter activity when compared with the control.

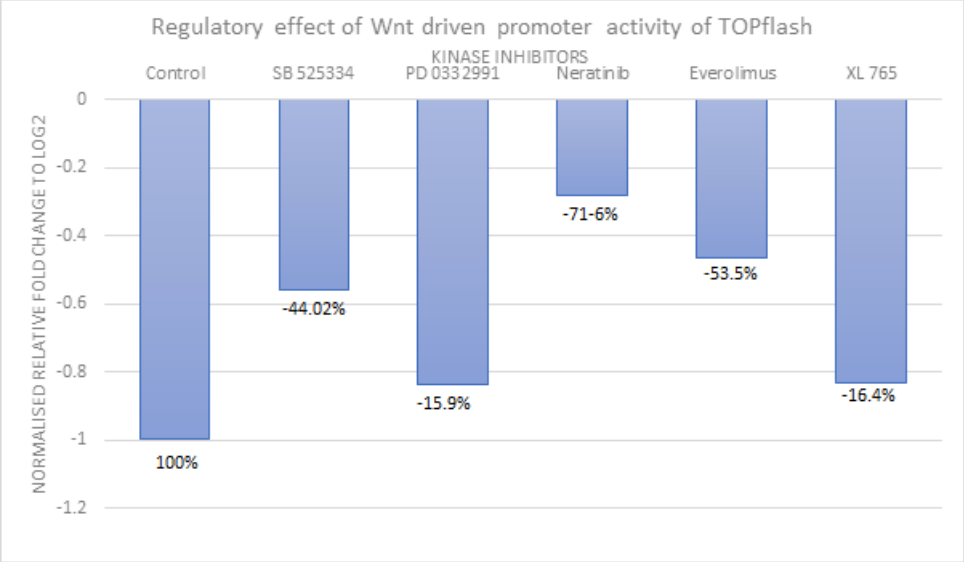


Figure 26 Regulatory effect on the Wnt driven promoter activity of TOPflash. The relative fold change (y-axis) was derived from the difference between 16 ng/ml DOX divided with 0 ng/ml DOX. Each KI was compared to the control, and the KIs that showed a further decrease in the promoter activity was considered relevant. None of the KIs showed a further decrease in Wnt driven promoter activity. The regulatory effect in percentage is shown above each KI and they are calculated based on the control being 100%.

To summarize, the results of screening the kinase inhibitor library showed that when inducing the CDX2 driven promoter activity of pGL4.10-IL-33-promoter-enhancer with 0 ng/ml and 16 ng/ml and stimulating LS174T7D9 with the four KIs, SB 525334, PD0332991, Everolimus and XL765, a regulatory effect was found on the CDX2 driven promoter activity of pGL4.10-IL-33-promoter-enhancer. However, the Wnt driven promoter activity of TOPflash assay did not find any KIs that shared the same promoter activity with the control at 0 ng/ml and was significantly difference when inducing LS174T7D9 with 16 ng/ml DOX. A further decrease was not found in the Wnt driven promoter activity of TOPflash, when comparing the KI with the control.

8.4 RT-qPCR of ABCB1, IL-33 and MYC.

Three target genes were chosen for the assessment of how CDX2 had a possible regulatory effect on respectively ABCB1, IL-33 and MYC. ABCB1 are the most widely known ABC transporters and they have been associated with Multi drug resistance (MDR) problems in relation to human cancers (An & Ongkeko, 2009; Litman et al., 2001; Shukla et al., 2021; Sun et al., 2012). There has been reported a correlation between the expression of CDX2 and the amount of expressed ABCB1 transporter, therefore the effect of expressed CDX2 on ABCB1 transporter is assessed by RT-qPCR. Due to the limited knowledge regarding how IL-33 is controlled, this gene was also assessed in the relation with CDX2 expression. As

mentioned earlier, CDX2 and Wnt signalling have seen to have a cross talk relation and one of the Wnt transduction pathways target genes is MYC. Therefore, RT-qPCR will help assess the crosstalk relation between CDX2 and Wnt signalling further by using MYC. The assays were conducted with inductions of 0 and 16 ng/ml DOX and then stimulated with 1 μM of either SB 525334, PD033299, Neratinib, Everolimus and XL765.

First to check if the qPCR machine worked optimally the positive GAPDH control (provided by the manufacturer) was analyzed (Figure 27). The results showed that of the two technical duplicates assessed, only one sample showed an amplification (Red) of the cDNA template of GAPDH. The amplification is set to start around cycle 25-26 and the change in the reference dye SYBR Green fluorescence was around 210. For the other sample, no amplification was observed (Blue).

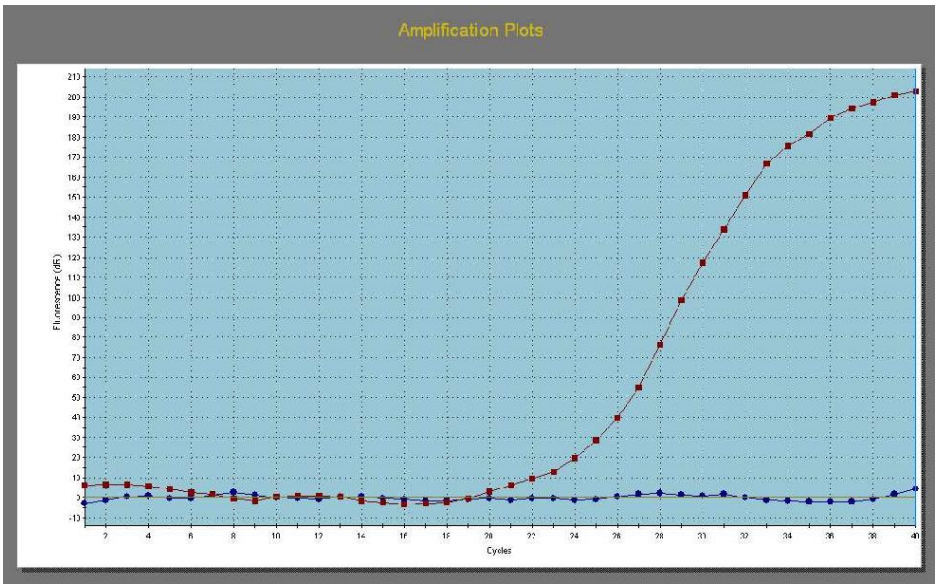


Figure 27 Amplification plot of GAPDH. y-axis show the change in reference dye SYBR Green (dR) pr cycle (x-axis). The fluorescens value peak at 200. It is also important to state that the y-axis is ΔR (delta R) and not Rn as used for other amplification plots. Instead of being normalized to the passive reference dye (ROX), it is the change in the reference reporter dye (SYBR Green), that is shown in the graph pr cycles.

After the assessment of the positive GAPDH control, a dilution series of 10^{-1}, 10^{-2} and 10^{-3} was made from the sample which was not stimulated by KIs but was induced with 16 ng/ml DOX to assess the optimal RNA concentration. The concentration of the sample was 74.7 ng/ul and 1860 ng for the whole sample. (The rest of the concentrations can be found in appendix 13.9). H_20 was used as a negative control and RPLP01 was used as a positive control (house-keeping gene), used for normalization of target gene expression. The assay will include samples receiving reverse transcriptase and samples not receiving reverse transcriptase. For the negative control with H_20, no reverse transcriptase nor cDNA is added.

The optimal concentration of RNA was tested twice due to a possible error in the first set up (Appendix 13.10), however, upon testing the optimal concentration again the result outcome was the same as the first time (Figure 28).

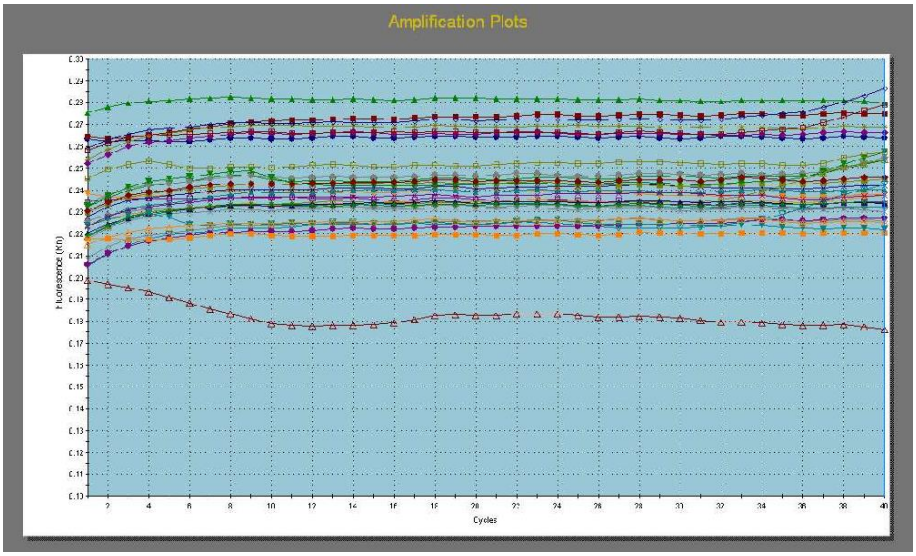


Figure 28 Amplification plot of ABCB1, IL33 and MYC. Samples contained reverse transcriptase and cDNA. X-axis shows cycles and y-axis show measured fluorescence normalized to the passive reference dye (Rn).

To summarize, these experiments did not yield useful data for the further assessment of CDX2s involvement in regulation of the three target genes *ABCB1, IL-33 and MYC,* because the qPCR did not work. However, what might be expected to see, if the results did yield useful data, will be discussed.

9 DISCUSSION

The following section will discussion if Ls174T7D9 has given the information wanted regarding investigate the regulatory mechanism behind CDX2. This section will further discussion which new pathways were found to regulate CDX2 gene regulatory effect in LS174T7D9 culminating in two overviews of where the KIs exert their inhibition and furthermore a figure illustrating where the new pathways regulated CDX2. Finally, the section will discussion what was expected to be found from the qPCR results, when cross referencing with previously published data.

9.1 LS174T7D9 AN MODEL ORGANISM TO INVESTIGATE REGULATORY MECHANISMS BEHIND CDX2

A model organism that creates a situation where no transcription of a given gene can simply be induce by adding a drug and then controlling the transcription has not to this date been created. However, Pinto et al., 2017 created a system where it was possible to avoid leakiness and ensure controllable gene expression. By using Precise integration of inducible transcriptional elements (PrIITE), they knocked out CDX2 with TetOn and then they inserted TRE and CDX2 into AAVS1 locus site. PhD student Stine Bull Jensen created a system in which cell viability served as a proxy for secreted *Lucia luciferase* transcribed through its promoter hEF1. Since TRE and CDX2 only occupied one allele in the AAVS1 locus, it was possible by homology recombination to insert hEF1 and the *Lucia luciferase* gene onto the second allele. The new model organism containing these two systems was LS174T7D9 and these modified cells made it possible to investigate and find the previously described results.

Based on the results it was found that when inducing and stimulating LS174T7D9 with DOX and KIs, it was possible to control and regulate CDX2. The optimal condition for the LS174T7D9 to induce and stimulate CDX2 driven promoter activity of pGL4.10-IL-33-promoter-enhancer construct was to incubate the cells for 72 hours, induce with 16 ng/ml DOX and stimulate with 1 μM of KI. The same condition was also applied when investigating the Wnt driven promoter activity of TOPflash. To check if CDX2 could drive the promoter activity of pGL4.10-IL-33-promoter-enhancer other previously published promoters were applied. CDX2 had been shown to regulate HEPH (Boyd et al., 2010; Coskun et al., 2012; Hinoi et al., 2005), YAP1 (Larsen et al., 2019) and GPA-33 (Hinio et al., 2002; Pinto et al., 2017), which made them good controls to check if the DOX induction work optimally.

LS174T7D9 also provided when to stimulate the cells with 1 μM KI. This was done by using two previously published KIs that had shown to have a regulatory effect on CDX2. The two KIs were U0126 (Rings et al., 2001) and SB203580 (Houde et al., 2001), which inhibits MEK1/2 in the Ras/Raf/MEK/Erk pathway and mitogen p38, respectively. The articles found that for U0126 the transactivation of CDX2 decreased when stimulated with U0126, whereas the transactivation of CDX2 increased when stimulated with SB203580. This does not consistent with the results found for U0126. During the optimization, it was found that the CDX2 driven promoter activity of pGL4.10-IL-33-promoter-enhancer increased when stimulated with U0126. For the Wnt driven promoter activity of TOPflash the results showed that when stimulating LS174T7D9 with SB203508 there were an increase in the promoter activity when compared with the control. The results showed that when inducing the LS174T7D9 cell with DOX, thereby increasing CDX2, the Wnt driven promote activity decrease, however this is not consistent with what figure 15B shows. Here the Wnt driven promoter activity of TOPflash increases when stimulated with SB203508 compared to the control. It would be expected to decrease the Wnt driven promoter activity because previous published data found that when downregulating or reducing CDX2 expression an increase of Wnt signalling occurred (Boyd et al., 2010; Coskun et al., 2014).

As mentioned, LS174T7D9 were equipped with a cell viability system, which were checked by the amount of secreted Lucia luciferase. If the cell is vial, the continuous transcription of *Lucia luciferase* gene will occur. The cell viability was first checked during the screening of the kinase inhibitor library, to make sure the cells kept proliferating when stimulated with 1 μM KI. For both pGL4.10-IL-33-promoter-enhancer and TOPflash assays, the cell viability did not remain constant when stimulating LS174T7D9 with the 19 KIs. The data showed that for both promoter constructs Flavopiridol, PHa-793887, Neratinib, AZD0530, Dastinib, BI 2536, Vinorelbine and Everolimus all had a decrease in secreted Lucia luciferase. The interesting point here is that it is the same KIs for both promoter construct showed a decrease in secreted lucia luciferase. When taking a closer look at what the different KIs target, it is cell cycle phases G1 and G2/M, cell proliferation and cell cycle progression. If the KIs inhibit pathways that control cell cycle, cell proliferation and cell cycle progression, the cells will not be able to secret the same amount of Lucia luciferase as the cells which do not have their cell cycles, cell proliferation of cell progression inhibited.

LS174T7D9 showed to be beneficial when assessing the 196 KIs from the commercially screening library. This was illustrated by three 3D graphs giving the total overview how the 196 KIs affected the CDX2 driven promoter activity of pGL4.10-IL-33-promoter-enhancer (Figure 16). However, it is important to notice that the whole screening was done in duplicates, whereas triplicate would have been optimal. But by applying CV% and other criteria listed in Table 4 it was possible to select the 19 KIs for further assessment and then compensating for the lack of a Student T-test. After validation, the LS174T7D9 helped provided information of the top five selected KIs regulatory effect on the CDX2 driven promote activity of pGL4.10-IL-33-promoter-enhancer. The results showed the promoter activity of pGL4.10-IL-33-promoter-enhancer increased in LS174T7D9 when induced with 16 ng/ml DOX and that when stimulated with SB525334, PD0332991, Everolimus and XL765 a further increase in the promoter activity happened. Since these assays were done in triplicates it was possible to verify a significant difference in promoter activity when inducing LS174T7D9 with 0 ng/ml and 16 ng/ml DOX by applying Student t-test. LS174T7D9 identified for the Wnt driven promoter activity of TOPflash two KIs that regulated CDX2 after selection of the 19 KIs. PD0332911 and PI-1003 was both significant and increase the promoter activity further. However, only PD0332991 was chosen since it was also found for the CDX2 driven promoter activity. Further validation of the five KIs stimulation on the Wnt driven promoter activity of TOPflash did show that SB525334 and Everolimus were significantly different in promoter activity when inducing the cells with DOX but did not show any further decrease in the promoter activity of TOPflash.

