TCF3 IN ESC SELF-RENEWAL AND DIFFERENTIATION

DELINEATING THE ROLE OF TCF3 IN MOUSE EMBRYONIC STEM CELL SELF-RENEWAL AND DIFFERENTIATION

By DEBORAH NG, B.MSc

A Thesis Submitted to the School of Graduate Studies in Partial Fulfillment of the

Requirements of the Degree Master of Science

McMaster University MASTER OF SCIENCE (2011) Hamilton, Ontario (BIOCHEMISTRY AND BIOMEDICAL SCIENCES)

TITLE: Delineating the Role of TCF3 in Mouse Embryonic Stem Cell Self-Renewal and Differentiation

AUTHOR: Deborah Ng, B.MSc (University of Western Ontario)

SUPERVISOR: Professor B.W. Doble

NUMBER OF PAGES: *xiv*, 85

Abstract

In addition to its roles in developmental and cancer-related processes, emerging evidence suggests that the canonical Wnt pathway is also involved in regulating stem cell self-renewal and differentiation. The ubiquitous serine/threonine kinase, Glycogen Synthase Kinase-3 (GSK-3) is a key regulator of this pathway. Genetic ablation of the two GSK-3 genes α and β (referred to as DKO, for **d**ouble **k**nock**o**ut) in mouse embryonic stem cells (mESCs), renders them incapable of efficiently differentiating into the three germ layers. In an attempt to rescue the differentiation blockade of DKO mESCs, we expressed dominant-negative forms of the TCFs (TCF1, LEF1, TCF3 and TCF4), the final effector molecules of the canonical Wnt pathway. The expression of dominant-negative TCF1, LEF1 or TCF4 was tolerated and resulted in attenuated expression of β-catenin/TCF target gene expression in cells that continued to self-renew and express pluripotency markers. By contrast, the expression of dominant-negative TCF3 resulted in cell differentiation/death. Thus, TCF3 appears to play a unique role in the regulation of mESC properties. To clarify the underlying mechanism of TCF3's action in mESCs, we examined the effects of expressing a dominant-negative TCF3 (TCF3DN) and full-length TCF3 (TCF3FL) in DKO and wild-type mESCs using an inducible doxycycline-regulated system. We found that expression of either TCF3DN or TCF3FL in DKO and wild-type mESCs down-regulates TCF activity and Nanog expression. However, expression of TCF3DN or TCF3FL in DKO mESCs was unable to rescue the neuroectoderm blockade, but may have directed cells to differentiate into the endoderm lineage. Moreover, we have

established a potential role for β-catenin in regulating the expression levels of TCF3 itself. The information obtained from these studies provides new insights into the mechanisms through which TCF3 regulates mESC self-renewal and differentiation.

Acknowledgements

First and foremost, I would like to thank my supervisor Dr. Brad Doble. Your continuous guidance, patience and support have made my grad school experience enjoyable. Not only have you taught me everything I know about stem cells, but you have also helped me become a better student and researcher. Thank you for the hours you spent editing all my reports and making my presentations better. I could not have asked for a better supervisor. I am also grateful for my committee members, Dr. André Bédard and Dr. Jon Draper for their time and helpful comments throughout this whole process.

I am fortunate to have worked with some amazing people in the Doble lab. Firstly, I would like to thank Dr. Kevin Kelly. Thank you for being my mentor for the past 2 years. I could always rely on you to answer my questions, talk about data or eat lunch with. Also, thank you to Sujeivan Mahendram who was always willing to help me with my lab work even if I asked you at the last minute. To the past and current members of the Doble lab, thank you for all your help and support, and more importantly, for all the laughs, usually at Kevin's expense.

I would also like to thank everybody at the Stem Cell and Cancer Research Institute (SCC-RI). Not only am I am grateful for your kinds words, encouragement and friendly smiles, but also for taking the time to teach me about your own work. I especially want to thank Ashley Calder, Branavan Manoranjan and Kyle Salci. Ashley, grad school would

definitely not have been as enjoyable without your friendship. Thank you for always being available to talk and listen even when you had a busy day planned. And to Branavan and Kyle, although I often complained about the two of you, thank you for making the late nights and weekends at the lab fun. Your friendships made me excited to come into work everyday.

Most of all, thank you to my parents who believed in me and supported me throughout this whole process. I couldn't have done it without your help

Table of Contents

Materials and Methods

List of Figures:

List of Tables:

List of Abbreviations

APC	Adenomatous Polyposis Coli
BMP4	Bone Morphogenetic Protein 4
ChIP	Chromatin immunoprecipitation
CK1	Casein Kinase 1
CRD	Context Regulatory Domain
Cys	Cysteine
DKO	Double Knockout (for GSK-3α and GSK-3β, in this thesis)
DMEM	Dulbecco's Modified Eagle Medium
DN	Dominant-Negative
Dvl	Dishevelled
EB	Embryoid Body
EpiSC	Epiblast Stem Cell
ERK	Extracellular-Signal-Regulated Kinases
ESC	Embryonic Stem Cell
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
FL	Full-Length
Fz	Frizzled
GSK-3	Glycogen Synthase Kinase-3
hESC	Human Embryonic Stem Cell
HIPK2	Homeodomain Interacting Protein Kinase 2
HMG	High Mobility Group
ICM	Inner Cell Mass
Id	Inhibitor of Differentiation
iPSC	Induced Pluripotent Stem Cell
JAK	Janus-associated Kinase
LIF	Leukemia Inhibitory Factor
LIFR	Leukemia Inhibitory Factor Receptor
LRP5/6	Low-Density Lipoprotein Receptor-Related Protein 5/6
MAPK	Mitogen-Activated Protein Kinase
mESC	Mouse Embryonic Stem Cell
NLK	Nemo-like Kinase

PBS	Phosphate Buffer Saline
rTTA	Reverse Tetracycline Transactivator
S/T	Serine/Threonine
Ser	Serine
SH2	Src Homology 2
STAT3	Signal Transducer and Activator of Transcription 3
TCF/LEF	T-Cell Factor/Lymphoid Enhancer Factor
TGFβ	Transforming Growth Factor β
TINK	Traf2 and Nck-Interacting Kinase
TLE	Transducin-Like Enhancer of Split
WRE	Wnt Responsive Element

Declaration of Academic Achievement

Deborah Ng created the TCF3 expression vectors, and all of the TCF3 cell lines and performed the majority of the experiments and analyses in this thesis (with some assistance as mentioned below).

Brad Doble provided funding and experimental design.

Sujeivan Mahendram assisted with some qRT-PCRs and TCF reporter assays.

Steve Moreira assisted with some Western blot experiments.

Introduction

Originally described by Till and McCulloch in 1961[1], stem cells, with their unique ability to self-renew while maintaining the potential to differentiate into specialized cell types, have become widely studied due to their promising potential application to regenerative medicine strategies. Stem cells can be classified based on the degree of their "potency". Multipotent stem cells can only give rise to a limited variety of cell types. For instance, haematopoietic stem cells can only differentiate into cells found in the blood system. By contrast, embryonic stem cells (ESCs) are considered to be pluripotent, since they have the potential to differentiate into any cell type of the adult animal. Thus, it is theoretically possible to use human pluripotent cells to generate cells or tissues that could be used to replace or repair damaged or diseased tissues. However, before stem cells can be used in the clinic, a basic understanding of the unique properties of these special cells is critical.

1 Embryonic Stem Cells

ESCs are defined by their unique ability to self-renew, while maintaing their pluripotency, the potential to differentiate into cells from the three germ layers, endoderm, mesoderm and ectoderm[2]. Pluripotency is regulated minimally by a core set of transcription factors comprising Oct4, Sox2 and Nanog[2].

1.1 Oct4/Sox2/Nanog transcriptional network

In ESCs, the pluripotent state is primarily governed by the transcription factors Oct4, Sox2 and Nanog. Knockout mice for each transcription factor display embryonic lethality[3-5]. Oct4, a member of the POU (PIT/OCT/UNC) class of homeodomain transcription factors binds to a 8 base-pair DNA sequence[6]. Maintenance of Oct4 levels in ESCs is critical, as either up- or down-regulation drives the cells out of the pluripotent state[7]. Sox2 is a SRY (Sex determining Region Y) related transcription factor that possesses a high mobility group (HMG) that binds DNA[4]. Sox2-null ESCs are prone to spontaneous differentiation[8]. Oct4 and Sox2 can heterodimerize in ESCs and Sox2 can regulate the expression of Oct4[8]. Nanog is a homeodomain transcription factor that functions as a dimer[9]. Overexpression of Nanog liberates mouse embryonic stem cells (mESCs) from requiring Leukemia inhibitory factor (LIF), a cytokine normally critical for mESC self-renewal[10]. Moreover, Nanog levels are down-regulated during differentiation[10]. Through genome-wide studies aimed at identifying the genes that are bound by these 3 factors, it was determined that Oct4, Sox2, and Nanog can bind each other's promoters and thereby form an interconnected auto-regulatory loop[11]. This suggests that the core factors rely on each other to mediate the transcriptional program that regulates pluripotency in ESCs[2]. These factors appear to target two sets of genes; one set that is actively expressed in ESCs and a second set of genes that is silent but poised for eventual expression when cells undergo differentiation[11]. Many of the

transcriptionally silent genes are bound by the Polycomb group proteins, which silence gene expression via epigenetic modifications[12].

1.2 Mouse embryonic stem cells

1.2.1 Derivation of pluripotent mESCs

In the 1980's mouse embryonic stem cells (mESCs) were derived from the inner cell mass (ICM) of preimplantation embryos at the blastocyst stage of development[13,14]. These cells were derived in culture conditions that employed a layer of fibroblast cells, referred to as "feeder cells", and growth media containing serum. These cells could generate teratomas when injected into mice and could form embryoid bodies when cultured in suspension[13,14].

1.2.2. Assays for assessing mESC function

To test whether an ESC is pluripotent, a number of different assays can be performed. Commonly, ESCs are assayed for their ability to differentiate into the 3 lineages, endoderm, mesoderm and ectoderm. Aggregation of ESCs when grown in suspension causes the formation of embryoid bodies (EBs). EBs are spherical structures that mimic the post-implantation embryo and when formed, they activate a rudimentary developmental program. Over time, EBs can differentiate into cells from the 3 germ layers[15]. When stem cells are injected into a syngeneic mouse, a teratoma is formed[2]. A teratoma is a benign tumour that contains cells from the endoderm, mesoderm and

ectoderm lineages. Finally, embryonic stem cells of the mouse are capable of widespread contribution to the tissues of chimeric animals that arise from "host" blastocysts injected with a small number of ESCs that are surgically implanted into the uterus of a pseudopregnant female mouse and allowed to develop to term[2].

1.3 Pathways involved in the regulation of mESCs

The feeders and fetal bovine serum in which mESCs were originally cultured provide a rich source of nutrients and growth factors for the cells. Several studies have identified the critical components supplied by serum and feeders that serve to maintain mESCs in a pluripotent state, which include leukemia inhibitory factor (LIF) and bone morphogenetic protein (BMP).

1.3.1 LIF/Stat3

Self-renewal and pluripotency of mESCs is dependent on the leukemia inhibitory factor (LIF), a member of the interleukin-6-cytokine family[16]. LIF signals through the gp130 and the LIF receptors (LIFR) which heterodimerize upon binding the LIF ligand. This in turn activates the Janus-associated tyrosine kinases (JAKs). Phosphorylation by JAK on LIFR and gp130 recruits proteins with a Src homology 2 (SH2) domain, such as the transcription factor Signal Transducer and Activator of Transcription 3 (STAT3)[17]. STAT3 is then phosphorylated, which triggers its dimerization. Dimerized STAT3 translocates into the nucleus and activates downstream signalling. STAT3 is believed to play an

important role in regulating mESC self-renewal and differentiation. Overexpression of a dominant-negative version of STAT3 in mESCs induces differentiation[17], whereas expression of an activated form of STAT3, in the absence of LIF, was sufficient for mESCs to remain undifferentiated[18]. Despite the importance of LIF/STAT3 signalling in mESCs, no single target downstream of STAT3 has been identified that is sufficient to replace the effects of LIF[19]. STAT3 can be detected in the regulatory regions of both active and inactive genes[20].

1.3.2 BMP4/SMAD

When mESCs are grown in serum-free conditions, LIF alone is insufficient to maintain pluripotency. mESCs also require Bone Morphogenetic Protein 4 (BMP4) for self-renewal[21]. BMP4 is a member of the bone morphogenetic protein family, which itself is part of the transforming growth factor β (TGF-β) family. BMP4 signals through the type I and type II serine/threonine protein kinase receptors which eventually trigger the phosphorylation of SMAD1 and the subsequent activation of inhibitor of differentiation (Id) proteins. When grown in serum free conditions, but in the presence of LIF, overexpression of Id liberates mESCs from their requirement for BMP4. Id specifically blocks neuronal differentiation, suggesting that LIF and BMP4 act synergistically in order to block differentiation and to maintain pluripotency in mESCs[21]. In addition, BMPs can further promote self-renewal by inhibiting ERK/MAPK signalling[22].

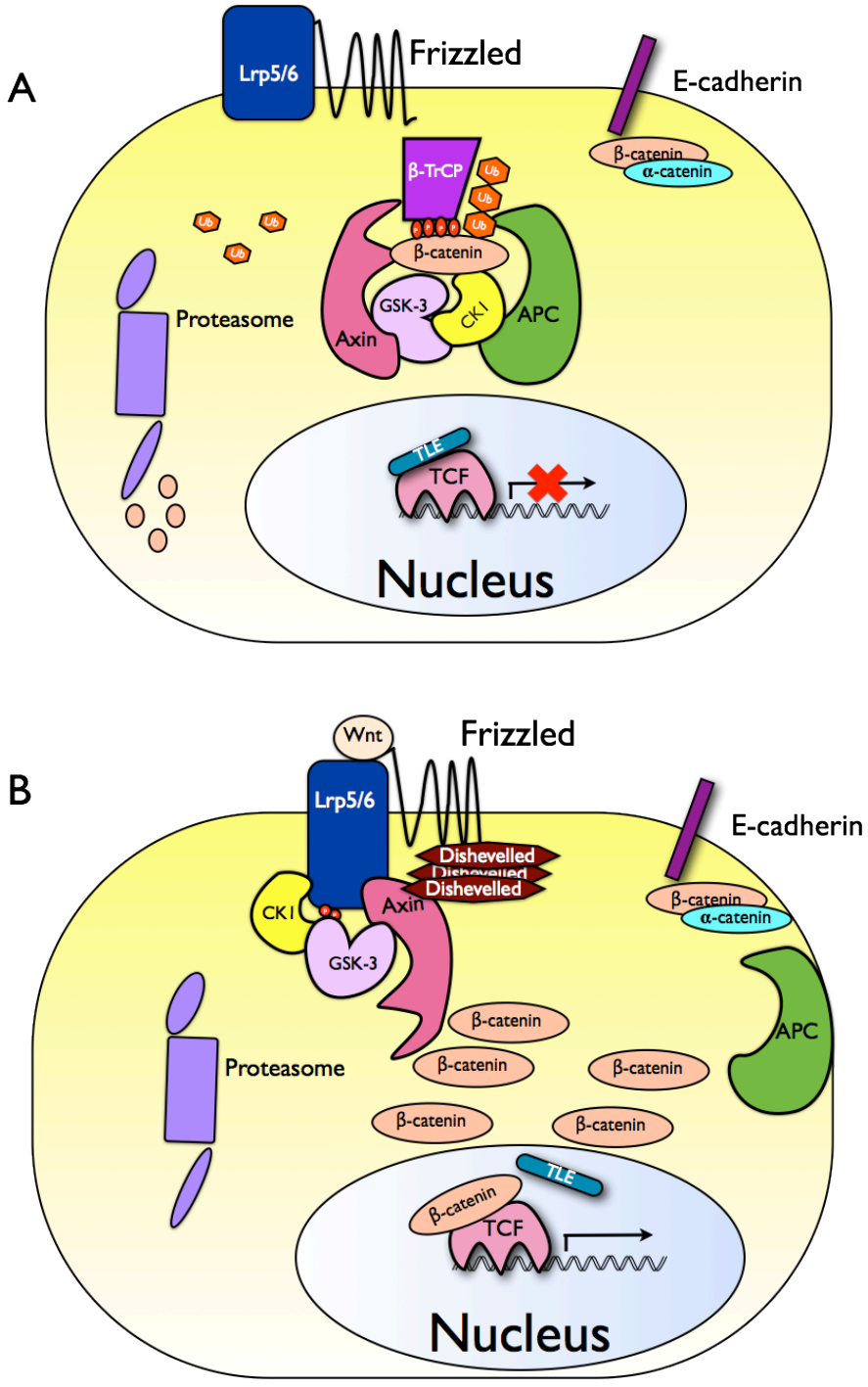


Figure 1. Schematic of canonical Wnt/β-catenin signalling.

The Wnt/β-catenin pathway exists in 2 states, "off" represented in panel A and "on" as depicted in panel B. (A) When the pathway is "off", cytoplasmic β-catenin is held in the β-catenin destruction complex where it is phosphorylated and thereby targeted for proteasomal degradation. The levels of β-catenin in the cytoplasm and nucleus are low and transcription of canonical Wnt target genes is repressed by TCF/LEF factors bound by TLE repressor proteins. (B) Activation of the pathway results in the dissociation of the β-catenin destruction complex, allowing β-catenin to escape phosphorylation and degradation. As a result, β-catenin accumulates in the cytoplasm, resulting in its translocation to the nucleus where it binds TCF/LEF factors to activate transcription of Wnt target genes.

1.3.3 Canonical Wnt/β-catenin

The canonical Wnt/β-catenin pathway has been implicated in many processes including normal development and when dysregulated, cancer. In the absence of Wnt, the cytoplasmic pool of β-catenin is constantly being degraded by the proteasome. However, when the pathway is activated, cytoplasmic β-catenin is stabilized, accumulating in the cytoplasm and subsequently translocating to the nucleus where it binds to the TCF/LEF transcription factors to activate the transcription of Wnt target genes[23] (Fig 1). The details of the Wnt/β-catenin pathway will be discussed below. Wnts have been strongly implicated in mESC self-renewal. Activation of the canonical Wnt pathway can maintain cells in a pluripotent state[24-28].

1.4 Human embryonic stem cells

1.4.1 Derivation of pluripotent hESCs

The first human embryonic stem cells (hESCs) were derived in 1998[29]. Similar to the culture conditions used in the derivation of mESCs, a layer of feeders and serum-containing media was used to propagate hESCs derived from inner cell mass outgrowths from cultured human blastocysts[29]. As with mESCs, hESCs can be maintained in an

undifferentiated state for a prolonged period of time and they can differentiate into all 3 germ layer lineages[29].

1.5 Pathways involved in the regulation of hESCs

Although both mESCs and hESCs were first derived in the presence of fibroblast feeder cells and serum-containing media and although both cell types require the Oct4/Sox2/ Nanog transcription factors to regulate pluripotency[2], the signalling pathways that are important for mESC self-renewal do not appear to be sufficient for hESC self-renewal[30].

1.5.1 FGF2

hESCs were originally derived on mitotically inactivated fibroblasts cells, which secrete a number of different growth factors that are important for hESCs to maintain an undifferentiated state. One such factor is fibroblast growth factor 2 (FGF2), a member of the fibroblast growth factor (FGF) family[30]. Binding of FGF2 to its receptors activates signalling through the extracellular-signal-regulated kinases (ERKs)[30]. Inhibition of this pathway in hESCs causes them to lose their ability to self-renew[31]. It has been shown that the addition of high amounts of FGF2 to hESC cultures mitigates the requirement for feeders[31-34]. The mechanism by which high amounts of FGF2 can retain hESC pluripotency in feeder-free conditions has been linked to the inhibition of the BMP signalling pathway[32,33]. In hESCs, the addition of BMP4 to culture conditions induces cells to undergo differentiation[35].

1.5.2 Activin/Nodal

Activin and Nodal, two growth factors in the TGFβ family, are important in regulating hESC pluripotency. In hESCs, activation of SMAD2/3 by the TGFβ/Activin/Nodal branch of TGFβ signalling is critical to the maintenance of an undifferentiated state[36]. TGFβ antagonists or small molecule inhibitors of TGFβ signalling promote the differentiation of hESCs[36-38]. SMAD2/3 proteins are bound at the Nanog promoter and are thought to play an important role in maintaining hESC pluripotency[39,40]. However, Activin/Nodal alone is insufficient for long term maintenance of hESCs[39,40].

1.6 Overview of the Differences between mESCs and hESCs

Although hESCs and mESCs self-renew indefinitely in culture and can differentiate into the 3 germ layers when prompted, there are clear differences between the two cell types, as suggested in the preceding sections. While mESCs require LIF and BMP4 to regulate self-renewal, these pathways are incapable of maintaining pluripotency in hESCs[41,42]. Similarly, inhibition of ERK signalling in mESCs enhanced self-renewal whereas FGF2 activated ERK signalling is required for hESC self-renewal[43]. One explanation for these differences is that although both human and mouse ESCs are derived from the inner cell mass of blastocysts they may reflect slightly different stages of development[44,45].

1.6.1 Mouse epiblast stem cells

Mouse epiblast stem cells (EpiSCs) are derived from the post-implantation mouse epiblast[46,47]. EpiSCs lines could not be derived in the presence of LIF and BMP4, growth factors important for mESC pluripotency, but could be generated in the presence of FGF and Activin/Nodal. They can differentiate into a variety of cell types when grown as embryoid bodies and can form teratomas comprising tissues from all three germ layers. However, when EpiSCs are injected into pre-implantation embryos, chimaeras cannot be generated. Although EpiSCs express Nanog and Oct4, the global gene expression profiles of EpiSCs and mESCs are quite different. Inhibition of the Activin/Nodal pathway promotes differentiation of EpiSCs and hESCs but not mESCs[46,47]. It has been proposed that mESCs and EpiSCs represent two different states in development, in which different types of pluripotent stem cells exist; so called naïve and primed pluripotent state[44,45]. The naïve LIF-pluripotent state is thought to reflect conditions within the preimplantation ICM, whereas the primed FGF-pluripotent state is more similar to the post implantation epiblast[44,45].

