INVESTIGATING TCF-INDEPENDENT β-CATENIN SIGNALLING AND ITS FUNCTION IN MOUSE ES CELL BIOLOGY

INVESTIGATING TCF-INDEPENDENT β-CATENIN SIGNALLING AND ITS FUNCTION IN MOUSE ES CELL BIOLOGY

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ABSTRACT

The Wnt/β-catenin pathway is a fundamental signalling pathway involved in a communicative process that regulates cellular function during development, immune responses, tissue homeostasis, cell fate determination and repair. It is a crucial pathway, with a multifunctional protein, β-catenin, acting as one of its key components. β-catenin's interaction with the TCF/LEF family of proteins is very well characterised, however little is known about its TCF/LEF independent roles in the nucleus. To investigate the TCF/LEF independent signalling roles of β-catenin, we employed previously generated TCF/LEF quadruple knockout (QKO) and newly developed, TCF/LEF, β-catenin knockout (TLB) cell lines (i.e. QKO lines that also lack β-catenin expression). In vitro, similarly to QKO cells, the TLB line displayed a clear neuroectodermal differentiation bias and also displayed surprising upregulation of Wnt responsive genes. The neuroectodermal bias in the absence of β-catenin reveals that the neuroectodermal differentiation program does not require β- catenin for its initiation. We describe a novel phenotype observed in embryoid body (EB) assays, where we observe that lack of β-catenin results in shedding of nonadherent cells from EBs. Based on our RNA-seq data analyses of WT, QKO and TLB lines, the AIRE transcription factor was found to be a potential β-catenin target that is regulated in a TCF/LEF-independent manner. We observed an upregulation in AIRE expression in the QKO cell line, which was not observed in the TLB cell line. We postulate that AIRE plays a role in a β-catenin-regulated pluripotency regulatory network and describe its importance in maintaining self-renewal and cell pluripotency.

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First and foremost, I would like to thank my supervisor, Dr. Bradley Doble. His continued guidance, patience and understanding really helped me grow as a student and added to my overall experience at McMaster as a graduate student. Dr. Doble's open door policy made for an ideal learning environment and I could have not asked for a better supervisor to do my grad studies under. The Doble lab environment was friendly and helpful, making it an ideal lab setting for any graduate student and was a huge contribution factor to positive experience I had at the SCCRI.

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LIST OF ABBREVIATIONS AND ACRONYMS

A	AER	Apical Ectodermal Ridge
B	BKO	β-catenin knockout
C	CK1-α C- CBP	Casein Kinase-α Carboxy-CREB-binding protein
D	DSH	Dishevelled
	Dickkopf Inhibitor 1	DKK1
E	ESCs	Embryonic Stem cells
	EBs	Embryoid Bodies
	EB assay	Embryoid body assay
G	GSK-3	Glycogen Synthase Kinase-3
K	Kb	Kilobases
	KDa	Kilodalton
L	LEF	Lymphoid enhancer factor
M	mESC	Mouse Embryonic Stem Cell
	MEF	Mouse embryonic fibroblast
N	N-	Amino-
P	PP2A	Protein Phosphatase 2A
	PKO	Penta Knockout/ TCF/LEF:β-catenin Knockout (Cell line made using Basler lab QKOs)
	PBS	Phosphate-buffered saline
Q	QKO	TCF/LEF quadruple Knockout
	qRT-PCR	Quantitative reverse transcription polymerase chain reaction
R	RIPA	Radioimmunoprecipitation assay
	RSpo	R-Spondin
	RSpo1-4	R-Spondin 1-4
T	TLB	TCF/LEF:β-catenin Knockout (Cell line made using Doble lab QKOs)
	TCF	T-cell Factor
	TLE	Transducer-like enchancer of split
W	WRE	Wnt Responsive element

DECLARATION OF ACADEMIC ACHIEVEMENT

This thesis was completed by Smarth Narula, with the following contributions from individuals in Dr. Bradley Doble's lab:

Steven Moreira developed the TCF/LEF quadruple knockout (QKO) cells;

Scott Poortinga helped with the development of the TCF/LEF - β-catenin knockout (TLB) cells.

CHAPTER 1:INTRODUCTION

1.1 Embryonic Stem Cells (ESCs)

Stem cells are distinguished from other cell types due to their unspecialized nature (Thomson, 1998; National Institutes of Health, 2019). They have two unique characteristics: the ability to self-renew and the ability to differentiate into specialized cell types (Thomson, 1998). Embryonic stem cells (ESCs) are essentially immortal, capable of unlimited proliferation *in vitro*, and are derived from the pluripotent inner cell mass of early preimplantation mammalian embryos, which are capable of generating all three germ layer lineages: endoderm, ectoderm and mesoderm (Thomson et al, 1998). Due to their pluripotent nature, mouse ESCs (mESCs) provide researchers with a powerful cellular tool to genetically alter the germ line of chimeric mice that can be generated by introducing genetically modified ESCs into preimplantation host embryos (Thomson et al, 1998; Martins, 1981; Kaufman et al, 1981). The characterization of resultant mutant animals, such as knockout mice, have been instrumental in advancing our understanding of targeted genes.

Given their ability to self-renew and differentiate into seemingly any cell type, pluripotent stem cells have transformed the biomedical industry. Embryonic stem cell (ESC) and induced pluripotent stem cell (iPSC) research is actively pursued by thousands of investigators worldwide, and although many advances have been made towards our understanding of these cells, mechanisms of self-renewal and cell fate determination remain unclear.

1.2 Naïve and primed states of pluripotency

Pluripotency describes the ability of cells having no fixed developmental potential to differentiate into different cell types. Pluripotent cells can be cultured indefinitely and can maintain their pluripotent state with the help of exogenous signaling factors. Thanks to advancements in the field of stem cell research; cells which previously required the use of mitotically inactivated mouse

fibroblast feeder cells (MEFs) with fetal bovine serum (FBS) can now be cultured with Leukemia Inhibitory Factor (LIF).

LIF activates the JAK-STAT3 pathway and is sufficient to keep ES cells in their pluripotent state without the need of MEFs (Smith et al, 1988; Niwa et al, 1998). Much of what we know about culturing stem cells is based on studies performed with serum-based media (media supplemented with fetal bovine serum containing purified or synthetic ingredients to provide cells with growth factors and nutrients). Thanks to the discovery of LIF, we now know that cells can be grown without serum, as LIF can act to inhibit differentiation and keep cells in a pluripotent state, in combination with other factors such as BMP-4 (Niwa et al, 1998). The lack of serum can also be overcome with the presence of two small-molecule inhibitors: one targeting the ERK1/2/mitogen-activated protein kinase (MAPK) signal transduction pathway (MEKi, PD0325901), and the other inhibiting glycogen synthase kinase-3 (CHIR99021). These two inhibitors prevent the cells from differentiating and select for undifferentiated cells (Ying et al, 2008).

Pluripotent cells can exist in two states of pluripotency termed the primed or naïve states (Evans et al, 1981). The major differences between the two states being the gene expression profile, the cellular morphology and the growth conditions required for maintenance of the particular pluripotent state. Cells derived from the inner cell mass of preimplantation embryos are considered to be naïve pluripotent stem cells, capable of differentiation into all three germ layers with unlimited self-renewal capacity. Furthermore, naïve stem cells can be injected back into pre-implantation embryos and contribute to all somatic cells and, more importantly, the germline, indicative of their pluripotent state in *vivo* (Huang et al, 2012).

Cells in the primed pluripotent state, on the other hand, are derived from the epiblast of post-implantation embryos, and, like naïve cells, have the same ability to self-renew indefinitely as well as to differentiate into all three germ layers, but they lack the ability to give rise to germline chimeras

in *vivo* (Huang et al, 2012). Furthermore, naïve state cells typically grow as small, dome-shaped colonies, whereas primed state cells are larger and grow as a monolayer (Tesar et al, 2007). Additionally, they are both different metabolically, as naïve cells generate energy by utilizing oxidative phosphorylation and glycolysis, whereas primed cells prefer to generate energy by using the glycolytic pathway (Tesar et al, 2007; Teitell et al, 2015).

1.3 Embryoid Bodies

Embryonic stem cells grown in the absence of factors supporting pluripotency form embryoid bodies (EBs). EBs are aggregates of pluripotent cells that undergo spontaneous differentiation to form poorly organized embryo-like structures containing cells of all three germ layer lineages (Doetschman et al, 1985; Schell, 2012). The interaction of the cells at a three-dimensional level allows for significant morphological change (Schell, 2012; Eldor et al, 2000). EBs provide a simple method for studying complex developmental processes as well as the ability to observe phenotypic attribute changes at an embryonic level (Doetschman et al, 1985; Desbaillets et al, 2000; Schell, 2012). EBs have been considered to represent post-implantation embryos, with three-dimensional cystic structures offering a model to study differentially expressed genes as differentiation takes place (Spangler et al, 2018; Doetschman et al, 1985).

Growing EBs, although strenuous, is a fairly straightforward process, and can be achieved by using a variety of different methods. One of the more common techniques is to remove the cells from mLIF and to culture them in methyl cellulose liquid in bacterial petri dishes (Keller, 1995). Using this technique, the cells are unable to adhere to the surface and form EBs. The second method is to specifically employ the use of stromal cells to form EBs. The use of stromal cells allows for a supportive environment for the cells as they start to differentiate and form EBs (Keller, 1995; Dang, 2002). EBs can also be formed by using the ‘hanging drop’ method, the method employed for this thesis. In this method, the cells are suspended in droplets and are in close proximity to one another.

The close association of cells allows for an efficient method of forming EBs. The 'hanging drop' method is advantageous for forming EBs when working with cell lines that may not form EBs efficiently (Keller, 1995).

As mentioned earlier, EBs are aggregates of cells clumped together, which form a ball-like structure. This phenomenon is observed when the cell-cell adhesion force is greater than that of cell-substratum (Martins & Evans, 1975). This adhesive force allows for the cells to come together and form a spheroid structure, growing larger in size as time progresses (Martins & Evans, 1975). As EBs grow, there is a clear visual disparity between the center of the EB and the outer layer; the inner clump of cells remains intact and displays characteristics similar to the ICM, whereas the outer layer of cells are indicative of primitive endoderm formation (Martins & Evans, 1975). This difference can be observed under the microscope, with a dark clump observed in the middle of an EB, which is surrounded by cystic features.

1.4 Introduction to Wnt signalling

Cells, being the building blocks of life, are governed by signalling molecules that provide instructions and coordinate basic cellular activities. Signalling molecules are part of a communicative process that perceives the environment around cells to help direct processes including development, immune responses, tissue homeostasis, and repair. One family of signalling proteins is the Wnt family.

An abnormality resulting in the absence of a wings and halters led to the discovery of the *Wingless* gene in the fruit fly *Drosophila* (De, 2011). A homolog of the fly *Wingless* gene was found in a conserved locus in mouse and was named *int.* The *int*-1 gene was originally discovered as a frequent insertion target site that was activated by the mouse mammary tumour virus.

As more studies looked into the function of *int-1*, it was apparent that other mouse loci were activated as well in various mammary tumours. These were named, *int-2* and *int-3*, it soon became

apparent that there is a clear difference between the three; with *int-1* encoding a set of glycoproteins, similar to that of the *Wingless* gene (Nusse et al, 1991). *Int-2* and *Int-3*, encode for members of the fibroblast growth factor family of protein and transmembrane receptor proteins, respectively (Nusse et al, 1991; Heinz et al, 2015). With *int-1* coding a homolog of *Wingless*, the combination of the two names led to the gene being named *Wnt.*

The Wnt gene products consist of lipid-modified glycoproteins involved in various events during embryogenesis and adult tissue homeostasis as well as cell fate determination and stem cell renewal (Logan et al, 2004; Komiya et al 2008). The Wnt pathway can be further divided, minimally, into three distinct pathways: the Wnt/β-catenin dependent pathway (often called the canonical Wnt pathway) and the non-canonical pathway or β-catenin-independent pathway, which can be further divided, broadly, into two categories, the Planar cell polarity pathway and the Wnt/Ca^{2+} pathway (De, 2011; Komiya et al, 2008; Devenport et al, 2014).

The initiation of canonical Wnt signalling is a result of an interaction between Wnt proteins, four secreted R-spondin proteins (Rspo 1-4), the cell surface receptor, Frizzled (Fz), and the co-receptor low-density-lipoprotein-related-protein (LRP5/6) (Komiya et al, 2008). R-spondins are found upstream of Wnt proteins and interact with LRP, acting to enhance Wnt activity, with studies showing a higher rate of β-catenin stabilization in the presence of R-spondins (Kazanskaya et al, 2004; Kim et al, 2008).

