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Glycogen Synthase Kinase 3 (GSK-3) involvement in regulation of mouse embryonic stem cell fate

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Glycogen Synthase Kinase 3 (GSK-3) involvement in regulation of mouse embryonic stem cell fate

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Abstract

Pluripotent embryonic stem cells (ESCs) have great potential for use in regenerative medicine and drug discovery. However, in order to harness this potential, we must understand the molecular mechanisms regulating self-renewal and differentiation. Previous studies had implicated Glycogen Synthase Kinase-3 (GSK-3) in both maintenance of pluripotency and neuronal differentiation. To investigate the role of GSK-3 in control of ESC fate further, we used GSK-3α/β double knock-out (DKO) cells and specific bis-indolylmaleimides that selectively inhibit GSK-3. Self-renewal of DKO GSK-3 ESCs and ESCs treated with GSK-3 inhibitors was enhanced in the presence of LIF and serum but not in the absence of LIF. On the other hand, GSK-3 inhibition during embryoid body differentiation promoted ESC differentiation towards mesendodermal lineage.

Several mechanisms of action by which GSK-3 inhibition enhances self-renewal has been proposed, most studies to date suggest that β-catenin mediates the effect of GSK-3 in self-renewal. However, β-catenin independent mechanisms including stabilisation of c-Myc and regulation of Nanog have also been proposed. In the present study, we investigated the effects that GSK-3 inhibition has on the levels, stability and synthesis of pluripotency-associated transcription factors, including Nanog, Tbx3, c-Myc, Zscan4c and Oct4. Levels of Nanog and Tbx3 were elevated following GSK-3 inhibition, c-Myc and Zscan4c levels were also up-regulated but to a lesser extent. Alternatively, Oct4 protein was not dramatically affected. Moreover, Nanog and Tbx3 levels were maintained when GSK-3 was inhibited upon removal of LIF, or in 2i conditions. These effects were not due to increase protein stability or entirely to increase in transcription, but instead arose as a result of enhanced protein synthesis, promoted by inhibition of GSK-3. Increased loading of mRNAs encoding pluripotency factors onto polysomes occurred following inhibition of GSK-3, supporting a role for GSK-3 inhibition in increasing translation of these mRNAs. Moreover, general or cap-dependent translation did not seem to be increased following GSK-3 inhibition suggesting that GSK-3 regulates translation of specific mRNAs. The present study supports a role for GSK-3 in fine-tuning the protein levels of transcription factors in pluripotent ESCs, which could play an important role in establishing the gene regulatory network in ESCs.

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Abbreviations

APS	Ammonium Persulphate
ADP	Adenosine Diphosphate
APC	Adenomatous polyposis coil
ATP	Adenosine Triphosphate
BIO	6-bromoindirubin 3'-oxime
BMP	Bone morphogenic protein
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
CKI	Casein kinase I
ChiP	Chromatin immunoprecipitation
CHIR	CHIRON99021
CHX	Cyclohexidime
CM	Conditioned Media
DKO	GSK-3α/β double knock-out
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
E	Stage of embryonic development, indicating days post coitum
EC	Embryonal Carcinoma
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic acid discodium salt
EGF	Epidermal Growth Factor
eIFs	eukaryotic translation initiation factors
EpiSC	Epiblast Stem Cell
ERK	Extracellular regulated kinase
ESC	Embryonic Stem Cell
FACS	Fluorescence Activated Cell Sorter
FBS	Foetal bovine serum
Fgf	Fibroblast Growth Factor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDP	Guanosine Diphosphate
GFP	Green Fluorescence protein
GMEM	Glasgow Minimum Eagle Medium
Grb2	Growth Factor Receptor bound protein 2
GSK-3	Glycogen synthase kinase 3
GTP	Guanosine Triphosphate
hESC	Human Embryonic Stem Cell
HRP	Horse Radish Peroxidase
ICM	Inner Cell Mass
Id	Inhibitor of differentiation
IGF	Insulin-like growth factor
IL	Interleukin
iPSC	Induced pluripotent stem cells
IRES	Internal Ribosomal Entry Site
JAK	Janus Kinase
kDa	Kilo Daltons
Klf	Kruppel-like family

Klf4	Krupple-like factor 4
KO SR	Knockout Serum Replacement
LIF	Leukemia inhibitory factor
LIFR	LIF Receptor
LRP	Low density lipoprotein
MAPK	Mitogen activated protein kinase
MEK	Mitogen-activated extracellular signal-regulated kinase
Met-tRNAi	Methione transfer RNA
mRNA	Messenger RNA
MTG	Monothioglycerol
mTOR	Mammalian target of rapamycin
NEAA	Non-essential amino acids
N2B27	Defined media, 1:1 Neurobasal:DMEM F12 plus N2 and B2 supplements.
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PD	PD0325901
PDK1	3-phosphoinositide-dependent protein kinase 1
p-Erk	Phospho-Erk
PH	Pleckstrin Homology
PI3K	Phosphoinositide 3-kinase
PI(3)P	Phosphatidylinositol-3-phosphate
PKB	Protein kinase B
PMSF	Phenylmethylsulphonylfluoride
POU	Pit Oct Unc
pSmad1	Phospho-Smad1
PTEN	Phosphatase and tensin homologue
PS	Primitive Streak
qPCR	Quantitative PCR
RT-PCR	Reverse Transcription PCR
S	SU5402
SDS-PAGE	Sodium Dodecyl Sulphate-Poly acrylamide gel electrophoresis
S.E.M.	Standard Error of the Mean
Shp2	Src-homology 2 containing phosphatase 1
shRNA	Short-hairpin Ribonucleic acid
siRNA	Short interfering Ribonucleic acid
Smad	*Caenorhabditis elegans* protein Sma, *Drosophila* mothers against
Stat3	Signal Transducer and Activator of Transcription 3
S6K1	p70 ribosomal S6 kinase (S6K)
TAE	Tris-acetate EDTA
TBS	Tris Buffered Saline
TBST	TBS plus 0.05%
TEMED	Tetramethylethylenediamine
Tet	Tetracycline
tTA	Tetracycline-sensitive transactivator
2i	2 inhibitors, GSK-3 and MEK
3i	3 inhibitors, GSK-3, MEK and FGFR
4-OHT	4-hydroximatoxifen
5’UTR	5’ untranslated region

1 CHAPTER: INTRODUCTION

1.1 Embryonic stem cells (ESCs) – an overview

ESCs are undifferentiated cells that have unique and remarkable properties. One of these properties is their self-renewal capacity, which is the capability to give rise to at least one daughter equivalent to the mother cell. ESCs can, therefore, proliferate in culture generating a large number of undifferentiated stem cells. The other remarkable property of ESCs is their pluripotency, which can be defined as the ability of ESCs to differentiate into derivatives of the three embryonic germ layers, ectoderm, mesoderm and endoderm (Figure 1.1). In addition, demonstration of pluripotency is the ability of ESCs to contribute to the formation of chimeras if injected back into a blastocyst (Smith, 2001).

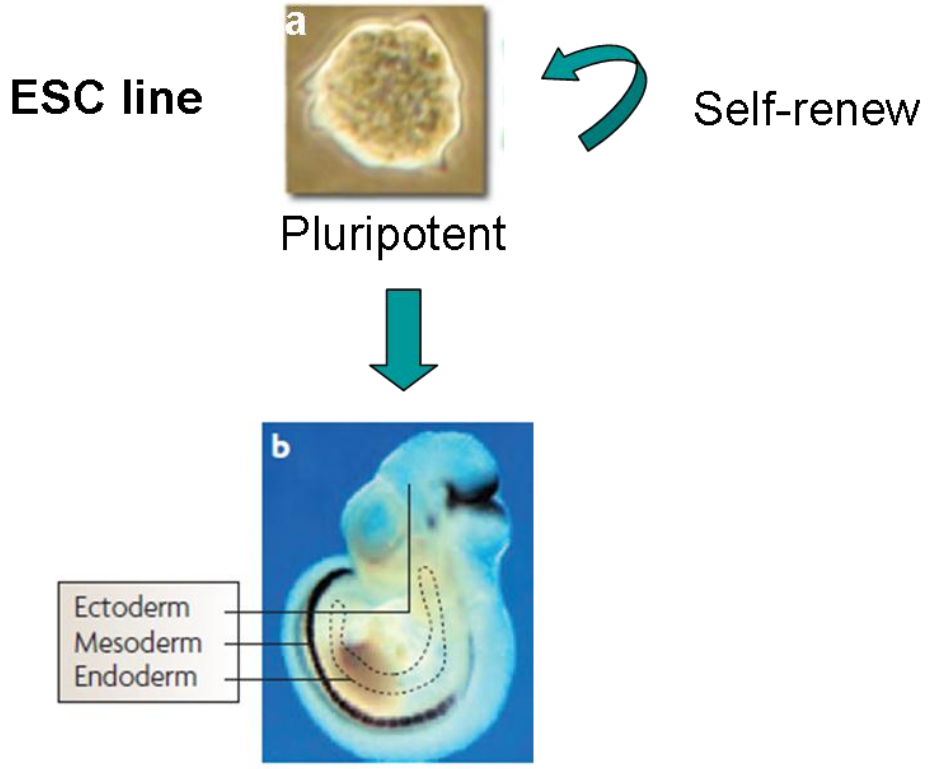

Figure 1.1 Properties of ESCs. ESCs have self-renewal (a) and pluripotency (b) properties. Self-renewal is the ability to give rise to at least one undifferentiated ESC daughter. (b) Pluripotency is the ability to give rise to derivates of the three germ layers, ectoderm, mesoderm and endoderm. In b, a post-gastrulated mouse embryo and location of the embryonic germ layers is shown (Modified from Tam and Loebel, 2007).

Due to their properties, ESCs are an attractive source of cells that can be used in different fields such as regenerative medicine, drug development and toxicity screening and as an *in vitro* system to study early development. Regarding regenerative medicine they have the potential to be used in cell-based therapies to treat diseases for which they are currently no effective treatments, such as Parkinson´s disease, diabetes, traumatic spinal cord injury and myocardial infarction, which arise by loss of cells. These diseases could be treated by transplanting specific cell types obtained *in vitro* following differentiation of ESCs. ESCs are also a potent tool in drug development and toxicity screening. Current methods to test drug safety involves toxicity screening in cell lines which may de-differentiate in culture such as in the case of hepatocytes and thus they do not precisely predict what will happen in the human body (Elaut et al., 2006). For this reason, many drugs currently on the market can have secondary toxic effects with hepatotoxicity being a very common side effect. Much effort is being put into directing differentiation of human ESCs into differentiate cell types, such as hepatocytes for toxicity screening. Recently, the laboratory in which I have been carrying out my PhD succeeded in generating definitive endoderm with hepatic potential from human embryonic stem cells by inhibiting GSK-3 (Bone et al., 2011). This is a breakthrough in stem cell research and it is likely to revolutionise the way drugs are currently tested. Finally, ESCs are a very good *in vitro* system to study early development. For instance, they can contribute to our understanding of the regulatory pathways that regulate lineage specification by studying their *in vitro* differentiation potential.

Despite the importance of understanding the signalling pathways governing stem cell fate to maintain ESCs in culture and control their differentiation towards a desired cell type, mechanisms controlling embryonic stem cell fate are not fully understood. Unravelling the multiple signals regulating stem cell fate remains one hurdle to be overcome before ESCs can fulfil their potential.

1.1.1 History of ESCs.

In 1970, two groups reported the remarkable finding that early mouse embryos could generate teratocarcinomas when implanted into adult mice (Solter et al., 1970; Stevens, 1970). Teratocarcinomas are malignant tumours that not only contain differentiated cell types from all the three germ layers but also undifferentiated cells which can be propagated in culture and are known as embryonal carcinoma (EC) cells. Previous to Stevens' and Solter's work, teratocarcinomas were known to occur spontaneously in testes and thought to be restricted to male germ cells (reviewed by Stevens 1983). Remarkably, EC cells derived from the teratocarcinomas generated by embryo injection into an adult mouse, could also be propagated *in vitro* and had the ability to differentiate into endoderm, mesoderm and ectoderm (Kleinsmith and Pierce, 1964; Martin and Evans, 1975). The fact that teratocarcinomas could only be generated by injecting pre-gastrula embryos or from grafts containing epiblast indicated that EC cells originated from the epiblast (Diwan and Stevens, 1976). In fact, EC cells are phenotypically similar to epiblast cells and some EC cell lines can contribute to the embryo giving rise to chimeras (Brinster, 1974). However, the majority of EC cells do not significantly contribute to chimeras, they are tumorigenic and frequently aneuploid so they cannot give rise to mature gametes. The work on EC cells led to the isolation of mouse ESCs by Evans and Kaufman in 1981. One of the important steps towards the isolation of mouse ESCs was the finding that EC cell cultures could be established by co-cultured with mitotically inactivated embryonic fibroblasts, which were thought to supply EC cells with nutrients supporting their growth and they were named feeder layers. EC cell cultures grown on feeder layers also have a high differentiation capacity (Martin et al., 1977). Hence, ESCs were derived from mouse by plating embryos after 3.5 days of fertilization (the blastocyst stage) or directly plating inner cell masses (ICM) onto a feeder layer of mitotically inactivated fibroblasts (Figure 1.2) (Evans & Kaufman 1981, Martin 1981, cited in Smith, 2001). ESCs, unlike EC cells, retain a diploid karyotype, they can integrate into the embryo, generating viable chimeras and they are able to produce functional gametes. Years of study of mouse ESCs led to the successful isolation of human ESCs for the first time in 1998 (Thomson et al., 1998).

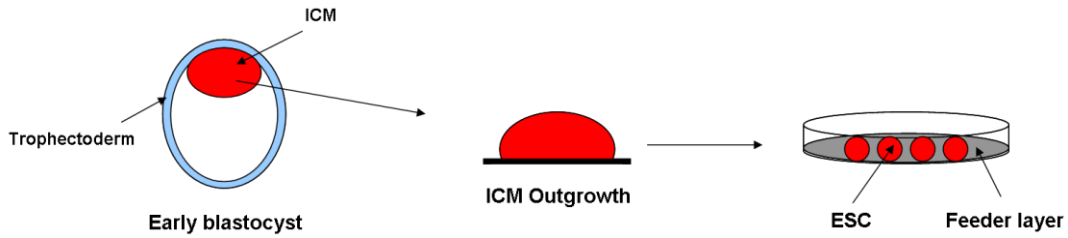

Figure 1.2 First protocol developed for ESC derivation. ESCs were derived by plating early blastocysts formed at E3.5 or the ICM onto a feeder layer of mitotically inactivated fibroblast. Modified from Nichols and Smith, 2011.

1.1.2 Early embryo development.

Embryonic development in mammals begins with cell divisions of the fertilised egg into an 8-cell stage-embryo, which has the same size as the zygote. At this stage all the cells of the embryo are equivalent and each blastomere has the potential to give rise to all the cell lineages (Johnson and McConnell, 2004). Embryonic development proceeds by compaction of the blastomeres, which become polarised and successive cell division generates the morula (16-cell stage) that has either outer or inner cells. The outer cells will form an epithelium, called the trophectoderm, and will give rise to the placenta and the inner cells will form the inner cell mass (ICM), which will give rise to the embryo and the yolk sack (Rossant and Tam, 2004). The trophectoderm secretes fluid internally leading to the generation of the blastocoel (a fluid filled cavity) and the ICM becomes restricted to one side of the hollow structure. 3.5 days after fertilization the blastocyst is formed (Figure 1.3). The trophectoderm and the ICM are not only different morphologically but also molecularly. The trophectoderm is characterised by the expression of the transcription factors Cdx2 and Eomes (Strumpf et al., 2005) and the ICM by the expression of Oct4 and Nanog (Chambers et al., 2003; Chazaud et al., 2006; Mitsui, 2003). Cdx2 and Oct4 are essential for the establishment of the trophectoderm and ICM respectively (Nichols, 1998; Strumpf et al., 2005). The ICM segregates into the hypoblast, also known as primitive endoderm, which will form the yolk salk, and the epiblast, that will give rise to the embryo. The hypoblast and the epiblast are clearly distinctive by the time of implantation (E4.5) and they are characterised by the expression of transcription factors Nanog in the case of the epiblast and Gata4 and Gata 6 in the hypoblast (Plusa et al., 2008). The epiblast is also characterised by the

reactivation of the inactive X paternal chromosome in female mouse embryos (Silva et al., 2009). The silent X chromosome is not reactivated in the trophectoderm or the primitive endoderm. The fact that reactivation of X chromosomes is a feature of successful reprogramming of somatic cells to induced pluripotent stem (iPS) cells (Silva et al., 2008) suggests that X chromosome reactivation may be an epigenetic event that facilitates chromatin accessibility to establish the pluripotent state in the epiblast (Nichols and Smith, 2011). After implantantion, the egg cylinder is formed which consist of trophectoderm, epiblast and hypoblast.

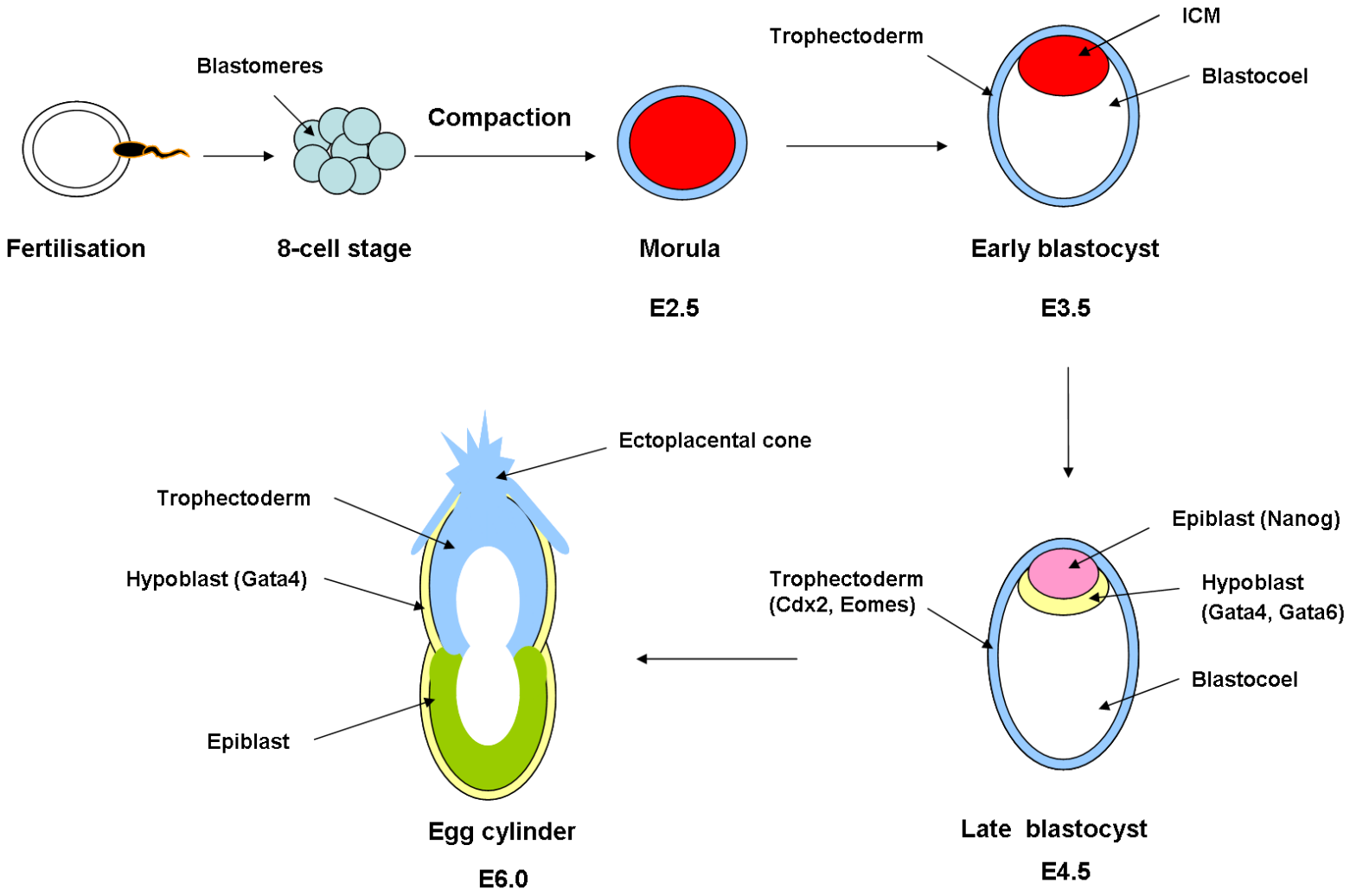
Blastomeres
Compaction
Trophectoderm
ICM
Blastocoel
Fertilisation
8-cell stage
Morula
E2.5
Early blastocyst
E3.5
Ectoplacental cone
Trophectoderm
Hypoblast (Gata4)
Epiblast
Egg cylinder
E6.0
Epiblast (Nanog)
Trophectoderm
(Cdx2, Eomes)
Hypoblast
(Gata4, Gata6)
Blastocoel
Late blastocyst
E4.5

Figure 1.3 Early development of mouse embryo. Embryonic development begins with cell divisions of the fertilised egg into an 8-cell stage-embryo, where all the cells of the embryo are equivalent and are named blastomeres (Johnson and McConnell, 2004). After compaction of the blastomeres and cell division the morula is formed at E2.5. Cells in the morula are either outer or inner cells. The outer cells will form the trophectoderm, and the inner cells will form the inner cell mass (ICM), (Rossant and Tam, 2004). The trophectoderm secretes fluid internally generating the blastocoel and the ICM becomes restricted to one side of the hollow structure forming the early blastocyst at E3.5. The ICM segregates into the hypoblast and the epiblast forming the late blastocyst at E4.5. By the time of implantation, the blastocyst is composed of three lineages, epiblast, hypoblast and trophectoderm, which are disctintive and characterised for the expression of different transcription factors. Nanog expression is restricted to the epiblast, Gata 4 and Gata 6 to the hypoblast and Cdx2 and Eomes to the trophectoderm. After implantation the egg cylinder is formed (Modified from Nichols and Smith, 2011).

1.1.3 ESC derivation.

ESCs are derived from the epiblast of the late blastocyst at day 3.5 of embryonic development (Evans and Kaufman, 1981; Martin, 1981). ESC derivation can be facilitated by making use of a natural event called diapause (Evans and Kaufman, 1981). This is a phenomenon whereby mice can delay implantation of embryos while they have another litter. Diapause can be experimentally induced by injecting mothers with tamoxifen when the developing embryos are at the morula stage. ESCs were originally derived by the plating of blastocysts, or ICMs isolated from blastocysts by immunosurgery, onto feeder layers in the presence of foetal calf serum (Figure 1.2). The cytokine leukaemia inhibitory factor (LIF) was later identified as the factor produced by feeder layers that contributes to maintenance of ESCs, and thus feeder layers were replaced by LIF (Smith et al., 1988; Williams et al., 1988). A few years ago, Bone morphogenetic protein 4 (BMP4) was found to be able to replace serum in culture allowing the derivation of ESCs in serum-free media supplemented with LIF and BMP4 (Ying et al., 2003a). However, until recently, ESC derivation was inconsistent and it was evident that ESCs could be more easily isolated from some mouse strains, such as 129, than others, such as CBA, C57BL/6 or NOD. LIF maintains pluripotency by activation of the STAT3 cascade (Niwa et al., 1998) but LIF also activates Erk MAP kinases, which directs differentiation. The variability in efficiency to derive ESCs from different mouse strains was thought to be due to variations in Erk signalling (Batlle-Morera et al., 2008; Wray et al., 2010). In accordance with this, inhibition of Erk signalling improved ESCs derivation from C57BL/6 and CBA strains (Batlle-Morera et al., 2008). However, the breakthrough in ESC derivation came with the development of the 2i media, which is a chemically defined media supplemented with two kinase inhibitors, one for the Mitogen-activated ERK kinase (MEK) and the other for the Glycogen Synthase Kinase (GSK-3) (Ying et al., 2008). The development of 2i media has allowed the derivation of ESCs from all mouse strains including the most refractory one, Non-obese diabetic (NOD) and also the derivation of ESCs from rats for the first time (Buehr et al., 2008; Li et al., 2008; Nichols et al., 2009). The fact that 2i media allowed efficient derivation of ESCs led to the idea that ESCs may in fact be identical to the epiblast cells rather than a tissue culture creation. This hypothesis was confirmed by studying the effect of blocking Erk signalling in the pre-implantation embryo (Nichols et al.,

2009). Blockade of Erk signalling at the 8-cell stage results in inhibition of hypoblast development and the whole ICM becomes epiblast and acquires pluripotency, confirmed by the expression of Nanog, reactivation of the X paternal chromosome and the contribution of epiblast cells to chimaeras with germline transmission. Blockade of Erk signalling after 3.75 days of fertilisation, when the hypoblast is thought to already be determined (Chazaud et al., 2006) did not prevent formation of the hypoblast, suggesting that the effect of the inhibitor is to divert the ICM into epiblast rather than discriminatory destruction of the hypoblast. The authors concluded that ESCs are indeed like naïve epiblast cells and both are highly susceptible to Erk signalling.

1.2 Other pluripotent cells.

1.2.1 Epiblast Stem cells.

ESCs were the only pluripotent cell lines to be derived from the early embryo until 2007 when Epiblast stem cells (EpiSCs) were derived from the mouse post-implantation epiblast (Brons et al., 2007; Tesar et al., 2007). Although EpiSCs have similarities with mouse ESCs (mESCs), such as expression of *Nanog* and *Oct4* and the ability to differentiate into somatic cell types and primordial germ cells, they were different to mESCs regarding morphology, cell culture requirements and methodology required to passage them. mESCs form rounded compact colonies, which can be passaged by dissociation to single cells using trypsin and they grow in the presence of LIF and Serum, LIF and BMP4 or 2i media. In contrast, EpiSCs grow as flattened cell monolayers rather than forming colonies, dissociation to single cells by trypsin results in extensive cell death meaning they need to be passage by mechanical dissociation, they have to be cultured in the presence of Activin A and FGF2, rather than LIF and they are unable to colonise the embryo. Moreover, the signals regulating differentiation, the epigenetic state and the gene expression of EpiSCs and mESCs are different. In fact, EpiSCs have more similarities with human ESCs than with mESCs and this suggests that human ESCs are more likely to correspond to the same developmental stage as EpiSCs. EpiSCs are certainly ideal to study whether the differences observed between mouse and human ESCs are due to

variation between species or to derivation from different stages of development (Brons et al., 2007; Tesar et al., 2007).

Recent studies showed that it is possible to convert EpiSCs to ESCs in response to LIF-STAT3 signalling or by forced expression of *Klf4* and culture in 2i media and LIF (Bao et al., 2009; Guo et al., 2009). In Bao´s study, opposite to Bron´s and Tesar´s, the authors dissociated epiblasts to single cells with trypsin, in their view, to disrupt cell interaction and thus to facilitate the stimulation of new transcriptional networks by LIF-STAT3 *in vitro*. STAT3 was phosphorylated in EpiSCs suggesting that they can indeed respond to LIF. Moreover, during conversion, epigenetic changes including, demethylation of *Rex1* and *Stella* and reactivation of the X chromosome took place. This so-called reprogrammed epiblast or ES-cell-like cells (rESCs) opposite to EpiSCs could contribute to germ cells and somatic tissues in chimaeras (Bao et al., 2009). In the second study, Guo et al., succeeded in converting EpiSCs into ESCs by forced expression of *Klf4*. They initially tested whether EpiSCs could be converted to ESCs by simply growing them in 2i and LIF, as this media improved iPS generation and ESC derivation. However, EpiSCs rather than converting into ESCs, differentiated and died. On the other hand, ESCs can become EpiSCs by growing them in EpiSCs culture conditions. The authors next tried to convert EpiSCs to ESCs by *Klf4* transgene expression but they were only able to succeed when *Klf4* transfected EpiSCs were transferred to 2i and LIF after 2-3 days of transfection and not if they were left in Activin and FGF2. This suggests that the conversion depends on the elimination of extrinsic stimuli (Guo et al., 2009). In summary, although ESCs can become EpiSCs by culturing in EpiSCs media, EpiSCs do not revert to ESC when only grown in media optimised for the growth of ESCs (2i plus LIF) but also require the force expression of *Klf4* (Guo et al., 2009). Moreover the frequency of conversion of EpiSCs to ESC by force expression of *Klf4* is very low with less than 1% of the cells fully converting (Guo et al., 2009).

1.2.2 Induced pluripotent Stem Cells (iPSCs).

Pluripotent stem cells cannot only be derived from the embryo but also by reprogramming adult somatic cells. The first report showing that such reprogramming was possible was made by the group of Shinya Yamanaka in Japan. In the study this team showed that mouse embryonic or adult fibroblasts could be reprogrammed to pluripotent cells, named Induced Pluripotent Stem cells (iPSCs) by retroviral-mediated introduction of *Oct4, Sox2, c-Myc* and *Klf4* (Figure 1.4), which are key transcriptions factors involved in the maintenance of self-renewal of ESCs (Section 1.3.1.1). iPSC show similarities with ESCs such as morphology and growth, expression of pluripotent markers and the ability to form teratomas and contribute to the generation of chimaeras (Takahashi and Yamanaka, 2006). However, iPSCs exhibit different gene expression and DNA methylation patterns than ESCs. One year later, the same group showed that reprogramming of adult fibroblast to iPSC could also be achieved in humans (Takahashi et al., 2007). This finding was a remarkable breakthrough in the field of stem cell biology, with impacts for biomedical research and drug development. iPSCs could potentially be used to study patient-specific disease, for cell therapy replacement without immune rejection and as a source to generate differentiated cells for toxicity screening without associated ethical issues. However, concerns about the use of iPSCs for human treatments arose as *c-Myc* and *Klf4* are oncogenes, in fact about 20% of the chimaeric mice developed tumours as a result of *c-Myc* transgene reactivation (Okita et al., 2007). Consequently many studies have sought to develop methods to create safer iPSC, such as transient expression of the factors by non-integrating vectors for example with adenovirus (Stadtfeld et al., 2010; Stadtfeld et al., 2008), plasmids (Okita et al., 2008), piggyback (PB) transposition (Woltjen et al., 2009) or avoiding *c-Myc* (Wernig et al., 2008). Despite of all this work to improve the safety of iPSC , recent studies suggest that iPSCs have mutations and they are genomically instable (Hussein et al., 2011; Pasi et al., 2011), which will hamper their use in regenerative medicine but they may still be valuable for drug development and to study mechanisms underlying specific diseases.

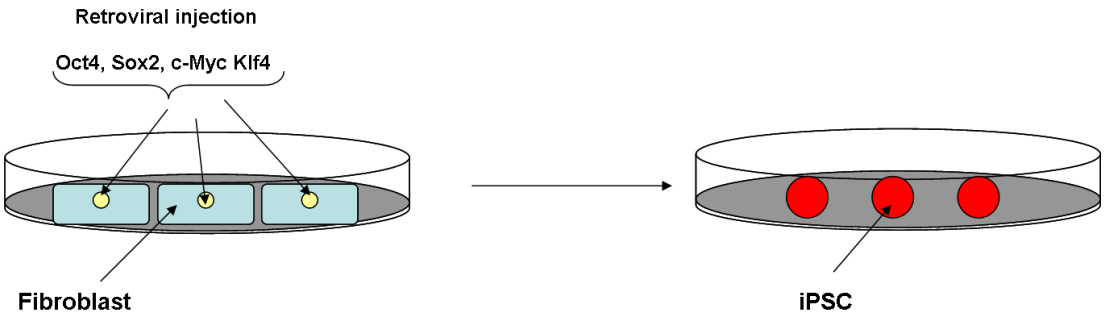

Figure 1.4 Reprogramming of somatic cells to iPSCs. Fibroblast can be reprogrammed by retroviral-mediated introduction of *Oct4, Sox2, c-Myc* and *Klf4* (Modified from Yamanaka and Blau, 2010).

1.3 Molecular mechanisms controlling self-renewal of mouse ESCs.

Under standard culture conditions, ESC pluripotency is controlled by the coordinated action of extrinsic factors, signalling pathways and transcription factors (Boiani and Scholer, 2005).

1.3.1 Intrinsic factors regulating mouse ESC self-renewal

ESCs retain their pluripotency capacity through the actions of a number of intrinsic factors in the form of transcription factors. Three transcription factors have been described as 'master regulators' of ESC pluripotency and include the POU domain-containing transcription factor Oct4, the homeobox transcription factor Nanog and a member of the HMG-domain DNA-binding-protein family Sox2 (Chambers et al., 2003; Mitsui et al., 2003; Boiani and Schoeler, 2005). In addition to their role in maintaining pluripotency in ESCs, Nanog and Oct4 also play an important role in formation and maintenance of the inner cell mass during mouse development (Avilion, 2003; Mitsui, 2003; Nichols, 1998; Niwa et al., 2000). Other transcription factors including c-Myc and Klf proteins also play a role in maintaining pluripotency of ESCs (Cartwright, 2005; Jiang et al., 2008).

1.3.1.1 Oct4, Sox2, c-Myc and Klf

The POU domain transcription factor Oct4 seems to be a key regulator of cell fate during development and for undifferentiated ESCs. It is expressed in early embryos in the morula, in all the cells of the ICM, the epiblast of the pre-implantation embryo, germ line cells and ESCs (Pesce et al., 1998). *Oct4* is essential for the establishment of the pluripotent lineage of the ICM as *Oct4* knockout embryos developed blastocysts but the cells of the ICM were not pluripotent (Nichols et al., 1998). In addition, repression of *Oct4* leads to loss of ESC self-renewal and expression of markers associated with trophectoderm. However, overexpression of *Oct4* results in differentiation of ESCs into primitive endoderm and mesoderm. Thus, the levels of Oct4 seem to be critical to sustain ESC self-renewal and to prevent differentiation of cells into trophectoderm (Niwa et al., 2000). Oct4 has also been shown to compete with Cdx2 which is important in the maintenance of trophectoderm. Overexpression of *Cdx2* in ESC has the same outcome as *Oct4* deletion leading to trophectoderm differentiation. Moreover, maintenance of *Oct4* expression from a transgene did not prevent trophectoderm differentiation when *Cdx2* was overexpressed (Niwa et al., 2005). Both Cdx2 and Oct4 are expressed in all the cells of the morula and then their expression become restricted to the trophectoderm in the case of Cdx2 and ICM in the case of Oct4. The temporal and spatial expression of Oct4 and Cdx2 together with the fact that Cdx2 and Oct4 can negatively regulate each other and as positively regulate themselves suggest that the segregation of the first lineage in the embryo depends on reciprocal inhibition between these two factors (Niwa et al., 2005).

Sox2 has also been proposed to play a role in maintenance of pluripotency as *Sox2* knockout leads to embryonic lethality shortly after implantation, suggesting that *Sox2* expression is essential for maintaining cells within the epiblast in an undifferentiated state. In the absence of *Sox2* cells of the epiblast become trophectoderm or extraembryonic endoderm (Avilion, 2003). Moreover, Sox2 expression overlaps with Oct4 during embryogenesis in the inner cell mass, epiblast and germ cells, and Oct4 has been shown to be able to heterodimerise with Sox2 to form Oct4/Sox2 complexes and regulate the expression of target genes (Boyer et al., 2005; Pesce and Scholer, 2001).

c-Myc, activated by the LIF/Stat3 pathway, also plays a role in maintaining pluripotency of mESCs. When ectopically expressed, *c-Myc* has been reported to be able to relieve the need for LIF/STAT3 signalling, and its inactivation results in loss of self-renewal (Cartwright et al., 2005).

The krüpel like factors (Klf) proteins, Klf2, 4 and 5 has also been implicated in regulation of pluripotency (Jiang et al., 2008). Individual knockdown of any of the three Klf proteins did not affect ESC self-renewal, double knockdown neither. However, triple knockdown resulted in loss of ESC morphology and reduction in the number of alkaline phosphatase colonies suggesting ESC differentiation. Co-expression of RNAi-resistant cDNA encoding *Klf2, Klf4* or *Klf5* reverted the effect of the knockdown and alkaline phosphatase positive colonies were generated (Jiang et al., 2008). Previous to this study, Klf4 was shown to be a transcription factor expressed in ESCs activated by the STAT3 pathway (Li et al., 2005).

In support of a role of Oct4, Sox2, Klf4 and c-Myc in regulating ESC self-renewal is the fact that expression of *Oct4*, *Sox2*, *Klf4* and *c-Myc* can reprogram mouse fibroblasts to iPSC (Section 1.2.2)(Takahashi and Yamanaka, 2006).

1.3.1.2 Nanog

Nanog was identified as an important regulator of pluripotency by two independent groups in 2003 using different approaches. Chambers et al., screened an ESCs cDNA library in the search for genes that could maintain ESC self-renewal in the absence of LIF and observed that this was the case for *Nanog* (Chambers et al., 2003). On the other hand, Mitsui et al., identified *Nanog* in an *in silico* differential display analysis and showed that is essential for establishment of pluripotency in ICM and ESCs (Mitsui et al., 2003). *Nanog* null embryos exhibited embryonic lethality, with embryos at E5.5 comprised of disorganised extra-embryonic tissue without an epiblast or extra-embryonic ectoderm. At E3.5 *Nanog* null blastocysts were morphologically similar to wild type blastocysts but nevertheless they failed to proliferate as undifferentiated cells *in vitro* and instead they differentiated into parietal-endoderm like cells. Hence, ESC could not be isolated from *Nanog* null

embryos. *Nanog* null ESCs produced by targeting of the Nanog locus, lost expression of pluripotent cell markers and differentiated into extra-embryonic endoderm, suggesting that *Nanog* expression is important in maintaining pluripotency of both ICM and ESCs (Matsui et al., 2003). The phenotype of *Nanog* null embryos is different to that of *Sox2* and *Oct4* null embryos. Sox2 null embryos lack epiblast but have extra-embryonic ectoderm (Avilion et al., 2003) and *Nanog* null blastocysts do not differentiate into trophectoderm as *Oct4* null embryos do (Nichols et al., 1998). Moreover, over-expression of *Nanog* and *Oct4* have different outcomes in ESCs; over-expression of *Oct4* drives ESC differentiation into primitive mesoderm and endoderm whereas over-expression of *Nanog* can maintain self-renewal in the absence of LIF. Hence, it seems that *Oct4* and *Nanog* have two different functions in ICM and ESCs, *Oct4* prevents differentiation into trophectoderm and *Nanog* into extra-embryonic endoderm, as well as contributing to maintenance of pluripotency (Matsui et al., 2003).

After the studes of Matsui *et al.* (Matsui et al., 2003) and Chambers *et al.* (Chambers et al., 2003), *Nanog* was considered to have a key role in maintaining pluripotency of both ICM and ESCs. However, later studies challenge this view as not all ESCs express *Nanog* and *Nanog* negative cells can re-express *Nanog* and form undifferentiated colonies (Chambers et al., 2007). The capacity of *Nanog* negative cells to form undifferentiated colonies is reduced compared to *Nanog* positive cells, indicating that although ESCs with low or no levels of *Nanog* are predisposed to differentiate they are not committed yet and can re-express *Nanog* and maintain an undifferentiated state. Furthermore, although conditional deletion of *Nanog* led to an increase in ESC differentiation and a reduction in colony numbers, some *Nanog* null cells could be propagated undifferentiated, keeping their ability for multilineage differentiation in teratomas, and were able to contribute to foetal and adult chimeras (Chambers et al., 2007). The observation that *Nanog* null ESCs can be maintained in an undifferentiated state contrasts with that of Matsui and colleagues who claimed that *Nanog* null cells differentiated into extra-embryonic endoderm. However, *Nanog* null cells were not able to contribute to the germ lineage after E11.5. *Nanog* expression is down-regulated after implantation and is re-expressed during germ line commitment (Yamaguchi et al., 2005) where it seems to be important for maturation of primordial germ cells (Chambers et al., 2007). This study suggested that *Nanog* is

not essential for maintaining pluripotency but it is necessary for establishing it (Chambers et al., 2007). A further study supports this hypothesis. Silva *et al.*, (Silva et al., 2009) investigated the reason why *Nanog* null embryos failed to develop pluripotent epiblast whereas ESCs without *Nanog* could be maintained undifferentiated. He observed that *Nanog* null cells in the ICM either die or differentiate into trophoblast around E3.5 and E4.5 and hypoblast cannot be formed. This observation, together with the expression pattern of Nanog, suggests that may specify the epiblast. Expression of during development begins at the morula stage, it is expressed in the ICM in a salt and pepper fashion and its expression is restricted to epiblast (Chazaud et al., 2006). On the other hand, Sox2 and Oct4 are expressed in the morula and in all the cells of the ICM and until the hypoblast has been segregated (Avilion, 2003; Chazaud et al., 2006; Palmieri et al., 1994). Hence, Nanog restricted expression in the epiblast, in comparison with a wider expression of Oct4 and Sox2, suggests that Nanog may specify epiblast in cells that already express Oct4 and Sox2 (Silva et al., 2009). Hence, in summary it appears that Nanog is required during embryonic development to specify pluripotent epiblast and later for correct development of germ cells.

The importance of Nanog for establishing pluripotency is further supported by studies in somatic reprogramming. Despite the fact that *Nanog* was shown to increase nuclear reprogramming after ESC fusion (Silva et al., 2006), *Nanog* was surprisingly not necessary for reprogramming somatic cells to iPSC with transcription factors (Takahashi et al., 2007; Takahashi and Yamanaka 2006). However, fully reprogrammed iPSC could be isolated more easily if colonies were selected by expression of endogenous *Nanog*, suggesting that expression of Nanog was essential for full reprogramming (Okita et al., 2007). The requirement of *Nanog* expression for reprogramming was shown by the inability of *Nanog* null neural stem cells to generate fully reprogrammed cells (Silva et al., 2009). The efficiency of reprogramming can be increased by using a two-step protocol, first step is transfection of cells with trangene factors which lead to loss of somatic markers and expression of some pluripotent markers but not *Nanog* and are called pre-iPSC (Silva et al., 2008). These pre-iPSC can be fully reprogrammed to iPSC by culture in 2i with LIF, which is an optimal medium for ESC propagation. The lack of *Nanog* does not seem to affect the first step of reprogramming to pre-iPSC but it affects the

progression of pre-iPSC to iPSC as cells die. Introduction of a *Nanog* transgene into *Nanog* null cells results in the survival of pre-iPSC in 2i+LIF and conversion to iPSCs. Furthermore, excision of the *Nanog* transgene after reprogramming has been completed does not affect iPSC pluripotency, shown by ability to colonise the embryo (Silva et al., 2009). Hence, these studies support the hypothesis of a role for Nanog in establishing the pluripotent state in both embryonic and induced pluripotency (Silva et al., 2009). Finally, similar to E3.5, where Nanog specifies the epiblast when other factors such as Oct4 and Sox2 are present, Nanog can specify induced pluripotency when other transcriptions such as Oct4, Sox2 or Klf4 are already expressed (Silva et al., 2009).

Chromatin immunoprecipitation studies have shed light on how Nanog may be regulating the change of pre-iPSC to iPSC (Sridharan et al., 2009). It seems that genes thought to be Nanog targets are co-occupied by Oct4, Sox2 and Klf4 in fully reprogrammed iPSC but not in pre-iPSC, suggesting that Nanog may promote binding of the reprogramming factors to these genes.

More recently, *Nanog* expression has been shown to promote the transition from pre-iPSC to iPSC in minimal conditions by overcoming several barriers including phospho-Erk (p-Erk) signalling and high levels of Oct4 (Theunissen et al., 2011). Transition of pre-iPSC to iPSC is facilitated by culture in 2i+LIF that leads to considerable activation of endogenous *Nanog* and down-regulation of *Oct4* transgene within 2 days. The down-regulation of *Oct4* transgene seems to be key for facilitating induced pluripotency as over-expression of *Oct4* results in decreased expression of *Nanog* and *Sox2* and up-regulation of *Brachyury* and *Gata6*. The increase in *Nanog* expression in 2i+LIF seems to be very important for the transition to iPSC as constitutive over-expression of *Nanog* facilitates the reprogramming in minimal conditions that do not normally support ESC or iPSC maintenance, such as serum-free medium with LIF. iPSC generated by over-expression of *Nanog* can contribute to the epiblast. The fact that p-Erk levels were unaffected in serum-free plus LIF with or without *Nanog* over-expression and that Oct4 levels do not change after 24 hours cultured in serum-free with LIF suggest that *Nanog* can overcome the negative effects of p-Erk and high levels of Oct4 and promote somatic reprogramming. To further characterise the ability of Nanog to overcome such

barriers, the authors over-expressed *Nanog* transgene in epiblast-stem cells, which can be reprogrammed to pluripotent cells by transgene over-expression and culture in 2i with LIF (Guo et al., 2009) and cultured them in serum-free media with or without LIF. Remarkably, iPSCs from *Nanog* over-expressing EpiSC were generated in both serum-free media with LIF and without LIF (Theunissen et al., 2011).

1.3.1.3 Zscan4c

Zscan4c is part of the Zscan4 family, which includes nine genes that were shown to play a role in early embryonic development as knockdown of *Zscan4* transcripts resulted in a 24 hour delay in the development from the two-cell to the four-cell stage, and failure of the blastocyst to implant or proliferate in *in vitro* outgrowths (Falco et al., 2007). A role for *Zscan4c* in maintenance of ESC pluripotency downstream of PI3K signalling was first reported by our laboratory. A microarray screen performed to investigate gene changes following PI3K inhibition with LY290024 identified *Zscan4c* as one of the genes rapidly down-regulated following PI3K inhibition. This rapid down-regulation, together with the fact that *Zscan4* expression is restricted to the early preimplantation embryo and ESCs (Falco et al., 2007), suggested that it may play role in ESC maintenance. siRNA knock-down of *Zscan4c* led to a decrease in the number of alkaline phosphatase positive self-renewing colonies and expression of pluripotency markers *Nanog*, *Rex1* and *Oct4* suggesting decreased self-renewal. On the contrary, over-expression of *Zscan4c* led to an increase in the formation of alkaline phosphatase positive colonies compared to control both in the presence and absence of LIF (Storm et al., 2009). The number of colonies in the absence of LIF was significantly reduced in comparison with LIF, suggesting that *Zscan4c* contributes to maintenance of self-renewal but it is not sufficient to support ESC proliferation. The fact that Zscan4c has a SCAN domain that mediates protein-protein interaction and also four zinc finger motifs, together with its enriched expression in the nucleus, suggest that Zscan4c may act as a transcription factor in ESCs (Storm et al., 2009). In fact, Zscan4c shares a feature with other pluripotency transcription factors including Nanog which is a heterogeneous expression (Falco et al., 2007). More recently, *Zscan4* has been reported to play an important role in ESC genomic stability and telomere elongation of ESCs (Zalzman et al., 2010). *Zscan4* knockdown led to karyotype aberrations,

telomeres shortening, decrease in cell proliferation and eventually to apoptosis indicating that *Zscan4* is important to maintain long-term culture of ESCs.

1.3.1.4 Pluripotency transcription factor network.

Several studies have shown that the pluripotency transcription factors cooperate with each other to activate or repress genes. Oct4 is known to heterodimerize with Sox2 to form Oct4/Sox2 complexes and the interaction between Oct4 and Sox2 is believed to play a key role in regulating gene expression (Boyer et al., 2005; Pesce and Scholer, 2001). Studies using chromatin immunoprecipitation coupled with DNA microarrays aimed to investigate target genes of Nanog, Oct4 and Sox2 in order to understand how these master regulators control pluripotency and self-renewal of human and mouse ESCs (Boyer et al., 2005; Loh et al., 2006). These studies showed that Oct4, Sox2 and Nanog co-occupy the promoters of many genes, including their own promoters, some of the genes are transcriptionally active and some inactive. Among the active genes were genes involved in maintenance of pluripotency of ESCs including transcription factors, *Nanog*, *Oct4*, *Sox2* and *Stat3*, components of the Wnt signalling pathway such as *Dkk1*, and of the TGF-β pathway such as *Lefty2* and chromatin modifying enzymes. This suggest that Nanog, Oct4 and Sox2 promote self-renewal and pluripotency by forming a regulatory circuitry with positive feedback loops to self-regulate themselves and positive regulation of genes that encode effectors of important signalling pathways. On the other hand, inactive genes co-occupied by Nanog, Oct4 and Sox2 included genes that encoded for transcription factors involved in developmental processes. Interestingly, these inactive genes were also co-occupied by Polycomb Repressive Complexes (PRC) that are known to be involved in transcriptional silencing in ESCs (Boyer et al., 2006; Lee et al., 2006). Hence, co-occupancy studies suggest that Oct4, Sox2 and Nanog are repressing expression of genes involved in development and activating themselves and key effectors of signalling pathways that promote self-renewal (Figure 1.5) (Boyer et al., 2005).

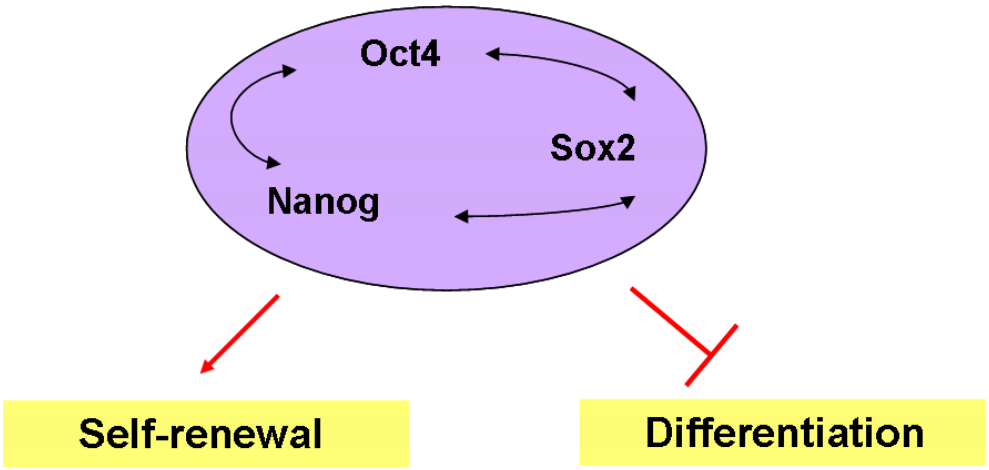

Figure 1.5 Pluripotency transcription factor network. Oct4, Sox2 and Nanog form part of a regulatory circuitry where they regulate each other expression, as well as positively regulate the expression of genes that promote self-renewal and repress expression of genes involved in development (after Boyer et al., 2005).

More recently the T-cell factor 3 (Tcf3), involved in the canonical Wnt pathway, was reported to co-occupy the promoters with Oct4, Nanog and Sox2 by using chromatin immunoprecipitation sequencing (Chip-seq) (Marson et al., 2008b) and ChIP-on-Chip experiments (Cole et al., 2008; Tam et al., 2008). Tcf3 seems to act to repress gene expression (Cole et al., 2008). In accordance with this, Polycomb Repressive Complexes were present in almost half of the genes co-occupied by Tcf3, Oct4 and Nanog. Tcf3, similarly to Oct4, Sox2 and Nanog, is bound to active and silenced genes suggesting that Wnt signalling may regulate pluripotency and self-renewal by regulating these genes. Cole *et al.*, (Cole et al., 2008) proposed a model to explain how Tcf3 may control the balance of pluripotency and differentiation in ESCs. They suggested that although Tcf3 may repress or activate genes under standard conditions, it is mainly repressive and thus induces differentiation. In favour of this, they argue that knockdown of Tcf3 led to increased expression of Nanog, Oct4 and Sox2. On the hand, activation of the Wnt pathway changes the main nature of the Tcf3 complexes from repressive to activating, favouring self-renewal. A previous study also using ChIP and promoter reporter assays had already demonstrated that *Nanog* gene expression can be repressed by Tcf3 (Pereira et al., 2006). The authors demonstrated that lack of Tcf3 leads to increased in *Nanog* promoter activity, level of mRNA and protein and resulted in ESCs that are more resistant to differentiation.

They showed that Tcf3 acts by binding to the *Nanog* promoter and repressing its transcriptional activity. They proposed that Tcf3 is controlling the balance between self-renewal and differentiation by limiting the levels of *Nanog* and thus creating a window for differentiation. Other two recently published papers are in agreement with Tcf3 acting as a negative regulator of pluripotency by repressing the pluripotency transcription network (Figure 1.6) (Wray et al., 2011; Yi et al., 2011). Tcf3 over-expression eliminates ESC capacity to generate alkaline phosphatase positive colonies, and this effect can be overcome with Wnt3a (Yi et al., 2011). Moreover, both groups observed that Tcf3 null cells can generate AP positive colonies when grown only with the MEK inhibitor (PD0325901). Wray et al., (2011) and Yi et al., (2011) proposed the following mechanism to explain how Wnt affect Tcf3 activity. Wnt signalling activation leads to β-catenin stabilisation, which in turn interacts with Tcf3 relieving Tcf3 repression in the pluripotency transcription network (Wray et al., 2011; Yi et al., 2011).

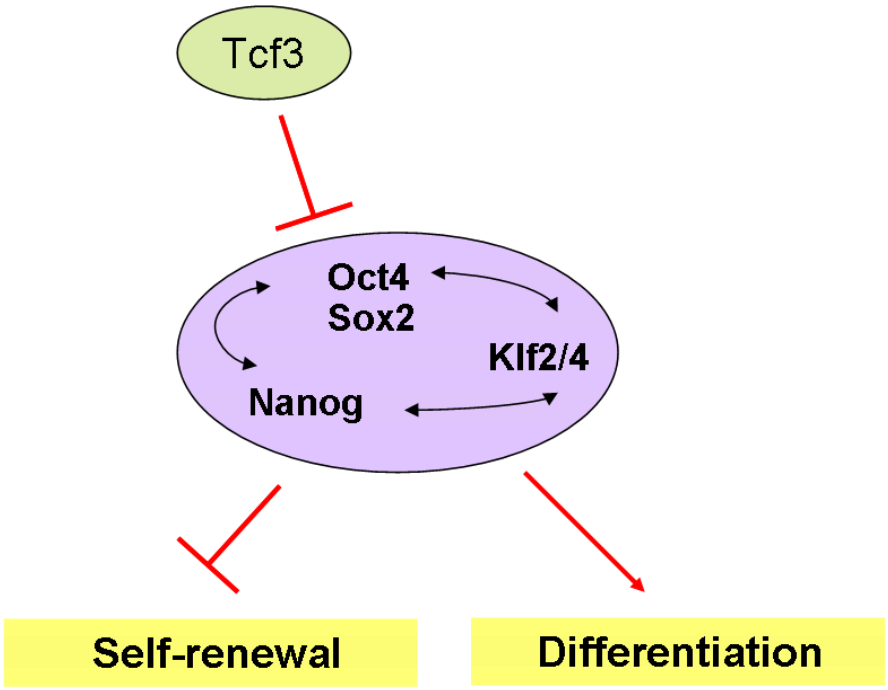

Figure 1.6 Tcf3 negatively regulates the pluripotency transcription factor network. Tcf3 promotes differentiation and decrease self-renewal by repressing the transcriptional activity of the pluripotency network (after Pereira et al., 2006; Wray et al., 2011; Yi et al., 2011).

Although Nanog, Oct4 and Sox2 are considered central regulators of pluripotency, further components of the network including Tcl1 (a cofactor of the Akt1 protein serine kinase), Tbx3, Esrrb, Dppa4 and Unigene Mm343880 were identified in a microarray screen (Ivanova et al., 2006). Consistent with a role in maintaining self-renewal of ESCs these genes were rapidly down-regulated following induction of differentiation with retinoic acid and their knockdown with shRNA led to a change of ESC morphology and also a reduction in alkaline phosphatase activity, suggesting ESC differentiation (Ivanova et al., 2006). Changes in global gene expression following knockdown of *Nanog*, *Oct4*, *Sox2*, *Esrrb*, *Tbx3*, *Tclf1* or *Dppa4* with shRNA suggested that there are 2 different pathways regulating self-renewal (Figure 1.7). One is through Nanog, Oct4 and Sox2 and the other through Esrrb, Tbx3 and Tclf1. However, the fact that over-expression of *Nanog* can overcome the effects of knocking down Tbx3, Esrrb or Tcl1 by restoring the levels of other pluripotency regulators suggest that Tbx3, Esrrb and Tcl1 are interconnected to Oct4, Nanog and Sox2 forming part of the transcriptional network.