1.6.2 mESC-like human pluripotent stem cells

Both hESCs and EpiSCs differentiate when the Activin/Nodal or FGF2 signalling pathway is inhibited, whereas mESCs remain pluripotent under the same conditions[48]. The similarities between hESCs and EpiSCs suggest that hESCs are in a primed pluripotent state and reflect a different point in development compared to mESCs.

Recently, there have been an number of studies examining whether human pluripotent stem cells can be altered so that they revert to a naïve state. Using induced pluripotent stem cell (iPSC) technology, where introduction of reprogramming factors into a somatic cell can reprogram it to an embryonic stem cell-like state[48], human fibroblast cells could be reprogrammed to a mESC-like state[49,50]. In the presence of LIF, human fibroblast cells were reprogrammed to iPSCs that displayed the properties mESCs. However, these cells were "metastable", as removal of ectopic reprogramming factors reverted the cells to a hESC- like state[49]. Hanna *et al.* also generated mESC-like human pluripotent stem cells from human fibroblast cells by reprogramming them in serum-free, LIF-supplemented conditions in the presence of ERK and GSK-3 inhibitors. Female mouse- like hiPSCs have two active X chromosomes, a property observed with mESCs but not hESCs and EpiSCs, suggesting that their epigenetic status has been "reset" to a naïve pluripotent state[50].

2 Wnt/β-catenin Signal Transduction Pathway

The name Wnt was derived from an amalgamation between wingless (wg) a *Drosophila* gene and Int-1, a mouse gene. In *Drosophila,* mutation of Wg results in a *Drosophila* mutant lacking wings. The mouse homologue of Wg Int-1, was activated in breast tumours of mice infected with the mouse mammary tumour virus.

2.1 Components of the Wnt/β-catenin signalling pathway

2.1.1 Ligands

Wnts, which are lipid-modified glycoproteins, are conserved in all metazoans and play a fundamental role in regulating many developmental processes[23]. There are 19 Wnts in mammals. All Wnts have an N-terminal signal sequence, a conserved region of cysteine residues and several N-glycosylation sites[51]. Wnt3a mutants that cannot undergo glycosylation are impaired in their ability to be secreted by the cell[52,53]. Wnts also undergo lipid modifications on cysteine (Cys) 77 and serine (Ser) 209[51]. Mutation of the cysteine residue on Wnt3a impaired its ability to activate β-catenin signalling[52,53], whereas mutation on serine 209 blocked its secretion into the cell culture media[54]. The acyltransferase Porcupine, may catalyze the addition of acyl groups to both Cys77 and Ser209[51].

2.1.2 Receptors

Activation of the Wnt pathway requires two receptors, Frizzled (Fz), a seven-pass transmembrane receptor and a co-receptor, low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6), which is a single-pass transmembrane[23]. There are 10 Fz genes in the mammalian genome and there is likely functional redundancy among Fz members. All Fz receptors have a cysteine-rich region in the extracellular domain, which Wnt ligands bind to directly[55]. The receptors LRP5/6 are a critical component of the canonical Wnt pathway. Although both LRP5 and LRP6 are ubiquitously co-expressed and share a 70%

amino acid sequence identity, knockout mice studies have revealed that ablation of LRP6 results in more defects than ablation of LRP5[56]. Furthermore, evidence suggests that LRP6 is the more potent of the two receptors in canonical Wnt signalling as overexpression of LRP6 exhibits stronger activity than overexpression of LRP5 in both *Xenopus* and mammalian cells[57,58].

2.1.3 Intracellular signalling components

2.1.3.1 β-catenin

β-catenin was initially identified in *Drosophila,* in which it is referred to as armadillo. The name stems from the observation that mutation of this gene in *Drosophila* results in an armadillo-like larval phenotype[59]. β-catenin has two main roles in cells: to act as a signalling molecule in the canonical Wnt pathway and to act as a structural adaptor protein linking cadherins at the plasma membrane to the actin cytoskeleton[60]. Knockout of β-catenin in mice results in embryonic lethality[61,62].

2.1.3.2 Casein Kinase 1

The protein kinase, Casein Kinase 1 (CK1) is a serine/threonine protein kinase implicated in a number of different processes including cell division and DNA repair. CK1 can phosphorylate a number of proteins involved in canonical Wnt signalling including β-catenin, Dishevelled, TCF/LEF factors, LRP5/6 and APC[63]. Interestingly, CK1 acts a positive and negative regulator in canonical Wnt signalling. It acts as a negative regulator

when it phosphorylates β-catenin marking it for proteasomal degradation. However, upon Wnt activation, it synergizes with GSK-3 to phosphorylate LRP5/6 to promote downstream canonical Wnt signalling[63].

2.1.3.3 Glycogen Synthase Kinase 3

The ubiquitous serine/threonine kinase, glycogen synthase kinase-3 (GSK-3) has many substrates in cells. GSK-3 prefers to phosphorylate serine/threonine residues that have been pre-phosphorylated by a priming kinase, four residues downstream of the GSK-3 target site. The GSK-3 consensus recognition site is **S/T**-X-X-X-pS/pT (S=Serine; T=Threnonine, X= any amino acid; p=phosohorylation) GSK-3's target is the bolded S/T, whereas the C-terminal pS/pT is the target of a priming kinase[64]. There are two GSK-3 homologues in mammalian cells, GSK-3α and GSK-3β. They are encoded on separate genes, and share an almost identical kinase domain, but differ at their N and C-termini[65]. In mESCs, there is a functional redundancy between GSK-3α and GSK-3β with respect to Wnt signalling[66], but the phenotypes of GSK-3α and GSK-3β knockout mice are quite different. Mice lacking GSK-3α are viable whereas GSK-3β knockout mice die prior to or immediately after birth[67,68].

2.1.3.4 Dishevelled

Dishevelled (Dvl) acts as a scaffolding protein and serves as a link between Fz and the downstream components of the Wnt pathway[69].

2.1.3.5 Adenomatous Polyposis Coli

Familial adenomatous polyposis, a subtype of colon cancer in which numerous polyps form in the colon and rectum, is caused by truncations in the Adenomatous polyposis coli (APC) protein. APC acts as a negative regulator of β-catenin mediated TCF signalling. Its link to the canonical Wnt pathway was first identified when it was found that it could bind β-catenin[70,71]. In APC$^{-/-}$ colon carcinoma cell lines, a stable β-catenin/TCF4 complex was shown to constitutively activate TCF activity, and this activity was abrogated when APC was re-introduced[72].

2.1.3.6 Axin

Axin, a negative regulator in the Wnt/β-catenin signalling pathway, also acts a scaffolding protein. Axin can interact with β-catenin, APC and GSK-3. Overexpression of Axin results in the degradation of β-catenin[73].

2.1.4 The β-catenin destruction complex

When the canonical Wnt pathway is not active, the cytoplasmic pool of β-catenin is held in a complex called the β-catenin destruction complex. Along with β-catenin, the complex contains the scaffolding proteins APC and Axin, and the kinases GSK-3 and CK1[74]. When bound in this complex, β-catenin is phosphorylated by CK1 at serine 45[75,76]. This phosphorylation event primes β-catenin to be phosphorylated by GSK-3 on serines 33, 37 and threonine 41[77]. These phosphorylation events are required for β-catenin to be

ubiquinated by an E3 ubiquitin ligase, β-TRCP, and subsequently degraded by the proteasome[78,79]. The cytoplasmic pool of β-catenin molecules is kept at low levels which preclude its nuclear translocation and activation of Wnt target genes. GSK-3 can also phosphorylate APC and Axin. These phosphorylation events increases their binding affinities for β-catenin[80,81].

2.2 Activation of the canonical Wnt/β-catenin pathway

Once the canonical Wnt pathway is activated, the cytoplasmic pool of β-catenin increases to high levels that are conducive to the nuclear translocation of signalling- competent β-catenin.

2.2.1 The LRP signalosome

The binding of Wnt ligands to the Fz/LRP receptors results in the aggregation of Axin, Dvl and GSK-3 with the Fz/LRP receptors to form what is termed the LRP-signalosome[82]. Dvl multimerizes adjacent to the intracellular domain of Fzs to form a scaffolding platform that binds proteins including the Axin-GSK-3 complex[83]. LRP5/6 is sequentially phosphorylated on a PPP(S/T)Px(S/T) (P, proline; S/T, serine/threonine; x, a variable residue) motif by GSK-3 and CKI[84]. There are 5 PPP(S/T)Px(S/T) motifs in both LRP5 and LRP6 which act synergistically, as mutations to even two of the motifs renders it inactive[85]. Phosphorylation of this motif provides a docking site for Axin[86]. GSK-3 is tethered to the membrane by Dvl, whereas CK1 is recruited to the membrane via the

transmembrane protein 198 (TMEM198), which specifically interacts with LRP6[87]. A proposed model of Wnt signal amplification is that after the initial phosphorylation of LRP5/6 by the Dvl bound GSK-3, the phosphorylated LRP5/6 recruits additional Axin-GSK-3 complexes to promote further phosphorylation on LRP5/6, thus stabilizing and sustaining the signal[83]. In addition, recruitment of GSK-3 to the membrane may funcution to inhibit GSK-3's phosphorylation of β-catenin allowing it to escape degradation[88-90].

2.2.2 Inactivation of GSK-3 via multi-vesicular endosomes

Recently, Taelman *et al.* proposed a new mechanism for GSK-3 inhibition in Wnt signalling, where upon Wnt stimulation, GSK-3 is sequestered in multi-vesicular endosomes[91]. When GSK-3 is contained in these vesicles, it is separated from its cytosolic substrates. After Wnt signalling initiation, β-catenin is initially translocated into the multi-vesicular endosomes along with GSK-3. However, as the pool of GSK-3 is depleted from the cytosol, newly synthesized β-catenin is no longer phosphorylated and is thus allowed to accumulate. Taelman *et al*. proposed that upon Wnt stimulation, the LRP5/6 signalosome is formed at the plasma membrane, but GSK-3 is eventually translocated into the vesicles allowing the Wnt signalling cascade to be propagated[91]. Wnt ligands and LRP6 have been shown to be internalized by endocytic trafficking and interfering with these events blocked β-catenin accumulation[92,93].

2.2.3 Regulation of β-catenin import into the nucleus

The mechanism in which β-catenin is shuttled to the nucleus is not well understood. β-catenin has no nuclear localization signal, and does not rely on importins or RanGTP[94,95] but instead, it can interact directly with nuclear pore proteins[94]. The C-terminus of β-catenin is important for localization as C-terminal deletions block its nuclear import[96]. A protein that has been implicated in the nuclear importation of β-catenin is Rac1, a member of the Rho family of small GTPases[97]. Up-regulation of the canonical Wnt pathway activates Rac1, which in turn triggers phosphorylation of β-catenin on serines 191 and 605 by the kinase JNK2, thereby increasing the nuclear translocation of β-catenin[97]. Recently, it was found that β-catenin interacts with the transcription factor forkhead box M1 (FoxM1)[98]. The FoxM1/β-catenin interaction is important for nuclear import of β-catenin as no nuclear translocation was observed in FoxM1$^{(-/-)}$ cells[98]. Retention of β-catenin in the nucleus is maintained by B cell lymphoma 9 (BCL9) and Pygopus, as both proteins reduce the rate at which β-catenin undergoes nucleo-cytoplasmic shuttling[99].

2.3 The TCF/LEF family of transcription factors

The final effector molecules of the canonical Wnt pathway are the T-Cell Factor/ Lymphoid Enhancer Factor (TCF/LEF) transcription factors. In vertebrates, there are four distinct TCF/LEF factors; TCF1, TCF3, TCF4 and LEF1[100]. While TCF3 mainly behaves

as a repressor and LEF1 as an activator, TCF4 and TCF1 can act as both an activator and repressor depending on the context[101].

LEF1 was identified in a screen looking for transcriptional regulators that were bound to an enhancer region controlling the T-cell receptor α gene[102]. TCF1 was discovered in a screen identifying transcription factors bound to the CD3-ε enhancer in T lymphocytes[103]. In an attempt to identify other proteins that contain the HMG box found in TCF1, TCF3 and TCF4 were discovered[104]. All TCFs have regions of high sequence identity, which include an N-terminal β-catenin binding domain, a high mobility group (HMG) DNA-binding domain and a nuclear localization signal (NLS). There are also regions of low similarity such as a context-dependent regulatory domain (CRD) and an extension that is found on some TCFs, known as an E-tail[105] (Fig 2).

A number of different isoforms of the TCF factors have also been identified. These isoforms are generated by alternative splicing and dual promoter usage[101]. For instance, both TCF1 and TCF4 undergo extensive alternative splicing to generate a number of different isoforms[106,107]. Furthermore, TCF4 isoforms with LVPQ and SXXSS motifs in the CRD act as repressors[108]. Interestingly, these motifs are always found in TCF3 and are essential for TCF3's repressive role[101,109]. Isoforms of TCF3 have also been identified in mESCs[110]. Another class of TCF/LEF isoforms are those that lack the β-catenin binding domain. These isoforms repress the activation of canonical Wnt target genes[111-113]. Both

LEF1 and TCF1 have two promoters. Initiation of transcription from the second promoter results in a TCF1/LEF1 isoform that lacks a β-catenin binding domain. Interestingly, in colon cancer, expression of the truncated TCF1/LEF1 is down-regulated while expression of full-length TCF1/LEF1 predominates[114,115].

Figure 2. The TCF/LEF transcription factors.
Schematic representation of the different domains found in TCF/LEF transcription factors. All TCF/LEF transcription factors have regions of high sequence identity, which include an N-terminal β-catenin binding domain, a high mobility group (HMG) DNA binding domain and a nuclear localization signal (NLS). However, there are also regions of low similarity such as a context-dependent regulatory domain (CRD) and an extension that is found on some TCFs, known as an E-tail.

2.3.1 Biological function of TCF/LEF factors

2.3.1.1 Target gene recognition

Regulation of Wnt target genes is achieved through the binding of TCF/LEF factors to Wnt responsive elements (WRE). TCF/LEF factors bind to the consensus sequence CCTTTGWW (W=A/T) via their HMG box domains. The HMG box binds to the minor groove of the DNA, which results in a sharp bend in the DNA[116,117]. For instance, binding by the HMG box of LEF1 kinks the DNA by 130°[116]. Structural analysis of the LEF1/ DNA complex revealed that LEF1 caused the DNA to bend severely towards the major groove and away from the bound protein[118]. This dramatic change in DNA conformation facilitates the recruitment of co-activators and co-repressors. Despite the fact that the HMG box is highly conserved between all four TCF/LEFs, not all factors have equivalent

affinities for the same Wnt target gene[119]. This phenomenon may be explained by the inclusion of the C-clamp at the C-terminus of certain TCF/LEF factors. A number of alternatively spliced isoforms of TCF1 and TCF4 have a C-clamp[119-121]. The C-clamp is a 30 amino acid cysteine-rich region adjacent to the HMG box that prefers a short GC-rich motif downstream of the WRE. Primary biding of the TCF factors occurs through the HMG box but auxiliary binding occurs via the C-clamp. A proposed function of the C-clamp may be to enhance the target gene recognition for Wnt target genes with suboptimal WREs[121]. In *Drosophila* it was discovered that TCF factors can recognize a unique site, AGAWAW. Intriguingly, binding of TCFs to this site appears to direct β-catenin to repress gene transcription, which is in contrast to its usual role in activating Wnt target genes[122].

2.3.1.2 Regulation of TCF/LEF factors

TCF/LEF factors have no inherent transactivation activity and rely on other proteins to help regulate their activity. Activation of the canonical Wnt pathway results in the translocation of β-catenin to the nucleus where it binds TCF/LEF factors[111,112,123]. β-catenin, a member of the Armadillo repeat protein superfamily features a central stretch of 12 Armadillo repeats. It is these Armadillo repeats that interact with the N-terminus of TCF/LEF factors. There are two lysine residues in β-catenin that are important for its interaction with TCF/LEFs, as mutation of either of these amino acids greatly attenuates the ability of β-catenin to associate with TCF/LEF factors[124]. The β-catenin/TCF complex

is dynamic as β-catenin's binding to the WRE has been shown to oscillate between on and off states[125,126].

When the Wnt pathway is inactive, repression of Wnt target genes is mediated by transducin-like enhancers (TLEs). Referred to as Groucho in *Drosophila,* Groucho/TLEs are transcriptional repressors that do not interact with the DNA directly, but are recruited to target gene promoters via transcription factors[127]. The interaction between Groucho and TCF factors was first identified in *Drosophila*[128,129]. It was subsequently found that in mammalian cells, all TCF/LEFs factors interact with TLEs[130]. TLEs interact with TCF/ LEF factors in the CRD and HMG box, and the repression is dependent on histone deacetylases[131]. How β-catenin and TLEs co-ordinate the regulation of Wnt target genes is not well understood. It has been proposed that β-catenin can bind to additional sites in the CRD which displaces TLEs from the TCF/LEF factor[132].

Additional methods of regulation occur through post-translational modifications. At the N-terminus, phosphorylation of LEF1 by casein kinase 2 (CK2) reduces its affinity for TLEs and promotes Wnt target gene activation[126]. Phosphorylation by CK1 has dual effects as it promotes the formation of the TCF3/β-catenin complex but disrupts the LEF1/β-catenin complex[133,134]. Whether these phosphorylation events occur in the same context is unknown. The CRD of TCF/LEF factors can be phosphorylated by Homeodomain Interacting Protein Kinase 2 (HIPK2), Nemo-like Kinase (NLK) and Traf2

and Nck-Interacting Kinase (TNIK)[101]. Whether these phosphorylation events occur simultaneously or concurrently is unknown. Upon Wnt stimulation, HIPK2 phosphorylates TCF3, TCF4 and LEF1. This results in the dissociation of the TCF protein from a target promoter. Dissociation of TCF3 from the promoter results in transcriptional activation while dissociation of LEF1 leads to transcriptional repression[135,136]. NLK phosphorylation on LEF1 and TCF4 down-regulates TCF/LEF transcription activity by preventing the formation of the β-catenin/TCF complex[137,138]. Phosphorylation by TNIK on TCF4 leads to transcriptional activation[139].

3 Wnt/β-catenin Pathway in mESCs

3.1 Activation of Wnt/β-catenin in mESCs

3.1.1 mESCs with mutant APC

Mutations of APC in mESCs alters its ability to regulate β-catenin levels. mESCs harboring APC mutants that result in highly elevated Wnt/β-catenin signalling, display severe differentiation defects. The severity of the impaired mESC differentiation was shown to correlate with the level of β-catenin signalling[140].

3.1.2 Inhibition/Ablation of GSK-3 in mESCs

Inhibition of GSK-3 with a small molecule, BIO, enhanced self-renewal of both mESCs and hESCs[141]. These studies are controversial since long-term analyses of self-renewal were not performed. Furthermore, it was later observed that in hESCs, activation of the

Wnt pathway actually enhances differentiation[142]. However, when grown in a fully defined serum-free media, the addition of the GSK-3 inhibitor CHIR-99021, and MAPK inhibitors SU5402 and PD184352, allowed for self-renewal of pluripotent mESCs[143]. In addition, ablation of both GSK-3 genes (GSK-3α and GSK-3β) in mESCs, enhances the self-renewal and pluripotency of mESCs[66]. When assayed for their differentiation capabilities, these GSK-3 double knockout cells (DKO) were unable to differentiate into the neuronal lineage. Still, when a dominant-negative version of TCF4 or TCF1 was introduced into the DKO cells, the cells remained blocked with respect to their neuroectoderm differentiation despite attenuation of hyperactive β-catenin signalling[144].

3.1.3 β-catenin's role in regulating mESCs properties

Expression of stabilized versions of β-catenin in mESCs enhances the ability of the cells to retain an undifferentiated phenotype even when LIF is withdrawn from culture conditions[25,145]. To better understand β-catenin's role in regulating pluripotency, various groups have made β-catenin deficient mESCs[27,146-148]. The effects of ablating β-catenin on stemness have been somewhat difficult to conclude as these studies have yielded inconsistent results. While some groups observed that β-catenin knockout mESCs have an altered gene expression profile[27,146], other groups have not[147,148]. Anton *et al.* found that their β-catenin$^{(-/-)}$ cells have a dramatic decrease in Rex-1 levels and an up-regulation of FGF5, suggesting that their cells were reminiscent of an epiblast stem cell[27]. Furthermore, when Wagner *et al.* assayed for the differentiation capabilities of their β-catenin deficient

cells through teratoma formation, they were unable to form tumours[146]. Wray *et al.* found that if β-catenin-deficient cells are grown in serum-free conditions, they require LIF and a MAP kinase inhibitor but not a GSK-3 inhibitor to retain stemness, suggesting that the effects of inhibiting GSK-3 in mESCs is mediated through β-catenin[148]. Finally, Lyashenko *et al* observed that ablation of β-catenin in mESCs blocked mesendoderm and neuronal differentiation[147].

C-terminal truncations of β-catenin abolish its ability to activate TCF/LEF-mediated transcription[113]. When either wild-type or C-terminally truncated β-catenin was expressed in β-catenin$^{(-/-)}$ mESCs treated with a GSK-3 inhibitor, the cells remained pluripotent[148]. Similarly, expression of either a stabilized version of β-catenin or a C-terminal truncation of stabilized β-catenin in wild-type mESCs enhanced self-renewal and delayed differentiation[144]. This suggests that β-catenin's role in regulating mESCs may be independent of TCF/LEF activity.

3.1.4 Activation of Wnt/β-catenin signalling promotes mesendoderm differentiation and blocks neuronal differentiation

In mESCs and hESCs, activation of the Wnt pathway or inhibition of GSK-3 in monolayer cultures promotes differentiation into multipotential mesendoderm progenitors[149]. These mesendoderm progenitors have an enhanced potential to differentiate into the endothelial, cardiac and skeletal lineages[149]. In addition, activation

of β-catenin signalling has been linked to a block in neuronal differentiation[140,144,150], whereas antagonism of Wnt signalling promoted neural differentiation in mESCs[151,152].