Finally, for the screening of the KIs the KIs stimulatory effect on CDX2 and the Wnt signalling were compared to the controls where no KI was added. All the experiment's controls, except for the regulation of the Wnt driven promoter activity of TOPflash with the five KIs, were not significantly different when inducing LS174T7D9 with DOX. Therefore, all the findings from the screening of the 196 KIs needs to be further validated to support the result outcomes.

Because the modified LS174T7D9 has not been tested before, it is not possible to compare with other published data and therefore it is necessary to investigate LS174T7D9 with other genes besides CDX2 and with other stimulants/drugs. But for this thesis aim, LS174T7D9

has been proven to aid with the necessary information to screen a possible regulatory effect on the CDX2 through stimulation with KIs.

9.2 A POSSIBLE REESTABLISHMENT OF CDX2 IN CRC CELLS AND DECREASE IN FURTHER PROGRESSION

From the screening of the kinase inhibitor library 5 KIs were selected for further validation, because they showed a further increase in the CDX2 driven promoter activity of pGL4.10-IL-33-promoter-enhancer. The five KIs were SB 525334, PD0332991, Neratinib, Everolimus and XL765. From these, all showed a significant difference when LS174T7D9 was induced with 16 ng/ml except from Neratinib. The target site for SB525334 is TGFβ-R1/ALK5, which is a part of the TGFβ pathway, which initiate transcription of targets genes responsible for cell proliferation, differentiation, cell survival and apoptosis. Neratinib target the HER2/EGF receptor, which through the binding of ligands activate the Raf/Ras/MEK/ERK pathway. This pathway leads to the activation of cell survival, cell proliferation and cell cycle progression. Furthermore, HER2/EGF also activate the PI3K pathway. In this pathway XL765 function as a dual inhibitor, which targets both PI3K and mTORC1, this leading to cell growth and protein synthesis activation. mTORC1 is also a target for Everolimus, which through the inhibition of FKBP12 bound with rapamycin inhibits mTORC1and thereby the cell growth and protein synthesis is also inhibited (Figure 29).

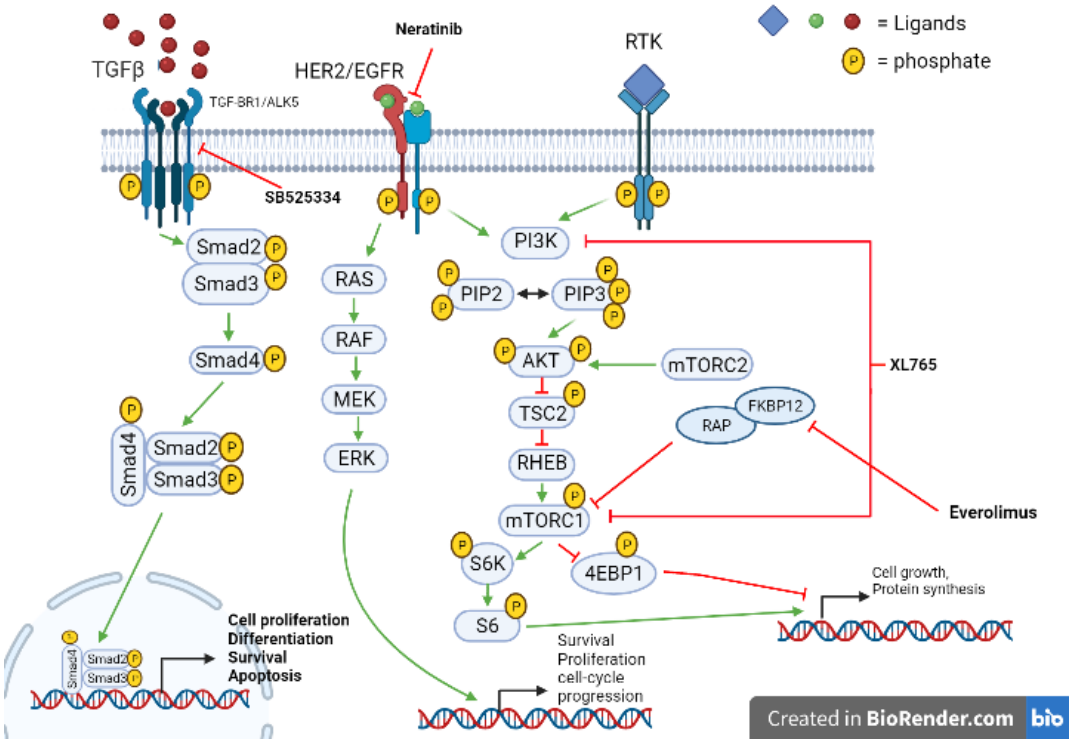


Figure 29 Overview of the top five KIs target sites in TGFB, HER2/EGFR and PI3K pathway. The figure shows that SB 525334 inibitbs the TGF-BR1/ALK5 receptor in the TGFB pathway. Neratinib inihibits the HER/EGR receptor in the Ras/Raf/MEK/Erk pathway. XL765 is a dual inibitor of both PI3K it self and mTORC1. Everolimus inhibits FKBP12 in a rapamycin complex, which together inhibits mTORC1. These pathways all lead to the transcription of target genes involved in cell proliferation, differntiation, cell survival, apotosis, cell cycle prograaion, cell growth and protein synthesis. The green arrows indicate activation and the red blunt end arrows indicate inhibition (own creation through BioRender).

The target site for PD0332991 is cycline dependent kinase 4/6, which is a part of the G1 phase of the cell cycle (Figure 30).

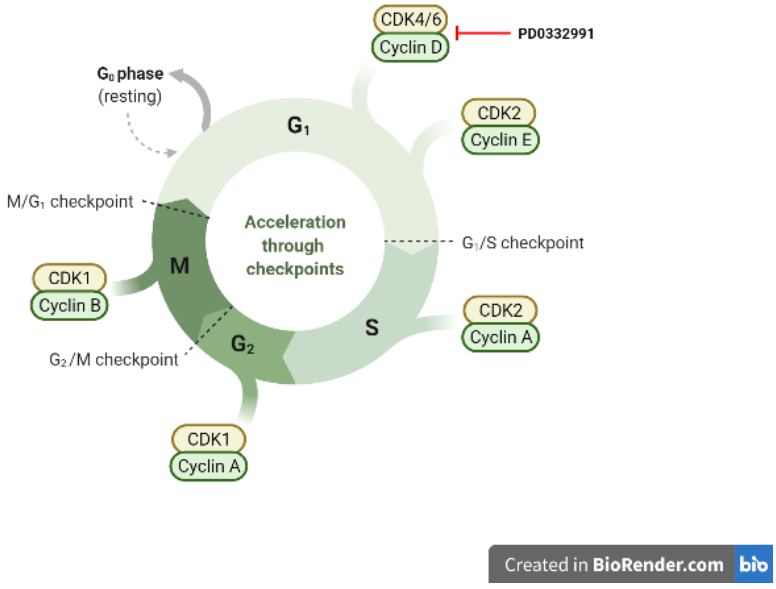


Figure 30 Overview of where PD0332991 targets the cell cycles. PD0332991 inhibits the cycline dependent kinase 4/6 in the G1 phase of the cell cycle. the red blunt end arrow indicates inhibition (own creation through BioRender).

Since only the CDX2 driven promoter activity of pGL4.10-IL-33-promoter-enhancer showed a further increase in the promoter activity and the Wnt driven promoter activity of TOPflash needed further assessment, only the CDX2 driven promoter activity of pGL4.10-IL-33-promoter-enhancer will be addressed in the next part of the discussion.

Previous data showed that CDX2 had been regulated both on a transcriptional and post translational level (Figure 4). Transcriptional regulation both inhibitory and activating occurred through GATA6, TCF7L2, HNFα, NKF-κB and promoter methylation (Benahmed et al., 2008; Boyd et al., 2009; Zheng et al., 2014; Kim et al., 2002; Graule et al., 2018). Post translational inhibition of CDX2 happened through phosphorylation of MAPK (Rings et al., 2001) and phosphorylation of Cdk2 (Boulanger et al., 2005) and CDX2 were activated by phosphorylation of mitogen p38 (Houde et al., 2001), which is also a component in the MAPK pathway. When comparing these previous published data with what my results have shown, all the 4 significant KIs increase CDX2, when assessing the CDX2 promoter activity. TGF-β, Raf/Ras/MEK/ERK, PI3K and mTOR pathways could by possible regulatory pathways by which CDX2 is regulated in LS174T7D9 (Figure 31). However, these previous published data have been shown through expression analysis, such as qPCR, western blotting, or northern blotting, whereas my results are solely based on the change in CDX2 promoter activity. Therefore, further testing is needed for the KIs regulatory effect on CDX2 at either mRNA or protein level through qPCR or western/northern blotting.

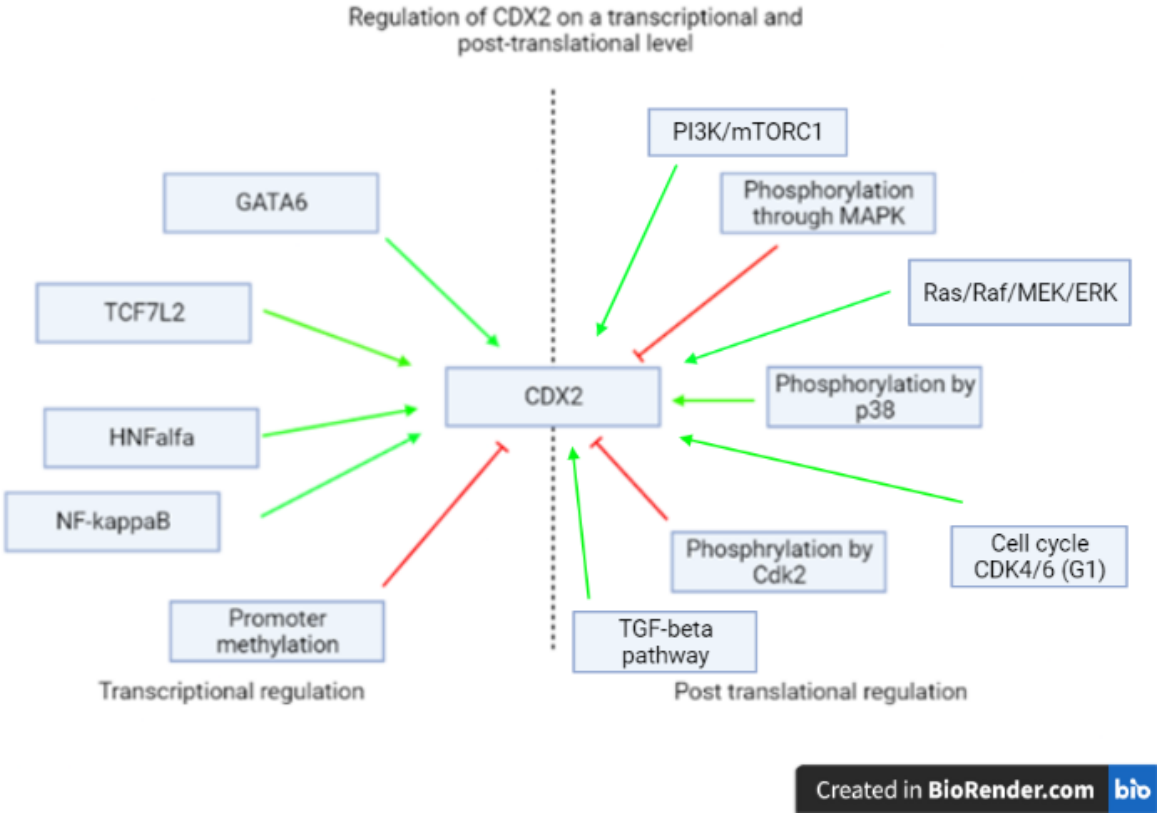


Figure 31 Transcriptional and post-translational regulation of CDX2 including the 4 new pathways. The figure illustrates CDX2 regualtion at a transcriptinal level by GATA6, TCF7L2, HNFα NF-κB and pormoter methylation and translational level by PI3K/mTORC1(new), MAPK, Ras/Raf/MEK/ERK (new), p38, Cell cycle CDK4/6 (new), CDK2 and TGF-β (new). (Own creation through BioRender).

As stated in the introduction, CRC cells lack or shown aberrant expression of CDX2 (Dalerba et al., 2016). The optimal situation would be to restore the CDX2 expression of the CRC cells and thereby limiting further cell proliferation and possible metastatic progression of the CRC. Interpretation of the CDX2 driven promoter activity of pGL4.10-IL-33-promoter-enhancer construct showed that when inducing LS174T7D9 with 16 ng/ml DOX there was a further increase in the CDX2 driven promoter activity and together with the cell viability check LS174T7D9 was still vial during the stimulation with the four kinases expect for Everolimus, which showed a lower amount of measured Lucia luciferase in the media (Figure 19). This scenario could possibly be beneficial when battling cancer and its progression. If these four KIs can increase the presence of CDX2 in the CRC cells, inhibit pathways which leads to activation of cell proliferation, cell survival, cell growth and etc, and still not kill the healthy tissue they would be KIs that should be further researched.

However, while the restoration of CDX2 in CRC seems to be beneficial in the battle of controlling and limiting CRC progression, a study investigating overexpression of CDX2 in

13 cancer cell lines, showed an increased in the development of chemoresistance towards chemotherapy drugs (Takakura et al., 2010). This will be discussed in the following section.

9.3 CDX2'S POSSIBLE INVOLVEMENT IN CHEMORESISTANCE, CROSSTALK WITH WNT AND LOCAL INFLAMMATION

The following discussion is solely based on assumption made from previous published data and what might had been expected to see if the qPCR had worked and given useful results.

In short, three genes, transporter gene ABCB1, alarmin IL-33 and oncogene MYC, were set to be amplified from RNA extract from samples containing DOX induction with 0 ng/ml and 16 ng/ml and stimulation with the five top KIs SB 525334, PD0332991, Neratinib, Everolimus and Xl765. However, no results were obtained from the qPCR machine, because of a malfunction of the machine. Only one sample with GAPDH control of a set of duplicates showed to be amplified at 25-26 cycles, with melting curve analysis showing a peak at around 86°C.