In another study, using chromatin immunoprecitipation coupled with microarray, it was shown that Klf2, Klf4 and Klf5 proteins co-occupied many target genes including *Nanog* and numerous Nanog target genes which are important for maintaining ESC pluripotency including *Oct4*, *Sox2*, *Sall4*, *Tcl1*, *Esrrb*, *Tbx3* and *Tcf3* (Jiang et al., 2008). In addition, the three Klf proteins can bind to the *Nanog* distal enhancer region and mutation in the Klf binding motif of *Nanog* showed that Klf proteins regulate Nanog protein enhancer activity. Klf proteins can compensate for the lack of each other and only triple knockdown of Klf abolished *Nanog* enhancer activity (Jiang et al., 2008). In summary, this study suggests that Klf proteins form also part of the pluripotency transcription network and control the expression of genes in the network including *Nanog*.

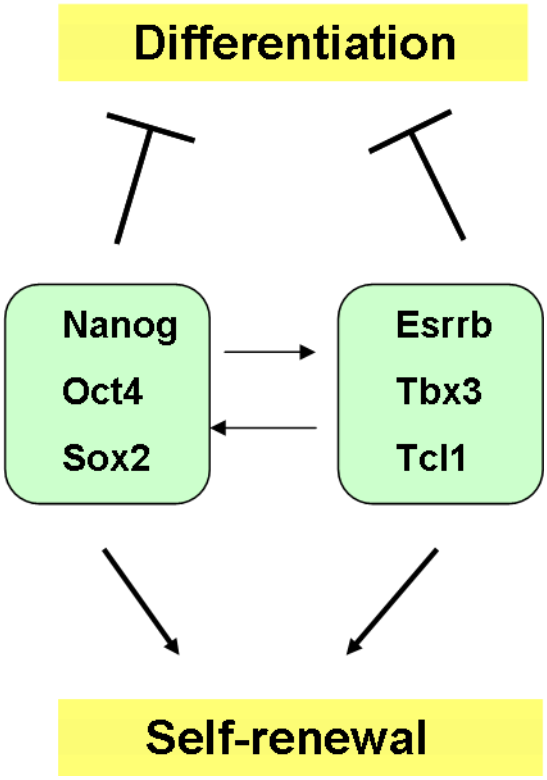

Figure 1.7 Two different pathways cooperate to maintain self-renewal. Two pathways contribute to maintenance of self-renewal by promoting the expression of genes related with self-renewal and repressing genes involved in differentiation. The two pathways are interconnected to each other (Modified from Ivanova et al., 2006).

One study has identified Nanog protein interacting partners including Oct4, Sall4, Dax1, Nac1 and Zfp281 by using affinity purification and mass spectrometry (Kim et al., 2008). All of these proteins were confirmed to have a role in maintaining pluripotency as their silencing with shRNA led to loss of pluripotency. Sall4 and Dax1 had already been reported to play an important role in ESCs (Niakan et al., 2006; Sakaki-Yumoto et al., 2006). Nac1 and Zfp281, together with Nanog were found to be enriched at the Gata6 promoter suggesting that these genes repress Gata6. Interacting partners of Oct4, Dax1, Nac1, Zfp281 and also Rex1 were identified using the same approach used with Nanog. Interestingly, numerous binding proteins were shared between the Oct4, Dax1, Nac1, Zfp281 and Rex1 complexes isolated and the authors proposed that these complexes interact with each and form a network (Kim et al., 2008). The network contained a high number of

proteins that are down-regulated during differentiation and are likely to play a role in maintaining ESC self-renewal. Furthermore, the network is linked to histone deacetylase NuRD, polycomb group and SWI/SNF chromatin remodelling complexes, all of them known to be involved in translational repression. e.g. Oct4 and Rex1 are linked to polycomb components whereas Nanog is linked to HDAC/NuRD (Wang et al., 2006). Further work from the same group showed that many genes are co-occupied by a combination of four or more of the following transcription factors: Oct4, Sox2, Klf4, Nanog, Dax1, Nac1, Zpf281, and they observed a relationship between the number of transcription factors bound to a gene and its expression in ESCs or repression during differentiation. Genes bound by four or more factors are expressed in ESCs and the ones bound by fewer factors are repressed, suggesting that these factors act by activating transcription of genes involved in self-renewal and repressing genes related to differentiation (Kim et al., 2008).

In summary, Oct4, Sox2 and Nanog are the core components of the pluripotency network and recently, other genes involved in regulating pluripotency and linked to the network have also been identified.

1.3.2 Extrinsic factors and signalling pathways regulating mouse ESC self-renewal.

Leukemia Inhibitory Factor (LIF) is one important extrinsic factor involved in maintaining self-renewal of murine ESCs (Smith et al., 1988). LIF binding to the gp130 receptor activates Stat3 (Matsuda et al., 1999), which in turn trans-activates c-Myc (Cartwright, 2005). LIF also activates a number of other signalling pathways including the Src kinases (Anneren et al., 2004), Ribosomal S6 kinases (Boeuf et al., 2001), Phosphoinositide 3-Kinase (PI3K) signalling (Paling et al., 2004) and Erk1 and Erk2, which seem to promote differentiation (Burdon et al., 1999b). In the presence of serum, LIF alone is able to maintain self-renewal of mouse ESCs. However, in serum-free conditions bone morphogenetic proteins 2 or 4 (BMPs) are also required. BMPs appear to cooperate with LIF to maintain self-renewal through induction of Id (Inhibitor of differentiation) proteins (Ying et al., 2003). Maintenance

of self-renewal in serum-free media in the absence of LIF and BMP4 can be achieved by simultaneous inhibition of Glycogen Synthase Kinase-3 (GSK-3) and MAP kinase/ERK kinase (MEK) (Ying et al., 2008). The canonical Wnt pathway has also been reported to play a role in maintenance of self-renewal of mESCs (Sato et al., 2004; Ogawa et al., 2006; Hao et al., 2006; Singla et al., 2006).

1.3.2.1 LIF/STAT3 signalling

mESCs were originally cultured on fibroblast feeder layers which provide stimuli to maintain them in a self-renewing condition (Evans and Kaufman, 1981; Martin, 1981). LIF was later identified as the factor produced by the feeder layers that could support growth and self-renewal of ESCs (Smith et al., 1988; Williams et al., 1988). Binding of LIF to its receptor (LIF receptor) induces heterodimerisation with the gp130 receptor and activation of Janus-associated kinase (JAK) (Narazaki et al., 1994; Stahl et al., 1994) which then phosphorylates tyrosine residues of the gp130 receptor. The phosphorylated residues serve as docking sites for the recruitment of proteins that contain SH2 domains, such as the signal transducer and activator of transcription 3 (STAT3), which in turn is phosphorylated by JAK (Burdon et al., 1999a). Phosphorylated STAT3 forms homodimers and translocates to the nucleus where they activate transcription of target genes (Figure 1.8) (Stahl et al., 1995). STAT3 has been shown to have a key role in maintaining ESCs in an undifferentiated state by using dominant-negative forms of STAT3 and a conditionally active form of STAT3 (Matsuda et al., 1999; Niwa et al., 1998). Niwa *et al.,* (Niwa et al., 1998) expressed a dominant negative mutant form of STAT3 name STAT3F, which has tyrosine 705 mutated to alanine. This tyrosine residue is known to be important for dimerisation and translocation to the nucleus and several studies had previously shown that activation of endogenous STAT3 can be blocked by expression of high levels of STAT3F (Fukada et al., 1996; Ihara et al., 1997; Kishimoto et al., 1994; Minami et al., 1996). Constitutive expression of STAT3F in ESCs led to growth arrest, cell death and differentiation. Hence, the authors developed an inducible STAT3F transgene to be able to investigate STAT3 function in ESCs. Expression of STAT3F resulted in loss of self-renewal and cell differentiation, highlighting the key role of STAT3 in maintenance of self-renewal.

The role of STAT3 in ESCs is opposite to the one in somatic cells where it acts mainly by promoting differentiation (Kishimoto et al., 1994). Matsuda *et al.* (Matsuda et al., 1999) used a conditionally active form of STAT3, STAT3ER, which is a fusion protein containing the whole coding region of STAT3 and the domain of the estrogen receptor where the ligand (4-hydroxytamoxifen) binds inducing STAT3 expression, to investigate the role of STAT3 in ESC. STAT3 activation was not only shown to be essential for self-renewal but also that it is on its own capable of sustaining self-renewal of ESCs in the absence of LIF. However, these experiments were performed in the presence of LIF and at high cell density so the presence of additional signals that maintain ESC proliferation cannot be ruled out. Although this study showed that STAT3 is important for maintaining self-renewal, the downstream effectors of LIF/STAT3 remained unknown for some years until 2005 when the oncogene *c-Myc* was identified as a direct transcriptional target of STAT3 by chromatin immunoprecipitation (Cartwright et al., 2005). Consistent with *c-Myc* being a target of STAT3, *c-Myc* mRNA levels were shown to be highly expressed in self-renewing ESC and down-regulated following LIF withdrawal.

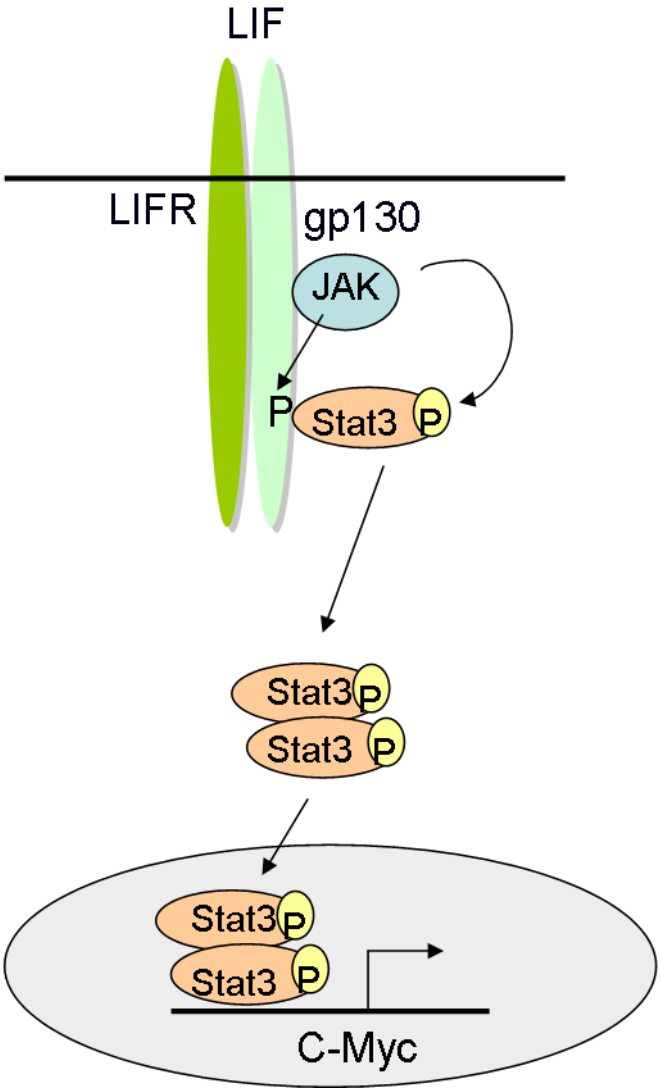

Figure 1.8 LIF activation of Stat3. Following binding of LIF, the LIFR heterodimerised with the gp130 receptor and activates JAK that in turn phosphorylates tyrosine residues of the gp130 receptor. Stat3 is then recruited to the gp130 receptor, through its SH2 domain, where it is phosphorylated by JAK. Phosphorylated Stat3 forms homodimers and translocates to the nucleus where it activates transcription of target genes including *c-Myc* (Modified from Cartwright et al., 2005).

1.3.2.2 LIF/ERK signalling and Fgf4

LIF signalling through gp130 can also activate the Ras/Mitogen-activated protein kinase (MAPK) pathway. This is mediated through the recruitment of tyrosine phosphatase (SHP2), which contains SH2 domains, to the phosphorylated tyrosines of gp130 where it is phosphorylated generating docking site for the binding of a complex composed of the Grb2 adaptor and the Sos guanine-nucleotide-exchange factor. This induces the activation of Ras and the initiation of a cascade of phosphorylation events leading to the activation of ERK (Figure 1.9) (Kolch, 2000). Several studies have shown that Erk activation promotes differentiation in ESCs (Burdon et al., 1999b; Cheng et al., 1998; Qu and Feng, 1998). ESC self-renewal can be enhanced by reducing the activation of the Ras/MEK signalling through ablation of the docking sites of SHP2 in gp130 (Burdon et al., 1999b). Inhibition of MEK activity or forced expression of ERK phosphatases had the same outcome (Burdon et al., 1999b). The pro-differentiation activity of ERK is further supported by showing that genetic alterations of either Grb2 or Shp2 lead to defects in differentiation (Burdon et al., 2002). ESC fate was proposed to be determined by the balance between Stat3 and Erk signals (Burdon et al., 2002) but this is a relatively old model as other pathways activated by LIF, such as the Src and Phosphoinositide 3-Kinase (PI3K) pathways have been shown to be required for the maintenance of ESC self-renewal through a Stat3 and ERK independent pathways (Anneren et al., 2004; Paling et al., 2004).

Undifferentiated ESCs produce Fgf4 in an autocrine fashion, which leads to activation of Erk signalling (Figure 1.9). Disruption of Fgf4 and Erk signalling with chemical inhibitors, or genetically, does not alter ESC propagation but these ESCs are unable to commit to differentiation. Inhibition of Fgf receptor or *Fgf4* knockout leads to ESCs more resistant to neural and mesodermal induction, and unable to respond to BMP inductive non-neural differentiation (Kunath et al., 2007). The fact that disruption of Fgf4 results in ESCs refractory to neural induction is in agreement with previous reports suggesting that Fgf4 promotes ESC commitment to neural lineages (Lowell et al., 2006) and also in accordance with the fact that Fgf signalling is required for neural induction in vertebrate embryos (Stavridis et al., 2007). Moreover, the authors suggested that the fact that ESCs with disrupted Fgf4 are not able to respond to BMP inductive non-neural differentiation but addition of Fgf

protein restored the ability indicates that Fgf plays a key role in the change of BMP signalling from maintaining self-renewal to promoting non-neural differentiation (Ying et al., 2003b).

The inability of null *Fgf4* ESCs or ESCs treated with FGF receptor inhibitors to differentiate to mesoderm lineages suggest that Fgf signalling has also a role in commitment to mesoderm differentiation. Hence, Fgf signalling may induce commitment of ESCs to various lineages. *Erk2* knockout ESCs failed to differentiate into neural or mesodermal lineages and maintained expression of *Nanog* and *Rex1* in differentiating conditions, suggesting that activation of Erk2 by Fgf4 may drive cells to a transient state where they are responsive to differentiating signals (Kunath et al., 2007).

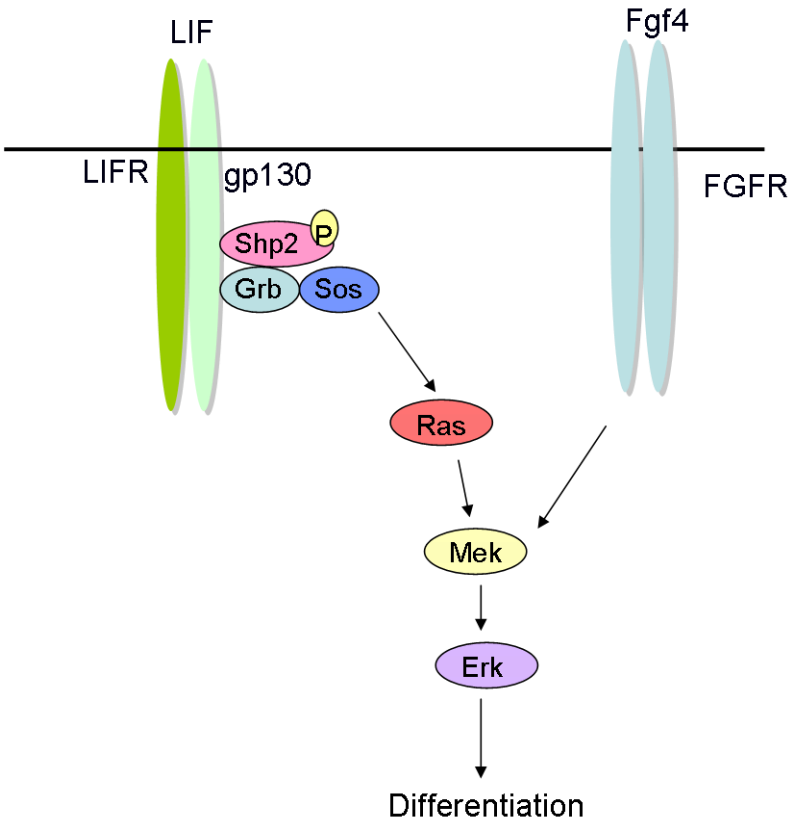

Figure 1.9 Activation of Erk by LIF signalling and Fgf4. Binding of LIF to the LIFR leads to heterodimerisation with the gp130 receptor and phosphorylation of tyrosine residues of the gp130 receptor. Shp2 is then recruited to the receptor through it SH2 domain, where it is phosphorylated generating docking site for the binding of Grb2 and Sos. This leads to activation of Ras and the initiation of a cascade of phosphorylation events leading to the activation of ERK. Erk signalling is also activated by Fgf4 secreted by undifferentiated ESCs (After Burdon et al., 1999b; Kolch.,2000; Ying et al., 2003; Kunath et al., 2007) .

1.3.2.3 LIF/PI3K signalling

PI3Ks are a family of lipid kinases reported to be involved in different cellular processes such as proliferation, cell survival, cell differentiation, migration and trafficking (Vanhaesebroeck et al., 1999; Vanhaesebroeck et al., 2001). They are divided into three classes (I, II, III) (Vanhaesebroeck et al., 1997). The $PI(3,4)P_2$ and $PI(3,4,5)P_3$, products of activated class I PI3Ks act as intracellular second messengers, recruiting proteins that contain pleckstrin homology (PH) domains to the cell membrane where they are activated through phosphorylation events.

Activation of PI3K by LIF in ESCs was first reported by Paling in our laboratory (Paling et al., 2004). Previous studies had shown that PI3K activation was coupled to gp130 receptors (Boulton et al., 1994) and that PI3K signalling was functioning in ESCs (Jirmanova et al., 2002). Hence, Paling et al. investigated a possible activation of PI3K by LIF in ESCs firstly by studying changes in phosphorylation of known PI3K downstream effectors such as serine 473 of PKB, serine 21, serine 9 of GSK-3 α and β and phosphorylation of S6 protein. The fact that LIF stimulation increased the phosphorylation of all of them and that the LIF-stimulated phosphorylation was reduced by treatment with a broad spectrum PI3K inhibitor (LY294002) suggested that LIF can activate PI3K in ESCs. Moreover, expression of a dominant negative form of p85α (termed Δp85), which is a regulatory subunit of class I_A PI3Ks, also reduced phosphorylation of PKB, GSK3α/β and S6 protein at PI3K-sensitive sites in LIF-stimulated samples and in basal conditions. Hence, in ESCs the PI3K pathway is activated upon binding of LIF to the LIF receptor resulting in activation of protein kinase B, also known as Akt, which in turn phosphorylates GSK-3 promoting self-renewal. Activated PI3K also inhibits the Erk pathway preventing differentiation (Figure 1.10) (Paling et al., 2004).

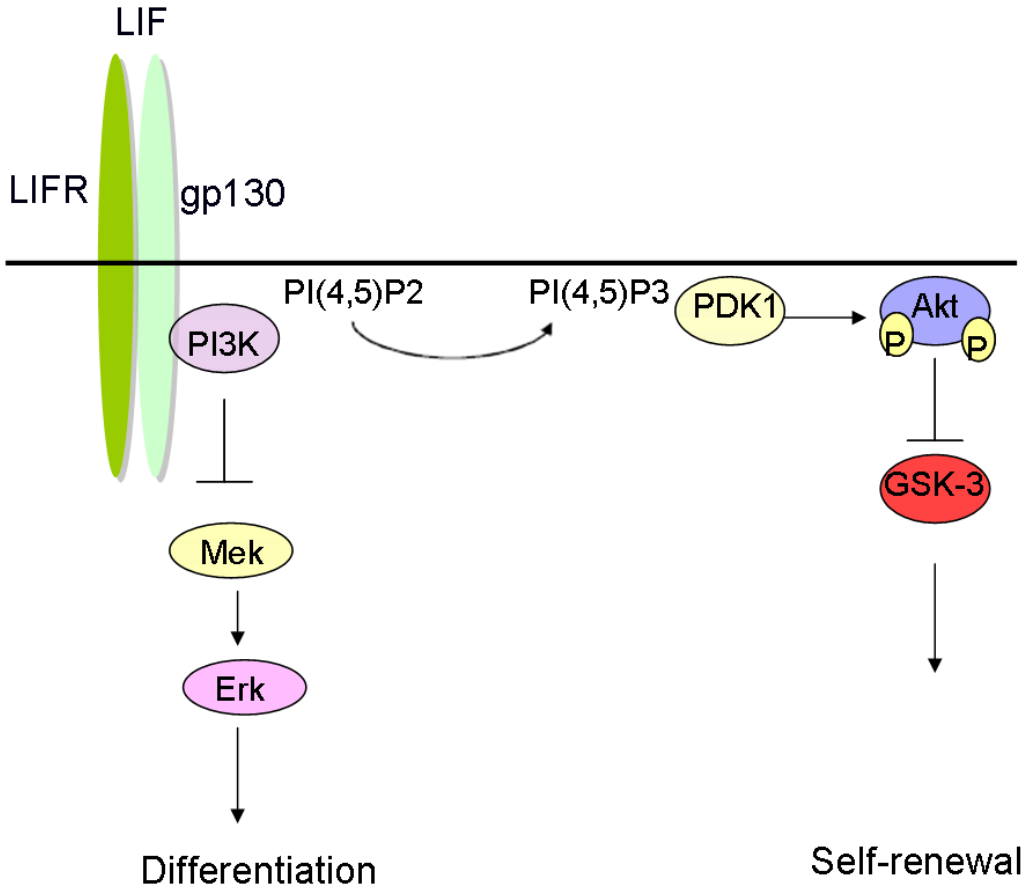

Figure 1.10. LIF activation of PI3K signalling. LIF binding to the receptor leads to activation of PI3K, activated PI3K phosphorylates PI(4,5)P2 in the membrane to form PI(3,4,5)P_3, PDK1 is then recruited and phosphorylates Akt in Thr308 and Ser473, which in turn phosphorylates GSK-3 promoting self-renewal. Activated PI3K also inhibits the Erk pathway preventing differentiation (After Paling et al., 2004).

Previous reports had suggested that the role of PI3K in ESCs was to regulate ESC proliferation (Jirmanova et al., 2002). However, our laboratory has also shown that the PI3K pathway is involved in regulating self-renewal of ESCs, and that this regulation is mediated at least partly by PI3Ks ability to maintain Nanog expression. Inhibition of PI3K by LY294002, a PI3K inhibitor, or by a dominant negative form of p85, the regulatory subunit of PI3K, lead to loss of ESC self-renewal and to a decrease in *Nanog* RNA levels and protein expression (Paling et al., 2004; Storm et al., 2007). Moreover, PI3K inhibition of GSK-3 seems to play an important role in regulation of Nanog expression shown by using small molecule inhibitors of GSK3, BIO or TD114-2 and GSK-3 mutants. Inhibition of GSK-3 reversed the effects of PI3K inhibition on *Nanog* RNA levels and protein expression and restored self-renewal (Storm et al., 2007). Moreover, two different GSK-3β mutants, GSK-3β S9A and R96E GSK-3β confirmed that the effect observed using GSK-3 inhibitors is due to selective inhibition of GSK-3. The GSK-3β S9A lacks Serine 9, which is the phosphorylation site of PKB/Akt leading to a constitutively active GSK-3β, as Akt-dependent phosphorylation of GSK-3 is inhibitory. The R96E GSK-3β is a dominant mutant where arginine 96 is exchanged for glutamic acid so GSK-3 is unable to recognise phosphorylated substrates. Expression of GSK-3β S9A mimicked PI3K inhibition and decreased the levels of Nanog. On the other hand R96E GSK-3β mimicked GSK-3 inhibition and led to increase in Nanog levels (Storm et al., 2007). The role of PI3K in maintaining self-renewal of ESCs is further supported by a report showing that activation of Akt downstream of PI3K is enough to support maintenance of ESC self-renewal in the absence of LIF. The authors used a myristoylated form of Akt to show this (Watanabe et al., 2006). Another group, using a gain of function screen, also identified Akt as a gene that can maintain self-renewal of ESCs in the absence of LIF when it is overexpressed (Pritsker et al., 2006). This study further supports the role of PI3K in supporting undifferentiated state of ESCs.

1.3.2.4 LIF/Src signalling

LIF can also activate members of the Src family of non-receptor protein tyrosine kinases such as Hck, Lck, Yes and Src, which are highly expressed in ESCs (Anneren et al., 2004). Hck, Yes and Lck have been reported to contribute to maintenance of ESC self-renewal. Expression of a mutant form of Hck, which is always active, can reduce the concentration of LIF needed to maintain ESC self-renewal (Ernst et al., 1996). Several studies suggested that Yes may have a role in maintaining ESC self-renewal. Yes was highly expressed in ESCs in comparison with differentiated cells (Ivanova et al., 2002), Yes was shown to be very responsive to LIF stimulation and serum and rapidly down-regulated upon differentiation (Annerén et al., 2004; Trouillas et al., 2009), transfection of *Yes* siRNA led to a decrease in the expression of pluripotency markers such as *Oct4* and *Nanog* (Annerén et al., 2004), and Oct4 binds to *Yes* promoter positively regulating it (Zhang et al., 2007). Moreover, inhibition of the Src family of tyrosine kinases did not have an effect on other pathways activated by LIF, such as STAT3 or MAPK, suggesting that the Src pathway is independently activated by LIF (Annerén et al., 2004). Although Yes was known to be activated by LIF via association of Yes to gp130 through its SH2 domain, the exact pathway downstream of Yes remained unknown until a recent study where they showed that Yes bound to gp130 is activated through autophosphorylation. Once activated Yes can phosphorylate the Yes-associated protein (YAP) in tyrosine residues, phosphorylated YAP binds to TEAD2 in the nucleus where it activates *Oct4* transcription (Tamm et al., 2011). Opposite to the role of Yes, Hck and Lck, Src is highly expressed in differentiating ESCs and it seems to induce differentiation into primitive ectoderm (Meyn and Smithgall, 2009).

In summary, LIF activation can contribute to maintenance of ESC self-renewal by activating STAT3, PI3K, Yes but also activates other pathways, such as MAPK and Src, that promote differentiation (Figure 1.11).

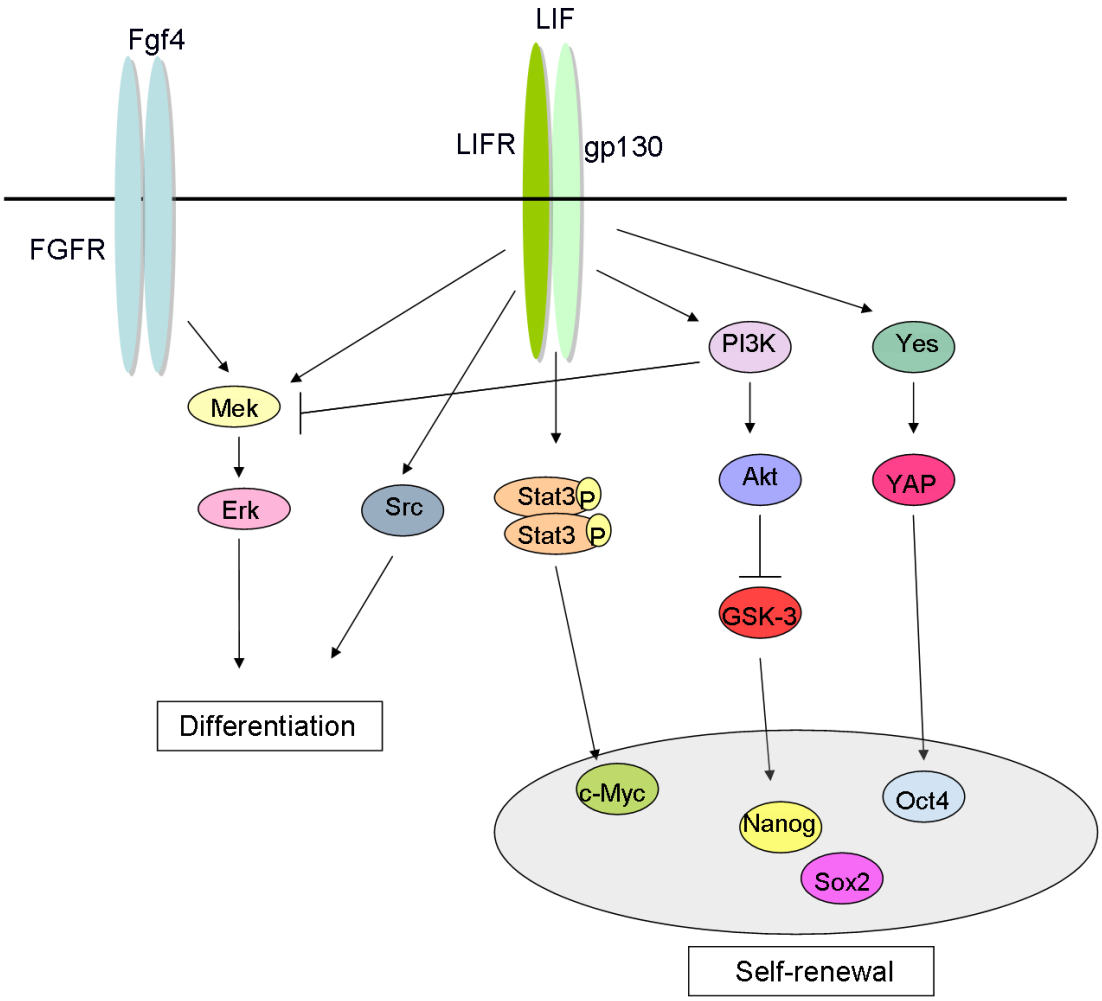

Figure 1.11 Summary of pathways regulated by LIF signalling. LIF can promote self-renewal of mouse ESCs by activating the Yes, PI3K and Stat3 signalling but it can also promote differentiation by activating MAPK and Src signalling (After Burdon et al., 1999b; Kolch, 2000; Ying et al., 2003b; Anneren et al., 2004; Paling et al., 2004; Cartwright et al., 2005; Kunath et al., 2007; Tamm et al., 2011).

1.3.2.5 BMP signalling.

Until 2003, ESCs were only cultured in the presence of LIF and Serum or in feeder layers, and it was obvious that some component of the serum was activating LIF-independent pathways as self-renewal of ESCs could not be maintained only with LIF but also required serum. Neural differentiation is not fully blocked in the absence of serum. Ying et al. reported that bone morphogenetic proteins (BMPs) were the component present in serum responsible for blocking neural differentiation and sustaining self-renewal in combination with LIF (Ying et al., 2003a). BMPs are members of the TGF-beta superfamily that act through binding to serine/threonine kinase receptors and downstream effectors, the Smad transcriptional regulators. Following activation of the receptor, receptor-activated Smads (R-Smads) 1, 5 and 8 are recruited to the receptor where they are phosphorylated, once phosphorylated they bind Smad4 and translocate to the nucleus where they regulate the expression of gene targets (Massagué et al., 2005; Shi and Massagué, 2003). *Induction of Inhibitor of differentiation (Id)* genes was found to be the key contribution of BMP as self-renewal of ESCs could be maintained in N2B27 serum-free medium with LIF but without BMP by forced expression of *Id*. ESCs with forced expression of *Id* were unable to differentiate into neural lineages when LIF was withdrawn suggesting that BMP contributes to maintenance of self-renewal by induction of *Id* genes, which in turn blocks the expression of transcription factors that specify neural lineages. Although BMP and LIF seem to cooperate to maintain self-renewal, following LIF withdrawal the role of BMP changes to promoting differentiation. Expression of a constitutively active BMP receptor or over-expression of Smad 1/4 overcomes the effect of LIF and leads to non-neural differentiation (Ying et al., 2003a).

One year layer another group reported that BMP4 could also contribute to self-renewal by inhibiting Erk and p38 MAPK pathways. Inhibition of these pathways with small inhibitors specific for Erk and p38 MAPK had the same effect as BMP4 (Qi et al., 2004).

Interestingly, the duration of the BMP signalling is regulated by other pathways that negatively regulated self-renewal such as MAPK or GSK-3 pathways. Phosphorylation of Smad1 by GSK-3 seems to suppress it transcriptional activity by increasing Smad1 proteosomal degradation. However, Smad1 has to be

phosphorylated in order for GSK-3 to recognise it, MAPK kinases such as Erk, p38 or JNK are responsible for this phosphorylation. As described above, following BMP receptor activation Smad1 is phosphorylated in the C-terminus, binds Smad4 and translocate to the nucleus. In the nucleus, MAPK kinases primes Smad1 for subsequent GSK-3 phosphorylation, at an unknown location and triply phosphorylated Smad1 is transported to the centrosome where it is proteosome degraded (Figure 1.12) (Fuentealba et al., 2007). The duration of a pulse of BMP7 was extended when inhibitors of the MAPK and GSK-3 kinases were used. Moreover, addition of Wnt3a decreased the phosphorylation of Smad1 at the GSK-3 sites leading to accumulation of phospho-Smad1 (pSmad1).

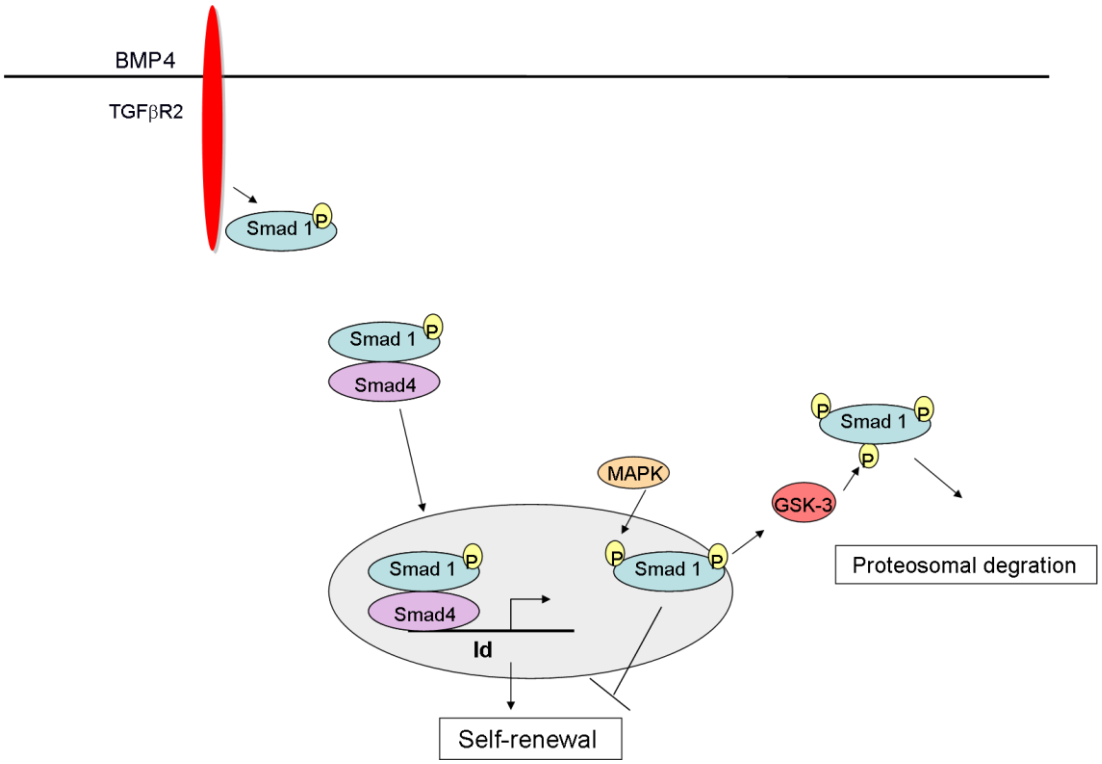

Figure 1.12. BMP signalling and regulation of its duration. Binding of BMP4 to the receptor leads to Smad1 phosphorylation that binds to Smad4 and translocate to the nucleus to activate Inhibitor of differentiation (Id) proteins. The duration of BMP signalling is regulated by phosphorylation of Smad1 first by MAPK in the nucleus that primes Smad1 for subsequent phosphorylation by GSK-3 in an unknown location. Triply phosphorylated Smad1 is transported to the centrosome and proteosomal degraded (After Fuentealba et al., 2007).

1.3.2.6 Wnt signalling

Wnts are a family of glycoproteins that play important roles in regulation of cell fate and proliferation (Moon et al., 2002). In the absence of Wnt, GSK-3 is associated with a multi-protein complex, including adenomatous polyposis coli (APC), axin and β-catenin forming the so-called "destruction complex". GSK-3 phosphorylates the three proteins present in the destruction complex leading to stabilisation of Axin, a likely enhancement of APC interaction with β-catenin and targeting of β-catenin for proteosomal degradation (Ding and Dale, 2002). Following Wnt binding to the seven transmembrane Frizzled (Fz) receptor and to the LRP (low density lipoprotein receptor protein) 5/6 receptors, a signal pathway involving dishevelled (DVL) and FRAT (Frequently rearranged in advanced T-cell lymphomas) leads to disruption of the destruction complex and GSK-3 is inactivated. Inactivation of GSK-3 leads to a decrease in phosphorylation of β-catenin resulting in accumulation of β-catenin in the cytosol and its later translocation to the nucleus. In the nucleus β-catenin associates with TCF/LEF transcription factors and becomes a transcriptional transactivator, activating transcription of Wnt target genes (Moon et al., 2002) (Figure 1.13).

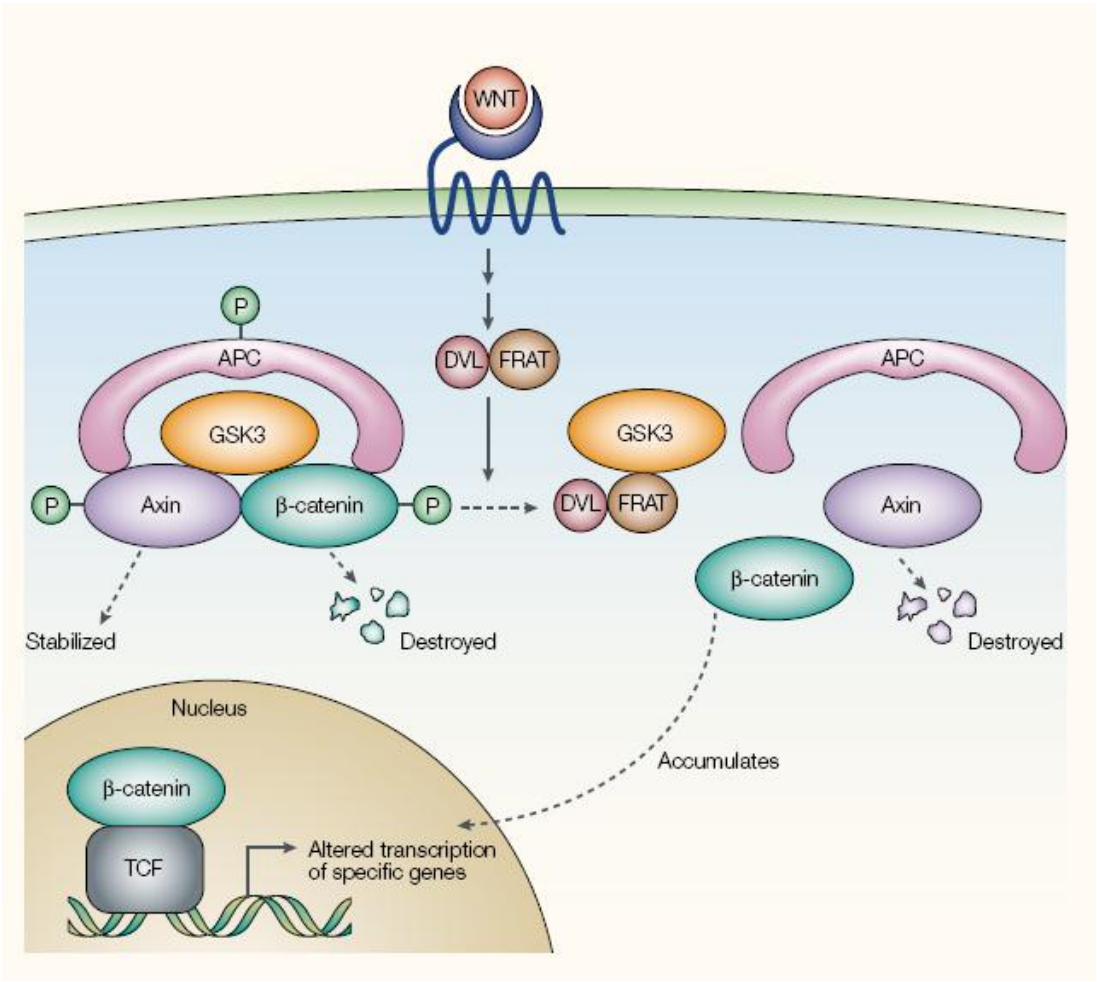

Figure 1.13 Wnt signalling pathway. In the absence of Wnt, GSK-3 is associated with APC, axin and β-catenin forming the "destruction complex". GSK-3 phosphorylates the three proteins present in the destruction complex leading to stabilisation of Axin, a likely enhancement of APC interaction with β-catenin and targeting of β-catenin for proteosomal degradation (Ding and Dale, 2002). Following Wnt binding to the Fz receptor and to the LRP 5/6 receptors, a signal pathway involving DVL and FRAT leads to disruption of the destruction complex and GSK-3 is inactivated. Inactivation of GSK-3 leads to a decrease in phosphorylation of β-catenin and consequent stabilisation of β-catenin that translocated to the nucleus. In the nucleus β-catenin associates with TCF/LEF transcription factors and becomes a transcriptional transactivator, activating transcription of Wnt target genes (reproduced with permission from Cohen and Frame, 2001).

Wnt signalling has been shown to play a role in maintenance of the undifferentiated stated of mouse ESCs. Several reports claimed that self-renewal of ESCs can be maintained by conditioned media (CM) containing Wnt3a (Ogawa et al., 2006; Singla et al., 2006). Feeders secreting Wnt5, Wnt6, Wnt3 and Wnt3a are able to prevent ESC differentiation in medium with serum in the absence of LIF. The effect of Wnt proteins was shown to be through β-catenin stabilisation as activation of β-catenin could mimic the effect of Wnt signalling. In addition to this, phosphorylation of β-catenin decreased in Wnt5a and Wnt3 conditioned media (Hao et al., 2006). However, the observation that recombinant Wnt3a alone or expression of constitutively active β-catenin can not support ESC self-renewal suggested that the Wnt signalling alone is not able to support undifferentiated ESCs and other components from the conditioned media were contributing (Ogawa et al., 2006). Indeed, low LIF activity was found to be present in Wnt3a CM by using a STAT3-responsive luciferase reporter. Wnt3a CM's ability to maintain self-renewal was reduced when an anti-LIF antibody was used to counteract LIF activity suggesting that ESC self-renewal may be supported by the synergistic action of Wnt3a and LIF present in the CM. Additional experiments proved that this was the case, e.g. addition of Wnt3a reduced the concentration of LIF required to have Oct4 positive expressing colonies from 10U to 6U, but Wnt3a alone could not maintain Oct4 positive colonies. In addition, expression of constitutively activated β-catenin was unable to maintain pluripotency alone but showed synergy with LIF (Ogawa et al., 2006). The report by Hao et al., (Hao et al., 2006) is in agreement with Wnt and LIF acting together to support ESC maintenance but the mechanism proposed is different to Ogawa's. Hao's group, similar to Ogawa's report, observed an increase in STAT3 transcription which they interpreted as a direct consequence of activation of Wnt signalling. The fact that Wnt proteins were unable to sustain pluripotency in the absence of serum and LIF but could in the presence of 10U/ml of LIF led to the hypothesis that Wnt's act in synergy with LIF to maintain pluripotency through conversion on STAT3. They proposed that Wnts increase STAT3 levels while LIF increases phosphorylation of STAT3 (Figure 1.14) (Hao et al., 2006). However, Ogawa et al., (Ogawa et al., 2006) showed that the effect on STAT3 is due to LIF present in the conditioned media which in the case of Hao could be due to LIF secreted by the feeders. Ogawa did not observe changes in phosphorylation of

STAT3 following Wnt3a activation and concluded that LIF and Wnt do not crosstalk but act synergistically to sustain self-renewal (Ogawa et al., 2006). A role of Wnt/ β-catenin in ESC self-renewal is further supported by studies showing that its expression promotes induction of pluripotency in somatic cells (Lluis et al., 2008; Marson et al., 2008a).

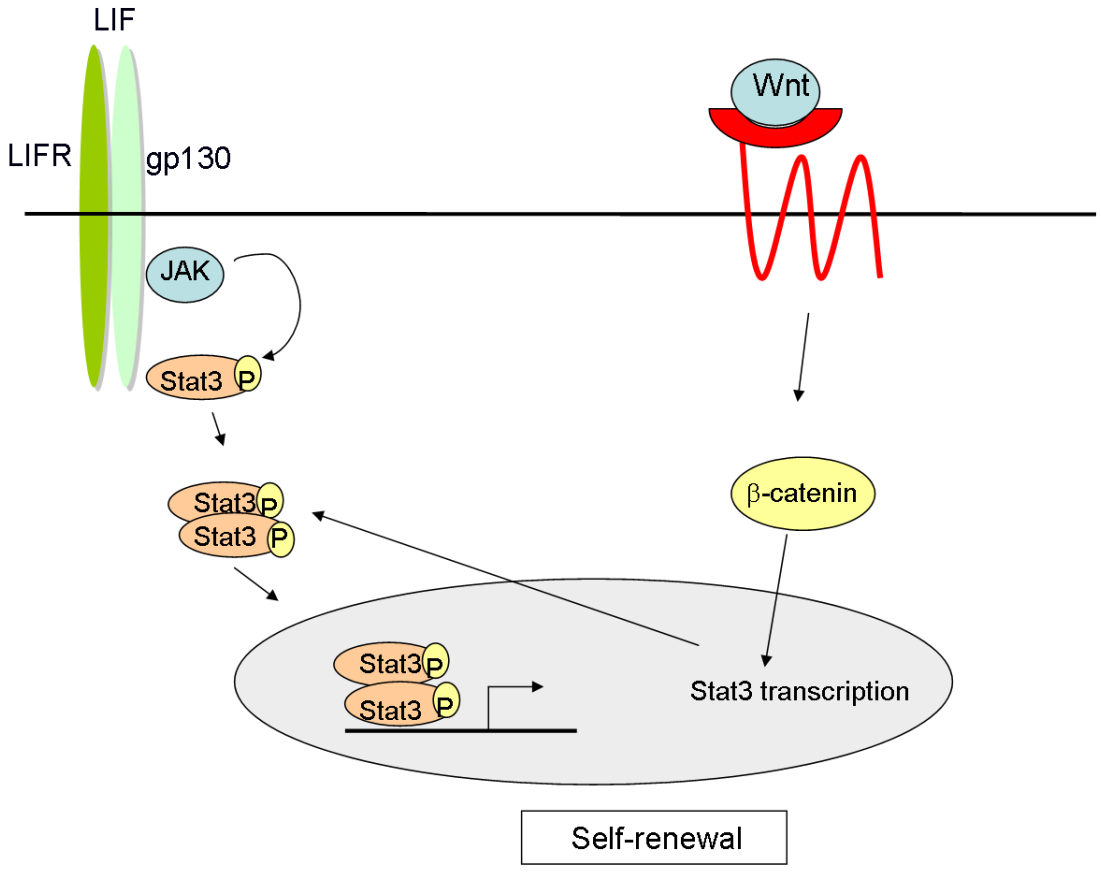

Figure 1.14 Proposed model of cooperation between Wnt and LIF to maintain self-renewal. Wnt binding to the receptor leads to stabilisation of β-catenin that translocate to the nucleus, bind to TCF/LEF transcription factors and activates Stat3 transcription increasing Stat3 levels. Activation of the LIF receptor results in phosphorylation of Stat3, translocation to the nucleus where activates transcription of target genes that promote self-renewal (Modified from Hao et al., 2006).

1.3.3 Glycogen synthase kinase-3 (GSK-3).

GSK3 was first associated with glycogen metabolism. It was identified as a kinase that inactivates glycogen synthase, which is the rate-limiting enzyme of glycogen synthesis (Embi et al., 1980). Today, GSK-3 is known to play a role in numerous signalling pathways activated by insulin, growth factors and nutrients and it is known to be important for different cellular processes such as protein synthesis, cell proliferation, cell division, differentiation, microtubule function and apoptosis (Cohen and Frame, 2001; Doble and Woodgett, 2003). GSK-3 has also been shown to be essential for establishing body pattern during embryonic development and it is also involved in ESC self-renewal and differentiation (Bakre et al., 2007; Bone et al., 2009; Doble and Woodgett, 2003; Sato et al., 2004; Ying et al., 2008). There are two GSK-3 isoforms in mammalian cells, GSK-3α and GSK-3β, which have almost identical kinase domains with 97% similarity. GSK-3α, with a mass of 51kDa, is slightly larger than GSK3-β which is 47kDa. The difference in size is due to GSK3-α having a glycine-rich extension at the N-terminus. Although they have similar kinase domains, they differ substantially in their termini (Woodgett, 1990).

GSK-3 is an unusual kinase because it is typically active in resting cells and it is inhibited upon activation of signalling pathways. Also opposite to many kinases, substrate phosphorylation leads to its inactivation. Regulation of GSK-3 is achieved through phosphorylation of Ser 21 on GSK-3α and Ser9 on GSK-3β; this phosphorylation is catalysed by protein kinase B (PKB, also known as Akt), which is activated by PI3K in response to insulin (Cross et al., 1995). GSK-3 can also be phosphorylated at the same serine residues by MAPK-activated protein kinase-1 (MAPKAP-K1), which is activated in response to growth factors or phorbol esters. Both PI3K and MAPK signalling can be activated by growth factors, for instance by the epidermal growth factor (EGF) (Shaw and Cohen, 1999). Finally, GSK-3 is also phosphorylated at Ser9/21 by p70 ribosomal S6 kinase-1 (S6K1), which is activated by mTOR in response to amino acids (Figure 1.15) (Armstrong et al., 2001; Cross et al., 1994)

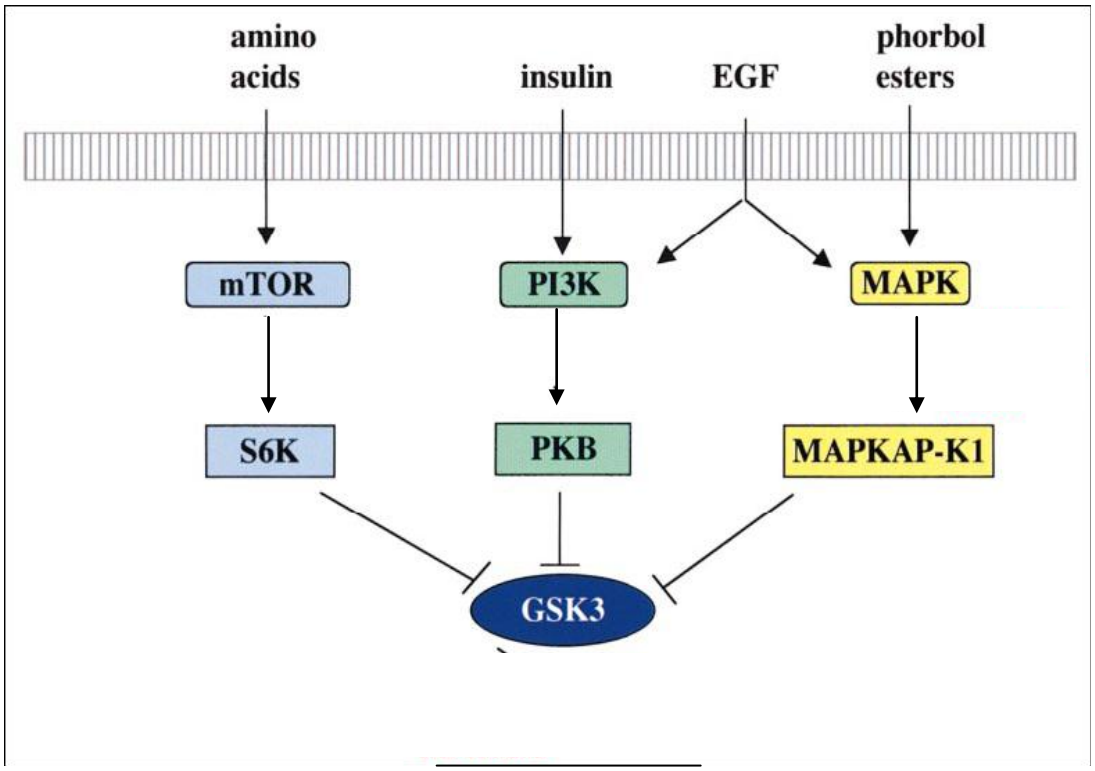

Figure 1.15 Regulation of GSK-3 by several signalling pathways. The PI3K, MAPK and the mTOR pathways can inhibit GSK-3 in response to insulin, phorbol esters and amino acids respectively. EGF can lead to inhibition of GSK-3 through activation of both PI3K and MAPK pathways (Modified from Frame and Cohen, 2001, reproduce with permission).

GSK-3 is also peculiar because it preferentially phosphorylates primed substrates that have been previously phosphorylated by another kinase at a serine or threonine residue located four residues carboxy-terminal of the GSK-3 phosphorylation site. The priming phosphate binds to a GSK-3 pocket containing arginine 96 (R96), arginine 180 (R180) and lysine 205 (K205), this binding is thought to align the substrates for subsequent GSK-3 phosphorylation. Regulation of GSK-3 by insulin and growth factors is related to its requirement for a primed substrate. In the absence of insulin or growth factor the priming phosphate is bound to the R96, 180 and K205 pocket, allowing alignment of the substrate and phosphorylation. However, following insulin or growth factor activation, GSK3 is phosphorylated at Ser 21 in GSK-3α and Ser 9 GSK-3β. These serine residues are near the amino terminal end and change the amino terminus into a pseudosubstrate which binds to its own priming phosphate pocket, impeding priming substrates binding and also blocking entry to the catalytic site (Figure 1.16) (Cohen and Frame, 2001; Frame et al., 2001).