3.2 TCF/LEF signalling in mESCs

In mESCs, it has been shown that they express all TCF/LEF factors. The transcript levels of TCF3 is 3- to 12-fold higher than that of the other TCF/LEF factors[153]. Western blot analysis also indicates that mESCs express all 4 factors at readily detectable levels[144]. In part due to its high level of expression in mESCs, there has been a focus on the role of TCF3 in mESC biology, despite the presence of the other TCF/LEF factors in these cells.

3.2.1 Regulation of pluripotency by TCF3

There have been various lines of evidence suggesting that TCF3 acts as a repressor and plays an important role in the regulation mESC pluripotency and differentiation[110,148,153-158]. In TCF3 knockout mESCs, Nanog levels are up-regulated by 2.5 fold. Moreover, when subjected to embryoid body differentiation assays, differentiation was delayed in TCF3$^{(-/-)}$ mESCs when compared to wild-type mESCs[153]. Whereas a wild-type mESC would lose its ability to self-renew in the absence of LIF, TCF3$^{(-/-)}$ mESCs can self-renew even after 10 passages when grown in these conditions[154]. Chromatin immunoprecipitation (ChIP) data suggest that TCF3 occupies a number of pluripotency associated genes, in concert with Nanog and Oct4[154-156]. Furthermore, the majority of TCF3-bound genes were up-regulated when TCF3 levels

were depleted[155,156]. TCF3 is also bound at transcriptionally inactive genes that are linked to differentiation. Many of these genes were also bound by TLE2 and an interaction between TCF3 and TLE2 was observed via co-immunoprecipitation experiments[156]. In mESCs, 2 isoforms of TCF3 have been identified, TCF3(s) and TCF3(l). The TCF(s) isoform has a 14 amino acid deletion in the TLE-binding domain. Upon differentiation, TCF3(l) expression is downregulated while the TCF3(s) level is unchanged. These two isoforms appear to regulate different target genes although the mechanism through which differential binding of the isoforms occurs is unclear.[110].

3.2.2 Regulation of TCF3 β-catenin

Up-regulation of the Wnt/β-catenin pathway enhances mESC self-renewal, but the downstream mechanism was not well understood. Recently, two independent groups found that TCF3 plays a key role in mediating the effects of β-catenin on mESCs. It was shown that activation of the Wnt/β-catenin pathway, either by the addition of a GSK-3 inhibitor[148] or Wnt3a[158], de-repressed the repressive role of TCF3. Using the two inhibitor (2i) system, which employs small molecule inhibitors of GSK-3 (CHIR-99021) and MAPK (PD98059) in serum-free medium lacking LIF, Wray *et al*. found that TCF3$^{(-/-)}$ mESCs expressing a wild-type copy of TCF3 could be maintained in the 2i conditions, whereas TCF3$^{(-/-)}$ mESCs expressing a TCF mutant lacking the β-catenin binding domain were unable to self-renew[148]. Thus, the derepression of TCF3 appears to be mediated through a β-catenin/TCF3 interaction [148,158].

4 Project Rationale

Studies performed in our lab have suggested that β-catenin's regulation of pluripotency may be independent of typical TCF signalling. However, since TCF3 is the most abundantly expressed TCF factor at the transcript level and is bound at many promoters in concert with Nanog and Oct4, the possibility that TCF3 is important in regulating pluripotency could not be ruled out. Thus, we were interested in better understanding how TCF3 is involved in regulating both the pluripotency and differentiation capabilities of mESCs. The experiments and data described in this thesis are focused on testing the following hypothesis:

Hypothesis: over expression of full-length and dominant-negative TCF3 in GSK-3 DKO and wild-type mESCs will promote the exit from the pluripotent state and drive specific types of differentiation.

Methods and Materials

1 Cell culture and transfection

mESCs cells were maintained in DMEM (Thermo Scientific) supplemented with 15% FBS (Thermo Scientific), 1x non-essential amino acids (Thermo Scientific), 1x L-glutamine (Thermo Scientific), 1x Sodium pyruvate (Thermo Scientific), 1x β-mercaptoethanol and 1000U/mL ESGRO (Millipore). Media was filter-sterilized with a 0.22μM Stericup® Filter (Millipore). Cells were grown on tissue culture-treated plates coated with 0.1% gelatin and maintained in a humidified incubator at 37˚C with CO_2 maintained at 5%. Cells were passaged every 2 days with Accutase (Innovative Cell Technologies), spun down and split at a 1:6 ratio. Doxycycline Hyclate (dox; Sigma) was reconstituted in distilled water and filter sterilized (0.22 μm) before storage at 4 °C.

mESCs were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Cells were counted with a Countess® Automated Cell Counter (Invitrogen) and 1-2 x 10^6 cells were transfected with 2 μg of DNA.

2 Mammalian expression constructs

The pCAG-RTTA-IRES-Puro, pB-TET and pDONR plasmid were gifts from the J. Draper lab (SCC-RI, McMaster) and the 8x TOPFlash plasmid was a gift from the lab of R. Moon (University of Washington).

The TCF3DN-V5 and TCF3FL-V5 transgenes were cloned into mammalian expression vectors (pFRT/TET) that allows for site-specific integration of transgenes into the genome and regulated expression of a transgene when doxycycline is present. To generate the pFRT/TET construct, a pB-TET plasmid was digested with PacI and ApaI to excise the Tet response elements and attL sites. This fragment was ligated with a pEF5/FRT plasmid (Invitrogen) that was digested with BglII and AgeI. The TCF3DN-V5 transgene was PCR amplified from a pEF-TCF3DN-V5 plasmid (primers: AttB1-kozak-TCF3DN-V5 5'ggggacaagtttgtacaaaaaagcaggcttacaccatggagaatcagag 3'; TCF3-AttB2 5'ggggaccactttgtacaagaaagctgggtactaaccggtacgcgtaga 3'). The TCF3FL-V5 transgene was PCR amplified from pEF-TCF3FL-V5 (primers: AttB1-kozak-TCF3FL-V5 5'ggggacaagtttgtacaaaaaagcaggcttacaccatgccccagctcggt 3'; TCF3-AttB2 (see above)). The amplified products were inserted into pDONR and subsequently subcloned into the pFRT/TET plasmid using the Gateway system (Invitrogen). Colonies were digested with NcoI to confirm the correct orientation of the transgene and were also sequenced to ensure the absence of mutations (MOBIX, McMaster). Plasmid stocks of positive clones were generated with an Invitrogen PureLink HiPure Plasmid Midi-prep kit. All restriction enzymes were obtained from Fermentas.

3 Generation of stable Flp-in and random integration mESC lines

E14K-FRT ($E14K^F$) cell lines were generated by transfecting 2 μg of linearized pFRT/lacZeo (Invitrogen) into mESCs using Lipofectamine 2000 (Invitrogen). 2 days after

transfection, 50 μg/mL of zeocin was added, and 7-10 days later, clones were isolated, expanded and characterized. To verify successful integration of pFRT/lacZeo into the genome of mESCs, the cells were assayed for β-galactosidase activity. In addition, Southern blots were performed in order to determine the number of pFRT/lacZeo integrations.

The DKO-RTTA and E14K-FRT-RTTA (DKO^{T} and $E14K^{FT}$) lines were generated by transfecting 2 μg of linearized pCAG-RTTA-IRES-Puro into mESCs. Puromycin (at 2 μg/ mL concentration) was used for selection and 7-10 days after selection, clones were isolated, expanded and characterized. To determine whether the cells express the reverse tetracycline transactivator, 2 μg of pFRT/TET-TCF3DN-V5 was transiently transfected into cells using Lipofectamine 2000. Dox was added to the cells and transgene expression was monitored via immunoblot analysis.

To generate Flp-in mESCs, cells were co-transfected with either pFRT/TET-TCF3DN-V5, pFRT/TET-TCF3FL-V5 or pFRT/TET and pOG44 plasmids at a 1:9 ratio using Lipofectamine 2000 (Invitrogen). Two days after transfection, 250 μg/mL of hygromycin was added to the cells. Approximately 7-10 days later, colonies were picked, expanded and screened for transgene expression by immunoblot analysis.

4 β-galactosidase Staining

Cells were washed twice with PBS before fixation with 4% paraformaldehyde/PBS for 5 minutes. The cells were then incubated with an X-gal solution, made in PBS and containing 1 mg/mL X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 2 mM magnesium chloride for 2 hours in a 37 °C humidified incubator before they were imaged using an Olympus IX51 Microscope.

5 Southern blotting

Genomic DNA was harvested from a confluent 60 mm plate of mESCs and purified with a Qiagen DNeasy blood and tissue kit. 10 μg of gDNA was digested with HindIII (Fermentas) overnight. The digested gDNA was then purified with QiaEx beads (Qiagen), and the samples were run on a 0.7% agarose gel at 15V overnight. The gel was then soaked in 0.25M HCl for 10 minutes and 0.4M NaOH for 30 minutes before transferring it to a BioTrace, Biodyne or UltraBind Transfer Membrane (Pall). A 0.4M solution of NaOH was used as the transfer buffer. A LacZ probe was generated by PCR amplification of the pFRT-lacZ plasmid (primers; LacZ Fwd- 5'-GGCAACTCTGGCTCACAGTACG-3' and LacZ Rev 5'-ACGCCAATGTCGTTATCCAGCG-3'). The LacZ probe was radio-labeled with ^{32}P-dCTP using Amersham's Rediprime™ II DNA Labeling System (GE Healthcare), according to the manufacturer's instructions. The probe was then purified with a G-50 sephadex spin column (Illustra ProbeQuant™ G-50 Micro Columns; from GE

Healthcare). The membrane was pre-hybridized with with PerfectHyb Plus Hybridization Buffer (Sigma) before hybridization with the ^{32}P-labeled LacZ probe overnight at 68°C. The following day, the membrane was washed with 2x SSC, 0.1% SDS at 68°Cs for 5 minutes followed with two washes with 0.5X SSC, 0.1% SDS also at 68°C. The signal was typically exposed on film for 4 days.

6 Cell lysate preparation

Cells for protein extraction were rinsed twice with PBS at room temperature before lysis. Either ice-cold 1x Radioimmunoprecipitation Assay buffer (150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris pH 8.0, 1 mM EDTA and 1x Halt Protease Phosphatase Inhibitor Cocktail (Pierce)) or 1x Gentle-Soft buffer (10 mM NaCl, 0.5% NP-40, 0.05% 2-mercaptoethanol, 5 mM EDTA, 20 mM Pipes and 1x Halt Protease Phosphatase Inhibitor Cocktail, final pH of 7.4) was added directly to the cells. After 5 minutes, the cell suspension was harvested into microcentrifuge tubes and centrifuged at 16 100 x g for 10 minutes at 4°C. The supernatant, which contains the soluble proteins, was retained and quantified using the Lowry method (DC Protein Assay from Bio-Rad). The samples were then normalized to 0.5 µg/µL - 1.0 µg/µL concentration and prepared in 1x LDS buffer (Invitrogen) with 5% TCEP Bond-Breaker solution (Thermo) and heated at 95°C for 5 minutes.

7 Antibodies

The following primary antibodies were used for Western blotting and/or immunofluorescent staining: mouse anti-V5 (Invitrogen); rabbit anti-V5 (Bethyl Laboratories); mouse anti-GAPDH (Abcam); rabbit anti-Nanog (Bethyl Laboratories); mouse anti-Oct3/4 (Santa Cruz); rabbit anti-Sox2 (Cell Signalling Technology); mouse anti-β-Tubulin I (Sigma-Aldrich); mouse anti-β-III-Tubulin (R&D Systems).

For secondary antibodies, fluorochrome-conjugated secondary antibodies were obtained from Invitrogen; goat anti-mouse Alexa Fluor 488 and goat anti-rabbit Fluor 647. Horseradish peroxidase-conjugated secondary antibodies for Western blotting were obtained from Bio-Rad (goat anti-mouse and goat anti-rabbit).

8 Immunoblotting

Equal amounts of protein lysates (typically 10 μg) were separated on 10% Bis-tris gels. Gels were transferred onto PVDF either using the Biorad Trans-Blot® Turbo™ System (25V for 30 mins) or the Hoefer Semi-Dry Transfer Unit (75 mAmp per gel for 30 mins). Membranes were then blocked in 5% skim milk powder/Tris Buffer Saline (SMP/TBS). Primary antibodies (diluted in 2-3% SMP/TBS-Tween 20 (TBST)) were either left overnight at 4 °C or for 1 hour at room temperature. The primary antibody dilutions used were: Oct-4 (1:5000); Nanog (1:5000); Sox2 (1:2000); β-III-tubulin (1:20000); V5-Invitrogen (1:10000); V5-Bethyl (1:10000); GAPDH (1:800 000); β-tubulin-1 (1: 3 200

000). The membranes were washed 6 times for 5 minutes per wash in 2-3% SMP/TBST, before incubation with horseradish peroxidase-conjugated secondary antibodies, diluted 1:20000 for 1 hour at room temperature. Membranes were then washed 6 times for 5 minutes with TBST. The membranes were submerged in Luminata Forte Western HRP substrate (Millipore) for 5 minutes before detecting the chemiluminscent signal with Amersham hyperfilm (or equivalent).

9 Immunoflourescence

9.1 IF of EBs

For immunofluorescence analysis of EBs, harvested EBs were washed twice with PBS before overnight fixation in 4% paraformaldeyhyde/PBS at 4°C on a rotator. The next day, EBs were permeabilized twice with 0.2% Triton X-100/PBS for 15 minutes at room temperature on a rotator. The EBs were then blocked with 2% BSA/PBS at 4°C for 2 hours on a rotator. Primary antibodies (diluted in 2% BSA/PBS) were incubated on a rotator overnight at 4°C. The primary antibody dilutions used were: Nanog (1:250); β-III-Tubulin (1:500); V5- Invitrogen (1:500). The following day, the EBs were rinsed three times in PBS for 15 minutes per wash. Fluorochrome-conjugated secondary antibodies at a dilution of 1:1000 (diluted in 2% BSA/PBS) were then incubated at 4°C overnight. DAPI (1 mg/mL) at a 1:1000 dilution was also added to the secondary antibody. The following day, the EBs were washed five times with PBS, 15 minutes per wash before mounting on standard glass slides with ProLong Gold antifade reagent with DAPI

(Invitrogen). Stained EBs were imaged using a standard epifluorescence microscope (Olympus IX-81).

9.2 IF of monolayer mESCs

The protocol used for IF staining for monolayer mESCs is similar to that described for EBs. The major differences were: the cells were fixed on ice for 10 mins, permeabilized once for five minutes at room temperature and blocked for 30 mins on ice. The primary antibody was left to incubate on the samples overnight at 4°C while the secondary antibody was incubated for 1 hour at room temperature. Cells were imaged using a spinning disk confocal microscope (Olympus IX-81 with spinning disk confocal accessories).

10 Sub-cellular localization

A coverslip was placed into a tissue culture 6-well dish and both were 0.1% gelatin coated. $1x10^6$ cells were resuspended in 150 µL of media, pipetted onto the coverslip and allowed to adhere. After 4 hours, an additional 2 mL of media was added to the well, drop wise. The following day, the cells were processed for immunofluorescence analysis.

11 RNA extraction, cDNA synthesis and Quantitative RT-PCR

Total RNA was extracted from cells using a Qiagen RNeasy kit as per the manufacturer's protocol. 1 µg of RNA was used for first-strand cDNA synthesis using a qScript cDNA

synthesis kit (Quanta) following the manufacturer's protocol. Quantitative RT-PCR reactions were run using 25-50 ng of cDNA (based on assumed equivalence with the amount of input RNA) with PerfeCTa®SYBR® Green FastMix® (Quanta) on a Stratagene Mx3000P real-time instrument. β-actin was used as the housekeeping gene. The MxPro software package was used to determine the relative gene expression levels using the delta-delta Ct method.

Table 1 Quantitative RT-PCR Primers

Primer	Sequence
Actin (β)	Fwd: 5'-TTGCTGACAGGATGCAGAAGGAGA-3' Rev: 5'-ACTCCTGCTTGCTGATCCACATCT-3'
Map-2	Fwd: 5'-CTGGATTTCAAGGAAAAGGCC-3' Rev: 5'-ATCTCAGCCCCGTGATCTA-3'
Nanog	Fwd: 5'-AACCAAAGGATGAAGTGCAAGCGG-3' Rev: 5'-TCCAAGTTGGGTTGGTCCAAGTCT-3'
Nestin	Fwd: 5'-AAGTTCCCAGGCTTCTCTTG-3' Rev: 5'-GTCTCAAGGGTATTAGGCAAGG-3'
Oct-4	Fwd: 5'-AGCTGCTGAAGCAGAAGAGGATCA-3' Rev: 5'-TCTCATTGTTGTCGGCTTCCTCCA-3'
β-III-Tubulin	Fwd: 5'-CGCCTTTGGACACCTATTCAG-3' Rev: 5'-TTCTCACACTCTTTCCGCAC-3'
Tcf3	Fwd: 5'-ACCAAATCCTGGGAAGAAAGTGGC-3' Rev: 5'-TTCCTGCTGAGACTGTGTCTGTGA-3'
Cardiac troponin T2	Fwd: 5'- GTAGAGGACACCAAACCCAAG-3' Rev: 5'-GAGTCTGTAGCTCATTCAGGTC-3'
Pax6	Fwd: 5' - CCCTCACCAACACGTACAG-3' Rev: 5'- TCATAACTCCGCCCATTCAC-3'
Eomes	Fwd: 5'-AGGCGCATGTTTCCTTTCTTGAGC-3' Rev: 5'- GCCCTGCATGTTATTGTCCGCTTT-3'
FoxA2	Fwd: 5'-AAGTATGCTGGGAGCCGTGAAGAT-3' Fwd: 5'-CGCGGACATGCTCATGTATGTGTT-3'

12 TOPFlash luciferase assay

mESCs were co-transfected with 1.9 μg of 8xTOPFlash which drives firefly luciferase production and 100 ng of pRL-CMV plasmid that constitutively expresses renillla luciferase (for normalization). For DKO^{T} cells, the day after transfection, the cells were lysed with 1x passive reporter lysis buffer provided by the Promega Dual-Luciferase® Reporter Assay System. For $E14K^{FT}$ cells, the day after transfection, the β-catenin/TCF pathway was stimulated with either Wnt3a (50:50 mix of Wnt3a conditioned media and mESC media) or CHIR99021 (15 μM) for an additional twenty-four hours before lysis. The firefly and renilla reporter activities were measured using the Promega Dual-Luciferase® Reporter Assay System and a 96-well-based luminometer.

13 Neural differentiation assay

The neuronal differentiation protocol was based on that of Ying and Smith[159]. mESCs were cultured for at least 1 passage prior to the initiation of neural differentiation. The cells were then cultured in media referred to as N2B27, which includes DMEM/F12 (50/50) (Invitrogen) supplemented with N2 (Gibco) and BSA (Wisent), combined 1:1 with Neurobasal media (Invitrogen) supplemented with B27 (Gibco). 2 x 10^5 cells were plated in N2B27 onto 1 well of a 0.1% gelatin-coated 6-well dish. The day after seeding, either 0 or 100 ng/mL of dox was added to the cells. The N2B27 culture medium was replenished every 2^{nd} day and the cells were imaged and harvested for RNA or protein analysis at the indicated time points.

14 Embryoid body assay

Cells were cultured for at least 1 passage prior to formation of embryoid bodies. EBs were maintained in DMEM (Thermo Scientific) supplemented with 5% FBS (Thermo Scientific), 1x non-essential amino acids (Thermo Scientific), 1x L-glutamine (Thermo Scientific), 1x sodium pyruvate (Thermo Scientific) and 1x β-mercaptoethanol (Sigma). Initially, EBs were propagated in hanging drops on 10 cm^2 Petri dishes (800 cells in 30 μL). The drops were incubated for 3 days before they were transferred to an ultra-low-binding 96-well plate (Corning). Once the EBs were moved to the 96-well plate, dox was added the media. The media was replenished every two days.

15 Wnt3a-conditioned media / CHIR-99021

Mouse L-Cells expressing a Wnt3a transgene were purchased from ATCC. Cells were cultured in DMEM supplemented with 15% FBS, 1x non-essential amino acids, 1x L-glutamine, 1x Sodium pyruvate and 1x β-mercaptoethanol. The cells were expanded onto five T-175 flasks, where after 2 days of growth, the media was collected and filtered.

CHIR-99021 (BioVision) was reconstituted in DMSO and subsequently stored at -30˚C, protected from light.

16 Statistical Significance

All statistical analyses were performed by using the GraphPad Prism software package. All comparisons were performed using ANOVA with the Tukey's post-hoc test. Statistical significance was set at 0.05.

Results

1 Generation and validation of GSK-3 DKO cells that induce expression of a TCF3 transgene upon addition of doxycycline

Previous attempts to stably express TCF3 lacking its β-catenin binding domain (dominant-negative TCF3, denoted TCF3DN) in GSK-3 DKO cells were unsuccessful. Thus, in order to study the effects of the expression of TCF3DN in the DKO cells, a doxycycline-regulated system was used to regulate the expression of TCF3 transgenes at the transcriptional level. Transcription of a gene of interest is activated in the presence of doxycycline (dox), which is a derivative of tetracycline. This system is commonly referred to as the "tet-on" system. This system contains two components, a plasmid that encodes the tetracycline-responsive regulatory protein and a response plasmid. The regulatory protein is the reverse tetracycline transactivator (rTTA). The response plasmid expresses a gene of interest under the control of tetracycline response elements. In the presence of dox, rTTA binds to the tetracycline response elements upstream of the gene of interest, and this interaction allows for transcriptional activation of the gene of interest (Fig 3a).

To generate this system, the rTTA was first stably integrated into the genome of DKO cells that were previously modified to contain a single genomic FRT site for Flp-mediated site-specific recombination (DKO-rTTA). The transgenes were cloned into mammalian expression vectors containing the tetracycline response elements and a FRT site for FLP-mediated integration of the transgene into the FRT site in the DKO-rTTA cells. The DKO-

RTTA cells, hereafter referred to as "DKO^{T}" cells, were used to generate isogenic FLP-in cell lines expressing (1) V5 epitope-tagged dominant-negative TCF3 (DKO^{T}-TCF3DN-V5), (2) V5 epitope-tagged full-length TCF3 (DKO^{T}-TCF3FL-V5) (Fig 3b) and (3) the empty vector (DKO^{T}-Vector). Two clones of each cell line were generated and used for further experiments.