9.3.1 ABCB1 transporter

Until now, CDX2 have proven to be a tumour suppressor in the development of CRC, however, Takakura et al., 2010 showed that CDX2 may function as a link in obtaining chemoresistance through the upregulation of ABCB1 transporters. The normal function of ABC transporters is to transport hormones, lipids, ions, xenobiotics, maintenance of osmotic homeostasis, antigen processing, cell division, cholesterol homeostasis, regulating central nervous system permeability and blood-brain barrier, which makes them responsible for a wide range of physiological functions (Gottsman et al., 2002; Liu et al., 2019; Robey et al., 2018). In human CRC cells the transcription of ABCB1 transporter was found to be initiated by CDX2. Different colorectal cell lines, such as Caco-2, DLD1, GCT116, HT-29, LoVo, LS174T, SW48, SW1116, SW480, Sw837, SW1463 and WiDr, was investigated to see the expression pattern of MDR1/ABCB1 and CDX2. The study found that *MDR1/ABCB1* was a primary target for CDX2 activity. Figure 32 shows a correlation between CDX2 expression and *MDR1/ABCB1* expression that when CDX2 was expressed, *MDR1/ABCB1* was expressed. However, for two of the eight cell lines, LS174 and SW48, an undetectable level of *MDR1/ABCB1* (Takakura et al., 2010). This indicating that most of the colorectal cancer cell lines showed an increase in the presences of MDR1/ABCB1 transporters. Hence, increasing the chemoresistance mechanisms of enhanced efflux of drug when CDX2

expression levels is increased as well. However, based on the LS174 and SW48 data, CDX2 activity may not be sufficient for the transcription of *MDR1/ABCB1*, and other factors may be involved in initiating the transcription.

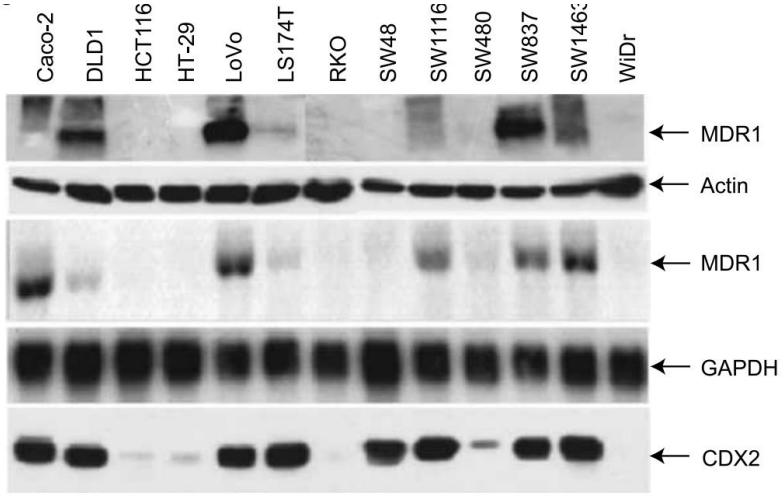


Figure 32 Correlation between CDX2 expression and MDR1/ABCB1 expression in 13 different colon cancer cell lines. A western blot was done, showing the CDX2 regulated protein expression level of MDR1 in Caco-2, DLD1, BCT116, HT-29, LoVo, LS174T, RKO, Sw48, SW1116, SW480, Sw837, SW1436 and WiDr. Control actin and GAPDH were used to check that the same amount is loaded every time and because they remain stable during treatment(Takakura et al., 2010).

These findings lead to the assumption that for the control samples where no stimulation is added, it was expected to see no increase in ABCB1 expression at 0 ng/ml DOX, but a small increase in the expression when the cells is induced with 16 ng/ml DOX. However, when LS174T7D9 is stimulated with SB 525334, PD0332991, Neratinib, Everolimus and Xl765 there could be a further increase in ABCB1 expression, since the KIs leads to an increase in CDX2 driven promoter activity of pGL4.10-IL-33-promoter-enhancer. This information leads to the speculation of how the increase of CDX2 could possibly increase the survival of CRC cells in that ABCB1 is upregulated when CDX2 is expressed. Therefore, these cells could progress into metastasis and thereby making it more difficult to target the cancer with chemotherapy. And if the CRC is targeted with chemotherapy that increases ABCB1 transporters through CDX2 it could lead to an increase of efflux of the drugs applied in the treatment.

9.3.2 Wnt pathway's target gene MYC

It has already been established that CDX2 crosstalk with the Wnt pathway by several studies and this crosstalk is the reason that Wnt's target gene oncogene MYC also were chosen for qPCR, which normally contributes to cell proliferation and the formation of cancer. A study showed that CDX2 is not directly regulating to MYC, but rather interact with component of the degradation complex of β-catenin and thereby regulates MYC (Yu et al., 2019). When

cells proliferate the expression of Wnt is high and for the cell to start differentiation, TCF7L2 triggers the shift in the two states (Kuhnert et la., 2004; Mariadason et al., 2001; Pinto et al., 2003). Based on the theory on how CDX2 crosstalk with Wnt pathway's components and how this leads to either an increase or a decrease in transcription of its target genes, it was expected that the outcome of the control only induced with 0 ng/ml DOX show an expression of MYC due to the lack of CDX2. However, when induced with 16 ng/ml of DOX it would be expected to see a decrease in MYC and furthermore when stimulating LS174T7D9 with the KIs, this would lead to a further decrease of MYC. By decreasing the expression of MYC by inducing the cells with DOX, this could lead to a decrease in cell proliferation, which would slow down a possible progression of CRC.

When screening the Wnt driven promoter activity of TOPflash and inducing LS174T7D9 with DOX there were a tendency of a decrease in the promoter activity, which could indicate that CDX2 affects the Wnt signalling and thereby its functions that the Wnt pathway controls. It would be beneficial when treating for CRC that by stimulating the cells with KIs and increasing CDX2, the progression of CRC could possibly be slowed down, due to the crosstalk CDX2 have with Wnt and furthermore, oncogenes such as MYC would not be able to aid the development and formation of CRC.

9.3.3 IL-33 a guardian of barriers and conductor of local inflammation

Due to IL-33's important role in initiating local immune response, its association with the development of CRC and the limited knowledge on its transcriptional regulation, it was chosen as the third gene to use in the qPCR. Larsen et al., 2021 found that by inducing LS174 with DOX and thereby increasing CDX2 the mRNA levels of IL-33 could be regulated through its enhancer region. When increasing the concentration of DOX, the mRNA levels of IL-33 rose to the levels of WT LS174T cells. Based on these findings it was expected to see an increase in IL-33 expression when inducing LS174T7D9 cell with 16 ng/ml DOX. And by further increasing the CDX2 by stimulation with the KIs, it would be expected to further increase IL-33. Studies have found that the IL-33 possess both pro- and anti-tumour properties. IL-33 increase the recruitment of immune cells that increase cytokines, inflammatory molecules and growth factors that lead to angiogenesis, invasion and migration, immune escape, and metastasis by providing the immune cells to the tumour microenvironment. Or Il-33 may recruit natural killer cells and cytotoxic T cells, which leads to an increase in Inf-γ and cytokines, which in return leads to tumour regression and tumour rejection. If CDX2 aids the increase in IL-33 it is not possible to say in which way the

outcome of the two possible ways IL-33 aids either the pro- (Maywald et al., 2015; Mertz et al., 2016; Zhang et al., 2017; Akitomo et al., 2016 (both mouse and patient samples); Fang et al., 2017; He et al., 2017 (both mouse and patient); Cui et al., 2015; Liu et al., 2014) or anti-tumour progression (O'Donnell et al., 2016; Eissmann et al., 2018) or if it leads to CRC development. However, by finding out if CDX2 regulates the expression of IL-33 it could give a deeper insight into how IL-33 is regulated at a transcriptional level.

10 CONCLUSION

LS174T7D9 has shown to be a potential model organism when investigating modulation of CDX2's gene regulatory activity through posttranscriptional modifications. This was shown through CDX2 driven promoter activity of pGL4.10-IL-33-promoter-enhancer construct. The LS174T7D9 gave the opportunity to research a system where CDX2 could be controlled by induction with DOX. Furthermore, by screening LS174T7D9 with 196 KIs, it was possible to identify 4 new pathways in which CDX2 is increased, SB 525334, PD0332991, Everolimus and XL765. These 4 pathways were TGF-β, Ras/Raf/MEK/ERK, PI3K/mTORC1 and through the cell cycles G1 phase component CDK4/6. These 4 pathways control important cellular processes, such as cell proliferation, cell differentiation, cell migration, cell growth and cell survival, which is all key components in the development and progression of CRC.

The assessment of CDX2 regulatory effect on the Wnt driven promoter activity found that when LS174T7D09 was induced with DOX, a decrease was shown in promoter activity of TOPflash, thereby concluding that Wnt signalling is lowered when CDX2 is present in the cell ls. From the validation of the 19 KIs, two KIs was found to decrease the promoter activity of TOPflash and when stimulating with the KIs, decreased the promoter activity further. The two KIs were PD0332991 and PI-103. These two KIs affect the cell cycles CDK4/6 and PI3K pathway, which control cell proliferation and cell survival. However, screening of the 4 KIs SB 525334, PD0332991, Everolimus and XL765 showed that when compared to the controls 0 ng/ml DOX promoter, it was not the same for the KIs at 0 ng/ml DOX. Additionally, they did not decrease the promoter activity further.

Nothing can be concluded on CDX2 regulatory effect on target genes *ABCB1, IL-33* or *MYC,* because the qPCR machine did not work.

In conclusion, LS174T7D9 is a powerful model when screening drugs and has the ability to give a full control on gene transcription because LS174T7D9 can be induce with DOX. This could be very beneficial when searching for new drugs in the treatment against CRC and maybe avoid relapses. LS174T7D9 can be applied to any gene/protein one wish to investigate when screening new drugs regulatory effect on said gene/protein of interest.

11 FUTURE RESEARCH

Since we were not able to obtain more material of the top 5 KIs SB5255334, PD0332991, Neratinib, Everolimus and XL765, it was not possible to do a validation experiment on the Wnt driven promoter activity of TOPflash again. Beside the CDX2 driven promoter pGL4.10-IL-33-promoter-enhancer and Wnt driven promoter TOPflash construct, promoter construct such as HEPH, GPA-33 and YAP1 could be applied to investigate their regulatory effect when stimulated with KIs. The concentration of 1 μM KIs were chosen based on the optimization experiment with the two KIs U0126 and SB203580. However, is could be more accurate to stimulate LS174T7D9 in a dose-dependent manner.

Due to the malfunction of the qPCR apparatus MX3005P, it was not possible to investigate CDX2 possible regulatory role in the transcription of ABC transporter ABCB1, inteleukin-33 and a possible crosstalk to Wnt signalling through its target gene MYC. Therefore, new experiments testing this regulatory effect would be beneficial for further knowledge regarding chemoresistance with CRC patients, transcriptional and translational regulation of IL-33 before release and in which way CDX2 affect Wnt signalling through its target gene MYC. Furthermore, it would be interesting to see how CDX2 regulated the expression of degradation component in the Wnt pathway, such as AXIN1/2, APC and GSK-3β.

Together with the assessment of chemoresistance and expression of transporter ABCB1, it would be interesting to analyse how CDX2 expression affect the amount of ABCB1 transporters through a dye efflux assay by using Hoechst. Together with this experiment, the cells could further be stimulated with SB5255334, PD0332991, Neratinib, Everolimus and XL765 found during the screening experiment to see the affect it will have on the efflux of Hoechst.

Lastly, it would be interesting to analyse LS174T7D9 induced with DOX, stimulated with the top five KIS and treated with 5-FU through a cell proliferation assay. The proliferation assay could give an indication on how the cells are affected by the drug 5-FU and to see how the effect is affected when the cells are stimulated with SB5255334, PD0332991, Neratinib, Everolimus and XL765 and induced with DOX.

12 REFERENCES

Aamodt, R., Bondi, J., Andersen, S. N., Bakka, A., Bukholm, G., & Bukholm, I. R. K. (2010). Clinical Study The Prognostic Impact of Protein Expression of E-Cadherin-Catenin Complexes Differs between Rectal and Colon Carcinoma. *Gastroenterology Research and Practice*, *2010*.

Ackerman, M. E., Chalouni, C., Schmidt, M. M., Raman, V. V., Ritter, G., Old, L. J., Mellman, I., & Wittrup, K. D. (2008). A33 antigen displays persistent surface expression. *Cancer Immunology, Immunotherapy*, *57*(7), 1017–1027.

Balbinot, C., Armant, O., Elarouci, N., Marisa, L., Martin, E., de Clara, E., Onea, A., Deschamps, J., Beck, F., Freund, J. N., & Duluc, I. (2018). The Cdx2 homeobox gene suppresses intestinal tumorigenesis through non-cell-autonomous mechanisms. *Journal of Experimental Medicine*, *215*(3), 911–926.

Barros, R., Freund, J. N., David, L., & Almeida, R. (2012). Gastric intestinal metaplasia revisited: Function and regulation of CDX2. In *Trends in Molecular Medicine* (Vol. 18, Issue 9, pp. 555–563). Elsevier Current Trends.

Barros, R., Teixeira Da Costa, L., Pinto-De-Sousa, J., Duluc, I., Freund, J.-N., David, L., & Almeida, R. (2011). *CDX2 autoregulation in human intestinal metaplasia of the stomach: impact on the stability of the phenotype*.

Benahmed, F., Gross, I., Gaunt, S. J., Beck, F., Jehan, F., Domon-Dell, C., Martin, E., Kedinger, M., Freund, J. N., & Duluc, I. (2008). Multiple Regulatory Regions Control the Complex Expression Pattern of the Mouse Cdx2 Homeobox Gene. *Gastroenterology*, *135*(4), 1238-1247.e3.

Bhat, A. A., Sharma, A., Pope, J., Krishnan, M., Washington, M. K., Singh, A. B., & Dhawan, P. (2012). Caudal homeobox protein Cdx-2 cooperates with Wnt pathway to regulate claudin-1 expression in colon cancer cells. *PLoS ONE*, *7*(6), 37174.

Bienz, M., & Clevers, H. (2000). Linking colorectal cancer to Wnt signaling. *Cell*, *103*(2), 311–320.

Boulanger, J., Vé, A., Bastien Mongrain, S., Boudreau, F., Perreault, N., Auclair, B. A., Lainé, J., Asselin, C., & Rivard, N. (2005). *Cdk2-dependent Phosphorylation of Homeobox Transcription Factor CDX2 Regulates Its Nuclear Translocation and Proteasome-mediated Degradation in Human Intestinal Epithelial Cells**.

Boyd, M., Bressendorff, S., Møller, J., Olsen, J., & Troelsen, J. T. (2009). *BMC Gastroenterology Mapping of HNF4α target genes in intestinal epithelial cells*.

Boyd, M., Hansen, M., Jensen, T. G. K., Perearnau, A., Olsen, A. K., Bram, L. L., Bak, M., Tommerup, N., Olsen, J., & Troelsen, J. T. (2010). Genome-wide analysis of CDX2 binding in intestinal epithelial cells (Caco-2). *Journal of Biological Chemistry*, *285*(33), 25115–25125.