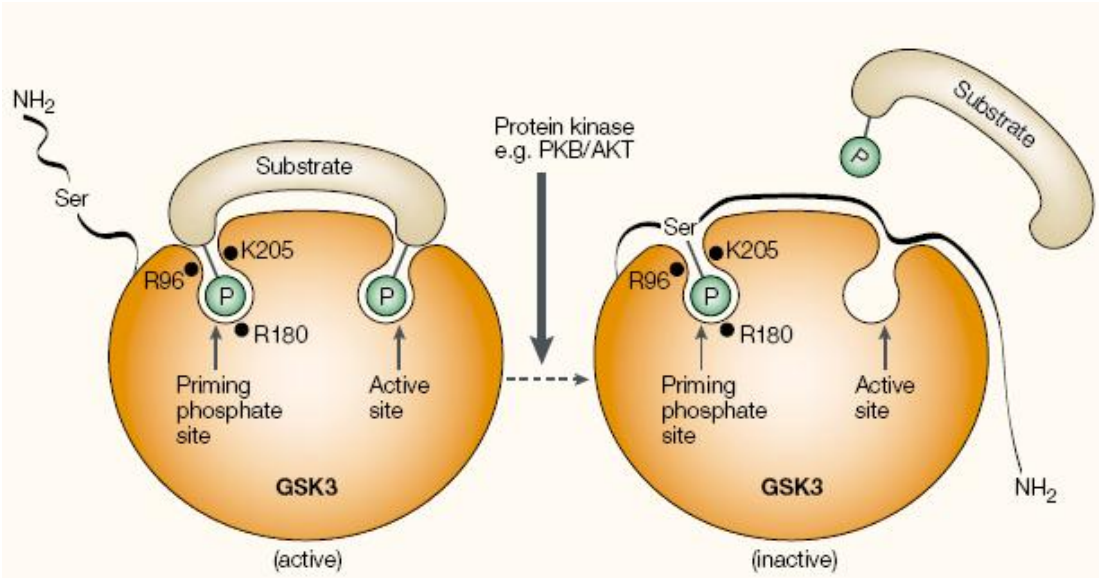

Figure 1.16. Mechanism of inhibition of GSK-3 by phosphorylation. In the absence of Insulin or growth factors, the priming phosphate is bound to a pocket containing R96, R180 and K205 which aligns the substrate for phosphorylation in the active site. Insulin or growth factors lead to phosphorylation at Ser 21 in GSK-3α and Ser 9 GSK-3β which are near the amino terminal end, changing the amino terminus into a pseudosubstrate that binds to its own priming phosphate pocket, impeding priming substrates binding and also blocking entry to the catalytic site (Reproduced with permission from Cohen and Frame, 2001).

In 1992, GSK-3 was identified to be important for cell fate specification in *Drosophila melanogaster*. The GSK-3 homologue in Drosophila, Shaggy, was discovered to be a mediator in a pathway that is activated by Wingless, a secreted glycoprotein homologue to vertebrate Wnts, which establishes the pattern of segment polarity in the fly consisting of naked band and denticle belts. Loss of function studies suggested that Wingless repressed Shaggy, e.g. loss of function of Wingless or armadillo, which is the homologue of vertebrate β-catenin , resulted in loss of the naked bands, whereas loss of function of Shaggy led to loss of the denticle belts (Siegfried et al., 1992).

The canonical Wnt pathway is very well conserved between Drosophila and vertebrates, including the mechanism of suppression of Shaggy or GSK-3 (reviewed

in Cohen and Frame, 2001). Opposite to the pool of GSK-3 downstream of PKB, MAPK or S6K1 which is free, the pool of GSK-3 involved in the Wnt signalling forms part of a destruction complex, that includes GSK-3, Axin, the adenomatous polyposis coli (APC) protein and β-catenin (Section 1.3.2.5). How GSK-3 is inactivated in response to Wnt signalling has already been discussed in section 1.3.2.5.

1.3.3.1 Involvement of GSK-3 in regulating of mouse ESC fate.

The first indication that GSK-3 may be important in maintaining ESC self-renewal came from a study carried out by Sato *et al.*, using the GSK-3 inhibitor 6-bromoindirubin-3'oxime (BIO) (Sato et al., 2004). Sato *et al.*, suggested that mouse and human ESCs could be maintained in an undifferentiated state by simply inhibiting GSK-3 with BIO, which activates Wnt signalling evidenced by activation of a promoter module that contained TCF binding sites. mESCs grown in conditioned media containing Wnt3a, similar to GSK3 inhibition, maintained transcriptional expression of *Rex1* after 5 days in the absence of LIF. In addition to this, transfection of ESCs with a dominant negative form of *Tcf3*, that impedes expression of Wnt target genes, reduced the transcriptional expression of *Rex1* in mESCs. However, the effect of GSK-3 inhibition was not assessed at a clonal density and several reports published after Sato's has shown that although GSK-3 inhibition can maintain short-term self-renewal of mESCs in the absence of LIF, addition of LIF is needed in order to support long-term self-renewal (Doble et al., 2007; Bone et al., 2009). Moreover, opposite to Sato's observation, inhibition of GSK-3 in human ESC promotes their differentiation to definitive endoderm (Bone et al., 2011).

More recent studies are in accordance with a role of GSK-3 in maintaining ESC self-renewal (Doble et al., 2007; Ying et al., 2008; Bone et al., 2009). Mouse ESCs with both isoforms α and β deleted, GSK-3α/β double knock-out (DKO), which have increase β-catenin, are more resistant to differentiation and maintained expression of markers of pluripotency, *Nanog*, *Rex1* and *Oct4*, under differentiation conditions (EB formation) (Doble et al., 2007). More recently, inhibition of GSK-3, together with

inhibition of differentiation inducing signals from Fgf4 and Erk, was shown to be sufficient to maintain ESC self-renewal in the absence of extrinsic stimuli (Ying et al., 2008). The media with the three inhibitors is designated 3i. Fgf4 is known to stimulate the Erk1/2 pathway in mESCs, promoting differentiation. PD184352 (PD) and SU5402 (S), small molecule inhibitors of the Erk cascade and FGF receptor tyrosine kinases respectively, were used to suppress differentiation signalling. Residual differentiation was suppressed by inhibition of GSK-3 with the selective GSK-3 inhibitor CHIR99021 (CHIR). mESC could also be maintained self-renewal by culturing in the presence of only CHIR and PD, this is referred as 2i media. Inhibition of GSK-3 alone could maintain short-term self-renewal but addition of an inhibitor of the Erk cascade is needed in order to support long-term self-renewal (Ying et al., 2008).

Few months after the Ying et al., report, we published that inhibition of GSK-3 with a novel selective GSK-3 inhibitor (1m) enhances self-renewal in the presence of LIF and serum. Inhibition of GSK-3 did not only increase the number of alkaline phosphatase positive colonies but also the percentage of pure self-renewing colonies. Moreover, Nanog was also increased after 2-3 days of GSK-3 inhibition. GSK-3 inhibition with 1m also decreased phosphorylation of β-catenin and increased activity of a TCF TopFlash luciferase reporter (Bone et al., 2009).

In summary, inhibition of GSK-3, which mimics activation of Wnt signalling, with small molecules inhibitors, BIO (Sato et al., 2004), 1m (Bone et al., 2009) or CHIR (Ying et al., 2008), can maintain short-term self-renewal of ESCs. However, additional factors are needed in order to support long-term self-renewal in the presence of serum is LIF and in chemically defined media (N2B27), such as inhibition of MEK.

1.3.3.2 Mechanism of action of GSK-3 inhibition in maintenance of mESC self-renewal.

Although GSK-3 has been shown to play a role in self-renewal and differentiation of ESCs (Doble et al., 2007; Ying et al., 2008; Sato el al., 2004; Bakre et al., 2007; Ding et al., 2003; Bone et al., 2009), its mechanism of action remains unclear because GSK-3 is involved in numerous pathways and regulates many biological processes including cell cycle, apoptosis, metabolism, translational and transcription (Jope et al., 2004). Despite of the fact that GSK-3 has many downstream effectors, most studies up to date suggest that the effect observed in GSK-3 inhibition is at least partly through Wnt/β-catenin. However, the role of Wnt/β-catenin remained controversial for several reasons, recombinant Wnt3a alone, or the expression of an activated β-catenin mutant, were not able to maintain self-renewal of ESCs (Ogawa et al., 2006; Singla et al., 2006), over-expression of β-catenin was not sufficient to maintain ESC self-renewal (Otero et al., 2004) and recombinant Wnt3a was not as efficient as CHIR in generating undifferentiated colonies when added to media containing the MEK inhibitor (PD184352) and the FGF inhibitor (SU5402). In addition to this, β-catenin transcriptional activity was not essential for generation of undifferentiated colonies in 3i (Ying et al., 2008). This was shown by introducing a dominant negative ΔNhLef1 construct into mESCs, which abolishes transcriptional activation as it is unable to bind β-catenin. The authors did not rule out the possibility of a contribution of β-catenin by TCF-independent mechanisms. On the other hand, over-expression of *β-catenin* can promote commitment to neuronal lineages (Otero et al., 2004) and inhibition of GSK-3 with TWS119, a 4,6-disubstituted pyrrolopyrimidine, which increased β-catenin levels as well as activating a luciferase TCF/LEF reporter, leads to neural differentiation of ESCs and embryonal carcinoma cells (Ding et al., 2003). Furthermore, GSK-3 inhibition also facilitates ESC differentiation toward mesendoderm lineages (Bakre et al., 2007).

To investigate the importance of Wnt/β-catenin in ESC self-renewal, *β-catenin* null cells were generated by two groups (Anton et al., 2007; Wray et al., 2011). The first report (Anton et al., 2007) suggested that β-catenin contributes to maintenance of ESC self-renewal as *β-catenin* null cells exhibited strong down-regulation of the pluripotency marker *Rex1* and *Nanog, Lefty1, Sox2* and *Klf2* were also down-

regulated, to a lesser extent though. Although the authors proposed that β-catenin is possibly not essential for maintenance of self-renewal because *Oct4*, *Sox2* and *Nanog* are still expressed, the fact that *Sox2* and *Nanog* are considerably reduced, *Rex1* disappeared, and *Fgf5* was up-regulated, suggests that β-catenin ESCs are likely to become epiblast stem cell- like suggesting that β-catenin is important for ESC self-renewal under regular culture conditions (serum plus LIF) (Anton et al., 2007).

Another report from Wray et al., (Wray et al., 2011) showed that β-catenin is not essential for maintenance of undifferentiated ESCs as *β-catenin* null cells can be grown in 2i+LIF and PD+LIF while retaining expression of pluripotency markers Nanog and Oct4, as well as their ability to give rise to alkaline phosphatase positive colonies when plated at clonal density. However, *β-catenin* null cells lose responsiveness to CHIR, evidenced by the lost of *Rex1* expression if they are cultured in CHIR+LIF, 2i or CHIR but not in 2i+LIF or PD+LIF. Moreover, they are unable to form colonies from single cells when grown in 2i or CHIR+LIF and stable expression of β-catenin can restore this ability. These experiments suggested that the effect of GSK-3 inhibition with CHIR in self-renewal is mainly mediated by β-catenin. However, the fact that colony formation was better in 2i+LIF than in PD+LIF suggested that β-catenin independent mechanisms downstream of GSK-3 can also contribute to the effect of CHIR in ESC self-renewal.

According to Anton et al., and Wray et al., reports, it seems that the importance of Wnt/β-catenin in regulating self-renewal may depend on the culture conditions. Several mechanisms of action of how Wnt/β-catenin is mediating GSK-3 inhibition have been proposed.

Anton et al., (Anton et al., 2007) suggested that the effect of β-catenin could be mediated partly through TCF/LEF independent mechanism as they observed minimal activation of TopFlash activity in ESCs. They argued that β-catenin actually was reported to interact with Klf4 (Zhang et al., 2006b), Sox2 (Mansukhani et al., 2005) and Oct4 (Takao et al., 2007) which cannot be monitored with Tcf/Lef TopFlash. A recent report supports the idea of β-catenin acting through a Tcf independent mechanism, Kelly and colleagues propose that stabilised β-catenin interacts with

Oct4 and enhances its activity (Kelly et al., 2011). On the other hand, several reports support the idea of β-catenin acting through a Tcf-dependent mechanism (Cole et al., 2008; Wray et al., 2011; Yi et al., 2011). Cole et al., proposed a model to explain how Tcf3 may control the balance of pluripotency and differentiation in ESCs. They suggested that although Tcf3 may repress or activate genes under standard conditions, it is mainly repressive and thus induces differentiation. In favour of this, they argue that knockdown of *Tcf3* led to increased expression of *Nanog*, *Oct4* and *Sox2*. Activation of the Wnt pathway changes the main nature of the Tcf3 complexes from repressive to activating, favouring self-renewal (Cole et al., 2008). Two more recent reports support the idea that the GSK-3 inhibition effect is mediated through stabilisation of β-catenin, which interacts with Tcf3 abrogating its repressive activity in the pluripotency transcriptional network (Wray et al., 2011; Yi et al., 2011). Wray and colleagues demonstrated that the effect of GSK-3 inhibition is not mediated through *β-catenin* transcriptional activation as expression of β-catenin that lacks the transactivation domain in *β-catenin* null cells restored responsiveness to GSK-3 inhibition similar to cells expressing a WT β-catenin (Wray et al., 2011). The fact that β-catenin without the transactivation domain can still interact with Tcf/Lef factors together with the fact that Tcf3 act as a repressor (Pereira et al., 2006) and that Tcf3 knockout delay differentiation (Guo et al., 2011; Pereira et al., 2006) led to the hypothesis that β-catenin may act by relieving Tcf3 repression. In accordance with this, expression of pluripotency markers such as *Klf4* and *Nanog* were shown to be maintained in conditions that will be lost such as when you grow cells in N2B27 alone simply by knocking down *Tcf3*. Moreover, *Tcf3* null cells can stay undifferentiated when grown only in the presence of MEK inhibitors (e.g. PD) for 5 passages. However, *Tcf3* null cells expressing either WT-Tcf3 or Tcf3 that lacks the β-catenin interacting domain (Tcf3-ΔN) failed to form undifferentiated colonies in PD only. Tcf3-WT cells were able to form undifferentiated colonies in 2i whereas Tcf3-ΔN formed very few colonies suggesting that β-catenin interaction with Tcf3 is essential to mediate the effect of GSK-3 inhibition. They concluded that GSK-3 inhibition may enhance self-renewal by alleviating Tcf3 repression in the pluripotency network (Yi et al., 2011).

As previously mentioned Yi et al., (Yi et al., 2011) report is also in accordance with a role of β-catenin interaction with Tcf3 relieving the Tcf3 repression activity. However, they also suggest a role for endogenous Tcf1 as a mediator of Wnt/β-catenin activation. They performed a series of experiments to test this idea. The repressive activity of Tcf3 in self-renewal was demonstrated by the fact that *Tcf3* over-expression eliminated self-renewal capacity, shown by the lack of alkaline phosphatase positive colonies and a shift from a cell cycle typical of ESCs, with a high percentage of cells in the S phase, to one typical of somatic cells, with higher percentage in G1. Moreover, Wnt3a could overcome the effect of *Tcf3* over-expression restoring alkaline phosphatase positive colonies and cell cycle profiles, thereby supporting the idea of Tcf3 as a negative regulator and Wnt3a as a positive regulator of self-renewal. They also confirmed the results from Wray and colleagues regarding the fact that *Tcf3* null cells gave rise to alkaline phosphatase positive colonies when cultured in serum free media supplemented with the MEK inhibitor (PD0325901) only and they also observed that ESC with intact Tcf3 can generate alkaline phosphatase positive colonies in 2i but not in PD only suggesting that Tcf3 ablation indeed mimicked GSK-3 inhibition. They also showed that interaction of β-catenin with Tcf3 was important for mediating the Wnt3a effect as the number of alkaline phosphatase positive colonies was reduced in cells with a dominant negative mutant Tcf3 ($Tcf3^{\Delta N/\Delta N}$) which can not bind to β-catenin following Wnt3a stimulation. However, the fact that the number of alkaline phosphatase positive colonies was reduced by not eliminated by Wnt3a in $Tcf3^{\Delta N/\Delta N}$ and that the number of alkaline phosphatase positive colonies could also be increased in *Tcf3* null cells stimulated with Wnt3a suggested that Tcf3-β-catenin independent mechanisms also contribute to Wnt3a effect. Tcf1 was shown to activate *Nanog* and superTOPFlash promoters and was thought to mediate Wnt effect. The role of Tcf1 as a mediator activator was shown through a genetic approach. Elimination of the Tcf3-β-catenin interaction and Tcf1 endogenous activity by using $Tcf3^{\Delta N/\Delta N}$ and short hairpin RNA (shRNA) targeting *Tcf1*, reduced the self-renewal response to Wnt3a. The authors proposed that Wnt3a can stimulate self-renewal by suppression of Tcf3 repressor transcriptional activation through Tcf3-β-catenin interaction but also by Tcf1-dependent mechanisms. They did not rule out the possibility of some contribution by other Tcf-independent mechanisms to Wnt effect in self-renewal as a small number

of alkaline phosphatase positive colonies were produced from ESCs expressing $Tcf3^{\Delta N/\Delta N}$ and with Tcf1 shRNA. In fact, Anton et al., and Kelly et al., (Anton et al., 2007; Kelly et al., 2011) support the idea of the existence of β-catenin-Tcf-independent mechanism, and Kelly and colleagues proposed that the effect of Wnt signalling is mediated by stabilisation of β-catenin which binds to Oct4 enhancing its activity (Kelly et al., 2011). However, Yi et al., did not agree with this paper as whereas they observed Tcf3 and Tcf1-dependent β-catenin recruitment to chromatin using ChiP-qPCR, they did not observed that Oct4 dependent β-catenin recruitment. They concluded that Tcf-independent mechanisms can have small contribution to the enhancement of self-renewal following Wnt activation but they do not think this is through β-catenin recruitment to the chromatin by Oct4.

In summary, most studies to date suggest a role for β-catenin as a mediator of the effects occurring following GSK-3 inhibition and most recently light has been shed regarding how β-catenin is mediating this effect. Although β-catenin TCF-dependent and TCF-independent mechanisms have been proposed, the controversy continues. Interestingly, Wray et al., and Yi et al., agree with the possibility of other TCF-independent mechanisms contributing to regulation of self-renewal, and Ying et al., and Wray et al., suggest that β-catenin-independent mechanisms may also contribute to the effect of GSK-3 inhibition in self-renewal.

Mechanisms of action of GSK-3 independent of β-catenin including regulation of c-Myc and Nanog have also been proposed (Bechard and Dalton, 2009). Both reports proposed a mechanism involving GSK-3 downstream of PI3K. PI3K has been shown to be important for maintaining ESC self-renewal (Paling et al., 2004) and this is partially by PI3K's ability to maintain Nanog expression, which is mediated by GSK-3 (Storm et al., 2007). Inhibition of GSK-3 can reverse the decrease in *Nanog* RNA levels and protein expression following inhibition of PI3K suggesting that PI3K regulates Nanog expression through inhibition of GSK-3 (Storm et al., 2007). In addition to this, inhibition of PI3K decreases phosphorylation of S21/9 of GSK-3 but there is no significant effect on phosphorylation of β-catenin or β-catenin levels suggesting that PI3K does not regulate the pool of GSK-3 involved in the Wnt signalling, and thus the effect observed in ESC self-renewal following GSK-3

inhibition may be mediated by GSK-3 downstream of PI3K as well as downstream of Wnt/β-catenin signalling.

1.3.3.3 GSK-3 downstream effectors.

The mechanism of action of GSK-3 inhibition that result in enhancement of mESC self-renewal have partly remained unclear because GSK-3 is involved in a number of signalling pathways and numerous downstream effectors have been identified in non-ESC types, including transcriptional regulators, components of the cell-division cycle and protein synthesis initiation factors,. Among transcriptional regulators known to be GSK-3 substrates are c-Jun, c-Myc, β-catenin, CCAAT/ enhancer binding protein α (C/EBPα) and Nuclear factor of activated T-cells (NFATc). Cyclin D1, a cell cycle regulator, is known to be a GSK-3 substrate (reviewed in Kim et al., 2006; (Doble and Woodgett, 2003; Frame and Cohen, 2001). Finally, regarding protein synthesis, GSK-3 can inhibit protein synthesis in eukaryotes through phosphorylation of the eukaryotic protein synthesis initiation factor 2B (eIF2B). This factor is critical for initiation of translation. Therefore, GSK-3 could potentially play a role in determining ESC fate at the translational level as well as the transcriptional level. A recent report suggested that regulation of translation may play a role in controlling self-renewal and fate choice in ESCs (Sampath et al., 2008). Moreover, Nanog protein is downregulated before *Nanog* mRNA following PI3K inhibition with LY294002 suggesting that Nanog may be regulated at translational level (Storm et al., 2007). Of relevance is the fact that translation initiation can be regulated by phosphorylation of Ser539 eIF2Bε by GSK-3 downstream of PI3K (Welsh et al., 1998; Welsh et al., 1997). In the present study, we investigated a possible role for GSK-3 inhibition in regulation of translation. Molecular mechanisms that regulate general or cap-dependent translation are described in the following Section.

1.4 Regulation of global protein synthesis

A tight regulation of general translation/protein synthesis is essential for maintaining cell and tissue homeostasis because it is a very demanding process from the energetic point of view. In response to changing conditions, overall protein synthesis can be rapidly, within minutes, modulated by changes in phosphorylation of translation factors and RNA-binding proteins that control translation. It is well known that two mRNAs present at the same level can generate different amounts of protein and this is partly due to the fact that ribosomes do not associate directly with mRNA but are recruited to it by eukaryotic translation initiation factors (eIFs) (Gingras et al., 2001). Protein synthesis is a complex process that is divided into three steps: initiation, elongation and termination. Although all the steps are characterised by the presence of translation factors that associate with the ribosomes, the initiation step is the one subjected to more regulation with at least nine initiation translation factors (eIFs) identified in eukaryotes (Jackson et al., 2010; Proud, 2007).

Initiation of translation is a complex process that requires several steps including the recruitment of the 40S ribosomal subunit to the mRNA, the identification of the initiation codon and the binding of the 60S ribosomal subunit with the 40S complexes to form the 80S ribosomes leading to the start of elongation. Translation of most mRNAs is carried out by a "cap-dependent" or "scanning" mechanism whereby the 40S subunit is recruited to the cap of the mRNA, which is a structure present at the 5' end of all mRNAs, in a process that requires the unwinding of the secondary structures present in the 5' end of the mRNAs, which is accomplished by the eIF4F complex. The structure of the cap is m7GpppN where guanine nucleotide methylated on the 7 position (m^7G) is attached to the mRNA by a 5' to 5' triphosphate linkage. Once attached to the cap, the 40S ribosomal subunit then starts "scanning" the 5' untranslated region (5'UTR) of the mRNA until it finds a start codon, once codon/anticodon interactions are created, the translation initiation factors dissociate from the 40S ribosomal subunit and the 60S subunit attaches to the 40S subunit, forming the 80S ribosome and elongation starts.

The 40S ribosomal subunit seems to need a single-stranded mRNA for optimal binding (Gingras et al., 1999; Sonenberg, 1993) and the unwinding of the secondary structures is carried out by eIF4F, which is a complex that binds the cap of the

mRNA, (Gingras et al., 1999), and is composed of three units, eIF4A, eIF4E, and eIF4G. eIF4E binds to the cap and together with eIF4G, which is a scaffolding protein, recruits the translational machinery. eIF4A is an ATP-dependent RNA helicase that binds and unwinds complex secondary structures in the 5´UTR (Methot et al., 1996). The activity of the helicase is stimulated by the RNA binding protein eIF4B. The 40S ribosome, which is bound to eukaryotic initiation factor 3 (eIF3), and the ternary complex (eukaryotic initiation factor 2 (eIF2)–GTP–Methione transfer RNA (Met-tRNAi)) is brought to the cap of the mRNA through the scaffolding protein eIF4G resulting in the formation of the pre-initiation complex (Figure 1.17). The binding of the Met-tRNAi to the 40S ribosome is regulated by the eukaryotic initiation factor 2 (eIF2). eIF2 binds GTP which is hydrolysed during translation initiation. Every round of translation initiation needs eIF2-GTP and the guanine nucleotide exchange factor eIF2Bε is involved in exchanging GDP for GTP. GSK-3 is known to phosphorylate and inactivate eIF2Bε, which slows general translation initiation (Welsh et al., 1998).

The level or activity of eukaryotic initiation factor 4 (eIF4F) is frequently associated with changes in translation rate. The formation of the eIF4F complex is regulated by the eukaryotic initiation factor 4E-binding protein (4EBP1) that competes with eIF4G for binding to eIF4E. Binding of 4EBP1 to eIF4E is regulated by phosphorylation. In resting cells, 4EBP1 binds and sequesters eIF4E inhibiting translation initiation (Gebauer and Hentze, 2004; Richter and Sonenberg, 2005). mTOR activation in response to growth factors leads to phosphorylation of 4EBP1 and release of eIF4E, which can then bind to eIF4G to form the eIF4F complex (Parsa and Holland, 2004).

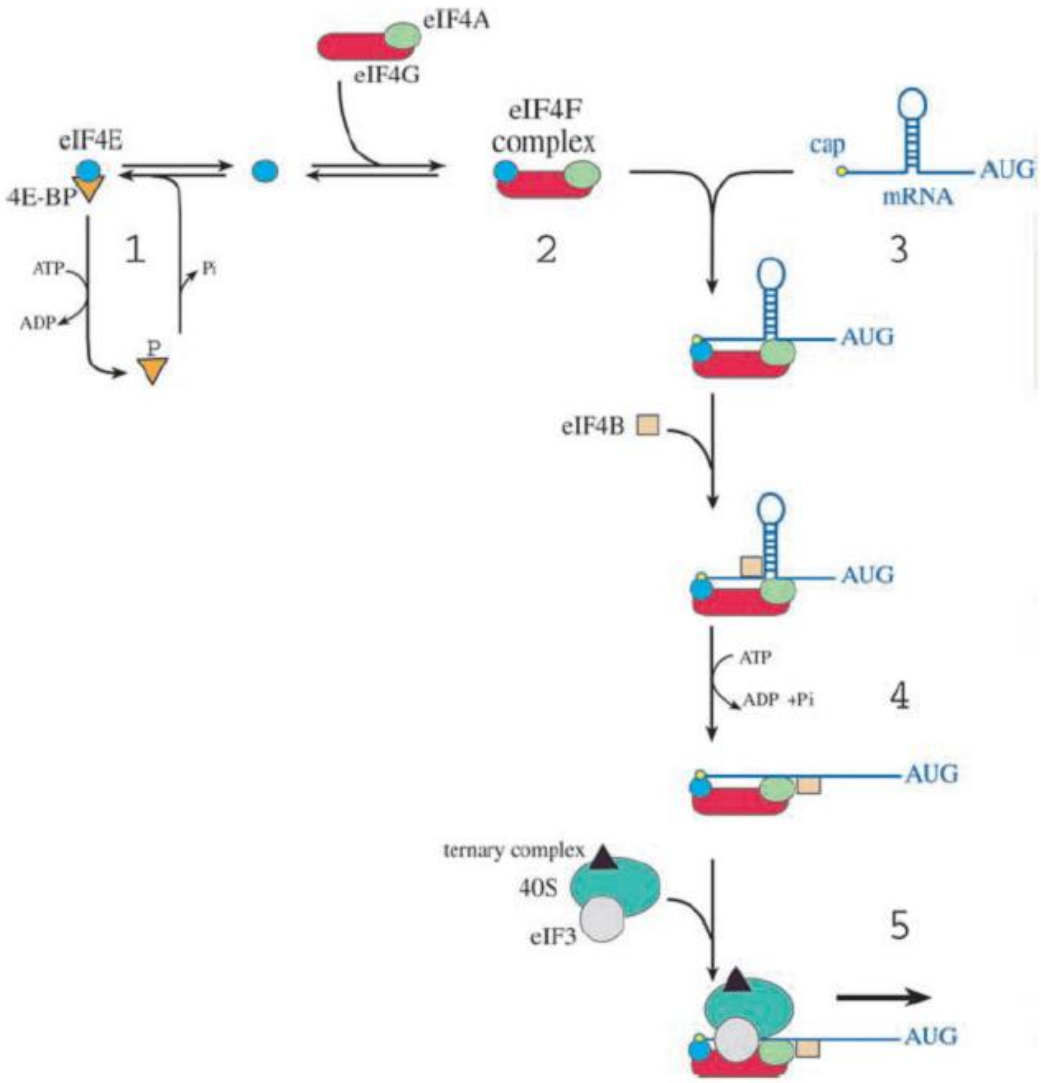

Figure 1.17. Regulation of protein synthesis initiation. In resting cells, 4EBP1 binds and sequesters eIF4E inhibiting translation initiation. Phosphorylation of 4EBP1 in response to growth factors release of eIF4E (1), which can then bind to eIF4G and eIF4A to form the eIF4F complex (2) that binds the cap (3). eIF4A together with eIF4B unwinds the secondary structure of the cap (4) and the 40s ribosomal subunit together with the ternary complex is subsequently recruited to the mRNA (Reproduced with permission from Gingras et al., 2001).

1.5 Background and aims of the study.

mESC self-renewal is regulated by the coordinated action of several pathways that are activated by extrinsic stimuli and control expression of transcription factors (Boiani and Scholer, 2005). Several studies, including ours, are in agreement with a role of GSK-3 in maintaining self-renewal of ESCs (Sato et al., 2004; Doble et al., 2007; Ying et al., 2008; Bone et al., 2009). We demonstrated that inhibition of GSK-3 with novel selective inhibitors bis-indolylmaleimides 1m and 1i enhances ESC self-renewal in the presence of Serum and LIF (Bone et al., 2009). Despite of the fact that GSK-3 has many downstream effectors, most studies to date suggest that the effect observed upon inhibition of GSK-3 is at least partly mediated through Wnt/β-catenin. Recently several proposals have been put forward regarding the underlying mechanism including Gsk3 inhibition acting via β-catenin-Tcf3 complexes to relieve the repressive effect of Tcf3 on pluripotency gene expression (Wray et al., 2011; Yi et al., 2011) and β-catenin-Tcf independent stabilisation of Oct4 enhancing its activity (Kelly et al., 2011). However, β-catenin-independent effects have been observed, raising the possibility that Gsk3 also acts via alternate mechanisms.

Mechanisms of action of GSK-3 independent of β-catenin including stabilisation of c-Myc and regulation of Nanog have also been proposed (Bechard and Dalton, 2009). Both reports proposed a mechanism involving GSK-3 downstream of PI3K. Interestingly, translation initiation can be regulated by phosphorylation at Ser^{539} of eIF2Bε by GSK-3 downstream of PI3K (Welsh et al., 1998; Welsh et al., 1997) and Nanog protein was observed to be down-regulated before its mRNA following PI3K inhibition suggesting that GSK-3 may regulate Nanog at the translational level (Storm et al., 2007). In contrast, other studies have reported that inhibition of GSK-3 leads to neuronal or mesendodermal differentiation of ESCs (Ding et al., 2003; Bakre et al., 2007). The present study intended to examine the importance and mechanism of action of GSK-3 in ESCs, which was performed by addressing the following aims:

- **Investigation of the maintenance of ESC pluripotency following treatment with novel GSK-3 inhibitors**.

  ESCs cultured in the presence of GSK-3 inhibitors, BIO or CHIR, have been shown to maintain their pluripotency by contributing to chimeras and generating teratomas containing derivates of the three germ layers following withdrawal of the inhibitors (Sato et al., 2004; Ying et al., 2008). However, DKO GSK-3 cells exhibited abnormal differentiation potential in EBs or teratocarcinomas (Doble et al., 2007). It was therefore essential to ensure that these novel GSK-3 inhibitors are not increasing ESCs self-renewal because they are irreversibly blocking the ability of ESCs to differentiate and this was investigated in Chapter 3.

- **Investigation of the effects of GSK-3 inhibitors on the multilineage differentiation of ESCs.**

  Several studies reported that GSK-3 inhibition had an effect on the multi-lineage differentiation of ESCs. Some reports suggest that GSK-3 inhibition promotes differentiation of ESCs into neuronal lineages (Ding et al., 2003) while others proposed that it induces differentiation of ESCs along mesendodermal lineages, probably at the expense of ectoderm/neuroectoderm. In order to resolve the importance of GSK-3 in ESC differentiation, effects of GSK-3 inhibition with 1i, 1m and BIO on the multi-lineage differentiation potential of ESCs was investigated on chapter 3.

- **To unravel the mechanism of action of GSK-3 in mouse ESCs**.

  GSK-3 has been shown to regulate expression of pluripotency-associated transcription factors including c-Myc and Nanog (Bechard and Dalton et al., 2009; Storm et al., 2007) raising the possibility that GSK-3 inhibition is regulating the expression of others transcription factors also. We investigated the effects that GSK-3 inhibition has on the levels, stability and synthesis of pluripotency-associated transcription factor proteins, including Nanog, Tbx3, c-Myc, Zscan4 and Oct4 and examined the influence of GSK-3 inhibition on pathways known to influence protein translation (Chapter 4 and 5).

2 CHAPTER: MATERIALS AND METHODS

2.1 Tissue culture consumables.

Table 2.1 Growth medium and supplements

CULTURE MEDIUM	SUPPLIER	CATALOG Nº
Glasgow's Minimal Essential Medium (GMEM)	Invitrogen	21710-025
Knockout Dulbecco's Modified Eagle Medium (DMEM)	Invitrogen	10829-018
Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM F-12 media)	Invitrogen	12634-010
Neurobasal media	Invitrogen	21103-049
Iscove´s Modified Dulbecco´s Medium (IMDM)	Invitrogen	21980-032
SERUM	**SUPPLIER**	**CATALOG Nº**
Knockout Serum Replacement	Invitrogen	10828-028
ES Screened Fetal Bovine Serum (FBS) (Hyclone)	Hyclone	SH30070.03E
MEDIUM SUPPLEMENTS	**SUPPLIER**	**CATALOG Nº**
Glutamine	Invitrogen	25030-024
2-mercaptoethanol	Bio-Rad	161-0710
100x Non-essential amino acids (NEAA)	Invitrogen	11140-050
Sodium Pyruvate	Fisher Scientific	11360
N2 Supplement (100X)	Fisher Scientific	07152
B27 Supplement (50x)	Fisher Scientific	07153
Bovine Serum Albumin (BSA) Fraction V	Invitrogen	15260
Monothioglycerol	Sigma	M6145

Table 2.2 Cytokines and growh factors

CYTOKINES AND GROWTH FACTORS	SUPPLIER	CATALOG Nº
ESGRO LIF	Chemicon	ESG1106
BMP4 Recombinant human	R&D systems	314-BP-010
Stemfactor BMP4, Human Recombinant	StemGent	03-007

Table 2.3 Inhibitors

INHIBITORS	SUPPLIER	CATALOG Nº
1m, 1i	School of Chemistry and Astbury Centre for Structural Molecular Biology, University of Leeds	
GSK-3 inhibitor IX (BIO)	Merk/Calbiochem	361550
CHIR99021	Axon MedChem	Axon 1386
PD0325901	Axon MedChem	Axon 1408

Table 2.4 Other consumables

OTHER CONSUMABLES	SUPPLIER	CATALOG Nº
Porcine Gelatine	Sigma	G1890-110G
Trypsin-Ethylenediaminetetraacetic acid (EDTA)	Fisher Scientific	25300-062
DMSO	Sigma	D2650
Phosphate Buffered Saline (PBS)	Invitrogen	14200-067

2.2 Cell Culture

2.2.1 Cell lines

E14tg2a

E14tg2a mouse embryonic stem cell line (clone R63), modified to maintain stable integration of a tetracycline-regulated transactivator construct pCAG20-1 (Era and Witte, 2000).

Brachyury-GFP

CGR8 ESCs stably expressing GFP under the control of the endogenous Brachyury promoter, given as a gift by Agapios Sachinidis (University of Cologne, Germany).

Sox1-GFP

E14 ESCs stably expressing GFP under the control of the endogenous Sox1 promoter. The coding sequence for GFP was knocked in the open reading frame of the Sox1 gene (Ying et al., 2003b).

GSK3 double knockout (DKO)

ESCs where both alleles of Gsk3α and β have been deleted by homologous recombination (Doble et al., 2007).

2.2.2 Culture of Embryonic Stem cells (ESC).

The murine embryonic stem cell lines, E14tg2a (clone R63), Brachyury-GFP and Sox1-GFP were routinely cultured on 92 x 17 mm NUNC culture plates coated with 0.1% (w/v) porcine gelatin in Knock-Out (KO) Dulbecco's modified Eagle medium in the presence of 15% (v/v) Knock-Out serum replacement, supplemented with 0.1mM 2-mercaptoethanol, 2mM glutamine and 0.1mM non-essential amino acids. We refer to the KO with all the supplements as complete KO media. Cultures were supplemented with 4µl/ml recombinant human LIF conditioned media from the HEK293LIFV5 cell line, which was generated by stable expression of a V5 epitope-tagged version of human LIF cDNA in HEK293 cells. In order to passage, cells were

washed twice with phosphate buffered saline (PBS) then dissociated with Trypsin/EDTA for 5 minutes at 37°C. Cells were resuspended in complete KO media, centrifuged at 1000 rpm for 5 minutes and the supernatant was removed. After resuspension, cell counts were performed using a Neubauer haemocytometer. ESCs were plated at densities of 5 x 10^5 cells/92 x 17 mm Nunc-tissue culture dish for passage every two days or 2 x 10^5 cells/dish for passage over the weekend. Cultures were maintained in humidified incubators at 37°C and 5% (v/v) CO_2.

E14tg2a ESC were also cultured in N2B27-defined media, which consists of 1 volume DMEM F-12 media: 1 volume Neurobasal media supplemented with N2 and B27 supplements (see below for composition), 0.0125% (v/v) Monothioglycerol, 50 μM bovine serum albumin (BSA) and 2mM Glutamine. 10ng/ml BMP4 (Stem Gen or R&D Systems) and 1000U/ml LIF (Chemicon) were added to the media as previously described (Ying et al., 2003a). ESCs were plated at 6 x 10^5 cells/92 x 17 mm dish.

E14tg2a ESC were also cultured in 2i media, consisting of N2B27 media supplemented with 1μM PD0325901 and 3μM CHIRON and plated at a minimum density of 6 x 10^5 cells/92 x 17 mm dish.

N2 supplement: 25μg/ml Insulin,100μ/ml Apo-transferrin, 30ng/ml Sodium Selenite,16μg/ml Putrescine and 6ng/ml Progesterone.

B27 supplement: Biotin, L-camitine, Corticosterone, Ethanolamine, D(+)-galactose, Glutathione, Linolenic acid, Linoleic acid, Progesterone, Putrescine, Retinyl acetate, Selenium, T3 (triodo-1-thyronine), DL-_-tocopherol (Vitamin E), DL-_-tocopherol acetate, Albumin (bovine), Catalase, Insulin, Superoxide dismutase, Transferrin.

2.2.3 Freezing and Thawing of ESCs.

ESCs were washed twice in PBS, trypsinised, resuspended in 10ml complete KO media and centrifuged at 1000rpm for 5 minutes. The supernatant was removed and cells resuspended in ice-cold Glasgow's Minimal Essential Medium supplemented

with 2mM glutamine, 50µM ß-mercaptoethanol, 0.1mM non-essential amino acids, 1mM sodium pyruvate and 10% Biosera serum. We refer to the ice-cold Glasgow's Minimal Essential Medium with all supplements as complete GMEM. 1ml of complete GMEM and 10% (v/v) Fetal Bovine Serum (Media A) was added per $2x10^6$ cells. After resuspending in media A, 1ml/$2x10^6$ cells of media B, consisting of media A with 20% (v/v) DMSO, was added drop-wise. 1ml of cells was aliquoted into NUNC (377224) Cryovials and placed at -80°C overnight before being transferred for long-term storage in liquid nitrogen.

To thaw cells, cryovials were placed in the water bath at 37°C until defrosted, and then cells transferred to 9ml of complete KO media and pelleted at 1000rpm for 5 minutes. Cell pellets were resuspended in complete KO media plus 4µl/ml recombinant human LIF conditioned media from the HEK293LIFV5 cell line and plated onto gelatin-coated 92 x 17mm NUNC dishes and placed in humidified incubators at 37°C with 5% CO_2 (v/v). After 24 hours incubation, media was changed and ESCs were cultured as described in 2.2.1.

2.2.4 Generation of Embryoid Bodies (EBs)

Embryoid bodies were formed from either ESCs grown in complete GMEM with 10% (v/v) Hyclone serum or ESCs grown in complete KO. ESCs were trypsinised to dissociate the cells and resuspended in complete KO media, centrifuged at 1000 rpm for 5 minutes and the cell pellet was resuspended in Iscove´s Modified Dulbecco´s Medium (IMDM). $1x10^5$ cells were added to 10ml of EB media. EB media consists of 5ml 2.5% (w/v) Methylcellulose (2.5g methylcellulose/100ml Baxter water, Sigma), 1.5ml Fetal Bovine Serum (Invitrogen, VX16000-044), 20µl transferin (100mg/ml), 30µl MTG, 10µl ascorbic acid (50mg/ml), 10µl insulin (10mg/ml) and 2.5ml IMDM. After addition of cells the mixture was vortexed, left for 10 minutes to allow bubbles to disappear and was then plated into non-gelatin coated dishes before incubating them at 37˚C for 2, 4 and 6 days.

2.3 Methods for protein analysis.

2.3.1 Generation of protein extract.

Two different protocols were followed to extract protein lysates. The NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce) were used for enrichment of nuclear extracts following the Manufacturer´s instructions. Briefly, dishes were washed twice with PBS, trypsinised, cells resuspendend in media containing serum to neutralise the supernatant, cell pellets were washed twice before resuspending in chilled cytoplasmic extraction reagent I (CERI) at 100µl CERI /2x10^6 cells and transfer to 1.5ml eppendorf tubes. Tubes were vortexed for 15 seconds on the highest setting to resuspend the cell pellet, incubated on ice for 10 minutes before adding 5.5µl cytoplasmic extraction reagent II (CERII) and vortexed again for 5 seconds on the highest setting. After vortexing, samples were incubated on ice for 1 minute and vortexed again for 5 seconds before centrifugation at 16,000x g. The supernatant, containing the cytoplasmic extract, was then transferred to a chilled tube and store at -20ºC until use. The pellet, containing the nuclei, was then resuspendend in 40µl of ice-cold nuclear extraction reagent (NER), vortexed for 15 seconds on the highest setting and incubated on ice for 10 minutes. Samples were vortexed for 15 seconds every 10 minutes for a minimum of 40 minutes and then centrifuged at 16,000 x g for 10 minutes. The supernatant, containing the nuclear extracts, was transferred to a chilled tube and stored at -20ºC until use.

When nuclear extracts were not required, another protocol was used to extract protein from cells, dishes were placed on ice and washed with PBS three times prior adding 80µl of solubilisation buffer/6x10^5 cells containing 50mM Tris- Hcl pH7.5, 150mM NaCl, 1%(v/v) Nonidet P40, 10% (v/v) glycerol, 5mM EDTA, 1mM Sodium Vanadate, 10mM sodium fluoride, 1mM Sodium Molybate, 40µg/ml PMSF, 0.7µg/ml pepstatin A, 10µg/ml Aprotinin, 10µg/ml Leupeptin, 10µg/ml Soyabean trypsin inhibitor and water. Sterile cell scrapers were used to harvest protein lysates. Insoluble material was removed by centrifugation at 13000rpm, 4ºC for 3 minutes. Supernatant was then removed and transferred to a chilled 1.5ml eppendorf tube. Protein concentrations of the clarified supernatants were determined by Bradford protein assay. Reagents for sample extraction are listed on Table 2.8.

2.3.2 Bradford protein assay.

Protein concentrations in samples were measured using the Bradford protein assay (Bradford, M. M., 1976). 1ml of Bio-Rad protein assay reagent, diluted 1 in 10, was added to 1.5ml tubes and 0, 2, 4, 6, 8 and 10 µl of 1mg/ml BSA was added to each tube to generate a standard curve. 5 µl of each sample, the concentration of which had to be determined, was added to 1ml of the diluted Bio-Rad reagent. After thorough mixing, 200 µl of each test solution and the standards were pipetted in triplicate into individual wells of a 96 well tray and the plate read at 595nm using a microplate reader (Versamax tunable, Molecular Devices). The concentration of protein in test samples were determined from the standard curve obtained with the BSA standards.

2.3.3 Sample preparation

20µg of cytosolic protein or 10µg of nuclear extract were boiled at 95˚C for 5 minutes, to denature protein, in an SDS buffer composed of 10% SDS (w/v), 50% Glycerol (v/v), 200mM Tris HCl pH6.8, Bromophenol blue and 5%(w/v) 2-mercaptoethanol. Protein samples were separated according to their molecular size using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

2.3.4 SDS-PAGE

7.5%, 10%, 12% or 15% (w/v) acrylamide separating gels were used for immunoblotting depending on the molecular weight of the protein to be resolved. For proteins of molecular weight between 40-200 kDa, 7.5% was used, between 30-150 kDa, 10% was used, between 20-120 kDa, 12% was used and finally between 10-100 kDa 15%.

SDS gels were prepared using mini protean III gel electrophoresis apparatus (Bio-Rad). The compositions of the 7.5%-15% resolving gels are shown in Table 2.5. The resolving gel was poured into the gel casting apparatus, milli-q H_2O was placed on top and the gel allowed to polymerise for 30 minutes. After polymerisation water was aspirated and 5% (w/v) stacking gel (1.67ml acrylamide, 6ml milli-Q H_2O, 1.25ml 1M Tris-HCl pH6.8, 0.15ml 10%(w/v) SDS, 50µl 10% (w/v) ammonium

persulphate (APS) and 20µl Tetramethylethylenediamine (TEMED) was poured on top. A fifteen well comb was used to create the wells. The wells were washed with milli-qH_2O before loading boiled samples and protein standards and then placed into a gel tank with 1x SDS-PAGE running buffer (25mM Tris, 0.1% (w/v) SDS, 192mM glycine).

A constant voltage of 80mV was applied to stack the proteins in the stacking gel and 180mV to resolve proteins in resolving gel.

Table 2.5 Composition of resolving gel according to its acrylamide percentage.

% Acrylamide Resolving gel*	7.5	10	12	15
Acrylamide/bisacrylamide 30% (w/v)/ 0.8% (w/v)	3.75	5	6	7.5
MilliQ H_2O	5.6	4.35	3.35	1.85
1M Tris-HCl pH 8.8	5.6	5.6	5.6	5.6
10% (w/v) SDS	0.25	0.25	0.25	0.25
10% APS	0.05	0.05	0.05	0.05
TEMED	0.02	0.02	0.02	0.02

*All volumes are ml.

2.3.5 Immunoblotting by Semi-Dry Transfer

After separation by SDS-PAGE, proteins were transferred from the gel to a nitrocellulose membrane by immunoblotting in semi-transfer buffer (39mM glycine, 48mM Tris base, 0.0375% (w/v) SDS, 20% (v/v) methanol). Gels were placed onto a piece of nitrocellulose, sandwiched between four buffer-soaked sheets of 3MM Whatman paper. The sandwich was placed between the lower (positive) and the upper (negative) graphite electrodes of the transfer apparatus, which was connected to a electrophoresis power supply (Amersham Biosciences). Immunoblotting was performed with a current of 0.8mA per cm^2 for 60 minutes.

After rinsing in distilled water, protein transfer to the nitrocellulose membrane was assessed by staining the nitrocellulose with the water-soluble stain Ponceau S (0.1%

Ponceau S (w/v) in 5% acetic acid). This procedure also revealed the molecular weight standards, the positions of which were subsequently marked. Blots were then washed in TBS (20mM Tris-HCl pH7.5, 150mM NaCl) and blocked for 1 hour either in ECL Advanced blocking solution (0.2g blocking agent in 10 ml TBS) or 5% BSA block (5% (w/v) BSA, 1% (w/v) ovalbumin, 0.05% (w/v) sodium azide in TBS) at room temperature. Nitrocellulose blots were incubated overnight with primary antibody (Table 2.6) at 4ºC. Following primary incubation, membranes were washed once in TBS, three times with TBS Tween (TBST) (0.05% (v/v) Tween in TBS) and a final TBS wash. All the washes were applied for 10 minutes. A polyclonal peroxidase-conjugated secondary antibody (Table 2.7) was then applied and blots were incubated for 1 hour. Blots were washed as before with an additional final TBS wash. ECL Advanced Western blotting detection reagent (GE Healthcare) or Chemiglow (Alpha Innotech) was applied for 1 minute. There are two reagents in these kits that had to be mixed 1:1 before adding them to the immunoblots. Blots were wrapped in Clingfilm and placed in an autoradiography cassette. Blots were developed with Fuji X-Ray film developer or in the ImageQuant RT-ECL system. Reagents used for resolution and immunoblotting are listed on Table 2.8.

For reprobing, blots were stripped in stripping buffer (6.25% (v/v) 1M Tris-HCl pH 7.5, 2% (w/v) SDS, 770µl of ß-mercaptoethanol) at 55°C for 45 minutes. After stripping blots were washed thoroughly in TBST and blocked again in 5% BSA+ 0.05% sodium azide for 1 hour. Immunoblotting with the appropriate antibodies was carried out as described above.

Table 2.6 Primary antibodies

PrimaryAntibodies	Source	Dilution	Supplier and Cat. No.
Nanog	Rabbit	1:2000	Abcam (ab80892)
Tbx3	Goat	1:750	Santa Cruz Biotechonology (17871)
c-Myc	Rabbit	1:1000	Cell Signalling (9402)
Oct4	Rabbit	1:1000	Santa Cruz Biotechnology (9081)
Zscan4	Rabbit	1:4000	Millipore
pSer539 eIF2Bε	Rabbit	1:1000	Chemicon International (07-822)
pSer51 eIF2α	Rabbit	1:1000	Cell Signalling Technologies(9721)
pThr389 S6K1	Rabbit	1:1000	Cell signalling Technologies (9205)
pSer366 eEF2K	Rabbit	1:1000	Cell signalling Technologies (3691)
pSer65 4E-BP1	Rabbit	1:1000	Cell signalling Technologies (9451)
pSer2448 mTOR	Rabbit	1:1000	Cell signalling Technologies (2971)
pSer2481 mTOR	Rabbit	1:1000	Cell signalling Technologies (2974)
mTOR	Rabbit	1:1000	Cell signalling Technologies (2972)
GAPDH	Mouse	1:15000	Ambion (AM4300)
SHP2	Mouse	1:1000	Santa Cruz Biotechonology (c-18)

Table 2.7 Secondary antibodies

Secondary Antibody	Source	Dilution	Supplier and Cat. No.
Rabbit-HRP	Goat	1:20000	Dako Cytomation (P0778)
Mouse-HRP	Goat	1:20000	Dako Cytomation (PO447)
Goat-HRP	Rabbit	1:20000	Dako Cytomation (PO449)

2.3.6 Immunochemistry

ESCs were cultured on Lumox (Sarstedt) trays for 5 days before fixing them with 4% (w/v) paraformaldehyde (PFA) for 45 minutes at room temperature. Cells were then permeabilised with PBS containing 0.2% (v/v) Triton X-100 and blocked with PBS containing 2% (v/v) FCS for 20 minutes. The primary antibody Oct4, diluted 1:200, in the blocking buffer was incubated overnight at 4°C. Samples were washed with PBS 3 x 10 minutes before incubating with the secondary antibody (1:200) for 1 hour at room temperature. After secondary antibody incubation, cells were washed again with PBS 3 x 10 minutes, counterstained for 10 minutes with 0.5μg/ml DAPI (Sigma) and mounted in MOWIOL. Images were acquired using a Zeiss 510 Meta confocal microscope in the University of Bath's Bioimaging Suite.

2.3.7 Fluorescence activated cell sorting (FACS).

Cells were grown in 6-well Nunc gelatin-coated dishes and plated at different densities varying from $2x10^5$ to $5x10^4$ cells/ well and were incubated at 37˚C for 1, 2, 3, 4, 5 or 6 days depending on the experiment. After incubation, cells were washed 3 times with PBS before adding trypsin and placing them for 5 minutes at 37˚C. Next, cells were resuspended in 0.5ml of FACS buffer composed of 5%(v/v) Fetal Bovine Serum, 0.1%(w/v) sodium azide and transferred into a 1.5ml tube before centrifuging at 5000rpm and 4˚C for 30 seconds. Supernatant was then removed and 1ml of FACS buffer added before centrifuging again using the conditions previously mentioned. This step was repeated once more. After removing the supernatant, cells were resuspended in 0.5ml FACS buffer and transferred into a FACS tube. 1μl of 7AAD (0.5mg/ml) was added to each sample, 10000 events were monitored in the FACSCanto™ flow cytometer and analysed using FACS Diva software.

2.3.7.1 Gating for GFP-positive cells.

Gate for GFP-positive cells was set by using wild-type cells. 7-AAD was used to analyse only alive cells.

Table 2.8 Western blot reagents

WESTERN BLOT REAGENTS-SAMPLE EXTRACTION AND PREPARATION		
PRODUCT	**SUPPLIER**	**CATALOG Nº**
NE-PER Reagents	Pierce	78833
Nonidet P40	VWR	560092-L
Glycerol	Sigma	G5150
SDS	VWR	442444-H
Trizma (Tris) base	Sigma	T8404
Sodium Chloride	Sigma	S7653
Aprotinin	Roche Biochemicals	236624
Leupeptin	Sigma	L8511
Pepstatin	Sigma	P5318
PMSF	Sigma	H0891
Sodium Molybdate	VWR	102542-Q
Sodium Vanadate	Sigma	S6508
Sodium Fluoride	Sigma	S6521
Soybean Trypsin Inhibitor	Sigma	T9003
Bromophenol blue	BD	44305
Bradford Reagent	Bio-Rad	500-006
WESTERN BLOT-PROTEIN RESOLUTION AND IMMUNOBLOTTING		
PRODUCT	**SUPPLIER**	**CATALOG Nº**
30% Acrylamide/bisacrylamide	Bio-Rad	161-0158
Ammonium Persulphate	Sigma	A7460
TEMED	Sigma	T9281
Glycine	Sigma	G8790
Hybond ECL Nitrocellulose membrane	Amersham/GE Healthcare	RPN203D
3MM Whatman paper	VWR	3030917
Methanol	Fisher	M-4056-17
Ponceau S	Sigma	P7170
Bovine Serum Albumin (BSA)	Roche Biochemicals	735-108
Ovalbumin	Sigma	A5378
Sodium azide	Fisher Scientific	S2380-48
ECL	Amersham/GE	RPN-2135

2.4 Molecular techniques

2.4.1 RNA Isolation

2.4.1.1 TRizol method

Cells were washed twice with PBS and 1ml Trizol (Invitrogen Life Technologies, Paisley, UK) reagent was added to each cell culture dish. Sterile cell scrapers were used to harvest RNA and the lysate was transferred to sterile RNase free 1.5ml tubes. For extraction of RNA from Embryoid Bodies (EBs), 3ml of PBS were added to 5ml dishes and the PBS with EB media transferred to a 50ml tube. 5ml more of PBS was added into the dish, mixed again with a Pasteur pipette and transferred to the same 50ml tube. PBS was added to the tube to a final volume of 50mls. Cells were pelleted by centrifugation at 1500rpm for 5 minutes. The supernatant was removed and 50ml of PBS added before centrifuging again at 1500rpm for 5 minutes to wash the EBs. After repeating this step again, the pellet was resuspended into 1ml of Trizol and lysates were transferred to sterile RNase free 1.5ml tubes. Lysates were then either stored at -80°C or the following protocol carried out. 200µl of Chloroform were added per 1.5ml tube and mixed vigorously. Tubes were centrifuged at 14000rpm at 4°C for 15 minutes. The upper aqueous phase, of approximately 500µl, was transferred to a new RNase-free 1.5ml tube. For RNA precipitation 500µl of 2-Propanol was added and incubated at room temperature for 10 minutes before centrifuging at 14000rpm at 4°C for 10 minutes. The supernatant was then removed and 1ml of 75% (v/v) ice cold Ethanol added to wash the RNA pellet. Tubes were centrifuged at 13000rpm, 4°C for 5 minutes and the ethanol was removed. The RNA pellet was allowed to air dry and resuspended in 15-40µl RNase free H_2O.