To test the inducibility of this system, 0 and 100 ng/mL of dox were added to cells for 24 hours before they were harvested for protein analysis. Based on Western blot analysis, when dox was added to the cells, the DKO^{T}-TCF3DN-V5 and DKO^{T}-TCF3FL-V5 mESCs displayed induced expression of the TCF3DN-V5 and TCF3FL-V5 transgenes. Furthermore, the expression level of the TCF3FL-V5 transgene was higher than that of the TCF3DN-V5 transgene (Fig 3c).

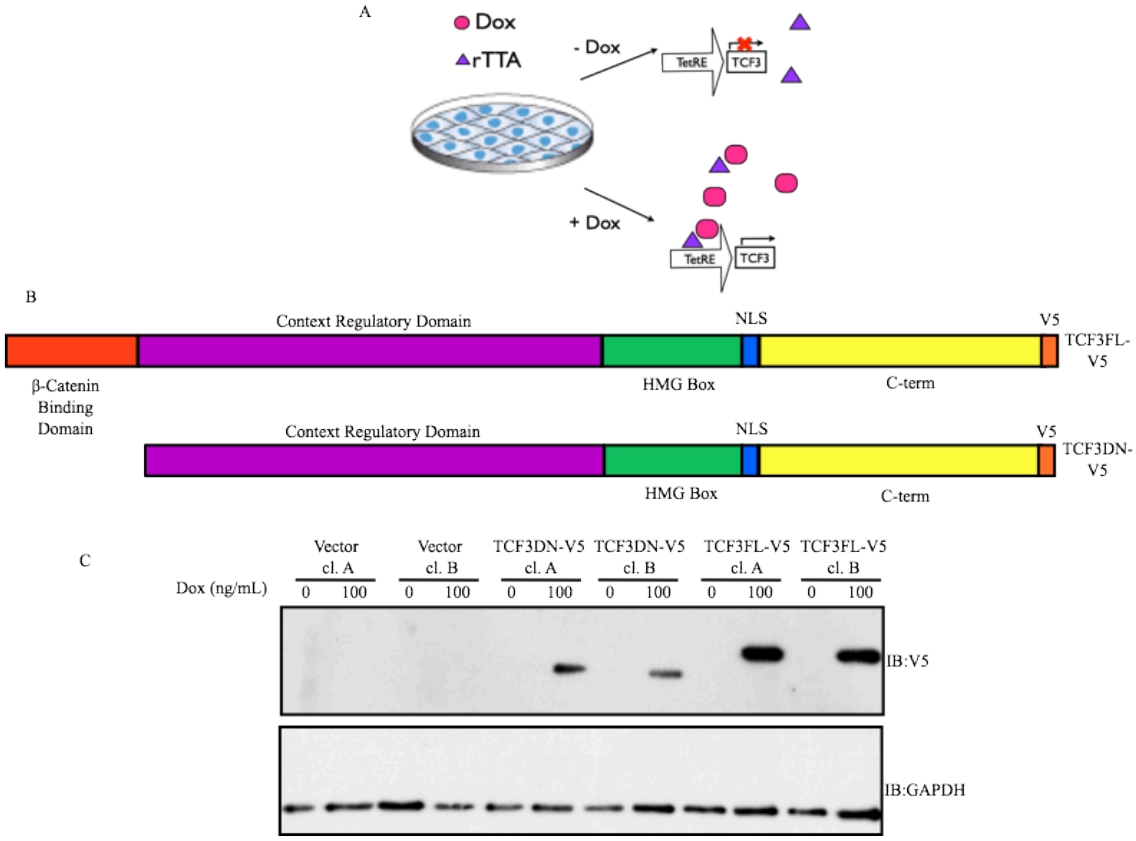


Figure 3. Generation of GSK-3 DKO mESCs that allow for doxycycline-inducible regulation of transgenes.

(A) A doxycycline regulated system to express the gene of interest. Cells were transfected with reverse tetracycline transactivator (rTTA) and a gene expression plasmid with tetracycline (Tet) response elements. Dox binds to the Tet response elements to activate transcription of the gene of interest.
(B) The TCF3DN-V5 and TCF3FL-V5 constructs used.
(C) Expression of TCF3DN-V5 and TCF3FL-V5 in DKO^{T}-TCF3DN-V5 and DKO^{T}-TCF3FL-V5 mESCs was induced with 100 ng/mL of dox.

TCF3 is normally localized to the nucleus[107]. To determine the localization of the TCF3DN-V5 and TCF3FL-V5 transgenes, cells were induced with dox for 24 hours before immunofluorescent analysis. A V5 immunofluorescent stain indicated that in the presence of dox, the TCF3DN-V5 and TCF3FL-V5 transgenes were localized to the nucleus. (Fig 4).

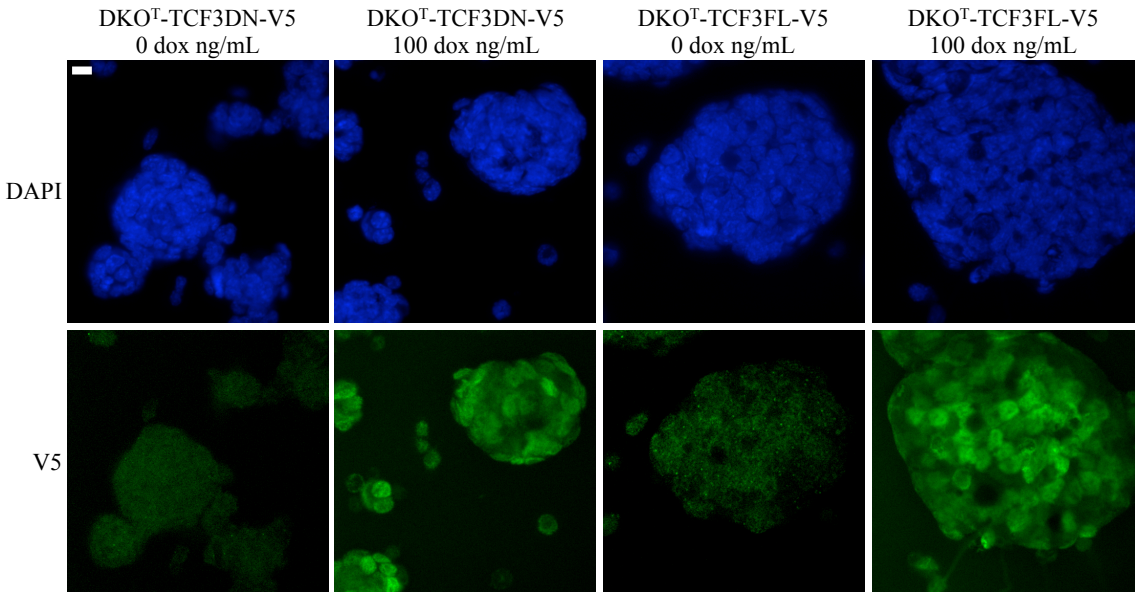


Figure 4. Nuclear localization of TCF3DN-V5 and TCF3FL-V5 proteins in DKO^{T}-TCF3DN-V5 and DKO^{T}-TCF3FL-V5 mESCs.
Dox was added to DKO^{T}-TCF3DN-V5 and DKO^{T}-TCF3FL-V5 for 24 hours. V5 immunofluorescent staining indicates that the TCF3-V5 transgenes were localized to the nucleus. Pictures were taken with a confocal microscope, 60x magnification. Bar = 10 μM.

To determine the TCF activity present in these cells, a TCF reporter assay was utilized. The TCF luciferase reporter plasmid Super 8X-TOPFlash, a plasmid containing 7 TCF/ LEF bindings sites driving firefly luciferase expression, and a plasmid that constitutively expresses renilla luciferase (pRL-CMV), were co-transfected into the stable cell lines: DKO^{T}-Vector, DKO^{T}-TCF3DN-V5 and DKO^{T}-TCF3FL-V5. As expected, TCF reporter activity was elevated in DKO cells harbouring the vector control (DKO^{T}-Vector). In the absence of dox, TCF reporter activity was similarly elevated in the DKO^{T}-TCF3DN-V5 and DKO^{T}-TCF3FL-V5 cell lines. The addition of dox to either the DKO^{T}-TCF3DN-V5 or DKO^{T}-TCF3FL-V5 cell lines significantly repressed TCF reporter activity ($p<0.05$, Fig 5).

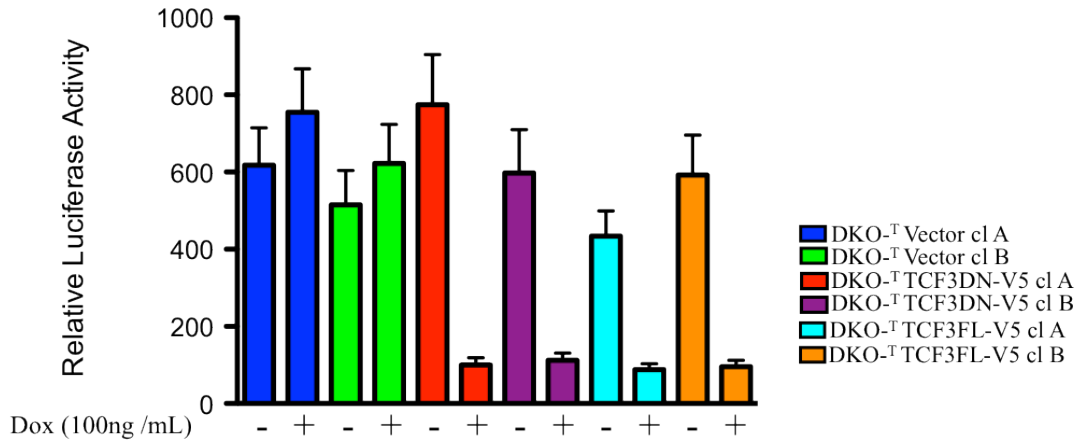


Figure 5. Expression of TCF3DN-V5 and TCF3FL-V5 in GSK-3-DKO cells represses TOPFlash Activity

DKO^{T} cells expressing the empty vector, TCF3DN-V5 or TCF3FL-V5 were transfected with with a TCF reporter plasmid. TCF reporter assay data suggests that expression of either TCF3DN-V5 or TCF3FL-V5 in the DKO background repressed TCF activity. Bars represent the mean of two independent experiments, ± SEM.

2 Short-term expression of TCF3DN-V5 and TCF3FL-V5 in DKO mESCs down-regulates the expression of Nanog

To study the short-term effects of expressing TCF3DN-V5 and TCF3FL -V5 in DKO mESCs, both DKO^{T}-TCF3DN-V5 and DKO^{T}-TCF3FL-V5 cells were grown in standard mESC media for 72 hours. Morphologically, after 72 hours of dox induction, the DKO^{T}-TCF3DN-V5 cells exhibited cell death/differentiation, which was not seen in DKO^{T}-TCF3DN-V5 cells that were uninduced (Fig 6a). In Figure 3b, through Western blot analysis, we observed that the expression levels of TCF3FL-V5 was higher than TCF3DN-V5. When the TCF3 transcript levels were measured using qRT-PCR analysis, after 72 hours of dox treatment, in DKO^{T}-TCF3DN-V5, there was a 7-fold increase in TCF3 expression. However, in DKO^{T}-TCF3FL-V5 mESCs, there was a 20-fold increase in TCF3 transcript levels. (Fig 6b). Western blot analysis was performed to examine the

expression levels of the pluripotency markers Nanog, Oct4 and Sox2. When grown in the presence of dox, both DKO^{T}-TCF3DN-V5 and DKO^{T}-TCF3FL-V5 mESCs downregulated the protein levels of Nanog. However, Sox2 and Oct4 levels appears to be unchanged (Fig 6c). When the steady-state level of Nanog was quantified through densitometry, the amount of Nanog in DKO^{T}-TCF3DN-V5 mESCs treated with dox was approximately 80% of the total amount of Nanog detected in uninduced DKO^{T}-TCF3DN-V5 mESCs. The steady-state level of Nanog in induced DKO^{T}-TCF3FL-V5 mESCs was approximately 70% of the amount of Nanog that was observed in uninduced DKO^{T}-TCF3FL-V5 mESCs.

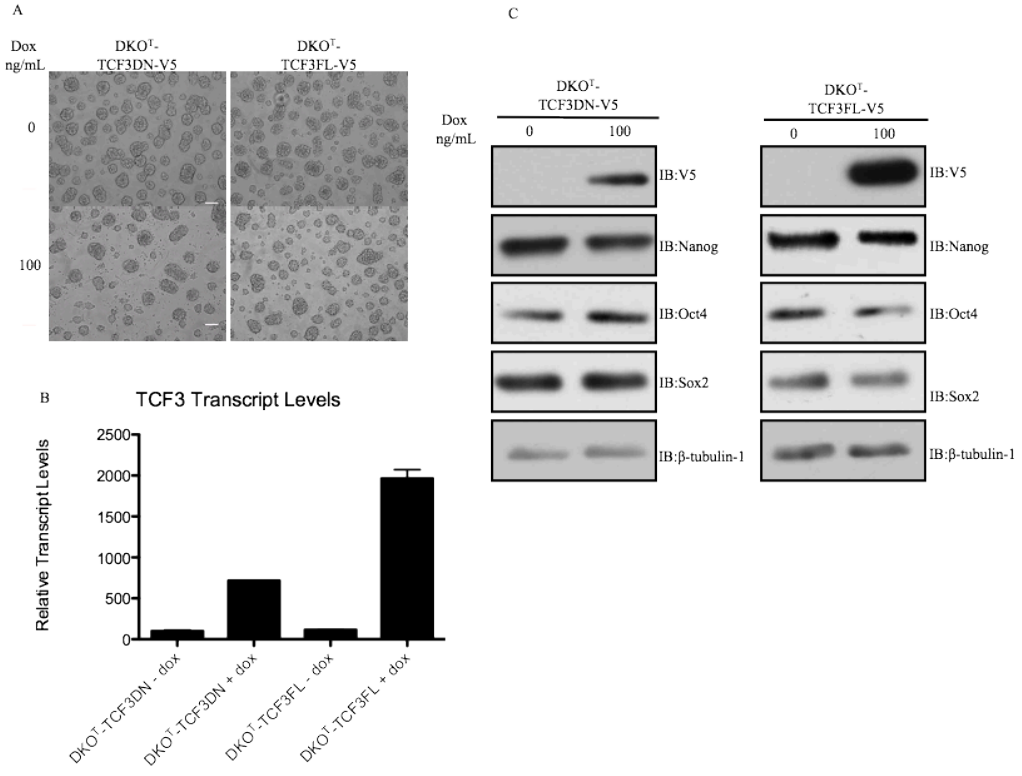


Figure 6. Induction of full-length or dominant-negative TCF3 (72 hours) results in downregulated expression of Nanog in DKO^{T}-TCF3DN-V5 and DKO^{T}-TCF3FL-V5 mESCs.

(A) Morphology of cells at the 72 hour time point suggests that expression of TCF3DN-V5 in DKO mESCs may promote death/differentiation.
(B) qRT-PCR analysis of TCF3 transcript level indicates that induction of dox up-regulates expression of TCF3 in mESCs. Bars represent mean ± s.d. (n=2).
(C) Western blot data suggests that either TCF3DN-V5 or TCF3FL-V5 expression down-regulates Nanog levels.

3 DKO mESCs expressing the TCF3DN-V5 or TCF3FL-V5 transgenes are unable to rescue the neuroectoderm blockade

When wild-type mESCs are grown in a basal medium supplemented with N2 and B27, over time the cells will undergo neural differentiation[159]. Previously, we found that both DKO-TCF1DN and DKO-TCF4DN mESCs were unable to differentiate into the neural lineage[144]. To assay whether expression of either TCF3DN-V5 or TCF3FL-V5 in the DKO background can remove the block to neuronal differentiation, DKO^{T}-TCF3DN-V5 and DKO^{T}-TCF3FL-V5 cells were grown in N2B27 media for 13 days. Induction with 100 ng/mL of dox in DKO^{T}-TCF3FL-V5 mESCs was highly detrimental as no viable cells were retained at the end of the assay. Conversely, DKO^{T}-TCF3DN-V5 mESCs induced with 100 ng/mL dox were viable. Although the cells were grown in the absence of drug selection throughout the assay, expression of the TCFDN-V5 transgene was retained. However, the transcript level of TCF3 in DKO^{T}-TCF3DN-V5 cells induced with dox was statistically insignificant compared to the -dox control ($p < 0.05$, Fig. 7b). Western blot analysis revealed that 13 days after the initiation of neural differentiation, wild-type cells lost expression of Nanog and Oct-4 but gained expression of β-III-tubulin. DKO^{T}-TCF3DN-V5 mESCs grown in either the absence or presence of dox retained Oct-4 and Nanog expression but had no readily detectable β-III-tubulin expression. There was a slight down-regulation of Nanog expression in DKO^{T}-TCF3DN-V5 mESCs

induced with dox (Fig 7c). The qRT-PCR analyses support the immunoblot results, which showed that wild-type cells had a significant decrease of Nanog and Oct4 expression ($p < 0.05$, Fig 7d). Additionally, the transcripts for the neural makers, β-III-tubulin, Nestin, Pax6 and Map2 were expressed only in the wild-type mESCs (Fig 7d). Although the transcript levels of Nanog and Oct4 were downregulated in DKO^{T}-TCF3DN-V5 + dox samples, the effect was not as dramatic as the down-regulation that occurs in wild-type cells. It appears that expression of TCF3DN-V5 in DKO cells had no effect on neuronal differentiation, since both DKO^{T}-TCF3DN-V5- dox and DKO^{T}-TCF3DN-V5 + dox failed to express β-III-tubulin, Nestin, Pax6 and Map2 transcripts (Fig 7d).

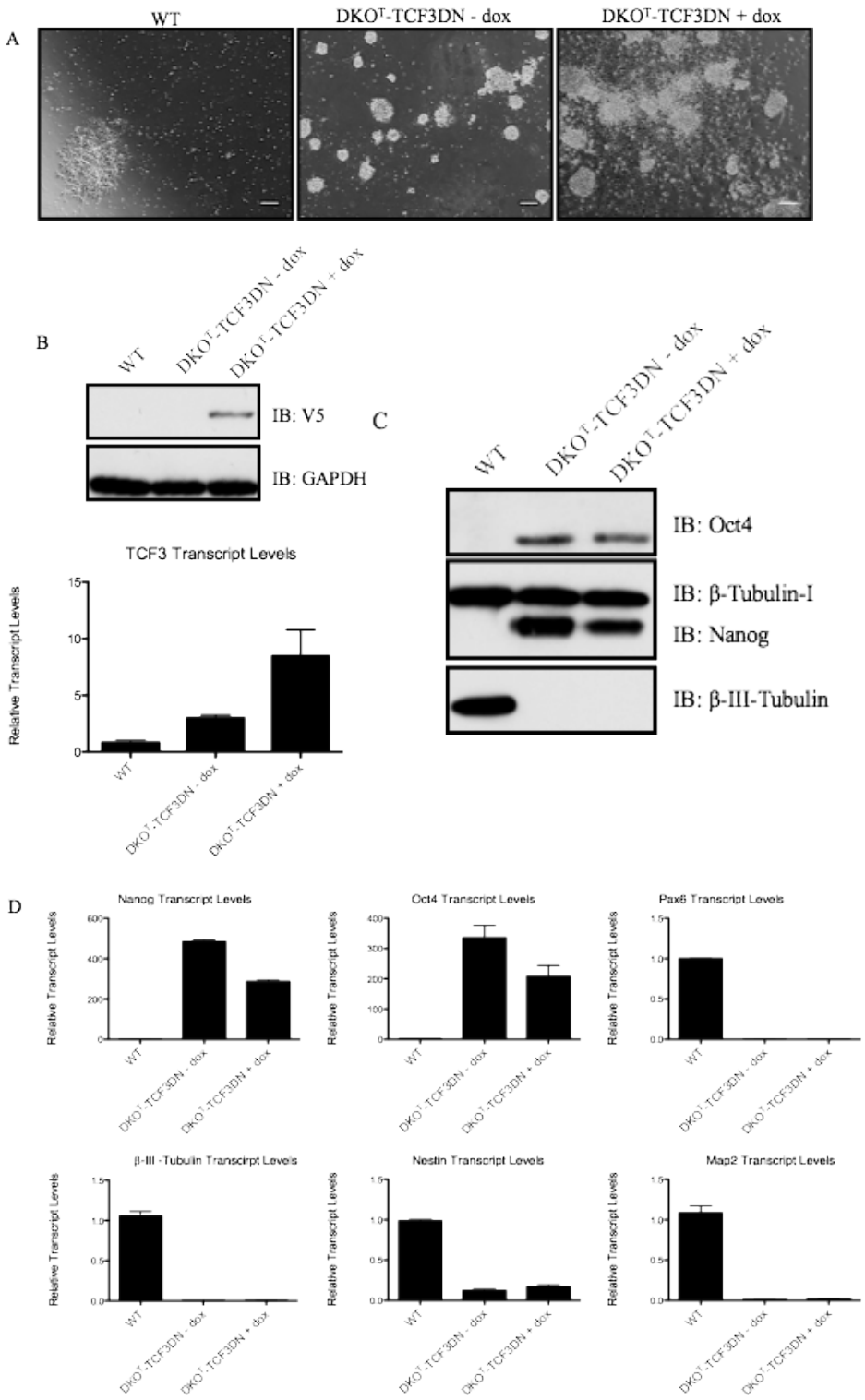


Figure 7. DKO mESCs expressing TCF3DN-V5 or TCF3FL-V5 are unable to undergo neuronal differentiation in a defined neural differentiation assay.