Although R-Spondins are often required for efficient activation of the Wnt pathway, and the four members share 40%-60% of their sequence identity, not all R-Spondins act in the same way. R-spondin1 (RSpo1) is less potent in its enhancing ability than R-Spondin 2-3 (RSpo2 and RSpo3); while R-Spondin 4 (RSpo4) remains mostly inactive (Kim et al, 2008). R-Spondins act by competing with dickkopf inhibitor 1 (DKK1) and reduce the internal accumulation of LRP6 and allowing it to

accumulate on the cell surface (Kim et al, 2008).

Wnt genes are found in both humans and mice, and deregulation of Wnt signalling has been found to be extremely deleterious to the developing embryo. Abnormal Wnt signalling has been noted to be a major factor for various human pathologies such as breast cancer, colon cancer, and skin and skeletal defects (Komiya et al, 2008).

1.5 The Wnt/β-catenin Pathway

1.5.1 Wnt Off State

One of the defining aspects of the Wnt/β-catenin pathway (often referred to as the canonical Wnt pathway) is the accumulation of the adherens junction-associated protein, β-catenin, in the nucleus. With no Wnt stimulus, the signalling pool (non-junctional, cytosolic) of β- catenin is degraded by the β-catenin destruction complex (Figure 1). The remaining β-catenin is found in adherens junctions (Komiya et al, 2008; Eastman & Grosschedl, 1999).

The β-catenin destruction complex consists of Axin, Adenomatosis polyposis coli (APC), Protein phosphatase 2A (PP2A), Glycogen synthase kinase-3 (GSK-3) and Casein kinase 1α (CK1-α) (Komiya et al, 2008). The degradation of β-catenin by the destruction complex is hindered by the binding of Wnt to Frizzled and LRP5/6 (Clevers et al, 2006). The interaction causes the disruption of APC/Axin/GSK-3, which in turn prevents the degradation of β-catenin (Habas, 2008; Komiya et al, 2008; Reya et al, 2005). In the absence of Wnt signalling, APC and Axin bind to a newly synthesized β-catenin molecule. The binding causes phosphorylation of conserved serines and a threonine, located near the amino terminus, by CK1 and GSK-3 (Behrens, 1998; Salic et al, 2000; Reya, 2005). The phosphorylation results in the recruitment of β-TrCP-containing E3 ubiquitin ligase, which in turn allows for proteasomal degradation of β-catenin (Macdonald et al, 2009; Reya, 2005).

The occupancy of the receptors Frizzed and LRP5/6 by Wnt ligand inhibits the degradation of β-

catenin through a poorly understood mechanism. It is thought that the interaction between Axin and LRP5/6 and/or the Axin-binding molecule, Dishevelled (DSH), inhibits kinase activity, which, in turn, inhibits the destruction complex. The inhibition of the destruction complex results in the accumulation of β-catenin in the nucleus, where it interacts with the N-terminus of TCF or LEF transcription factors (Reya, 2005).

1.5.2 Wnt On State

Wnt/β-catenin signalling is in its active state in the presence of a Wnt ligand. Binding occurs between the ligand and the transmembrane receptors LRP5/6 and Frizzled (Clevers & Nusse, 2012). The presence of the Wnt ligand results in a ligand-induced conformational change of the two receptors, resulting in the activation of DSH. The activated DSH works to recruit Axin and the destruction complex to the membrane (Figure 1). Furthermore, activated DSH leads to the inhibition of GSK-3, leading to further destabilization of the destruction complex (Clevers & Nusse, 2012; Re2005; Moon et al, 2004). The destabilization of the destruction complex ultimately allows for cytosolic β-catenin to accumulate and make its way to the nucleus. The exact mechanism as to how β-catenin translocates to the nucleus is not completely understood, but once inside the nucleus, β-catenin interacts with a variety of transcription factors, most notably the TCF/LEF family (Yokoya et al, 1999; Reya, 2005; Clevers & Nusse, 2012; Moon et al, 2004).

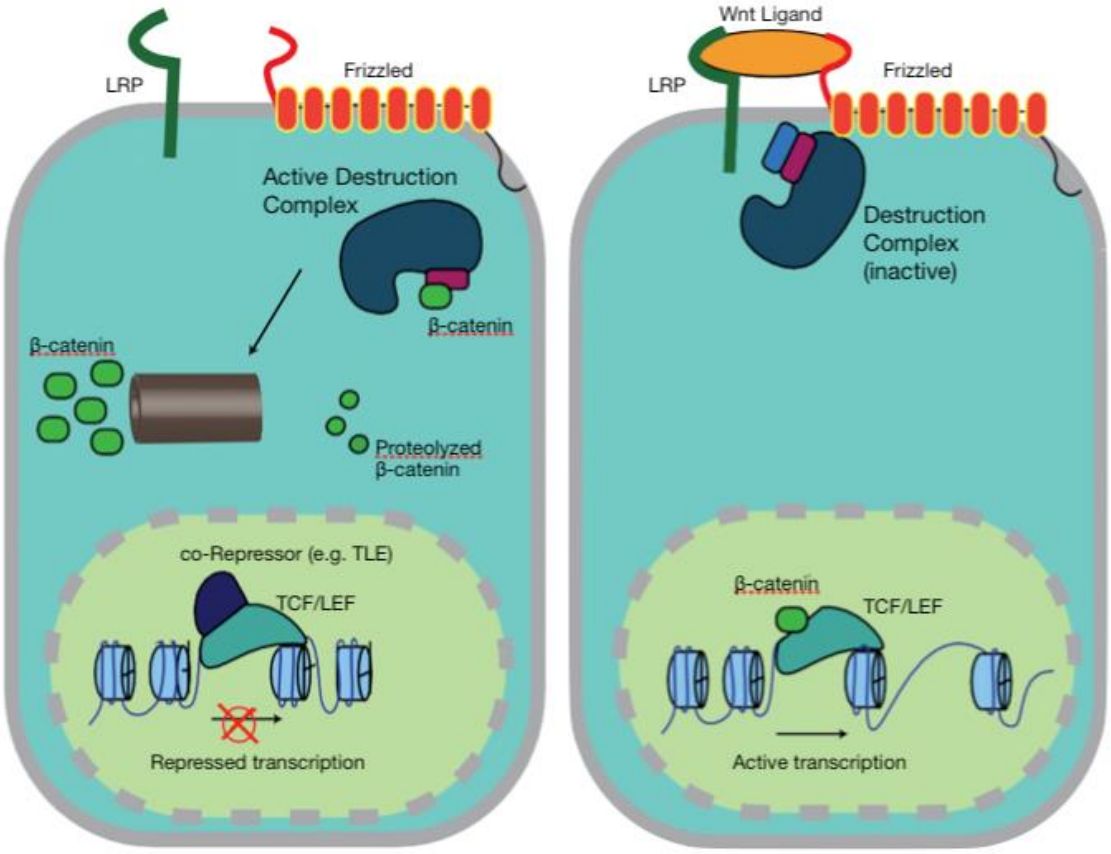

Figure 1: Schematic diagram of Wnt/β-catenin (canonical) signalling.

(A) In the absence of Wnt ligand, phosphorylation of β-catenin takes place in the destruction complex. Phosphorylated β-catenin is then marked for proteasomal degradational, and transcription of Wnt-related genes is blocked. (B) In the presence of a Wnt Ligand, the destruction complex is recruited to the membrane, leading to destabilization of the heterocomplex. β-catenin accumulates and translocates to the nucleus leading to transcription of Wnt-regulated genes. Illustration adapted from one provided by Pratik Joshi.

1.6 T-Cell factors and Lymphoid Enhancer Factor

TCF and LEF acting as mediators of Wnt signal transduction came as a major surprise when discovered, as LEF-1 was previously thought to act only as a factor facilitating the assembly of multiprotein enhancer complexes (Bienz, 1998). Their involvement as Wnt responsive factors allowed to them be a part of a unique group of molecules known for their multipurpose usage in mammalian cells, like β-catenin (Bienz, 1998).

All invertebrates studied to date possess a single TCF/LEF (Cadigan et al, 2012). This is not the case for higher-level vertebrates, which produce four different *TCF/LEFs: Lef-1, TCF-1(TCF7), TCF-3 (TCF7l1)* and *TCF-4(TCF72)* (Bienz, 1998). Knock-out experiments have helped to determine that each of the four family members has its own unique function that cannot be mirrored or relayed by another. TCF-1 and Lef-1 have been determined to be predominantly transcriptional activators, whereas TCF-3 typically acts as a repressor. TCF- 4 can act as a repressor or an activator depending on the environment and requirements of a cell (Bienz, 1998).

All the members of the TCF/LEF family have a highly conserved DNA-binding domain known as an HMG domain, which is located towards the C-terminus (Graham, 2000). TCF/LEFs, on their own, possess no transcriptional activity but rather repress Wnt target genes through interactions with co-repressors such as the Groucho/Transducer-like enchancer of split (TLE) family, with Groucho being a corepressor found in *Drosophilia* and its mammalian counterpart being TLE (Chen et al, 2000; Graham, 2000).

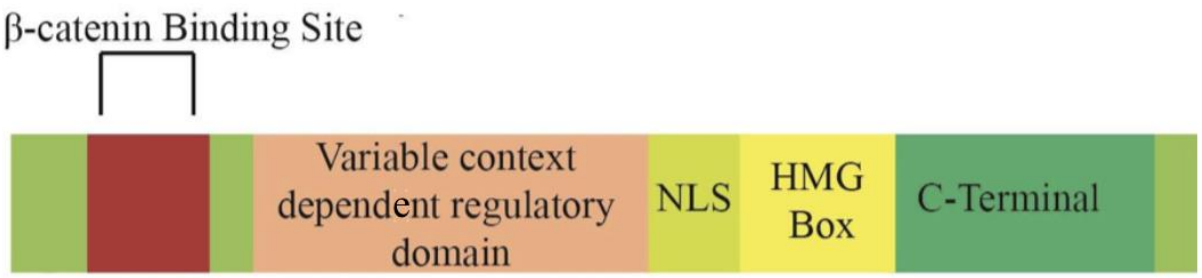

Figure 2: Schematic diagram of TCF/LEF protein structure

TCF/LEF isoforms can be divided into five main domains; β-catenin binding domain, a highly variable context dependent regulatory domain, a nuclear localization signal domain (NLS), a highly conserved DNA-binding domain (HMG), and the C-terminus.

The structure of TCF contains a β hairpin module, an extended region, and an α-helix region. Although all three are important in terms of the binding of TCF to β-catenin, it has been concluded that the β-hairpin is largely dispensable (Graham, 2000). The critical residues Arg-474, Arg-612 and Arg-582 in the β-hairpin help to initiate binding between TCF and the β-hairpin, but based on mutagenesis experiments, it has been concluded that the extended region of TCF is the minimal unit required for binding. Lastly, the α-helix is crucial for the binding to take place, as it was determined that mutations in the α-helix reduced the binding affinity drastically (Graham, 2000).

Lymphoid enhancer-binding factor (LEF), is very closely related to its counter-part, TCF1, both of whom share similar expressional profiles during development at sites of organogenesis (Hsu, 1998). The amino-terminal region of LEF, like that of the TCFs, is capable of associating directly with β-catenin, and, with an abundance of β-catenin in the system, the formation of LEF-β-catenin complexes can occur (Hsu, 1998). LEF-1 also interacts with the protein AML-1, which can help LEF-1 to stimulate the T-cell receptor α (TCR α) enhancer (Hsu, 1998).

TCF/LEFs are architectural transcription factors that are capable of introducing a sharp bend in the DNA after they bind it, which allows for regions of DNA widely separated, based on their linear

sequences, to be brought together to form enhanceosome complexes containing multiple DNA-binding and associated factors (Hsu, 1998).

In the absence of β-catenin, TCF binds with a transcriptional repressor, Groucho/TLE, and histone deacetylase to form a complex, which is functionally responsible for the repression of Wnt target genes. On the other hand, in the presence of the nuclear β-catenin, β-catenin can overcome the repressive function of Groucho, binding with TCFs to convert the complex from a transcriptional repressor to an activator, thus allowing for the transcription of the Wnt genes (Eastman et al, 1998; Gordon et al, 2006). There are various other factors essential for transcriptional activation that bind to TCF-β-catenin-complexes. Most notably are Legless (*Lgs)* and Pygopus (*Pygo). Lgs* binds to the N-terminus of β-catenin and acts as an adaptor for *Pygo*, which plays a role in chromatin remodelling (Thompson et al, 2002; Gordon et al, 2006)

1.7 β-catenin

β-catenin, first discovered by McCrea in 1991, is a 90kD protein that has received much attention due to its critical involvement in the highly evolutionarily conserved Wnt/β- catenin signalling pathway (McCrea et al, 1991; Averett et al, 2014; Yan et al, 2017). Under normal physiological conditions, levels of β-catenin are kept low through continuous degradation by a destruction complex (Aberle et al, 1997; Angers et al, 2009). β-catenin, being an essential part of the "canonical" pathway, plays an important role in stem cell renewal and organ regeneration (Valenta et al 2012a; Valenta et al 2012b; Averett et al, 2014). Abnormal activity of β-catenin can induce many malignant transformations, and irregular levels of β-catenin have been reported in many cancer types (Averett et al, 2014; Yan et al, 2017; Valenta et al 2012).