In order to remove any contaminating genomic DNA, RNA samples were treated with RQ1 DNase. Up to 1µg of RNA was incubated with 1U DNase (Promega) in DNase buffer (400mM Tris-HCl pH8.0, 100mM $MgSO_4$, 10mM $CaCl_2$) (Promega) at 37°C for 30 minutes. DNase was heat inactivated at 65°C for 10 minutes with 1µl DNase stop solution (20mM EDTA pH8.0). RNA was quantified using a GeneQuant II spectrophotometer.

2.4.1.2 Polysomal fractioning and RNA isolation from fractions

40-50 x 10^6 ESC were grown in GMEM supplemented with LIF and Serum. Cells were treated with 100μM/ml Cycloheximide for 15 minutes to stop ribosome movement before lysing (Sampath et al., 2008). Cells were lysed using lysis buffer described previously (Welham et al., 1994) supplemented with 2mM DTT, 150ug/ml cycloheximide and 80U/ml RNAsin. Cell lysates were centrifuged for 3 minutes at 4ºC at 6000rpm for the removal of nuclei and supernatant transferred to a new tube and spun again at 13000rpm at 4ºC for 5-10 minutes for removal of mitochondria and membrane particles. The supernatant was loaded into a sucrose gradient column made in DEPC-treated water with 10mM Tris-Hcl pH 7.5, 140mN NaCl, 1.5mM MgCl2 and 10%, 15%, 20%, 35%, 40% and 50% sucrose, 1mM DTT and 100μM Cyclohexidime and centrifuged at 130,000 g for 1.5hr at 4ºC in an SW40-Ti swinging bucket rotor (Beckman). After centrifugation, fractions from the column were collected and transferred to 1.5ml tubes containing 0.5 % (v/v) SDS, 12μl EDTA (stock 0.5M) and 10μl proteinase K (stock mg/ml), mixed immediately and incubated at 37ºC for 30 minutes. After incubation 200ng yeast tRNA was added to assist with precipitation. 600ul phenol:chloroform 1:1 was added to each tube, tubes vortexed and spun for 5 minutes at 13000rpm at 4ºC. The upper phase, containing the RNA, was transferred to a tube containing 0.3M NaCl and 800μl 100% ethanol added and left overnight at -20ºC. Tubes were then centrifuged at 13000rpm for 30 minutes at 4ºC, the pellet washed with 1ml 75% (v/v) ethanol and spun at 13000rpm for 5 minutes. The dry RNA pellet was resuspendend in 20μl DEPC-treated water. OD at 260nm was measured to determine polysomal distribution and corresponding fractions pooled. LiCl precipitation was performed in the pooled fractions.

2.4.1.3 LiCl precipitation

The pooled fractions were subjected to LiCl precipitation. 0.67 X Volume of 10M LiCl was added to RNA, mixed and incubated at -20ºC for 20 minutes. RNA was then centrifuged at 13,000rpm for 20 minutes, supernatant discarded and RNA pellet resuspended in 100ml DEPC-treated milli q H_2O. 12.5μl 5M Potassium Acetate and 400μl of cold 100% ethanol was added to the tubes and tubes were centrifuged at 13000rpm for 10 minutes. After centrifugation the supernatant was discarded, the

pellet washed in 1m of cold 80% ethanol, centrifuged at 13000rpm for 5 minutes, the supernatant discarded and pellet air dried before resuspending in 15-30 µl DEPC-treated milli q H_2O.

2.4.1.4 RNA quantification

RNA was quantified by measuring absorbance at 260nm using a GeneQuant II spectrophotometer.

2.4.2 Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

RNA samples treated with RQ1 DNase were incubated at 65°C for 5 minutes with 0.5µl of 500µg/ml Oligo dT (Promega) and then kept on ice for at least 1 minute. RT-PCR was performed using first strand buffer (Invitrogen), 5mM DTT (Invitrogen), 2U/µl Rnasin plus (Promega), 0.5mM dATP, 0.5mM dTTP, 0.5mM dGTP, 0.5mM dCTP (Invitrogen) and 10U/µl SuperscriptIII (Invitrogen). Samples were incubated at 42°C for 50 minutes for elongation, then at 70°C for 15 minutes to denature. The resulting cDNA was used as the template for PCR or stored at -20° for later use.

2.4.2.1 Standard PCR

PCR was carried out to investigate expression of markers of interest. 2µl cDNA were added to a master mix composed of 14.6µl water, 5µl of 5x colorless gotaq flexi buffer, 0.5µl of dNTPs, 2µl of Mg_2Cl (25mM), 0.4µl of 5' and 3´ primers (25pmol/µl) and 0.1µl Go Taq. PCR reactions were run according to the following parameters: the number of cycles was dependent on the primer combinations used (Table 2.9), 5 minutes at 94°C, 30 seconds at 94°C for denaturing, 30 seconds at the appropriate annealing temperature that depends on the primer used (Table 2.9), 45 seconds at 72°C for elongation, final extension 5 minutes at 72°C. β-actin was used to check the quantity of cDNA in the samples as it is ubiquitously expressed in cells.

Table 2.9: Primers sequences. The table shows primer sequences used in this study as well as the annealing temperature and the number of cycles.

Gene	Primer Sequences		Annealing Temperature	Number of cycles
β-actin	sense	5'-TAGGCACCAGGGTGTGATGG	62° C	25
	anti-sense	5'-CATGGCTGGGGTGTTGAAGG		
Nanog	sense	5'-CTCTTCAAGGCAGCCCTGAT	60°C	30
	anti-sense	5'-CCATTGCTAGTCTTCAACCAC		
Oct4	sense	5'-GGCGTTCTCTTTGGAAAGGTGTTC	58°C	30
	anti-sense	5'-CTCGAACCACATCCTTCTCT		
Rex1	sense	5'-CGTGTAACAACACCATCCG	58°C	30
	anti-sense	5'-GAAATCCTCTTCCAGAATGG		
Nodal	sense	5'-ACGTTCACCGTCATTCCTTC	62°C	30
	anti-sense	5'-TCAGCTTCCCAAAGCAAAGCAAAGT		
Fgf5	sense	5'-AAAGTCAATGGCTCCCACGAA	62°C	30
	anti-sense	5'-CTTCAGTCTGTACTTCACTGG		
Brachyury	Sense	5'-CATGTACTCTTCTTGCTGG	58°C	30
	anti-sense	5'-GGTCTCGGGAAAGCAGTGGC		
Flk1	Sense	5'-CACCTGGCACTCTCCACCTTC	60°C	30
	anti-sense	5'-GATTTCATCCCACTACCGAAAG		
Sox1	Sense	5'-CACAACTCGGAGATCAGCAA	60°C	30
	anti-sense	5'-GTCCTTCTTGAGCAGCGTCT		
N-cam	Sense	5'-CTCGAAGTTCAATATGCTCC	59°C	30
	anti-sense	5'-CTGAATCAGGGGTCACCTCC		
AFP	Sense	5'-TCCAGACAAAGAGAGCATCC	59°C	30
	anti-sense	5'-AGTTTCCTTGGCAACACTCC		
Nestin	Sense	5'-AGGAACCAAAAGAGGCAGGT	60°C	35
	anti-sense	5'-TTGGGACCAGGGACTGTTAG		

2.4.2.1.1 Gel electrophoresis

Separation of DNA was performed using agarose gel electrophoresis. Agarose gels (normally 2% (w/v)) were made by melting agarose in Tris-acetate EDTA (TAE) buffer (50x TAE buffer: 2M Tris, 50mM Na2 EDTA (pH8.0) adding glacial acetic acid (to pH 7.6)). After cooling down the agarose was poured into gel casting trays and allowed to set before placing it into an electrophoresis tank with TAE. DNA loading buffer (6x loading buffer: 30% (v/v) glycerol, 0.05% (w/v) bromophenol) was added to the PCR products, samples were loaded into the wells and gels were run at 80V-110V. Agarose gels were placed into a tray containing a solution of 0.5µg/ml ethidium bromide in TAE and left for 30 minutes before visualising the DNA under the UV light. Images of the gels were taken in a Syngene UV transluminator using Genesnap software.

2.4.2.2 Quantitative real-time PCR (qRT-PCR)

Quantitative RT-PCR was carried out using the Roche Molecular Biochemical LightCycler. A master mix containing 0.5µM sense and antisense primers of interest (Table 2.10), 2.5mM $MgCl_2$, 1µl SYBR Green and sterile water to a final volume of

8µl was pipetted to pre-chilled LightCycler capillaries before adding 2µl of 1:10 dilute cDNA sample. Capillaries were next centrifuged at 4000rpm for 20 seconds at 4°C before loading into the Roche Molecular Biochemical LightCycler. A negative control that does not contain cDNA template was also loaded and the program shown in Table 2.10 run. After the run, melting curves were studied to ensure that only one product was amplified. Primer specific annealing temperatures are shown in Table 2.11.

SYBR Green dye binds to double stranded DNA leading to fluorescence, which can be monitored using the Lightcycler. The intensity of the fluorescence reflects the quantity of double stranded DNA. Increase of DNA amount was monitored for 40 cycles by measuring the fluorescence at 530nm and crossing point values were calculated by the LightCycler version 4.0 software (Roche, Idaho Technology Inc.). The crossing point is the cycle at which the fluorescence from the sample of interest is higher than the background fluorescence. Relative quantification was calculated by normalising the crossing point of the target gene to the housekeeping gene β-actin.

Table 2.10 qPCR program

STAGE	TEMPERATURE	DURATION	CYCLES
Hot Start	95ºC	10 minutes	
Initial Denaturation	95ºC	30 seconds	
Denaturation	95ºC	10 seconds	40 Cycles
Annealing	Primer specific (Table 2.11)	5 seconds	
Elongation	72ºC	15 seconds	
Melting curve	65-95ºC	0.1 ºC/sec	

Table 2.11. Primers used in the qPCR and annealing temperatures.

Gene	Primer Sequences		Annealing Tª
β-actin	sense	5'-TAGGCACCAGGGTGTGATGG	62ºC
	anti-sense	5'-CATGGCTGGGGTGTTGAAGG	
Nanog	sense	5'-CTCTTCAAGGCAGCCCTGAT	60ºC
	anti-sense	5'-CCATTGCTAGTCTTCAACCAC	
Oct4	sense	5'GGCGTTCTCTTTGGAAAGGTGTTC	58ºC
	anti-sense	5'-CTCGAACCACATCCTTCTCT	
Tbx3	sense	5´-AGGAGCGTGTCTGTCAGGTT	58ºC
	anti-sense	5´-GCCATTACCTCCCCAATTTT	
c-Myc	sense	5´CAGAGGAGGAACGAGCTGAAGCGC	58ºC
	anti-sense	5´-TTATGCACCAGAGTTTCGAAGCTGTTCG	
CyclinD1	sense	5´-CTACACTGACAACTCTATCC	56ºC
	anti-sense	5´-GGCTTCAATCTGTTCCTGGC	
Zscan4	sense	5´-TTGAAGCCTCCTGTCATGGTCC	61ºC
	anti-sense	5´-TCCATTTCATTTCCACTACAGC	

Standard curves for the target and reference genes were generated in order to calculate relative quantification from unknown samples. Serial dilutions of a cDNA template were run in triplicate and the standard curve for each gene generated by plotting the cell cycle number at the crossing point versus the log concentration of the starting cDNA (Figure 2.1). PCR efficiencies can be calculated using the formula $E=10^{-1/slope}$. The maximum PCR efficiency is 2. PCR efficiency values are used to correct data from unknown samples.

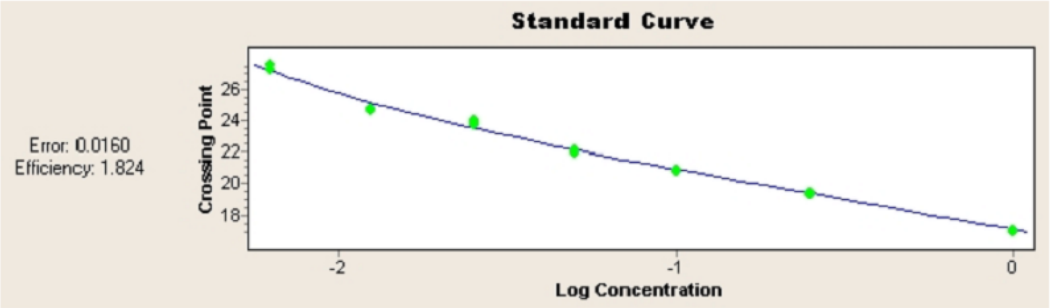

Figure 2.1: Example of a standard curve. Standard curves were generated for each target and reference gene in this study. The LightCycler Version 4.0 software was used to create a Standard curve by plotting the crossing point versus the log concentration of serially diluted cDNA template. PCR efficiency values are generated from the slope of the curve, which are then used to correct data obtained from the samples of interest. The error that shows variations between sample replicates is also calculated from the standard curve. Typically, an error <0.1 is adequate.

Calibrator cDNA samples were also run in all the qPCRs in this study. The calibrator cDNA was made from RNA obtained from undifferentiated ESCs. The calibrator cDNA was made at the same time as cDNA from the unknown samples. The use of a calibrator permits comparison between different experiments because the data from each experiment can be compensated to account for differences in RT-PCR efficiencies.

2.4.2.3 qPCR relative quantification

qPCR data presented in this study was examined by relative quantification and was generated by dividing the ratio of target to reference gene (β-actin) relative concentrations by the ratio of target calibrator to reference calibrator relative concentration to allow comparison between experiments.

Table 2.12 Molecular reagents

MOLECULAR REAGENTS		
PRODUCT	**SUPPLIER**	**CATALOGUE Nº**
TRIzol	Invitrogen	15596-018
DNase	Promega	M6101
dNTPs	Invitrogen	10297-018
Oligo (dT)15 Primer	Promega	C1101
Ethidium Bromide	Bio-Rad	1610433
Agarose	Invitrogen	15510-027
RNase Free Eppendorfs	Eppendorf	3810X
Superscript III reverse transcriptase (including RT buffer and DTT)	Invitrogen	18080-044
RNasin Plus	Promega	N2611
Taq Polymerase	Promega	M1661

3 CHAPTER: ROLE OF GSK-3 IN mESC

3.1 Introduction and aims

Several groups, including ours, have reported that inhibition or deletion of GSK-3 contributes to maintenance of self-renewal in both serum and serum-free conditions (Bone et al., 2009; Doble et al., 2007; Sato et al., 2004; Ying et al., 2008). The first evidence implicating GSK-3 in control of ESC self-renewal came from a study where a small molecule inhibitor of GSK-3, referred to as BIO, was used (Sato et al., 2004). The importance of GSK-3 in self-renewal has been further demonstrated by showing that self-renewal of ESCs is enhanced in GSK-3α/β double knockout cells (Doble et al., 2007). Inhibition of both the Erk1/2 MAPK pathway and GSK-3 in serum-free conditions, and in the absence of any extrinsic stimuli, has been reported to be sufficient to maintain self-renewal of ESCs. GSK-3, under these conditions, is thought to be essential for maintaining cell viability (Ying et al., 2008).

Work previously carried out in the laboratory where I have been studying for my PhD showed that inhibition of GSK-3 can overcome the decrease in *Nanog* RNA levels and protein expression that is observed following inhibition of PI3K, suggesting that PI3K regulates Nanog expression through inhibition of GSK-3 (Storm et al., 2007). More recently, Several groups, including ours, have reported that inhibition or deletion of GSK-3 contributes to maintenance of self-renewal in both serum and serum-free conditions (Bone et al., 2009; Doble et al., 2007; Sato et al., 2004; Ying et al., 2008). In contrast, other studies reported that inhibition of GSK-3 leads to neuronal or mesendodermal differentiation of ESCs (Bakre et al., 2007; Ding et al., 2003; Thomson et al., 2011).

The aims of this part of the study were:

- To investigate maintenance of ESC pluripotency following treatment with GSK-3 inhibitors.
- To investigate the effects of GSK-3 inhibitors on the multi-lineage differentiation of ESCs

A number of GSK-3 inhibitors, including our novel selective inhibitors 1m and 1i, were used in this part of the study to address the aims stated above.

3.1.1 GSK-3 selective inhibitors.

Several structurally different small molecule inhibitors of GSK-3 were used in our study, 6-bromoindirubin-3'oxime (BIO), the novel bis-indolylmaleimides 1m and 1i and an aminopyrimidine derivative CHIR99021 (CHIR).

BIO selectivity for GSK-3 was tested in vitro against a panel of 20 kinases (Meijer et al., 2003) and it was used to study the role of GSK-3 in self-renewal of ESC (Sato et al., 2004). mESC grown in the presence of BIO could activate the Wnt signalling shown by an increase in TopFlash reporter activity (Sato et al., 2004). Although we used BIO in our initial studies, we discontinued its use because we discovered that BIO has off-target kinases evidenced by decrease of phosphorylation of Stat3 and Erk1 and 2 (Bone et al., 2009).

CHIR was shown to selectively inhibit GSK-3 against a panel of kinases (Cline et al., 2002) and it has also been shown to inhibit GSK-3 in mESC (Ying et al., 2008). Opposite to BIO we have not observed decrease in phosphorylation of Stat3 and Erk1 and 2 (results not shown) suggesting that CHIR does not have off-targets.

The bisindolylmaleimide 1i was identified as a selective small molecule inhibitor of GSK-3β which is ATP-competitive (Bartlett et al., 2005). 1i was tested for activity against a panel of 29 protein kinases in an *in vitro* screen and were shown to be GSK-3 selective inhibitors (Bartlett et al., 2005). Moreover, a kinase assay showed that 1i also inhibits GSK-3 kinase activity with an IC_{50} of 250 nM in ESC lysates and IC_{50} of 20nM using a recombinant GSK-3β protein. An interaction between 1i and ATP was shown by affinity purification. Using 1i as a lead molecule a panel of bisindolylmaleimides was synthesised, the bisindolylmaleimide 1m with an IC_{50} of 3nM was more potent than 1i (Bartlett et al., 2005). GSK-3 inhibition with 1m in intact ESCs was shown by a decrease of β-catenin phosphorylation levels and an increased in β-catenin-mediated TCF/LEF transcriptional activity using a TOPFlash luciferase reporter assay. 1m and 1i specifically inhibit GSK-3 in mESC and does not perturb the Stat3 or Erk pathways (Bone et al., 2009).

3.2 Investigation of maintenance of ESC pluripotency following treatment with novel GSK-3 inhibitors.

As discussed in Section 1.1, one of the unique properties of ESCs is their pluripotency, which is defined as the ability to differentiate into derivates of all the three germ layers, ectoderm, mesoderm and endoderm. Pluripotency of ESCs can be demonstrated by their ability to contribute to viable chimeras when injected into the blastocyst of the pre-implantation mouse embryo (Bradley et al., 1984), but it can also be shown by their capacity to form teratocarcinomas, which are tumours that contain derivatives of the three germ layers and are generated by injecting ESCs into an adult mouse. Finally, pluripotency of ESCs can also be studies *in vitro* by Embryoid Bodies (EB) formation (Martin and Evans, 1975), which are generated by cell aggregation when ESCs are cultured in suspension.

ESCs cultured in the presence of GSK-3 inhibitors, BIO or CHIR, have been shown to maintain their pluripotency by contributing to chimeras and generating teratomas containing derivates of the three germ layers following withdrawal of the inhibitors (Sato et al., 2004; Ying et al., 2008). However, DKO GSK-3 cells exhibited abnormal differentiation potential in EBs or teratocarcinomas. EBs generated from DKO ESCs retained high levels of pluripotency markers even after 7 days of EB formation and the teratocarcinomas generated were mainly undifferentiated, with only bone differentiation observed. One of the aims of this study was to show that ESCs treated with novel GSK-3 inhibitors (Bone et al., 2009) kept their pluripotency following withdrawal of the inhibitors as it was essential to ensure that these novel GSK-3 inhibitors are not increasing ESCs self-renewal because they are irreversibly blocking the ability of ESCs to differentiate.

In order to study the ability of ESCs to undergo multi-lineage differentiation following treatment with GSK-3 inhibitors, embryoid-body-based differentiation was used.

3.2.1 Embryoid-body-based differentiation system.

As previously mentioned, ESCs can be maintained in an undifferentiated state in culture by the addition of LIF. Removal of LIF results in spontaneous differentiation towards derivatives of the three germ layers. ESCs can be triggered to differentiate *in vitro* by cell aggregation, whereby the ESCs form a multi-differentiated cell mass called an embryoid body (EB) (Martin and Evans 1975). The progression of cellular differentiation in these EBs resembles differentiation in the embryo, but without axial organisation (Doetschman et al., 1985). In order to favour formation of EBs, ESCs were placed in a media called methylcellulose, which does not contain LIF. This media is gelatinous, maintaining cells in suspension and allowing cells to aggregate, grow and thus form EBs (Figure 3.1).

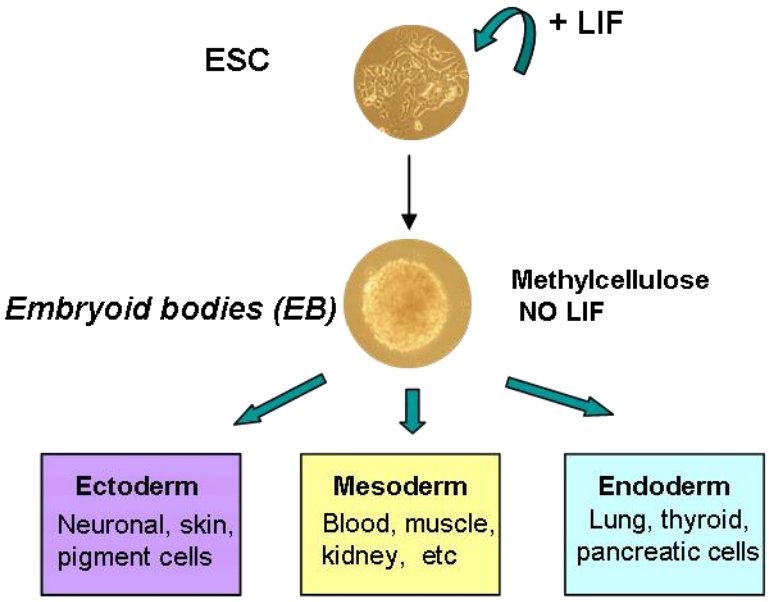

Figure 3.1. Embryoid body differentiation system. ESC cultured with LIF remain undifferentiated. Removal of LIF and placement of ESCs in methylcellulose results in formation of Embryoid bodies (EB). In these EB, cells differentiate into ectoderm, mesoderm and endoderm in a manner that resembles early embryonic differentiation but without axial organisation. The tissues listed are examples of ectoderm, mesoderm or endoderm derivates.

3.2.2 ESCs maintain pluripotency following short-term GSK-3 inhibition.

To ascertain if ESCs maintain pluripotency following treatment with GSK-3 inhibitors, ESCs were cultured in the presence of the GSK-3 inhibitors BIO or 1i for 48 hours before plating them into methylcellulose for formation of EBs. EBs were generated and allowed to form for 4 to 6 days. The experimental procedure described in Fig 3.2 was followed to investigate maintenance of ESC pluripotency. Pluripotency and lineage markers were analysed by RT-PCR, Oct4 expression was investigated by immunostaining and the number of EBs formed from ESCs treated with GSK-3 inhibitors was also studied.

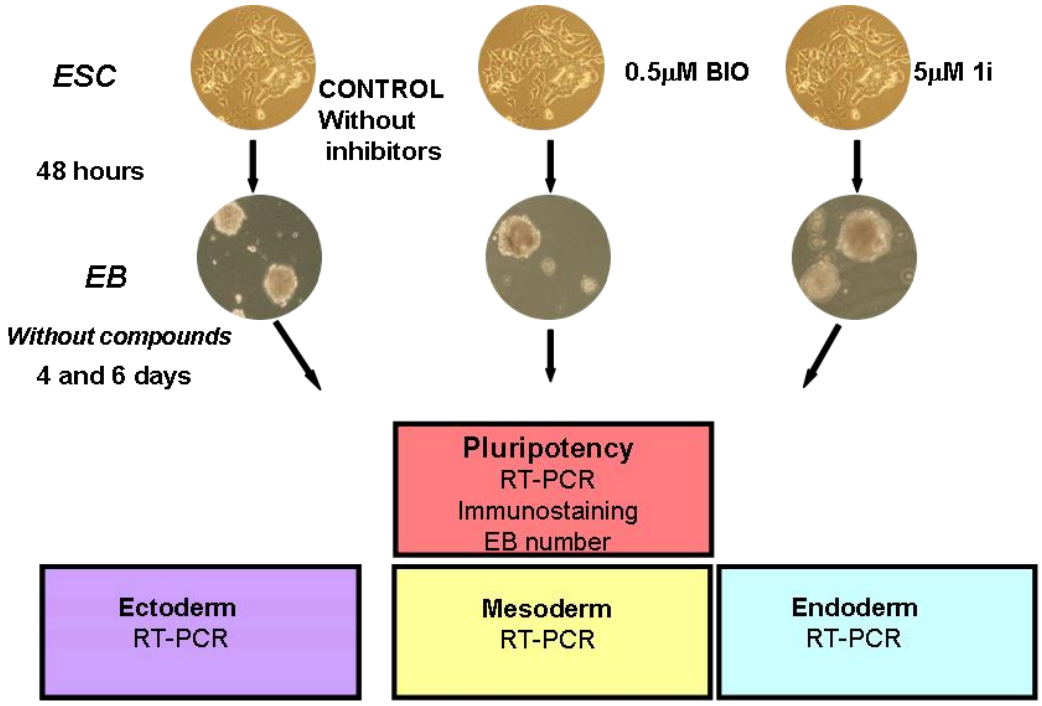

Figure 3.2 Experimental procedure to investigate maintenance of ESC pluripotency following treatment with the GSK-3 inhibitors, BIO and 1i. Cells were cultured in the presence of 0.5µM BIO, 5µM 1i or DMSO (Control) for 48 hours. Cells were then plated into EBs in methylcellulose and grown for 4 and 6 days without the GSK-3 inhibitors. RNA was extracted from these EBs, and from ESC treated for 48 hours with the inhibitors and cDNA made by RT-PCR. Expression of pluripotent markers was studied by RT-PCR, Oct4 expression analysed by immunostaining and number of 6 day-old EB recorded. Pluripotency was further investigated by analysing expression of ectoderm, mesoderm and endoderm markers.

3.2.2.1 ESCs treated with GSK-3 inhibitors express pluripotency markers.

Undifferentiated ESCs are characterised by the expression of transcription factors which have a role in maintaining their self-renewal capacity. These transcription factors have already been extensively discussed in Section 1.3.1. Consistent with their roles, they are expressed in ESCs and their expression is downregulated upon differentiation. *Rex1* (Rogers et al., 1991), *Oct4* (Rosner et al., 1990) and *Nanog* (Chambers et al., 2003) are well known pluripotency transcription factors in ESCs.

Oct4 is expressed in the morula, in all the cells of the ICM, the epiblast of the pre-implantation embryo, germ line cells and ESCs (Pesce et al., 1998). Oct4 is essential for the establishment of the pluripotent lineage of the ICM (Nichols et al., 1998) and in addition, repression of Oct4 leads to differentiation of ESCs into trophectoderm (Niwa et al., 2000).

Nanog is expressed at the morula stage, in the ICM in a salt and pepper fashion and its expression is restricted then to epiblast (Chazaud et al., 2006). Nanog is essential for establishment of the pluripotency in the ICM (Chambers et al., 2007; Silva et al., 2009).

The zinc finger protein Reduced Expression-1 (Rex1) is expressed in ESCs and in the ICM but it is down-regulated in the epiblast and primitive ectoderm in the embryo and at the beginning of ESC differentiation (Rogers et al., 1991; Toyooka et al., 2008). Although Rex1 can be used as a marker of pluripotency it is not essential for the maintenance of pluripotency of ESC or ICM (Masui et al., 2008).

Expression of *Nanog*, *Oct4* and *Rex1* in ESCs treated with the GSK-3 inhibitors and in day 4 and day 6 EBs grown in absence of the inhibitors were analysed by RT-PCR (Figure 3.3 A).

The data presented in Figure 3.3A show that ESCs maintain expression of pluripotency markers following short-term treatment with BIO and 1i. Expression of *Rex1* and *Nanog* is down-regulated in EBs, and it is almost non-existent in 4 and 6 day-old EBs. On the other hand, *Oct4* expression is maintained for longer than *Rex1* and *Nanog*, being down-regulated in 6 day-old EBs. The patterns of marker

expression observed were in accordance to what would be expected as ESCs within the EBs start to differentiate. These data suggest that ESCs in fact maintain pluripotency upon treatment with the compounds and following removal of inhibitors and induction of differentiation, expression of pluripotency markers declines, indicative of differentiation.

Immunostaining for Oct4 was carried out in order to confirm maintenance of pluripotency. ESCs were grown in the presence of the GSK-3 inhibitors, BIO, 1i and another bisindolylmaldeimide 1m, which is more potent than 1i, for 5 days and Oct4 expression analysed. Expression of Oct4 was observed in all the cells in the colony in control as well as in cells grown in the presence of BIO, 1m and 1i suggesting that all or at least the majority of ESCs within the colony are pluripotent (Fig. 3.3. B).

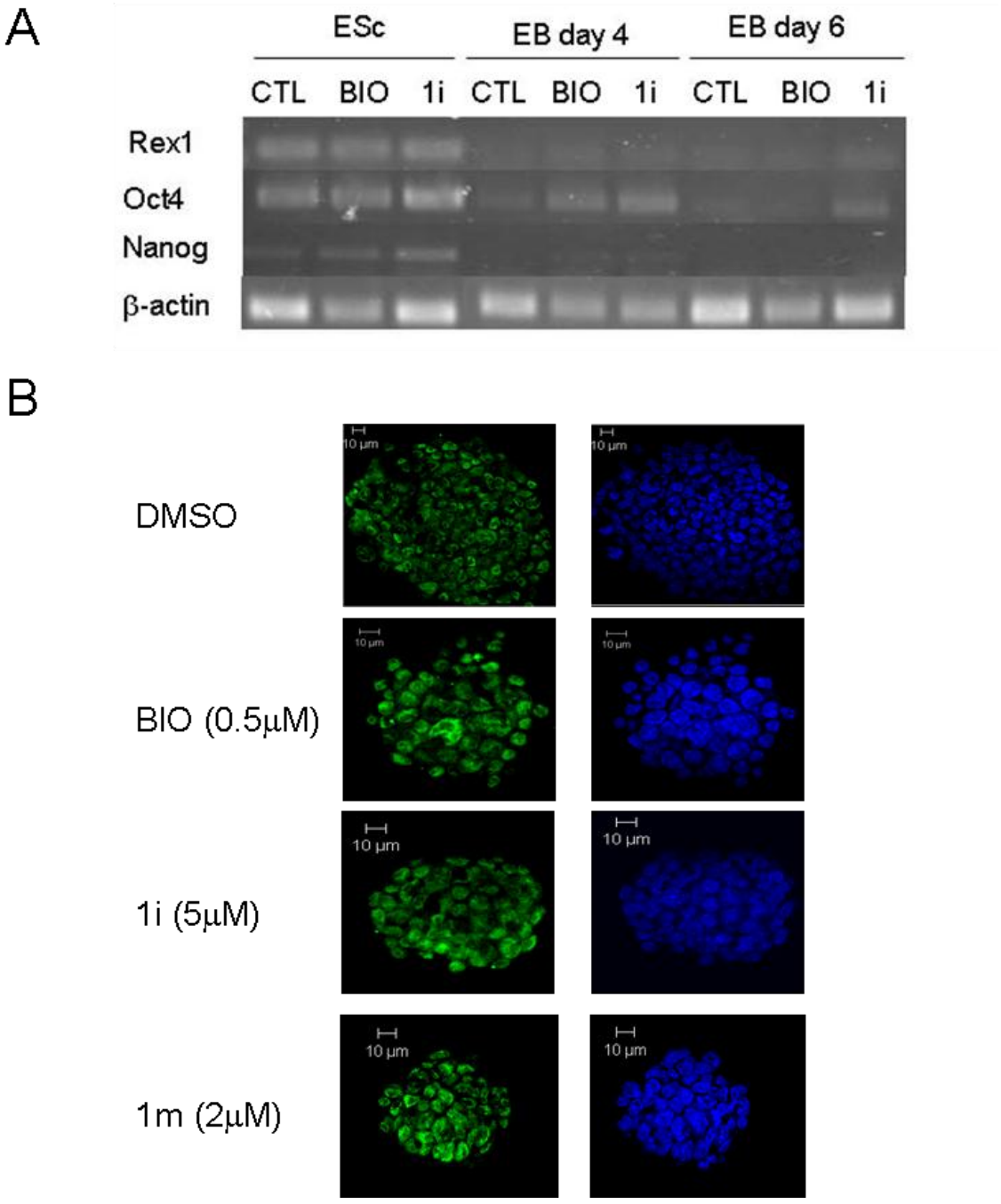

Figure 3.3: Expression of pluripotency markers is maintained in ESCs following treatment with the GSK-3 inhibitors, BIO and 1i. The experimental procedure described in Figure 3.2 was followed to analyse expression of markers of the pluripotent ESC state, *Rex1, Nanog and Oct4*. A. RT-PCR analysis of Pluripotent marker expression in ESC treated with GSK-3 inhibitors for 48 hours and in EB after 4 and 6 days of EB formation. Time-course experiment was performed twice. B Immunohistochemical staining with Oct4 antibody shown in green and nuclear staining with DAPI in blue after 5 days in cultured with the indicated GSK-3 inhibitors. This experiment was carried out twice and data shown are representative.

3.2.2.2 EB formation capacity does not change following GSK-3 inhibition.

EBs are formed from pluripotent cells, so one way to assess maintenance of pluripotency following GSK-3 inhibition would be to count the number of EBs formed. Hence, if pluripotency is maintained the number of EBs formed from ESCs grown in the presence of GSK-3 inhibitors should be comparable to the number of EBs formed from control untreated ESCs. The number of 6 day old EBs formed from $2x10^5$ ESCs that had been cultured in the presence of BIO or 1i for 48 hours were counted (Figure 3.4).

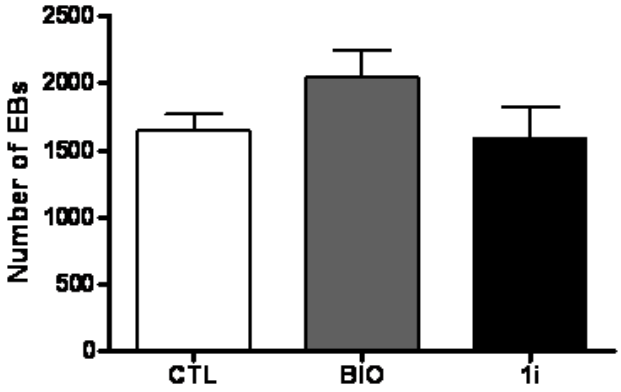

Figure 3.4 ESCs maintain capacity to form EBs. ESC were grown in the presence or absence of BIO or 1i for 48 hours prior EB formation. Number of EBs were counted after 6 days of EB formation. The average and S.E.M from 4 independent experiments (n=4) are shown.

The numbers of EBs formed from ESCs treated with 0.5μM BIO or 5μM 1i were comparable to those formed from the controls. Results from the studies presented in Section 3.2.2.1 and this Section suggest that pluripotency is maintained following removal of GSK-3 inhibitors. However, pluripotency has to be confirmed by showing that cells can differentiate into the three germ layers

3.2.2.3 ESCs treated with GSK-3 inhibitors can differentiate into the three germ layers.

In order to study the ability of ESCs to differentiate into the three germ layers, after having been cultured with BIO or 1i for 48 hours, expression of endodermal, mesodermal and ectodermal lineage markers were investigated. Primers detecting lineage markers including *Hnf4α , Afp, Nodal, Fgf5, Brachyury, Flk1, Nestin* and *N-cam* were used.

Hnf4α is a transcription factor expressed in primary endoderm at E4.5 and in visceral endoderm from E5.5 to E8.5 and thus it is considered a specific primitive endoderm marker in the implanting blastocyst (Duncan et al., 1994). However Hnf4α is also expressed in the definitive endoderm and it is essential for hepatocyte differentiation (Li et al., 2000). Afp is expressed in extraembryonic and embryonic endoderm during embryogenesis, and it is first detected in visceral endoderm at E7.0. It is considered a marker of definitive endoderm marking specification to hepatic lineage and it is expressed later than Hnf4α during mouse development *in vivo* (Abe et al., 1996).

Nodal is a transforming factor-β-related gene with a role in formation of the primitive streak (PS) evidenced by the inability of nodal null embryos to form primitive streak. In agreement with this role, Nodal is expressed in the ectoderm of pre-streak embryos and during initiation of primitive streak formation (Conlon et al., 1994). Primitive streak formation is essential for later development of mesoderm and definitive endoderm as they originate from a mesendodermal population located in the anterior region of the PS (Tada et al., 2005). Nodal signalling intensity specifies the anterior PS to either mesoderm at lower levels or endoderm at higher levels (Lowe et al., 2001; Vincent et al., 2003). Nodal can be used as marker of primitive ectoderm and primitive streak formation.

Fibroblast growth factor 5 (Fgf5) is expressed in several locations between E5.5 and E15.5 of mouse embryogenesis. Fgf5 is first expressed in the post-implantation embryo in the embryonic ectoderm and visceral endoderm at E5.5 and its expression continues at E7.0. Fgf5 is normally used as a primitive ectoderm maker. Fgf5 is later

expressed at E9.5 in derivates of lateral mesoderm and paraxial mesoderm (Haub and Goldfarb, 1991; Loebel et al., 2003).

Brachyury is an early mesoderm maker which is expressed in the primitive streak, mesendoderm and in the early mesoderm (Kubo et al., 2004; Wilkinson et al., 1990). Flk1 is a Vascular endothelial growth factor (VEGF) receptor expressed in a mesodermal progenitor that gives rise to myocardial, endothelial, and smooth muscle lineages (Kattman et al., 2006).

Nestin is expressed in central nervous system (CNS) progenitors. At E7.75 its expression can be detected in the columnar neuroepithelial cells of the neural plate and at E8.5 in the developing CNS (Dahlstrand et al., 1995). The neuronal cell-adhesion molecule (N-cam) is a marker of primitive neuroectoderm formation (Jacobson and Rutishauser, 1986).

The expression of the markers describe above in EBs formed from ESC cultured with BIO and 1i for 48 hours prior EB formation are shown in Figure 3.5. Following treatment with BIO or 1i, the expression of the ectodermal markers *N-cam* and *Nestin* can be seen at all stages of EB formation, suggesting the ESCs treated in this way have the ability to differentiate into ectoderm (Fig. 3.5). *Fgf5* expression can be observed at all stages of EB formation and its expression is higher in EB formed from ESC treated with GSK-3 inhibitors in comparison with control. *Nodal* expression can also be seen in 4 day old EBs from ESC pretreated with 1i and BIO with the latter showing very low levels of expression at day 4 but higher at day 6. *Nodal* expression can not be observed in untreated EBs in 4 or 6 day old EBs suggesting that both 1i and BIO increase expression of *Nodal. Fgf5* and *Nodal* expression suggests differentiation of ESCs into primitive ectoderm and probably induction of PS (Fig. 3.5).

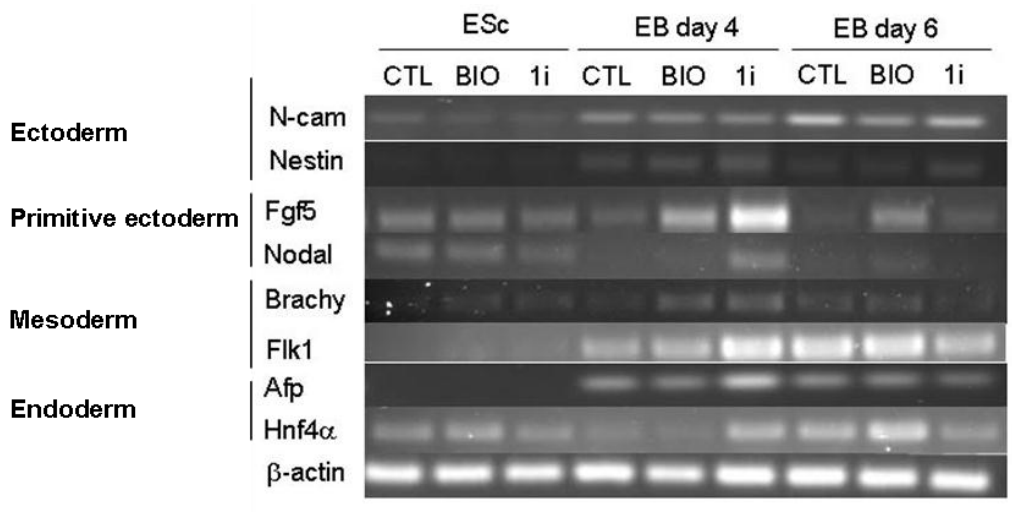

Figure 3.5. ESCs can still undergo differentiation into the three germ layers. The experimental procedure described in Figure 3.2 was followed to investigate ESC differentiation into three germ layers. RT-PCR was performed to analyse expression of the indicated markers. CTL: control, BIO: bio and 1i:1i. *N-cam* and *Nestin* were used as ectodermal markers, *Nodal* and *Fgf5* as a marker of primitive ectoderm, *Nodal* also marks mesoderm induction and *Fgf5* marks lateral and paraxial mesoderm, *Brachyury* and *Flk1* are mesodermal markers and *Afp* and *Hnf4α* endodermal markers. This experiment was perfomed twice and results shown are representative.

Expression of the mesodermal markers *Flk1* and *Brachyury* can be observed in 4 and 6 day old EBs. Expression of the endodermal markers *Afp* and *Hnf4α* can be observed in EBs after 4 and 6 days of formation suggesting that ESCs can in fact differentiate into endoderm (Fig. 3.5).

These results suggest that ESCs can differentiate into the three germ layers following GSK-3 inhibition. However, some markers, including *Fgf5*, *Nodal*, *Brachyury*, *Hnf4α, Flk1* and *N-cam* are expressed at low levels in undifferentiated ESCs. Expression of low levels of *Brachyury* following treatment with BIO or 1i is in agreement with previous studies suggesting *Brachyury* is a target of Wnt signalling (Arnold et al., 2000). Expression of the other markers in undifferentiated ESCs could be due to spontaneous differentiation of ESCs in culture or the presence of lineage-

biased populations within the ESC culture. This will be further discussed in Section 3.4.1.

Nestin and *Afp* seems to be better lineage markers as they are not expressed in undifferentiated ESCs. This is probably because they are expressed later during embryogenesis.

To summarise, following short-term treatment with BIO or 1i, ESCs maintain expression of pluripotency markers, suggesting that cells can differentiate into the three germ layers. In addition, based on expression of lineage markers, pluripotency was further supported by data demonstrating the ability of ESCs to differentiate into cells comprising all three germ layers.

3.2.3 ESC maintain pluripotency after long-term culture in GSK-3 inhibitors.

The previous Section showed that ESCs can maintain pluripotency following short-term (48 hours) treatment with GSK-3 inhibitors. In this Section the aim was to investigate whether pluripotency can be maintained following long-term culture in the presence of GSK-3 inhibitors. ESCs were grown in the presence of serum supplemented with LIF and GSK-3 inhibitors BIO or 1m for 19 days (8 passages) prior to EB formation. Analysis of pluripotency and lineage markers was performed by RT-PCR in ESCs and EBs after 2, 4 and 6 days of EB formation.

ESCs grown for 19 days in the presence of BIO or 1m express the pluripotency markers *Rex1, Oct4* and *Nanog* prior EB formation and these markers are down-regulated as cells differentiate. *Nanog* and *Rex1* expression are almost extinct by day 6, although low level expression of *Oct4* and *Nanog* remain (Fig. 3.6A). The maintenance of *Oct4* expression for longer could be due to the fact that the kinetics of *Oct4* down-regulation is slower than *Nanog* and *Rex1*. Others possible explanation will be discussed in Section 3.4.1.

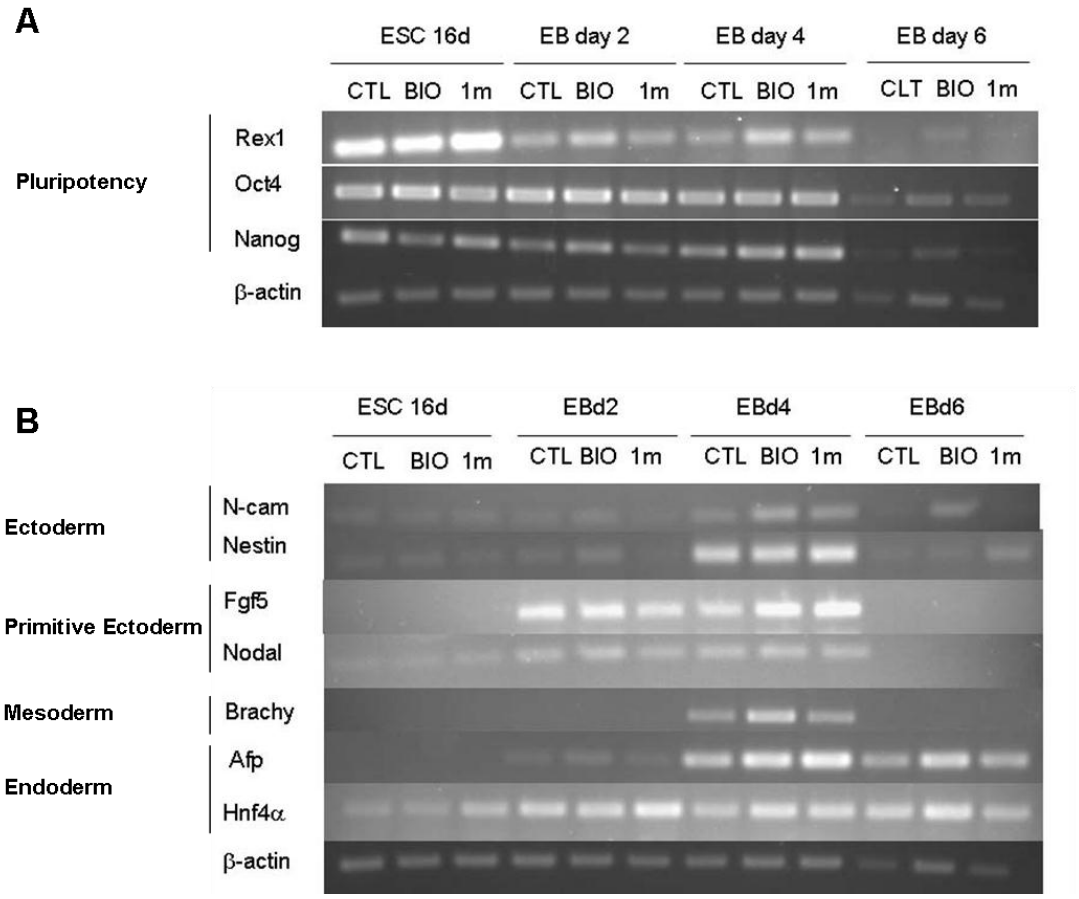

Figure 3.6 ESCs maintain pluripotency and undergo multilineage differentiation after long-term culture with GSK-3 inhibitors. ESCs were grown in the presence of GSK-3 inhibitors, BIO or 1m for 19 days prior EB formation. Expression of the pluripotency (A) and the lineage markers (B) indicated was analysed by RT-PCR. *N-cam* and *Nestin* were used as ectodermal markers, *Nodal* and *Fgf5* as a marker of primitive ectoderm, nodal also marks mesoderm induction and *Fgf5* marks lateral and paraxial mesoderm, *Brachyury* and *Flk1* are mesodermal markers and *Afp* and *Hnf4α* endodermal markers. This experiment was perfomed twice and results shown are representative.

ESCs cultured long-term in BIO or 1m can also undergo differentiation into ectoderm, endoderm and mesoderm, shown by the expression of lineage markers as cells differentiate in the EBs (Fig. 3.6B). *N-cam* and *Nestin* expression indicates differentiation into ectodermal lineages. Differentiation of ESCs into primitive ectoderm is evidenced by the expression of *Fgf5* and *Nodal* in 2 and 4 day-old EBs. *Nodal* expression is involved in induction of PS (Conlon et al., 1994) and promotion

of differentiation of the anterior region of the PS to mesoderm and endoderm (Tada et al., 2005). Expression of *Brachyury*, *Hnf4α* and *Afp* suggest differentiation into mesoderm and endoderm.

In summary, pluripotency of ESCs is also maintained in cells grown with GSK-3 inhibitors for longer periods of time.

3.3 Effects of GSK-3 inhibitors on the multi-lineage differentiation potential of ESCs.

Prior to this work, several studies reported that GSK-3 inhibition had an effect on the multi-lineage differentiation of ESCs. Ding et al., (2003) showed induction of differentiation of ESCs into neurons by TWS119, which inhibits GSK-3. However, other studies are in disagreement with the Ding report. Doble et al., (2007) demonstrated that EBs formed from GSK-3α/β double-knockout (DKO) ESCs totally fail to differentiate into neuronal tissue and differentiated mainly into bone. This work is supported by other studies that demonstrated that sustained Wnt signalling activation, through GSK-3 inhibition by lithium chloride or a small molecule inhibitor of GSK-3β, had a negative effect on neuro-differentiation (Aubert et al., 2002). Moreover, Bakre *et al.*, (Bakre et al., 2007) showed that sustained activation of Wnt signalling, using a small molecule inhibitor of GSK-3β or Wnt3a, induced differentiation of ESCs along mesendodermal lineages, probably at expense of ectoderm/neuroectoderm.

In order to resolve the importance of GSK-3 in ESC differentiation, effects of GSK-3 inhibition with 1i and BIO on the multi-lineage differentiation potential of ESCs was investigated. EBs were formed from ESCs that had been cultured without inhibitors and GSK-3 inhibitors (BIO and 1i) were added to the EB media at the time of plating and EBs were allowed to develop in the presence of BIO or 1i for 4 days. Expression of pluripotency and lineage markers were analysed by RT-PCR, and the numbers of EBs formed counted. Figure 3.7 depicts a schematic summarising the approach used.

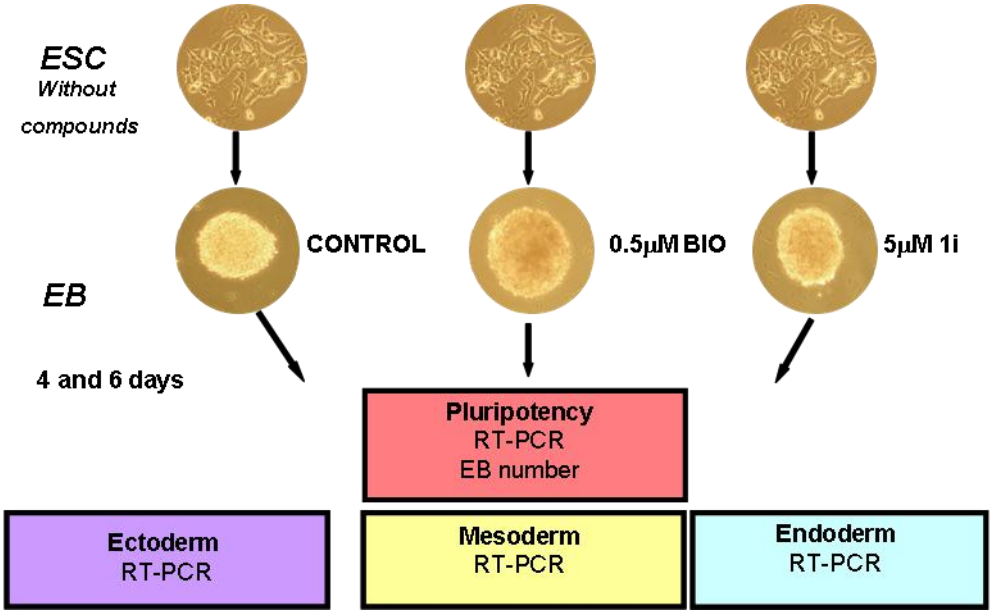

Figure 3.7 Experimental procedure to investigate the effects of GSK-3 inhibitors in the multi-lineage differentiation of ESCs. ESC that had been cultured without GSK-3 inhibitors were plated into EB media, which contained 0.5µM BIO, 5µM 1i or DMSO (Control), and EBs grown for 4 and 6 days. RNA was extracted from these EBs, and from the starting population of non-treated ESC, and cDNA made by RT-PCR. Expression of pluripotent markers, as well as markers of the three germ layers, ectoderm, mesoderm and endoderm, was studied by RT-PCR.

3.3.1 ESCs grown in the presence of GSK-3 inhibitors maintain pluripotency for longer.

In order to assess pluripotency we investigated the expression of *Rex1*, *Nanog* and *Oct4* in ESCs that had been grown in the absence of GSK-3 inhibitors, and in EBs that had been generated in the presence of BIO and 1i for 4 and 6 days.

3.3.1.1 ESCs grown in the presence of GSK-3 inhibitors are more resistant to differentiation.

Expression of *Rex1, Oct4* and *Nanog* can be seen in the starting population of ESCs. The levels of *Nanog* and *Rex1* are higher in 4 day-old EBs grown in the presence of BIO and 1i and in 6 day-old EBs grown in 1i than in control untreated EBs, suggesting than ESCs are more resistant to differentiation when cultured with GSK-3 inhibitors (Figure 3.8). This is in agreement with the observation that GSK-3 DKO cells retain expression of pluripotency makers such as *Oct4* and *Nanog* after 12 days of EB formation (Doble et al., 2007).

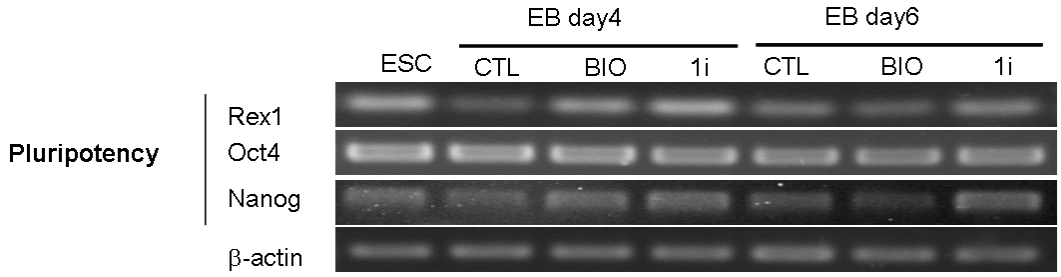

Figure 3.8 ESCs retained pluripotency marker expression when GSK-3 is inhibited. Expression of pluripotency markers indicated were analysed by RT-PCR in EB grown with 0.5μM BIO, 5μM 1i or DMSO for 4 days and in untreated ESCs. CTL: Control, BIO: Bio and 1i:1i. This experiment was performed twice and results shown are representative.

Although expression of pluripotency markers is decreased in EBs, their levels are still higher that would be expected after 6 days of EB formation suggesting that the cells in this experiment differentiated slower than normal.

3.3.1.2 BIO increases the numbers of EBs at day 6 of EB formation.

In order to ascertain whether the GSK-3 inhibitors have an effect on EB formation, and thus on differentiation of ESCs, the number of EBs formed in the presence of BIO, 1i or DMSO were counted at day 6 (Figure 3.9).