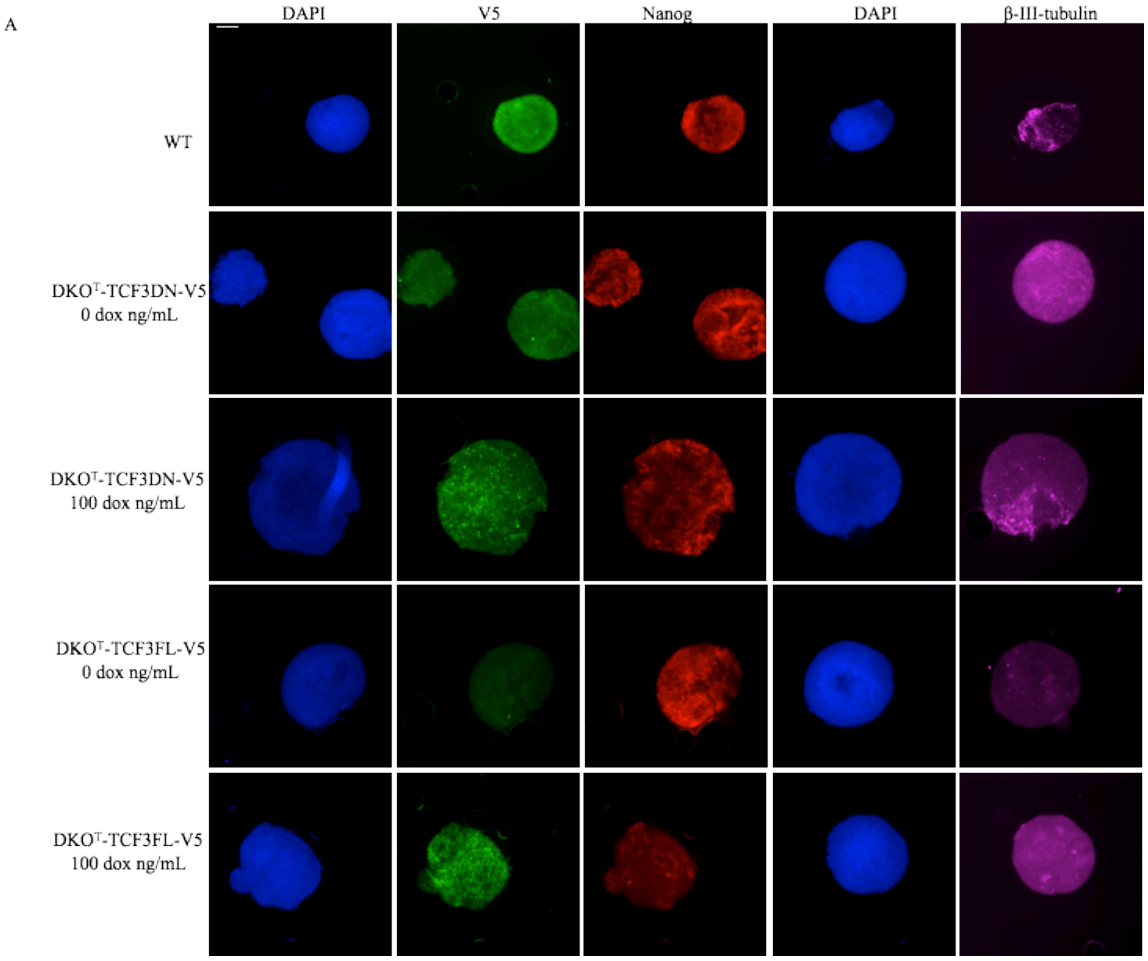
(A) Morphology of WT, DKO^{T}-TCF3DN-V5 - dox and DKO^{T}-TCF3DN-V5 + dox mESCs 13 days after initiation of neural differentiation in N2B27 media. Bar, 100 μM.
(B) Even in the absence of drug selection, TCF3DN-V5 is expressed after 13 days as measured with a V5 immunoblot. Furthermore, TCF3 transcript levels are higher in DKO^{T}-TCF3DN-V5 + dox compared to DKO^{T}-TCF3DN-V5 - dox and wild-type cells.
(C and D) Western blot (C) and qRT-PCR (D) analysis after a 13 day neuronal differentiation assay indicated that wild-type cells had no Oct4 and Nanog expression but expressed β-III tubulin, Nestin, Pax6 and Map2. DKO^{T}-TCF3DN-V5 - dox and + dox cells retained expression of Nanog and Oct4 but failed to express β-III-tubulin, Nestin, Pax6 and Map2. Bars represent mean ± s.e.m. (n=2).

4 DKO^{T}-TCF3DN-V5 and DKO^{T}-TCF3FL-V5 EBs are unable to differentiate into the neuronal lineage but may differentiate into the endoderm lineage

To further study the differentiation capabilities of DKO^{T}-TCF3DN-V5 and DKO^{T}-TCF3FL-V5 mESCs, an embryoid body (EB) experiment was performed. EBs are aggregates of embryonic stem cells, which upon aggregation spontaneously differentiate into cells of the three germ layers. During the initial 3 days of the assay, mESCs were allowed to form aggregates in hanging drops in the absence of dox. When the EBs were moved onto 96-well low attachment plates, they were grown in either the absence or presence of 100 ng/mL dox for an additional 13 days.

Addition of dox induced the expression of the TCF3-V5 transgenes in the EBs. There was more V5 staining observed in the DKO^{T}-TCF3FL-V5 EBs than in the DKO^{T}-TCF3DN-V5 EBs (Fig 8a), which is consistent with previous work suggesting that TCF3FL-V5 is expressed at a higher level than TCF3DN-V5 in DKO mESCs (Fig 3c). However, the V5 staining was did not appear to be uniformly distributed in all of the cells of DKO^{T}-TCF3FL-V5 and DKO^{T}-TCF3DN-V5 EBs (Fig 8a). The expression of Nanog and β-III-tubulin was also monitored by immunofluorescence staining. The addition of dox to both DKO-TCF3DN-V5 and DKO-TCF3FL-V5 EBs reduced the detectable protein expression of Nanog, but β-III tubulin staining was not detected. Control wild-type EBs however,

lacked Nanog staining but contained regions of intense β-III-tubulin expression (Fig 8a). We then measured the transcript levels of pluripotent and lineage specific markers in the experimental transgenic DKO EBs using qRT-PCR. Expression of TCF3FL-V5 and TCF3DN-V5 in DKO EBs significantly down-regulated the expression of Sox2 and Nanog ($p<0.05$). Similar to the defined neuronal differentiation assay, DKO^{T}-TCF3DN-V5 and DKO^{T}-TCF3FL-V5 EBs lacked expression of the neuroectoderm markers β-III-tubulin and Pax6, while wild-type EBs expressed these markers in abundance (Fig 8b).



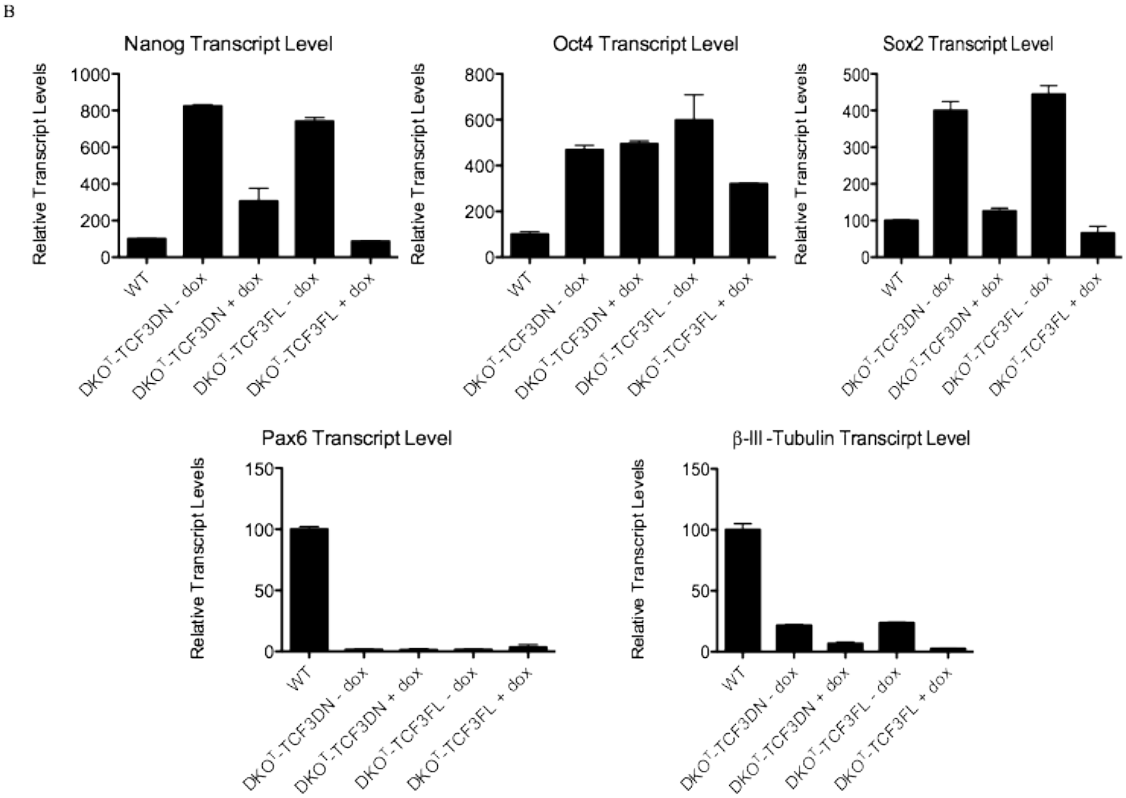


Figure 8. DKO mESCs expressing TCF3DN-V5 or TCF3FL-V5 are unable to undergo neuronal differentiation in a embryoid body differentiation assay.

(A) Immunofluorescent staining for the V5 epitope tag in EBs suggests that some DKO^{T}-TCF3DN-V5 and DKO^{T}-TCF3FL-V5 mESCs express detectable levels of the transgene. Immunofluorescent staining data also suggest that expression of TCF3DN-V5 and TCF3FL-V5 in DKO EBs down-regulate the level of Nanog. No β-III-tubulin staining was detected in any of the DKO^{T}-TCF3DN-V5 and DKO^{T}-TCF3FL-V5 EBs. 4x magnification. Bar = 200 μM.

(B) Nanog and Sox2 transcript levels were downregulated upon expression of either TCF3DN-V5 or TCF3FL-V5 in DKO EBs. Furthermore, transcript levels for neuroectoderm makers β-III-tubulin and Pax6 were also downregulated when TCF3DN-V5 or TCF3FL-V5 were expressed in DKO EBs Bars represent mean ± S.D. (n = 2).

Eomes and Cardiac troponin, markers for the mesoderm lineage were also downregulated or not detected in dox induced DKO^{T}-TCF3DN-V5 and DKO^{T}-TCF3FL-V5 EBs. However, markers for the endoderm lineage, FoxA2 and Gata4, were up-regulated in

DKO^{T}-TCF3DN-V5 and DKO^{T}-TCF3FL-V5 EBs treated with dox to levels similar to those observed in wild-type EBs (Fig 9).

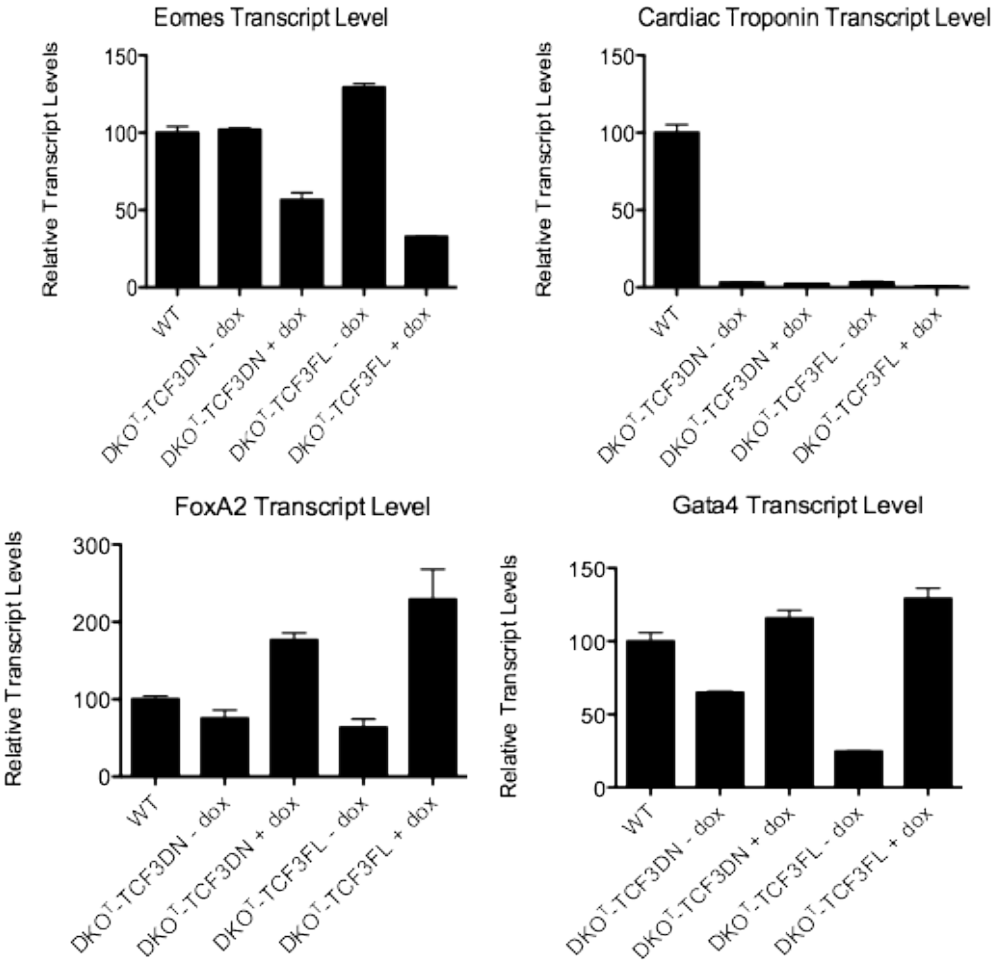


Figure 9. Endoderm lineage markers are up-regulated in DKO^{T}-TCF3DN-V5 and DKO^{T}-TCF3FL-V5 EBs

Eomes and Cardiac Troponin transcript levels were downregulated whereas FoxA2 and Gata4 transcript levels were up-regulated upon upon expression of either TCF3DN-V5 or TCF3FL-V5 in DKO EBs. Bars represent mean ± S.D. (n = 2).

5 Generation and validation of wild-type mouse embryonic stem cells that induce expression of TCF3 transgenes upon addition of doxycycline

In addition to studying the effects of expressing TCF3DN-V5 and TCF3FL-V5 in the GSK-3 DKO cells, we were also interested in studying the effects of expressing TCF3DN-V5 and TCF3FL-V5 transgenes in wild-type mESCs. The FLP-In system from Invitrogen was used to generate a single-copy, isogenic transgenic mESC line. Wild-type mESCs were modified such that an FRT site was randomly integrated into the genome, which allows for site-specific integration of a transgene into a single genetic locus. Using a wild-type mESC line called E14K, a plasmid containing a FRT site along with a lac-Zeocin fusion gene (pFRT/lacZeo) was randomly integrated into the genome of E14K cells. In order to determine the number of integrated FRT sites in the clones that were generated, Southern blot analysis with a probe against a fragment of the lacZ gene was performed (Fig 10a). Clones that have successfully integrated the lac-Zeocin plasmid also expressed the enzyme β-galactosidase. The addition of X-gal to these cells resulted in an insoluble blue product (Fig 10b).

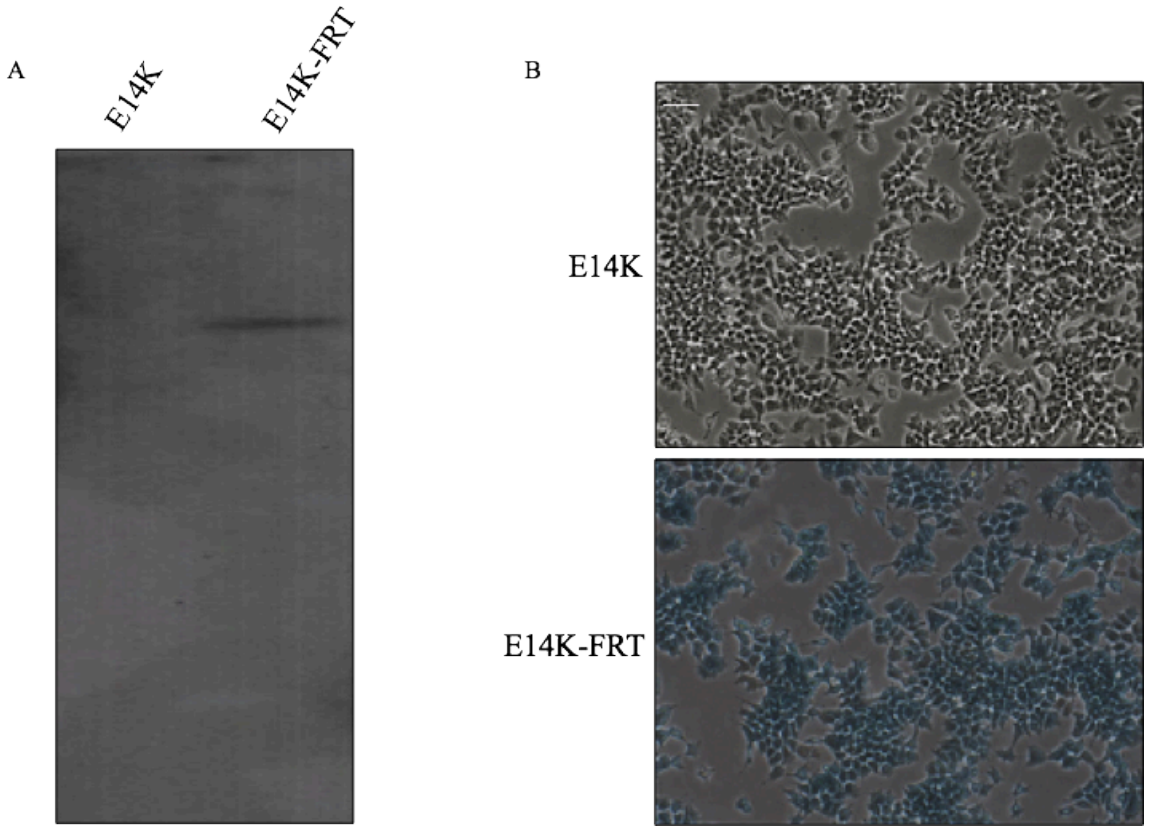


Figure 10. Generation of wild-type (E14K) mESCs with a single FRT integration site.

(A) Wild-Type mESCs (E14K) were stably transfected with a pFRT/lacZeo plasmid which introduces a FRT recombination site for the Flp-in^{TM} system randomly into the genome. In order to determine the number of FRT integration sites in the E14K-FRT cell lines, a Southern blot was performed using a LacZ probe.

(B) Clones that have successfully integrated the plasmid into the genome produce β-galactosidase. The addition of X-gal to cells expressing β-galactosidase resulted in an insoluble blue product. Bar = 100 μM

An E14K-FRT clone that had one FRT site was stably transfected with the reverse tetracycline transactivator (E14K-FRT-rTTA; E14K^{FT}). The TCF3DN-V5 transgene (E14K^{FT}-TCF3DN-V5), TCF3FL-V5 transgene (E14K^{FT}-TCF3FL-V5) and the empty vector (E14K^{FT}-vector) were then integrated into the E14K^{FT} cells at the FRT site via transient expression of Flp recombinase. Addition of dox to E14K-TCF3FL-V5 and E14K-TCF3DN-V5 cells induced expression of V5-tagged TCF3 transgenes (Fig 11).

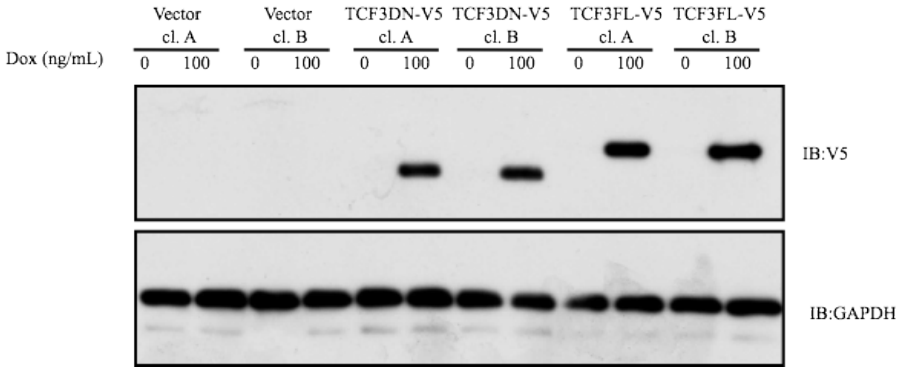


Figure 11. Generation of E14K^{FT} mESCs that allows for doxycycline-inducible regulation of transgenes.
Empty vector, TCF3DN-V5 and TCF3FL-V5 transgenes were introduced into E14K^{FT} mESCs. Cells were induced with 0 or 100 ng/mL dox for 24 hours before they were harvested for Western blot analysis. The addition of dox induced the expression of the TCF3-V5 transgenes.

The E14K^{FT}-TCF3DN-V5 and E14K^{FT}-TCF3FL-V5 mESCs were induced with dox for 24 hours to determine the localization of the expressed transgenic protein. Immunofluorescent visualization of the V5-epitope-tagged proteins revealed that the TCF3DN-V5 and TCF3FL-V5 proteins were localized to the nucleus, as expected. Furthermore, not all cells appeared to express the TCF3-V5 proteins (Fig 12).