Brabletz, T., Spaderna, S., Kolb, J., Hlubek, F., Faller, G., Bruns, C. J., Jung, A., Nentwich, J., Duluc, I., Domon-Dell, C., Kirchner, T., & Freund, J.-N. (2004). Down-Regulation of the Homeodomain Factor Cdx2 in Colorectal Cancer by Collagen Type I: An Active Role for the Tumor Environment in Malignant Tumor Progression. In *CANCER RESEARCH* (Vol. 64).

Bruun, J., Sveen, A., Barros, R., Eide, P. W., Eilertsen, I., Kolberg, M., Pellinen, T., David, L., Svindland, A., Kallioniemi, O., Guren, M. G., Nesbakken, A., Almeida, R., & Lothe, R. A. (2018). Prognostic, predictive, and pharmacogenomic assessments of CDX2 refine stratification of colorectal cancer. *Molecular Oncology*, *12*, 1639–1655.

Cai, L., Bennedsen, A. L. B., Qvortrup, C., & Gögenur, I. (2019). Increasing incidence of colorectal cancer in young patients. *Ugeskrift for Læger*, *1*.

Cattin, A.-L., Le Beyec, J., Barreau, F., Saint-Just, S., Houllier, A., Gonzalez, F. J., Robine, S., Pinçon-Raymond, M., Cardot, P., Lacasa, M., & Ribeiro, A. (2009). Hepatocyte Nuclear Factor 4α, a Key Factor for Homeostasis, Cell Architecture, and Barrier Function of the Adult Intestinal Epithelium. *Molecular and Cellular Biology*, *29*(23), 6294–6308.

Cayrol, C., & Girard, J. P. (2014). IL-33: An alarmin cytokine with crucial roles in innate immunity, inflammation, and allergy. *Current Opinion in Immunology*, *31*, 31–37.

Chan, D. S. M., Lau, R., Aune, D., Vieira, R., Greenwood, D. C., Kampman, E., & Norat, T. (n.d.). *Red and Processed Meat and Colorectal Cancer Incidence: Meta-Analysis of Prospective Studies*.

Chawengsaksophak, K., James, R., Hammond, V. E., Köntgen, F., & Beck, F. (n.d.). *Homeosis_and_intestinal_tumour.pdf*.

Cong, F., & Varmus, H. (2004). Nuclear-cytoplasmic shuttling of Axin regulates subcellular localization of β-catenin. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(9), 2882–2887.

Coskun, M., Olsen, A. K., Bzorek, M., Holck, S., Engel, U. H., Nielsen, O. H., & Troelsen, J. T. (2014). Involvement of CDX2 in the cross talk between TNF-α and Wnt signaling pathway in the colon cancer cell line Caco-2. *Carcinogenesis*, *35*(5), 1185–1192.

Coskun, M., Olsen, A. K., Holm, T. L., Kvist, P. H., Nielsen, O. H., Riis, L. B., Olsen, J., & Troelsen, J. T. (2012). TNF-α-induced down-regulation of CDX2 suppresses MEP1A

expression in colitis. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, *1822*(6), 843–851.

Dalerba, P., Sahoo, D., Paik, S., Guo, X., Yothers, G., Song, N., Wilcox-Fogel, N., Forgó, E., Rajendran, P. S., Miranda, S. P., Hisamori, S., Hutchison, J., Kalisky, T., Qian, D., Wolmark, N., Fisher, G. A., van de Rijn, M., & Clarke, M. F. (2016). CDX2 as a Prognostic Biomarker in Stage II and Stage III Colon Cancer. *New England Journal of Medicine*, *374*(3), 211–222.

Danielsen, E. T., Olsen, A. K., Coskun, M., Nonboe, A. W., Larsen, S., Dahlgaard, K., Bennett, E. P., Mitchelmore, C., Vogel, L. K., & Troelsen, J. T. (2018). Intestinal regulation of suppression of tumorigenicity 14 (ST14) and serine peptidase inhibitor, Kunitz type -1 (SPINT1) by transcription factor CDX2. *Scientific Reports*, *8*(1), 11813.

Davidsen, J., Larsen, S., Coskun, M., Gögenur, I., Dahlgaard, K., Bennett, E. P., & Troelsen, J. T. (2018). The VTI1A-TCF4 colon cancer fusion protein is a dominant negative regulator of Wnt signaling and is transcriptionally regulated by intestinal homeodomain factor CDX2. *PLoS ONE*, *13*(7).

Dawson, H. ;, Galván, J. A. ;, Helbling, M. ;, Muller, D.-E. ;, Karamitopoulou, E. ;, Koelzer, M. ;, Hammer, C. ;, Lugli, A. ;, & Zlobec, I. (2014). *Possible role of Cdx2 in the serrated pathway of colorectal cancer characterized by BRAF mutation, high-level CpG Island methylator phenotype and mismatch repair-deficiency*.

DRUMMOND, F., PUTT, W., FOX, M., & EDWARDS, Y. H. (1997). Cloning and chromosome assignment of the human CDX2 gene . *Annals of Human Genetics*, *61*(5), 393–400.

er Vuik, F., aV nieuwenburg, S., Bardou, M., lansdorp-Vogelaar, iris, Dinis-ribeiro, M., Bento, M. J., Zadnik, V., Pellisé, M., esteban, laura, Kaminski, M. F., Suchanek, S., ngo, O., Májek, O., leja, M., Kuipers, ernst J., & cW Spaander, M. (2019). Increasing incidence of colorectal cancer in young adults in Europe over the last 25 years. *Gut*, *0*, 1–7.

Fagotto, F., Glück, U., & Gumbiner, B. M. (1998). Nuclear localization signal-independent and importin/karyopherin-independent nuclear import of β-catenin. *Current Biology*, *8*(4), 181–190.

Fedirko, V., Tramacere, I., Bagnardi, V., Rota, M., Scotti, L., Islami, F., Negri, E., Straif, K., Romieu, I., La Vecchia, C., Boffetta, P., & Jenab, M. (2011). Alcohol drinking and colorectal cancer risk: An overall and dose-Response meta-analysis of published studies. In *Annals of Oncology* (Vol. 22, Issue 9, pp. 1958–1972). Oxford University Press.

Fevr, T., Robine, S., Louvard, D., & Huelsken, J. (2007). Wnt/β-Catenin Is Essential for Intestinal Homeostasis and Maintenance of Intestinal Stem Cells. *Molecular and Cellular Biology*, *27*(21), 7551–7559.

Forbyggende kemoterapi ved tyktarmskræft - Kræftens Bekæmpelse. (n.d.). Retrieved July 2, 2021, from https://www.cancer.dk/tyktarmskraeft-coloncancer/behandling-tyktarm/forbyggende-kemoterapi/

Grady, W. M., & Markowitz, S. D. (n.d.). *The molecular pathogenesis of colorectal cancer and its potential application to colorectal cancer screening*.

Graule, J., Uth, K., & Fischer, E. (2018). *CDX2 in colorectal cancer is an independent prognostic factor and regulated by promoter methylation and histone deacetylation in tumors of the serrated pathway*.

Gross, I., Duluc, I., Benameur, T., Calon, A., Martin, E., Brabletz, T., Kedinger, M., Domon-Dell, C., & Freund, J.-N. (2008). The intestine-specific homeobox gene Cdx2 decreases mobility and antagonizes dissemination of colon cancer cells. *Oncogene*, *27*, 107–115.

Guo, R.-J., Funakoshi, S., Lee, H. H., Kong, J., & Lynch, J. P. (2010). The intestine-specific transcription factor Cdx2 inhibits b-catenin/TCF transcriptional activity by disrupting the b-catenin-TCF protein complex. *Carcinogenesis*, *31*(2), 159–166.

Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. In *Cell* (Vol. 100, Issue 1, pp. 57–70). Elsevier.

Hansen, T. F., Kjaer-Frifeldt, S., Eriksen, A. C., Lindebjerg, J., Jensen, L. H., Sørensen, F. B., & Jakobsen, A. (2018). Prognostic impact of CDX2 in stage II colon cancer: results from two nationwide cohorts. *British Journal of Cancer*, *119*, 1367–1373.

Hao, H. X., Xie, Y., Zhang, Y., Zhang, O., Oster, E., Avello, M., Lei, H., Mickanin, C., Liu, D., Ruffner, H., Mao, X., Ma, Q., Zamponi, R., Bouwmeester, T., Finan, P. M., Kirschner, M. W., Porter, J. A., Serluca, F. C., & Cong, F. (2012). ZNRF3 promotes Wnt receptor turnover in an R-spondin-sensitive manner. *Nature*, *485*(7397), 195–202.

Hinkel, I., Duluc, I., Martin, E., Guenot, D., Freund, J.-N., & Gross, I. (2012). Cdx2 Controls Expression of the Protocadherin Mucdhl, an Inhibitor of Growth and β-Catenin Activity in Colon Cancer Cells. *YGAST*, *142*, 875-885.e3.

Hinoi, T., Gesina, G., Akyol, A., Kuick, R., Hanash, S., Giordano, T. J., Gruber, S. B., & Fearon, E. R. (2005). CDX2-regulated expression of iron transport protein hephaestin in intestinal and colonic epithelium. *Gastroenterology*, *128*(4), 946–961.

Hinoi, T., Lucas, P. C., Kuick, R., Hanash, S., Cho, K. R., & Fearon, E. R. (2002). CDX2 regulates liver intestine-cadherin expression in normal and malignant colon epithelium and intestinal metaplasia. *Gastroenterology*, *123*(5), 1565–1577.

Hoppler, S. P., & Kavanagh, C. L. (2007). Wnt signalling: Variety at the core. *Journal of Cell Science*, *120*(3), 385–393.

Houde, M., Laprise, P., Jean, D., Ne Blais, M., Asselin, C., & Rivard, N. (2001). *Intestinal Epithelial Cell Differentiation Involves Activation of p38 Mitogen-activated Protein Kinase That Regulates the Homeobox Transcription Factor CDX2*.*

Hryniuk, A., Grainger, S., Savory, J. G. A., & Lohnes, D. (2014). Cdx1 and Cdx2 Function as Tumor Suppressors. *Journal of Biological Chemistry*, *289*, 33343–33354.

Hryniuk, A., Grainger, S., Savory, J. G. A., & Lohnes, D. (2012). Cdx function is required for maintenance of intestinal identity in the adult. *Developmental Biology*, *363*(2), 426–437.

Imai, Y., Yamagishi, H., Fukuda, K., Ono, Y., Inoue, T., & Ueda, Y. (2013). Differential mucin phenotypes and their significance in a variation of colorectal carcinoma. *World J Gastroenterol*, *19*(25), 3957–3968.

Jess, T., Rungoe, C., & Peyrin-Biroulet, L. (2012). *Risk of Colorectal Cancer in Patients With Ulcerative Colitis: A Meta-analysis of Population-Based Cohort Studies BACKGROUND & AIMS: Patients with ulcerative colitis*.

Jiang, Y., Ben, Q., Shen, H., Lu, W., Zhang, Y., & Zhu, J. (2011). Diabetes mellitus and incidence and mortality of colorectal cancer: A systematic review and meta-analysis of cohort studies. *European Journal of Epidemiology*, *26*(11), 863–876.

Johnstone, C. N., Tebbutt, N. C., Abud, H. E., White, S. J., Stenvers, K. L., Hall, N. E., Whitehead, R. H., Cody, S. H., Catimel, B., Nice, E. C., Burgess, A. W., & Heath, J. K. (2000). Characterization of mouse A33 antigen, a definitive marker for basolateral surfaces of intestinal epithelial cells. *American Journal of Physiology - Gastrointestinal and Liver Physiology*, *279*(3 42-3).

Kannarkatt, J., Joseph, J., Kurniali, P. C., Al-Janadi, A., & Hrinczenko, B. (2017). Adjuvant chemotherapy for stage II colon cancer: A clinical dilemma. *Journal of Oncology Practice*, *13*(4), 233–241.

Komiya, Y., & Habas, R. (2008). Wnt signal transduction pathways. *Organogenesis*, *4*(2), 68–75.

Korinek, V., Barker, N., Moerer, P., Van Donselaar, E., Huls, G., Peters, P. J., & Clevers, H. (1998). Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. *Nature Genetics*, *19*(4), 379–383.

Kriegl, L., Horst, D., Reiche, J. A., Engel, J., Kirchner, T., & Jung, A. (2010). LEF-1 and TCF4 expression correlate inversely with survival in colorectal cancer. *Journal of Translational Medicine*, *8*(1), 1–8.

Krishn, S. R., Kaur, S., Smith, L. M., Johansson, S. L., Jain, M., Patel, A., Gautam, S. K., Hollingsworth, M. A., Mandel, U., Clausen, H., Lo, W. C., Fan, W. T. L., Manne, U., & Batra, S. K. (2016). Mucins and associated glycan signatures in colon adenoma-carcinoma sequence: Prospective pathological implication(s) for early diagnosis of colon cancer. *Cancer Letters*, *374*(2), 304–314.

Krishn, S. R., Kaur, S., Smith, L. M., Johansson, S. L., Jain, M., Patel, A., Gautam, S. K., Hollingsworth, M. A., Mandel, U., Clausen, H., Lo, W.-C., Louis, W.-T., & Batra, S. K. (2016). Mucins and associated glycan signatures in colon adenoma-carcinoma sequence: prospective pathological implication(s) for early diagnosis of colon cancer HHS Public Access. *Cancer Lett*, *374*(2), 304–314.

Kuhnert, F., Davis, C. R., Wang, H. T., Chu, P., Lee, M., Yuan, J., Nusse, R., & Kuo, C. J. (2004). Essential requirement for Wnt signaling in proliferation of adult small intestine and colon revealed by adenoviral expression of Dickkopf-1. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(1), 266–271.

Larsen, K. M., Minaya, M. K., Vaish, V., & Peña, M. M. O. (2018). The role of IL-33/ST2 pathway in tumorigenesis. *International Journal of Molecular Sciences*, *19*(9), 1–28.

Larsen, S., Seidelin, J. B., Davidsen, J., Dahlgaard, K., Nielsen, C. H., Bennett, E. P., Pedersen, O. B., Coskun, M., & Troelsen, J. T. (2021). CDX2 regulates interleukin-33 gene expression in intestinal epithelial cells (LS174T). *FEBS Open Bio*, *11*(6), 1638–1644.

Liang, K., Zhou, G., Zhang, Q., Li, J., & Zhang, C. (2014). Expression of hippo pathway in colorectal cancer. *Saudi Journal of Gastroenterology*, *20*(3), 188–194.

Liang, P. S., Chen, T.-Y., & Giovannucci, E. (n.d.). *Cigarette smoking and colorectal cancer incidence and mortality: Systematic review and meta-analysis*.

Ma, Y., Yang, Y., Wang, F., Zhang, P., Shi, C., Zou, Y., & Qin, H. (n.d.). *Obesity and Risk of Colorectal Cancer: A Systematic Review of Prospective Studies*.

MacDonald, B. T., Tamai, K., & He, X. (2009). Wnt/β-Catenin Signaling: Components, Mechanisms, and Diseases. In *Developmental Cell* (Vol. 17, Issue 1, pp. 9–26). Elsevier.