β-catenin is composed of 781 amino acids, with highly conserved armadillo repeats at its core (Averett et al 2014; Yan et al, 2017; Valenta et al 2012a). Each armadillo repeat consists of 40 amino acids in three α-helices (Figure 2). The distal regions of β-catenin, the N-terminus and the C-

terminus, are very unstructured and are not as conserved as the core armadillo repeats (Yan et al, 2017; Davidson et al, 2014). The N-terminal region contains a unique armadillo repeat, featuring an extended region with a kink formed by the merging of α-helices 1 and 26 (Graham, 2000 et al; Yan et al, 2017). The kink allows for the binding of β-catenin partners. The region also features a motif specific to the E3 ubiquitin ligase that helps control the levels of β-catenin through degradation mediated by phosphorylation (Graham, 2000 et al; Yan et al, 2017). The C-terminal region (residues 665-781) contains an α helix, Helix C, (Graham, 2000 et al; Yan et al, 2017). The armadillo repeats, along with Helix C, contribute to β-catenin's transcriptional activity by recruiting both effectors and inhibitors (Behrens et al, 1996; Yan et al, 2017). At its core, the armadillo repeats found at the center of β-catenin are highly conserved and are responsible for the binding and interaction with transcription factors as well as some small ligands (Yan et al, 2017; Orsulic et al, 1996).

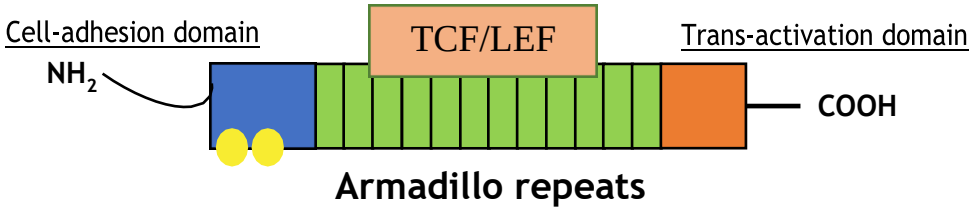

Figure 3. Schematic representation of β-catenin

Wild type β-catenin can be divided into three distinct regions: The N-terminal responsible for the cell-adhesion role of β-catenin as well as the site for phosphorylation by the destruction complex; the C-terminal being the binding site of various transcriptional co-activators; and a central rigid structure containing an armadillo repeat region acting as the binding site for the TCF/LEF factors.

1.8 β-catenin and Cell pluripotency

Embryonic stem cells (ES cells) are obtained from the inner cell mass (ICM) of early preimplantation embryos called blastocysts. Inner cell mass cells only transiently possess the property of pluripotency, which is lost as they choose their fate and commit to one of the three germ

layers (endoderm, ectoderm, mesoderm) (Sokol et al, 2011). Pluripotency is regulated by a transcription factor regulatory network, which can be sustained with appropriate cell culture conditions (Schwarz et al, 2007; Sokol et al, 2011). This network includes the DNA-binding transcription factors NANOG, OCT4, and SOX2. It is important to note that the conditions required in culture to maintain pluripotency and long-term self-renewal differ greatly between mouse ES cells and human ES cells (Hoffmeyer et al; Sokol et al, 2011).

In addition to its involvement in various functions during embryogenesis, β-catenin has also been proposed to be involved in maintaining cell pluripotency (Schwarz et al, 2007; Song et al, 2003). Studies have shown that a β-catenin deficiency disrupted ES cell self-renewal and pluripotency within differentiating cells (Sokol et al, 2011).

Before delving deeper into understanding the role of β-catenin in pluripotency, it is important to mention the role of glycogen synthase kinase-3 (GSK-3), a protein kinase that is responsible for the phosphorylation of β-catenin and, ultimately, its proteasomal degradation (Schwarz et al, 2007; Sokol et al, 2011; Kelly et al, 2011). It was shown that by inhibiting GSK-3, high levels of β-catenin were observed and the ability of mESCs to differentiate was greatly inhibited (Kelly et al, 2011). Similarly, the same result was observed with disruption of destruction complex function, allowing for high levels of β-catenin to build up. This correlation has been noted in various models where overexpression of β-catenin led to a severe block in the ability of cells to differentiate (Lyashenko et al, 2011; Kelly et al, 2011).

To determine the role of the β-catenin/TCF complex in pluripotency, ES cells overexpressing dominant-negative TCF7L2 or TCF1 in a background where GSK- 3α/β were both knocked out (Double knockout, DKO) were employed. The dominant-negative TCF/GSK-3 DKO line resulted in low levels of β-catenin/TCF gene transactivation, yet the cells retained makers of pluripotency and self-renewal was not disrupted (Kelly et al, 2011). An experiment was conducted to examine

the differentiation capacity of GSK-3 DKO mESCs with reduced β-catenin levels (via shRNA knockdown), and it was determined that these cells were able to differentiate out of the pluripotent state, providing further evidence of the involvement of β-catenin in retaining mESC pluripotency (Sokol et al, 2011; Kelly et al, 2011).

The literature suggests that β-catenin, in part, acts independently of TCF/LEFs to help to maintain the pluripotent state of ES cells. There is some evidence suggesting that β-catenin forms a complex with Oct-4 to promote retention of mESC pluripotency (Kelly et al, 2011; Chatterjee et al, 2015).

1.9 Cell fate determination

Lineage specification begins in a mouse embryo around 3.5 days post coitum, at the blastocyst stage. Within the blastocyst, there exists a cluster of cells known as the inner cell mass (ICM) (Gadue et al, 2005). The Wnt pathway is heavily involved in various embryonic processes, including determination of cellular fate. It was determined that in the absence of Wnt3 (Wnt3-null embryos), the epiblast (one of the two layers arising from the inner cell mass, also known as the primitive ectoderm) remains undifferentiated. Identical observations were made in β-catenin null mutants, implying that β-catenin's specific role in the Wnt pathway is involved in determining cell fates (Gadue et al, 2005; Miller et al, 1996). Furthermore, to the test the effects of Wnt signalling on gastrulation, mutations in the co-receptors, LRP5/6, were performed. The mutations led to reduced Wnt signalling and pushed the ES cells towards generating more neuroectoderm, suggesting that Wnt pathway in wild-type cells inhibits neuroectoderm formation (Gadue et al, 2005).

To test further the possibility that Wnt pathway activation inhibits neuroectoderm formation, the Wnt inhibitor, secreted frizzled relation protein 2 (sFRP2) was used. With the inhibition of the Wnt pathway via sFRP2 administration, it was observed that neural development was enhanced (Gadue et al, 2005). Elevated levels of Wnt3a (expressed at high levels during embryogenesis) displayed a neuronal differentiation blockage and induced the expression of *brachyury* (needed for defining the

midline of organisms displaying bilateral symmetry) (Gadue et al, 2005).

β-catenin has another unique and important feature, which is its role in the regulation of cell-cell adhesion. The dual function of β-catenin leads to the obvious question: are both features of β-catenin important in its role in determining cell fate?

To answer this question, β-catenin$^{(-/-)}$ mESCs were made by Wagner et al (Wagner et al, 2010). ESCs lacking β-catenin displayed a defect in endoderm and mesoderm formation (Wagner et all, 2010). They also had elevated levels of ectodermal apoptosis (Lickert et al, 2005; Lyashenko et al, 2011). β-catenin-rescued mESCs expressing wild type or a TCF/LEF signalling-defective β-catenin variant were also examined. The purpose of using a signalling-defective variant was to re-establish cadherin-mediated cell adhesion in the absence of signalling (Lyashenko et al, 2011).

It is important to note that the signalling-defective variants of β-catenin were established via the truncation of the C-terminal region, the region involved in Wnt-mediated transcriptional activation. By comparing the results from WT and mutant- rescued lines, whether cell-adhesion plays a role in determining cell-fate could be evaluated. It was determined that the signalling-defective β-catenin restored endodermal layer formation, which was verified by immunofluorescent staining for Gata4, Fox2, and Cxcr4, as well as the neuroectodermal layer, verified by β-III-tubulin immunofluorescent staining (Lyashenko et al, 2011). Cells of the mesodermal germ layer lineage were not observed, possibly because, unlike the other two germ layers, mesoderm requires the transcriptional activity of β-catenin rather than the cell-cell adhesion function, which seems to be needed for the formation of endoderm and neuroectodermal layers (Lyashenko et al,2011). It is possible that the results of the signalling-defective β-catenin variant experiments were misinterpreted, as β-catenin was not completely removed from the system and it is also known that the C-terminus of β-catenin is not the only region to which co-activators can bind (Hoffman et al, 2004; Sampietro et al, 2006; Lyashenko

et al, 2011). The results observed from these experiments might be due to the residual nuclear effects of the presumed inactive β-catenin variant.

1.10 Non-TCF/LEF binding partners for β-catenin

Previously, it has been reported that TCF/LEF is required for Wnt/ β-catenin signalling to occur (Schuijers, 2014). These reports were largely based on cell models with truncated versions of TCF/LEF, resulting in somewhat biased conclusions. Our cell model system, lacking all four full-length TCF/LEFs provides us with a unique opportunity to gain a better understanding of how β-catenin interacts and functions in the absence of TCF/LEFs. Many β-catenin interacting DNA-binding partners have been identified, which are involved in activating or repressing Wnt/β-catenin signalling (Table 2; MacDondald, 2010). What is yet unclear is whether the involvement of TCF/LEF is required for these Non-TCF/LEF β- catenin interacting partners to have an effect on Wnt/ β-catenin signalling.

Finding binding partners of β-catenin is not an easy task, as endogenous β-catenin has been postulated to bind with various proteins to form a multi-protein complex that could cause the accessibility of antibody binding sites to be sterically blocked, which makes it difficult to use common techniques such as co-immunoprecipitation (Traenkle et al, 2015).

In order to bypass this issue, Traenkle et al, developed a set of nanobodies specifically designed to target specific areas of β-catenin. The areas targeted were the C-terminus, N-terminus and the core (Traenkle et al, 2015). The results from their experiments showed that β-catenin forms complexes with binding partners in the plasma membrane, cytoplasm and the nucleus (Traenkle et al, 2015). Furthermore, the nanobody specific for the N- terminal region identified another new binding partner (Traenkle et al, 2015). The study focused on developing better methods to study β-catenin's dynamic changes in protein stability and thus, does not elaborate on the possible identity of the targets found. It is important to note that the study was performed using human ES cells (Traenkle

et al, 2015). There have been other studies done to identify TCF/LEF-independent β-catenin binding partners and in fact, our lab and others have shown an interaction taking place between β-catenin and Oct-4, a crucial part of the transcriptional network involved in regulating pluripotency (Kelly et al, 2011; Kelly et al, 2010; Chaterjee et al, 2015).

These findings suggest an intricate network of Wnt/ β-catenin signalling which works independent of TCF/LEF, providing evidence that the previously thought notion of the dependence of Wnt/ β-catenin signalling on TCF/LEF is not entirely correct.

1.11

Non-TCF β-catenin interacting binding partners	Effect on Wnt/β-catenin signalling	Non-TCF β-catenin interacting binding partners	Effect on Wnt/β-catenin signalling
AP-1[26]	+	GR[27]	-
AR[29]	-	HIF1α[30]	-
E2F1[32]	-	KLF4[33]	-
ERα[35]	+	Lrh-1[36]	-
Foxo4[38,39]	-	Mitf [40]	-
Foxo3a[38,39]	-	Oct4[42]	-
FoxM1[44]	+	PPARγ[45]	-
Gli3[47]	-	RAR[48]	-
RXR[28]	-	TRβ[41]	-
Sox6[31]	-	VDR[43]	-
Sox9[34]	-	Xsox3[46]	-
Sox17[37]	-		

Table 1: TCF independent β-catenin binding partners

1.11 Media conditions: Serum vs 2i media

Mouse embryonic stem cell maintenance in media containing serum and murine LIF (mLIF) is a

well-known and -characterized method (Sim et al, 2017). It is a very effective method of maintaining self-renewal as well as pluripotency. Although effective, there are some drawbacks to serum-containing media formulations supplemented with mLIF. Colonies of cells display a heterogeneous population within them and variation in terms of the expression of pluripotency-associated factors (Sim et al, 2017).