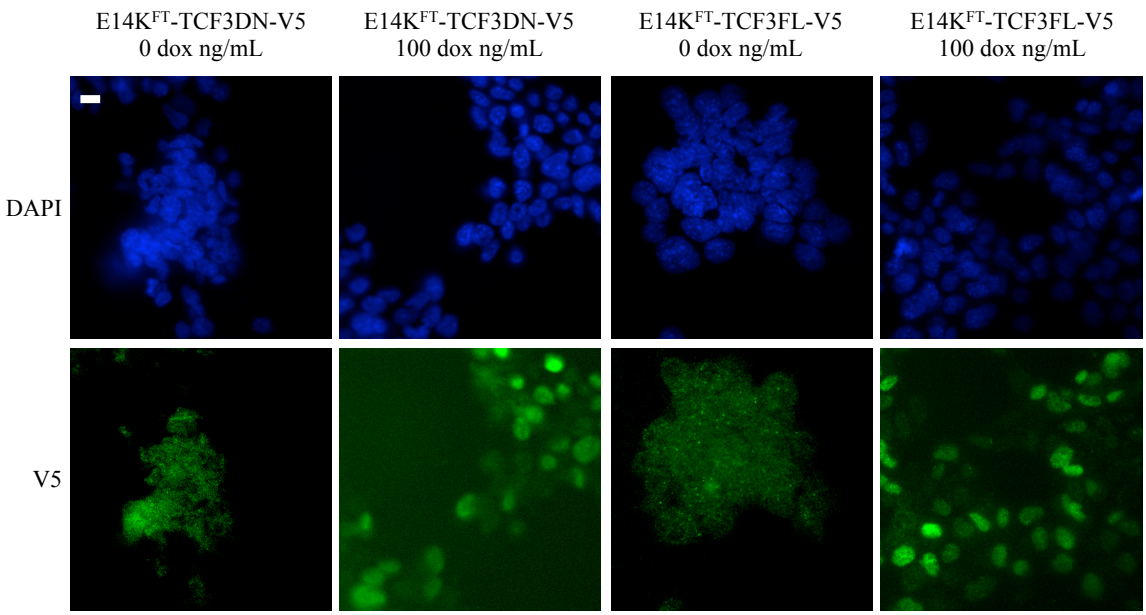


Figure 12. Nuclear localization of TCF3DN-V5 and TCF3FL-V5 proteins in E14K^{FT}-TCF3DN-V5 and E14K^{FT}-TCF3FL-V5 mESCs.
When dox was added to E14K^{FT}-TCF3DN-V5 and E14K^{FT}-TCF3FL-V5 mESCs for 24 hours, expression of the TCF3DN-V5 and TCF3FL-V5 transgenes were localized to the nucleus. In addition, there is a heterogenous mixture of cells that express and do not express the TCF3-V5 transgene. Pictures were taken with a confocal microscope, 60x magnification. Bar = 10 μM.

Since the canonical Wnt pathway is not highly activated in wild-type mESCs, expression of the TCF3DN-V5 or TCF3FL-V5 transgene has minimal effect on TCF activity ($p<0.05$ Fig 13a and 13b, first 6 columns). However, the Wnt pathway can be activated through the addition of Wnt3a conditioned medium or inhibitors of GSK-3. When the TCF3DN-V5 or TCF3FL-V5 transgene was expressed in wild-type cells treated with Wnt-3a CM, TCF activity was attenuated (Fig 13a). A number of GSK-3 inhibitors have been developed as potential therapeutics for diseases such as diabetes, stroke and Alzheimer's[160]. Through *in vitro* studies, the aminopyridine CHIR-99021 was found to be the most potent and specific inhibitor[161]. Similar to the addition of Wnt3a conditioned

medium, the addition of CHIR-99021 results in an activation of TCF activity in WT mESCs (Fig. 13b). However, dox-mediated induction of the expression of TCF3DN-V5 or TCF3FL-V5, results in significant down-regulation of TCF reporter activity ($p<0.05$ Fig. 13a and 13b). In addition, there appears to be an unexpected significant up-regulation of TCF reporter activity, which is dox-dependent as the addition of dox markedly up-regulated the TCF activity in E14K$^{\mathbf{FT}}$-vector mESCs in both the Wnt3a and CHIR-99021 treated cells ($p<0.05$ Fig. 13a and 13b).

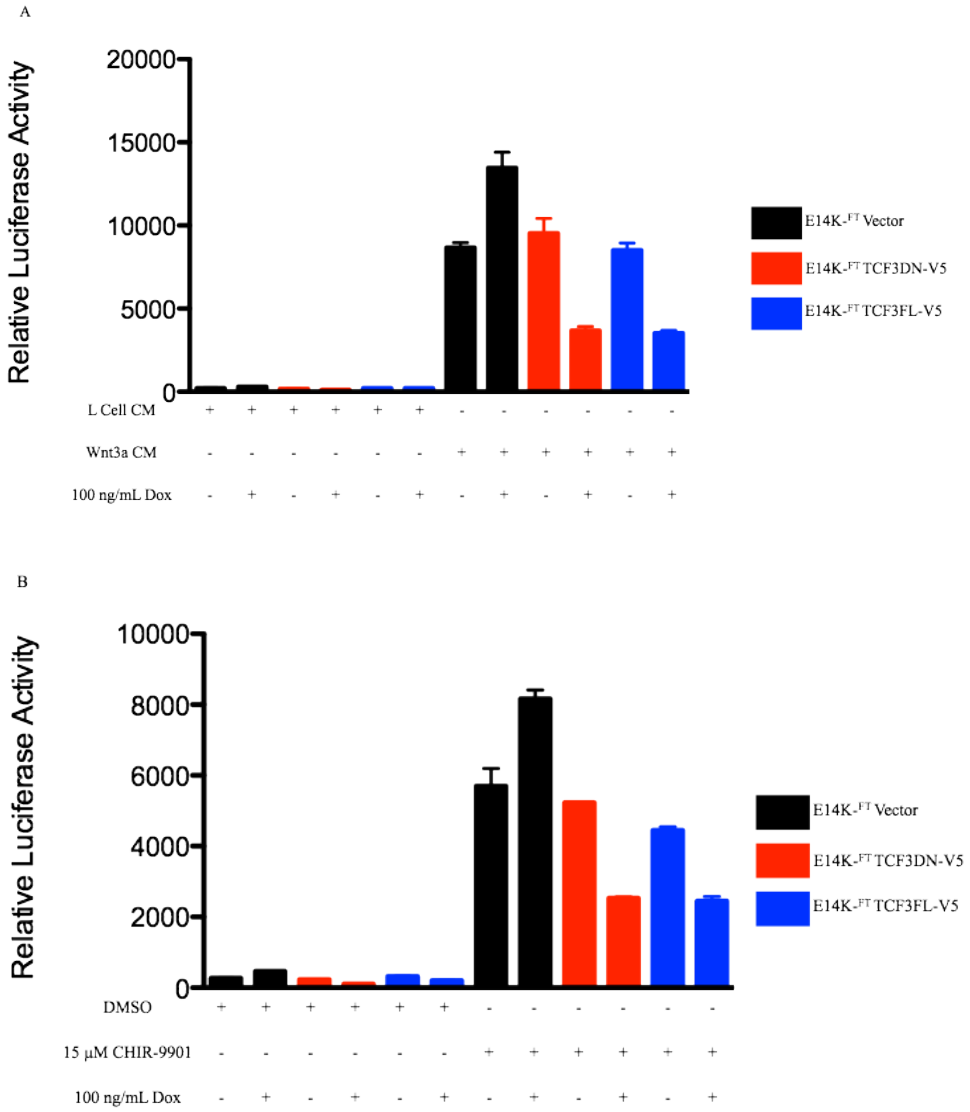


Figure 13. Full-length or dominant-negative TCF3 represses TCF reporter activity in E14K cells treated with Wnt3a-conditioned media or CHIR-99021.

E14K^{FT} cells expressing an empty vector or TCF3DN-V5 or TCF3FL-V5 transgenes were transfected with a TCF reporter plasmid (super8XTOP-flash). 24 hours after transfection, either Wnt3a-conditioned medium (A) or CHIR-99021 (B) was used to activate β-catenin signalling. Expression of TCF3DN-V5 or TCF3FL-V5 attenuated TCF activity by approximately 2-fold. Bars represent mean ± s.e.m. (n=2).

6 Addition of Wnt3a-conditioned medium or CHIR-99021 upregulates the expression of TCF3FL-V5 in E14K^{FT} mESCs.

We examined the short-term effects of expressing the TCF3DN-V5 and TCF3FL-V5 transgenes in wild-type mESCs with activated canonical Wnt signalling. E14K^{FT}-TCF3DN-V5 and E14K^{FT}-TCF3FL-V5 mESCs were grown in either a 50:50 mix of mESC media:Wnt3a -conditioned media or 10 μM of the GSK-3 inhibitor CHIR-99021. Although 15 μM of CHIR-99021 was used in the TCF reporter assays described in Figure 13b, previous studies in our lab have indicated that 10 μM CHIR-99021 is sufficient to up-regulate the canonical Wnt pathway in wild-type mESCs. After 48 hours, we observed that cells grown in Wnt3a-conditioned medium or CHIR-99021-containing medium were highly refractile and individual cells were tightly packed into colonies. Cells grown in L cell-conditioned medium or DMSO control medium remained less refractile and remained loosely associated to each other (Fig. 14a). Induced expression of either TCF3DN-V5 or TCF3FL-V5 downregulated Nanog protein levels, which is consistent with what we observed in the DKO cells. Surprisingly, we also observed that upon activation of β-catenin signalling, the protein level of TCF3FL-V5 was up-regulated compared to that observed in the control conditions (Fig. 14b). However, the levels of TCF3DN-V5 transgenic protein were similar when E14K^{FT} -TCF3DN-V5 mESCs were grown in L-cell-conditioned medium, Wnt3a-conditioned medium, DMSO control medium, or CHIR-99021-treated medium (Fig 14b).

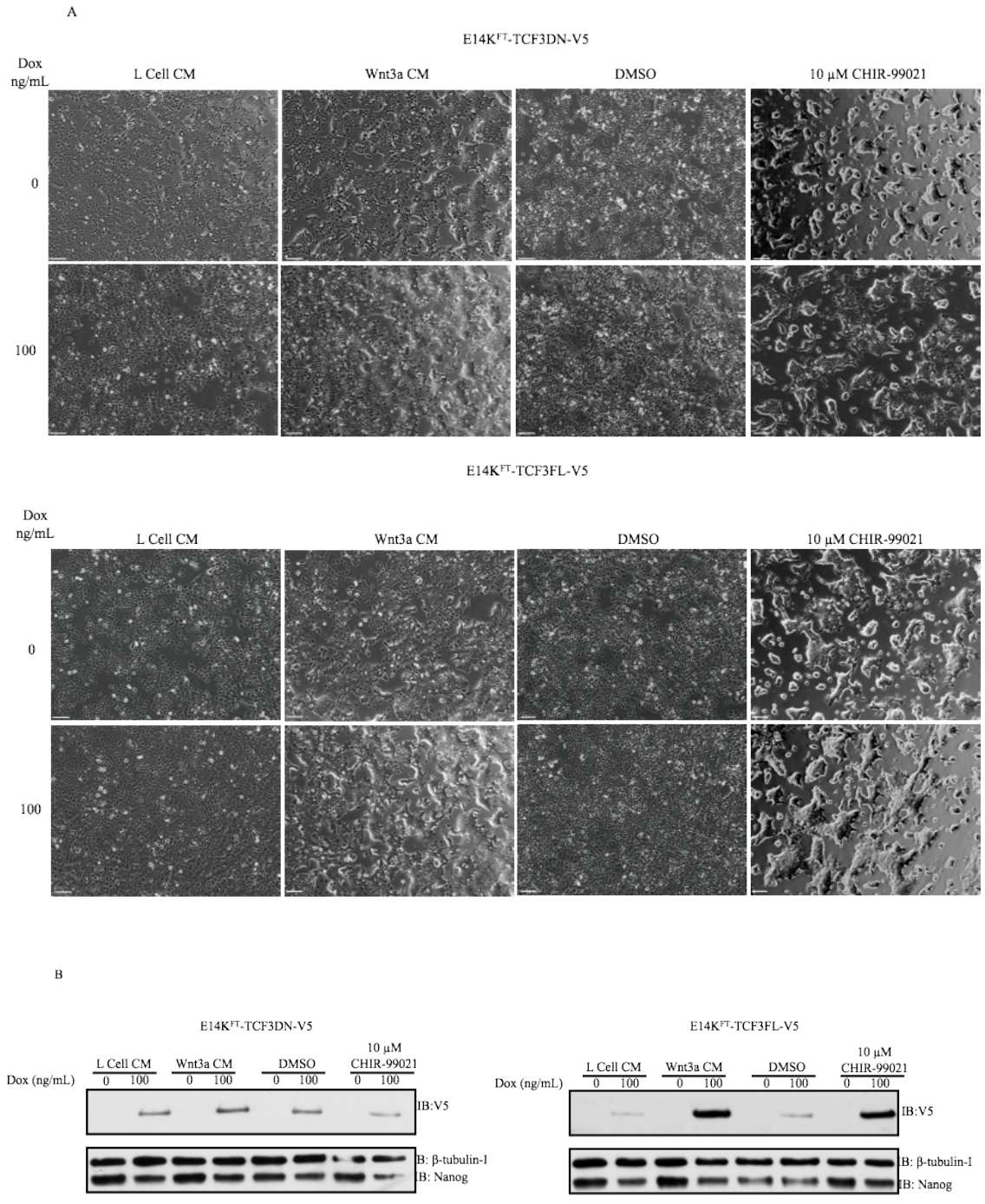


Figure 14. The consequences of overexpressing full-length or dominant-negative TCF3 in wild-type mESCs when the canonical Wnt pathway is activated with Wnt3a CM or CHIR 99021.

(A) Morphology of E14K^{FT} -TCF3DN-V5 and E14K^{FT} -TCF3FL-V5 mESCs grown in Wnt3a conditioned media or CHIR-99021 for 48 hours at 10x magnification. Bar = 100 μM.
(B) Nanog levels are downregulated when TCF3DN-V5 and TCF3FL-V5 transgenes are expressed in E14K^{FT} -TCF3DN-V5 and E14K^{FT} -TCF3FL-V5 mESCs. TCF3FL-V5 levels are up-regulated when grown in Wnt3a conditioned media or CHIR-99021.

7 E14K^{FT} -TCF3DN-V5 and E14K^{FT} -TCF3FL-V5 mESCs do not retain expression of TCF3-V5 transgene after a 10 day EB assay

To assay whether expression of TCF3DN-V5 or TCF3FL-V5 in the wild-type mESCs has an effect on differentiation, an embryoid body experiment was performed with E14K^{FT}-TCF3DN-V5 and E14K^{FT}-TCF3FL-V5 mESCs. After 10 days, the EBs were harvested. However, we noticed the expression of the transgene was lost, as there was no V5 staining in both E14K^{FT}-TCF3DN-V5 and E14K^{FT}-TCF3FL-V5 EBs treated with dox (Fig 15).

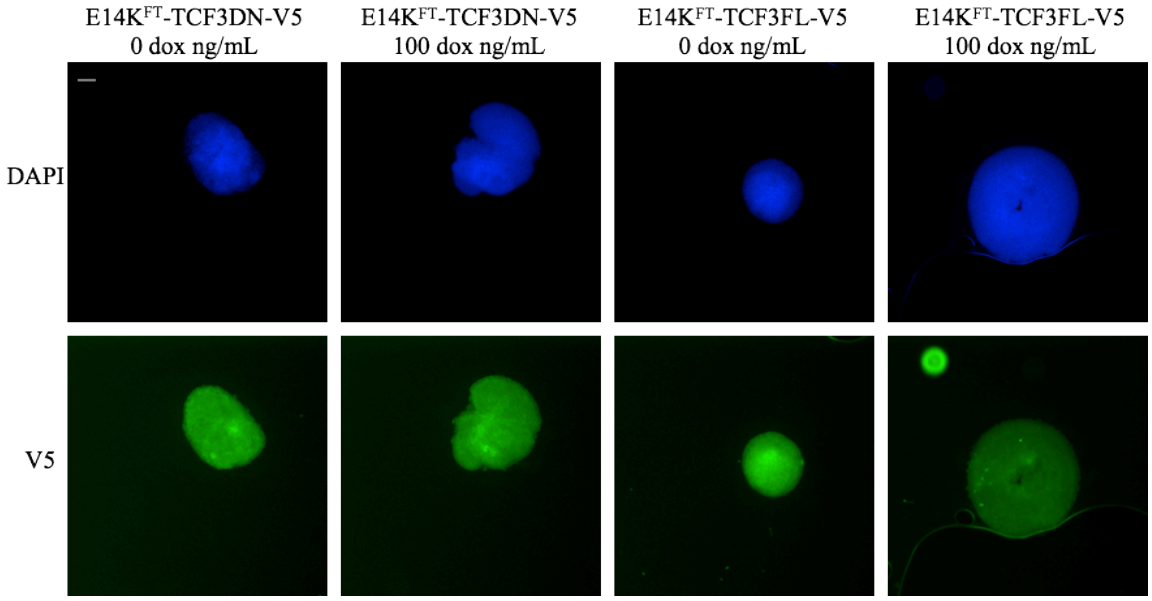


Figure 15. Transgenic expression of full-length or dominant-negative TCF3 is lost after 10 days of differentiation in embryoid bodies derived from E14K^{FT} -TCF3DN-V5 and E14K^{FT} -TCF3FL-V5 mESCs.
Ten day E14K^{FT} -TCF3DN-V5 and E14K^{FT} -TCF3FL-V5 EBs lost expression of TCF3DN-V5 and TCF3FL-V5 transgene. 4x magnification. Bar = 200 μM.

Discussion

1 Summary

Ablation of GSK-3 in mESCs renders them unable to differentiate into the neuroectoderm lineage. Furthermore, expression of a dominant-negative version of TCF1 or TCF4 is unable to rescue this blockade. Therefore, we were interested in determining whether TCF3, the most abundant TCF factor in mESCs based on transcript levels could rescue the neuroectoderm blockade.

Here, we have generated DKO and E14K mESCs that can inducibly express transgenes upon the addition of doxycycline. We have utilized these cell lines to study both the short-term and long-term effects of expressing TCF3DN-V5 and TCF3FL-V5 transgenes in mESCs. Using these cell lines, we have found that expression of either transgene is sufficient to down-regulate the expression of Nanog, a key pluripotent transcription factor (Fig. 6c and 14b). However, expression of TCF3DN-V5 or TCF3FL-V5 in DKO mESCs was unable to rescue the block to neuroectoderm differentiation displayed by these cells (Fig. 7 and 8). Moreover, we have also found that when TCF3DN-V5 or TCF3FL-V5 in DKO cells were assayed for their differentiation potential using an embryoid body assay, there was an up-regulation of endodermal lineage markers (Fig 8b). Furthermore, we observed that β-catenin appears be important in the regulation of TCF3 expression in both DKO and wild-type mESCs (Fig 3c and 14b).

2 The role TCF3 in self-renewal and differentiation

We used a doxycycline regulated system in order to express the TCF3DN-V5 and TCF3FL-V5 transgene in DKO mESCs. These DKO cells were previously modified to contain a single genomic FRT site for Flp-mediated site-specific recombination[144]. The transgenes, which were cloned into mammalian expression vectors containing tetracycline response elements and a FRT site were then introduced into the DKO cells at the FRT site. The major advantage to using the FRT/Flp-mediated system is that transgenes are introduced as a single copy in the genome of the cell. Furthermore, all transgenes are targeted to the same site. Western blot analysis indicated that 100 ng/mL of dox was sufficient to up-regulate the expression of the TCF3 transgene (Fig 3c). Moreover, when dox was not added, expression of TCF3-V5 transgene was not detected, suggesting that the system tightly controls transgene expression and is not detectably "leaky" (Fig 3c). To further validate our DKO^{T}-TCF3DN-V5 and DKO^{T}-TCF3FL-V5 system, we determined that the TCF3DN-V5 and TCF3FL-V5 transgenes both localized to the nucleus (Fig 4), which is consistent with the localization pattern of endogenous TCF3[107]. When TCF reporter activity in DKO transgenic cell lines was monitored, expression of either TCF3DN-V5 or TCF3FL-V5 repressed TCF activity (Fig 5). This is consistent with published data in which the introduction of TCF3 or a N-terminal truncation of TCF3 into $TCF3^{(-/-)}$ mESCs was able to repress basal TCF activity[153]. The repressive function of TCF3 is likely linked to its TLE binding domain as introduction of a TCF3 transgene lacking a TLE binding site into $TCF3^{(-/-)}$ mESCs up-regulated TCF activity[153].

The link between TCF3 and Nanog expression has been well established as it has been previously shown that TCF3$^{(-/-)}$ mESCs have a higher level of Nanog expression compared to wild-type cells[153]. Knockdown of TCF3 for 48 hours also had the same effect[155]. In our DKO^{T}-TCF3DN-V5 and DKO^{T}-TCF3FL-V5 mESCs, induction of the transgene for 72 hours downregulated the expression of Nanog at the protein level (Fig 5c). However, expression of Oct4 and Sox2 appears to be unchanged (Fig 5c). Although there is strong evidence that TCF3 represses Nanog, the link between Oct4 expression and TCF3 is less solid. Yi *et al*. have shown that knockdown of Oct4 or Nanog both reduced the levels of TCF3 and that the overlap between TCF3 and Oct4 bound genes is greater than that observed for TCF3 and Nanog. However, they also found that compared to wild-type cells, the transcript levels of Oct4 in TCF3 $^{(-/-)}$ mESCs remained unchanged[154]. Cole *et al*. found that TCF3 is associated with sites on the genome that are bound by Nanog and Oct4. In contrast to the study by Yi *et al.,* when TCF3 was knocked down, there was an up-regulation of Nanog, Oct4 and Sox2 protein expression[155]. Tam *et al.* found that overexpression of TCF3 in mESCs repressed an Oct4 promoter-luciferase construct[156]. The precise manner by which TCF regulates Oct4 remains to be fully elucidated.