Mallo, G. V, Rechreche, H., Frigerio, J.-M., Rocha, D., Zweibaum, A., Lacasa, M., Jordan, B. R., Dusetti, N. J., Dagorn, J.-C., & Iovanna, J. L. (1996). MOLECULAR CLONING, SEQUENCING AND EXPRESSION OF THE mRNA ENCODING HUMAN Cdx1 AND Cdx2 HOMEOBOX. DOWN-REGULATION OF Cdx1 AND Cdx2 mRNA

EXPRESSION DURING COLORECTAL CARCINOGENESIS. In *Int. J. Cancer (Pred. Oncol*. Wiley-Liss, Inc.

Mariadason, J. M., Bordonaro, M., Aslam, F., Shi, L., Kuraguchi, M., Velcich, A., & Augenlicht, L. H. (2001). Down-regulation of β-catenin TCF signaling is linked to colonic epithelial cell differentiation. *Cancer Research*, *61*(8), 3465–3471.

Martin, N. T., & Martin, M. U. (2016). Interleukin 33 is a guardian of barriers and a local alarmin. *Nature Immunology*, *17*(2), 122–131.

Mattiuzzi, C., Sanchis-Gomar, F., & Lippi, G. (2019). Concise update on colorectal cancer epidemiology. *Ann Transl Med*, *7*(21), 609.

Merlos-Suá rez, A., Barriga, F. M., Jung, P., Iglesias, M., Virtudes Cé spedes, M., Rossell, D., Sevillano, M., Hernando-Momblona, X., da Silva-Diz, V., Muñ oz, P., Clevers, H., Sancho, E., Mangues, R., & Batlle, E. (2011). Cell Stem Cell The Intestinal Stem Cell Signature Identifies Colorectal Cancer Stem Cells and Predicts Disease Relapse. *Stem Cell*, *8*, 511–524.

Natoli, M., Christensen, J., El-Gebali, S., Felsani, A., & Anderle, P. (2013). The role of CDX2 in Caco-2 cell differentiation. *European Journal of Pharmaceutics and Biopharmaceutics*, *85*(1), 20–25.

Neeman, E., & Eliyahu, S. (2013). The perioperative periode and promotion of cancer metastasis: New outlooks on mediating mechanisms and immune involvement. *Bone*, *23*(1), 1–7.

Nordcan 2.0. (n.d.). Retrieved June 20, 2021, from https://nordcan.iarc.fr/en/dataviz/age_specific?years=2018&years_available=1943_2018&sexes=1_2&cancers=520&mode=year&group_populations=1&multiple_cancers=0&key=crude_rate&show_bar_mode_prop=0&types=0_1&show_map_view_per_prop=0&populations=208&multiple_populations=1&sort_by=value1&dual_position=1&survival=5&age_end=17&map_view_per=countries&group_cancers=0&pop_indicator=num&values_position=0&group_years=0&years_multiple=2011_2018

Novellasdemunt, L., Antas, P., & Li, V. S. W. (2015). Targeting Wnt signaling in colorectal cancer. A review in the theme: Cell signaling: Proteins, pathways and mechanisms. *American Journal of Physiology - Cell Physiology*, *309*(8), C511–C521.

Olsen, A. K., Coskun, M., Bzorek, M., Kristensen, M. H., Danielsen, E. T., Jørgensen, S., Olsen, J., Engel, U., Holck, S., & Troelsen, J. T. (2013). Regulation of APC and AXIN2 expression by intestinal tumor suppressor CDX2 in colon cancer cells. *Carcinogenesis*, *34*(6), 1361–1369.

Olsen, J., Espersen, M. L. M., Jess, P., Kirkeby, L. T., & Troelsen, J. T. (2014). The clinical perspectives of CDX2 expression in colorectal cancer: a qualitative systematic review. *Surgical Oncology*, *23*(3), 167–176.

Piñeros, M., Parkin, M., Ward, K., Chokunonga, E., Ervik, M., Farrugia, H., Gospodarowicz, M., O'sullivan, B., Soerjomataram, I., Swaminathan, R., Znaor, A., Bray, F., & Brierley, J. (1997). Review Essential TNM: a registry tool to reduce gaps in cancer staging information.

Pinto, D., Gregorieff, A., Begthel, H., & Clevers, H. (2003). Canonical Wnt signals are essential for homeostasis of the intestinal epithelium. *Genes and Development*, *17*(14), 1709–1713.

Pinto, D., Gregorieff, A., Begthel, H., & Clevers, H. (2003). Canonical Wnt signals are essential for homeostasis of the intestinal epithelium. *Genes and Development*, *17*(14), 1709–1713.

Pinto, R., Hansen, L., Hintze, J., Almeida, R., Larsen, S., Coskun, M., Davidsen, J., Mitchelmore, C., David, L., Troelsen, J. T., & Bennett, E. P. (2017). Precise integration of inducible transcriptional elements (PrIITE) enables absolute control of gene expression. *Nucleic Acids Research*, *45*(13), 1–15.

Rawat, V. P. S., Keith Humphries, R., & Buske, C. (2012). *Beyond Hox: the role of ParaHox genes in normal and malignant hematopoiesis*.

Rings, E. H. H. M., Boudreau, O., Taylor, J. K., Moffett, J., & Traber, P. G. (2001). *Phosphorylation of the Serine 60 Residue Within the Cdx2 Activation Domain Mediates Its Transactivation Capacity*.

Saito-diaz, K., Chen, T. W., Wang, X., Thorne, C. A., Wallace, H. A., Page-mccaw, A., & Lee, E. (2013). *NIH Public Access*. *31*(1), 1–31.

Sakaguchi, T., Gu, X., Golden, H. M., Suh, E., Rhoads, D. B., & Reinecker, H. C. (2002). Cloning of the human claudin-2 5′-flanking region revealed a TATA-less promoter with conserved binding sites in mouse and human for caudal-related homeodomain proteins and hepatocyte nuclear factor-1α. *Journal of Biological Chemistry*, *277*(24), 21361–21370.

Sakuma, K., Aoki, M., & Kannagi, R. (n.d.). *Transcription factors c-Myc and CDX2 mediate E-selectin ligand expression in colon cancer cells undergoing EGF/bFGF-induced epithelial-mesenchymal transition*.

Satake, S., Semba, S., Matsuda, Y., Usami, Y., Chiba, H., Sawada, N., Kasuga, M., & Yokozaki, H. (2008). *Cdx2 transcription factor regulates claudin-3 and claudin-4 expression during intestinal differentiation of gastric carcinoma*.

Schwarz-Romond, T., Fiedler, M., Shibata, N., Butler, P. J. G., Kikuchi, A., Higuchi, Y., & Bienz, M. (2007). The DIX domain of Dishevelled confers Wnt signaling by dynamic polymerization. *Nature Structural and Molecular Biology*, *14*(6), 484–492.

Simmini, S., Bialecka, M., Huch, M., Kester, L., Van De Wetering, M., Sato, T., Beck, F., Van Oudenaarden, A., Clevers, H., & Deschamps, J. (2014). Transformation of intestinal stem cells into gastric stem cells on loss of transcription factor Cdx2. *Nature Communications*, *5*.

Spit, M., Koo, B. K., & Maurice, M. M. (2018). Tales from the crypt: Intestinal niche signals in tissue renewal, plasticity and cancer. In *Open Biology* (Vol. 8, Issue 9). Royal Society Publishing.

Statistik om kræft i tyktarmen - Kræftens Bekæmpelse. (n.d.). Retrieved June 20, 2021, from https://www.cancer.dk/tyktarmskraeft-coloncancer/statistik-tyktarmskraeft/?gclid=EAIaIQobChMIxcK0h7fl3wIV9RXTCh1UPAaoEAAYASAAEgIImfD_BwE

Steinhardt, A. A., Gayyed, M. F., Klein, A. P., Dong, J., Maitra, A., Pan, D., Montgomery, E. A., & Anders, R. A. (2008). Expression of Yes-associated protein in common solid tumors. *Human Pathology*, *39*(11), 1582–1589.

Subtil, C., Guérin, E., Schneider, A., Chenard, M. P., Martin, E., Domon-Dell, C., Duluc, I., Brabletz, T., Kedinger, M., Duclos, B., Gaub, M. P., & Freund, J. N. (2007). Frequent rearrangements and amplification of the CDX2 homeobox gene in human sporadic colorectal cancers with chromosomal instability. *Cancer Letters*, *247*(2), 197–203.

Tabariès, S., & Siegel, P. M. (2017). The role of claudins in cancer metastasis. *Oncogene*, *36*(9), 1176–1190.

Takakura, Y., Hinoi, T., Oue, N., Sasada, T., Kawaguchi, Y., Okajima, M., Akyol, A., Fearon, E. R., Yasui, W., & Ohdan, H. (2010). CDX2 regulates multidrug resistance 1 gene expression in malignant intestinal epithelium. *Cancer Research*, *70*(17), 6767–6778.

Tamagawa, Y., Ishimura, N., Uno, G., Yuki, T., Kazumori, H., Ishihara, S., Amano, Y., & Kinoshita, Y. (2012). *Notch signaling pathway and Cdx2 expression in the development of Barrett's esophagus*.

Taylor, D. P., Burt, R. W., Williams, M. S., Haug, P. J., & Cannon-Albright, L. A. (n.d.). *Population-based family-history-specific risks for colorectal cancer: a constellation approach*.

Tóth, C., Sükösd, F., Valicsek, E., Herpel, E., Schirmacher, P., & Tiszlavicz, L. (2018). Loss of CDX2 gene expression is associated with DNA repair proteins and is a crucial

member of the Wnt signaling pathway in liver metastasis of colorectal cancer. *Oncology Letters*, *15*(3), 3586–3593.

Tschaharganeh, D. F., Chen, X., Latzko, P., Malz, M., Gaida, M. M., Felix, K., Ladu, S., Singer, S., Pinna, F., Gretz, N., Sticht, C., Tomasi, M. L., Delogu, S., Evert, M., Fan, B., Ribback, S., Jiang, L., Brozzetti, S., Bergmann, F., … Breuhahn, K. (2013). Yes-associated protein up-regulates jagged-1 and activates the NOTCH pathway in human hepatocellular carcinoma. *Gastroenterology*, *144*(7), 1530-1542.e12.

van den Born, M., van Oudenaarden, A., Itzkovitz, S., Koo, B.-K., Boj, S. F., Korving, J., van Es, J. H., Haegebarth, A., & Kujala, P. (2012). Robine and Hans Clevers Adult Intestinal Homeostatic Self-Renewal A Critical Role for the Wnt Effector Tcf4 in. *Mol. Cell. Biol*, *32*(10).

Veeman, M. T., Slusarski, D. C., Kaykas, A., Louie, S. H., & Moon, R. T. (2003). Zebrafish prickle, a modulator of noncanonical Wnt/Fz signaling, regulates gastrulation movements. *Current Biology*, *13*(8), 680–685.

Wager, M. G. T. and J. F. S. (2011). Hepatocyte Nuclear Factor 4α Is Essential for Embryonic Development of the Mouse Colon. *Bone*, *23*(1), 1–7.

Wang, H., Burnett, T., Kono, S., Haiman, C. A., Iwasaki, M., Wilkens, L. R., Loo, L. W. M., Van Den Berg, D., Kolonel, L. N., Henderson, B. E., Keku, T. O., Sandler, R. S., Signorello, L. B., Blot, W. J., Newcomb, P. A., Pande, M., Amos, C. I., West, D. W., Bézieau, S., … Le Marchand, L. (2014). *Trans-ethnic genome-wide association study of colorectal cancer identifies a new susceptibility locus in VTI1A*.

Wenzel, J., Rose, K., Haghighi, E. B., Lamprecht, C., Rauen, G., Freihen, V., Kesselring, R., Boerries, M., & Hecht, A. (2020). Loss of the nuclear Wnt pathway effector TCF7L2 promotes migration and invasion of human colorectal cancer cells. *Oncogene*, *39*(19), 3893–3909.

Williams, B. B., Tebbutt, N. C., Buchert, M., Putoczki, T. L., Doggett, K., Bao, S., Johnstone, C. N., Masson, F., Hollande, F., Burgess, A. W., Scott, A. M., Ernst, M., & Heath, J. K. (2015). Glycoprotein A33 deficiency: A new mouse model of impaired intestinal epithelial barrier function and inflammatory disease. *DMM Disease Models and Mechanisms*, *8*(8), 805–815.

Xia, X., Xu, E., Quan, S., Huang, Q., & Lai, M. (2009). No association between the polymorphisms in CDX2 coding regions and colorectal cancer in Chinese. *Molecular and Cellular Biochemistry*, *331*(1–2), 27–30.

Yan, K. S., Janda, C. Y., Chang, J., Zheng, G. X. Y., Larkin, K. A., Luca, V. C., Chia, L. A., Mah, A. T., Han, A., Terry, J. M., Ootani, A., Roelf, K., Lee, M., Yuan, J., Li, X., Bolen, C. R., Wilhelmy, J., Davies, P. S., Ueno, H., … Kuo, C. J. (2017). Non-

equivalence of Wnt and R-spondin ligands during Lgr5 + intestinal stem-cell self-renewal. *Nature*, *545*(7653), 238–242.

Yu, J., Liu, D., Sun, X., Yang, K., Yao, J., Cheng, C., Wang, C., & Zheng, J. (2019). CDX2 inhibits the proliferation and tumor formation of colon cancer cells by suppressing Wnt/β-catenin signaling via transactivation of GSK-3β and Axin2 expression. *Cell Death and Disease*, *10*(1), 1–14.

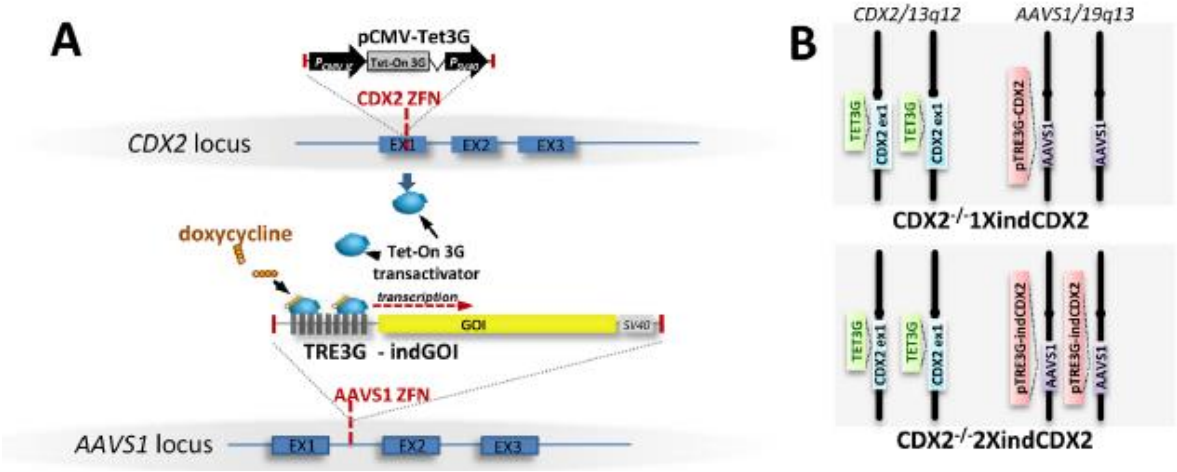
Yu, J., Liu, D., Sun, X., Yang, K., Yao, J., Cheng, C., Wang, C., & Zheng, J. (2010). *CDX2 inhibits the proliferation and tumor formation of colon cancer cells by suppressing Wnt/β-catenin signaling via transactivation of GSK-3β and Axin2 expression*.