Much more cellular homogeneity is acquired by using a fully-defined alternative to serum medium, commonly referred to as "2-inhibitor medium" (2i medium; 2i medium employs defined serum-free conditions and supplementation with small molecule kinase inhibitors of GSK3 (CHIR99021) and MEK (PD0325901) (Doble et al, 2007; Wray et al, 2010). The inhibition of GSK3 and MEK via CHIR99021 and PD0325901, respectively, results in the stabilization of exogenous signals which have been known to have a destabilising effect on the pluripotency network (Kawano et al, 2003; Doble et al, 2007; Sim et al 2017; Wray et al, 2010). Furthermore, Kalkan et al showed that cells with prolonged exposure to 2i medium displayed a more "naïve" cell state type and displayed a dramatic decrease in the number of partially differentiated cells observed in mESC colonies. The data further suggest that cells grown in 2i conditions experience optimal self-renewal and colony formation that is limited to cells in a naïve state (Kalkan et al, 2017).

1.12 Project Rationale and Hypothesis

The overall goal of this project is to identify novel targets that are β-catenin dependent and act in a TCF/LEF independent manner. While the implications of TCF/LEF-β-catenin interactions are well documented, little is known about TCF/LEF independent Wnt/β- catenin signalling.

In order to elucidate the TCF/LEF independent targets, we are currently in collaboration with the laboratory of Dr. Konrad Basler (Institute of Molecular Life Sciences, University of Zurich, Switzerland). The Basler lab has extensive expertise in generating and analyzing RNA-seq and ChIP-seq data.

Our lab previously designed a cell line lacking all four TCF/LEF factors (QKO mESCs). In this thesis, I describe the generation of QKO mESC lines in which β-catenin has been knocked out by using CRISPR/Cas9 methodology, as well as a control line in which WT parental cells have had β-catenin knocked out. These cell lines have been interrogated by using RNA-seq in collaboration with the Basler lab. Given the crucial role that β-catenin plays in determining cell fate, I have also undertaken embryoid body differentiation assays with the cell lines I developed to determine the cell fate choice limitations that occur in the absence of TCF/LEFs and β-catenin.

The overall goals of this project will help to increase our understanding of TCF/LEF independent mechanisms of β-catenin nuclear function that are currently poorly understood. It will also shed light on the function of such TCF/LEF-independent β-catenin activities in the nucleus.

HYPOTHESES:

- The TLB cell line will be indistinguishable from the wild type in terms of morphology and cell culture requirements (e.g. media, frequency of passaging, adhesion to gelatin-coated plates).
- The TLB cell line will favour neuroectoderm differentiation, as observed with QKO mESCs.
- RNA-seq experiments will yield TCF/LEF-independent transcriptional targets that act in a β-catenin dependent fashion.

CHAPTER 2: MATERIALS AND METHODS

2.1 Cell Culture

For the purposes of this thesis, E14TG2a (ATTC® CRL-182™) mESCs were used to generate all derivative Doble lab cell lines as well as any assays that were performed. Cell lines created for collaborative work with the Basler lab used wild type and the QKO cells provided by them, which were of different parental WT mESCs than the E14TG2a line. The cells were cultured on tissue culture specific plates coated with 0.1% gelatin and were maintained in a humidified incubator kept at a constant 37ºC, 5% CO2. For overall cell maintenance, standard serum culturing media was used, containing: Dulbecco's Modified Eagle Medium (DMEM, Sigma D5671), 15% FBS (Gibco), 1X GlutaMAX (Gibco), 1% non-essential amino acids (Gibco), 55 μM β-mercaptoethanol (Sigma), 1 μM sodium pyruvate (Gibco), and 1000 U/mL mLIF (Miltenyi Biotec). For media being used for differentiation purposes, standard culturing medium was used that lacked supplementation with mLIF (Miltenyi Biotec). For any experiments that were performed under 2i serum free conditions, cells were first plated onto 0.1% gelatin coated plates with a monolayer of feeders, using standard serum culturing media. After two passages on the monolayer of feeders, they were passaged onto 0.1% gelatin coated plates for another two passages. Thereafter, they were plated onto 0.1% gelatin with N2B27 medium containing 3 μM CHIR99021 and 1 μM PD0325901, lacking mLIF (as in Moreira et al, 2017).

Cells were maintained regularly with a media change occurring once every 2 days and subculturing also typically occurring every 2 days, although the frequency of repassaging was dependent on the confluency of the cultures. Dissociation of cells was accomplished by using Accutase (37˚C for 5 minutes, following manufacturer's instructions), which liberated them from the coated plate surface. Once cells were dislodged, they were carefully collected by resuspending the cell/accutase mixture with standard culture medium and collecting them by centrifugation at 200 g for 3 minutes and were

then re-plated with an appropriate pre-determined split ratio. As a rule, the mESCs were maintained for at least two passages before being used for any experiments.

2.2 Generation of β-catenin knockout in QKO and Wild Type mESC line

The β-catenin knockout cell line was created by using pSpCas9-PX459 V2.0 plasmid. 200 000 cells were transfected by using Lipofectamine® 2000 DNA Transfection Reagent protocol (Invitrogen). The cells were split onto 4 separate 0.1% gelatin coated plates, with two of the plates containing 50 000 cells and the other two containing 100 000 cells. The left-over cells were discarded. After 24 hours, the cells were subjected to 2 µM puromycin in order to kill off the cells that had not been successfully transfected with the vector. The cells were kept on puromycin only for 24 hours and were then allowed to grow standard culturing media. They were maintained until single colonies were visible. Once the colonies were of good size for picking, the colonies were isolated and allowed to expand.

2.3 Protein Lysate preparation

One million cells were collected, washed with phosphate buffered saline (PBS) to get rid of media and allowed to sediment using centrifugation. PBS was aspirated, and the cells were suspended with radioimmunoprecipitation assay (RIPA) buffer (50 µM NaCl, 1% NP-40, 0.1% SDS, 1%NP-40, 1 µM EDTA, 50 µM Tris pH 8.0, 1X Halt Protease Inhibitor Cocktail; Thermo Scientific). The cells were kept in RIPA buffer for 25 min. on ice and were pelleted by centrifugation at 15,000 x g for 12 minutes. The supernatant was then collected, and the protein concentration was determined by using DC Protein Microplate Assay Protocol (Bio-rad). Once the protein concentrations were determined, the proteins were diluted down to 1µg/µl in 5% Bond-Breaker (Novex) and 1x NuPAGE LDS Sample Buffer (Novex) and stored at -80ºC.

2.4 Western Blot Analysis

Protein samples (15 µg/well) were loaded onto 10% Bis-Tris gels and were subjected to 180V for 50 minutes to allow for protein separation. The gel was then submerged in running buffer composed of 0.5% NuPAGE® MOPS SDS Running Buffer (20X) solution. The separated protein was then transferred to a polyvinylidene fluoride (PVDF) membrane. The transfer of protein was mediated by Towbin transfer buffer (25 mM Tris, 192 mM glycine, and 20% (v/v) methanol) and was subjected to 200 mA for 2 hours. Once the transfer of protein was complete, the membrane was blocked with 3% skim milk/tris-buffered saline (TBS) solution for 45 min at room temperature. The membrane was then submerged with primary antibody diluted in 5% skim milk/TBS-T solution (Tris-buffered solution with 0.5% TWEEN®20, Sigma) and kept overnight at 4˚C. Post overnight incubation, the membrane underwent multiple wash steps (5x) with 3% skim-milk/TBS-T solution each lasting 10 minutes. The membrane was then subjected to secondary antibody diluted in horseradish peroxidase substrate (HRP) for 45 minutes at room temperature. After the secondary antibody, the membrane underwent 4 washes in TBS-T, each lasting 10 minutes. After the final wash step, the membrane was submerged in 25% Luminata Forte Western HRP Substrate (Millipore) for 5 minutes and kept away from direct light. The membrane was then carefully dried off and imaged using the ChemiDoc Imaging System (Bio-Rad).

2.5 Antibodies

The following primary antibodies were used for western blots: mouse anti-β-catenin (AF1329:R&D system); mouse anti-β-catenin carboxy (SC-7963: Santa Cruz); mouse anti-Nestin (MAB353: Millipore Sigma); goat anti-HNFβ (SC-6554: Santa Cruz); rabbit anti-Brachyury (SC-20109: Santa Cruz); mouse anti-βIII Tubulin (SC-51670:Santa Cruz); rabbit anti-β-Actin (13E5:Cell signaling technologies); rabbit anti-Sox17 (09-308: Sigma- Aldrich).

The following horseradish peroxidase-conjugated secondary antibodies were used: goat- anti mouse (170-6516; Bio-Rad); goat anti-rabbit (170-6515; Bio-Rad) and donkey anti- goat (HAF109; R&D

Systems).

2.6 Ponceau S Staining of PVDF Blots

PVDF membrane was submerged in 5 mL of Ponceau S solution (0.1% w/w Ponceau S dye – 0.5g, 1% v/v acetic acid – 5 mL and 500 mL of dH_2O) for 10 minutes and placed on rocking platform shaker. Thereafter, the membrane was rinsed with dH_20 for 2 minutes and imaged. After imaging, the membrane was de-stained using 0.1 M NaOH solution.

2.7 EB Assay

Cells were plated on a monolayer of feeders in standard culturing media. They were maintained on feeders for 2 passages. Thereafter, the cells were pelleted and counted. The pelleted cells were then resuspended in EB medium and droplets of cells each containing 800 mESCs / 30 µL EB medium were plated on the lid of 10 cm^2 petri dishes (non-gelatin coated) containing 6 mL of PBS. The petri dish was placed in incubator (37ºC, 5% CO2) for 3 days. Afterwards, each droplet was transferred to ultra-low adhesion 96-well plates, each well containing 200 µL of EB media. The EB medium was replenished carefully every two days by tilting the plate to one side, thus moving the forming EBs to one side of the well and collecting the used media from the other side. Images were taken of the forming EBs everyday using an Evos Microscope (Thermo-Fisher). EBs were collected at two time points, day 7 and 14. On day 7, 20 EBs were collected and 10 EBs were collected on day 14. Thereafter, they were washed with PBS and pelleted via centrifugation at 4,000 x g for 3 min. All liquid was removed, leaving the pellet intact.

2.8 Quantitative RT-PCR

500,000 cells were collected and pelleted. RNA isolation was performed by using Monarch® Total RNA Miniprep Kit and concentration of RNA samples was determined. 1 µg of total RNA was used for cDNA synthesis using LunaScript™ RT SuperMix Kit. qRT- PCR was performed using Luna® Universal qPCR Master Mix and 2 µL of previously synthesized cDNA. All primers used were

obtained from previous publications (Table 2) and RPL13a was used as the reference housekeeping gene for all qRT-PCRs. Relative gene expression was calculated using the delta-delta Ct method.

Table 2: Primers used for qRT-PCR

Primer	Sequence
RPL13a	Fwd: 5'-TCCCTCCACCCTATGACAAG-3' Rev: 5'-GTCACTGCCTGGTACTTCC-3'
Axin2	Fwd: 5'-AAAGAAACTGGCAAGTGTCCACGC-3' Rev: 5'-GGCAAATTCGTCACTCGCCTTCTT-3'
Wnt3a	Fwd: 5'- TGGAACTGTACCACCATAGATGAC-3' Rev: 5'- ACACCAGCCGAGGCGATG-3'
Nestin	Fwd: 5'-AAGTTCCCAGGCTTCTCTTG-3' Rev: 5'-GTCTCAAGGGTATTAGGCAAGG-3'
FoxA2	Fwd: 5'-AAGTATGCTGGGAGCCGTGAAGAT-3' Rev: 5'-CGCGGACATGCTCATGTATGTGTT-3'
Brachyury	Fwd: 5'-AGCTCTCCAACCTATGCGGACAAT-3' Rev: 5'-TGGTACCATTGCTCACAGACCAGA-3'
Pax6	Fwd: 5'-CCCTCACCAACACGTACAG-3' Rev: 5'-TCATAACTCCGCCCATTCAC-3'
Sox17	Fwd: 5'-CGATGAACGCCTTTATGGTG-3' Rev: 5'-TTCTCTGCCAAGGTCAACG-3'
Aire	Fwd: 5' –TGGCAGGTGGGGATGGAA- 3' Rev: 5' -GGAGGGATGGAAGGGGAGGA- 3'

2.9 Adapting to 2i Serum-Free conditions

One million mESCs were plated per 10 cm^2 cell culture plate (0.1% gelatin-coated) and were maintained for two passages using standard culture medium. Thereafter, the cells were collected, pelleted and plated on a monolayer of feeders. They were maintained on feeders for two passages. Once cells reached confluency, they were collected and split onto a new monolayer of feeder cells. The cells were maintained on feeders for another two passages and upon confluency, were removed off feeder cells. Cells were grown without feeders and maintained using standard culturing medium for two passages before being introduced to 2i serum-free media. The cells were carefully monitored and maintained for 4 passages on 2i media, with a 1:5 split each passage. After 4 passages on 2i media, the cells were frozen down, with each vial containing 3-5 million cells.

After 4 passages in 2i media, the cells were fully adapted to 2i conditions, at which point, the concentration of CHIR99021 could be altered depending on experimental needs. qRT-PCR analysis of *AIRE* required two separate 2i conditions, one with 3 µM CHIR99021 and the other with 10 µM CHIR9902. The concentration of PD0325901 remained the same.