We were interested in examining the effects on differentiation when TCF3DN-V5 and TCF3FL-V5 are expressed in DKO mESCs. Hyperactive Wnt/β-catenin signalling has been linked to a blockade in neuroectoderm differentiation[140,144,150]. Additionally,

expression of either a TCF1 or TCF4 dominant-negative in DKO cells was unable to rescue their differentiation into the neuronal lineage when they grown in N2B27 medium[144]. Normally, when grown in N2B27 medium, wild-type cells differentiate into neuroectoderm[159]. Expression of TCF3FL-V5 in DKO^{T}-TCF3FL-V5 resulted in cell death and this was observed for two independent DKO^{T}-TCF3FL-V5 clones. Expression of TCF3DN-V5 in DKO^{T}-TCF3DN-V5, however, was tolerated. Western blot and qRT-PCR analysis indicated that expression of TCF3DN-V5 in DKO mESCs downregulated the levels of Nanog and Oct4. The levels of the pluripotent markers Nanog and Oct4 in the wild-type control mESCs, maintained for 13 days in N2B27 medium were not detected (Fig. 7c and 7d). However, when markers for the neuroectoderm lineage were examined, wild-type mESCs yielded cells that robustly expressed β-III-tubulin, Pax6, Nestin and Map2, whereas both the uninduced and induced DKO^{T}-TCF3DN-V5 cells did not yield cells with detectible expression of these neuroectoderm markers. (Fig 7d). This suggests that expression of TCF3DN-V5 is unable to rescue the differentiation blockade in preventing cells from generating neuronal lineage. However, the up-regulation of TCF3 transcript levels is statistically insignificant and it is possible that the TCF3DN-V5 transgene was not expressed at high enough levels throughout the duration of this assay and as a result, the blockade to neuroectoderm differentiation was not rescued (Fig 7b).

To further test the differentiation capabilities of the DKO cells expression TCF3FL-V5 and TCF3DN-V5, embryoid bodies were generated. In contrast to what was observed in

the defined neuronal differentiation assay, the DKO^{T}-TCF3FL-V5 mESCs survived the 13 day EB assay. Similar to the N2B27 assay, expression of TCF3DN-V5 or TCF3FL-V5 was unable to promote DKO mESCs to differentiate into the neuronal lineage. No β-III-tubulin staining and low levels of β-III-tubulin and Pax6 transcript levels were observed in DKO^{T}-TCF3DN-V5 and DKO^{T}-TCF3FL-V5 mESCs in contrast to the wild-type EBs (Fig 8 and b). Furthermore, Eomes and cardiac troponin, markers of the mesoderm lineage were also downregulated in both DKO^{T}-TCF3DN-V5 and DKO^{T}-TCF3FL-V5 EBs when dox was added (Fig 9). Interestingly, FoxA2 and Gata4 transcript levels, which are markers for the endoderm lineage, were up-regulated in in both DKO^{T}-TCF3DN-V5 and DKO^{T}-TCF3FL-V5 EBs (Fig 9). It has been demonstrated that activation of the canonical Wnt pathway in mESCs, resulted in the derivation of a multipotential mesendodermal progenitor which have increased levels of mesoderm and endoderm markers[149]. TCF3 is bound to many promoters in mESCs including genes related to differentiation[154-156]. Through ChIP on chip studies, TCF3 was found to bind to Pax6, β-III-tubulin, Eomes, and FoxA2 but not to Cardiac Troponin or Gata4[156]. A more defined endoderm differentiation assay would need to be performed in order to determine whether expression of TCF3DN-V5 or TCF3FL-V5 can accelerate endodermal differentiation[162,163].

In addition to studying the effects of TCF3DN-V5 and TCF3FL-V5 in DKO cells, we were also interested in expressing TCF3DN-V5 and TCF3FL-V5 in E14K mESCs, a

wild-type line. Initially, an E14K mESC line was modified with a single FRT site to allow for site specific integration of the TCF3DN-V5 and TCF3FL-V5 transgene (Fig 10). Initial characterization of the E14K^{FT}-TCF3DN-V5 and E14K^{FT}-TCF3FL-V5 mESCs determined that expression of TCF3DN-V5 and TCF3FL-V5 could be obtained with 100 ng/mL dox and the transgenes were localized to the nucleus (Fig 11 and 12). When TCF reporter activity in E14K transgenic cell lines was monitored, expression of either TCF3DN-V5 or TCF3FL-V5 significantly repressed TCF activity when the canonical Wnt pathway was activated (Fig 13 a and b). In addition, we also observed a significant up-regulation of TCF reporter activity in E14K$^{\mathbf{FT}}$-vector mESCs when dox was added. This trend was observed for both the Wnt3a and CHIR-99021 treated cells. Potentially, in the presence of β-catenin, the reverse tetracycline transactivator may bind TCF/LEF factors to further activate the reporter (Fig 13 a and b). Furthermore, expression of TCF3DN-V5 or TCF3FL-V5 resulted in a down-regulation of Nanog expression even when the canonical Wnt pathway was not activated (Fig 14b).

3 β-catenin's role in TCF3 expression

Until recently, how TCF3 influences stemness was not well understood. The current model of TCF/LEF signalling where upon activation of the canonical Wnt pathway, β-catenin binds to TCF/LEF factors to activate transcription of Wnt target genes appears not to be the case for all TCF/LEF members. Emerging evidence suggest that for TCF3, the responses elicited upon Wnt signalling may be primarily mediated by derepression

instead of activation. This is supported by data indicating that the loss of TCF3, inhibition of GSK-3 or addition of Wnt3a all produce the same phenotype[148,158]. Moreover, β-catenin is important for this de-repression, as N-terminal mutations diminish the effect that activation of the canonical Wnt pathway has on mESC self-renewal[148,158]. Our data further suggests that the β-catenin may also play a role in regulating TCF3 expression levels.

We observed that in DKO mESCs, expression of TCF3FL-V5 was higher than TCF3DN-V5 (Fig 3c). Since the TCF3 transgenes were introduced into a single location in the genome, the variation in TCF3-V5 expression was not due to differences in the copy number of the transgene. In addition, two independent clones for each type of cell line were assayed. When cell lysates were compared in side-by-side analysis, the levels of TCF3FL-V5 were always higher than TCF3DN-V5 (Fig 3c). The major difference between the two constructs is the absence of the β-catenin binding domain in TCF3DN-V5. This suggests that the β-catenin interaction is important in modulating the protein levels of TCF3. In the presence of high Wnt/β-catenin signalling, β-catenin will interact with the TCF factors to regulate the transcription of Wnt target genes. However, β-catenin does not act alone, as a plethora of other proteins have been identified that interact with β catenin. For instance the histone acetylases, p300 and CREB-binding protein (CBP) interact with β-catenin to activate transcription of Wnt target genes[164,165]. To further validate that the β-catenin/TCF3 interaction is important for the regulation of TCF3

levels, small molecule inhibitors that disrupt the β-catenin/TCF complex can be utilized[166].

In wild-type cells, which have a low level of canonical Wnt signalling, expression levels of both TCF3DN-V5 and TCF3FL-V5 transgenes is similar. In TCF3$^{(-/-)}$ mESCs, introduction of a wild-type version or a N-terminal truncation version of TCF3 were both expressed at equivalent values[148]. However, from our data, we noticed that upon activation of the Wnt pathway either by growing the cells in Wnt3a conditioned media or via inhibition of GSK-3 with the small molecule inhibitor CHIR-99021, there was an up-regulation of the protein expression of the TCF3FL-V5 transgene. This was not observed in the E14K^{FT}-TCF3DN-V5 cells, further suggesting that the β-catenin interaction with TCF3 important in regulating the protein levels of TCF3. Since the TCF3-V5 transgenes are expressed at such low levels in wild-type cells, this could also explain why after a 10 day EB experiment, no V5 staining was detected for either E14K^{FT}-TCF3DN-V5 and E14K^{FT}-TCF3FL-V5 EBs. Throughout the duration of the assay, the E14K^{FT}-TCF3DN-V5 and E14K^{FT}-TCF3FL-V5 mESCs may have lost expression of the TCF3-V5 transgene (Fig 15).

TCF3 can also bind to it's own promoter, suggesting that it can regulate its own transcriptional activity of itself[155] . Thus, upon activation of the canonical Wnt pathway, β-catenin and its associated proteins will interact with TCF3 to regulate the expression of

TCF3. In addition, it has been recently shown that the inhibition of GSK-3 via lithium upregulated the mRNA levels of TCF4 in HEK293 cells[167]. LEF1 is also a well-known β-catenin target gene[120]. Thus, positive-feedback regulation of TCF expression may occur in several scenarios and may apply to all TCF/LEF family members in specific contexts.

4 Future work

As observed with over-expression of TCF1DN or TCF4DN, over-expression of TCF3DN-V5 is unable to rescue the neuroectoderm blockade in DKO mESCs. However, over-expression of TCF3DN-V5 and TCF4DN have different effects on mESCs, as DKO cells expressing TCF4DN could be readily propagated whereas DKO cells expression TCF3DN can not. Two potential reasons why TCF4DN and TCF3DN act differently in DKO mESCs are differential protein interactions or differential binding affinities for specific genomic targets.

Although TCF/LEFs have no inherent transactivation activity, they have the ability to interact with a wide range of proteins with gene regulatory activities. In the largest domain of TCF/LEF factors, the context regulatory domain, there is a low level of amino acid sequence similarity between the 4 factors[105], providing a mechanism by which different proteins can interact with the various TCF/LEF factors to exert different effects on the transcription of target genes. Currently, a number of different proteins have been identified to interact that TCF/LEFs but an unbiased TCF3 or TCF4 interaction screen has

not been published to date. Thus, it would be interesting to identify proteins that interact with TCF3DN and TCF4DN in order to determine whether there are proteins that uniquely interact with one TCF factor and not the other.

However, another possible explanation is that different TCF/LEF factors can bind to different Wnt responsive elements. Supporting this hypothesis is the presence of the auxiliary DNA binding domain, the C-Clamp found at C-terminus of TCF1 and TCF4 but not TCF3 and LEF1[120,121]. Inclusion of the C-clamp allows the TCF factor to bind to suboptimal WREs. The dominant-negative TCF4 variant that Kelly *et al.* used in their studies contain an E-tail[144]. In addition, differential DNA promoter binding may also be regulated by post-translational modifications of the TCF/LEF factors, such as phosphorylation by HIPK2 on TCF3, TCF4 and LEF1 (but not TCF1)[135,136]. Utilizing technologies such as ChIP on chip or ChIP Seq could identify TCF3DN and TCF4DN binding sites globally and answer the question as to whether TCF4DN and TCF3DN have differential binding affinities for specific target sequences.

Although TCF3 is the most widely studied TCF factor in mESCs, Western blot analysis revealed that all four TCF/LEF factors are expressed at readily detectible levels[144]. We have shown that expression of either TCF3DN (this report) or TCF4DN/TCF1DN[144] is unable to rescue the neuroectoderm blockade in GSK-3 DKO mESCs, suggesting that β-catenin uses a TCF-independent pathway to regulate pluripotency. However, recent

evidence has emerged suggesting that in certain contexts, TCF/LEF factors can synergistically work together to regulate target gene expression. For instance, in mESCs, when an N-terminal truncation of TCF3 was expressed and TCF1 was knocked down, self-renewal was substantially reduced upon Wnt3a treatment compared to that observed in cells that expressed TCF3DN in which normal TCF1 expression was retained[158]. The combined results of the study by Yi *et al.* suggests that activation of the canonical Wnt pathway stimulates self-renewal by suppressing TCF3's repressive nature but also requires TCF1 activity[158]. Furthermore, conditional ablation of either TCF3 or TCF4 in epithelial stem cells has no deleterious effects on epithelial homeostasis, but a conditional TCF3/TCF4 double-knockout mouse was impaired in long-term epidermal homeostasis and proper hair follicle formation[168]. In normal colon crypts, both a full-length TCF4 and a dominant-negative TCF1 is expressed in cells to help balance cell growth[115]. However, upon carcinogenesis, a full-length isoform of TCF1 is abundantly expressed in concert with the full-length TCF4. As a result, the balance between Wnt promotion and repression in colon crypts is lost[115]. Thus, focusing on a single TCF/LEF factor in mESCs is insufficient to elucidate the function of TCF/LEF signalling in mESC self-renewal and differentiation which is likely an interplay between various forms of the TCF/LEF family members.

5 Conclusions

Here were have established DKO and wild-type mESCs cell lines that can inducibly regulate the expression of a transgene through doxycycline administration. This system allows us to study both the short and long term effects of expressing TCF3DN-V5 and TCF3FL-V5 in mESCs. We have found that expression of TCF3DN-V5 and TCF3FL-V5 in both GSK-3 DKO and wild-type mESCs can repress TCF activity. Furthermore, although expression of TCF3DN-V5 or TCF3FL-V5 represses the levels of Nanog, it is unable rescue the neuroectoderm blockade associated with hyperactive Wnt/β-catenin signalling, but may poise the cells to differentiate into the endoderm lineage. Moreover, using this system we have also established a potential role for β-catenin in regulating the expression levels of TCF3 itself. From our results presented in this thesis and recent published reports, the enigmatic role of TCF3 in regulating pluripotency have begun to be clarified. This new insight into TCF/LEF signalling in mESCs will allow us to better understand the mechanisms by which mESCs regulate their self-renewal and and differentiation.

References

1. Till, J.E. & McCulloch, E.A. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat. Res.* **14**, 213–222 (1961).
2. Jaenisch, R. & Young, R. Stem Cells, the Molecular Circuitry of Pluripotency and Nuclear Reprogramming. *Cell* **132**, 567–582 (2008).
3. Nichols, J. et al. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell* **95**, 379–391 (1998).
4. Avilion, A.A. et al. Multipotent cell lineages in early mouse development depend on SOX2 function. *Genes & Development* **17**, 126–140 (2003).
5. Mitsui, K. et al. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell* **113**, 631–642 (2003).
6. Rosner, M.H. et al. A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo. *Nature* **345**, 686–692 (1990).
7. Niwa, H., Miyazaki, J. & Smith, A.G. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat Genet* **24**, 372–376 (2000).
8. Masui, S. et al. Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells. *Nat Cell Biol* **9**, 625–635 (2007).
9. Mullin, N.P. et al. The pluripotency rheostat Nanog functions as a dimer. *Biochem. J.* **411**, 227–231 (2008).
10. Chambers, I. et al. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell* **113**, 643–655 (2003).
11. Boyer, L.A. et al. Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell* **122**, 947–956 (2005).
12. Boyer, L.A. et al. Polycomb complexes repress developmental regulators in murine embryonic stem cells. *Nature* **441**, 349–353 (2006).
13. Evans, M.J. & Kaufman, M.H. Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292**, 154–156 (1981).
14. Martin, G.R. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci USA* **78**, 7634–7638 (1981).
15. Desbaillets, I., Ziegler, U., Groscurth, P. & Gassmann, M. Embryoid bodies: an in vitro model of mouse embryogenesis. *Exp. Physiol.* **85**, 645–651 (2000).
16. Smith, A.G. et al. Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature* **336**, 688–690 (1988).
17. Niwa, H., Burdon, T., Chambers, I. & Smith, A. Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. *Genes & Development* **12**, 2048–2060 (1998).

18. Matsuda, T. et al. STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells. *EMBO J.* **18**, 4261–4269 (1999).
19. Wray, J., Kalkan, T. & Smith, A.G. The ground state of pluripotency. *Biochem. Soc. Trans* **38**, 1027 (2010).
20. Kidder, B.L., Yang, J. & Palmer, S. Stat3 and c-Myc genome-wide promoter occupancy in embryonic stem cells. *PLoS ONE* **3**, e3932 (2008).
21. Ying, Q.L., Nichols, J., Chambers, I. & Smith, A. BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell* **115**, 281–292 (2003).
22. Qi, X. et al. BMP4 supports self-renewal of embryonic stem cells by inhibiting mitogen-activated protein kinase pathways. *Proc Natl Acad Sci USA* **101**, 6027–6032 (2004).
23. MacDonald, B.T., Tamai, K. & He, X. Wnt/beta-Catenin Signaling: Components, Mechanisms, and Diseases. *Developmental Cell* **17**, 9–26 (2009).
24. Hao, J., Li, T.-G., Qi, X., Zhao, D.-F. & Zhao, G.-Q. WNT/beta-catenin pathway up-regulates Stat3 and converges on LIF to prevent differentiation of mouse embryonic stem cells. *Developmental Biology* **290**, 81–91 (2006).
25. Ogawa, K., Nishinakamura, R., Iwamatsu, Y., Shimosato, D. & Niwa, H. Synergistic action of Wnt and LIF in maintaining pluripotency of mouse ES cells. *Biochem Biophys Res Commun* **343**, 159–166 (2006).
26. Singla, D.K., Schneider, D.J., LeWinter, M.M. & Sobel, B.E. wnt3a but not wnt11 supports self-renewal of embryonic stem cells. *Biochem Biophys Res Commun* **345**, 789–795 (2006).
27. Anton, R., Kestler, H.A. & Kühl, M. Beta-catenin signaling contributes to stemness and regulates early differentiation in murine embryonic stem cells. *FEBS Lett* **581**, 5247–5254 (2007).
28. Berge, ten, D. et al. Embryonic stem cells require Wnt proteins to prevent differentiation to epiblast stem cells. *Nat Cell Biol* **13**, 1070–1075 (2011).
29. Thomson, J.A. et al. Embryonic stem cell lines derived from human blastocysts. *Science* **282**, 1145–1147 (1998).
30. Pera, M.F. & Tam, P.P.L. Extrinsic regulation of pluripotent stem cells. *Nature* **465**, 713–720 (2010).
31. Xu, C. et al. Basic Fibroblast Growth Factor Supports Undifferentiated Human Embryonic Stem Cell Growth Without Conditioned Medium. *Stem Cells* **23**, 315–323 (2005).
32. Wang, G. et al. Noggin and bFGF cooperate to maintain the pluripotency of human embryonic stem cells in the absence of feeder layers. *Biochem Biophys Res Commun* **330**, 934–942 (2005).
33. Xu, R.-H. et al. Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. *Nat Meth* **2**, 185–190 (2005).

34. Levenstein, M.E. et al. Basic Fibroblast Growth Factor Support of Human Embryonic Stem Cell Self-Renewal. *Stem Cells* **24**, 568–574 (2006).
35. Xu, R.-H. et al. BMP4 initiates human embryonic stem cell differentiation to trophoblast. *Nat Biotech* **20**, 1261–1264 (2002).
36. James, D., Levine, A.J., Besser, D. & Hemmati-Brivanlou, A. TGFbeta/activin/nodal signaling is necessary for the maintenance of pluripotency in human embryonic stem cells. *Development* **132**, 1273–1282 (2005).
37. Beattie, G.M. et al. Activin A Maintains Pluripotency of Human Embryonic Stem Cells in the Absence of Feeder Layers. *Stem Cells* **23**, 489–495 (2005).
38. Smith, J.R. et al. Inhibition of Activin/Nodal signaling promotes specification of human embryonic stem cells into neuroectoderm. *Developmental Biology* **313**, 107–117 (2008).
39. Xu, R.-H. et al. NANOG Is a Direct Target of TGFβ/Activin-Mediated SMAD Signaling in Human ESCs. *Cell Stem Cell* **3**, 196–206 (2008).
40. Vallier, L. et al. Activin/Nodal signalling maintains pluripotency by controlling Nanog expression. *Development* **136**, 1339–1349 (2009).
41. Dahéron, L. et al. LIF/STAT3 signaling fails to maintain self-renewal of human embryonic stem cells. *Stem Cells* **22**, 770–778 (2004).
42. Humphrey, R.K. et al. Maintenance of pluripotency in human embryonic stem cells is STAT3 independent. *Stem Cells* **22**, 522–530 (2004).
43. Burdon, T., Stracey, C., Chambers, I., Nichols, J. & Smith, A. Suppression of SHP-2 and ERK signalling promotes self-renewal of mouse embryonic stem cells. *Developmental Biology* **210**, 30–43 (1999).
44. Nichols, J. & Smith, A. Naive and primed pluripotent states. *Cell Stem Cell* **4**, 487–492 (2009).
45. Blair, K., Wray, J. & Smith, A. The Liberation of Embryonic Stem Cells. *PLoS Genet* **7**, e1002019 (2011).
46. Brons, I.G.M. et al. Derivation of pluripotent epiblast stem cells from mammalian embryos. *Nature* **448**, 191–195 (2007).
47. Tesar, P.J. et al. New cell lines from mouse epiblast share defining features with human embryonic stem cells. *Nature* **448**, 196–199 (2007).
48. Hanna, J.H., Saha, K. & Jaenisch, R. Pluripotency and Cellular Reprogramming: Facts, Hypotheses, Unresolved Issues. *Cell* **143**, 508–525 (2010).
49. Buecker, C. et al. A Murine ESC-like State Facilitates Transgenesis and Homologous Recombination in Human Pluripotent Stem Cells. *Cell Stem Cell* **6**, 535–546 (2010).
50. Hanna, J. et al. Human embryonic stem cells with biological and epigenetic characteristics similar to those of mouse ESCs. *Proceedings of the National Academy of Sciences* **107**, 9222–9227 (2010).