Zheng, J. B., Sun, X. J., Qi, J., Li, S. S., Wang, W., Ren, H. L., Tian, Y., Lu, S. Y., & Du, J. K. (2011). Effects of homeodomain protein CDX2 expression on the proliferation and migration of Lovo colon cancer cells. *Pathology and Oncology Research*, *17*(3), 743–751.

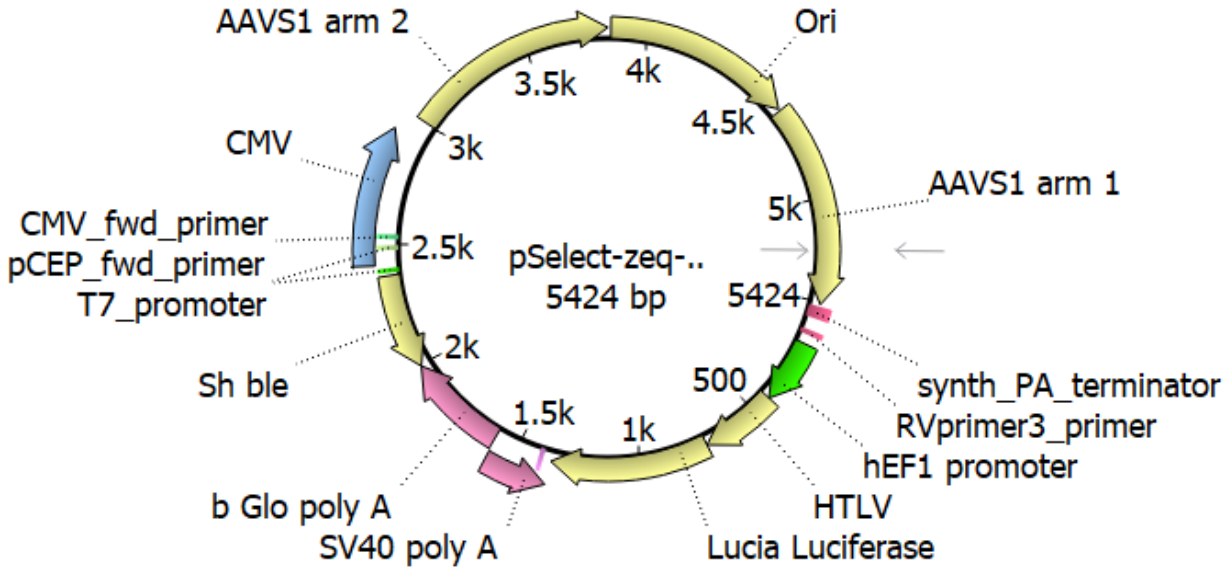
Zhang, J. F., Qu, L. S., Qian, X. F., Xia, B. L., Mao, Z. B., & Chen, W. C. (2015). Nuclear transcription factor CDX2 inhibits gastric cancer-cell growth and reverses epithelial-to-mesenchymal transition in vitro and in vivo. *Molecular Medicine Reports*, *12*(4), 5231–5238.

13 APPENDIX

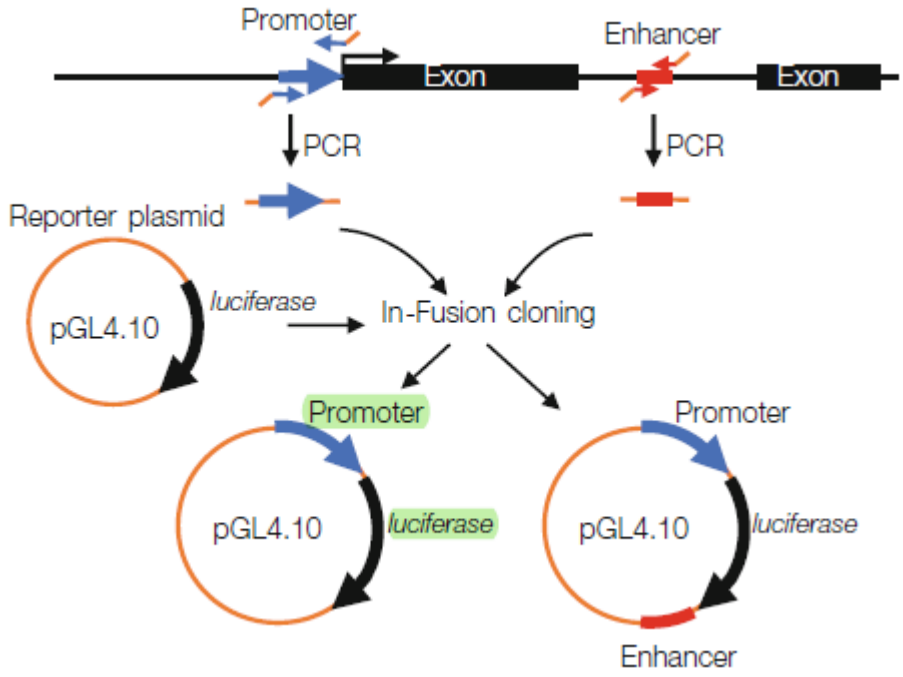
13.1 OVERVIEW OF KNOCKOUT OF CDX2 BY TETON AND INTEGRATION INTO THE AAVS1 LOCUS BY ZFN (PINTO ET AL., 2017)



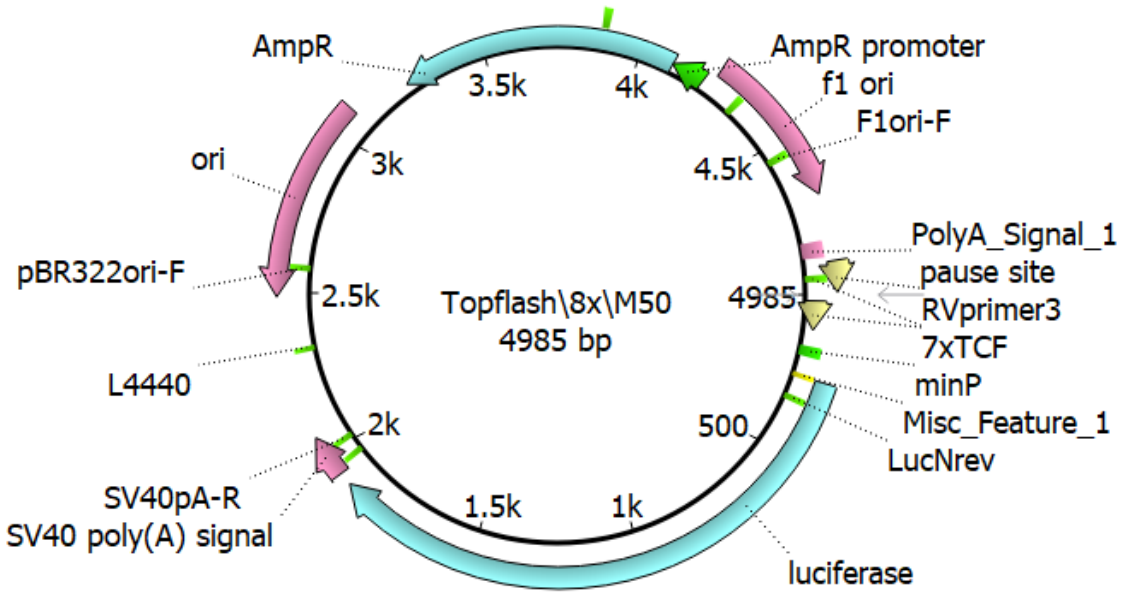
13.2 PSELECT-ZEOCIN-LUCIA PROMOTER CONSTRUCT FOR RECOMBINANT INSERTION INTO AAVS1 LOCUS SITE



13.3 In-Fusion cloning of pGL4.10-IL-33+promoter+enhancer (Larsen et al., 2021)



13.4 TOPFLASH promoter construct



13.5 OVERVIEW OF SELLECK CHEMICALS KINASE INHIBITOR SCREENING LIBRARY

Plate layout:L1200-01

	1	2	3	4	5	6	7	8	9	10	11	12
a	Empty	BMS-599626	Erlotinib Hydrochloride	Gefitinib(Iressa)	Neratinib	PD153035 hydrochloride	Pelitinib	Vandetanib	WZ3146	WZ4002	WZ8040	Empty
b	AV-951(Tivozanib)	Axitinib	BIBF1120(Vargatef)	BMS 794833	Empty	Cediranib(AZD2171)	Empty	CYC116	Empty	Imatinib(STI571)	Imatinib Mesylate	Empty
c	Ki8751	KRN 633	Masitinib(AB1010)	MGCD-265	Motesanib Diphosphate	MP-470	OSI-930	Pazopanib Hydrochloride	Sorafenib Tosylate	Sunitinib Malate	TSU-68	Empty
d	Vatalanib	XL880(GSK1363089)	PHA-739358(Danusertib)	AT9283	AZD0530(Saracatinib)	Bosutinib(SKI-606)	Dasatinib	Nilotinib	Quercetin(Sophoretin)	NVP-ADW742	AC-220	Empty
e	AP24534	Tandutinib (MLN518)	KW 2449	CI-1033(Canertinib)	CP-724714	BAY 73-4506(Regorafenib)	JNJ-38877605	PF-04217903	PF-2341066	Empty	SGX-523	Empty
f	SU11274(PKI-SU11274)	NVP-TAE684	SB 525334	R406	R406(free base)	XL184	BI 2536	GSK461364	HMN-214	ON-01910	AT7519	Empty
g	Flavopiridol(Alvocidib)	BS-181 hydrochloride	PD0332991	PHA-793887	Roscovitine(CYC202)	SNS-032(BMS-387032)	AZD7762	Aurora A Inhibitor I	AZD1152-HQPA(Barasertib)	CCT129202	ENMD-2076	Empty
h	Hesperadin	MLN8237	Empty	PHA-680632	SNS-314 Mesylate	VX-680	ZM-447439	AS703026	AZD6244(Selumetinib)	AZD8330	BIX 02188	Empty

Plate layout:L1200-02

	1	2	3	4	5	6	7	8	9	10	11	12
a	BIX 02189	BMS 777607	CI-1040 (PD184352)	PD318088	PD0325901	PD98059	U0126-EtOH	LY2228820	BIRB 796	SB 202190	SB 203580	Empty
b	Vinorelbine(Navelbine)	VX-702	VX-745	GDC-0879	Empty	PLX-4720	RAF265	SP600125	AZD6482	AS-605240	GDC-0941	Empty
c	IC-87114	LY294002	PIK-293	PIK-90	PIK-93	TG100-115	TGX-221	XL147	XL765	ZSTK474	AZD8055	Empty
d	Deforolimus(MK-8669)	Everolimus(RAD001)	KU-0063794	Rapamycin(Sirolimus)	Temsirolimus	WYE-354	Empty	PIK-75 Hydrochloride	CHIR-99021	Indirubin	SB 216763	Empty
e	KU-55933	KU-60019	MK-2206	Empty	AT7867	Empty	AZD1480	Empty	LY2784544	Empty	Enzastaurin	Empty
f	Empty	Empty	SB 431542	ABT-869(Linifanib)	AEE788	BIBW2992(Tovok)	Lapatinib Ditosylate	JNJ-7706621	Empty	BEZ235	GSK1059615	Empty

g	PI-103	AG-490	CP-690550(Tofacitinib)	Crenolanib (CP-868569)	GSK1838705A	KX2-391	NVP-BSK805	PCI-32765	PF-562271	DCC-2036	LDN193189	Empty

Plate layout:L1200-03

	1	***2***	***3***	***4***	***5***	***6***	***7***	***8***	***9***	***10***	***11***	***12***
a	AZD8931	Raf265 derivative	NVP-BHG712	OSI-420	R935788 (Fostamatinib)	AZ 960	Mubritinib (TAK 165)	PP242	Cyt387	Apatinib	CAL-101	Empty
b	PIK-294	VX-765	Telatinib (BAY 57-9352)	BI6727 (Volasertib)	WP1130	BKM120 (NVP-BKM120)	CX-4945	Phenformin hydrochloride	TAK-733	AZD5438	PP-121	Empty
c	OSI-027	LY2603618 (IC-83)	PKI-587	CCT128930	A66	NU7441	GSK2126458	WYE-125132	WYE-687	A-674563	AS-252424	Empty
d	GSK1120212 (JTP-74057)	Flavopiridol hydrochloride	AS-604850	WAY-600	TG101209	GDC-0980 (RG7422)	A-769662	TAK-901	AMG900	ZM336372	Empty	Empty
e	PH-797804	PF-04691502	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty

13.6 CONCENTRATIONS OF PURIFIED RNA AND TOTAL CONCENTRATION IN 2

Concentration pr. µl (ng/µl).

Kinase inhibitors	0 DOX (ng/ µl)	16 DOX (ng/ µl)
U KI	157.7	74.4
SB	210.7	191.5
PD	92.7	71.4
Ner	24.6	58.1
Ever	73.3	44.8
Xl 765	204	160.9

Total concentration of RNA in one sample containing 25 µl (ng).