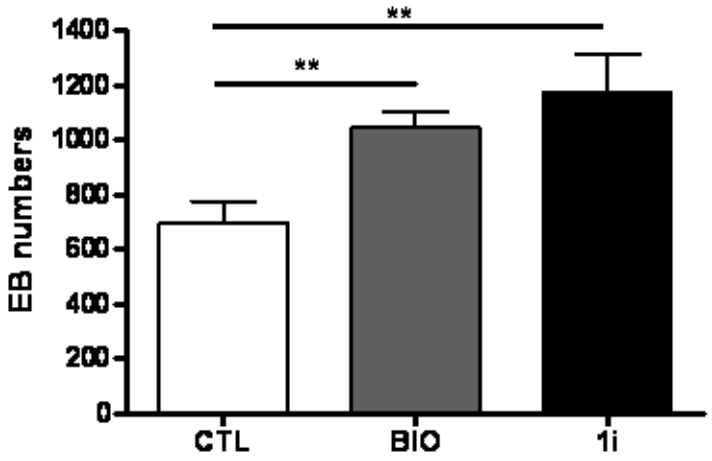

Figure 3.9. Numbers of EBs after 6 days of EB formation. EB were formed from ESCs that had been grown without inhibitors with 0.5μMBIO, 5μM 1i or DMSO added to the EB media at the time of plating. Number of EBs were counted following 6 days of EB formation. The average and S.E.M of four independent experiments (n=4) are shown. **indicates $P<0.01$ in a student test.

The number of EBs formed from ESCs treated with BIO and 1i during EB formation was significantly higher than those formed from the control (Figure 3.9), indicating that GSK-3 inhibitors are not stopping but rather improving ESC efficiency to form EBs. An increase in the number of EBs suggests an increase in pluripotency. Although ESCs differentiate upon removal of LIF, this increase in numbers of EBs suggests that GSK-3 inhibition results in ESCs maintaining self-renewal for a period of time before they start to differentiate. This is agreement with maintenance of pluripotency markers *Nanog* and *Rex1* observed in EB treated for 4 days with GSK-3 inhibitors (Figure 3.8). An alternative explanation would be that cell survival is increased in the presence of GSK-3 inhibitors. During EB formation cell death seems to be very high since approximately only 0.5% of the control cells plated are able to form EBs, thus if GSK-3 inhibition increase survival of undifferentiated ESC the number of EBs formed will also be higher.

3.3.2 Inhibition of GSK-3 drives differentiation towards mesodermal lineages.

If cells are pluripotent, we expect them to differentiate into three germ layers. However, as previously mentioned, some studies (Ding et al., 2003; Bakre et al., 2007) have shown that inhibition of GSK-3 promotes differentiation into a particular lineage. Thus, next it was investigated whether a similar effect can be observed when EBs are treated with BIO or 1i. Expression of ectodermal, mesodermal and endodermal markers in EBs grown in the presence of GSK-3 inhibitors were analysed. For this experiment, the early ectodermal marker *Sox1*, which has a role in inducing ectodermal cells to neural fate, was also analysed (Pevny et al., 1998).

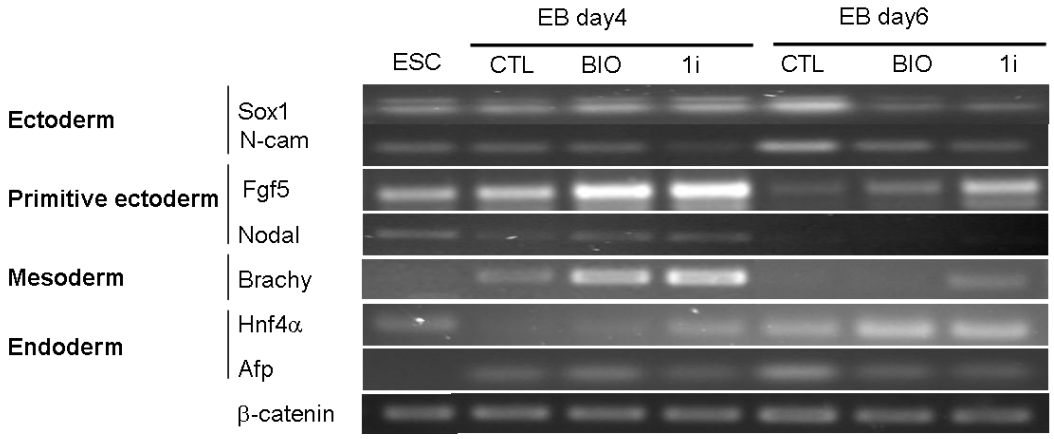

Figure 3.10 GSK-3 inhibition promotes ESC differentiation towards mesendodermal lineage. Expression of lineage markers indicated were analysed by RT-PCR in EB grown with 0.5μM BIO, 5μM 1i or DMSO for 4 and 6 days and in untreated ESCs. CTL: Control, BIO: bio and 1i: 1i. This time course experiment was performed once. Analysis of 6 day old EBs was performed one more time.

Both *Sox1* and *N-cam* have similar pattern of expression, their expression is elevated in 6 day-old EBs in comparison with ESC suggesting differentiation into ectoderm. Opposite to this, *Sox1* and *N-cam* expression in EB treated with GSK-3 inhibitors is lower than in untreated EBs and it is similar to ESC indicating that 1i and BIO is preventing or slowing down differentiation into ectodermal lineages. This would be in accordance with previous reports (Bakre et al., 2007; Doble et al., 2007; Ying et al., 2008).

Up-regulation of *Fgf5* in EBs grown in the presence of GSK-3 inhibitors can be observed at day 4 and 6, in comparison with untreated controls, suggesting promotion of primitive ectoderm following GSK-3 inhibition (Fig. 3.10). *Nodal*, which marks primitive ectoderm and PS formation, is transiently increased at day 4 in inhibitor-treated EBs, suggesting PS formation (Fig 3.10). Consistent with this, *Brachyury* expression, which marks PS, mesendoderm and mesoderm, is up-regulated when GSK-3 is inhibited at day 4 with either inhibitor and day 6 with 1i. *Hnf4α* expression is also up-regulated in 4 day-old EBs treated with 1i and in 6 day-old EBs with either inhibitor. *Afp* expression is the same in control and treated EBs (Fig. 3.10). Although up-regulation of *Hnf4α* would suggest promotion of endodermal differentiation after GSK-3 inhibition, a conclusion can not be drawn as *Afp* expression does not change. Previous reports have suggested that high levels of Nodal signalling in PS promotes differentiation to endoderm (Lowe et al., 2001; Vincent et al., 2003). Moreover, increase of *Nodal* expression in mouse ESCs has been shown to promote definitive endoderm specification and its later down-regulation induces definitive endoderm maturation (Takenaga et al., 2007). GSK-3 inhibition considerably increased Nodal signalling, so it may be possible that it promotes differentiation into endoderm. Other endodermal markers such as *Sox17* would help to elucidate whether GSK-3 inhibition promotes ESC differentiation towards the endodermal lineage.

Collectively, the data suggest that GSK-3 inhibition promotes ESC differentiation towards mesendoderm progenitors, as *Brachyury* expression is up-regulated. However, from the present study is not clear whether GSK-3 inhibition also promotes mesoderm or endoderm differentiation. *Brachyury* up-regulation could suggest induction of mesoderm but its expression is not restricted to mesoderm since it is also expressed in the mesendoderm and primitive streak. Other mesoderm markers, expressed later during embryogenesis, should be used in order to investigate promotion of mesodermal differentiation.

Expression of *Fgf5, Nodal, Hnf4α, Sox1* and *N-cam* in ESCs, as mentioned already in Section 3.2.2.3, could be due to spontaneous differentiation or the presence of lineage-biased populations within the ESC culture.

In summary, GSK-3 inhibition seems to bias differentiation of ESCs towards mesendodermal lineages, which is in accordance with a previous report (Bakre et al., 2007) This mesendodermal up-regulation may be at the expense of ectodermal lineages.

3.3.3 Further investigation of the effects of GSK-3 inhibition on the multi-lineage differentiation potential of ESCs.

To further investigate the effects observed on multi-lineage differentiation of ESCs using RT-PCR, *Brachyury*-GFP and *Sox1*-GFP ESC reporter lines were used. In these cell lines expression of GFP is under the control of the *Brachyury* or *Sox1* promoter. *Brachyury*-GFP ESCs were obtained from Agapios Sachinidis (University of Cologne, Germany), and *Sox1*-GFP was developed by Ying et al. (2003). The coding sequence for GFP was knocked in the open reading frame of the *Sox1* gene (Ying et al., 2003b).

3.3.3.1 Mesendodermal lineage differentiation.

Results from Section 3.3.2 suggest that inhibition of GSK-3 facilitates ESC differentiation towards mesendodermal lineages (Figure 3.10). In order to study in more detail whether inhibition of GSK-3 promotes mesendodermal differentiation, *Brachyury*-GFP ESCs were grown in the presence of serum with BIO or 1m and in the absence of LIF for 1, 2, 3 and 4 days. LIF maintains ESCs in an undifferentiated state, and removal of LIF leads to spontaneous differentiation. The percentage of GFP expressing cells was analysed by Fluorescence activated cell sorting (FACS) and results are summarised in Table 3.1. A specific example of the types of FACS plots obtained is shown in Figure 3.11, with the percentage of *Brachyury*-GFP positive cells after 48 hours treatment with GSK-3 inhibitors indicated.

Table 3.1. Percentage of *Brachyury*-GFP positive cells present over time. ESC were grown in GMEM supplemented with serum with or without LIF and in the presence or absence of GSK-3 inhibitors BIO or 1m at the concentrations and time indicated. This experiment was repeated twice and the results shown are representative.

Treatment/Time	24 hrs	48hrs	72hrs	96hrs
+LIF	5.6%	6.5%	5.2%	4.9%
-LIF	4.8%	4.9%	8.0%	9.9%
-LIF+ 0.5μM BIO	6.5%	10.7%	18%	11.3%
-LIF+ 2μM BIO	17.7%	48%	28.6%	20.1%
-LIF+ 0.5μM 1m	11.2%	10.1%	19.2%	13%
-LIF+ 2μM 1m	27.4%	38%	35.8%	18.1%

Data shown in Table 3.1 suggests that ESCs start spontaneously differentiating approximately 72 hours after LIF removal, which is evidenced by an increase in the percentage of GFP-*Brachyury* expressing cells grown in the absence of LIF in comparison with control cells grown with LIF. A considerable up-regulation in *Brachyury* expression, as early as 24 hours after addition of 2μM BIO or 1m in the absence of LIF, was observed. *Brachyury* expression appeared to be transient (Table 3.1), maximum up-regulation was achieved following 48 hours of treatment at higher concentration of inhibitors and 72hr at lower concentrations.

This experiment was repeated once more and results were consistent. Hence, these results support the idea that inhibition of GSK-3 promotes differentiation of ESCs towards mesendodermal lineages.

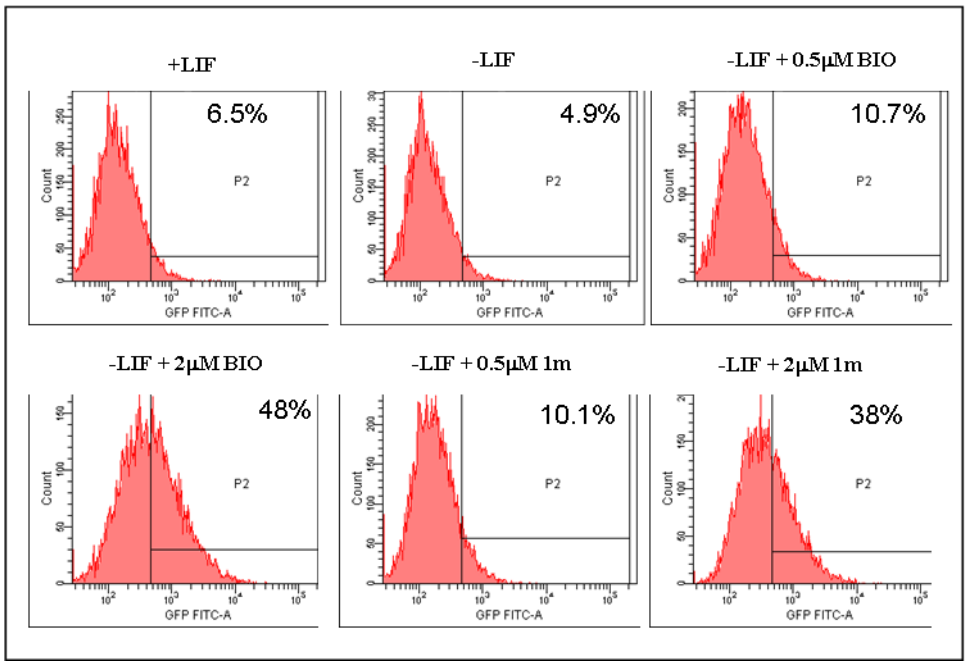

Figure 3.11. GSK-3 inhibition leads to upregulation of *Brachyury* expression. *Brachyury*-GFP ESC were grown in the presence of 0.5μM, 2μM BIO or 1m for 48 hours, and in the absence of LIF. A control with LIF was also set up. Percentage of GFP positive cells was assessed by FACS.

3.3.3.2 Ectodermal lineage differentiation.

Results from Section 3.3.2 suggest that ectoderm differentiation may be blocked or slowed down upon inhibition of GSK-3. The *Sox1*-GFP ESC reporter line was used to further study a possible effect of GSK-3 inhibition on differentiation of ESC towards the ectodermal lineage. *Sox1*-GFP ESCs were grown in the presence of serum with BIO or 1m and in the absence of LIF for 3, 4, 5 and 6 days. *Sox1* expression was slightly down-regulated in ESCs treated with the GSK-3 inhibitors at all time points investigated. The percentage of GFP expressing cells was analysed by FACS and the data is summarised in Table 3.2. Primary data showing the percentage of *Sox1*-GFP positive cells from EBs grown in the presence of inhibitors for 4 days are presented in Figure 3.15. This experiment was repeated twice and the results were reproducible.

Table 3.2 Percentage of *Sox1*-GFP positive cells over time-course. ESC were grown in GMEM supplemented with serum with or without LIF and in the presence or absence of GSK-3 inhibitors BIO or 1m at the concentrations and time indicated. This experiment was repeated twice and the results shown are representative.

Treatment/Time	3 days	4 days	5 days
+LIF	5.1%	4.3%	6.2%
-LIF	4.6%	6%	3.9%
-LIF+ 0.5µM BIO	1.5%	3.3%	1.2%
-LIF+ 2µM BIO	0.5%	0.9%	1.1%
-LIF+ 0.5µM 1m	2%	2.3%	2%
-LIF+ 2µM 1m	0.4%	1.8%	1%

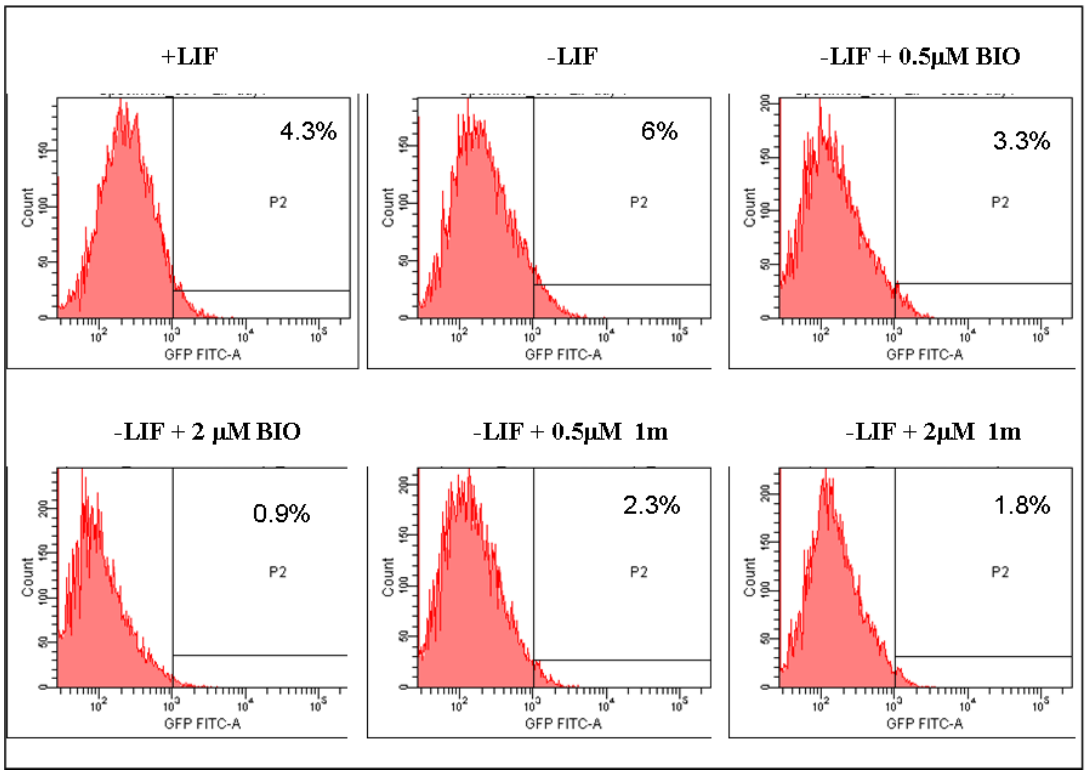

Figure 3.12. *Sox1* expression is downregulated following treatment of ESC with GSK-3 inhibitors. ESC were grown in the presence of 0.5µM and 2µM BIO or 1m for 4 days, and in the absence of LIF. A control with LIF was also set up. Percentage of GFP positive cells was investigated by FACS.

The fact that the percentage of *Sox1*-GFP in plus LIF and minus LIF conditions is similar at all times studied suggest that the majority of cells are not differentiating into ectoderm. Therefore, down-regulation of *Sox1*-GFP observed in cell treated with GSK-3 inhibitors may be due to possible decrease in spontaneous differentiation. A way to promote differentiation into ectoderm would be to grow the cells in N2B27 without any extrinsic stimuli, this has been shown to promote ESC differentiation towards ectoderm (Ying and Smith, 2003; Ying et al., 2003). Repetition of the experiment in these conditions may show higher differences between the percentage of *Sox1*-GFP expressing cells upon GSK-3 inhibition and in untreated control.

3.4 DISCUSSION

3.4.1 ESC maintain pluripotency following GSK-3 inhibition.

One of the aims of this study was to test whether ESCs treated with novel GSK-3 inhibitors (Bone et al., 2009) retain their pluripotency following withdrawal of the inhibitors. This was considered essential to ensure that these novel GSK-3 inhibitors were not increasing ESC self-renewal because they were irreversibly blocking the ability of ESCs to differentiate. Expression of pluripotency markers by ESCs grown in the presence of GSK-3 inhibitors, for both short (48h) and long (19 days) periods of time, and their down-regulation in differentiating conditions suggested that ESC maintained their pluripotency. ESCs treated with GSK-3 inhibitors also retained the capacity to form EBs, following removal of the inhibitors. Maintenance of pluripotency was further supported by the ability of ESC to differentiate into the three germ layers, judged on the basis of lineage marker expression. These findings are in agreement with previous reports showing that ESCs maintained their pluripotency after GSK-3 inhibition with BIO or CHIR (Sato et al., 2004; Ying et al., 2008).

Although *Rex1, Nanog* and *Oct4* were down-regulated under differentiating conditions, *Oct4* expression was retained for longer (Fig. 3.3A, and 3.7A). A possible explanation, discussed already in Section 3.2.2.3, is that the kinetics of *Oct4* down-regulation are slower than *Rex1* and *Nanog*. However, alternative explanations

exist such as the possibility that the ESCs under these conditions are differentiating into primordial germ cells or primitive ectoderm. *Oct4* seems to be down-regulated during embryogenesis as cells differentiate towards more committed cell types. However, primordial germ cells continue to express *Oct4* (Rosner et al., 1990) and ESCs have been reported to generate germ cells *in vitro* (Toyooka et al., 2003). Moreover, *Oct4* is also expressed in the primitive ectoderm until gastrulation (Rosner et al., 1990). Recently, Oct4 expression has been linked to promotion of differentiation of ESC into mesendodermal lineages (Thomson et al., 2011).

We observed that some of lineage markers examined, including *Fgf5, Nodal, Hnf4α, Brachyury* and *N-cam*, were expressed in populations of undifferentiated ESCs. There are two possible explanations that may account for these observations. One possibility is that ESCs spontaneously differentiate and express these markers as a result since these are early lineage markers. *Fgf5* and *Nodal* are markers of primitive ectoderm (Haub and Goldfarb, 1991; Loebel et al., 2003; Zhou et al., 1993) and *N-cam* marks primitive neuroectoderm. *Brachyury and Hnf4α* are early mesodermal and endodermal markers respectively. However, an alternative explanation, supported by several recent reports (Canham et al., 2010; Hayashi et al., 2008; Toyooka et al., 2008), is that undifferentiated ESC populations are not homogeneous but are instead heterogeneous consisting of pluripotent cells in different stages of development including the ICM, the epiblast and the primitive ectoderm (Toyooka et al., 2008). In conditions that support ESC self-renewal these populations are morphologically undifferentiated, express Oct4 and can fluctuate between each other. Canham et al., (Canhan et al., 2010) recently reported the existence of a population of cells that express low levels of the primitive endodermal gene *Hex1* which is in equilibrium with Nanog positive cells that resemble the ICM. They proposed that ESC cultures have lineage-biased populations but they are not committed toward any lineage.

3.4.2 Effects of GSK-3 inhibitors on the multilineage differentiation potential of ESCs.

Prior to this study there was controversy about the role of GSK-3 in differentiation. Some reports have reported that GSK-3 inhibition promote ESC differentiation to neuroectoderm lineages (Ding et al., 2003), while others observed the opposite, i.e., induction of mesendodermal differentiation (Bakre et al., 2007). To investigate the effects of GSK-3 inhibition on differentiation outcome, the EB differentiation system was used as a model.

3.4.2.1 ESCs treated with GSK-3 inhibitors retain pluripotency for a short-period.

Retention of the pluripotency markers *Nanog* and *Rex1* in EBs grown in the presence of BIO and 1i in comparison with control untreated (Fig.3.8), accompanied by an increase in the number of EBs generated when BIO or 1i was present during EB formation (Fig. 3.9) suggest that ESC are more resistant to differentiate when GSK-3 is inhibited. This is in agreement with the observation that GSK-3 DKO cells retain expression of pluripotency makers such as *Oct4* and *Nanog* after 12 days of EB formation (Doble et al., 2007).

3.4.2.2 Inhibition of GSK-3 drives differentiation towards mesendodermal lineages.

Upregulation of *Brachyury* expression in the presence of GSK-3 inhibitors, observed by both RT-PCR from EBs-derived RNA and using a *Brachyury*-GFP reporter cell line grown in monolayer differentiating conditions, suggest that GSK-3 inhibition promotes ESC differentiation towards mesendodermal lineages. GSK-3 inhibition mimics activation of Wnt signalling and hence our results are in agreement with previous reports showing that Wnt signalling is important for development of the primitive streak and mesoderm as mice with components of the Wnt signalling disrupted cannot form PS or mesoderm (Huelsken et al., 2000; Kelly et al., 2004; Liu et al., 1999; Yoshikawa et al., 1997).

Expression of *Brachyury*-GFP was considerably up-regulated following addition of 2μM 1m or BIO, at times as early as 24 hours after addition. The highest percentage of GFP positive cells were observed after 48 hours and then gradually decreased at 72 and 96h. Maximum expression of *Brachyury* after 48 hours and not earlier could be due to a requirement for down-regulation of *Nanog* in order for the cells to proceed to lineage differentiation. This has been reported recently, the authors observing that activation of *Brachyury* in response to CHIR could only occur in cells where *Nanog* had been down-regulated (Thomson et al., 2011). The reduction in numbers of *Brachyury*-GFP positive cells at 72 and 96 hours compared to 48 hours (with 2μM GSK-3 inhibitors) may be due to progression of differentiation of mesendoderm/early mesodermal cells (positive *Brachyury*-GFP) into more mature mesodermal cells. Transient expression of *Brachyury* during differentiation of embryonal carcinoma cell, mouse and human ESC has been previously observed (Davis et al., 2008; Holley et al., 2011; Kennedy et al., 2007; Vidricaire et al., 1994)

Hence, GSK-3 seems to promotes differentiation towards mesendoderm lineages and may prevent or slow down differentiation into ectoderm as *Sox1* and *N-cam* expression was similar in 6 day-old EB grown with GSK-3 inhibitors than in ESCs, but their expression was elevated in 6 day-old untreated EBs indicating ectoderm differentiation (Fig 3.10). Our results are in agreement with several reports published before our work commenced (Bakre et al., 2007; Aubert et al., 2002) and two reports published after this study (Thomson et al., 2011; Ying et al., 2008). Ying et al., showed that inhibition of GSK-3 with CHIR promotes non-neural differentiation, as well as blocking neural differentiation (Ying et al., 2008). More recently, inhibition of GSK-3 following 48 hours of withdrawal of LIF and BMP4, which lead to differentiation, was shown to promote mesendoderm differentiation with about 70% of the cells expressing *Brachyury* after 36 hours of CHIR addition, and inhibit neuroectoderm lineage differentiation (Thomson et al., 2011). Surprisingly, *Brachyury* positive cells also express the pluripotency marker *Oct4* at higher levels than in the initial ESC population and they observed that Oct4 binds to DNA regions associated with neuroectoderm differentiation where it acts as a repressor. This is in agreement with the fact that *Oct4* overexpression can block induction of neuroectoderm differentiation. In addition to this, the levels of Oct4 decrease following withdrawal of LIF and BMP4 and addition of CHIR leads to re-expression

of Oct4 and decrease of Sox2 levels. Sox2 seems to bind to DNA regions associated with mesendoderm differentiation where it acts as a repressor. Hence, decrease of Sox2 levels following CHIR addition alleviates Sox2 repression of genes associated with mesendoderm differentiation.

3.4.3 Conclusions

GSK-3 inhibition has different outcomes depending on the developmental stage of the cells. Inhibition of GSK3 in ESCs cultured under self-renewing conditions leads to enhanced self-renewal, whereas its inhibition during differentiation promotes ESC towards mesendoderm lineages. A recent report (Thomson et al., 2011) proposed that the presence of Nanog is key for the different outcomes. Decrease of *Nanog* (using siRNA) in conditions that maintain pluripotency led to a consequent down-regulation of other pluripotency markers, such as *Oct4* and *Sox2*, which in the authors' opinion leave the cells responsive to differentiating stimuli. They proposed that the pluripotency circuit represses all lineage differentiation in ESC but some markers, such as *Oct4* and *Sox2* have a lineage specific role, Oct4 represses Neuroectoderm differentiation and thus promoting Mesendoderm differentiation and Sox2 has the opposite role.

4 CHAPTER: GSK-3 MECHANISM OF ACTION

4.1 Introduction and aims

ESCs are very attractive as a source of differentiated cell types for use in regenerative medicine due to their properties, which have already been discussed in Section 1. We must understand the molecular mechanisms governing stem cell fate in order for the potential of ESCs to be realised since ESC pluripotency needs to be maintained in culture and differentiation towards a desired cell types tightly controlled.

Under standard culture conditions, mESC pluripotency is controlled by the coordinated action of extrinsic factors, signalling pathways and transcription factors (Boiani and Scholer, 2005), which have been extensively described in Section 1.3. ESC can be maintained in cultured in the presence or absence of serum. In the presence of serum, LIF alone is able to maintain self-renewal of mESCs. However, in serum-free conditions, Bone morphogenetic proteins 2 or 4 (BMPs) are also required. BMPs appear to cooperate with LIF to maintain self-renewal through induction of Id (Inhibitor of differentiation) proteins (Ying et al., 2003). Maintenance of self-renewal in serum-free media in the absence of LIF and BMP4 can be achieved by simultaneous inhibition of Glycogen Synthase Kinase-3 (GSK-3) and MAP kinase/ERK kinase (MEK) (Ying et al., 2008), often referred to a 2i conditions.

The canonical Wnt pathway has also been reported to play a role in maintenance of self-renewal of mESCs (Hao et al., 2006; Ogawa et al., 2006; Sato et al., 2004; Singla et al., 2006; Ying et al., 2008). Inhibition of GSK-3, which mimics activation of Wnt signalling, with small molecules inhibitors, BIO (Sato et al., 2004), 1m (Bone et al., 2009) or CHIRON99021 (CHIR; Ying et al., 2008), can maintain short-term self-renewal of ESCs. Furthermore, mESCs with both GSK-3 isoforms, α and β deleted (GSK-3 DKO) (Doble et al., 2007) have Wnt/β-catenin signalling hyperactivated and are more resistant to differentiation but cannot self-renew in longer-term cultures in the absence of additional factors. However, either addition of LIF in the presence of serum (Bone et al, 2009), or inhibition of MEK in chemically defined media (N2B27) media completely blocks differentiation and robust long-term self-renewal is achieved (Ying et al., 2008).

The mechanism of action of GSK-3 in regulating mESC self-renewal and pluripotency is still not fully understood. As previously discussed in Section 1.3.3.1, GSK-3 is known to play a role in different cellular processes such as apoptosis, cell survival metabolism and translation (Jope and Johnson, 2004). GSK-3 downstream effectors in non-ESC type include protein synthesis initiation factors, transcriptional regulators and components of the cell-division cycle (reviewed in Frame and Cohen 2001; Doble and Woodgett, 2003). Despite the fact that GSK-3 has many downstream effectors, most studies to date suggest that the effects observed upon GSK-3 inhibition is at least partly via Wnt/β-catenin-dependent mechanisms. However, mechanisms of action of GSK-3, independent of β-catenin, including regulation of c-Myc and Nanog, have also been proposed (Bechard and Dalton, 2009; Storm et al., 2007). Both reports proposed a mechanism involving GSK-3 downstream of PI3K.

Using clonal assays, we have previously demonstrated that GSK-3 inhibition enhances self-renewal of mESC in the presence of LIF and Serum (Bone et al., 2009) and that GSK-3 downstream of PI3K can regulate *Nanog* RNA expression (Storm et al., 2007). However, the precise mechanism of action of GSK-3 in these situations is unknown. If GSK-3 inhibition contributes to maintenance of self-renewal, it is reasonable to hypothesise that GSK3 may regulate other transcription factors as well as *Nanog*. Due to the fact that GSK-3 not only regulates transcription factors in non-ESC types but also protein synthesis factors, GSK-3 could also potentially play a role in determining ESC fate at the translational level, as well as the transcriptional level.

The aim of this study was to investigate whether GSK-3 regulates expression of pluripotency markers and, if so, the mechanism of action of GSK-3. We examined the possible regulation of Nanog, Tbx3, c-Myc, Zscan4 and Oct4 by GSK-3 at transcriptional, protein stability and translational levels. For our study we used a chemical genetic approach, including selective small molecules inhibitors of GSK-3 (3.1.1), and DKO GSK-3 cells (Doble et al., 2007).

4.2 Effect of GSK-3 inhibition on cell proliferation in different culture conditions.

Our group, together with others, have demonstrated a role for GSK-3 inhibition in contributing to maintaining ESC self-renewal (Sato et al., 2004; Doble et al., 2007; Ying et al., 2008; Bone et a., 2009). Ying *et al.,* showed that inhibition of both GSK-3 and MEK could sustain ESC self-renewal in the absence of BMP4 and LIF. Under these defined conditions the authors proposed that the main role of GSK-3 inhibition was to restore growth and metabolic capacity. However, in the presence of LIF and serum, ESC metabolism is well supported and we previously observed that inhibition of GSK-3 in the presence of LIF and serum did not seem to alter cell growth (results not shown). Hence, we first sought to investigate whether inhibition of GSK-3 affected the proliferation of ESCs cultured under different conditions and whether in serum-free media GSK-3 inhibition would have a positive effect on cell growth as previously reported (Ying et al., 2008).

Cells were grown in N2B27 (defined media 1:1 Neurobasal to DMEM F12 media supplemented with N2 and B27 supplements) medium alone, or in the presence of GSK-3 inhibitor (CHIR), MEK inhibitor (PD) or both and their growth was monitored for 3 days. GSK-3 inhibition with CHIR increased cell growth compare to N2B27 alone, as shown in Figure 4.1(i). Addition of PD to N2B27 alone led to a slight increase in cell growth, which was smaller than with CHIR. Cells grown in both CHIR and PD grew at the same rate as cells inhibited only with CHIR (Figure 4.1 (i)). No significant differences in cell growth were observed with only PD or CHIR in the presence of LIF and Serum, but combinations of both (2i) slightly reduced cell growth (Fig. 4.1 (ii)). In the presence of LIF and BMP4, inhibition of MEK seems to decrease cell growth and inhibition of GSK-3 restores it (Fig 4.1 (iii)). It seems, therefore, that the effect of the inhibitors differs depending on the culture milieu. In the presence of serum no major effect on cell growth is observed when GSK3 or MEK is inhibited. However, in serum-free conditions GSK-3 seems to restore cell growth when added to MEK inhibitor, which in accordance with Ying *et al*., report (Ying et al., 2008).

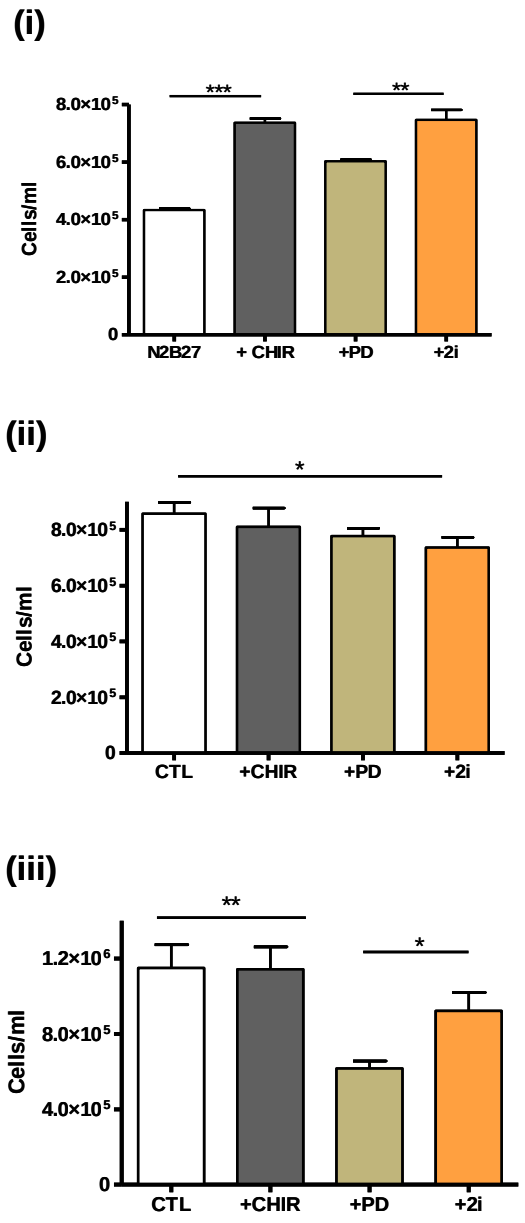

Figure 4.1. GSK-3 inhibition increases cell growth in the absence of serum but not in the presence. ESCs were grown in N2B27 alone without extrinsic stimuli (i), in GMEM supplemented with Serum and LIF (ii) or in N2B27 with BMP4 and LIF (iii) for 3 days in the presence or absence of 3μM CHIR, 1μM PD or both (2i) and their growth monitored. CTL: DMSO treated cells. The data are the average and S.E.M of triplicate experiment. *, <*p* 0.05; **, p<0.01, ***, *P*<0.005. Two-way anova, Bonferroni posttests.

4.3 GSK-3 inhibition regulates expression of pluripotency-associated transcription factors.

The key aim of this part of the study was to investigate changes in pluripotency-associated transcription factors following GSK-3 inhibition in different culture conditions, including serum plus or minus LIF, N2B27 plus LIF and BMP4 and ground state conditions (N2B27 without LIF or BMP4 but plus MEK and GSK-3 inhibitors).

4.3.1 Regulation of pluripotency-associated transcription factors by GSK-3 in the presence of serum.

A possible regulation of pluripotency-associated transcription factors by GSK-3 in the presence of serum and LIF or in the absence of LIF was investigated.

4.3.1.1 GSK-3 inhibition or knockout regulates the expression of Nanog, Tbx3 and c-Myc in the presence of serum and LIF.

We investigated early changes in the levels of both protein and RNA for the transcription factors Nanog, Tbx3, Oct4, c-Myc and Zscan4 following inhibition of GSK-3 with 1m (wild-type ESCs) and in GSK-3 DKO ESCs, grown in serum plus LIF. ESCs grown in the presence of 1m, as well as the DKO ESCs, exhibited a more compact colony morphology, reminiscent of highly self-renewing cells, compared to wild-type controls (Fig 4.2 (i)). We consistently observed an increase in Nanog, Tbx3 and c-Myc protein levels as early as 6-8 hours following initiation of GSK3 inhibition and their elevated levels were maintained at 24 hours (Fig. 4.2 (ii)). Nanog protein levels more than doubled in both 1m treated ESCs and GSK-3 DKO ESCs, and Tbx3 protein also showed approximately a 2-fold increase in samples grown in 1m after 8hours and almost a 3-fold increase in DKO cells in serum conditions (Fig. 4.2 (iii)). However, the levels of Oct4 protein did not show any consistent changes at the investigated times (Fig. 4.2 (iii)) and Zscan4 protein was consistently higher in DKO cells compared to WT (Fig 4.2. (ii)).

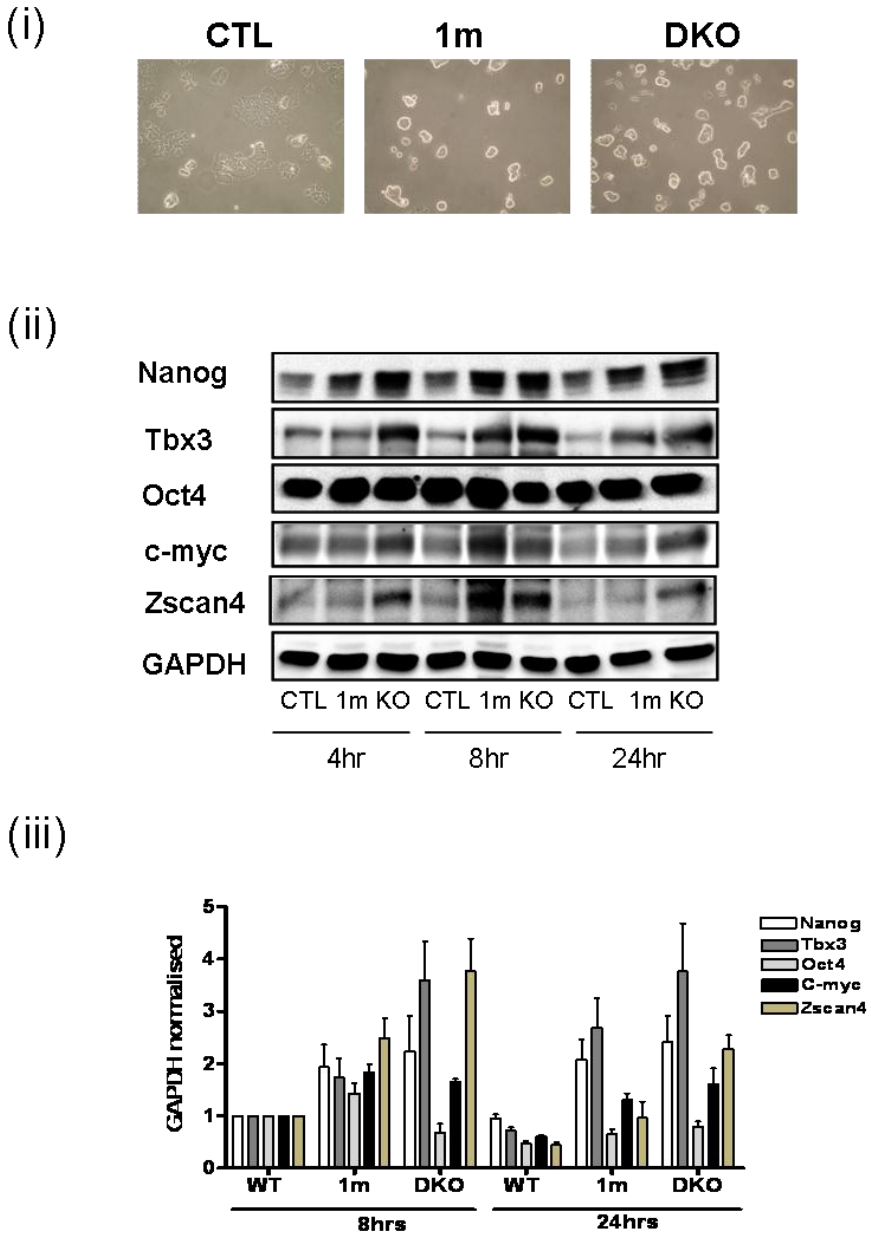

Figure 4.2. GSK-3 regulates protein expression of transcription factors in mESCs. E14tg2a wild-type (WT) and GSK-3α/β double knockout (DKO) mESCs were cultured in the presence of Serum plus LIF. GSK-3 inhibitor 1m was added to WT cells at 2 μM, as indicated. (i) Images show colonies formed from untreated WT ESCs (CTL), DKO ESCs, and WT ESCs cultured in the presence of 2μM 1m for 48h. Protein (ii) was extracted at the times indicated. 12μg of nuclear protein extracts were immunoblotted with the antibodies specified, antibody signals were quantified and normalised to GAPDH (loading control) (iii). A value of one was given to WT 8 hours. The experiment was performed three times and the data are the average of and SD of duplicate representative experiments.

To accompany analysis of protein levels, the levels of mRNA expression for pluripotency markers was also investigated. *Nanog* and *Tbx3* mRNA levels were slightly elevated after 8 hours in the presence of 1m or in GSK-3 DKO ESCs. Their levels were further increased after 24 hours of GSK-3 inhibition with 1m (Figure 4.3). *c-Myc* RNA levels were slightly decreased after 8 hours in 1m and DKO cells in the presence of serum, contrasting to the modest increase in c-Myc protein levels observed (Figures 4.2 and 4.3). *Oct4* mRNA level did not change significantly following GSK-3 inhibition either at 8 or 24 hours (Figure 4.3).

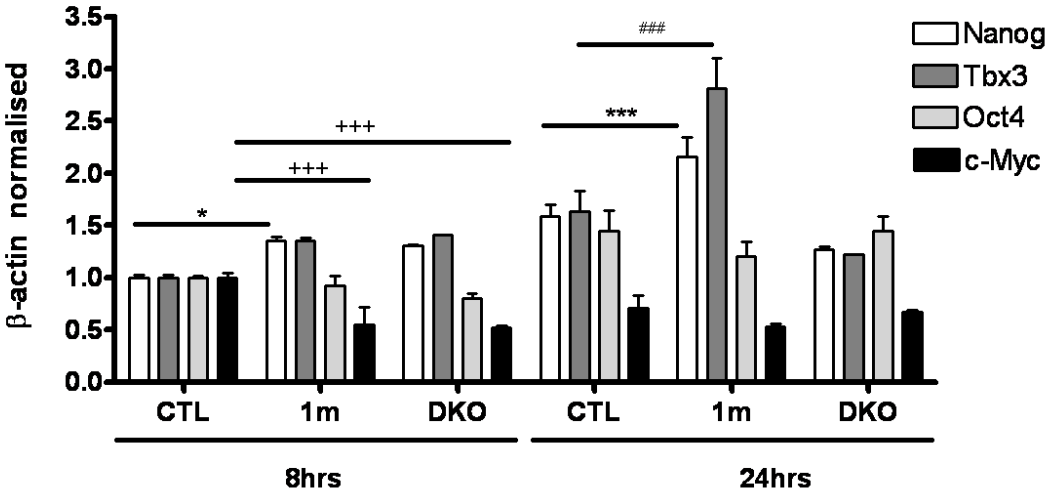

Figure 4.3. GSK-3 inhibition increases transcription of *Nanog* and *Tbx3* in ESCs. E14tg2a wild-type (WT) and GSK-3α/β double knockout (DKO) mESCs were cultured in the presence of Serum plus LIF. GSK-3 inhibitor 1m was added to WT cells at 2 μM. RNA was extracted at the times indicated, quantitative RT-PCR was carried out and gene expression normalized relative to β-actin levels. The data are the average and S.E.M of quadruplicate samples. *, <*p* 0.05; **, p<0.01, ***, *P*<0.005. * for *Nanog*, #for *Tbx3* and + for *c-Myc*. Two-way anova, Bonferroni posttests. A value of 1 was given to WT 8hours.

These data indicate that GSK-3 inhibition or knockout regulates the expression of *Nanog*, *Tbx3* and *c-Myc* in the presence of LIF and Serum. We also observed consistent increases in Zscan4 protein in DKO ESCs, but not after 8 or 24 hours of GSK-3 inhibition, suggesting that Zscan4 may not be a direct downstream effector of

GSK-3. Interestingly, changes in *Nanog* and *Tbx3* mRNA levels after 8 hours of initiation of GSK-3 inhibition are modest compared to changes in protein levels, suggesting that although transcription may account for some of the increase observed in their protein levels, other mechanisms are also likely to contribute.

4.3.1.2 GSK-3 inhibition can maintain Nanog and Tbx3 expression after LIF withdrawal for short-term in serum conditions.

In Section 4.2.1.1 GSK-3 inhibition or DKO has been shown to be able to regulate Nanog and Tbx3 expression in the presence of LIF and Serum. The next aim was to investigate whether GSK-3 could also regulate Nanog and Tbx3 expression in the absence of LIF. WT and DKO GSK-3 were grown overnight in the presence of LIF. LIF was then withdrawn and cells were grown without LIF for 1, 2 or 3 days. One control plus LIF was also grown for 3 days. Nanog and Tbx3 protein levels were elevated in cells grown in the absence of LIF and presence of 1m compared to grown only in the absence of LIF for 1, 2 and 3 days (Fig. 4.4 (i) (ii)). The fact that Nanog levels in ESC cultured without LIF but in the presence of 1m are similar to Nanog levels in WT cells grown for 3 days plus LIF suggests that GSK-3 can maintain the expression of Nanog in the absence of LIF. On the other hand, Tbx3 levels in the presence of LIF in WT ESCs were not evident, so the same conclusion cannot be drawn (Fig. 4.4 (i) (ii)). Interestingly, the levels of *Nanog* mRNA do not always correlate with Nanog protein, i.e. the levels of *Nanog* mRNA are similar in DKO cells grown with or without LIF for 1 and 2 days despite that Nanog protein is higher in DKO with LIF. This uncoupling of RNA and protein levels is even more evident for Tbx3, where the RNA levels are similar in WT ESCs grown in minus LIF and 1m and in DKO cells with or without LIF at day 2 despite of differences in protein levels (Fig. 4.4 (iii)).

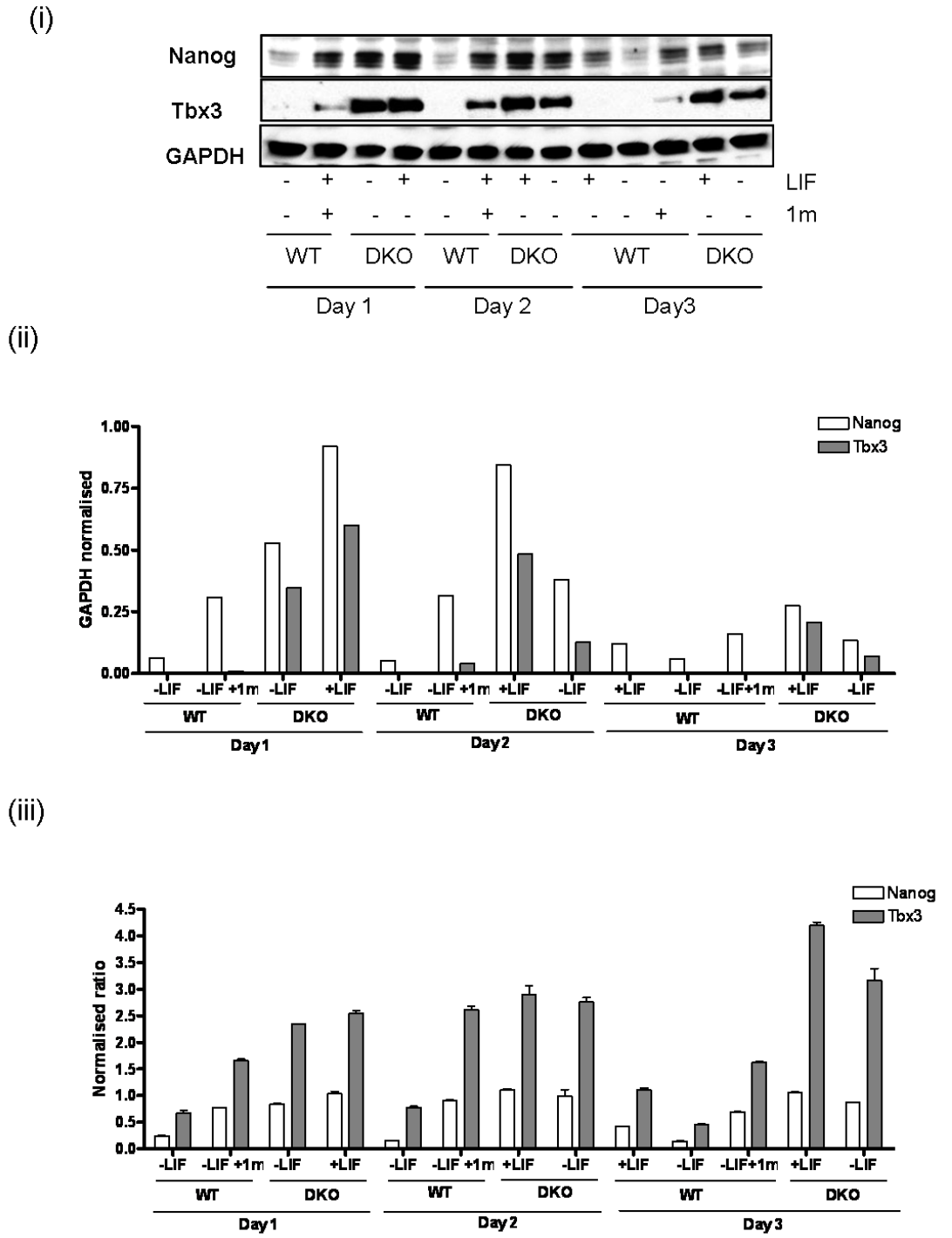

Figure 4.4 GSK-3 inhibition or DKO regulates Nanog and Tbx3 expression. WT and DKO ESCs were grown in absence of LIF for the times indicated, and in the presence of LIF for 3 days. WT incubated with 2μM 1m (1m) was also cultured in the absence of LIF. Protein and RNA were extracted at the times indicated. Immunoblotting was performed with the indicated antibodies (i) and antibody signals quantified and normalised to GAPDH (loading control)(ii). Quantitative RT-PCR was carried out and gene expression normalized relative to β-actin levels. The data are the average and S.D of one experiment run in duplicate (iii).

The experiment was repeated again to further investigate whether GSK-3 can regulate Nanog and Tbx3 in the absence of LIF, in this experiment a plus LIF control was included for 1, 2 and 3 days and Zscan4 was also investigated (Fig 4.5).

Nanog and Tbx3 protein levels seem to be tightly controlled and they are rapidly downregulated after 1 day of LIF withdrawal in WT cells. GSK-3 inhibition with 1m seems to maintain expression of Nanog and Tbx3 protein in WT ESCs in the absence of LIF compared to cells cultured without LIF or GSK-3 inhibiton (Fig. 4.5 (i) (ii)). The fact that *Nanog* mRNA does not seem to change dramatically in DKO cells after 1 day of LIF withdrawal (Fig. 4.5 (iii)) but protein is downregulated suggests that in the presence of LIF Nanog may be translated at higher rate. Interestingly, in the absence of LIF and presence of 1m, the levels of Nanog and Tbx3 protein and mRNA are maintained similar to those observed in WT ESCs grown in the presence of LIF, even after 2 days of LIF withdrawal, suggesting that GSK-3 inhibition can maintain Nanog and Tbx3 expression in the absence of LIF, at least for a short period of time. Finally, Zscan4 protein does not seem to be as tightly regulated as Nanog and Tbx3 because it is still expressed in cells cultured without LIF for 1 and 2 days and protein levels are similar in cells cultured with or without 1m. However, Zscan4 levels are higher in GSK-3 DKO cells than in WT cells even in the absence of LIF.

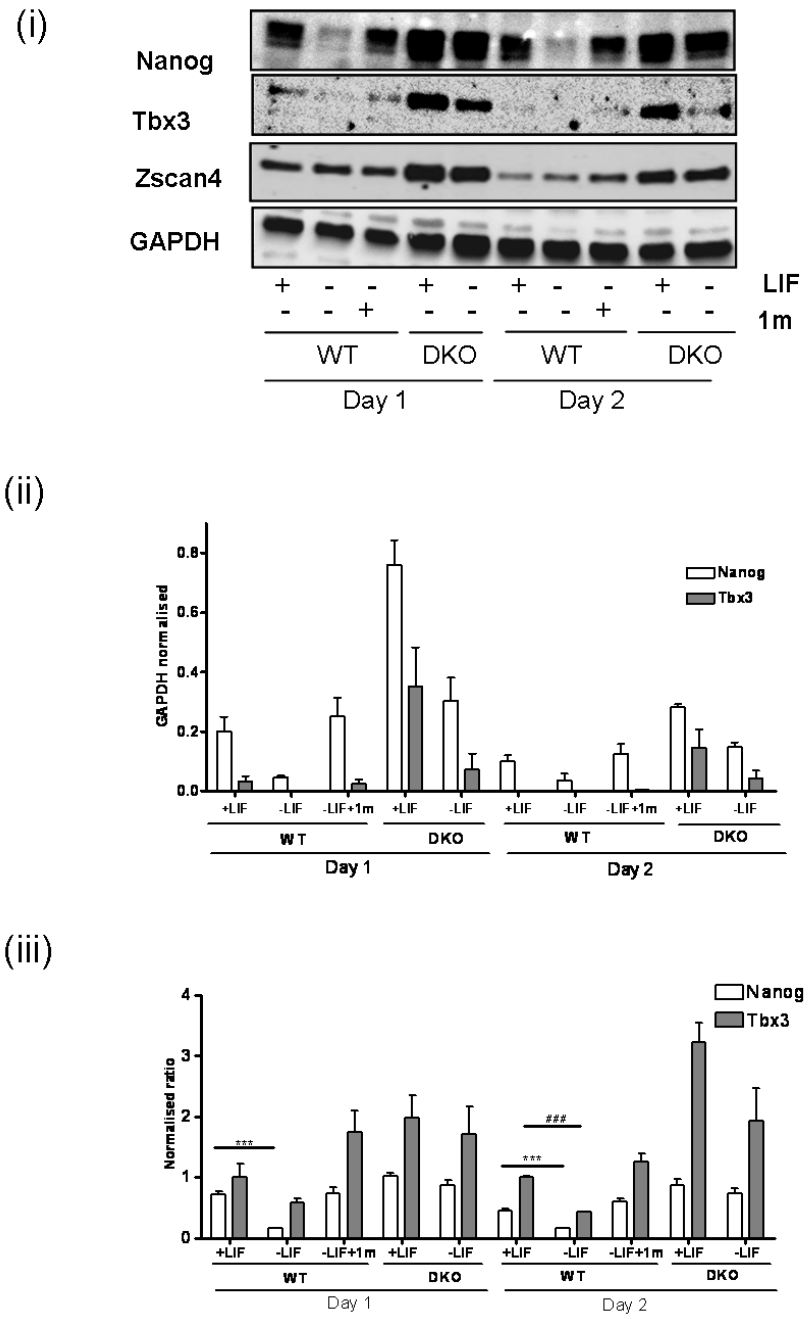

Figure 4.5. GSK3 inhibition or DKO maintain the expression of pluripotent markers upon LIF withdrawal. WT and DKO ESCs cells were grown in the presence or absence of LIF for the times indicated. WT ESCs incubated with 2μM 1m were also cultured in the absence of LIF. Protein and RNA were extracted at the times indicated. (i) Immunoblotting of 15μg protein was performed with the indicated antibodies and values normalised to GAPDH (loading control) (ii). Data are the average and S.D of duplicate experiments. (iii) Quantitative RT-PCR was carried out and gene expression normalized relative to β-actin levels. The data are the average and S.E.M of quadruplicate samples. ***, $P<0.005$. * for *Nanog* and # for *Tbx3*.