2.10 RNA-seq Analysis

The cells were cultured by the Basler lab in LIF + 2i medium and two separate conditions were tested; with/without CHIR99021. BAM files (aligned RNA-seq files) containing data comparing the three cell lines (WT, QKO and TLB) were then provided to us by the Basler lab. The files were uploaded to the open web-based bioinformatics platform, GALAXY (usegalaxy.org). GALAXY was then used to convert BAM files to htseq-files which helped to align features that overlap within the mouse genome file (GRCm38.84.gtf). The files obtained from GALAXY were also used to conduct principle component and clustering analysis (PCA plot).

CHAPTER 3: RESULTS

3.1 Generation of QKO cell line with additional β-catenin knockout (TCF/LEF:β-catenin Knockout; TLB)

The TLB cell line was generated by employing the CRISPR/Cas9 system. sgRNAs were designed to target Exon 4 and Exon 12 of *Ctnnb1* (Figure 3). 16 PKO clones were picked and grown. The clones were validated by performing Western Blots with antibodies detecting whole β-catenin protein, as well as antibodies directed to the N-terminus or C-terminus. The western blot revealed successful β-catenin knockout in all tested clones (Figure 3B)

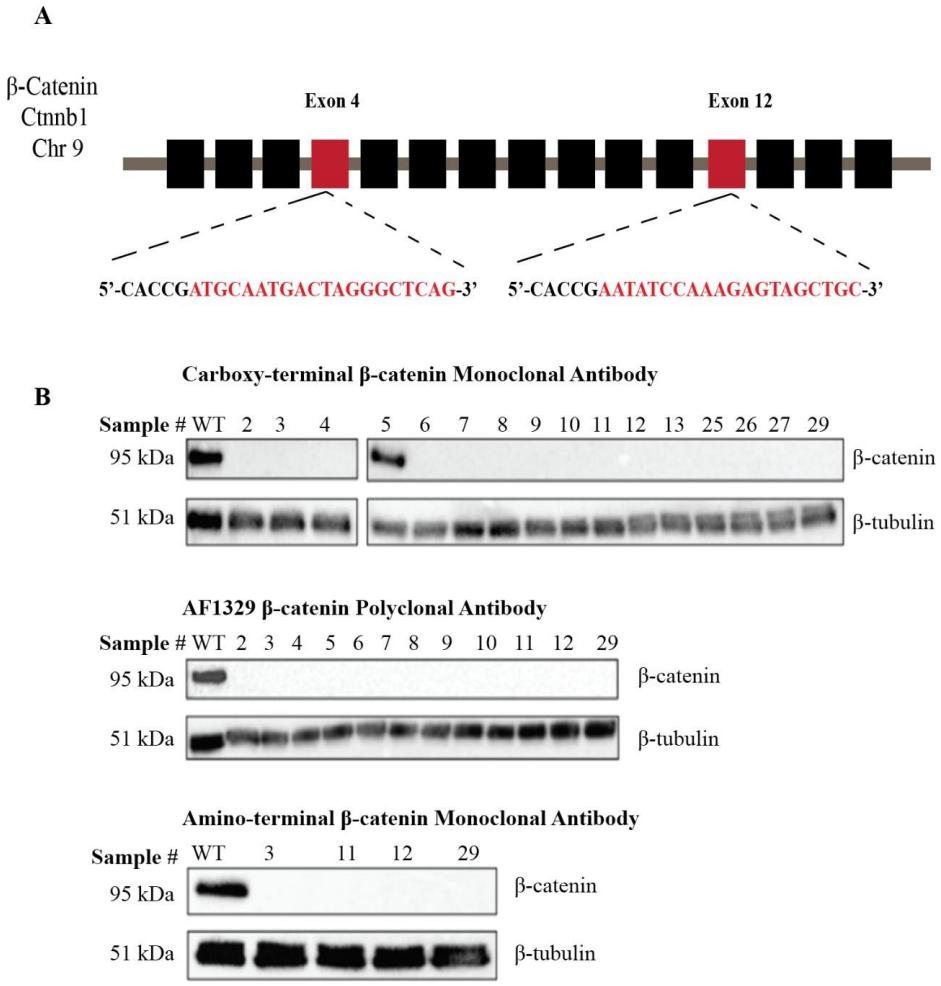

Figure 4: Validation of the β-catenin knockout line.

(A) Schematic overview of CRISPR target site for Exon 4 and Exon 12 in β-catenin shown in red. (B) Western blot validating the 16 clones, probing for the Carboxy-terminal using a monoclonal antibody, the whole β-catenin protein, using a polyclonal antibody, and lastly, the Amino-Terminal using a monoclonal antibody.

3.2 Assessing trilineage differentiation capabilities of TCF/LEF-β-catenin (TLB) knockout cell line by using the embryoid body assay.

Three different embryoid body assays were conducted to test the capabilities of trilineage potential in a cell line lacking TCF/LEF and β-catenin. Embryoid bodies were grown up until day 14 and pictures were taken regularly. The embryoid bodies were also collected on day 7 and day 14 for further analyses by qRT-PCR and Western blotting. By day 6-8, wild type EBs displayed cystic structures and clear signs of fluid accumulation (Figure 4). Wild type EBs were observed to have regions beating in a synchronised manner, with the beating starting anywhere from day 8-10. This was not observed in the other cell lines suggesting a mesodermal blockade. The cystic feature observed in the wild type EBs was not seen in EBs generated with the QKO cell line, as it remained intact as the days progressed, with very limited protrusions observed by day 14. These morphological characteristics were not shared by the TLB cell line as it displayed a clear shedding of cells and complete lack of structural integrity. By day 8, the TLB cell line had started to shed significantly with a central core remaining somewhat intact. By day 14, the TLB cell line seemed to have lost most of its structural integrity with most of the cells floating free around a minimal central core.

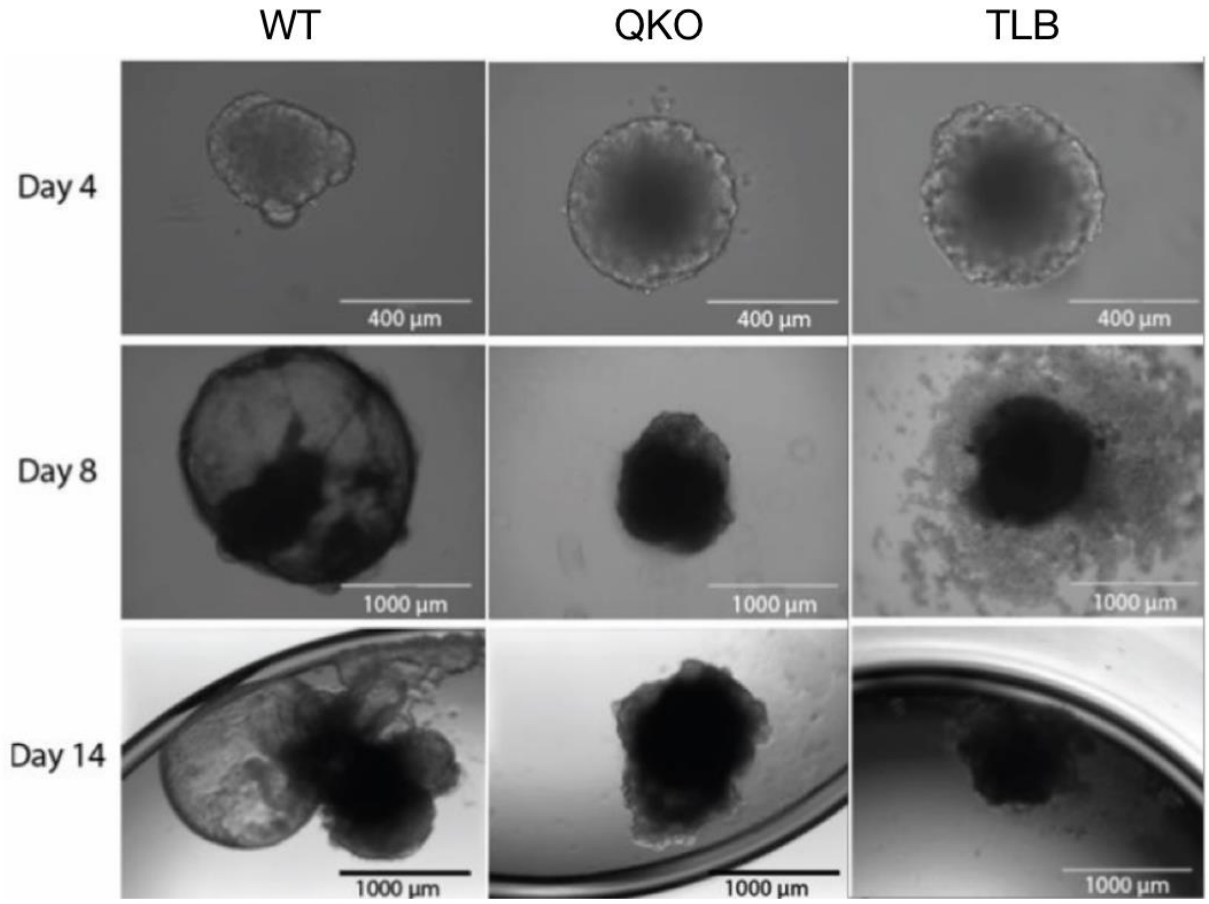

Figure 5: Morphological difference during various time points between different cell types.

Embryoid bodies were derived starting with 800 cells / 30 µL of EB medium. The above pictures were taken at different time points throughout the 14-day period. EBs were collected at time points: day 7 and 14. Cystic features were observed in Wild type cells starting from day 6-8, QKOs retained their core structure whereas TLB EBs started to show clear signs of cell shedding.

3.3 Establishing a β-catenin knockout cell line in a Wildtype and QKO background for the Basler lab

In part for our collaborative project with the Basler group, a working cell line had to be created which lacked β-catenin in a wild type background and one in QKO background. The wild type cells and the QKOs were provided by the Basler group. CRISPR constructs which were previously used to create the TLB cell line for the Doble lab were used and employed to knock β-catenin out (Figure 3A & 5). Polyclonal antibody was used to verify the picked clones, two clones were chosen for the putative wild type- β-catenin knockout (BKO) and putative QKO- β-catenin knockout (PKO). Verified by western blot, the clones were confirmed to have β-catenin knocked out. It is important to note that these wild type and QKO vary from the ones seen in figure 3 and 4 as they are from different parental WT mESCs and were provided by the Basler lab.

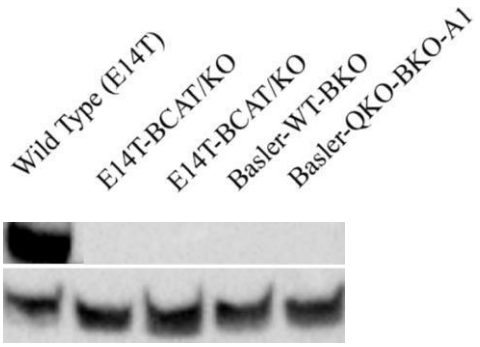

Figure 6: Validation of β-catenin knockout in Basler lab-wild type cells and QKO cells.

Western blot validating the successful knockout of β-catenin, creating two separate cell lines; one with a wild type background with a β-catenin knockout and QKO background with a β-catenin knockout.

3.4 Comparing morphological differences in an embryoid body setting in wild type, QKO, BKO, PKO

Our lab created two cell lines for our collaborative lab, the Basler lab. The two cell lines were the BKO and PKO, wild type with β-catenin knockout and TCF/LEF- β-catenin knockout, respectively. It is important to note that the background cell line for both the labs is E14, but the strains differ. Due to the difference in strain, the Doble lab cell line is being referred to as TLB and the Basler lab cell line is being referred to as PKO. The EB assay was performed by the Basler lab, and with their permission, the results are shown below (Figure 6). Wild type, QKO, PKO cell lines display similar morphological characteristics throughout the 14-day time period. The compact structure is not observed in the BKO cell line, displaying a clear shedding of cells starting somewhere between days 6 and 9.