Kinase inhibitors	0 ng	16 ng
U KI	3942	1860
SB	5267	4787.5
PD	2317.5	1785
Ner	615	1452.5
Ever	1832.5	1120
Xl 765	5100	4022.5

13.7 RT-QPCR PROGRAM

Step	Temperature (°C)	Time (sec)	Cycles
Preincubation	95	600	1
3 step amplification	95	20	40
	58	20	
	72	20	
Melting	95	10	1
	65	60	
	95	0.1	

13.8 PLACEMENT OF PRIMER AND THE DIFFERENT VARIANT OF THE TARGET GENES USED FOR RT-QPCR.

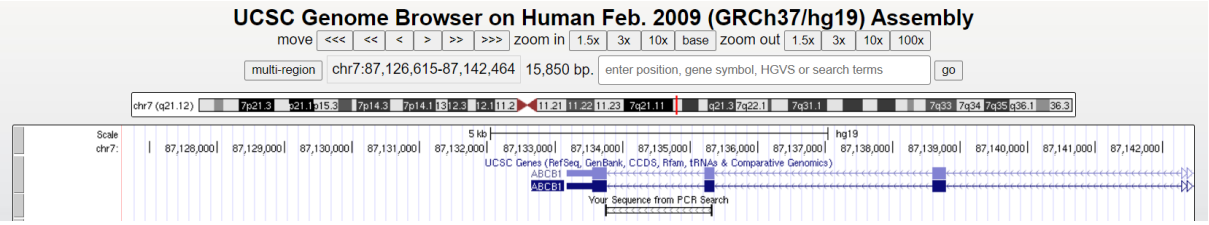


*Figure 33 **ABCB1** transcript variants and primer position base between 1 and second exons*

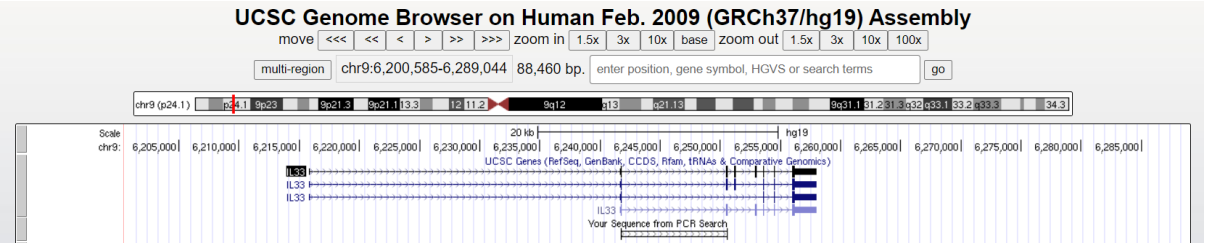


*Figure 34 **IL-33** transcript variants and primer position based between exon 1 and exon 3*

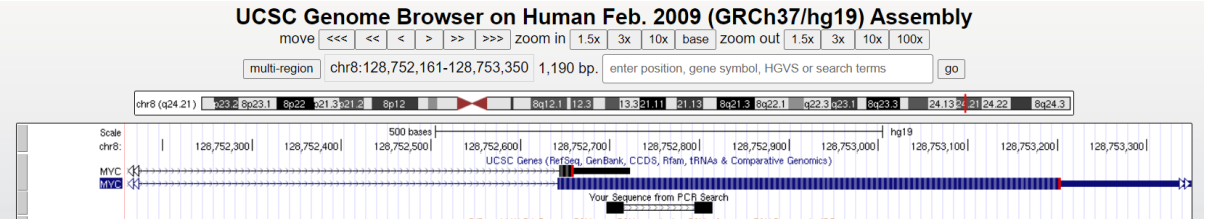


*Figure 35 **MYC** transcript variants and primer position based inside exon 3.*

13.9 DATA FROM KINASE INHIBITOR SCREEN WITH LS174T7D9

Table 5 Data from kinase inhibitor screen with LS174T7D9. Duplicate samples of 0 and 16 ng/ml DOX induced LS174T7D9 and stimulated with 1 µM kinase inhibitors incubated for 72 hours were done. The inhibitory effect in the CDX2 driven promoter activity of pGL4.10-IL-33-promoter-enhancer construct, fold change between 0 ng/ml and 16 ng/ml DOX (log2) and target sites for the kinase inhibitors are shown here.

Inhibitor	Regulatory effect on CDX2 driven promoter activity		Fold change (LOG2)	Target
	0 ng/ml DOX	16 ng/ml DOX		
AV-951(Tivozanib)	1.51	2.72	0.255	**VEGFR1/2/3**
Ki8751	1.41	1.42	0.003	**VEGFR2**
Vatalanib	1.78	2.11	0.074	**VEGFR2/KDR (kinase insert domain receptor).**
AP24534	4.63	4.40	-0.023	**Bcr-Abl and Scr**
SU11274(PKI-SU11274)	1.44	1.27	-0.055	**c-Met**
Flavopiridol(Alvocidib)	11.81	4.08	-0.461	**CDK1, CDK2, CDK4, CDK6, and CDK9.**
Hesperadin	9.61	3.71	-0.413	**Aurora B**
BMS-599626	1.31	2.18	0.219	**HER1 and HER2**
Axitinib	1.62	2.80	0.236	**VEGFR1, VEGFR2, VEGFR3,**
KRN 633	1.29	1.30	0.001	**VEGFR1/2/3**
XL880(GSK1363089)	0.63	1.56	0.392	**Met (c-Met) and KDR**
Tandutinib (MLN518)	1.47	2.05	0.144	**FLT3**
NVP-TAE684	0.32	0.95	0.465	**ALK**
BS-181 hydrochloride	1.50	2.05	0.136	**CDK7**
MLN8237	0.59	1.52	0.411	**Aurora A**
Erlotinib Hydrochloride	1.35	0.75	-0.255	**EGFR**
BIBF1120 (Vargatef)	1.59	2.41	0.180	**VEGFR1/2/3, FGFR1/2/3 and PDGFRα/β**
Masitinib (AB1010)	2.32	1.16	-0.302	**Kit (c-Kit) and PDGFRα/β**

PHA-739358(Danusertib)	1.25	1.49	0.076	**Aurora A/B/C**
KW 2449	1.55	1.99	0.108	**Flt3**
SB 525334 (F3)	1.71	1.82	0.028	**TGFβ receptor I (ALK5)**
PD0332991	0.88	1.84	0.321	**CDK4/6**
Gefitnib (Iressa)	1.95	2.25	0.062	**EGFR**
BMS 794833	2.47	2.00	-0.090	**Met (c-Met)/VEGFR 2**
MGCD-265	2.42	2.18	-0.045	**c-Met and VEGFR1/2/3**
AT9283	1.40	1.58	0.052	**JAK2/3**
CI-1033(Canertinib)	1.13	0.63	-0.253	**EGFR and ErbB2**
R406	1.37	1.93	0.148	**Syk**
PHA-793887	3.10	1.24	-0.400	**CDK2, CDK 5 and CDK7**
PHA-680632	0.64	1.54	0.383	**Aurora A/B/C**
Neratinib	1.64	2.44	0.173	**HER2 and EGFR**
Motesanib Diphosphate	2.24	2.30	0.012	**VEGFR1/2/3**
AZD0530(Saracatinib)	1.71	0.75	-0.354	**Src**
CP-724714	1.34	2.82	0.321	**HER2/ErbB2**
R406(free base)	1.19	1.09	-0.039	**Syk**
Roscovitine(CYC202)	2.04	2.03	-0.002	**Cdc2, CDK2 and CDK5**
SNS-314 Mesylate	1.36	1.16	-0.070	**Aurora A/b/C**
PD153035	1.53	4.49	0.468	**EGFR**
Cediranib(AZD2171)	1.01	1.06	0.021	**VEGFR (KDR)**
MP-470	1.18	1.70	0.157	**c-Kit, PDGFRalfa and Flt3**
Bosutinib(SKI-606)	0.80	0.89	0.050	**Scr/Abl**
BAY 73-4506(Regorafenib)	1.53	1.69	0.044	**VEGFR1-3, PDGFRbeta, Kit (c-Kit), RET (c-RET)**

				and Raf-1
XL184	1.06	1.86	0.242	**VEGFR2**
SNS-032(BMS-387032)	9.58	3.25	-0.469	**CDK2**
VX-680	0.86	1.92	0.352	**Aurora A**
A7 Pelitinib	0.97	0.87	-0.048	**Scr, MEK/ERK and ErB2.**
OSI-930	1.29	0.94	-0.139	**Kit, (c-Kit), KDR and CSF-1R**
Dasatinib	0.56	3.66	0.818	**Abl, Scr and c-Kit**
JNJ-38877605	1.07	1.23	0.061	**c-MET**
BI 2536	1.56	1.49	-0.019	**Plk1**
AZD7762	0.86	0.31	-0.450	**Chk1**
ZM-447439	0.57	1.64	0.461	**Aurora A/B**
A8 Vandetib	0.61	0.68	0.050	**VEFGR2**
CYC116	0.67	0.85	0.104	**Auroroa A/B inhibitors**
Pazopanib Hydrochloride	1.63	1.77	0.035	**VEGFR 1-3, PDGFR, EGFR, c-Kit and c-FMS.**
Nilotinib	1.50	3.23	0.334	**Bcr-Abl**
PF-04217903	1.33	1.17	-0.055	**c-Met**
GSK461364	1.87	1.83	-0.008	**Plk1.**
Aurora A Inhibitor I	0.95	1.72	0.259	**Aurora A**
AS703026	1.00	0.67	-0.173	**MEK1/2.**
A9 WZ3146	0.78	1.05	0.129	**EGFR**
Sorafenib Tosylate	2.97	3.41	0.061	**Raf-1, B-Raf**
Quercetin (Sophoretin)	1.64	1.84	0.050	**SIRT1**
PF-2341066	0.80	1.01	0.100	**c-Met, ALK**
HMN-214	0.62	1.06	0.234	**PLK-1**
AZD1152-HQPA(Barasertib)	0.60	0.94	0.193	**Aurora B**
AZD6244(Selumetinib)	1.51	0.26	-0.764	**MEK, ERK1/2**
A10 Wz4002	0.31	0.56	0.256	**EGFR**
Imatinib(STI571)	0.65	1.55	0.376	**v-Abl, c-Kit and PDGFR**
Sunitinib Malate	0.41	0.51	0.090	**VEGFR2 (Flk-1), PDGFRβ**
NVP-ADW742	0.27	0.64	0.382	**IGF-1R**

ON-01910	0.43	2.52	0.770	**PLK1 and PI3K/akt**
CCT129202	0.11	1.07	1.006	**Aurora A/B/C.**
AZD8330	0.41	0.25	-0.209	**MEK1/2**
A11 Wz8040	0.71	1.31	0.265	**EGFR**
Imatinib Mesylate	1.07	3.16	0.470	**v-Abl, c-Kit and PDG FR**
TSU-68	0.77	1.22	0.197	**PDGFR**
AC-220	3.01	1.45	-0.318	**FLT3**
SGX-523	2.47	1.56	-0.198	**c-Met**
AT7519	7.80	5.61	-0.143	**CDK1, 2, 4, 6 and 9**
ENMD-2076	0.42	1.10	0.417	**Aurora A, Flt3**
BIX 02188	1.32	1.43	0.034	**MEK5.**
Bix 02189	1.38	1.94	0.150	MEK5
Vinorelbine(Navelbine)	2.79	10.19	0.562	Inhibits mitosis through interaction with tubulin
IC-87114	1.13	2.94	0.413	PI3Kdelta
Deforolimus(MK-8669)	1.39	2.30	0.219	mTOR
KU-55933	1.79	1.55	-0.064	ATM (ataxia-telangiectasia mutated) .
G1 PI-103	1.10	3.19	0.464	PI3K p110 alfa, beta, delta and gamma
BMS 777607	1.16	2.05	0.248	c-Met, Axl, Ron and Tyro3
VX-702	1.52	5.69	0.573	P38alfa MAPK
LY294002	1.24	1.52	0.086	PI3K,alfa,gamme,beta
Everolimus(RAD001)	0.80	1.70	0.325	FKBP12
KU-60019	0.28	1.47	0.727	ATM (ataxia-telangiectasia mutated)
AG-490	0.82	2.00	0.389	EGFR and JAK2
A3 CI-1040 (PD184352)	2.11	1.20	-0.248	MEK

VX-745	2.56	2.96	0.062	P38 alfa
PIK-293	1.52	1.18	-0.111	PI3K
KU-0063794	0.74	1.28	0.237	mTOR
MK-2206	0.34	0.29	-0.075	AKT
SB 431542	2.66	1.12	-0.376	ALK receptors, 5, 4 and 7.
CP-690550(Tofacitinib)	1.23	1.21	-0.009	JAK3.
A4 PD318088	0.91	0.75	-0.084	MEK1/2
GDC-0879	0.62	1.01	0.216	B-Raf
PIK-90	1.04	1.51	0.161	PI3K alfa, gamma delta
Rapamycin(Sirolimus)	0.87	1.85	0.329	mTOR
ABT-869(Linifanib)	1.97	0.90	-0.340	VEGFR/PDGFR
Crenolanib (CP-868569)	0.43	0.84	0.287	PDGFR-alfa
A5PD0325901	1.09	0.59	-0.265	MEK/ERK
PIK-93	0.83	0.52	-0.200	PI4K (PI4KIII-beta)
Temsirolimus	1.40	0.58	-0.383	mTOR
AT7867	0.27	0.14	-0.298	AKT1-3 and p70s6k/PKA
AEE788	0.31	0.36	0.075	HER1/2 and VEGFR1/2
GSK1838705A	0.04	0.30	0.891	IGF-1R
A6 PD98058	0.62	1.22	0.294	MAPK
PLX-4720	1.46	1.74	0.075	B-Raf
TG100-115	2.00	2.31	0.063	PI3K gamma/delta
WYE-354	0.84	0.90	0.033	mTOR inhibitor
BIBW2992(Tovok)	0.42	0.42	0.003	EGFR and HER2
KX2-391	0.21	0.56	0.439	Scr and tubulin.
A7 U0126-EtOH	0.74	1.36	0.264	MEK1/2
RAF265	0.99	1.27	0.110	C-Raf/B-Raf/B-Raf
TGX-221	0.71	2.29	0.510	P110-beta
AZD1480	0.60	1.12	0.273	JAK2
Lapatinib Ditosylate	0.48	0.65	0.126	EGFR and ErbB2