4.3.2 Regulation of pluripotency-associated transcription factors by GSK-3 in serum-free conditions.

Results from the previous Section suggested that inhibition of GSK-3 or its knockout in the presence of LIF and Serum can regulate the expression of Nanog, Tbx3 and c-Myc. Zscan4 was also increased in DKO cells but not after inhibition of GSK-3 for 8 or 24 hours. This Section aimed to investigate whether GSK-3 inhibition also moderate the expression of pluripotency-associated transcription factors in the absence of serum.

We investigated early changes in the levels of both protein and RNA for the same pluripotency transcription factors as in the previous Section in serum-free media (N2B27) plus LIF and BMP4, following inhibition of GSK-3 with 1m, CHIR (wild-type ESCs) and in GSK-3 DKO ESCs. After 48 hours culture in the presence of 1m, CHIR or in DKO, ESC morphology changed compared to control and colonies became more round and compact (Fig. 4.6 (i)), similar to what it was observed in the presence of serum.

An increase in Nanog and Tbx3 proteins, 8 hours after addition of 1m or CHIR, was consistently observed (Fig 4.6 (ii)). Nanog protein increased between 3 and 5-fold in 1m, CHIR treated or GSK-3 DKO cells after 8 and 24 hours, whereas Tbx3 increased between 3 and 6-fold (Fig. 4.6 (iii)). On the other hand, the levels of Oct4 and c-Myc did not consistently change and Zscan4 was sometimes, but not always, elevated in DKO cells in comparison to control (Fig 4.6 (ii)).

Nanog and *Tbx3* mRNA levels were slightly elevated after 8 hours in the presence of 1m or in GSK-3 DKO ESCs, but not in CHIR, and their levels were maintained after 24 hours. After 24 hours of inhibition with CHIR Nanog and Tbx3 levels are as high as 1m or DKO (Fig 4.7). An increase in RNA levels of less than 2-fold (and in most cases of less than 50%) for *Nanog* and *Tbx3* is relatively low in comparison with protein changes of 3-5 fold for Nanog and 3-6 fold for Tbx3 (Figure 4.3 (iii)). *c-Myc* and *Oct4* mRNA levels were not significantly altered following GSK-3 inhibition (Fig. 4.7).

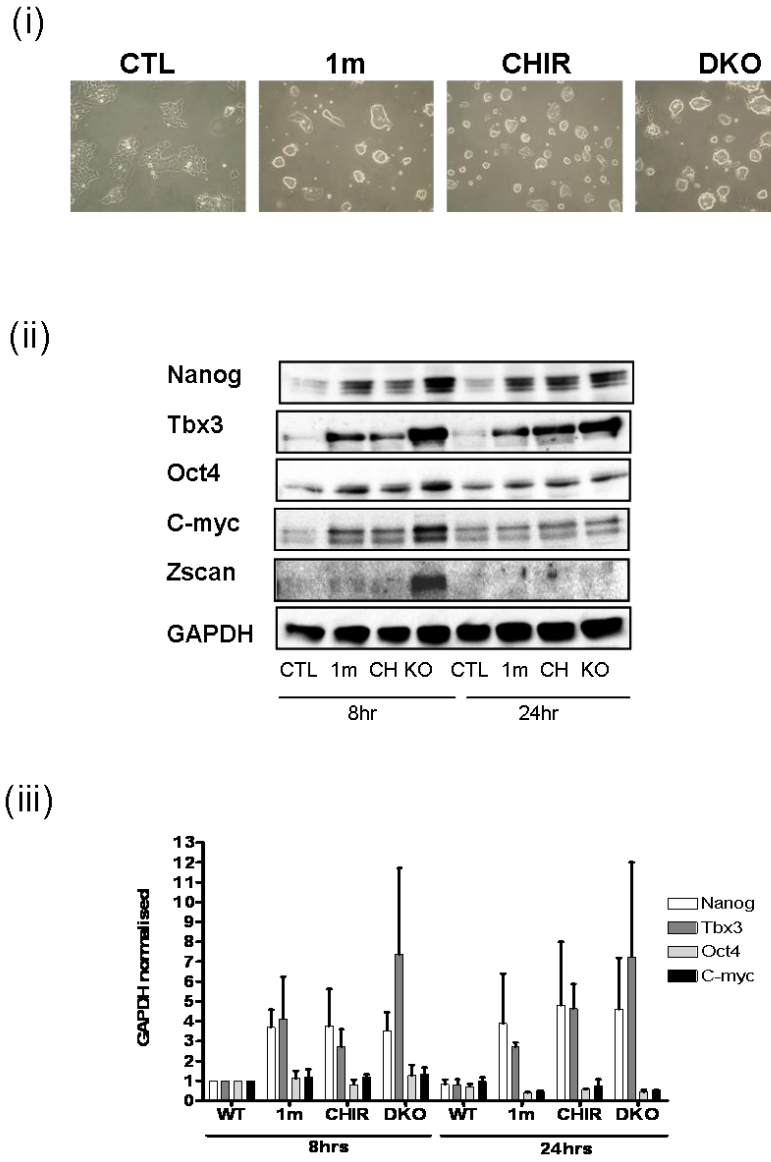

Figure 4.6. GSK-3 also regulates protein expression of transcription factors in serum-free media. E14tg2a wild-type (WT) and GSK-3α/β double knockout (DKO) mESCs were cultured in chemically defined medium (N2B27) plus LIF and BMP4. GSK-3 inhibitors 1m or CHIR99201 were added to WT cells at 2 μM and 3μM respectively. (i) Images show colonies formed from untreated WT ESCs (CTL), DKO ESCs, and WT ESCs cultured in the presence of 1m or CHIR99201 for 48h. Protein (ii) was extracted at the times indicated and 12μg of nuclear protein extracts were immunoblotted with the antibodies specified, antibody signals were quantify and normalised to GAPDH (loading control) (iii). A value of one was given to WT 8 hours. The experiment was performed three times and the data are the average and SD of duplicate experiments.

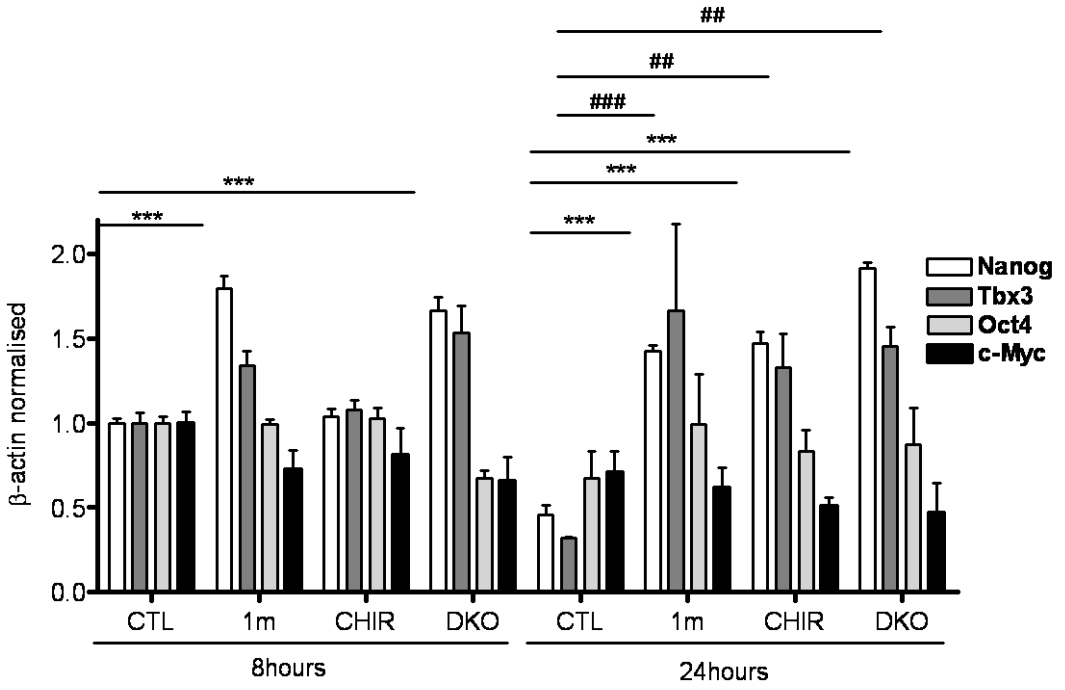

Figure 4.7. GSK-3 inhibition increases transcription of *Nanog* and *Tbx3* in serum-free conditions. E14tg2a wild-type (WT) and GSK-3α/β double knockout (DKO) mESCs were cultured in chemically defined medium (N2B27) plus LIF and BMP4. GSK-3 inhibitors 1m and CHIR were added to WT cells at 2 μM and 3μM respectively. RNA was extracted at the times indicated, quantitative RT-PCR was carried out and gene expression normalized relative to β-actin levels. The data are the average and S.E.M of quadruplicate samples. *, <*p* 0.05; **, p<0.01, ***, *P*<0.005. * for *Nanog*, and #for *Tbx3*. Two-way anova, Bonferroni posttests. A value of 1 was given to WT 8 hours.

These data suggest that GSK-3 inhibition has similar outcomes in serum and serum-free media including change of colony morphology and regulation of Nanog and Tbx3, but there are also some differences, for example in regulation of c-Myc and Zscan4. Moreover, changes in *Nanog* and *Tbx3* mRNA levels after GSK-3 inhibition are modest compared to changes in protein levels in both serum and serum-free conditions, suggesting that although transcription may partially account for the increases observed in their protein levels, it is plausible that other mechanisms also contribute.

4.3.3 GSK-3 inhibition can regulate Nanog and Tbx3 expression in the absence of extrinsic stimuli.

We were interested to investigate whether inhibition of GSK-3 in serum-free media, without extrinsic stimuli, could also regulate expression of Nanog and Tbx3. ESCs were grown for 16 hours in N2B7 alone before adding GSK-3 (CHIR or 1m) or MEK (PD0325901 (PD)) inhibitors or both. Tbx3 and Nanog protein levels were more elevated after GSK-3 inhibition compared to cells grown in N2B27 or in the presence of only MEK inhibitor after 24 hours. In the case of Nanog, protein was even higher when both GSK-3 and MEK were inhibited. However, Tbx3 protein was not further increased in samples extracted from cells incubated with two inhibitors (Fig. 4.8 (i)). ESCs also showed more compact colony morphology after 24 and 48 hours growth in the presence of GSK-3 inhibitor or both inhibitors, in comparison with cells grown only with MEK inhibitor or with no inhibitor (Fig. 4.8 (ii)). This suggests that GSK-3 inhibition may not only contribute to maintenance of the ground state of pluripotency, by restoring metabolic capacity as previously reported (Ying et al., 2008), but also by regulating expression of pluripotency regulators such as Nanog and Tbx3.

When cells were grown long-term in only MEK or GSK-3 inhibitors they eventually differentiated, however, GSK3 inhibition seemed to keep a higher proportion of ESCs self-renewing for longer. Robust long-term self-renewal was observed when both MEK and GSK-3 inhibitors were present and this is in accordance with Ying *et al.*, report (Ying et al., 2008).

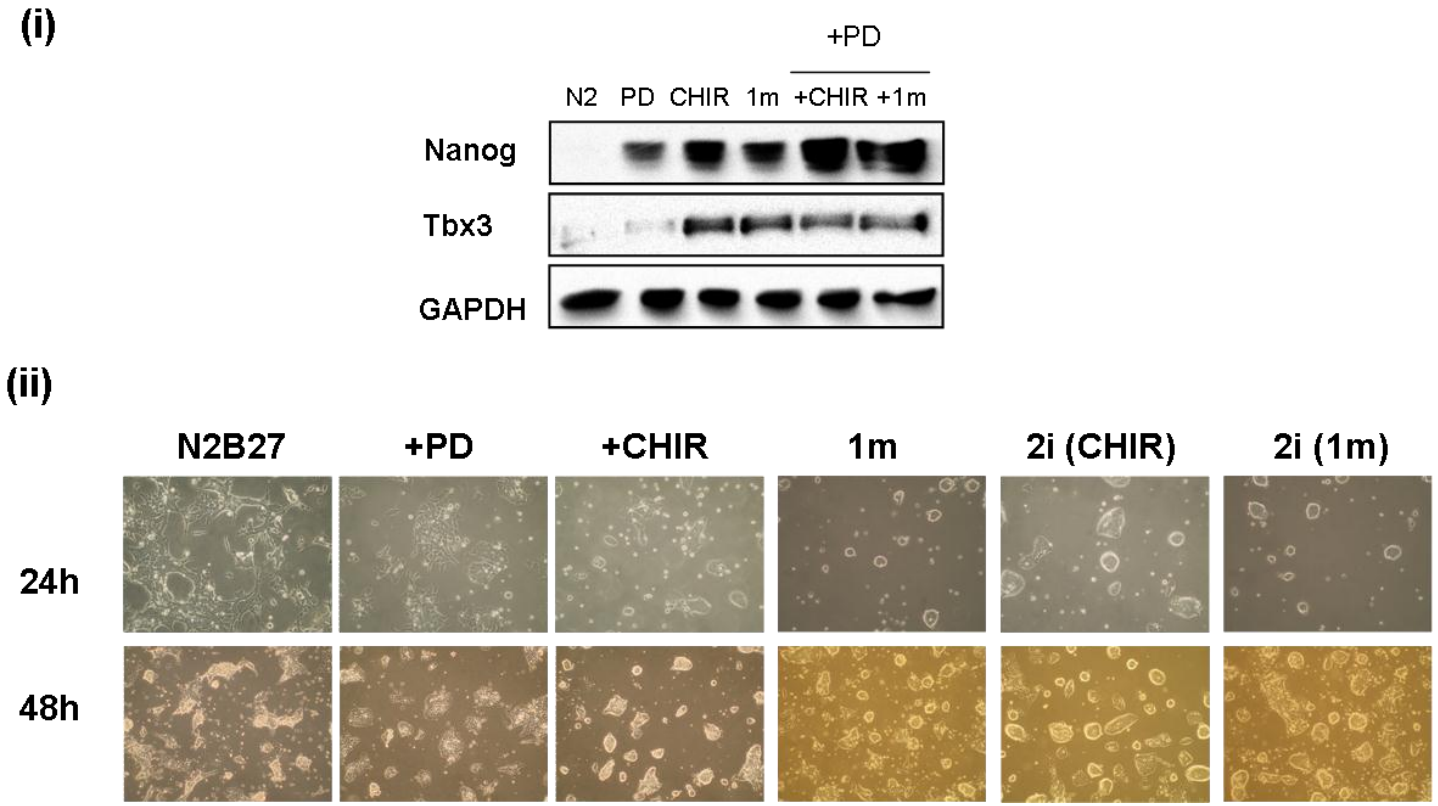

Figure 4.8. GSK-3 inhibition can regulate Nanog and Tbx3 expression in the absence of extrinsic stimuli. E14tg2a mESCs were cultured in N2B27 without LIF or BMP4 and in the presence of 1μM PD, 3μM CHIR, 2μM 1m, 3μM CHIR+1mM PD and 2μM 1m+ 1μM PD. Immunoblotting was performed after 24 hours with the antibodies specified (i) and images taken after 24 or 48 hours (ii). The experiment was repeated three times and data shown is representative

This part of the study next aim was to investigate whether inhibition of GSK-3 in cells pre-treated overnight with a MEK inhibitor would lead to early increases in Nanog and Tbx3 protein expression. Possible changes in Oct4 and Zscan4 were also investigated.

Results from a preliminary experiment (Fig 4.9 (i)) suggested that Nanog protein levels modestly increased after 4 hours of GSK-3 inhibition with CHIR and Tbx3 levels were considerably elevated in comparison to cells without GSK-3 inhibitors. The ability of GSK-3 to regulate Nanog was more evident after 24 and 48 hours of CHIR addition, where Nanog protein levels were considerably higher in cells with CHIR than in cells grown only with MEK inhibitor. The levels of Nanog and Tbx3 protein seemed to decrease overtime in cells cultured with MEK inhibitor only, whereas Nanog levels was maintained and the decrease of Tbx3 was less dramatic in cells cultured with CHIR. On the other hand, Oct4 levels was also reduced overtime in cells grown with MEK inhibitor only and addition of CHIR did not appear to have a significant effect. Finally, Zscan4 was different, with a modest decrease 24 and 48 hours after CHIR addition, in comparison with MEK only. Moreover, ESC colony morphology changed from differentiating looking cells to round compact self-renewing colonies after 24 h of GSK-3 inhibition (Fig. 4.9 (ii)). This experiment was repeated twice more and all results were reproducible, except the increase in Tbx3 and Nanog protein after 4hr of GSK-3 addition.

The fact that Nanog and Tbx3 proteins seem to decrease in cells grown in MEK inhibitor after 24 and 48 hours, compared to 4 hours, suggests either that GSK-3 inhibition acts either by preventing the loss of cells expressing Nanog and Tbx3 or actively maintains their levels.

To summarise, the data presented in Fig 4.8 and 4.9 suggest that GSK-3 may not only play a role in restoring metabolic capacity and growth in 2i conditions, but it may also contribute to self-renewal by regulating the expression of Nanog and Tbx3.

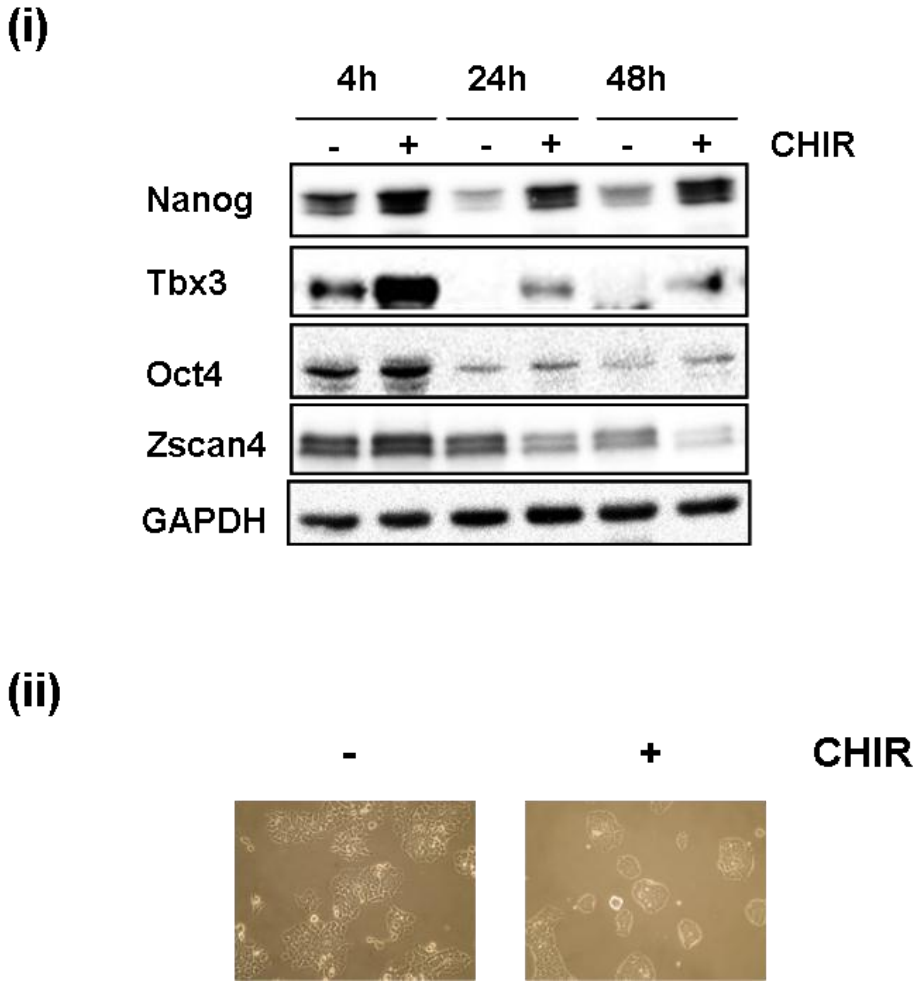

Fig 4.9 GSK-3 inhibition contributes to maintenance of Nanog and Tbx3 levels when added in combination with MEK inhibitor. ESCs were grown overnight in N2B27 without LIF and BMP4 and with 1μM PD before incubating with 3μM CHIR for 4, 24 and 48 hrs. Samples were immunoblotted with the antibodies specified (i) and images taken after 24 hours of CHIR addition (ii).

4.4 GSK3 inhibition does not alter Nanog, Tbx3, c-Myc, Oct4 or Zscan4 protein stability.

In Section 4.2 we have shown that the expression of certain pluripotency-associated transcription factors including Nanog, Tbx3, c-Myc and Zscan4 can be regulated by genetical ablation or pharmacological inhibition of GSK-3. GSK-3 is known to regulate β-catenin protein stability via phosphorylation and proteosomal degradation (Moon et al., 2002). Regulation of c-Myc protein stability has also been reported (Cartwright et al., 2005). We were interested to examine whether increases in protein stability could contribute to the increased levels of Nanog, Tbx3, c-Myc, and Zscan4 proteins observed following GSK-3 inhibition or in GSK-3 DKO cells. Initially, we studied protein degradation by using cycloheximide (CHX) treatment to block new protein synthesis and following protein levels over a time-course. GSK3 inhibition did not alter the stability of any of the proteins investigated in cells grown in the presence of 2μM 1m or in GSK-3 DKO compared to WT ESCs in medium containing serum (Fig 4.10 (i), 4.11 (i)) or in serum-free media (Fig. 4.12 (i), 4.13 (i)). Antibodies signals were quantified and normalised to GAPDH in order to estimate half-life. Figures 4.12 (ii) and 4.13 (ii) show the average of 2 or 3 experiments, whereas the Figure 4.10 and 4.11 shows only one experiment because of technical problems with reprobing including uneven stripping and photo-bleaching of the reprobe.

Nanog was the transcription factor with the shortest half-life, of approximately 1 hour, in serum with or without 2μM 1m (Fig 4.10 (ii)) and serum-free conditions with or without 2μM 1m and in WT and DKO cells (Fig 4.12 (ii), Fig 4.13 (ii)). On the other hand, the estimated Nanog half-life in WT and GSK-3 DKO cells in serum from one experiment was around 2 hours (Fig 4.11 (ii)). The samples from the WT and GSK-3 DKO experiment where the reprobe did not work should be run again. In brief, GSK-3 inhibition or DKO does not alter Nanog protein stability in serum or serum-free conditions and Nanog protein has a short half-life of between 1 and 2 hours.

Tbx3 and Oct4 proteins had estimated half-lives of about 3 and more than 6 hours respectively in serum (Fig. 4.10 (ii), Fig 4.11 (ii)) and in serum-free media (Fig 4.12 (ii), Fig 4.13 (ii)).

Zscan4 protein had an estimated half-life of around 3-6 hours in serum (Fig 4.10, Fig 4.11) and 3 hours in serum-free media (Fig 4.12 (ii), Fig 4.13 (ii)). Finally, c-Myc protein stability was roughly 4 hours in serum-free conditions (Fig 4.12 (ii) and Fig 4.13 (ii)), and about 2-3 hours in serum+1m (Fig 4.10 (ii)). c-Myc protein stability in DKO cells in serum was not investigated.

In conclusion, GSK-3 inhibition or DKO does not dramatically seem to affect stability of any of the protein studied in serum or serum-free media.

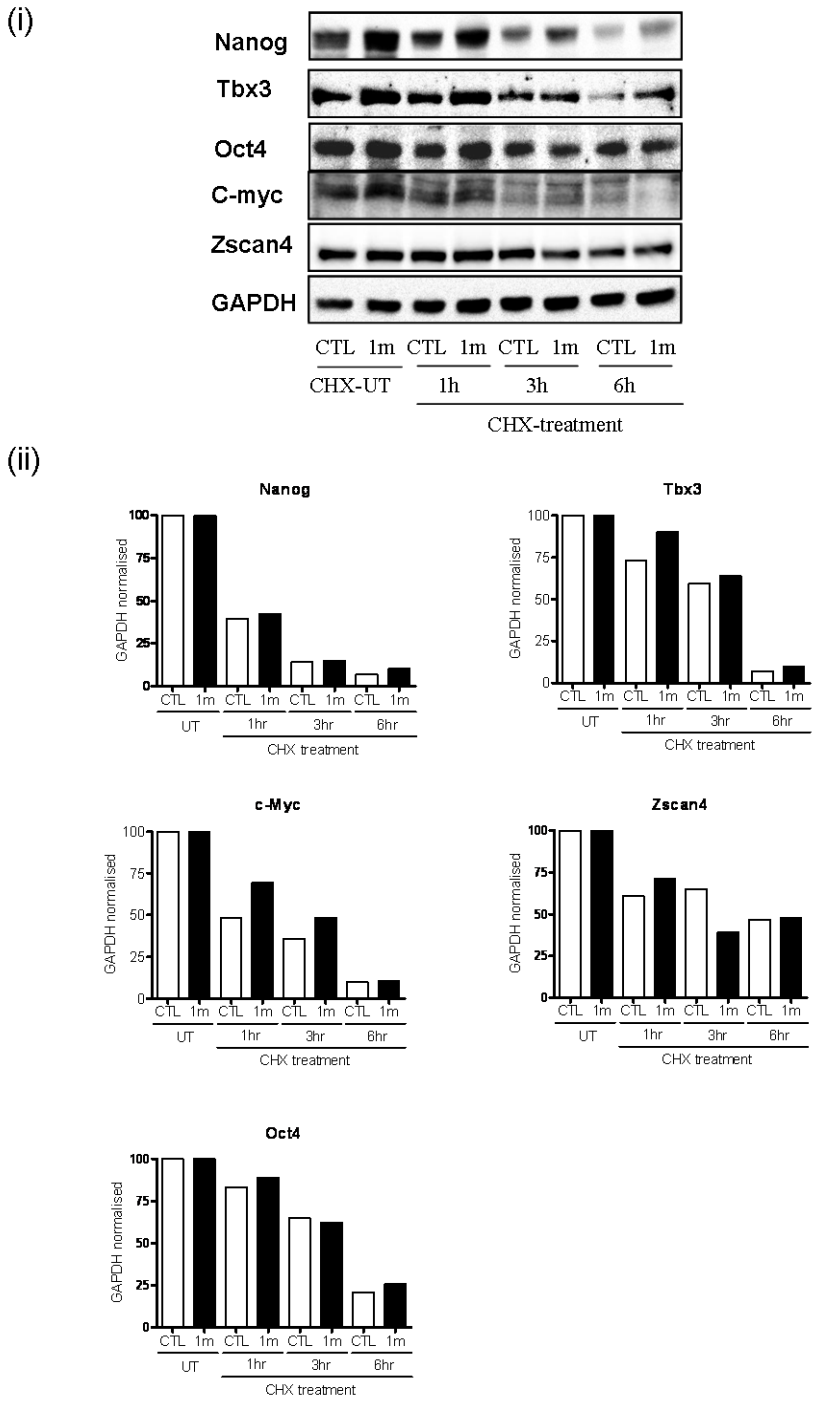

Figure 4.10. GSK-3 inhibition does not alter protein stability of Nanog, Tbx3, c-Myc, Oct4 and Zscan4 in serum conditions. mESCs (CTL) or preincubated with 2μM 1m for 24 hours grown in the presence of LIF and Serum were incubated with Cycloheximide (CHX) to halt protein synthesis. Protein samples were extracted after 1, 3 and 6 hours CHX treatment and from CHX-Untreated samples, and immunoblotting performed with the indicated antibodies (i). A value of 100 was given to the untreated samples and protein levels normalised to GAPDH (loading control) (ii). The experiment was performed twice and results shown are representative.

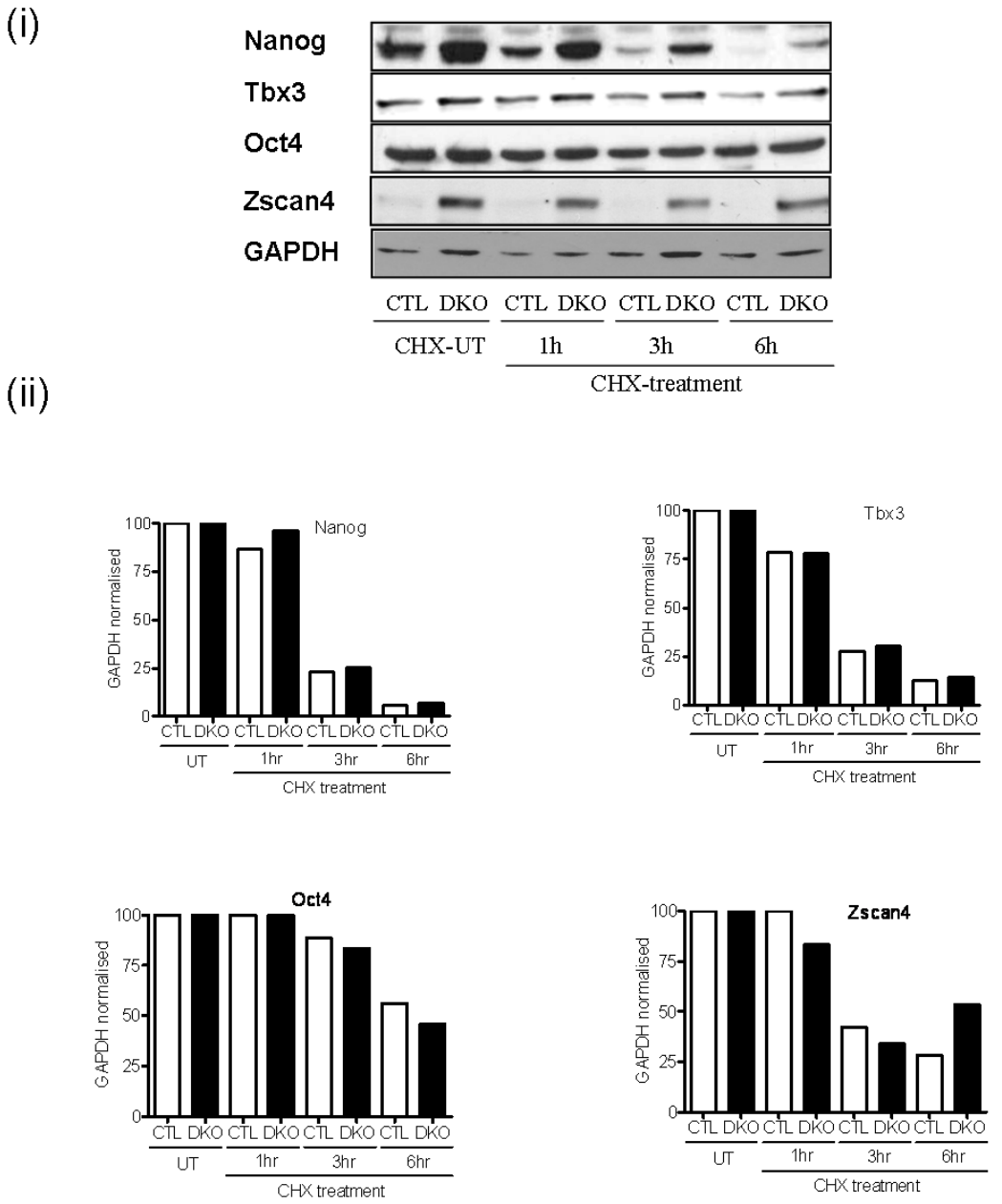

Figure 4.11. GSK-3 KO does not alter Nanog, Tbx3, Oct4, c-Myc or Zscan4 protein stability in serum-containing conditions. WT and GSK-3 DKO ESCs grown in the presence of LIF and Serum were incubated with Cycloheximide (CHX) to stop protein synthesis. Protein samples were extracted after 1, 3 and 6 hours CHX treatment and from CHX-Untreated samples, and immunoblotting performed with the indicated antibodies (loading control) (i). A value of 100 was given to the untreated samples and protein levels normalised to GAPDH (ii). The experiment was performed three times and data shown is representative.

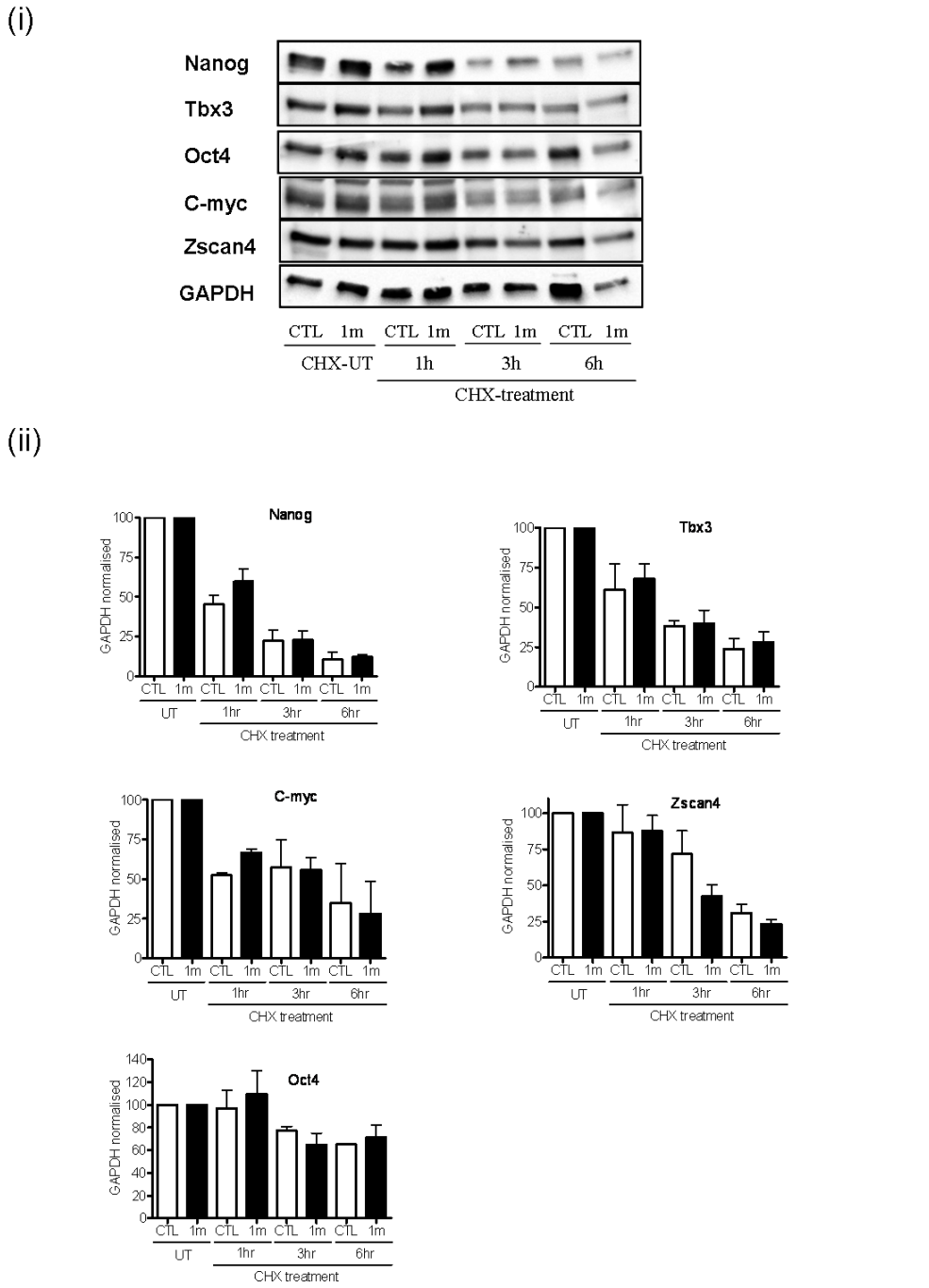

Figure 4.12. GSK-3 inhibition does not alter protein stability of Nanog, Tbx3, c-Myc, Oct4 and Zscan4 in serum-free conditions. mESCs (CTL) or preincubated with 2μM 1m for 24 hours grown in N2B27 plus LIF and BMP4 were incubated with Cycloheximide (CHX) to stop protein synthesis. Protein samples were extracted after 1, 3 and 6 hours CHX treatment and from CHX-Untreated samples, and immunoblotting performed with the indicated antibodies (i). A value of 100 was given to the untreated samples and protein levels normalised to GAPDH (loading control) (ii). The graphs show the average and S.E.M of triplicate experiments.

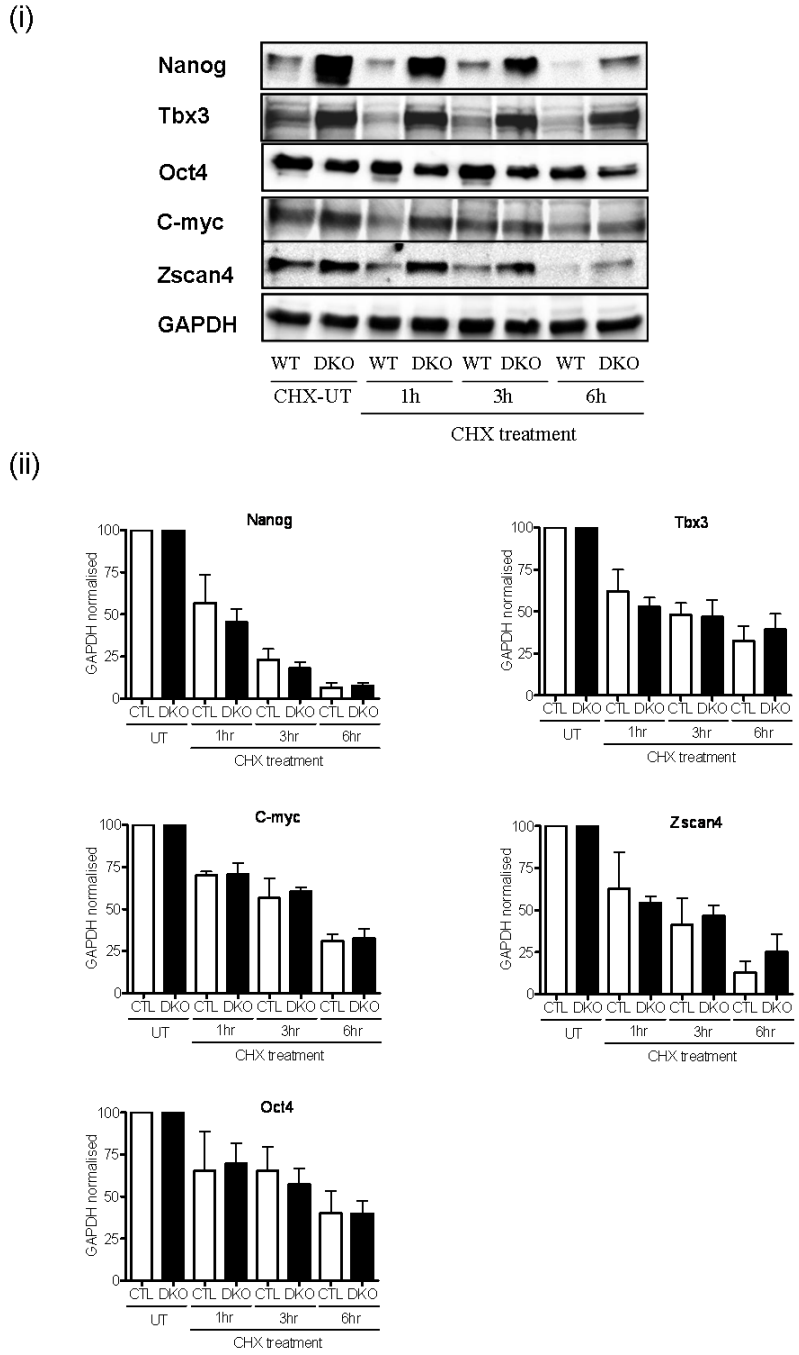

Figure 4.13. GSK-3 DKO does not alter Nanog, Tbx3, Oct4, c-myc or Zscan4 protein stability in serum-free conditions. WT and GSK-3 DKO ESCs grown in the presence of N2B27 plus LIF and BMP4 were incubated with Cycloheximide (CHX) to stop protein synthesis. Protein samples were extracted after 1, 3 and 6 hours CHX treatment and from CHX-Untreated samples, and immunoblotting performed with the indicated antibodies (i). A value of 100 was given to the untreated samples and protein levels normalised to GAPDH (loading control) (ii). The data are the average and S.E.M of triplicate experiments.

4.5 GSK-3 - a possible role in regulating translation of Tbx3 and Nanog.

Results from Section 4.1 suggested that additional mechanisms, in addition to transcriptional regulation, could account for the increased protein levels of pluripotency transcription factors, including Nanog and Tbx3, observed upon inhibition of GSK-3. In the previous Section, we investigated whether GSK-3 inhibition had any effect on protein stability, but no effects were observed. GSK-3 is known to control factors that regulate protein synthesis (Welsh et al., 1998; Welsh et al., 1997) and it could be possible that GSK-3 inhibition contributes to self-renewal by controlling protein translation.

Regulation of translation is known to be important in early development and differentiation (Mathews et al., 2000), where it can play a part in proteome constitution by fine tuning gene expression. Translational control allows for a quicker response than transcriptional control since mRNA does not need to be synthesised, processed or transported (Weyrich et al., 1998). Regulation of translation has been recently reported as a possible mechanism that controls stem cell fate (Sampath et al., 2008). Moreover, we have previously observed that Nanog protein down-regulation precedes decreases in *Nanog* RNA when ESCs are treated with the broad spectrum PI3K inhibitor LY294002 (Storm et al., 2007). These data suggests that Nanog, and possibly other transcription factors, may be regulated at the level of translational. To investigate this possibility we performed protein re-synthesis experiments and investigated mRNA translational state of *Nanog, Tbx3, Zscan4* and *C-myc* in order to investigate a possible role for GSK-3 in *de novo* protein synthesis.

4.5.1 Protein resynthesis experiments.

Protein resynthesis experiments were performed in E14tg2a cells and WT and GSK-3 DKO cells in either serum-containing or serum-free media. The experimental design was as follows, protein synthesis was halted by addition of CHX, and protein re-synthesis initiated by removing CHX after 4hours, washing ESCs extensively and adding back fresh medium, containing inhibitors to E14tg2a cells, or without inhibitors to DKO cells.

4.5.1.1 GSK-3 inhibition accelerates recovery of Nanog protein.

We initially investigated protein resynthesis of Nanog and Tbx3 in E14tg2a cells using GSK-3 inhibitors. Degradation of Nanog and Tbx3 were observed after 4 hours of CHX treatment (Fig 4.14 (i)). Nanog protein recovery was observed after 8 hours in control as well as samples grown in 2μM BIO or 1m in media containing serum. Interestingly, Nanog recovery was quicker in samples incubated with GSK3 inhibitors. Tbx3 recovery can also be observed after 8 hours of CHX wash-out in both control and samples treated with GSK-3 inhibitors with a modestly higher recovery in samples treated with 1m or BIO (Fig 4.14 (i)). RNA was also extracted to investigate whether the increase in Nanog protein correlated with an increase in *Nanog* mRNA (Fig 4.14 (ii)). Interestingly, despite of a considerable increase in Nanog protein recovery in samples treated with 1m or BIO as early as 8 hours after CHX washout, *Nanog* mRNA levels did not significantly increase in comparison with control (without inhibitors), suggesting that accelerated Nanog protein resynthesis in samples with 1m or BIO may be due to at least partly to a different mechanism than transcription, potentially an increase in translation. Consistent with increased Nanog and Tbx3 protein, colonies showed a more compact and self-renewing morphology when grown with GSK-3 inhibitors. Compaction of the colonies is evident after 16 hours of GSK-3 inhibition (Fig. 4.14 (iii)). This experiment was repeated three times but Tbx3 resynthesis was only investigated in one experiment.

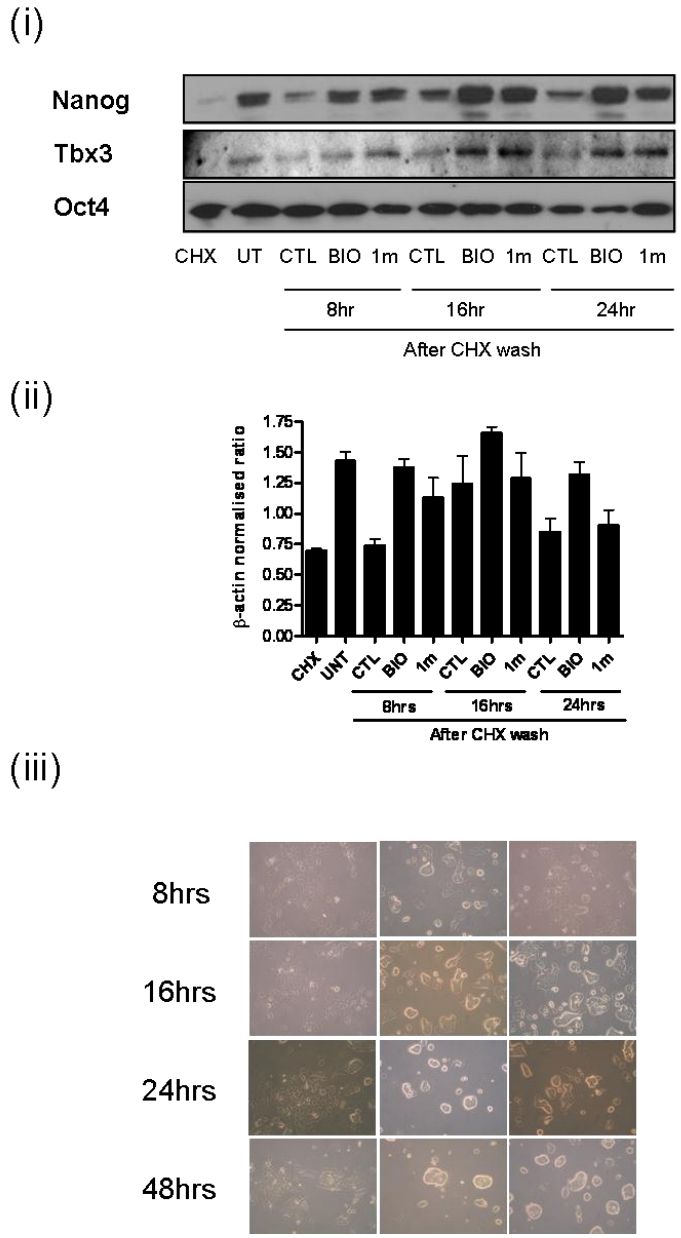

Figure 4.14 GSK-3 inhibition increases Nanog and Tbx3 protein synthesis in serum. E14tg2a mESCs were incubated with CHX for 4 hours to halt protein synthesis, CHX was then washed out and fresh media with serum and LIF supplemented with either 2μM BIO or 1m added back. UT: CHX-untreated. Protein (i), RNA (ii) and images (iii) were taken at the times indicated after CHX washing. (i) Immunoblotting was performed with the antibodies indicated (ii), quantitative RT-PCR was carried out and *Nanog* expression normalized relative to β-actin levels. The data are the average and S.E.M of quadruplicate samples. No significant differences observed between variations. Two-way anova, Bonferroni posttests.A value of 1 was given to untreated samples in (ii). Bright field microscopy images (iii). This experiment was performed 3 times and results shown are representative with the exception of Tbx3 that was only studied in one experiment.

4.5.1.2 Nanog and Zscan4 protein resynthesis is quicker in GSK-3 DKO cells.

The same experimental approach, described in the previous Section (4.4.1.1) using CHX to stop protein synthesis, was employed to investigate protein resynthesis of Nanog, Tbx3, Zscan4 and c-Myc in WT and GSK-3 DKO cells.

Nanog protein recovery can be observed as early as 1-2 hours after CHX washout in DKO cells, whereas Nanog recovery is not observed in the WT cells even after 4 hours (Fig 4.15 (i), (ii)). *Nanog* mRNA decreased after CHX treatment and it did not start increasing until 4 hours of CHX wash-out (Fig 4.15 (iii)). Thus, remarkably, Nanog protein re-synthesis in cells grown with 1m or in DKO cells occurs without measurable increases in *Nanog* mRNA levels suggesting that GSK-3 may regulate the translation of *Nanog* mRNAs. This experiment was also performed in serum-free media and the results were similar (Fig. 4.16). Nanog protein recovery was evident 2 hours after CHX wash-out, whereas Nanog mRNA did not increase in comparison with CHX-treated (Fig 4.16).

Tbx3 protein re-synthesis also occurred after CHX wash-out in media containing serum and serum-free media, re-synthesis rate seemed to be similar in WT and DKO cells in both media conditions (Fig 4.15 (i), (ii), Fig 4.16 (i), (ii)) suggesting that GSK-3 does not regulate Tbx3 protein synthesis. However, this experiment was initially optimised for investigating Nanog protein resynthesis, which has a shorter half-life than Tbx3 and Nanog protein is considerably reduced after 4 hours CHX treatment making it easy to study its recovery after CHX wash-out. On the other hand, Tbx3 protein has a longer half-life, so its levels are not reduced to such a significant extent after 4 hours of CHX treatment and thus the window to look at protein resynthesis is smaller. Moreover, preliminary results, already discussed in Section 4.4.1.1 (Fig. 4.14 (i)), suggest that Tbx3 may be resynthesised quicker when GSK-3 is inhibited. Further analysis of Tbx3 protein recovery in cells treated with the inhibitors should be performed in order to elucidate whether GSK-3 controls Tbx3 protein synthesis, using conditions optimised for examination of Tbx3 protein such as longer CHX treatment to further decrease protein or using radioisotopes.

Zscan4 protein resynthesis also seems to be accelerated in DKO cells compared to WT in serum conditions (Fig 4.15 (i) (ii)). It would be interesting to investigate the dynamics of *Zscan4* mRNA following CHX wash-out because it may be regulated at translational level.

c-Myc protein re-synthesis seemed to be slower in GSK-3 DKO cells compared to WT (Fig 4.15 (i), (ii)).

In summary, Nanog protein resynthesis seems to be accelerated when GSK-3 is inhibited or in GSK-3 DKO cells. Furthermore, the early increase in protein levels observed, does not seem to be due to corresponding increases in RNA. Zscan4 protein resynthesis also seems to be quicker in GSK-3 DKO cells.

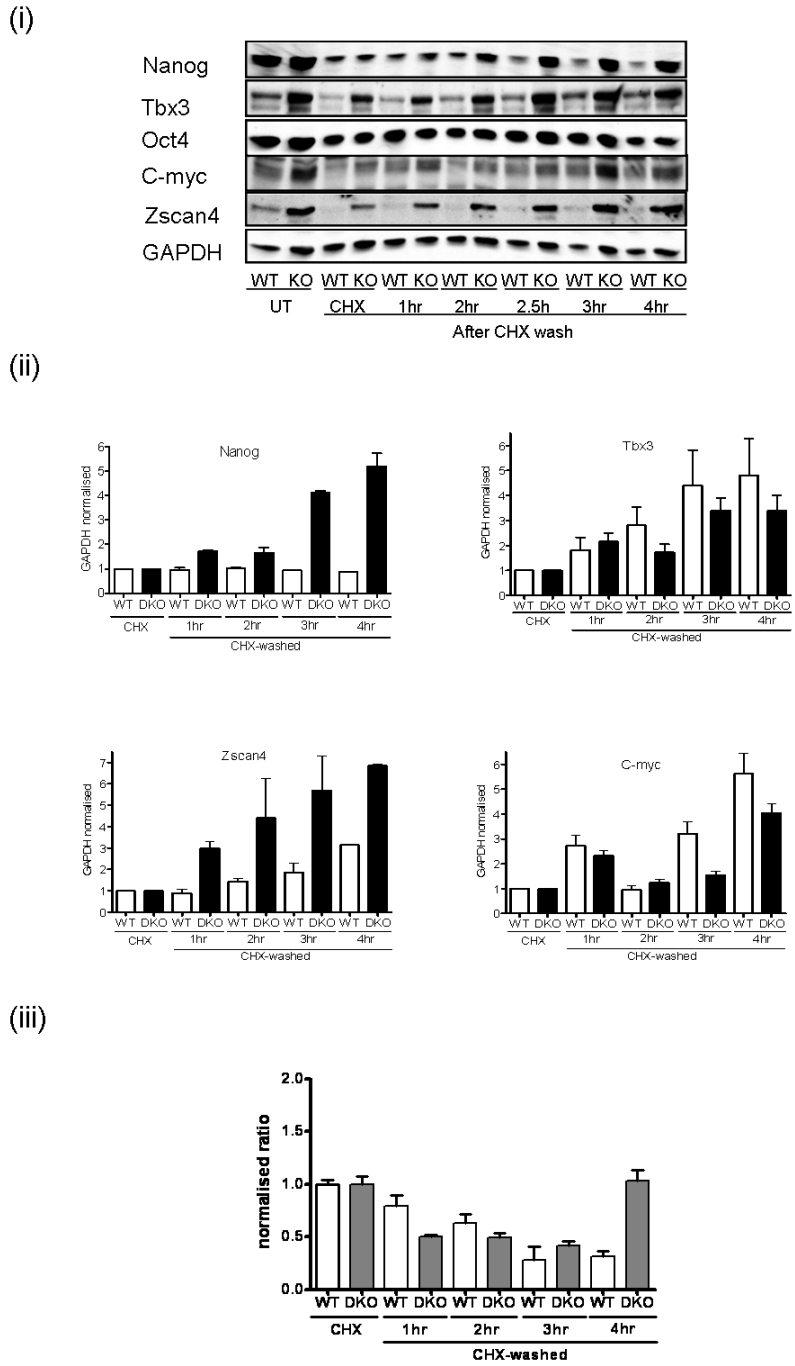

Figure 4.15 GSK-3 DKO accelerates Nanog and Zscan4 protein synthesis in serum-containing media. WT and GSK-3 DKO mESCs were incubated with CHX for 4 hours to stop protein synthesis, CHX was then washed out and fresh media with serum and LIF added back. Protein and RNA samples were taken at the times indicated after CHX washing. (i) Immunoblotting was performed with the antibodies indicated. (ii) A value of 1 was given to CHX treated and samples normalised to GAPDH (loading control). The data are the average and S.D of duplicate experiments. (iii), quantitative RT-PCR was carried out and Nanog expression normalized relative to β-actin levels. The data are the average and S.E.M of quadruplicate samples. A value of 1 was given to CHX treated samples.