51. Hausmann, G., Bänziger, C. & Basler, K. Helping Wingless take flight: how WNT proteins are secreted. *Nat. Rev. Mol. Cell Biol.* **8**, 331–336 (2007).
52. Willert, K. et al. Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature* **423**, 448–452 (2003).
53. Komekado, H., Yamamoto, H., Chiba, T. & Kikuchi, A. Glycosylation and palmitoylation of Wnt-3a are coupled to produce an active form of Wnt-3a. *Genes Cells* **12**, 521–534 (2007).
54. Takada, R. et al. Monounsaturated Fatty Acid Modification of Wnt Protein: Its Role in Wnt Secretion. *Developmental Cell* **11**, 791–801 (2006).
55. Bhanot, P. et al. A new member of the frizzled family from Drosophila functions as a Wingless receptor. *Nature* **382**, 225–230 (1996).
56. He, X., Semenov, M., Tamai, K. & Zeng, X. LDL receptor-related proteins 5 and 6 in Wnt/beta-catenin signaling: arrows point the way. *Development* **131**, 1663–1677 (2004).
57. Tamai, K. et al. LDL-receptor-related proteins in Wnt signal transduction. *Nature* **407**, 530–535 (2000).
58. Holmen, S.L., Salic, A., Zylstra, C.R., Kirschner, M.W. & Williams, B.O. A novel set of Wnt-Frizzled fusion proteins identifies receptor components that activate beta -catenin-dependent signaling. *J Biol Chem* **277**, 34727–34735 (2002).
59. Riggleman, B., Schedl, P. & Wieschaus, E. Spatial expression of the Drosophila segment polarity gene armadillo is posttranscriptionally regulated by wingless. *Cell* **63**, 549–560 (1990).
60. Nelson, W.J. & Nusse, R. Convergence of Wnt, beta-catenin, and cadherin pathways. *Science* **303**, 1483–1487 (2004).
61. Haegel, H. et al. Lack of beta-catenin affects mouse development at gastrulation. *Development* **121**, 3529–3537 (1995).
62. Huelsken, J. et al. Requirement for beta-catenin in anterior-posterior axis formation in mice. *J Cell Biol* **148**, 567–578 (2000).
63. Price, M.A. CKI, there's more than one: casein kinase I family members in Wnt and Hedgehog signaling. *Genes & Development* **20**, 399–410 (2006).
64. Cohen, P. & Frame, S. The renaissance of GSK3. *Nat. Rev. Mol. Cell Biol.* **2**, 769–776 (2001).
65. Woodgett, J.R. Molecular cloning and expression of glycogen synthase kinase-3/ factor A. *EMBO J.* **9**, 2431–2438 (1990).
66. Doble, B.W., Patel, S., Wood, G.A., Kockeritz, L.K. & Woodgett, J.R. Functional Redundancy of GSK-3α and GSK-3β in Wnt/β-Catenin Signaling Shown by Using an Allelic Series of Embryonic Stem Cell Lines. *Developmental Cell* **12**, 957–971 (2007).
67. Hoeflich, K.P. et al. Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. *Nature* **406**, 86–90 (2000).

68. Force, T. & Woodgett, J.R. Unique and overlapping functions of GSK-3 isoforms in cell differentiation and proliferation and cardiovascular development. *J Biol Chem* **284**, 9643–9647 (2009).
69. Gao, C. & Chen, Y.-G. Dishevelled: The hub of Wnt signaling. *Cellular Signalling* **22**, 717–727 (2010).
70. Rubinfeld, B. et al. Association of the APC gene product with beta-catenin. *Science* **262**, 1731–1734 (1993).
71. Su, L.K., Vogelstein, B. & Kinzler, K.W. Association of the APC tumor suppressor protein with catenins. *Science* **262**, 1734–1737 (1993).
72. Korinek, V. et al. Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma. *Science* **275**, 1784–1787 (1997).
73. Behrens, J. et al. Functional interaction of an axin homolog, conductin, with beta-catenin, APC, and GSK3beta. *Science* **280**, 596–599 (1998).
74. Kishida, S. et al. Axin, a negative regulator of the wnt signaling pathway, directly interacts with adenomatous polyposis coli and regulates the stabilization of beta-catenin. *J Biol Chem* **273**, 10823–10826 (1998).
75. Liu, C. et al. Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism. *Cell* **108**, 837–847 (2002).
76. Amit, S. et al. Axin-mediated CKI phosphorylation of beta-catenin at Ser 45: a molecular switch for the Wnt pathway. *Genes & Development* **16**, 1066–1076 (2002).
77. Yost, C. et al. The axis-inducing activity, stability, and subcellular distribution of beta-catenin is regulated in Xenopus embryos by glycogen synthase kinase 3. *Genes & Development* **10**, 1443–1454 (1996).
78. Aberle, H., Bauer, A., Stappert, J., Kispert, A. & Kemler, R. beta-catenin is a target for the ubiquitin-proteasome pathway. *EMBO J.* **16**, 3797–3804 (1997).
79. Marikawa, Y. & Elinson, R.P. beta-TrCP is a negative regulator of Wnt/beta-catenin signaling pathway and dorsal axis formation in Xenopus embryos. *Mech. Dev.* **77**, 75–80 (1998).
80. Ikeda, S. et al. Axin, a negative regulator of the Wnt signaling pathway, forms a complex with GSK-3beta and beta-catenin and promotes GSK-3beta-dependent phosphorylation of beta-catenin. *EMBO J.* **17**, 1371–1384 (1998).
81. Jho, E.H., Lomvardas, S. & Costantini, F. A GSK3beta phosphorylation site in axin modulates interaction with beta-catenin and Tcf-mediated gene expression. *Biochem Biophys Res Commun* **266**, 28–35 (1999).
82. Bilic, J. et al. Wnt Induces LRP6 Signalosomes and Promotes Dishevelled-Dependent LRP6 Phosphorylation. *Science* **316**, 1619–1622 (2007).
83. Zeng, X. et al. Initiation of Wnt signaling: control of Wnt coreceptor Lrp6 phosphorylation/activation via frizzled, dishevelled and axin functions. *Development* **135**, 367–375 (2008).

84. Zeng, X. et al. A dual-kinase mechanism for Wnt co-receptor phosphorylation and activation. *Nature* **438**, 873–877 (2005).
85. MacDonald, B.T., Yokota, C., Tamai, K., Zeng, X. & He, X. Wnt signal amplification via activity, cooperativity, and regulation of multiple intracellular PPPSP motifs in the Wnt co-receptor LRP6. *J Biol Chem* **283**, 16115–16123 (2008).
86. Tamai, K. et al. A mechanism for Wnt coreceptor activation. *Mol Cell* **13**, 149–156 (2004).
87. Liang, J. et al. Transmembrane Protein 198 Promotes LRP6 Phosphorylation and Wnt Signaling Activation. *Molecular and Cellular Biology* **31**, 2577–2590 (2011).
88. Cselenyi, C.S. et al. LRP6 transduces a canonical Wnt signal independently of Axin degradation by inhibiting GSK3's phosphorylation of beta-catenin. *Proceedings of the National Academy of Sciences* **105**, 8032–8037 (2008).
89. Piao, S. et al. Direct Inhibition of GSK3β by the Phosphorylated Cytoplasmic Domain of LRP6 in Wnt/β-Catenin Signaling. *PLoS ONE* **3**, e4046 (2008).
90. Wu, G., Huang, H., Abreu, J.G. & He, X. Inhibition of GSK3 Phosphorylation of β-Catenin via Phosphorylated PPPSPXS Motifs of Wnt Coreceptor LRP6. *PLoS ONE* **4**, e4926 (2009).
91. Taelman, V.F. et al. Wnt signaling requires sequestration of glycogen synthase kinase 3 inside multivesicular endosomes. *Cell* **143**, 1136–1148 (2010).
92. Blitzer, J.T. & Nusse, R. A critical role for endocytosis in Wnt signaling. *BMC Cell Biol.* **7**, 28 (2006).
93. Yamamoto, H., Komekado, H. & Kikuchi, A. Caveolin Is Necessary for Wnt-3a-Dependent Internalization of LRP6 and Accumulation of β-Catenin. *Developmental Cell* **11**, 213–223 (2006).
94. Fagotto, F., Glück, U. & Gumbiner, B.M. Nuclear localization signal-independent and importin/karyopherin-independent nuclear import of beta-catenin. *Curr Biol* **8**, 181–190 (1998).
95. Yokoya, F., Imamoto, N., Tachibana, T. & Yoneda, Y. beta-catenin can be transported into the nucleus in a Ran-unassisted manner. *Mol. Biol. Cell* **10**, 1119–1131 (1999).
96. Suh, E.-K. & Gumbiner, B.M. Translocation of β-catenin into the nucleus independent of interactions with FG-rich nucleoporins. *Experimental Cell Research* **290**, 447–456 (2003).
97. Wu, X. et al. Rac1 Activation Controls Nuclear Localization of β-catenin during Canonical Wnt Signaling. *Cell* **133**, 340–353 (2008).
98. Zhang, N. et al. FoxM1 Promotes β-Catenin Nuclear Localization and Controls Wnt Target-Gene Expression and Glioma Tumorigenesis. *Cancer Cell* **20**, 427–442 (2011).
99. Krieghoff, E., Behrens, J. & Mayr, B. Nucleo-cytoplasmic distribution of beta-catenin is regulated by retention. *Journal of Cell Science* **119**, 1453–1463 (2006).

100. Sokol, S.Y. Maintaining embryonic stem cell pluripotency with Wnt signaling. *Development* **138**, 4341–4350 (2011).
101. Mao, C.D. & Byers, S.W. Cell-Context Dependent TCF/LEF Expression and Function: Alternative Tales of Repression, De-Repression and Activation Potentials. *Crit. Rev. Eukaryot. Gene Expr.* **21**, 207–236 (2011).
102. Travis, A., Amsterdam, A., Belanger, C. & Grosschedl, R. LEF-1, a gene encoding a lymphoid-specific protein with an HMG domain, regulates T-cell receptor alpha enhancer function [corrected]. *Genes & Development* **5**, 880–894 (1991).
103. van de Wetering, M., Oosterwegel, M., Dooijes, D. & Clevers, H. Identification and cloning of TCF-1, a T lymphocyte-specific transcription factor containing a sequence-specific HMG box. *EMBO J.* **10**, 123–132 (1991).
104. Castrop, J., van Norren, K. & Clevers, H. A gene family of HMG-box transcription factors with homology to TCF-1. *Nucleic Acids Research* **20**, 611 (1992).
105. Arce, L., Yokoyama, N.N. & Waterman, M.L. Diversity of LEF/TCF action in development and disease. *Oncogene* **25**, 7492–7504 (2006).
106. van de Wetering, M., Castrop, J., Korinek, V. & Clevers, H. Extensive alternative splicing and dual promoter usage generate Tcf-1 protein isoforms with differential transcription control properties. *Molecular and Cellular Biology* **16**, 745–752 (1996).
107. Weise, A. et al. Alternative splicing of Tcf7l2 transcripts generates protein variants with differential promoter-binding and transcriptional activation properties at Wnt/ -catenin targets. *Nucleic Acids Research* **38**, 1964–1981 (2010).
108. Pukrop, T. et al. Identification of two regulatory elements within the high mobility group box transcription factor XTCF-4. *J Biol Chem* **276**, 8968–8978 (2001).
109. Liu, F., van den Broek, O., Destrée, O. & Hoppler, S. Distinct roles for Xenopus Tcf/Lef genes in mediating specific responses to Wnt/beta-catenin signalling in mesoderm development. *Development* **132**, 5375–5385 (2005).
110. Salomonis, N. et al. Alternative splicing regulates mouse embryonic stem cell pluripotency and differentiation. *Proceedings of the National Academy of Sciences* **107**, 10514–10519 (2010).
111. Behrens, J. et al. Functional interaction of beta-catenin with the transcription factor LEF-1. *Nature* **382**, 638–642 (1996).
112. Molenaar, M. et al. XTcf-3 transcription factor mediates beta-catenin-induced axis formation in Xenopus embryos. *Cell* **86**, 391–399 (1996).
113. van de Wetering, M. et al. Armadillo coactivates transcription driven by the product of the Drosophila segment polarity gene dTCF. *Cell* **88**, 789–799 (1997).
114. Hovanes, K. et al. Beta-catenin-sensitive isoforms of lymphoid enhancer factor-1 are selectively expressed in colon cancer. *Nat Genet* **28**, 53–57 (2001).
115. Najdi, R. et al. A Wnt kinase network alters nuclear localization of TCF-1 in colon cancer. *Oncogene* **28**, 4133–4146 (2009).

116. Giese, K., Cox, J. & Grosschedl, R. The HMG domain of lymphoid enhancer factor 1 bends DNA and facilitates assembly of functional nucleoprotein structures. *Cell* **69**, 185–195 (1992).
117. van de Wetering, M. & Clevers, H. Sequence-specific interaction of the HMG box proteins TCF-1 and SRY occurs within the minor groove of a Watson-Crick double helix. *EMBO J.* **11**, 3039–3044 (1992).
118. Love, J.J. et al. Structural basis for DNA bending by the architectural transcription factor LEF-1. *Nature* **376**, 791–795 (1995).
119. Hecht, A. & Stemmler, M.P. Identification of a promoter-specific transcriptional activation domain at the C terminus of the Wnt effector protein T-cell factor 4. *J Biol Chem* **278**, 3776–3785 (2003).
120. Atcha, F.A., Munguia, J.E., Li, T.W.H., Hovanes, K. & Waterman, M.L. A new beta-catenin-dependent activation domain in T cell factor. *J Biol Chem* **278**, 16169–16175 (2003).
121. Atcha, F.A. et al. A Unique DNA Binding Domain Converts T-Cell Factors into Strong Wnt Effectors. *Molecular and Cellular Biology* **27**, 8352–8363 (2007).
122. Blauwkamp, T.A., Chang, M.V. & Cadigan, K.M. Novel TCF-binding sites specify transcriptional repression by Wnt signalling. *EMBO J.* (2008).doi:10.1038/emboj.2008.80
123. Huber, O. et al. Nuclear localization of beta-catenin by interaction with transcription factor LEF-1. *Mech. Dev.* **59**, 3–10 (1996).
124. Graham, T.A., Weaver, C., Mao, F., Kimelman, D. & Xu, W. Crystal structure of a beta-catenin/Tcf complex. *Cell* **103**, 885–896 (2000).
125. Sierra, J., Yoshida, T., Joazeiro, C.A. & Jones, K.A. The APC tumor suppressor counteracts beta-catenin activation and H3K4 methylation at Wnt target genes. *Genes & Development* **20**, 586–600 (2006).
126. Wang, S. & Jones, K.A. CK2 controls the recruitment of Wnt regulators to target genes in vivo. *Curr Biol* **16**, 2239–2244 (2006).
127. Jennings, B.H. & Ish-Horowicz, D. The Groucho/TLE/Grg family of transcriptional co-repressors. *Genome Biol* **9**, 205 (2008).
128. Cavallo, R.A. et al. Drosophila Tcf and Groucho interact to repress Wingless signalling activity. *Nature* **395**, 604–608 (1998).
129. Roose, J. et al. The Xenopus Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. *Nature* **395**, 608–612 (1998).
130. Brantjes, H., Roose, J., van de Wetering, M. & Clevers, H. All Tcf HMG box transcription factors interact with Groucho-related co-repressors. *Nucleic Acids Research* **29**, 1410–1419 (2001).
131. Arce, L., Pate, K.T. & Waterman, M.L. Groucho binds two conserved regions of LEF-1 for HDAC-dependent repression. *BMC Cancer* **9**, 159 (2009).

132. Daniels, D.L. & Weis, W.I. Beta-catenin directly displaces Groucho/TLE repressors from Tcf/Lef in Wnt-mediated transcription activation. *Nat Struct Mol Biol* **12**, 364–371 (2005).
133. Lee, E., Salic, A. & Kirschner, M. Physiological regulation of ss-catenin stability by Tcf3 and CK1 epsilon. *J Cell Biol* **154**, 983–993 (2001).
134. Hämmerlein, A., Weiske, J. & Huber, O. A second protein kinase CK1-mediated step negatively regulates Wnt signalling by disrupting the lymphocyte enhancer factor-1/β-catenin complex. *Cell. Mol. Life Sci.* **62**, 606–618 (2005).
135. Hikasa, H. et al. Regulation of TCF3 by Wnt-Dependent Phosphorylation during Vertebrate Axis Specification. *Developmental Cell* **19**, 521–532 (2010).
136. Hikasa, H. & Sokol, S.Y. Phosphorylation of TCF Proteins by Homeodomain-interacting Protein Kinase 2. *Journal of Biological Chemistry* **286**, 12093–12100 (2011).
137. Ishitani, T. et al. The TAK1-NLK-MAPK-related pathway antagonizes signalling between beta-catenin and transcription factor TCF. *Nature* **399**, 798–802 (1999).
138. Ishitani, T., Ninomiya-Tsuji, J. & Matsumoto, K. Regulation of lymphoid enhancer factor 1/T-cell factor by mitogen-activated protein kinase-related Nemo-like kinase-dependent phosphorylation in Wnt/beta-catenin signaling. *Molecular and Cellular Biology* **23**, 1379–1389 (2003).
139. Mahmoudi, T. et al. The kinase TNIK is an essential activator of Wnt target genes. *EMBO J.* **28**, 3329–3340 (2009).
140. Kielman, M.F. et al. Apc modulates embryonic stem-cell differentiation by controlling the dosage of β-catenin signaling. *Nat Genet* **32**, 594–605 (2002).
141. Sato, N., Meijer, L., Skaltsounis, L., Greengard, P. & Brivanlou, A.H. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nat Med* **10**, 55–63 (2004).
142. Dravid, G. et al. Defining the Role of Wnt/β-Catenin Signaling in the Survival, Proliferation, and Self-Renewal of Human Embryonic Stem Cells. *Stem Cells* **23**, 1489–1501 (2005).
143. Ying, Q.-L. et al. The ground state of embryonic stem cell self-renewal. *Nature* **453**, 519–523 (2008).
144. Kelly, K.F. et al. β-catenin enhances Oct-4 activity and reinforces pluripotency through a TCF-independent mechanism. *Cell Stem Cell* **8**, 214–227 (2011).
145. Takao, Y., Yokota, T. & Koide, H. Beta-catenin up-regulates Nanog expression through interaction with Oct-3/4 in embryonic stem cells. *Biochem Biophys Res Commun* **353**, 699–705 (2007).
146. Wagner, R.T., Xu, X., Yi, F., Merrill, B.J. & Cooney, A.J. Canonical Wnt/β-Catenin Regulation of Liver Receptor Homolog-1 Mediates Pluripotency Gene Expression. *Stem Cells* **28**, 1794–1804 (2010).

147. Lyashenko, N. et al. Differential requirement for the dual functions of β-catenin in embryonic stem cell self-renewal and germ layer formation. *Nat Cell Biol* **13**, 753–761 (2011).
148. Wray, J. et al. Inhibition of glycogen synthase kinase-3 alleviates Tcf3 repression of the pluripotency network and increases embryonic stem cell resistance to differentiation. *Nat Cell Biol* **13**, 1–9 (2011).
149. Bakre, M.M. et al. Generation of multipotential mesendodermal progenitors from mouse embryonic stem cells via sustained Wnt pathway activation. *J Biol Chem* **282**, 31703–31712 (2007).
150. Haegele, L. et al. Wnt signalling inhibits neural differentiation of embryonic stem cells by controlling bone morphogenetic protein expression. *Mol. Cell. Neurosci.* **24**, 696–708 (2003).
151. Aubert, J., Dunstan, H., Chambers, I. & Smith, A. Functional gene screening in embryonic stem cells implicates Wnt antagonism in neural differentiation. *Nat Biotech* **20**, 1240–1245 (2002).
152. Čajánek, L. et al. Wnt/beta-catenin signaling blockade promotes neuronal induction and dopaminergic differentiation in embryonic stem cells. *Stem Cells* **27**, 2917–2927 (2009).
153. Pereira, L., Yi, F. & Merrill, B.J. Repression of Nanog Gene Transcription by Tcf3 Limits Embryonic Stem Cell Self-Renewal. *Molecular and Cellular Biology* **26**, 7479–7491 (2006).
154. Yi, F., Pereira, L. & Merrill, B.J. Tcf3 Functions as a Steady-State Limiter of Transcriptional Programs of Mouse Embryonic Stem Cell Self-Renewal. *Stem Cells* **26**, 1951–1960 (2008).
155. Cole, M.F., Johnstone, S.E., Newman, J.J., Kagey, M.H. & Young, R.A. Tcf3 is an integral component of the core regulatory circuitry of embryonic stem cells. *Genes & Development* **22**, 746–755 (2008).
156. Tam, W.-L. et al. T-Cell Factor 3 Regulates Embryonic Stem Cell Pluripotency and Self-Renewal by the Transcriptional Control of Multiple Lineage Pathways. *Stem Cells* **26**, 2019–2031 (2008).
157. Guo, G., Huang, Y., Humphreys, P., Wang, X. & Smith, A. A PiggyBac-Based Recessive Screening Method to Identify Pluripotency Regulators. *PLoS ONE* **6**, e18189 (2011).
158. Yi, F. et al. Opposing effects of Tcf3 and Tcf1 control Wnt stimulation of embryonic stem cell self-renewal. *Nat Cell Biol* **13**, 1–11 (2011).
159. Ying, Q.-L. & Smith, A.G. Defined conditions for neural commitment and differentiation. *Meth. Enzymol.* **365**, 327–341 (2003).
160. Cohen, P. & Goedert, M. GSK3 inhibitors: development and therapeutic potential. *Nat Rev Drug Discov* **3**, 479–487 (2004).

161. Bain, J. et al. The selectivity of protein kinase inhibitors: a further update. *Biochem. J.* **408**, 297 (2007).
162. Kubo, A. et al. Development of definitive endoderm from embryonic stem cells in culture. *Development* **131**, 1651–1662 (2004).
163. Yasunaga, M. et al. Induction and monitoring of definitive and visceral endoderm differentiation of mouse ES cells. *Nat Biotech* **23**, 1542–1550 (2005).
164. Takemaru, K.I. & Moon, R.T. The transcriptional coactivator CBP interacts with beta-catenin to activate gene expression. *J Cell Biol* **149**, 249–254 (2000).
165. Hecht, A., Vleminckx, K., Stemmler, M.P., van Roy, F. & Kemler, R. The p300/CBP acetyltransferases function as transcriptional coactivators of beta-catenin in vertebrates. *EMBO J.* **19**, 1839–1850 (2000).
166. Lepourcelet, M. et al. Small-molecule antagonists of the oncogenic Tcf/beta-catenin protein complex. *Cancer Cell* **5**, 91–102 (2004).
167. Struewing, I. et al. The balance of TCF7L2 variants with differential activities in Wnt-signaling is regulated by lithium in a GSK3beta-independent manner. *Biochem Biophys Res Commun* **399**, 245–250 (2010).
168. Nguyen, H. et al. Tcf3 and Tcf4 are essential for long-term homeostasis of skin epithelia. *Nat Genet* **41**, 1068–1075 (2009).