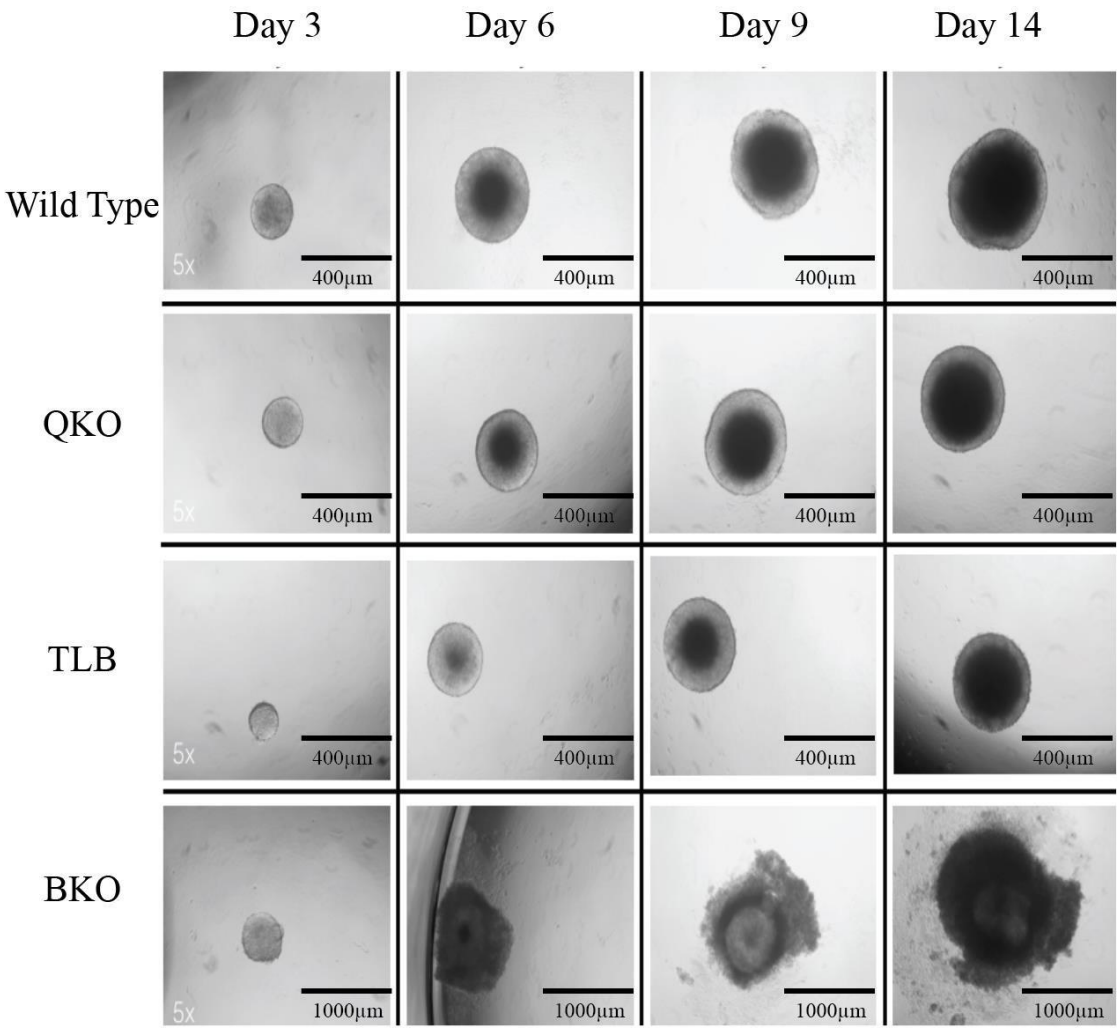

Figure 7: Wild type, QKO, BKO, TLB mESCs were assessed in their ability to differentiate.

Four separate EB assays were performed and were monitored over a 14-day time span. Based on the data, it appears that Wild Type, QKO, and TLB EBs display similar morphological characteristics whereas the BKO EBs display shedding of cells and structural instability.

3.5 Comparing the gene expression profile of *AIRE* in a wild type, QKO and TLB background under 2i conditions.

AIRE was a target gene obtained from the RNA-seq data (RNA-seq performed by Basler lab), where significant expression changes were observed via computational analysis between the three cell lines. All three cell lines were adapted to 2i serum-free conditions and were then subjected to two different CHIR99021 concentrations, 3 μM and 10μM, to mimic Wnt activity. qRT-PCR was performed checking for the expressional changes of *AIRE* depending on cell line and CHIR9902 concentration. Based on the data (Figure 7), it appears that *AIRE* is upregulated with TCF/LEF knocked out (QKO), returning to wild type expression levels with the additional knockout of β-catenin (TLB).

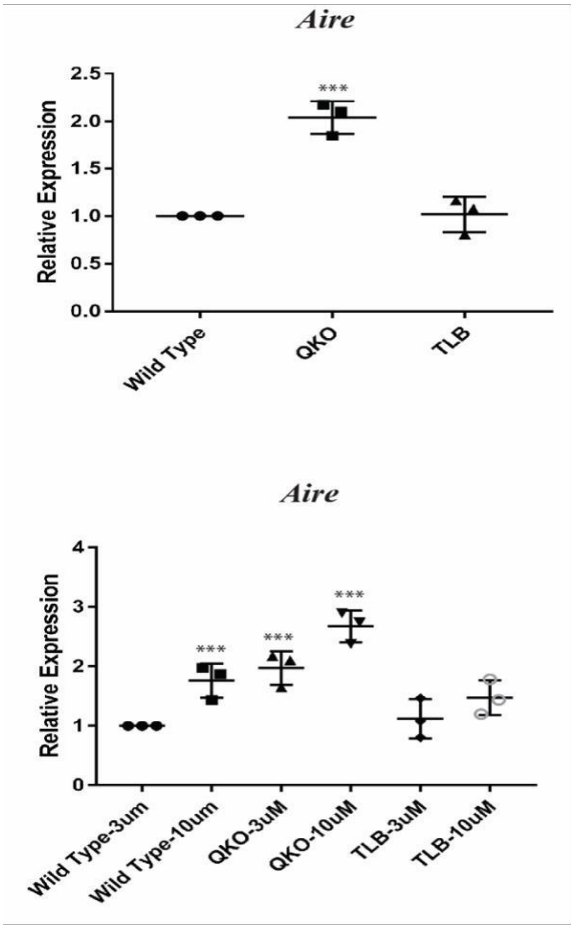

Figure 8: qRT-PCR performed to check for expressional changes of AIRE.

(A). Relative *AIRE* expression based on cell line. All values are relative to wild type expression which has been normalized to a value of 1. Mean ± SEM (n = 3). *** $p < 0.05$. (B). Relative expression of *AIRE* based on cell type and CHIR9902 levels. All values are relative to wild type expression which has been normalized to a value of 1. Mean ± SEM (n = 3). *** $p < 0.05$.

3.6 qRT-PCR assessing tri-lineage differentiation at day 7

EBs from wild type, QKO and TLB were collected on day 7. RNA extraction was performed, and cDNA was transcribed. The germ layer markers being tested were: *Brachyury, FoxA2, Sox17, Axin2, Nestin, Pax6, Wnt3a*. The housekeeping gene used for all qRT-PCR is *RPL13a.*

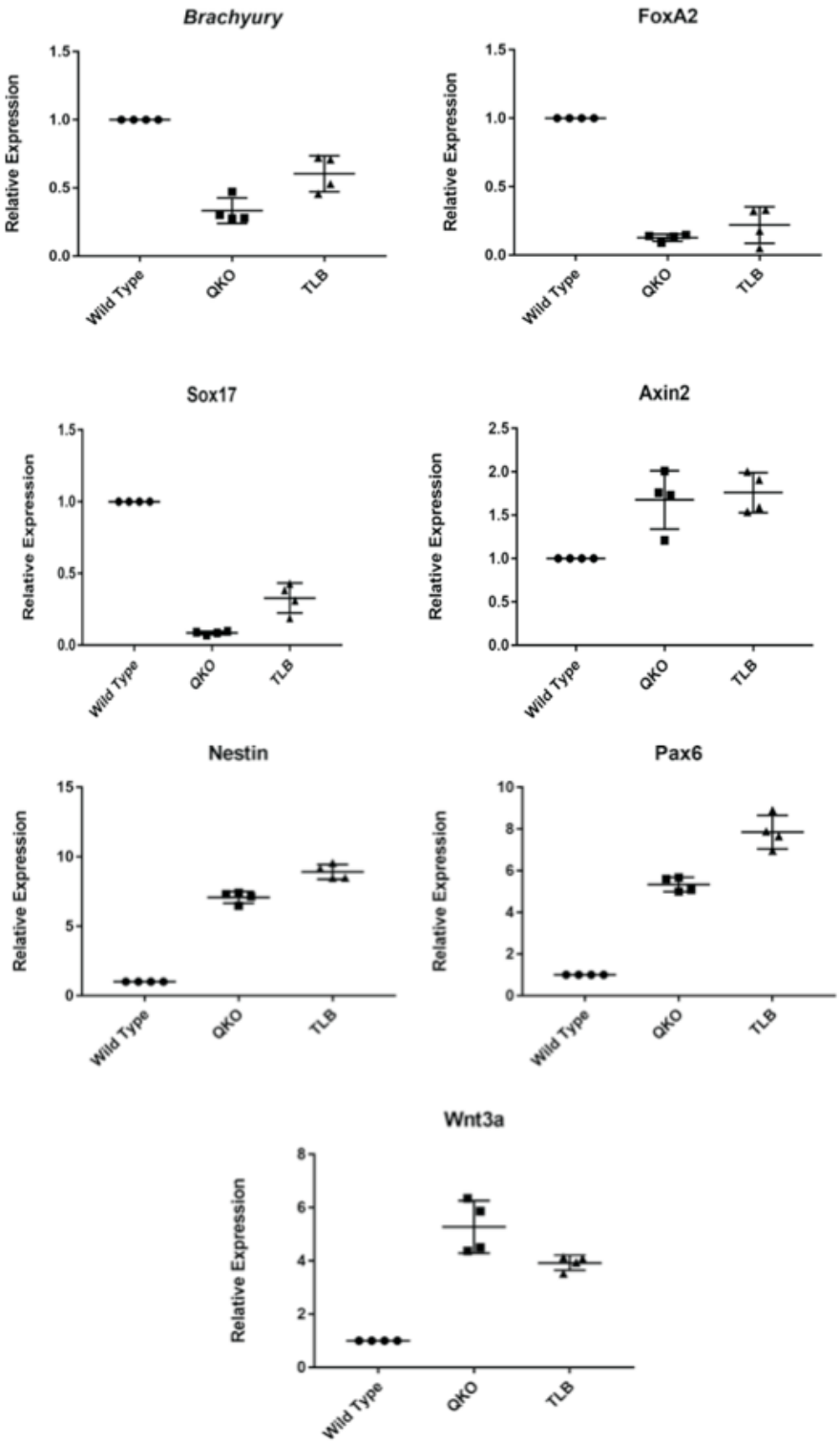

Figure 9: qRT-PCR to assess germ layer lineage potential on day 7.

Three cell lines were tested in their ability to differentiate to all three germ layers on day 7. All values are relative to wild type expression which has been normalized to a value of 1. Mean ± SEM (n = 4). *** $p < 0.05$.

3.7 qRT-PCR assessing tri-lineage differentiation at day 14

EBs from wild type, QKO and TLB mESCs were collected on day 14. RNA extraction was performed, and cDNA was transcribed. The germ layer markers being tested were: Brachyury, FoxA2, Sox17, Axin2, Nestin, Pax6, Wnt3a. The housekeeping gene used for all qRT-PCR experiments was RPL13a.

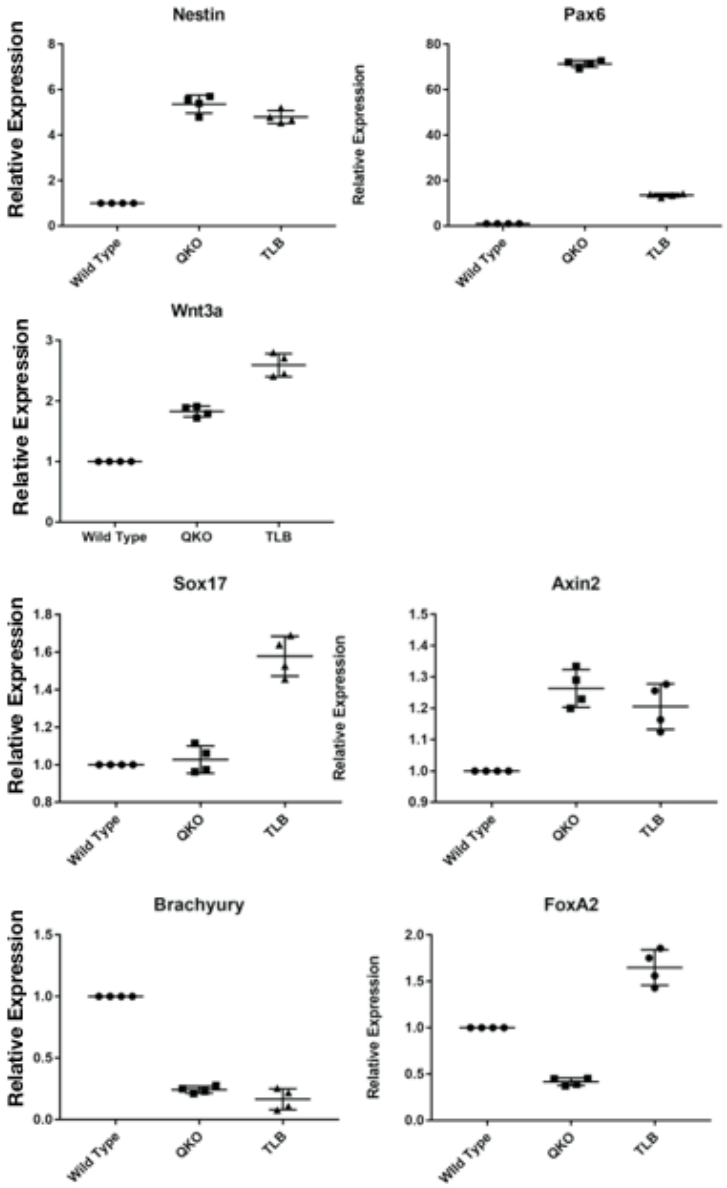

Figure 10: qRT-PCR to assess germ layer lineage potential on day 14

Three cell lines were tested in their ability to differentiate to all three germ layers on day fourteen. All values are relative to wild type expression which has been normalized to a value of one. Mean ± SEM (n = 4). *** $p < 0.05$.