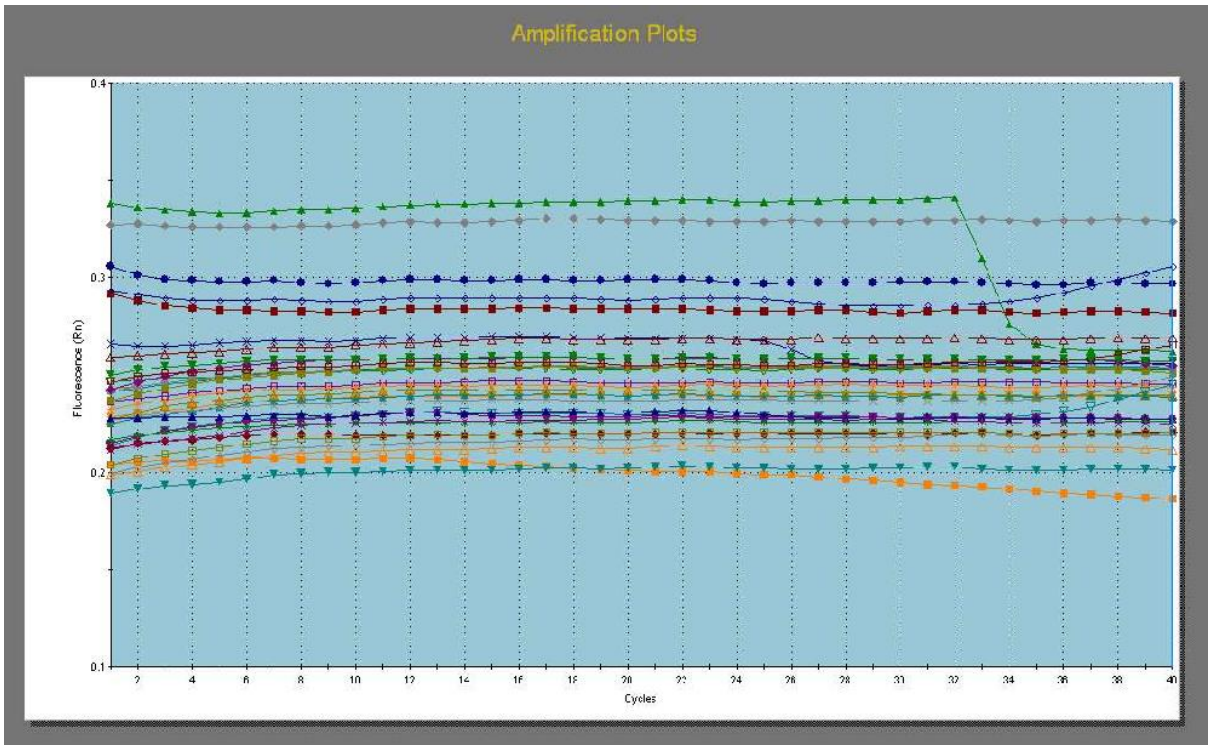
NVP-BSK805	0.94	0.71	-0.121	JAK2
A8 LY2228820	3.57	1.69	-0.324	P38MAPK
SP600125	0.80	1.04	0.114	c-Jun.
XL147	1.16	1.20	0.016	PI3Kalfa, delta and gamma.
PIK-75 Hydrochloride	9.75	5.03	-0.288	P110alfa
JNJ-7706621	0.80	1.35	0.230	CDK
PCI-32765	0.74	1.29	0.240	Brutons tyrosine kinase (Btk)
A9 BIRB 796	2.28	2.50	0.040	P38 MAPK
AZD6482	0.46	4.35	0.973	PI3K
XL765	1.31	2.16	0.219	mTOR, PI3K
CHIR-99021	0.84	1.65	0.291	GSK-3
LY2784544	0.64	1.33	0.316	JAK2
PF-562271	0.56	1.28	0.358	FAK
A10 SB 202190	1.16	4.72	0.611	P38
AS-605240	1.01	2.00	0.298	PI3K gamma
ZSTK474f	0.65	1.66	0.405	p110 beta/gamma and delta
Indirubin	0.44	1.07	0.380	CDK, GSK3
BEZ235	0.48	2.54	0.719	PI3Kalfa/beta /gamma/delt a and mTOR
DCC-2036	5.16	6.84	0.122	Bcr-Abl
A11 SB203580	2.44	3.44	0.149	P38 MAPK
GDC-0941	1.04	2.01	0.284	p110alfa/beta /delta/gamm a
AZD8055	1.21	1.66	0.137	mTOR
SB 216763	1.14	1.91	0.224	GSK3
Enzastaurin	0.84	2.39	0.454	PKCbeta, alfa, gamma and epsilon.
GSK1059615	0.61	1.33	0.335	PI3Kalfa/beta /delta/gamm a and mTOR
LDN193189	1.36	2.27	0.222	ALK2 and ALK3.
A1 AZD8931	0.98	1.44	0.165	EGFR and ErbB2
PIK-294	1.32	2.18	0.216	P110delta
OSI-027	2.12	1.78	-0.076	mTORC1/2
GSK1120212	0.53	2.69	0.709	MEK1/2

(JTP-74057)				
PH-797804	0.57	1.75	0.484	p38alfa
A2 Raf265 derivative	0.97	3.85	0.599	RAF and VEGFR
VX-765	0.85	2.12	0.395	Caspase-1
LY2603618 (IC-83)	1.01	1.44	0.153	Chk1
Flavopiridol hydrochloride	9.30	10.35	0.046	CDK1, 2 , 4 and 6.
PF-04691502	0.81	2.71	0.526	PI3K (alfa/beta/delta/gamma), mTOR
A3 NVP-BHG712	1.18	1.08	-0.037	EphB4
Telatinib (BAY 57-9352)	0.83	1.93	0.366	VEGFR2, VEGFR3, PDGFα, and c-Kit
PKI-587	9.43	9.76	0.015	PI3K alfa, gamma and mTOR
AS-604850	1.51	4.11	0.435	PI3Kgamma
A4 OSI-420	5.10	0.61	-0.922	EGFR
BI6727 (Volasertib)	19.92	3.14	-0.802	Plk1
CCT128930	2.55	1.00	-0.408	Akt2
WAY-600	9.04	1.85	-0.689	mTORC1/2
A5 R935788	3.04	1.37	-0.347	Syk
WP1130	2.05	2.69	0.117	Deubiquitinase, Bcr/Abl
A66	1.97	3.52	0.252	P110alfa
TG101209	1.52	0.51	-0.473	JAK
A6 AZ 960	1.45	0.12	-1.097	JAK2
BKM120 (NVP-BKM120)	3.70	0.89	-0.619	PI3K
NU7441	1.92	2.92	0.183	DNA-PK inhibitor
GDC-0980 (RG7422)	2.97	1.15	-0.413	PI3Kalfa, beta, delta and gamma, mTOR
A7 Mubritinib	2.86	1.61	-0.251	HER2, ErbB2
CX-4945	2.31	3.35	0.162	CK2
GSK2126458	6.37	5.68	-0.050	PI3K, mTOR
AMPK activator	1.71	1.55	-0.041	
A8 PP242	1.80	0.99	-0.260	mTOR

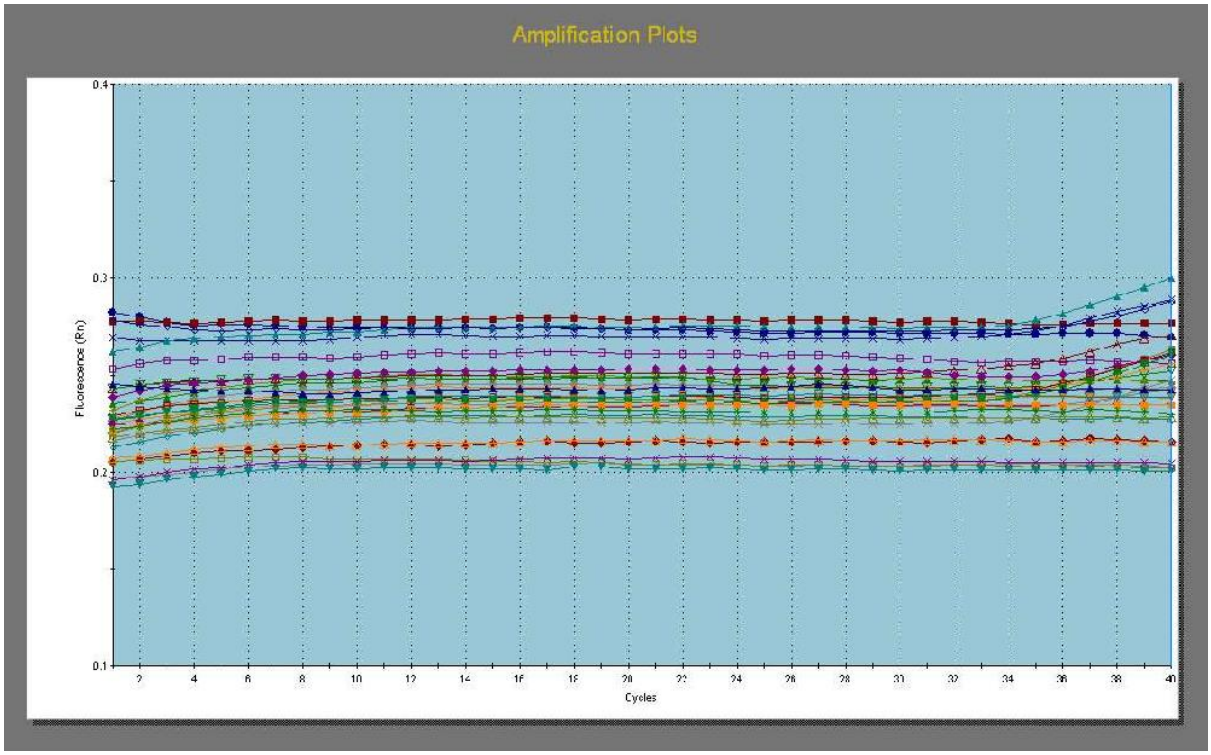
Phenformin hydrochloride	1.28	2.71	0.326	AMPK
WYE-125132	10.95	10.99	0.002	mTORC1/2
TAK-901	0.71	1.78	0.395	Aurora A/B.
A9 Cyt387	0.49	1.11	0.359	JAK1/2
TAK-733	1.89	4.84	0.407	MEK1.
WYE-687	0.77	1.38	0.252	mTOR
AMG900	0.57	2.02	0.547	Aurora A/B/C.
A10 Apatinib	2.03	1.17	-0.241	**VEGFR inhibitor.**
AZD5438	7.65	2.84	-0.431	CDK
A-674563	10.86	6.09	-0.251	Akt1
ZM336372	1.45	1.48	0.011	Raf
A11 CAL-101	1.95	1.89	-0.013	P110alfa
PP-121	1.93	0.90	-0.329	PDGFR
AS-252424	2.22	0.85	-0.419	PI3K gamma

13.10 qPCR AMPLIFICATION PLOT AND MELTING CURVE ANANLYSIS ON FIRST SET OF DATA

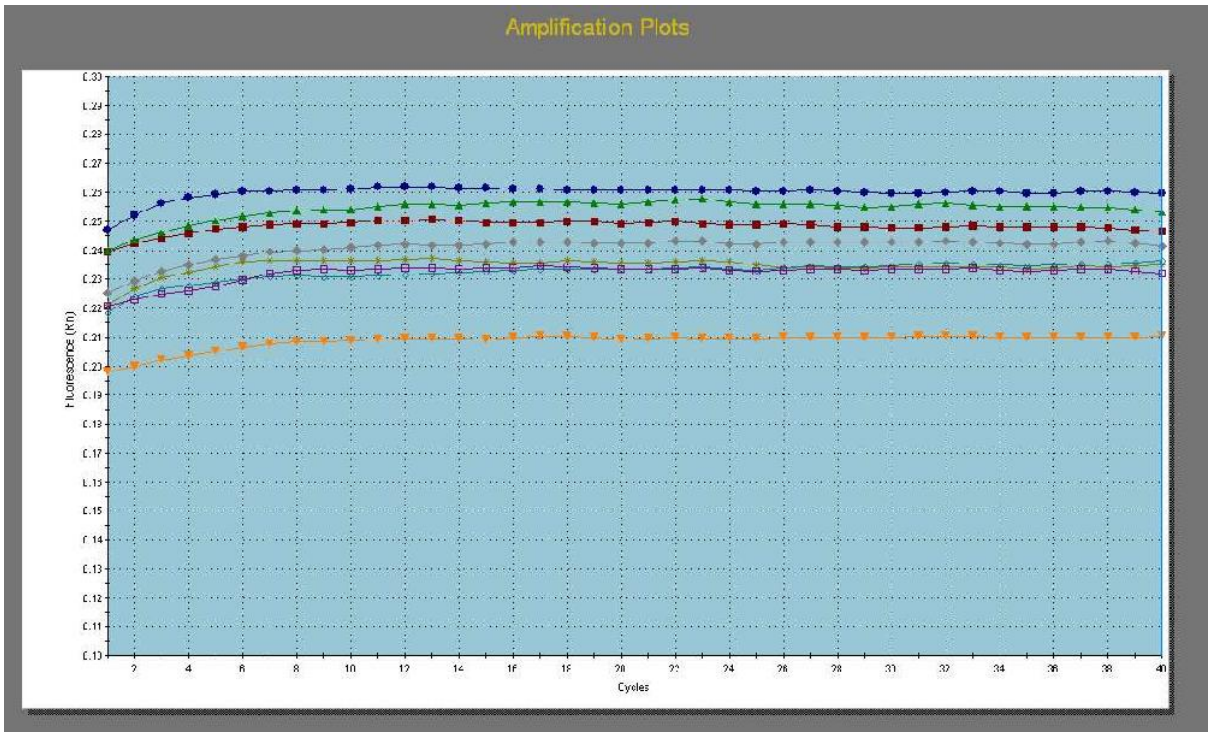
Amplification plot containing sample with 16 ng/ml DOX induction, no KIs, and with reverse transcriptase and ABCB1, IL-33 and MYC primer sets.



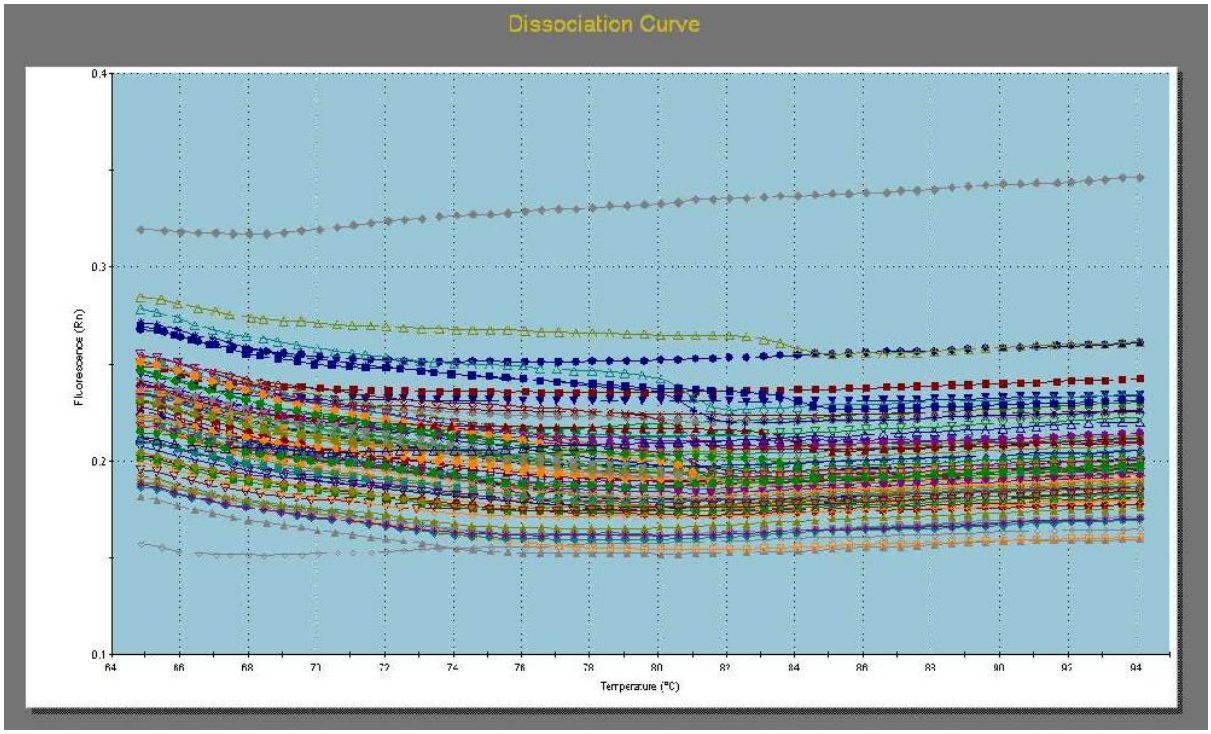
Amplification plot containing sample with 16 ng/ml DOX induction, no KIs, and without reverse transcriptase and ABCB1, IL-33 and MYC primer sets.



H_20 controls for ABCB1, IL-33 and MYC, containing 16 ng/ml DOX, no cDNA and no reverse transcriptase.



Melting curve analysis/specificity on sample for the three primer sets on ABCB1, IL-33 and MYC.


Dissociation Curve
Fluorescence (Rn)
Temperature (°C)