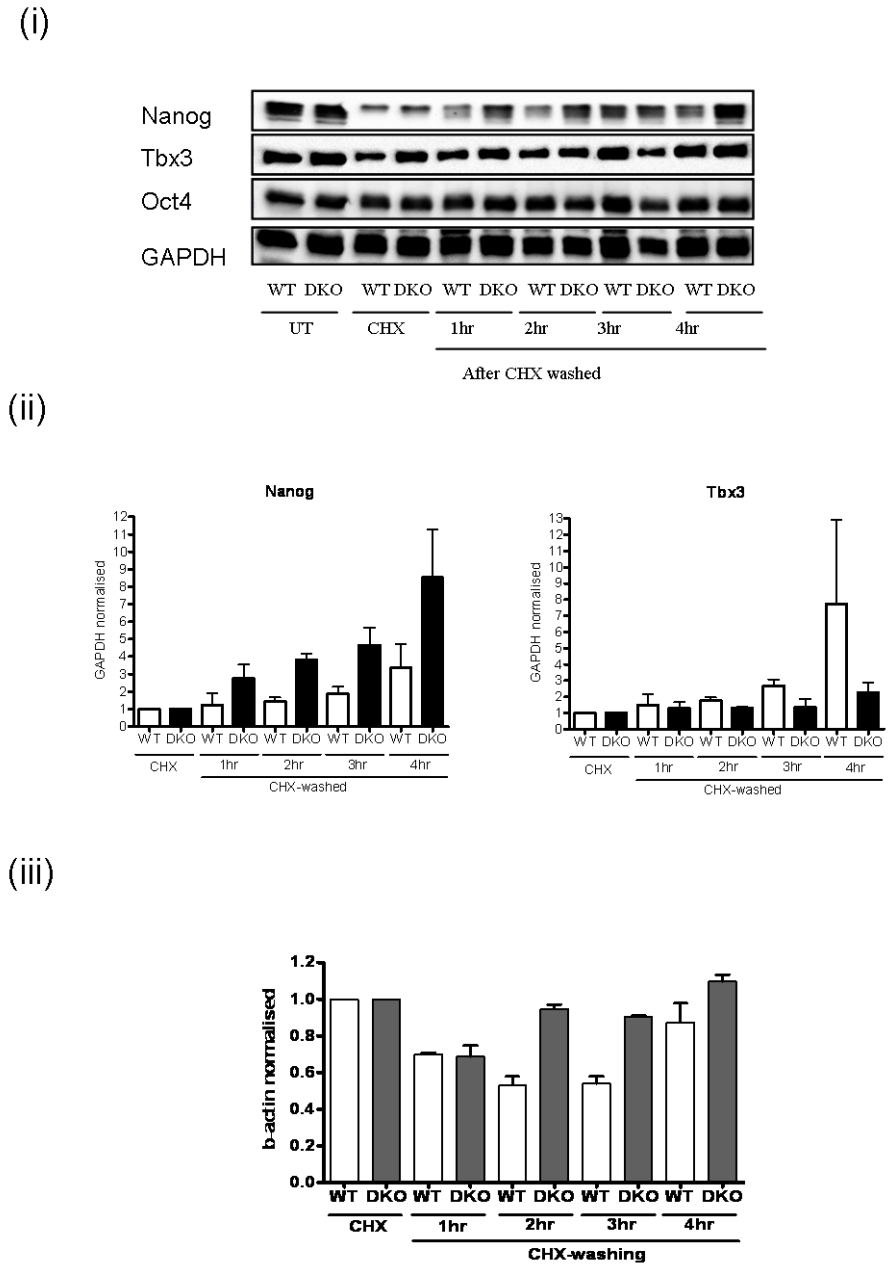

Figure 4.16 GSK-3 DKO accelerates Nanog protein synthesis in serum-free media. WT and GSK-3 DKO mESCs were incubated with CHX for 4 hours to stop protein synthesis, CHX was then washed out and fresh media with serum and LIF added back. Protein and RNA samples were taken at the time indicated after CHX washing. (i) Immunoblotting was performed with the antibodies indicated. (ii) A value of 1 was given to CHX treated and samples normalised to GAPDH (loading control). The data are the average and S.D of duplicate experiments. (iii), quantitative RT-PCR was carried out and *Nanog* expression normalized relative to β–actin levels. The data are the average and S.E.M of quadruplicate samples. A value of 1 was given to CHX treated samples.

4.5.2 GSK-3 inhibition increases translation state of Nanog, Tbx3 and Zscan4.

In the previous Section Nanog protein recovery was shown to be accelerated in GSK-3 DKO cells (Fig 4.15, Fig 4.16) or in cells grown in GSK-3 inhibitors (Fig 4.14) and Zscan4 protein recovery is also quicker in DKO cells in serum conditions (Fig 4.15). Moreover, Nanog protein recovery occurs without a previous increase in the mRNA levels (Fig 4.15 (iii)) suggesting that other mechanisms, apart from transcription may account for the increase in Nanog recovery when GSK-3 is inhibited. Previous data, shown in Section 4.2.1 and 4.2.2, also suggest that Nanog expression can be regulated by additional mechanisms. Although Nanog protein levels were elevated following 6-8 hours of GSK-3 inhibition, increases in *Nanog* mRNA were modest compared with that of the protein. The aim of this part of the study was to investigate a possible role of GSK-3 in regulating translation of Nanog, and other pluripotent markers, by studying changes in the rate at which these transcripts are translated after treatment with GSK-3 inhibitors and in GSK-3 DKO cells. This can be investigated by studying the mRNA levels of the gene of interest bound to polysomes. A molecule of mRNA that is being actively translated has several ribosomes attached, this is referred to as polysomal RNA. Polysomal-enriched fractions of RNA can be obtained by loading cell lysates (containing the mRNA) onto a sucrose gradient, followed by centrifugation. Briefly, cell lysates are loaded into a 10-50% sucrose gradient, ultracentifruge and fractions collected. After centrifugation, RNA is distributed in the sucrose gradient according to their weight, therefore, polysomal-bound RNA is heavier and it will be found in the bottom layers of the sucrose column whereas monosomes (single ribosomes) are found at the top layers. The absorbance at 260nm of each of the fraction was measured and fractions enriched in polysomes or monosomes were pooled (Fig. 4.17).

Initially the proportion of mRNA bound to polysome after 8 and 24 hours of GSK-3 inhibition with 1m and in DKO cells was investigated. Results suggested that the proportion of *Nanog* mRNA bound to polysomes is higher after 8 and 24 hours of GSK-3 inhibition or in DKO cells (Fig. 4.18).

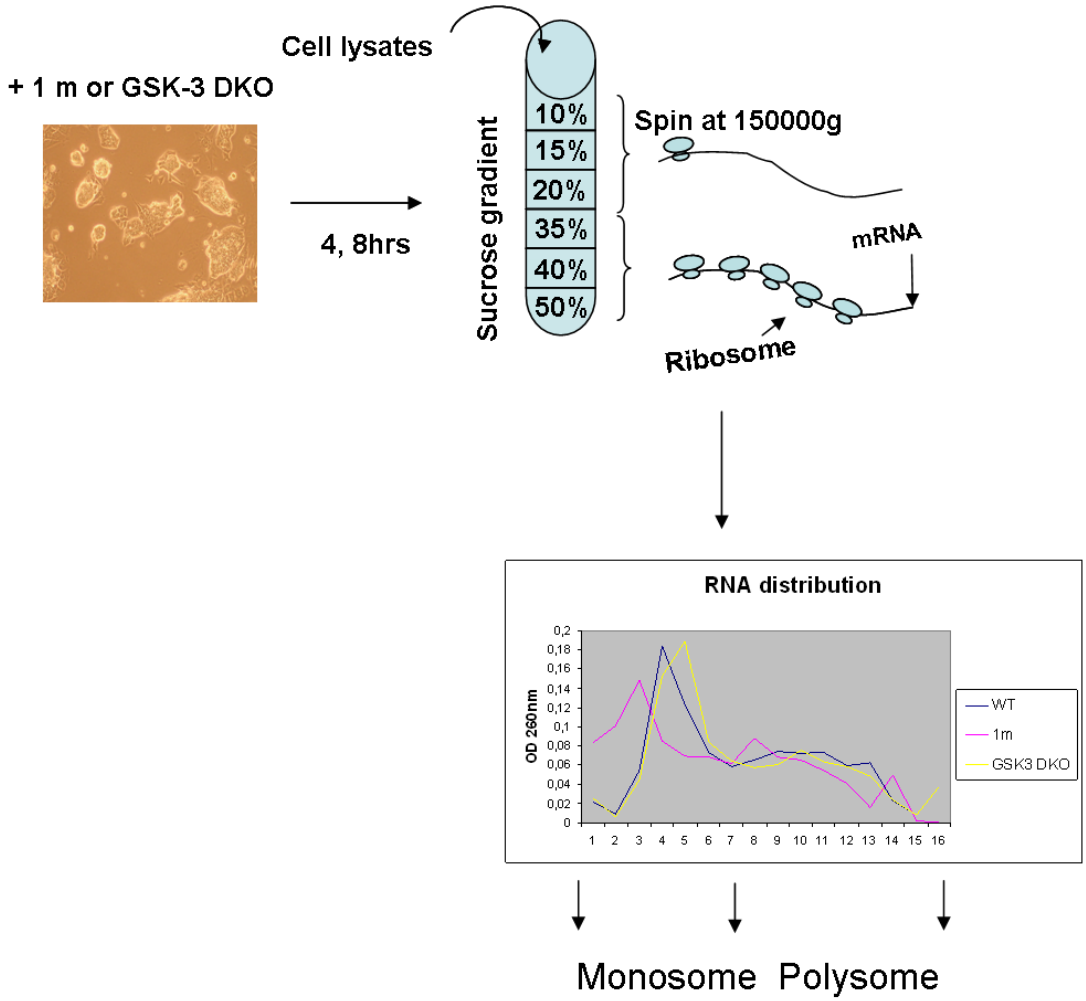

Fig. 4.17 Separation of polysome-enriched RNA fractions. E14tg2a wild-type (WT) and GSK-3α/β double knockout (DKO) mESCs were cultured in the presence of serum and LIF. The GSK-3 inhibitor 1m was added to WT cells at 2 μM and cells lysed after 4 and 8 hours of 1m addition. Cell lysates were loaded into a 10-50% sucrose gradient and ultracentrifuge at 150.000g for 1 hour and a half. After centrifugation, RNA was distributed in the sucrose gradient according to their weight. Polysomal-bound RNA is heavier and it was found in the bottom layers of the sucrose column whereas monosomes were found at the top layers. The absorbance at 260nm of each of the fraction was measured and fractions enriched in polysomes or monosomes were pooled. The graph shows the RNA distribution of polysome and monosomes. The experiment was performed three times and the graph shown is representative.

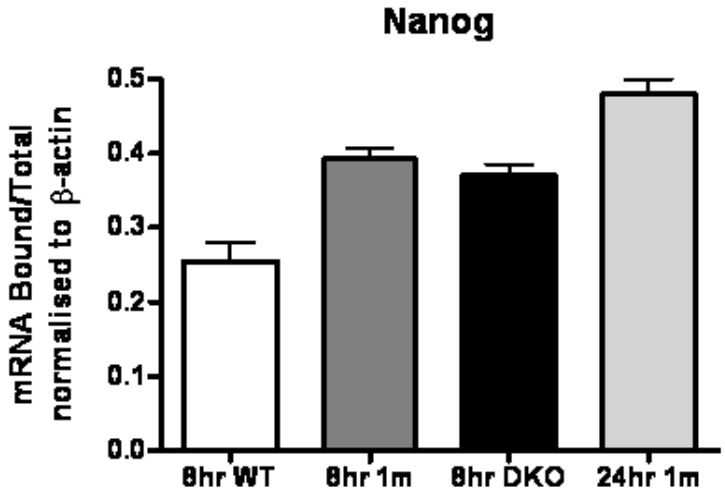

Figure 4.18. The proportion of *Nanog* mRNA bound to polysomes is higher following GSK3 inhibition and in DKO GSK-3 cells. WT cells grown in the presence of 2μM 1m and DKO cells were cultured in serum supplemented with LIF before extracting cell lysates at the time indicated. Cell lysates were run through a sucrose gradient to separate the polysomal-enriched fraction. The levels of mRNA bound to the polysome were investigated by quantitative PCR. Gene expression was normalized relative to β-actin levels. Values show the proportion of mRNA bound to the polysome fraction (Bound/Total mRNA). The data are the average and S.D of one experimen run in duplicate.

It was next examined whether the proportion of *Nanog* mRNA bound to polysomes was also higher after 4 hours of initiation of GSK-3 inhibition, as well as after 8 and 24 hours. There were technical problems while isolating the RNA from the 24 hours time point so reliable data was not obtained. Results from three independent experiments are plotted in Figure 4.19. The proportion of *Nanog* mRNA bound to polysomes was increased in cells grown in the presence of 1m for 4 and 8 hours. Changes in the proportion of mRNA bound to polysomes of other genes including *Tbx3, c-Myc, Oct4* and *Cyclin D1* was also investigated after 4 and 8 hours (Fig 4.19) and *Zscan4* and *β-catenin* only after 8 hours (Fig. 4.20). The proportion of *Nanog* and *Tbx3* mRNA bound to polysomes showed a significant increase, of approximately 30% and 40-50% respectively, following 4 and 8 hours of initiation of treatment with 2μM 1m and in GSK-3 DKO cells (Fig. 4.19). On the other hand, although the proportion of *c-Myc* mRNA bound to polysomes did not change

following 4 or 8 hours of GSK-3 inhibition, a significant increase in polysomally bound was observed in GSK-3 DKO cells (Fig. 4.19). This suggests that a period of GSK-3 inhibition longer than 8 hours is required in order to increase the proportion of *c-Myc* mRNA bound to polysomes and thus the early changes in c-Myc protein previously observed (Fig 4.2) may not be due to an increase in *c-Myc* translation. Finally, the polysomal distribution of *Oct4* and *Cyclin D1* mRNA did not change in cells grown in 1m for 4 and 8 hours or in GSK-3 DKO cells (Fig. 4.19).

The proportion of *Zscan4* and *β-catenin* mRNA bound to polysome, which was only investigated after 8 hours, also showed a significant increase of approximately 45 and 40% respectively in cells grown in 1m or in GSK-3 DKO cells (Fig.4.20).

The data shown in Figure 4.19 suggest that GSK-3 inhibition leads to effects on the translation of *Nanog* and *Tbx3* as early as 4 hours following GSK-3 inhibition. This early increase in the proportion of *Nanog* and *Tbx3* mRNA bound to polysomes after GSK-3 inhibition suggests that an elevation in their rate of translation may account for the early increases observed in their protein levels, which are not be entirely explained by changes in mRNA levels. The ability of GSK-3 to regulate *β-catenin* and *Zscan4* translation state after 8 hours of inhibition has also be shown (Fig. 4.20) and it would be interesting to investigate a possible increase in the proportion of their mRNA bound to polysome after 4 hours of 1m treatment.

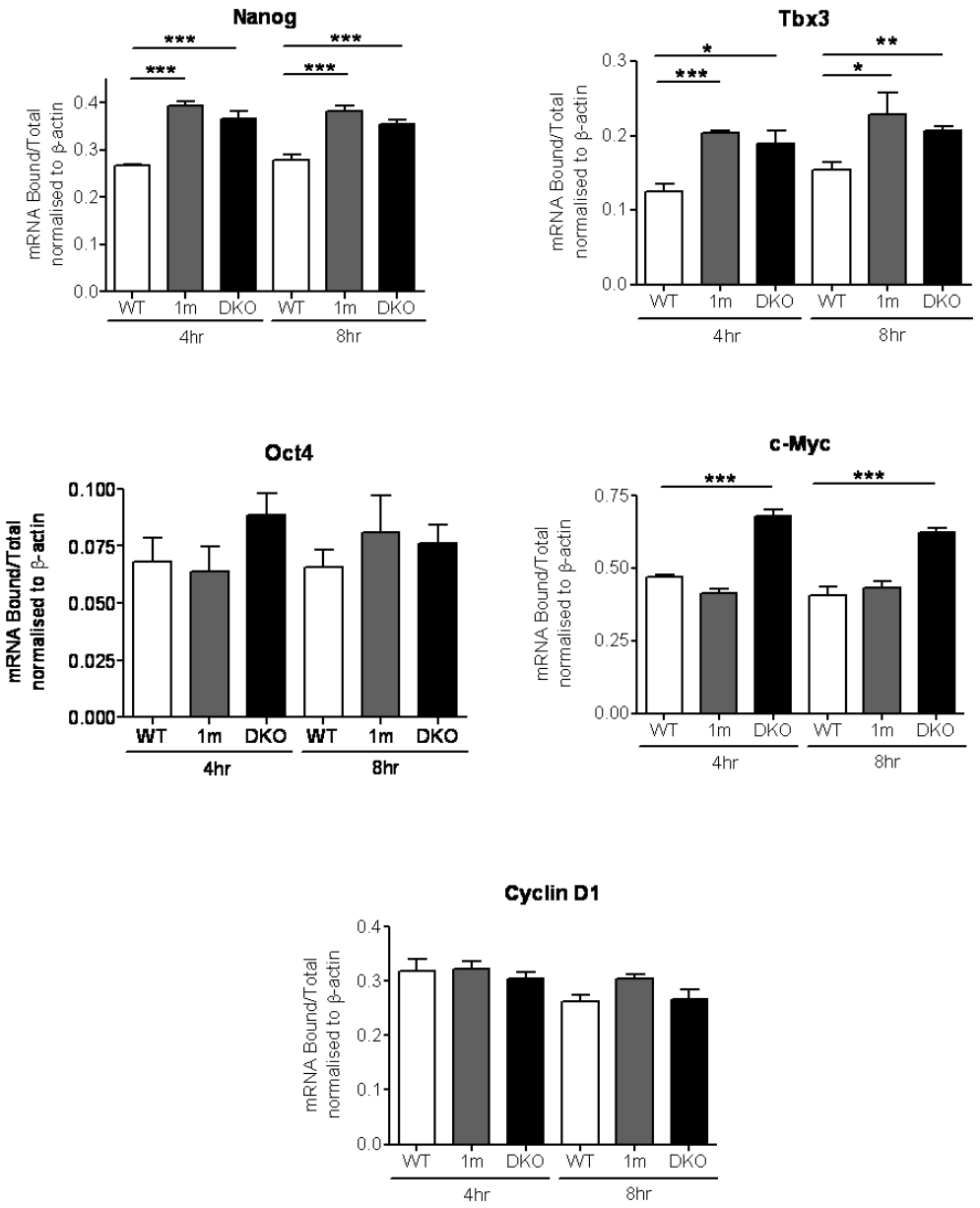

Figure 4.19. ***Nanog*** **and** ***Tbx3*** **mRNA translation are increased following GSK-3 inhibition and in DKO GSK-3 cells.** WT cells grown in the presence of 2μM 1m and DKO cells were cultured in serum supplemented with LIF for 4 and 8 hours before extracting cell lysates. Cell lysates were run through a sucrose gradient to separate the polysomal-enriched fractions from the monosomal fractions. The levels of mRNA bound to polysome or monosome were investigated by quantitative PCR. Gene expression was normalized relative to β-actin levels. Values show the proportion of mRNA bound to the polysome fraction (Bound/Total mRNA). The data are the average and S.E.M of three independent experiments run in duplicate for the 8 hours time point and the average and S.E.M of two independent experiments run in duplicated for the 4 hour time point. *, $<p$ 0.05; **, $p<0.01$, $p<0.005$. Student T-test.

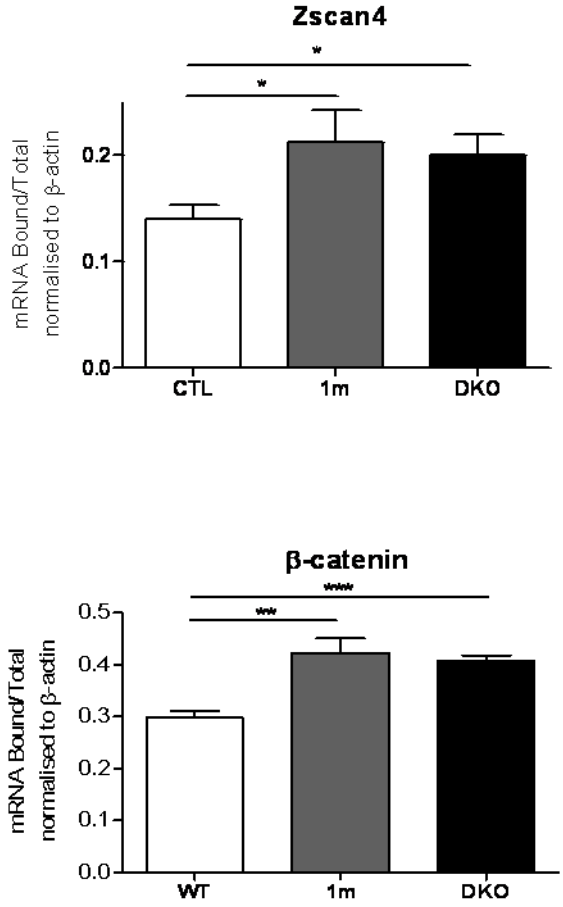

Figure 4.20. *Zscan4* and *β-catenin* mRNA translation are increased following GSK-3 inhibition and in DKO GSK-3 cells. WT cells grown in the presence of 2μM 1m and DKO cells were cultured in serum supplemented with LIF for 8 hours before extracting cell lysates. Cell lysates were run through a sucrose gradient to separate the polysomal-enriched fraction. The levels of mRNA bound to polysome and monosome were investigated by quantitative PCR. Gene expression was normalized relative to β-actin levels. Values show the proportion of mRNA bound to the polysome fraction (Bound/Total mRNA). The data are the average and S.E.M of three independent experiments run in duplicate. *, $<p$ 0.05; **, p<0.01, *** p<0.005. Student T-test.

4.6 Discussion

Several reports agree that GSK-3 inhibition can contribute to maintenance of ESC self-renewal. Despite the fact that GSK-3 has many downstream effectors, most studies to date suggest that the effect observed upon inhibition of GSK-3 is at least partly mediated through Wnt/β-catenin-dependent signalling. Indeed, several mechanisms of action of β-catenin have been recently proposed (Yi et al., 2011; Wray et al., 2011; Kelly et al., 2011), two of them agree that the major mechanism of GSK-3 inhibition is β-catenin stabilisation and interaction with Tcf3 abrogating its repressing activity in the pluripotency network. However, they also agreed in that Tcf-independent mechanisms can have a small contribution in the effect of GSK-3 inhibition/Wnt activation (Yi et al., 2011; Wray et al., 2011). For example, addition of CHIR to PD plus LIF increased the number of undifferentiated colonies formed from Tcf-3 null cells that express Tcf3-WT or Tcf3-ΔN cells, which lack the β-catenin interacting domain (Wray et al., 2011). If the only effect of GSK-3 inhibition was to abrogate Tcf3 repression, addition of CHIR to Tcf3-ΔN cells would not increase colony formation. Moreover, the number of alkaline phosphatase positive colonies generated in response to Wnt3a in Tcf3-ΔN cells is reduced but not eliminated and Wnt3a also increase the number of alkaline phosphatase positive colonies when added to Tcf3 null cells suggesting Tcf3-independent mechanism. Although Tcf1 was shown to mediate Wnt/β-catenin activation, β-catenin seems to bind Tcf1 leading to activation of Wnt target genes, a small number of colonies were also produced from Tcf3-ΔN cells with Tcf1 knocked down (Yi et al., 2011). A recent report propose that the effect of Wnt signalling is mediated by a Tcf-independent mechanism by which stabilisation of β-catenin binds to Oct4 enhancing its activity (Kelly et al., 2011). However, Yi *et al.,* did not observed Oct4–β-catenin dependent recruitment to chromatin (Yi et al., 2011). Thus, it could be possible that GSK-3 inhibition acts through an alternative Tcf-independent mechanism, which could be β-catenin dependent or independent.

The three recent reports described above (Wray et al., 2011; Yi et al., 2011; Kelly et al., 2011) agree with β-catenin regulating expression of the pluripotency factor network at the transcriptional level. However, there is also evidence suggesting that β-catenin-independent mechanisms downstream of GSK-3 may also play a part in

maintaining self-renewal (Ying et al., 2008; Wray et al., 2011; Storm et al., 2007; Bechard and Dalton et al., 2009). For example, recombinant Wnt3a does not fully replicate the effects of CHIR in 2i media (Ying et al., 2008) suggesting that CHIR has broader effects than simply activating Wnt/β-catenin signalling. Moreover, colony formation from β-catenin null cells is better in 2i+LIF than in PD+LIF suggesting that β-catenin independent mechanisms downstream of GSK-3 can also contribute to the effect of CHIR on ESC self-renewal (Wray et al., 2011).

To summarise, most studies to date suggest a role for β-catenin as a mediator of the effects occurring following GSK-3 inhibition either mainly through Tcf-dependent (Wray et al., 2011; Yi et al., 2011) or independent mechanisms (Kelly et al., 2011) but there is evidence indicating that β-catenin-independent mechanisms may also contribute to the effect of GSK-3 inhibition on ESC self-renewal.

Among the mechanisms of action of GSK-3 that may be independent of β-catenin, the regulation of c-Myc and Nanog are of particular interest (Bechard and Dalton, 2009; Storm et al., 2007). Both reports proposed a mechanism involving GSK-3 downstream of PI3K. Storm *et al.,* showed that inhibition of GSK-3 can reverse the decrease in *Nanog* RNA levels and protein expression following inhibition of PI3K suggesting that PI3K regulates Nanog expression through inhibition of GSK-3 (Storm et al., 2007). Inhibition of PI3K decreases phosphorylation of S21/9 of GSK-3 but there is no significant effect on phosphorylation of β-catenin or β-catenin levels (Paling et al., 2004) suggesting that PI3K does not regulate the pool of GSK-3 involved in Wnt signalling, and thus the effect observed on ESC self-renewal following GSK-3 inhibition may be mediated by GSK-3 downstream of PI3K as well as downstream of Wnt/β-catenin signalling.

The mechanism of action of GSK-3 inhibition and Wnt activation that result in enhancement of mESC self-renewal have partly remained unclear because GSK-3 is involved in a number of signalling pathways and numerous downstream effectors have been identified in non-ESC types, including protein synthesis initiation factors, transcriptional regulators and components of the cell-division cycle.

This study aimed to investigate a possible role of GSK-3 in regulating pluripotency-associated transcription factors including Nanog, Tbx3, C-myc, Oct4 and Zscan4.

4.6.1 Effect of GSK-3 inhibition in cell proliferation in different culture conditions.

GSK-3 has been reported to contribute to maintenance of self-renewal by blocking residual neural differentiation and mainly by sustaining cell viability in chemically defined 2i media (Ying et al., 2008). However, changes in cell growth following GSK-3 inhibition in serum and LIF were previously investigated and cell growth did not seem to be affected (results not shown). Changes in cell growth in different culture conditions, including serum plus LIF, serum-free media plus LIF plus BMP4 and 2i media were investigated and the effect of the inhibitors in the cells varied depending whether there is serum in the media. Cell growth was not affected following GSK-3 inhibition in the presence of serum. In contrast, GSK-3 inhibition was shown to have a positive input, restoring cell growth, when added to cells with MEK inhibitor in serum free conditions, which is in accordance with the report of Ying *et al*., (Ying et al., 2008). We have also shown (Section 4.2.3) that GSK-3 inhibition seems to regulate Nanog and Tbx3 expression, highlighting the pleiotrophic effect of inhibiting the kinase, which is not surprising considering that GSK-3 is involved in numerous pathways and has multiple downstream effectors (Doble and Woodgett, 2003).

4.6.2 Regulation of pluripotency-associated transcription factors by GSK-3.

The possibility that some of pluripotency-associated transcription factors including Nanog, Tbx3, c-Myc, Zscan4 and Oct4 are downstream effectors of GSK-3 was tested by using small molecule inhibitors (1m, BIO and CHIR) or DKO GSK-3 cells (Doble et al., 2007).

Increases in Nanog, Tbx3 and c-Myc protein levels, as early as 6-8 hours following GSK-3 inhibition, were observed in serum plus LIF (Fig 4.2). Zscan4 protein was

elevated in GSK-3 DKO cells but not in cells grown in 1m for 24 hours (Fig 4.2) suggesting that Zscan4 is not a direct downstream effector of GSK-3 but its elevated levels in DKO cells are rather due to GSK-3 DKO cells having self-renewal enhanced compared to WT cells. Finally, the levels of Oct4 did not consistently change (Fig 4.2). GSK-3 inhibition seems to regulate Nanog and Tbx3 expression also in the absence of LIF, at least in short-term experiments (Fig 4.4, Fig 4.5). Nanog and Tbx3 proteins were also up-regulated after GSK-3 inhibition in N2B27 plus LIF and BMP4. However, in contrast to the results observed in serum-containing conditions, c-Myc and Zscan4 protein levels did not consistently change (Fig 4.6). Hence, it seems that GSK-3 can control Nanog and Tbx3 expression in both serum and serum-free conditions.

The possibility that GSK-3 can regulate Nanog and Tbx3 expression in the ground state conditions, described by Ying *et al.,* (Ying et al., 2008), was also explored. Nanog protein was elevated when GSK-3 inhibitor (CHIR) was added alone or in combination with MEK inhibitor (PD) in comparison with no inhibitor or only MEK inhibition. Tbx3 was also increased when GSK-3 inhibitor was present. Moreover, the fact that Nanog and Tbx3 levels were maintained at higher levels in cells with both inhibitors in comparison with only MEK inhibitor suggests that GSK-3 is able to maintain the levels of Nanog and Tbx3 expression in the absence of any extrinsic stimuli. However, inhibition of GSK-3 is not sufficient to maintain robust long-term self-renewal and MEK inhibitor is also necessary. These data are in agreement with the report of Ying *et al.,* (Ying et al., 2008).

The results presented here show that GSK-3 can regulate Nanog and Tbx3 expression in all culture conditions tested and so it was of considerable interest to investigate the mechanism of action by which GSK-3 regulates these changes. First, we investigated whether the elevated levels of Nanog, Tbx3 and c-Myc proteins in cells grown in the presence of 1m or in GSK-3 DKO cells in serum correlated with an increase in their mRNA levels. Although RNA levels were elevated for *Tbx3* and *Nanog*, they were relatively small increases compared with changes in levels of protein (Fig 4.3). Moreover, the fact that Nanog protein is down-regulated in DKO cells after 1 and 2 days in the absence of LIF whereas *Nanog* mRNA levels are maintained (Fig 4.4) suggests that in the presence of LIF either Nanog protein is

more stable or *Nanog* mRNA is more actively translated. On the other hand, *c-Myc* RNA levels were modestly decreased following GSK-3 inhibition (Fig 4.3), suggesting that the increase in c-Myc protein is not due to increases in transcription. Moreover, similar to what it was observed in serum, *Nanog* and *Tbx3* RNA levels were modestly increased in comparison with the increase in protein in serum-free conditions (Fig 4.7). Results from serum and serum-free media conditions indicate that other mechanisms, apart from transcriptional regulation, are likely to contribute to the increases in Nanog and Tbx3 protein levels observed. Therefore, a possible role for protein stabilisation following GSK-3 inhibition was examined.

4.6.3 GSK-3 inhibition or DKO does not change protein stability of pluripotency-associated transcription factors.

GSK-3 is known to regulate the stability of several proteins including β-catenin, c-Myc and cyclinD1 (Cartwright, 2005; Diehl et al., 1998) by phosphorylating and marking them for proteosomal degradation. Inhibition of GSK-3 leads to decrease in phosphorylation leading to protein stabilisation. Therefore, an investigation to find out whether an increase in protein stability upon GSK-3 inhibition or in GSK-3 DKO cells could contribute to the increase in protein levels observed in pluripotency-associated transcription factors was conducted (Fig. 4.2, Fig 4.6).

Protein stability of Nanog, Tbx3 and other pluripotency markers, including c-Myc, Zscan4 or Oct4 did not dramatically change when GSK-3 was inhibited or in GSK-3 DKO cells in either serum (Fig. 4.10, Fig 4.11) or serum-free media (Fig 4.12, Fig 4.13) indicating that the increase in protein observed (Fig 4.2, Fig 4.6) may be due to an alternative mechanism to protein stabilisation. The half-lives of the transcription factors studied varied, Nanog had the shortest half-life, which was between 1-2 hours whereas Oct4 with a half-life of more than 6 hours was the transcription factor with the longest half-life. Tbx3 and c-Myc had similar half-life about 3 hours and finally Zscan4 half-life was between 3-6 hours.

Most studies to date have focused in investigating transcriptional regulation of pluripotency-associated transcription factors, and how they interact with each other

to form a network. However, there are no studies about protein turnover, which is also dynamic and of great relevance because this can also influence the transcription factor network.

4.6.4 GSK-3-a possible regulator of translation.

Regulation of translation plays a key role in early development and differentiation (Mathews et al., 2000) and it has been recently reported as a possible mechanism that can control stem cell fate (Sampath et al., 2008). GSK-3 can inhibit protein synthesis in eukaryotes through phosphorylation of the eukaryotic protein synthesis initiation factor 2B (eIF2B) (Welsh et al., 1998), which is critical for initiation of translation. Therefore, inhibition of GSK-3 would lead to an increase in general translation. Furthermore, Storm *et al.,* observed that Nanog protein is downregulated earlier than *Nanog* RNA when cells are treated with the PI3K inhibitor LY294002 and PI3K is known to regulate GSK-3 (Storm et al., 2007). Inhibition of PI3K leads to activation of GSK-3 and maybe to a subsequent phosphorylation of eIF2B, this would explain the decrease in Nanog protein before its RNA. A possible role of GSK-3 in controlling translation of *Nanog* and other transcription factors including *Tbx3* and *Zscan4* was investigated by performing protein recovery experiments and by looking at the translation state of their mRNAs.

The results obtained with Tbx3 were not conclusive, as preliminary data suggest that Tbx3 protein resynthesis is accelerated when GSK-3 is inhibited and Tbx3 seems to be more actively translated. However, results using DKO cells suggested that protein resynthesis is not quicker in DKO cells. One consideration is that the protein resynthesis experiments were optimised initially to investigate Nanog protein recovery and our data then demonstrated that Tbx3 has a longer half-life than Nanog. Further analysis of Tbx3 protein recovery in cells treated with the inhibitors should be performed in order to elucidate whether GSK-3 controls Tbx3 protein synthesis. One way to study Tbx3 protein recovery would be to optimise the protein resynthesis experiments for Tbx3, for example by longer CHX treatment in order to reduce its protein prior CHX washed-out. Alternatively, Tbx3 protein synthesis following GSK-3 inhibition could be investigated using radioisotopes.

Zscan4 protein resynthesis was accelerated in DKO cells and its translational state is also increased after 8 hours of GSK-3 inhibition. Finally, Nanog protein recovery was faster in ESC treated with GSK-3 inhibitors and in DKO cells. Moreover, Nanog protein recovery in GSK-3 DKO cells occurred without an increase in *Nanog* mRNA suggesting that GSK-3 may regulate Nanog protein resynthesis by an alternative mechanism to transcription, possibly translation. This is further supported by the fact that the proportion of *Nanog* RNA bound to polysome is higher after 4 and 8 hours of GSK-3 inhibition and in GSK-3 DKO cells, indicating increase translation. In summary, these data suggest that GSK-3 can regulate *Nanog* and maybe also *Zscan4* and *Tbx3* translation. However, further experiments are needed to test whether GSK-3 can regulate *Zscan4* and *Tbx3* translation. In the case of Tbx3, as mentioned above, protein resynthesis experiments optimise for Tbx3 should be performed. Moreover, the dynamics of *Tbx3* mRNA in these experiments should also be investigated in order to study whether Tbx3 protein recovery can take place without a previous increase in *Tbx3* mRNA. Finally, *Zscan4* mRNA dynamics should also be investigated in protein resynthesis experiments.

4.7 Summary and conclusions.

The ability of GSK-3 to regulate pluripotency-associated transcription factors was investigated. GSK-3 was shown to regulate the expression of Nanog and Tbx3 in all the culture conditions tested. Although both *Nanog* and *Tbx3* transcription can be controlled by GSK-3, the increase in transcription is modest compared with the increase in the levels of their proteins and GSK-3 seems to regulate *Nanog* also at translational level. However, further experiments are needed to test whether GSK-3 can also regulate *Tbx3* translation. GSK-3 downstream of PI3K has been reported to regulate translation by phosphorylating eIF2Bε in non-ESC types; therefore inhibition of GSK-3 may contribute to enhancement of self-renewal by regulating translation. Hence, GSK-3 inhibition could contribute to enhancement of self-renewal by a β-catenin dependent mechanism, which would involve inhibition of Tcf-3 and alleviation of its transcriptional repression of the pluripotency network (Wray et al., 2011), and by a β-catenin dependent or independent mechanism through increase in translation of specific pluripotency-associated transcription factors

including *Nanog* and *Tbx3*, maybe by increasing eIF2Bε activity, which in turn would feed into the pluripotency network (Figure 4.21). It would be interesting to investigate whether GSK-3 inhibition could regulate translation of other pluripotency markers including *Sox2* or *Klf4*.

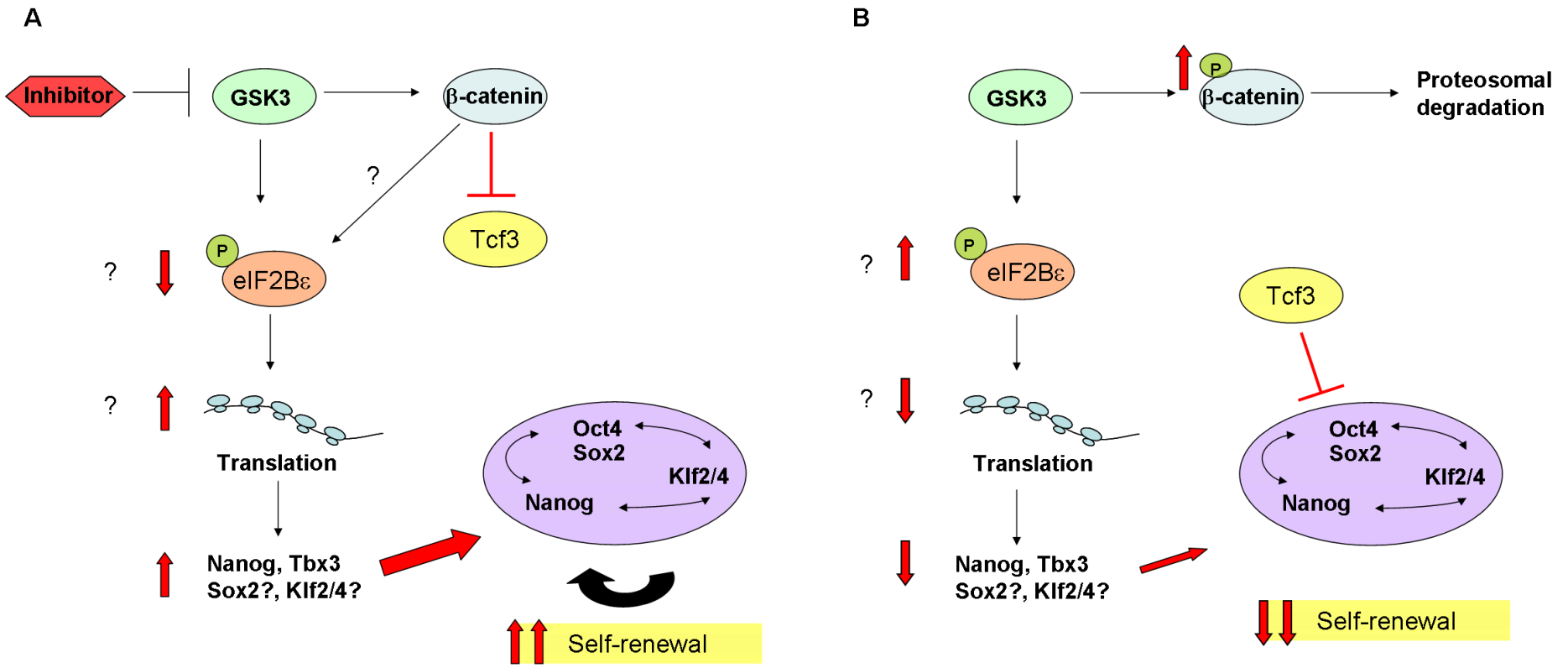
A
Inhibitor
GSK3
β-catenin
?
Tcf3
?
P
eIF2Bε
?
Translation
Oct4
Sox2
Klf2/4
Nanog
Nanog, Tbx3
Sox2?, Klf2/4?
Self-renewal
B
GSK3
P
β-catenin
Proteosomal
degradation
?
P
eIF2Bε
Tcf3
?
Translation
Oct4
Sox2
Klf2/4
Nanog
Nanog, Tbx3
Sox2?, Klf2/4?
Self-renewal

Figure 4.21. GSK-3 inhibition may contribute to enhancement of self-renewal by a β-catenin independent mechanism through increase of translation of the pluripotency-associated factors. A. GSK-3 inhibition and β-catenin stabilisation leads to inhibition of Tcf3 that alleviates its transcriptional repression in the pluripotency network (Wray et al., 2011). GSK-3 inhibition may also decrease phosphorylation of Ser539 resulting in increase of general translation and hence of *Nanog* and *Tbx3*. Nanog and Tbx3 would then feed into the pluripotency network. *Sox2* and *Klf2/4* translation could also be increased. **B.** In the absence of GSK-3 inhibitor, GSK-3 phosphorylates β-catenin leading to its proteosomal degradation. Thus, β-catenin can not inhibit Tcf3, which repress transcriptional activity of the pluripotency network. GSK-3 also phosphorylates Ser539 eIF2Bε leading to decrease translation of *Nanog* and *Tbx3*. The decrease in transcription and translation of pluripotency-associated transcription factors leads to decrease in self-renewal.

5 CHAPTER: IS CAP-DEPENDENT TRANSLATION AFFECTED BY GSK-3?

5.1 Introduction and aims

In the previous chapter, GSK-3 inhibition has been shown to increase the expression of pluripotency-associated transcription factors Nanog and Tbx3 and this effect is not due to enhanced protein stability, but instead occurred as a result of enhanced protein synthesis, promoted by inhibition of GSK3. Furthermore, increased loading of RNAs encoding pluripotency factors onto polysomes occurred following inhibition of GSK3, supporting a role for GSK3 inhibition in increasing translation of these RNAs.

This next part of the study sought to investigate whether the increase in mRNA translation observed in pluripotency-associated transcription factors following GSK-3 inhibition was due to increases in general (cap-dependent) translation. Changes in general translation following ESC differentiation into EBs have previously been reported (Sampath et al., 2008). As previously explained in Section 1.4, cap-dependent translation is mainly regulated at the initiation stage by changes in phosphorylation of eukaryotic translation initiation factors (eIFs). There are several eIFs that can regulate initiation of translation including eIF2Bε, eIF2α and eIF4F. The main steps in translation initiation are depicted in Figure 5.1.

GSK-3 downstream of PI3K is known to be able to regulate cap-dependent translation by regulating the activity of the guanine nucleotide exchange factor eIF2Bε via phosphorylation of Ser539 resulting in eIF2Bε inactivation (Figure 5.1) (Welsh et al., 1998; Welsh et al., 1997). eIF2Bε is involved in exchanging eIF2-GDP for GTP (Fig 5.1). The eukaryotic translation factor 2 (eIF2), as previously described in Section 1.4, controls translation initiation by binding of the Met-tRNAi to the 40S ribosome (Fig 5.1 (2)). Every round of translation initiation requires eIF2 bound to GTP and the GTP is hydrolysed to GDP during translation initiation (Fig 5.1 (4)). Phosphorylation of Ser51 of the α subunit of eIF2 increases its affinity for eIF2Bε that can only exchange eIF2-bound GDP for GTP if eIF2 is unphosphorylated. Therefore, phosphorylation of eIF2 leads to inhibition of translation initiation of most mRNAs (Day and Tuite, 1998; Goss et al., 1984). The exchange of GDP for GTP is not possible either if eIF2Bε is phosphorylated. Hence, phosphorylation of eIF2Bε by GSK-3 leads to its inactivation and in turn slows general translation

initiation (Welsh et al., 1998). On the other hand, although there is no evidence that eIF2α can be directly phosphorylated by GSK-3, inhibition of GSK-3 could indirectly affect its phosphorylation. Indeed, a decrease in phosphorylation of Ser51 of eIF2α was suggested following 24hour treatment with the GSK-3 inhibitor 1i in a Kinexus antibody microarray previously performed (Bone et al., 2009). However, this result has not been validated by immunoblotting.

Relevant to this study is the fact that Wnt signalling, through inhibition of GSK-3, has been implicated in indirect regulation of mTOR through TSC2 (Goss et al., 1984; Inoki et al., 2006). This raises the possibility that the increase in translation of *Nanog* and *Tbx3* observed following GSK-3 inhibition is due to an increase in cap-dependent translation through stimulation of mTOR activity.

mTOR activity can regulate the formation of the eukaryotic initiation factor 4 (eIF4F) complex, which is frequently associated with changes in translation rate by regulating the 4E-binding protein (4E-BP1). As previously explained in Section 1.4, eIF4F is important for binding the cap of the mRNA and recruiting the translation machinery and it is composed of three proteins, eIF4E that bind to the cap, a scaffolding protein eIF4G and the helicase eIF4A, which unwinds complex secondary structures in the 5'UTR (Fig 5.1(1)). 4EBP1 regulates the formation of the eIF4F complex by competing with eIF4G for binding to eIF4E. Binding of 4EBP1 to eIF4E is regulated by phosphorylation. In resting cells, 4EBP1 binds and sequesters eIF4E, preventing it from binding eIF4G, thus inhibiting translation initiation (Gebauer and Hentze, 2004; Richter and Sonenberg, 2005). FRAP/mTOR activation in response to growth factors leads to phosphorylation of Ser65 4EBP1 and release of eIF4E, which can then bind to eIF4G to form the eIF4F complex (Fig 5.2) (Parsa and Holland, 2004). mTOR has also been shown to activate the p70 ribosomal protein S6 Kinase 1 (S6K1) by phosphorylating Th389, which seems to regulate ribosomal biogenesis by phosphorylating S6 ribosomal protein in response to serum, amino acids or insulin.

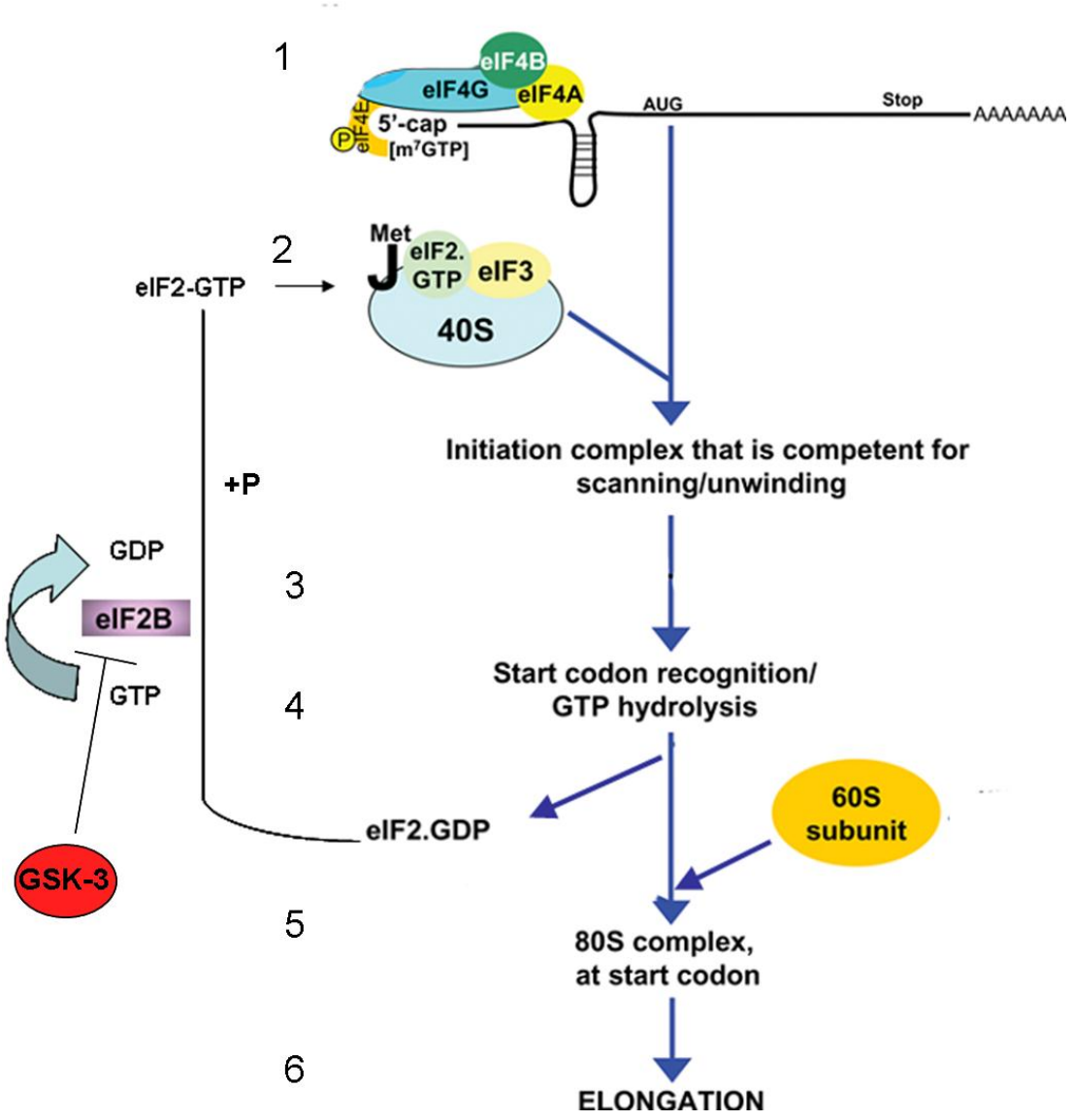

Figure 5.1 Main steps in initiation of translation. (1) eIF4E binds to the cap and together with eIF4G, will recruit the translational machinery. eIF4A is an ATP-dependent RNA helicase that binds and unwinds complex secondary structures in the 5´UTR binds to mRNA and eIF4G. The activity of the helicase is stimulated by the RNA binding protein eIF4B. (2) The 40S ribosome, which is bound to eukaryotic initiation factor 3 (eIF3), and the ternary complex (eukaryotic initiation factor 2 (eIF2)–GTP–Met-tRNAi) is brought to the cap of the mRNA through the scaffolding protein eIF4G resulting in the formation of the pre-initiation complex. (3) The start codon is recognise and (4) eIF2-GTP hydrolyse to eIF2-GDP which is release together with other initiation factors. (5) 60 S ribosomal subunit binds and (6) elongation starts (Kleijn and Proud, 2000; Proud, 2007).

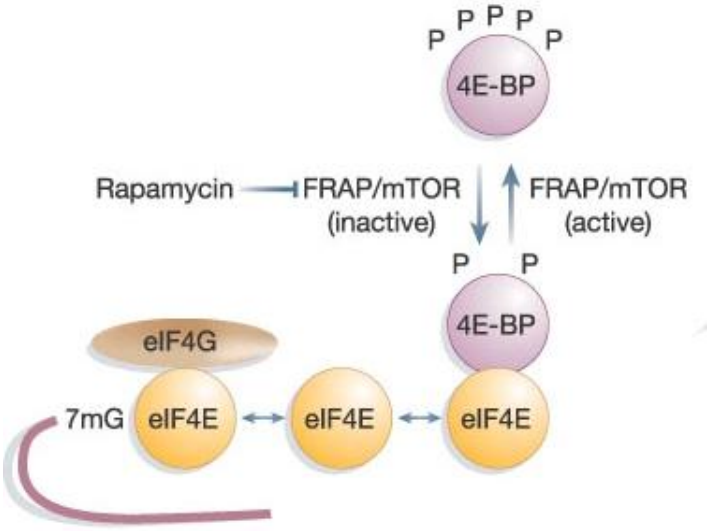

Figure 5.2 FRAP/mTOR regulates phosphorylation of 4E-BP. Unphosphorylated 4E-BP binds and sequesters eIF4E so that is unable to bind eIF4G. 4E-BP is inactivated by mTOR phosphorylation in response to growth factors releasing eIF4E which can then bind with eIF4G and initiate translation. (Modified from Richter and Sonenberg, 2005).

The aims of this study were to investigate a possible increase in cap-dependent translation following GSK-3 inhibition by examining the phosphorylation status of a number of the regulators of initiation described above, including pSer539 eIF2Bε, Ser51 eIF2α as well as the mTOR downstream targets Ser65 4EBP1 and Th389 S6K1.

5.2 Phosphorylation of Ser539 eIF2Bε does not change dramatically following GSK-3 inhibition.

As previously mentioned in Section 5.1, GSK-3 is known to phosphorylate Ser539 of eIF2Bε leading to its inactivation and a subsequent decrease in cap-dependent translation. This study aimed to investigate possible changes in phosphorylation of Ser539 eIF2Bε in ESCS grown with GSK-3 inhibitor or GSK-3 DKO for 4-24 hours. Phosphorylation of Ser539 did not seem to change at any of the time points examined (Fig 5.3).

We next investigated possible changes in phosphorylation after 5, 10, 20 and 30 minutes of LIF stimulation in samples pre-treated with 1m or untreated samples. There did not seem to be dramatic changes in LIF-stimulated phosphorylation of Ser539 in samples inhibited with 1m in comparison with untreated (Fig 5.4). Indeed there were not considerable changes in LIF-stimulated versus unstimulated. This experiment was only performed once at these time-points but in another experiment, samples were stimulated for 30 minutes, 2h and 24 hours and changes in phosphorylation Ser539 were not observed, indicating that inhibition of GSK-3 does not affect phosphorylation of Ser539 eIF2Bε in ESCs.

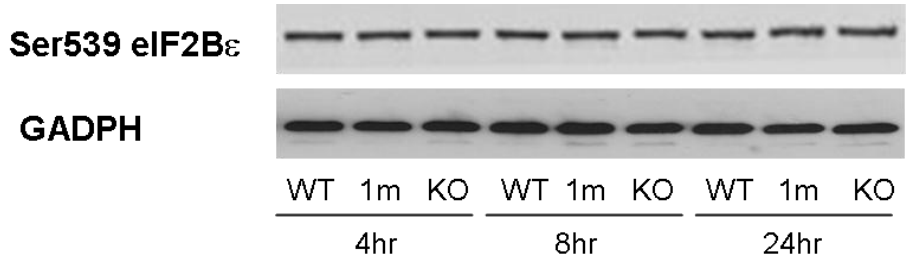

Figure 5.3. GSK-3 inhibition or knock-out does not affect phosphorylation of Ser539 of eIF2Bε. E14tg2a wild-type (WT) and GSK-3α/β double knockout (DKO) ESCs were cultured in the presence of Serum plus LIF. GSK-3 inhibitor 1m was added to WT cells at 2 μM and protein samples taken at the time indicated. Cell lysates were blotted with an antibody against phosphorylated Ser539 eIF2Bε and GAPDH. GAPDH was used as a loading control. This experiment was repeated twice and the blot shown is representative.

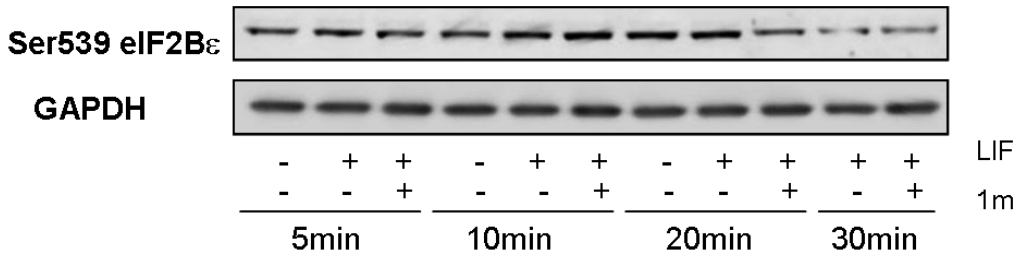

Figure 5.4. LIF-stimulated phosphorylation of Ser539 eIF2Bε does not considerably changed when GSK-3 is inhibited. E14tg2a were grown in N2B27+LIF+BMP4 for 48 hours before 4 hours starvation and cells stimulated with LIF for the time indicated. 1m was added 30 minutes before LIF stimulation. Cell lysates were blotted with an antibody against phosphorylated Ser539 eIF2Bε and GAPDH. GAPDH was used as a loading control Experiment performed once.

5.3 Short-term GSK-3 inhibition may increase phosphorylation of Ser51 eIF2α.

This part of the study aimed to investigate a possible change in phosphorylation of Ser51 of eIF2α following GSK3 inhibition because a Kinexus antibody microarray (kinexus.ca) previously performed suggested a decrease of pSer51 eIF2α after 24 hours of GSK-3 inhibition (Bone et al., 2009).