CHAPTER 4: DISCUSSION

4.1 Summary of Findings

In total, three cell lines were created, the TLB cell line (QKO/ β-catenin knockout) with QKO cells from our lab; BKOs (wild type mESCs with β-catenin knocked out) and PKO (QKO mESCs with β-catenin knocked out), the latter two being made in different parental WT mESC cells provided by the Basler lab.

All three cell lines were indistinguishable when grown during cell culturing and behaved like wildtype mESCs. The cell lines were all validated via western blotting as well as PCA plots of gene expression data. PCA plots provided us with a powerful method to analyse complex patterns amongst the biological data sets that were provided. PCA has a unique function where it does not disregard any samples or variables, but rather it considers all the characteristics of a given data set. The clustering of the samples indicates the variability of each given data point and compares it with the other data points. The clustering of multiple points together indicates a strong similarity between those points/data sets (Shlens, 2005). Our data indicated that the three cell lines clustered separately from each other, indicating a clear variability amongst the three and further validating that we had successfully generated three distinct cell lines (See Appendix).

We observed shedding of cells with the knockout of β-catenin in the TLB and the BKO cell line. The shedding can be attributed to the loss of adhesive function in the cells, a role which is carried out by β-catenin. Furthermore, we saw that the TLB and QKO mESCs followed a similar trend in terms of germ layer lineage differentiation, with both showing a clear neuroectodermal bias. The increase in expression levels of *Wnt* responsive genes can be attributed to the "β-catenin-ghost response" (Doumpas et al, 2018).

Additionally, our findings would suggest that AIRE is a potential downstream target of Wnt/β-catenin signalling with levels of AIRE being affected by the loss of β-catenin. Previously published

data, along with our findings would also suggest that there is a mechanism in which AIRE takes part in the pluripotency network either via the interaction of *AIRE* with *Oct4* or CBP

4.2 Aire as a potential member of the pluripotency network

4.2.1 RNA-seq targets

RNA-seq was performed by the Basler lab on wild type, QKO and the TLB cell lines, resulting in a BAM file containing sequence alignment data. The BAM files were uploaded to a web-based platform, USEGALAXY.org. The platform provided us with a list of potential targets which experience a difference in gene expression when compared over the three cell lines (Robinson et al, 2010). In order to shorten the list, criteria were set; the expressional difference had to be at least 2 folds and there had to be some indication in previously published papers regarding a potential gene's involvement with Wnt. Based on these criteria, two targets were chosen; *PLAGL1* and *AIRE*. Furthermore, RNA-seq analysis showed that the data between the RNA-seq conducted with our cell lines and a separate RNA-seq conducted with the Basler lab cell lines were very similar and yielded almost identical results; with the same gene hits showing up. Based on qRT-PCR analysis, it was determined that *PLAGL1* had no significant differences between wild type, QKO and TLB mESCs (See appendix). This was not the case for *AIRE*.

4.2.2 AIRE

The *AIRE* gene consists of 14 exons, encoding a protein with a molecular mass of 57.5 kDA (Org et al, 2008; Kumar et al, 2002). Mutations in the gene result in autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED). The protein is known to have several transcription regulatory domains and has been hypothesized to have a domain largely responsible for acting as a transcriptional coactivator (Kumar et al, 2002; St-Pierre et al, 2015). AIRE has been long thought to be involved solely in the immune system, but studies have shown the presence of AIRE protein in tissues outside the immune system, suggesting an alternative role for AIRE (Matsumoto et al, 2009;

Kumar et al, 2002). Subcellularly, AIRE is shown to be translocated to the nucleus and its interaction with CREB-binding protein (CBP) has been shown (Kumar et al, 2002; St-Pierre et al, 2015; Fierabracci, 2011). Its interaction with CBP, a common coactivator suggests that AIRE plays a regulatory role (Kumar et al, 2002).

4.2.3 Possible inhibitory role of TCF/LEF on AIRE

Based on our data (Figure 7A & 7B), AIRE seems to be upregulated in the QKO cell line, indicating a possible inhibitory role of TCF/LEF on AIRE. Furthermore, in the TLB cell line, the expression levels of AIRE seem to return to wild type levels, suggesting that β- catenin might play a role in the upregulation of AIRE. Additionally, it was observed in the presence of 10 μM CHIR9902, the relative expression in all three cell lines is slightly elevated. CHIR99021, a known WNT/β-catenin pathway activator, increased AIRE expression, suggesting a possible link between WNT/β-catenin signalling pathway and AIRE.

4.2.4 AIRE's involvement in maintaining cell pluripotency

As mentioned before, β-catenin plays a significant role in maintaining a cell's pluripotency. Based on the paper by Gu et al, it appears that AIRE also plays an important role in self- renewal and proliferation. Although an exact mechanism is not yet defined as to how AIRE helps to keep a cell in a pluripotent state, it is thought that AIRE promotes global gene transcription by employing a variety of transcriptional and post-transcriptional methods (Gu et al, 2010). Knockdown studies of AIRE demonstrated a significant decrease in the expression of known pluripotency factors, Oct4 and Nanog (Gu et al, 2010; Lewitzky et al, 2007). Additionally, it was observed that the transcript levels of AIRE decreased significantly as differentiation increased in cells. Together, these findings suggest a clear role for AIRE in pluripotency (Gu et al, 2010).

4.2.5 AIRE's connection with the pluripotency network

Lin28 is a highly conserved RNA binding protein, and it has been linked to regulating the timing of

development in mammalian cells (Viswanathan and Daley, 2010). Lin28 expression has been shown to be linked to AIRE. With an overexpression of AIRE, it was determined that Lin28 expression followed suit, showing a significant increase (Gu et al, 2012). Additionally, as mentioned previously, it was determined that self-renewal capabilities of ES cells were significantly attenuated with a knockdown of AIRE. Similar downregulation was observed for Lin28 in a model of AIRE knockdown, clearly providing evidence of an interaction between AIRE and Lin28 (Gu et al, 2010; Gu et al, 2012' Thornton et al, 2012). Studies have suggested that Lin28 plays an inhibitory role in regulating let-7, a microRNA responsible for promoting differentiation (Reinhart et al, 2000). This was confirmed by overexpression of AIRE, which resulted in a significant downregulation of let-7, confirming a role for AIRE in maintaining a pluripotent state and inhibiting differentiation (Gu et al, 2010; Gu et al, 2012).

As shown previously, the WNT/β-catenin pathway plays a role in regulating AIRE, which has been shown to regulate Lin28 (Figure 7; Gu et al, 2012). Lin28 has also been recently identified as a novel downstream target of WNT/β-catenin, where it was determined that Lin28 is needed for WNT/β-catenin pathway involvement in reprogramming cells to pluripotency (Cai et al, 2013). Furthermore, it was determined that WNT/β-catenin directly regulates the levels of *let-7* by transactivation of Lin28, similarly to AIRE (Cai et al, 2013). Lastly, AIRE has been shown to control the levels of known pluripotency factors, Oct4 and Nanog (Gu et al, 2010; Thomson et al, 2011). Oct4 has also been shown to form a complex with β-catenin, where it has been suggested to play a supportive role in helping maintaining pluripotency (Kelly et al, 2010).

Our data suggests that AIRE is upregulated in the QKO cell line, whereas its levels return to those of WT mESCs with the removal of β-catenin (TLB; Figure 7A). Furthermore, our data also suggests that an increase in WNT activity affects the levels of *AIRE* with an increase in expression seen in all cell lines maintained in a higher concentration of CHIR9902, a known Wnt/β-catenin pathway

activator. This would suggest a possible involvement of AIRE with the WNT/ β-catenin pathway. This would be in accordance with the fact that β-catenin and AIRE both help to maintain pluripotency by targeting Let-7 through the promotion of Lin28. Additionally, β-catenin has been shown to form a complex with Oct4, which is a known pluripotency factor, a factor whose protein levels are directly affected by AIRE. Lastly, β-catenin and AIRE are both known interactors of CBP, further suggesting involvement of AIRE with the WNT/ β-catenin pathway.

In conclusion, our data, along with previously published work, suggest that AIRE plays a pivotal role in maintaining pluripotency. While the exact mechanism is unknown, our data, along with previously published work, suggest a possible involvement of AIRE within the pluripotency network as well as AIRE being a potential downstream target of the WNT/ β-catenin pathway.

4.2.6 The loss of β-catenin results in the loss of structural integrity and shedding of cells

Based on our results (Figure 4), while the wild type EBs start forming cystic structures and fluid filled cavities, this is not the case for the QKO EBs. The QKO EBs were observed to maintain a compact-round structure throughout the 14-day period. These results are consistent with previous EB assays performed in our lab, where similar morphology was observed (Moreira et al, 2017).

The TLB EBs were observed to display a novel characteristic previously unobserved in an EB assay. The TLB line started to display "shedding" of cells where clear cellular debris was observed. Initially, it was thought that the shedding of the cells indicates cellular death with the EBs dying due to the lack of TCF/LEF and β-catenin. We soon determined that this is not the cause, as the TLB cell line is indistinguishable with the wild type morphologically and can be cultured with the same culturing conditions leading to self-renewal.

The more like scenario explaining the shedding of the cells is the knockout of β- catenin. β-catenin is involved in cadherin-catenin complexes, where the armadillo repeats of the β-catenin bind directly to the cadherins, and this binding is needed for the adhesive functionality in cells (Hartsock et al,

2008; Niessen, 2007). The other two cell lines (WT and QKO) possess β-catenin and can form cadherin-catenin complex, thus retaining their ability to form cadherin junctions and retain their overall structure (Hulsken et al, 1994). This would also help to explain why the shedding of the cells is not observed instantaneously and is only observed at day 8 of EB differentiation (Figure 4).

The latent effect can be explained by the presence of Plakoglobin, an adapter protein (also known as γ-catenin), found in adherent junctions (Niessen, 2007). Literature would suggest that Plakoglobin is a close relative of β-catenin and can take the place of β- catenin in the cadherin-catenin complex for a limited time (Niessen, 2007; Shimizu, 2007; Huelsken et al, 2000). The cadherin-catenin complex formed with Plakoglobin is sustainable only for a short period of time as β-catenin is preferred by the cell. The preference for β-catenin is due to the fact that, although Plakoglobin can help form adherens junctions, it is not able to mediate Wnt signalling, and its binding with TCF/LEF, although possible, is not sufficient enough to drive the transcription of WNT target genes (Shimizu, 2007).

Two separate cell lines were made for the Basler lab, the BKO and PKO lines, which were made in different parental WT mESC cells than the ones used in our lab. With their permission, this thesis includes their EB assay (Figure 6). Comparing our data to theirs, there are some obvious differences. Their wildtype EBs do not form any cystic structures and no fluid cavities are observed over a 14-day period, unlike what was seen in our assays (Figure 4). Additionally, the Basler PKO cells look almost indistinguishable from their wildtype and QKO cells. There are striking differences between the BKO cell line and the other three, with the BKO cell line showing clear signs of shedding of cells that is more representative of what we observed with our TLB cell line. With both the BKO cell line and the TLB cell line experiencing the loss of β-catenin, this would provide further evidence that the EBs undergo a latent effect that is due to the loss of β-catenin, where they start to lose their

structural integrity and experience shedding of cells.

The reason for the morphological differences observed between our wild type and TLB cells compared to the Basler lab's wild type and PKO mESCs can potentially be attributed to the assay conditions. We do not yet have the details of their conditions as it was their first time performing the experiment, and they are in the process of repeating it. It is likely that the conditions used to culture the mESCs before being exposed to EB conditions, or the handling of the EBs once plated, contributed to the differing results.

4.3 Germ-layer differentiation in the absence of β-catenin

4.3.1 Neural differentiation bias in QKO mESCs

Based on the previous work done in our lab, it was observed that QKO mESCs are biased towards neural differentiation. This is consistent with the literature, as the lack of TCF/β-catenin results in the inhibition of mesodermal and endodermal lineages. The idea that the lack of TCF/β- catenin signalling results in a bias towards neural differentiation is well noted (Kelly et al, 2007; Moreira et al, 2017). To help determine the involvement of β-catenin in neuroectodermal bias, we created the TLB cell line. Once the cell line was created, we collected EBs at day 7 and 14, and performed qRT-PCR to test for germ layer markers and their expression levels at the different time points (Figures 7 and 8).

4.3.2 TLB differentiation potential

Based on previous published work, the lack of β-catenin results in the inhibition of a mesodermal layer (Huelsken et al, 2000). It was further suggested that Wnt signalling, specifically the involvement of β-catenin is needed for the establishment of anterior-posterior polarity as well as for the formation of the primitive streak. The lack of β-catenin thus results in a differentiation bias towards neuroectoderm (Huelsken et al, 2000). This is consistent with our data, as the qRT-PCR data for the cell line lacking β-catenin shows a clear neuroectoderm bias with ectodermal markers,

Nestin and Pax6, showing a significant increase in relative expression to that of wild type (Figure 7 and 8). This is also evident when looking at other germ layer markers showing a low relative expression for Brachyury, FoxA2 and Sox17.

These trends are consistent for both days 7 and 14, with day 14 varying slightly from that of day 7; with the notable exception being Pax6, which was observed to be expressed very highly by day 14. The differentiation potential tested in the TLB EBs displayed expression trends like that of the QKO cell line, suggesting that the presence of β-catenin is crucial for proper germ layer differentiation; with the lack of β-catenin resulting in a clear neuroectodermal bias. Interestingly, the Wnt-responsive genes, Wnt3a and Axin2 also showed an upregulation in the QKO and the TLB cell lines at both time points. This was unexpected, as one would expect Wnt-responsive targets to be down regulated in the absence of important Wnt signalling factors, TCF/LEF and β-catenin.

Recent work done by Doumpas et al, suggested a mechanism termed "β-catenin-ghost response" (Doumpas et al, 2018). They describe a mechanism whereby an increase in transcriptional activity of β-catenin is observed in the absence of TCF/LEF, with β-catenin targeting a new set of transcriptional targets (Doumpas et al, 2018). This "β-catenin ghost response" would help explain our data, with an increase in Wnt responsive gene expression observed in the absence of TCF/LEFs (Figure 7 and 8).

There is a slight increase in Wnt3a expression observed in the TLB cell line at day 7 compared to that of the QKO line (Figure 7). Surprisingly, the opposite trend was observed when comparing both cell lines at day 14 (Figure 8). It is not yet understood why this occurs. One possible explanation is that in a similar fashion to plakoglobin, another protein acts as a temporary replacement for the lack of the β-catenin to drive transcriptional activity.

Additionally, previously published work has demonstrated the need for β-catenin for neuroectodermal formation (Moreira et al, 2017; Barrow et al, 2003). Surprisingly, this is not

observed in our data, as the lack of β-catenin does not hinder the neuroectodermal bias. The previously proposed mechanism detailed a communicative process which takes place between the Wnt and FGF signalling pathways, with the FGF pathway interacting with β-catenin for the development of the apical ectodermal ridge (AER; Barrow et al, 2003). It was determined that the removal of Wnt3 resulted in the complete disruption of the formation of the AER, whereas the removal of β-catenin resulted in limb defects (Barrow et al, 2003). This would suggest that although crucial for proper formation, β-catenin is not necessarily needed for the formation of the AER. This would help support our observed data and suggests that an alternate Wnt pathway factor is involved in pushing the cell fate towards neuroectoderm in the absence of β-catenin.

4.4 Potential pitfalls and alternative approaches

4.4.1 β-catenin knockout in a wild type background

Unfortunately, due to time constraints, we were unable to perform an EB assay with our wildtype cells with β-catenin knocked out along with the qRT-PCR to test alongside wild type, QKO and TLBs. Conducting an experiment with our own β-catenin knockout line would have provided us with a clear role for β-catenin in a TCF/LEF independent manner.

4.4.2 RNA-seq

We obtained preliminary data from the Basler lab in which we looked at WT, QKO and TLB data +/- CHIR treatment. The cells used for this experiment were maintained in naïve pluripotent conditions (mLIF + 2i) and stimulated with 10 μM CHIR. The cells were kept in these conditions, as naïve conditions allow for a homogeneous expressional profile. Unfortunately, the 2i conditions contains 3μM CHIR and it is possible that even the small levels of CHIR attenuated the overall results and affected the expression profiles. Since then, we have revised and redeveloped a strategy in which we will be removing CHIR from 2i medium for 24 hours before collecting the cells. We have also optimized the conditions and have adapted the cells to 2i conditions lacking mLIF. This

will keep the cells in a pure naïve pluripotent state and will allow for a complete homogeneous gene expression profile.

The Basler lab is currently in the process of re-performing RNA-seq with optimized conditions. It is possible that the results provided to us in the preliminary data are skewed and the gene expression profile is not completely accurate. Keeping that in mind, *AIRE* was one of those targets, and based on our data, there is significant difference in *AIRE* levels in the absence of TCF/LEF.

4.4.3 EB Assay

Cells were treated with MEFs prior to being used for EB assay. By using a feeder layer of MEFs, the cells plated on the MEFs are healthier and "happier". When repeating this experiment in the future, the cells should be adapted to the 2i condition, similarly to the cells that were used in the qRT-PCR experiment. Cells that are cultured in 2i conditions are at their most naïve state, with no cells undergoing differentiation. Since the idea behind an EB assay is to allow the cells to differentiate, by starting the cells off at a naïve state, we can ensure that all the cells are at the same starting point and no other biases are involved.

4.5 Future directions

4.5.1 Moving forward with AIRE

Based on our observed data, *AIRE* is upregulated in the absence of TCF/LEF and downregulated with the removal of β-catenin. It would be interesting to see if any interactions take place between β-catenin and AIRE. A co-immunoprecipitation assay would help to determine if any interactions take place between β-catenin and AIRE, and would identify AIRE as a novel target of β-catenin, which acts in a TCF/LEF independent fashion. Furthermore, it would be interesting to see the result on *AIRE* expression in conditions of β-catenin overexpression. Previously published data suggests that levels of *AIRE* play an important role in regulating the levels of *Pou5f1* (Oct4), which is an important pluripotency factor. It would be interesting to modulate *AIRE* levels to observe, not only

the levels of Oct4 in response, but also the levels of other pluripotency factors; this would have to be checked via qRT-PCR and western blotting, as AIRE has been known to cause some post transcriptional changes. Lastly, it would be interesting to observe changes in *AIRE* levels as the cells start to differentiate in all 3 cell lineages, with literature suggesting that as differentiation progresses, the levels of AIRE decrease as well, it would be interesting to test this in a cell line lacking β-catenin.

4.5.2 Knockout of Plakoglobin and β-catenin

The role of Plakoglobin in the absence of β-catenin is well documented. It would be worthwhile to observe the effect of a Plakoglobin/β-catenin double-knockout. Plakoglobin has been documented to take over the role of β-catenin in its absence, with publications clearly stating that there is competition between β-catenin and Plakoglobin for the binding sites of TCF/LEF (Huelsken et al, 2000). Although, it can take over the role that β-catenin plays in the adhesive function, the thought is that it is not able to take over the transcription role of β-catenin. This previous notion can be challenged on the fact that both β-catenin and Plakoglobin are extremely close family members and based on our data, we do observe Wnt activity in the absence of β-catenin, which could be explained with the Plakoglobin taking over the role of β-catenin for a short time. Creating a cell line which lacks both β- catenin and Plakoglobin would help us truly understand what takes place in the absence of TCF/LEF- β-catenin. Furthermore, it is likely that if an EB assay were performed with such a line, with a severe lack of adhesive functionality in the system, we would see shedding of the cells much earlier (Figure 7).

4.5.3 Moving forward with a β-catenin knockout line

For future directions regarding the importance of β-catenin in determining cell fate determination, it would be crucial to develop EBs with the newly developed β-catenin knockout in a wild type background. Additionally, it would be vital to test for lineage markers using qRT-PCR as well as to

probe for lineage markers using western blotting. Based on the literature, β-catenin serves a crucial role in determining cell fates. Based on that, one should see early signs of lineage differentiation into neuroectoderm with no actual formation of the ectodermal layer.

4.5.4 BioID

In order to find the mechanism of TCF/LEF independent β-catenin signalling on mouse ES cells, it is imperative to find potential binding partners of β-catenin. With the cell lines that we have developed, it would be interesting to screen for β-catenin protein interactions occurring in living cells via BioID (Kim et al, 2016). Knowing the binding partners of β-catenin would help find more TCF/LEF independent factors.

4.6 Concluding remarks

The findings in this thesis help to provide a crucial step forward in understanding TCF/LEF independent Wnt/β-catenin signalling, with the introduction of a novel β-catenin dependent pluripotency factor, AIRE. Together, our data summarizes the possibility of AIRE being involved in the pluripotency network and working alongside β-catenin to inhibit differentiation and promote self-renewal. Additionally, we observed the differentiation potential of a cell line lacking TCF/LEF-β-catenin, with a clear neuroectodermal bias observed. Lastly, the findings of this paper outline a novel phenotype observed in EB assays with cells lacking β-catenin, in which EBs revealed a latent shedding of cells. Overall, the findings of this project provide proof of the importance of looking into TCF/LEF independent Wnt/ β-catenin signalling, the findings of which could lead to the discovery of important therapeutic targets.

CHAPTER 5: REFERENCES

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CHAPTER 6: APPENDIX

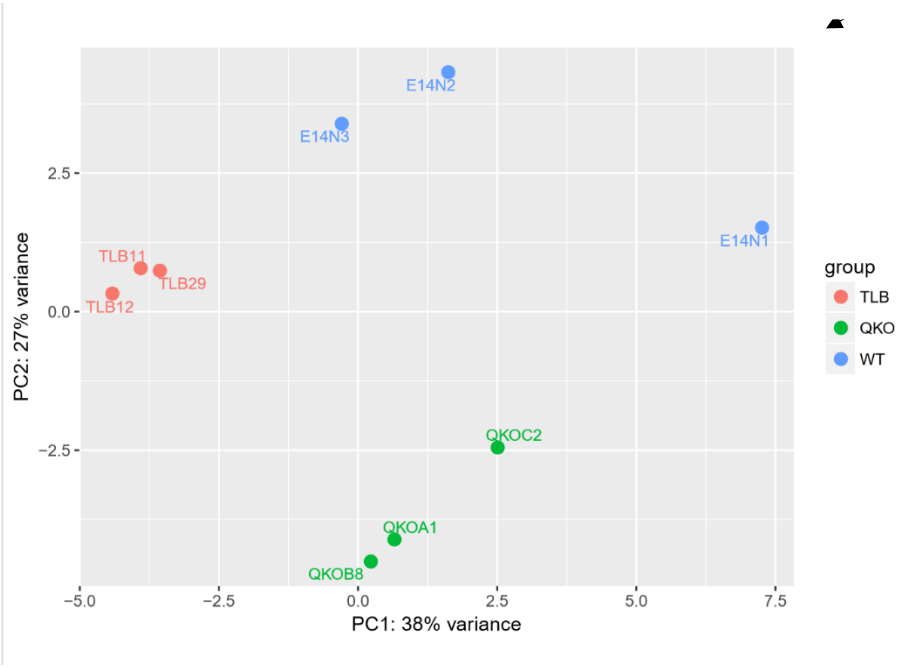

Figure 1: PCA plot showing the clustering of the three cell lines.

This plot was made via UseGalaxy.org with the data provided by the Basler lab. As seen above, the three cell lines are clustering separately.

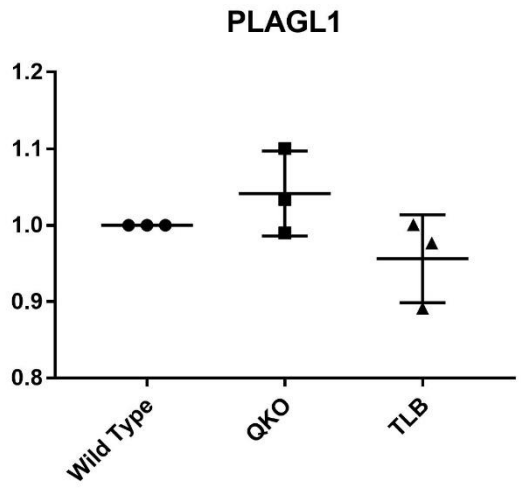

Figure 2: qPCR data displaying expression of *PLAGL1* in cells grown in 2i conditions.

As seen above, the values were all extremely close to that of the wild type levels. With the lowest expression recorded at 0.9, and the highest expression recorded at 1.1.