Initially, changes in phosphorylation of Ser51of eIF2α following GSK-3 inhibition over time or in GSK-3 DKO cells were investigated. Phosphorylation of Ser51 of eIF2α did not seem to change in either WT cells grown with 1m or in DKO cells (Fig 5.5). Earlier changes in pSer51 were investigated next by treating ESCs for 30 minutes with GSK-3 inhibitors, 1m or CHIR. Preliminary results showed that phosphorylation of Ser51 is modestly increased following 2μM 1m or 5μM CHIR (Figure 5.6). These data suggest that initiation of general translation maybe decreased but further repeats should be carried out to confirm this finding.

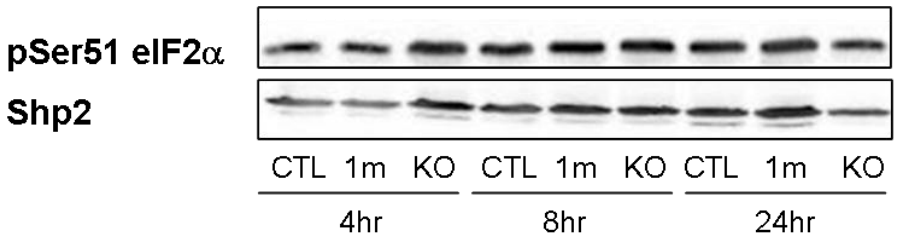

Figure 5.5. GSK-3 inhibition or knock-out did not dramatically change phosphorylation of Ser51 eIF2α. E14tg2a wild-type (WT) and GSK-3α/β□double knockout (DKO) ESCs were cultured in the presence of Serum plus LIF. GSK-3 inhibitor 1m was added to WT cells at 2 μM and protein samples taken at the time indicated. Cell lysates were blotted with an antibody against phosphorylated Ser51 eIF2α and Shp2. Shp2 was used as a loading control. This experiment was repeated twice and the blot shown is representative.

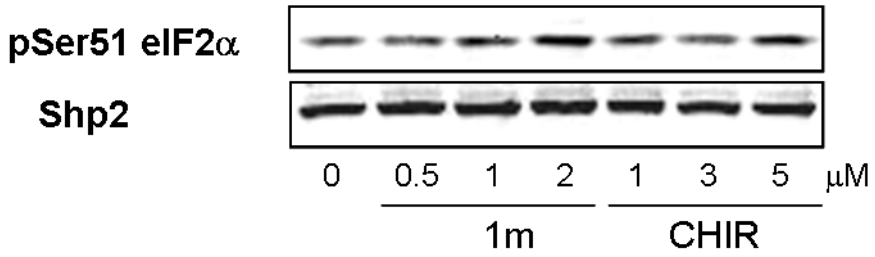

Figure 5.6. GSK-3 inhibition modestly increased phosphorylation of Ser51 eIF2α. E14tg2a mESCs grown in LIF plus Serum were treated for 30minutes with GSK-3 inhibitors, 1m or CHIR at the concentrations shown, before cell lysates were extracted and immunoblotting performed using antibodies against phosphorylated Ser51 eIF2α and Shp2. Shp2 was used as a loading control. This experiment was performed once.

Data from Section 5.2 and 5.3 suggest that cap-dependent translation is not dramatically affected by GSK-3 inhibition but may be slightly decreased (Fig 5.6). Further analysis of Ser51 eIF2α should be performed to investigate this in more detail. Based on these results, possible changes in cap-dependent translation through activation of mTOR, indirectly by GSK-3 inhibition, were examined.

5.4 Is GSK-3 acting through TSC2/mTOR to stimulate protein synthesis?

mTOR plays a role in regulation of protein synthesis through phosphorylation of factors that control translation. mTOR can associate with proteins forming two different complexes, mTORC1 and mTORC2, with the former being involved in regulating translational machinery (Kleijn and Proud, 2000).

mTORC1 regulates protein translation by phosphorylating the 4E-binding protein (4E-BP1) and p70 ribosomal protein S6 Kinase 1 (S6K1) (Fig 5.7). mTORC1 activity can be promoted by the small protein Rheb bound to GTP and can be

negatively regulated by TSC2 through hydrolysis of Rheb-GTP to GDP (Fig 5.7). TSC2 activity can be inhibited though phosphorylation in Ser939 and Th1462 by PKB, which is activated by PI3K signalling in response to insulin or growth factors. TSC2 inactivation by PKB leads to active Rheb that consequently activates mTOR leading to an increase in ribosome biogenesis and protein synthesis (Proud 2007). Interestingly for this study, Wnt signalling has also been implicated in regulation of TSC2 through GSK-3 inhibition (Inoki et al., 2006)(. GSK-3 can inhibit the mTOR pathway by phosphorylating TSC2 in Ser1337 and Ser1341 leading to its activation, subsequent inhibition of Rheb activity and mTOR (Inoki et al., 2006). GSK-3-dependent phosphorylation of TSC2 requires an AMPK-priming phosphorylation at Ser1345 (Inoki et al., 2003). AMPK is activated by AMP when the cellular energy levels are low (Inoki et al., 2006).

In order to elucidate whether GSK-3 inhibition decreases phosphorylation of TSC2 in ESCs, a phospho-specific antibody should have been used. However, antibodies against phosphorylated Ser1337 or Ser1341 were not commercially available and changes in phosphorylation of mTOR downstream effectors 4EBP1 and S6K1 were, therefore, studied as a read-out of mTOR activity.

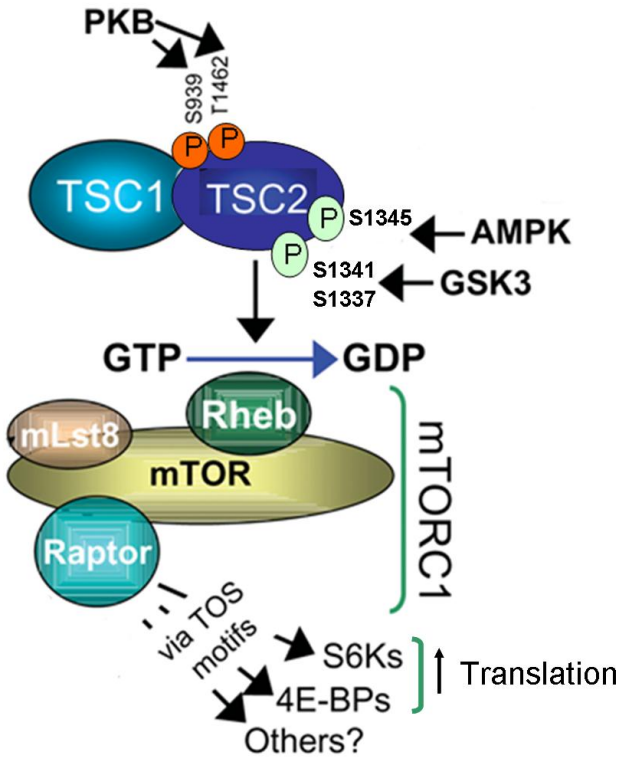

Fig 5.7 GSK-3 can regulate mTOR activity through phosphorylation and activation of TSC2. mTOR activity can be promoted by Rheb-GTP and TSC2 negatively regulates mTOR by hydrolysis of Rheb-GTP to GDP. TSC2 is inhibited by PKB phosphorylation in S939 and T1462 and it is activated by GSK-3 phosphorylation of S1337 and S1341 but it requires priming phosphorylation in S1345 by AMPK. Once activated mTOR can promote translation by phosphorylating S6Ks, 4EBPs and maybe others (Modified from Proud 2007).

5.4.1 Changes in phosphorylation of 4EBP1 following GSK-3 inhibition.

As previously mentioned in 5.1, 4EBP1 can regulate translation initiation by competing with eIF4G for binding to eIF4E. Phosphorylated 4EBP1 is unable to bind eIF4E and as a result translation increases. mTOR phosphorylates 4E-BP1 on different sites including Thr37, Thr46 and Ser65. The first two sites are thought to be priming sites and the latter is thought to interfere with binding to eIF4E (Fadden et al., 1997, 1998; Heesom et al., 2001). An increase in phosphorylation of Ser65 would be expected if mTOR activity is elevated following GSK-3 inhibition.

Changes in phosphorylation of Ser65 4EBP1 were investigated following GSK-3 inhibition and preliminary results suggest that pSer65 is not dramatically affected by GSK-3 inhibition (Fig 5.8). Although pSer65 seems to be slightly increased after 4 hours treatment with 1m or CH (Fig 5.8 (B)), the antibody did not work very well so it is not clear whether this result is representative. Indeed due to technical problems with the antibody, further results from repetition could not be obtained.

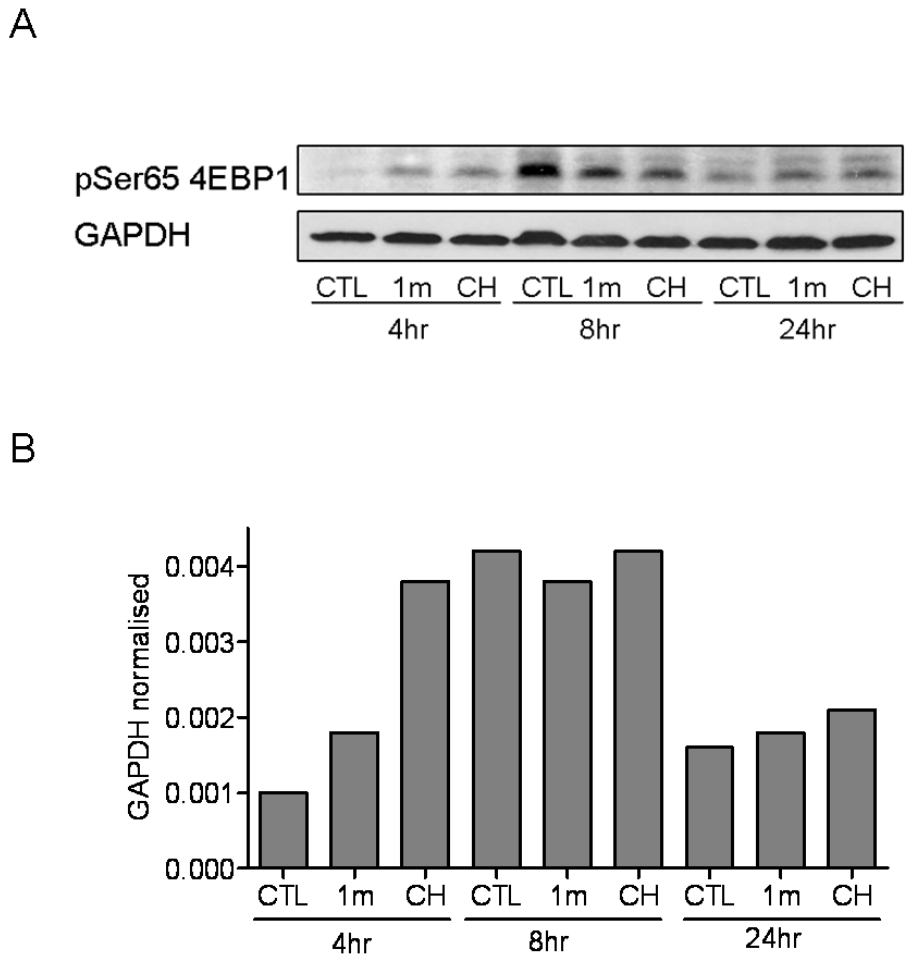

Figure 5.8. GSK-3 inhibition does not dramatically affect phosphorylation of Ser65 4EBP1. E14tg2a mESCs were cultured in the presence of Serum plus LIF in the presence of 2μM 1m or 3μM CH for the times indicated. (A) Cell lysates were blotted with an antibody against phosphorylated Ser65 4EBP1 and GAPDH. (B) Antibody signals were quantified and normalised to GAPDH. This experiment was performed once.

5.4.2 Phosphorylation of Thr389 S6K1 seems to decrease following GSK-3 inhibition.

The next aim was investigate changes in phosphorylation of other mTOR downstream effectors, this time S6K1. S6K1 is known to be regulated through phosphorylation of Thr389 by mTOR (Brown et al., 1995; Kim et al., 2002).

S6K1 plays a role in phosphorylating S6 ribosomal protein in response to serum, amino acids or insulin. Phosphorylated S6 ribosomal proteins rapidly increase the translation of 5´terminal oligopyrimidine tract (TOP) mRNA transcripts, commonly found in ribosomal proteins and elongation factors, and phosphorylation of Thr389 S6K1 is frequently associated with increased translation of ribosomal proteins (Jefferies et al., 1997; Jefferies et al., 1994). S6K1 can also phosphorylate and regulate the eukaryotic elongation factor 2 kinase (eEF2K) and the eukaryotic translation initiation factor 4B (eIF4B). Phosphorylation of eEF2K at Ser366 results in inhibition of kinase activity and thus increased translation elongation (Wang et al., 2001). The phosphorylation of eIF4B at Ser422 (Raught et al., 2004) increases the protein levels recruited to eIF4A, this results in increased scanning ability of ribosomes. Thus, S6K1 can regulate cap-translation through eIF4B increasing scanning of the ribosomes and eEF2K regulating elongation. S6K also regulates ribosome biogenesis by controlling ribosomal S6 protein (Fig 5.9).

Phosphorylation of Thr389, which is the mTOR phosphorylation site in S6K1 (Brown et al., 1995; Kim et al., 2002), was investigated following GSK-3 inhibition in order to further study a possible activation of mTOR.

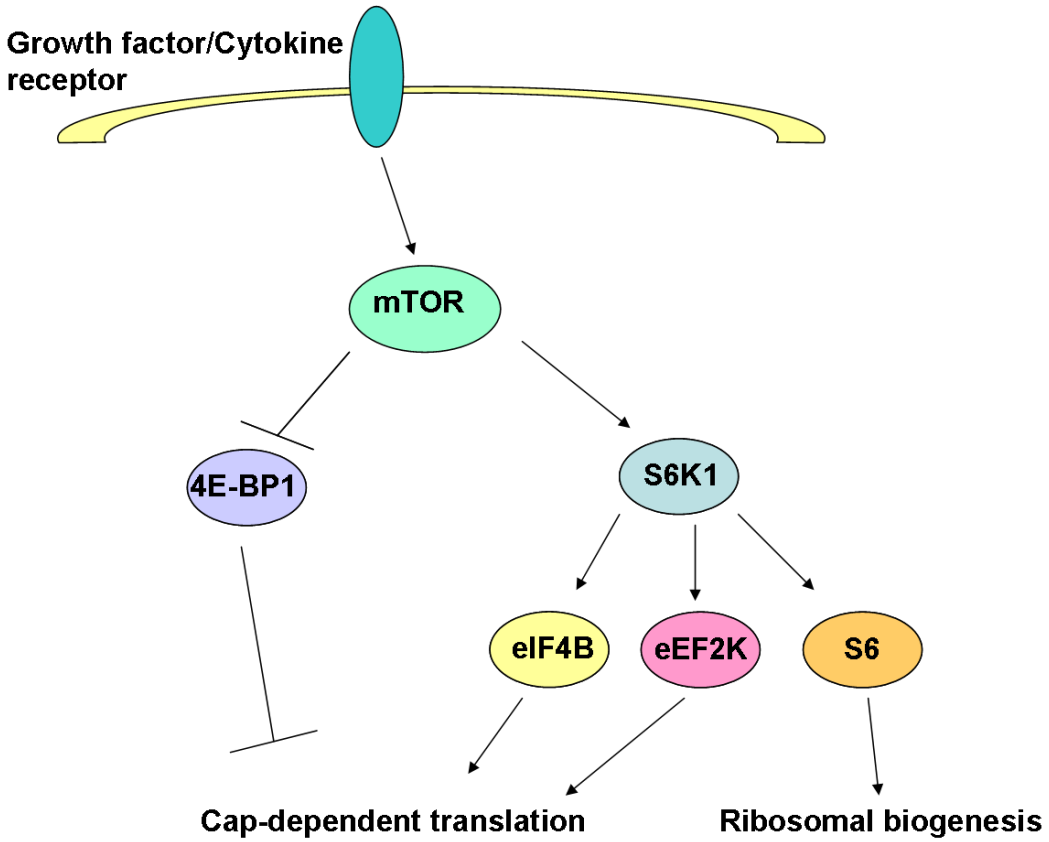

Figure 5.9. FRAP/mTOR can phosphorylate both 4E-BP1 and S6K1. mTOR activation in response to growth factor, amino acids or insulin leads to activation of S6K1 by phosphorylation in Th389, which in turn phosphorylates and activates S6 ribosomal protein promoting ribosomal biogenesis. Inactivation of 4E-BP1 by phosphorylation in Ser64 results in inactivation of 4E-BP1 and promotion of cap-dependent translation (After Gingras et al., 2001; Raught et al., 2004; Wang et al., 2001).

5.4.2.1 Phosphorylation of Thr389 of S6K1 decreases following GSK-3 inhibition.

Initially, changes in levels of Thr389 phosphorylation of S6K1 after 8 and 24 hours of GSK-3 inhibition were investigated because levels of Nanog and Tbx3 protein were shown to increase at these time points. Phosphorylation of S6K1 at Th389 seemed to be decreased after 24 hours of GSK-3 inhibition or in DKO cells. Changes in phosphorylation after 8 hours of GSK-3 inhibition were not consistently observed

(Fig 5.10). These data suggest that GSK-3 inhibition does not increase mTOR activity but rather may decrease it.

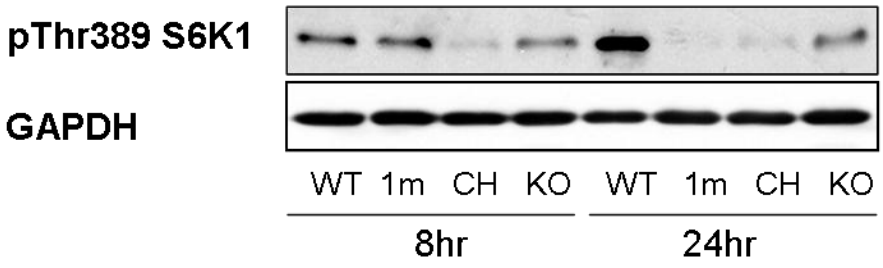

Figure 5.10. GSK-3 inhibition or Knock-out decreases phosphorylation of Thr389 S6K1. WT and DKO ESCs were grown in N2B27 plus BMP4 and LIF. WT were treated with 2μM 1m or 3μM CHIR for 8 and 24 hours before lysing. Immunoblotting was performed with antibodies against pThr389 S6K1 and GAPDH. This experiment was repeated three times and the blot shown is representative.

In order to further investigate a possible change in S6K1 activity due to GSK-3 inhibition, LIF stimulation experiments were performed. ESCs were starved of LIF for 4 hours and GSK-3 inhibitors added 30 minutes before LIF stimulation. GSK-3 inhibition significantly abolished LIF-stimulated phosphorylation of Thr389 of S6K1 (Fig 5.11).

In summary, GSK-3 inhibition does not only seem to decrease phosphorylation of Thr389 S6K1 but also abolished its phosphorylation following LIF stimulation. This reduction in phosphorylation of Thr389 suggests a decrease in protein synthesis and a possible decrease in mTOR activity. Although this is consistent with preliminary results observed for phosphorylation of Ser51 on eIF2α (Fig 5.6), which indicates a decrease in cap-translation, it is somehow opposite to what was expected, as GSK-3 inhibition increases translation of Nanog and Tbx3 (Chapter 4). However, translation of specific mRNAs through different mechanisms can take place under conditions where general translation is reduced. This will be discussed further in Section 5.5.3.

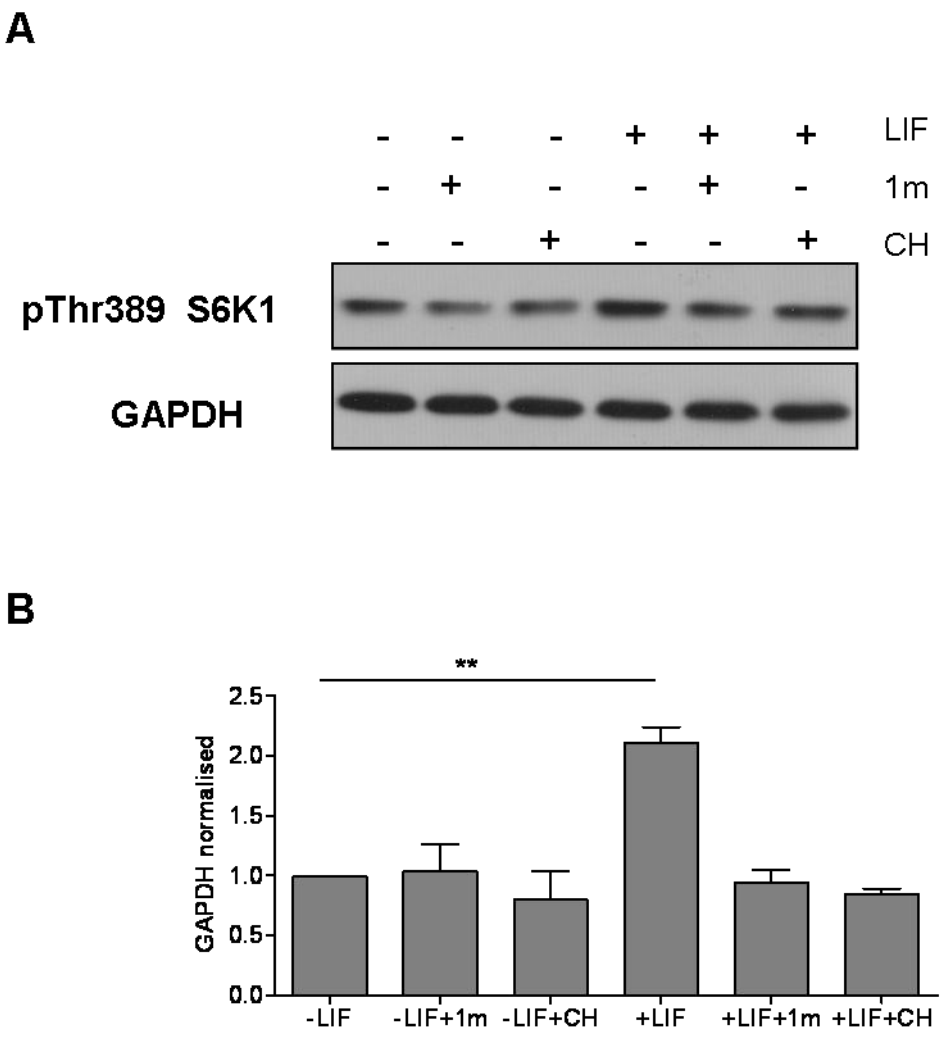

Figure 5.11. GSK-3 inhibition reduces LIF-stimulated phosphorylation of Thr389 S6K1. E14tg2a ESCs were cultured in N2B27+LIF+BMP4 for 48 hours and starved for 4 hours before stimulation with 1000U/ml LIF for 10 minutes. GSK-3 inhibitors, 1m and CH were added at 2μM and 3μM respectively 30 minutes before LIF stimulation and cell lysates immunoblotted with antibodies against pThr389 S6K1 and GAPDH. GAPDH was used as loading control. The values are the average and S.E.M from three independent experiments. **p<0,005. A value of 1 was given to –LIF.

5.4.2.2 GSK-3 inhibition also decreases pThr389 S6K1 in 2i media.

Data presented in Section 4.2.3 suggested that GSK-3 inhibition can regulate Nanog and Tbx3 expression in the presence of PD (2i conditions) and so it was also investigated whether GSK-3 inhibition would also lead to a reduction in pThr389 S6K1 in 2i conditions.

Phosphorylation of Thr389 S6K1 was reduced in cells grown in the presence of GSK-3 inhibitor alone or in combination with PD in comparison with PD alone (Fig 5.12). Phosphorylation of Ser366 on eEF2K, a downstream effector of S6K1, was also modestly reduced in the same conditions. These conditions were previously shown to result in higher levels of Nanog and Tbx3 proteins (Fig 4.8). Moreover, consistent with elevated levels of Tbx3 and Nanog protein, ESC colonies showed a more compact and self-renewing morphology in the presence of GSK-3 inhibitor (Fig 4.8). Therefore, there seems to be a correlation between a decrease in pThr389 of S6K1 and increase in ESC self-renewal.

In order to further investigate a correlation between a decrease in phosphorylation of Thr389 S6K1 and an increase in ESC self-renewal, changes in levels of Thr389 phosphorylation following GSK-3 inhibition in cells pre-treated with MEK inhibitor overnight were investigated. Results suggest that phosphorylation of Thr389 decreases following inhibition of GSK3 after 4 hours (Fig 5.13), conditions where Nanog and Tbx3 were shown to be increased (Fig 4.9). Decreases in Thr389 phosphorylation can also be observed after 8 and 24 hours. The data presented indicate that there is a correlation between the increase in self-renewal and decrease in phosphorylation of Thr389 on S6K1.

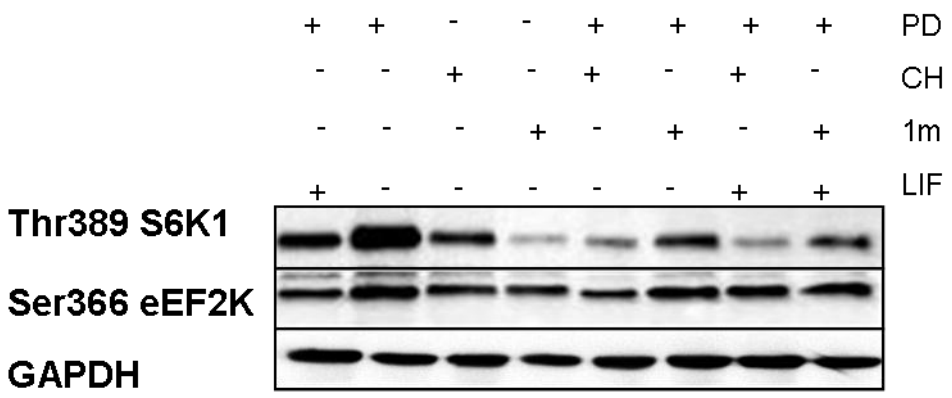

Figure 5.12. Phosphorylation of Thr389 p70S6K correlates with self-renewal. E14tg2a ESCs were grown for 48 hours in chemically defined media N2B27 with the inhibitors indicated. Cell extracts were immunoblotted with antibodies specific to either pThr389 S6K1, pSer366 eEF2K or GAPDH. GAPDH was used as a loading control. This experiment was performed twice and results shown are representative.

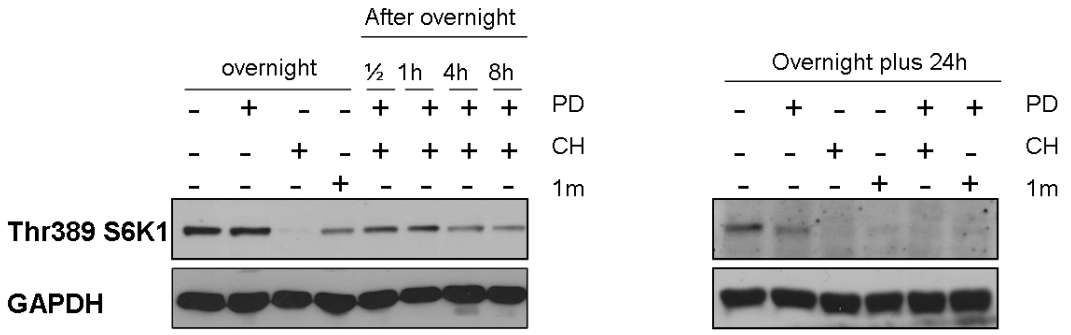

Figure 5.13. GSK-3 inhibition reduces Thr389 phosphorylation in the absence of extrinsic stimuli. E14tg2a mESCs were grown overnight in the presence of MEK inhibitor (PD) before treating them with 3μM CHIR or 2μM 1m for the timed indicated. mESCs were also grown in CHIR and 1m overnight before cell lysates were prepared. Immunoblotting to detect phosphorylation of Thr389 on S6K1 and GAPDH was performed. GAPDH was used as a loading control. This experiment was repeated three times and results shown are representative.

5.4.2.3 Decrease in phosphorylation of Thr389 on S6K1 following GSK-3 inhibition is mTOR-independent.

Results from previous Sections 5.4.2.1 and 5.4.2.2 suggest that S6K1 Thr389 phosphorylation decreases following GSK-3 inhibition. However, there is no evidence that S6K1 is a direct substrate of GSK-3 and Thr389 of S6K1 is known to be phosphorylated by mTOR. As previously mentioned in 5.1 and 5.4 (Fig 5.8), mTOR has been reported to be negatively regulated by GSK-3 through TSC2. Therefore, it was somewhat surprising that phosphorylation of Thr389 on S6K1, the mTOR phosphorylation site, was decreased following GSK-3 inhibition. This was investigated further to determine whether GSK-3 inhibition decreases mTOR activity by looking at changes in phosphorylation of Ser2448 on mTOR, which is known to be phosphorylated by activated Akt/PKB downstream of PI3K in response to insulin. Changes in phosphorylation of Ser2481 on mTOR, which is the autoregulatory phosphorylation site, were also investigated.

In order to investigate this, cells were grown in Serum plus LIF supplemented with LY294002, which is a broad spectrum PI3K kinase inhibitor; in Rapamycin, which is known to inhibit mTOR; in PI-103 that inhibits mTOR, PI3K and DNA-PK, and finally also in 1m, that inhibits GSK-3. Phosphorylation of Thr389 of S6K1 decreased after 24 and 40 hours of LY, PI-103, Rapamycin and 1m treatment (Figure 5.14). However, the decrease in phosphorylation was higher when either LY or PI-103 were used in comparison with the decreases observed in Rapamycin and 1m-treated samples. Although phosphorylation of Ser2448 of mTOR does not seem to change dramatically after any treatment, there is a modest decrease after LY and PI-103 treatment (Fig 5.14 (B)). On the other hand, changes in phosphorylation of Ser2481 of mTOR seem to be high, with a reduction of approximately 50% in samples grown in the presence of LY and PI-103. Opposite to this, rapamycin and 1m treatment seemed to modestly increase Ser2481 phosphorylation (Fig 5.14 (B)). These data suggest that the effects observed on Thr389 phosphorylation following 1m inhibition are not due to a decrease in mTOR activity. In support of this, is the fact that preliminary results looking at changes in phosphorylation of Ser65 on 4EBP1, which is downstream of mTOR, does not seem to be dramatically affected by GSK-3 inhibition. This raised the possibility that the decrease observed in phosphorylation of Thr389 on S6K1 was due to 1m and CHIR off-target effects, for

example inhibition of AGC family of kinases, that have also been implicated in phosphorylation of Thr389 of S6K1 (Foster and Fingar, 2010). However, the decrease in Thr389 S6K1 is unlikely to be due to off-target effect because it can be observed following treatment with both 1m and CHIR and they are structurally unrelated.

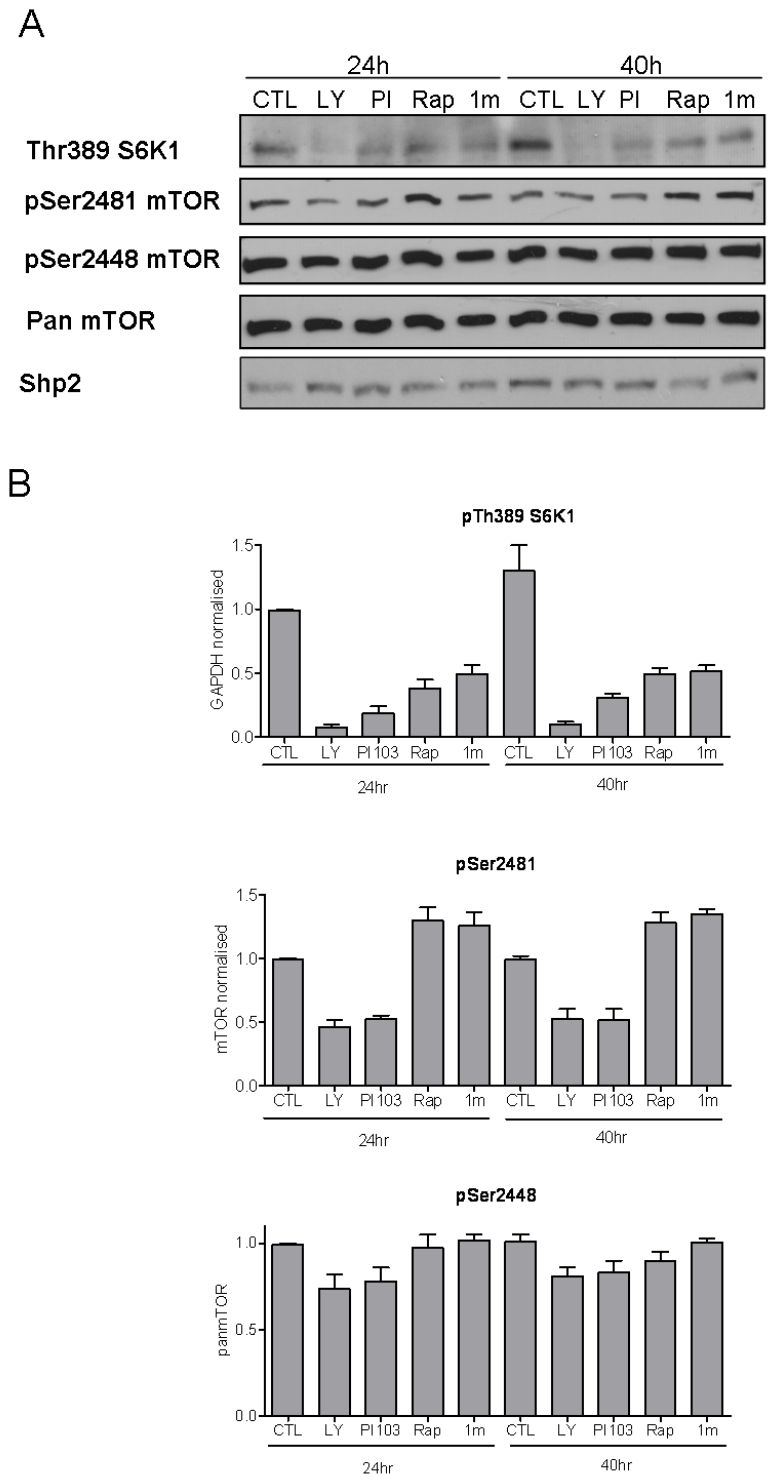

Figure 5.14. Decrease of phosphorylation of Thr389 on S6K1 following GSK-3 inhibition is not mTOR-dependent. E14tg2a ESCs were cultured in the presence of Serum and LIF supplemented with 5μM LY294002, 100nM PI-103, 1nM Rapamycin and 2μM 1m for 24 and 40 hours before extracting cell lysates. (A) Immunoblotting was performed with the antibodies indicated. (B) Antibody signals were quantified and normalised to Shp2.Shp2 were used as a loading control. The values in B are the average and S.D of duplicate experiments.

5.4.2.4 Decreased phosphorylation of Thr389 on S6K1 is observed following GSK-3 inhibition with 1m is not due to off-target effects.

To rule out the possibility that the effect observed in pThr389 following GSK-3 inhibition is due to 1m off-target effects, GSK-3 DKO cells were used. We investigated whether addition of 1m to DKO cells would decrease LIF-stimulated phosphorylation of Thr389 on S6K1. As a control, cells were pre-treated with LY and rapamicin. LIF-stimulated phosphorylation of Thr389 on S6K1 decreased in GSK-3 DKO cells incubated with LY and rapamycin but not with 1m (Figure 5.15). These data suggest that the decrease in Thr389 phosphorylation observed in WT ESCs treated with 1m is due to GSK-3 inhibition and not to off-target effects.

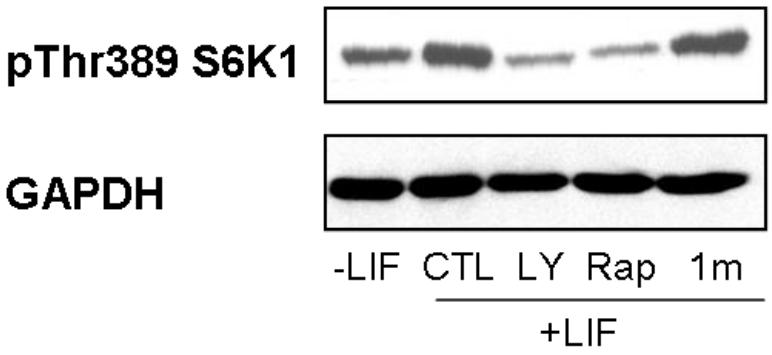

Figure 5.15 Reduction in LIF-stimulated phosphorylation of Thr389 on S6K1 is not due to 1m off-target effects. GSK-3 DKO cells were LIF-starved for 4 hours before pre-treating them for 30 minutes with 5μM LY, 10nM Rapamycin and 2μM 1m. Cell lysates were obtained 10 minutes after LIF stimulation and immunoblotting performed with the antibodies indicated. GAPDH was used as a loading control. The experiment was repeated twice and the blot is representative.

5.5 DISCUSSION

The aim of the studies presented in this Chapter was to investigate whether the increase in mRNA translation observed for *Nanog* and *Tbx3* following GSK-3 inhibition (Chapter 4) was due to an increase in general (cap-dependent) translation. Changes in cap-dependent translation following ESC differentiation into EBs has previously been reported (Sampath et al., 2008). Changes in phosphorylation of several factors that regulate translation were investigated.

5.5.1 GSK-3 inhibition does not alter phosphorylation of Ser539 of eIF2Bε or Ser51 of eIF2α.

The first factor to be studied was the guanine nucleotide exchange factor eIF2Bε because it is known to be negatively regulated by GSK-3 downstream of PI3K and it is important for controlling translation initiation (Welsh et al., 1997; Welsh et al., 1998). PI3K activation in response to insulin inhibits GSK-3 resulting in dephosphorylation of Ser539 eIF2Bε and in its subsequent activation promoting translation initiation. No dramatic changes in phosphorylation of Ser539 on eIF2Bε were observed following GSK-3 inhibition suggesting that cap-dependent translation may not be altered. However, there are several factors regulating translation so an increase or decrease in activity of another factor may have an effect on translation.

The next factor to be investigated was eIF2, which recruits the Met-tRNA to the 40S ribosomal subunit, and thus is a key regulator of translation initiation. As previously mentioned in 5.1, eIF2 only binds Met-tRNA if it is itself bound to GTP. eIF2-GTP is hydrolysed to GDP in each round of initiation of translation and GTP is exchanged for GDP by eIF2Bε which can only bind eIF2 in its unphosphorylated state. Phosphorylation of Ser51 of eIF2α inhibits eIF2 activity and decreases translation initiation. A Kinexus antibody microarray previously performed suggested a decrease of phosphorylation of Ser51 on eIF2α after 24 hours of GSK-3 inhibition (Bone et al., 2009). However, this result was not further investigated by immunoblotting until this study. Although phosphorylation of Ser51 was not

dramatically affected by GSK-3 inhibition (Fig 5.3), preliminary results suggest that Ser51 phosphorylation may be modestly increased after 30 minutes of initiation of GSK-3 inhibition (Fig 5.6). Changes of about 30% in the level of phosphorylation of eIF2α at Ser51 are thought to be enough to inhibit all of the eIF2Bε, as it is present at lower levels than eIF2. Thus, small increases in phosphorylation of Ser51 could inhibit cap-dependent protein synthesis (Block et al., 1998). More samples should be examined to determine whether GSK-3 inhibition consistently increases phosphorylation of Ser51 of eIF2α and if so to what extent.

5.5.2 GSK-3 does not seem to act through TSC2/mTORC1 to control translation in ESCs.

Most studies to date support a role for Wnt signalling in maintaining ESC self-renewal by β-catenin-dependent transcriptional activation of target genes (Wray et al., 2011; Yi et al., 2011; Kelly et al., 2011). However, a role for Wnt in promoting translation and cell growth in other cell types has been reported (Inoki et al., 2006). GSK-3 inhibition by Wnt stimulation was shown to increase mTOR activity and translation by decreasing the phosphorylation and activation of the Tuberous sclerosis complex 2 (TSC2), which inhibits Rheb activity required for mTOR activation (Inoki et al., 2006). The present study investigated whether GSK-3 in ESC would also regulate mTOR activity. Ideally changes in GSK-3 target phosphorylation sites of TSC2 (Ser1337 and Ser1341) would have been investigated but suitable antibodies were not commercially available. As an alternative approach, changes in phosphorylation of Ser65 on 4E-BP1 and Thr389 on S6K1, which are known mTOR target phosphorylation sites, were investigated following GSK-3 inhibition to assess mTOR activity.

Although preliminary results suggest that GSK-3 inhibition did not seem to dramatically change levels of 4E-BP1 Ser65 phosphorylation (Fig 5.9), a conclusion can not be drawn without analysing further experimental repeats, which will first require optimisation of the antibody. On the other hand, GSK-3 inhibition led to a decrease in phosphorylation of S6K1 at Thr389 (Fig 5.10, 5.11, 5.12, 5.13), which was at least partly mTOR independent (Fig 5.14). Interestingly, there seems to be a

correlation between a decrease in Thr389 phosphorylation and an increase in self-renewal (Fig 5.12, 5.13). A decrease in Thr389 phosphorylation suggests that ribosomal biogenesis may be reduced following GSK-3 inhibition, which in turn will affect general translation. This would be in accordance with the work of Sampath *et al.,* because they observed an increase in general translation during ESC differentiation (Sampath et al., 2008). Therefore, it is reasonable to think that an enhancement of self-renewal observed following GSK-3 inhibition could lead to a decrease in general translation.

It has been proposed that S6K1 may not be essential for ribosomal biogenesis because S6K1 knock-out cells or knock-in of mutant S6K1 (that cannot be phosphorylated) exhibit normal translation of ribosomal proteins (Pende et al., 2004; Ruvinsky et al., 2005). However, S6K1 is also known to promote translation initiation by phosphorylating eIF4B at Ser422, which promotes its recruitment to eIF4A where it stimulate eIF4A activity and thus mRNA with complex secondary structures would be translated more efficiently. A decrease in S6K1 activity would potentially lead to a decrease in phosphorylation of eIF4B at Ser422 and consequently a decrease in translation initiation. Changes in phosphorylation of Ser422 on eIF4B should be studied in order to test this. S6K1 can also control translation elongation by phosphorylating Ser366 on eEF2K and Ser366 phosphorylation was decreased following GSK-3 inhibition in 2i conditions (Fig 5.12) suggesting a possible reduction of translation elongation. To summarise, mTOR activity does not appear to increase following GSK-3 inhibition because the decrease in S6K1 Thr389 phosphorylation suggests a decrease in activity. However, preliminary results suggest that phosphorylation of Ser65 of 4E-BP1, another downstream effector of mTOR, is not dramatically altered by GSK-3 inhibition. Moreover, phosphorylation of mTOR itself at Ser2481, the autoregulatory phosphorylation site that reflects mTOR catalytic activity (Soliman et al., 2010), was modestly increased following inhibition of GSK-3 indicating that mTOR activity maybe slightly increased. Although the decrease in phosphorylation of Thr389 of S6K1 suggests a decrease in mTOR activity, Thr389 can be phosphorylated by other kinases apart from mTOR (Fig 5.16).

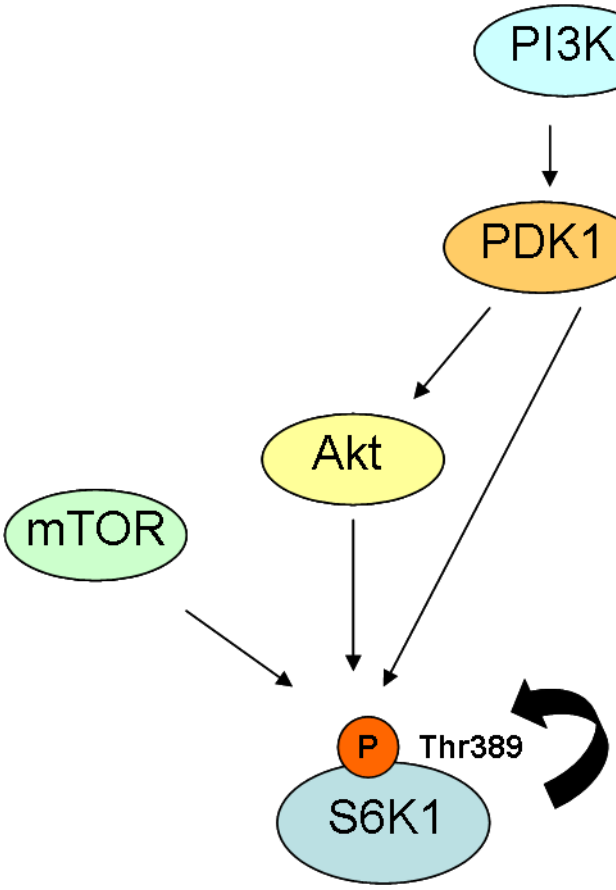

Fig 5.16 S6K1 Thr389 can be phosphorylated by several kinases. S6K1 is phosphorylated by mTOR on Thr389 but this site can also be phosphorylated by PDK1 and Akt/PKB downstream of PI3Ks. Phosphorylation by Akt/PKB requires S6K1 activity and S6K1 is thought to autophosphorylate itself.

S6K1 is known to be regulated through phosphorylation of Thr389 by mTOR (Kim et al., 2002). PDK1 can also phosphorylate Thr389 *in vivo* and *in vitro* (Balendran et al., 1999). PDK1 null ESCs cannot phosphorylate Thr389 on S6K1 in response to insulin-like growth factors (Williams et al., 2000). Akt/PKB downstream of PI3K has also been shown to phosphorylate Thr389 on S6K1 (Romanelli et al., 2002). Although PDK1 is able by itself to phosphorylate Thr389 on S6K1, Akt/PKB phosphorylation depends on S6K1 activity. After the initial phosphorylation by mTOR, Thr389 phosphorylation is maintained by autophosphorylation (Romanelli et

al., 2002). Therefore, the decrease in phosphorylation of Thr389 on S6K1 observed following GSK-3 inhibition could be due to a decrease in activity of, for example, PDK1 or Akt/PKB and not mTOR. This is supported by the fact that Ser2481 is not decreased but modestly increased after GSK-3 inhibition. Phosphorylation of Akt at Ser473 did not change following GSK-3 inhibition (results not shown) indicating that Akt/PKB activation is not altered. However, phosphorylation of Akt at Th308 should also be examined as it is also needed for full activation. A decrease in PDK1 activity should be investigated because it could be possible that there is a feedback regulatory loop between PDK-1 and GSK-3.

Another possible explanation for the decrease in S6K1 Thr389 phosphorylation could be that GSK-3 is directly phosphorylating Thr389 S6K1. Although GSK-3 is not known to regulate S6K1, S6K1 can phosphorylate GSK-3 under certain conditions (Zhang et al., 2006a). It could, therefore, be possible, similar to what I proposed for PDK1, that there is a feedback regulatory mechanism whereby GSK-3 phosphorylates S6K1.

While the mechanism whereby levels of S6K1 Thr389 phosphorylation decrease following GSK-3 inhibition is unclear, it is evident that it is likely to have an effect on cap-dependent translation either directly, by decreasing activity of eIF4B and eEF2K, or indirectly by potentially decreasing ribosomal biogenesis. This is the opposite what was expected since *Nanog* and *Tbx3* translation seem to be increased following GSK-3 inhibition. However, translation of specific mRNA transcripts, without an increase in general translation or in conditions where the cap-dependent translation is compromised, can occur via a number of different mechanisms. The fact that translation of other genes, including *Oct4* and *Cyclin D1*, are not increased following GSK-3 inhibition (Chapter 4) suggest that the increase in translation observed with *Nanog* and *Tbx3* is specific. The next Section will discuss mechanisms whereby *Nanog* and *Tbx3* mRNA translation could be specifically increased.

5.5.3 Nanog and Tbx3 may be translated by a specific mechanism.

Until the 1980s, cap-dependent or scanning translation was thought to be the only mechanism whereby an mRNA could be translated. However, studies of viral gene expression led to the discovery of another mechanism of translation initiation whereby the 40S ribosomal subunit can be recruited to the proximity of the start codon without the need of attaching to the cap and scanning the 5'UTR until it finds an initiation codon. The regions of the mRNA where the ribosome attached were named Internal Ribosome Entry Sites (IRES) (Komar and Hatzoglou, 2011). A large number of mRNAs containing IRES are less dependent on signals that inhibit cap-dependent translation, such as increased phosphorylation of eIF2α, than mRNAs that lack IRES (Clemens, 2001; Komar and Hatzoglou, 2005; Tinton et al., 2005). In addition, IRES translation can be regulated by proteins that bind the internal initiation site and are named IRES trans-acting factors (ITAFS) (Komar and Hatzoglou, 2011). The mechanisms that control ITAF concentrations are largely unknown.

IRES-translation is thought to play a role in promoting translation of mRNAs that have complex structures in the 5'UTR, which are more difficult to translate by the cap mechanisms. Furthermore, IRES-translation can promote translation of mRNAs under conditions where the cap-dependent translation is compromised, for example during cell differentiation or nutrient limitation. Although IRES are known to be highly structured, with stem loops and pseudo knots, a common sequence or structure for identification of IRES elements has not yet been discovered and the presence of an IRES in an mRNA has to be experimentally tested (reviewed in Komar and Hatzoglou, 2005).

One mRNA containing an IRES that is relevant to this work is c-Myc. ITAFs that associate with the IRES of c-Myc, including P54nrb, YB-1 (Y-box binding protein) and GRSF-1 (guanine-rich RNA sequence binding factor 1) were identified by affinity chromatography (Cobbold et al., 2008). Knock-down of YB-1 and p54nrb were shown to lead to a decrease in c-Myc protein expression. The same effect, but to a lesser extent, was observed following knock-down of GRSF-1. Importantly, GRSF-1 was shown to promote the translation of specific mRNAs by associating to

the following target sequences in the 5'UTR: AGGU, AGGGU, and AGGGGU with the 3' G and U being the most important for the binding (Kash et al., 2002). Of relevance to this study is the fact that GRSF-1 has been identified as a Wnt/β-catenin downstream target (Lickert et al., 2005) raising the possibility that GSK-3 inhibition, which mimics Wnt activation, leads to up-regulation of GRSF-1 promoting translation of mRNA targets. Interestingly, Tbx3 has a binding site for GRSF-1 in its 5'UTR and thus *Tbx3* may be a GRSF-1 target. In addition to this, YB-1 was also shown to play a part in recruitment of c-Myc to polysomes, also raising the possibility that YB-1 regulates *Tbx-3* mRNA recruitment to polysomes. Moreover, *Tbx3*, similar to *c-Myc*, has complex secondary structures in the 5'UTR and it could be translated through IRES. On the other hand, *Nanog* mRNAs does not have GRSF1 binding sites and the 5'UTR is much simpler than those of *c-Myc* and *Tbx3*. Figure 5.17 shows the number of secondary structures or stem loops present in the 5´UTR of Nanog, Tbx3 and c-Myc RNA, as well as the energy required to unwind them. The 5´UTR of Nanog only has 18 stem loops whereas both c-Myc and Tbx3 has 100. The simplicity of Nanog 5´UTR compared to Tbx3 and c-Myc makes it unlikely to be translated through IRES. However, its translation could be regulated by other mechanisms, which will be further described below.

Translation of specific mRNAs independently for the cap-translation or IRES-mediate translation is possible due to structural features and regulatory sequences in the 5' and 3' untranslated region of the mRNA (Gray and Wickens, 1998). Upstream open reading frames (uORFs), the presence of specific sequences for mRNA binding-proteins, the length of the poly(A) tail, number of secondary structures in the 5'UTR and the presence of miRNA target sequence can modulate the translation efficiency of mRNAs (de Moor et al., 2005; Gingras et al., 2001; Jackson et al., 2010).

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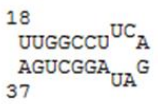
A Nanog

Molecule: Nanog-UTR-5` 215 bps
Analysis: Dyad Symmetries; Num Stem-Loops = 18 (<= 0.0 kcals)

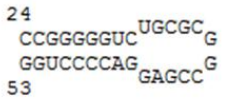
Energy	First	Repeat	Sequence
-6.3	18	37	UUGGCCU
-3.2	65	85	GGAAG
-2.7	49	67	UCUUGC
-2.6	60	76	CUGUGG
-2.5	66	88	GAAGG
-2.4	62	81	GUGGG
-2.1	13	35	AGCC
-2.0	16	48	CGUUGGCC
-2.0	7	22	GCC
-1.7	164	180	GGUU
-1.4	25	37	UCAG
-0.9	7	34	GCCU
-0.8	160	178	UUUUGG
-0.8	56	70	CUUUC
-0.7	21	71	GCCUUC
-0.5	11	80	UGAGCCG
-0.3	152	166	ACUGA
-0.2	48	71	GUCUU



B C-Myc

Molecule: UTR-5 626 bps
Analysis: Dyad Symmetries; Num Stem-Loops = 100 (<= -0.6 kcals)

Energy	First	Repeat	Sequence
-14.1	24	53	CCGGGGGUC
-13.9	284	319	CGCUCCGGGGC
-13.5	34	60	GCGCGGCC
-9.9	381	407	CAGCGGC
-8.7	212	234	CUCGCUG
-8.6	477	495	CUCCCC
-8.6	104	123	CCUGCC
-7.9	414	435	UUUGCCC
-7.5	97	162	CUCCCCUCC
-7.4	383	402	GCGGC
-6.7	545	563	AGCUCU
-5.8	103	145	UCCUGCC
-5.4	573	598	GUUUGAAGG
-5.4	402	430	CCGCUG
-5.1	44	80	GGACCC
-5.0	254	270	ACGGUU
-4.3	478	517	UCCCCA
-4.2	544	573	CAGCUC
-4.2	463	488	GAGCC
-3.8	325	341	GGCU
-3.5	406	421	UGGG
-3.3	308	328	AGCUC
-3.1	275	332	GCAGAGCC
-3.1	36	63	GCGGC
-2.9	128	142	CGCCGA
-2.9	9	28	CCCG
-2.9	478	498	UCCC
-2.8	321	335	GAGG
-2.7	587	606	UUUCC



C

Tbx3

Molecule: NoName 1347 bps

Analysis: Dyad Symmetries; Num Stem-Loops = 100 (<= -1.1 kcals)

Energy	First	Repeat	Sequence
-10.9	1139	1164	GCCGCUUU
-9.2	959	975	CCGGGG
-8.7	1012	1034	GGGCUUG
-8.5	929	956	CUUGCGCUUU
-8.4	374	409	GGAGGAGGGUG
-8.2	845	882	CGCCUCCU
-7.7	1113	1140	GCAGCGG
-6.7	1115	1144	AGCGGC
-6.5	20	44	CGCGGC
-6.5	302	316	GGGCU
-6.2	310	326	GCAGC
-5.9	1304	1334	GGAGCCA
-5.5	61	106	AGCCACC
-5.5	21	40	GCGGC
-5.3	385	401	GCGAG
-5.2	1002	1019	CUAAGCC
-5.0	1018	1034	GGGC
-4.8	298	361	CCUCGGGC
-4.5	1311	1332	AGCCAG
-4.5	562	591	UAGGAAAGCG
-4.5	283	307	GAGCC
-4.2	935	964	CUUUGGC
-4.1	848	886	CUCCUGC
-4.1	738	760	AAAGGGGC
-4.0	150	167	GCCG
-4.0	691	711	GCGAA
-4.0	51	73	CUUCUGG
-4.0	1116	1140	GCGGC
-3.9	1106	1121	AGCC
-3.8	4	20	GGCU
-3.5	1041	1077	GCUGCU
-3.5	8	31	GAGCU
-3.3	100	113	GGUGG
-3.3	16	31	GAGC
-3.2	1323	1341	CGGAC
-3.2	523	536	GCGU
-3.2	341	375	CCGCC
-3.1	965	984	AGCC
-2.9	1136	1164	GCUGC
-2.8	657	675	CUCUG
-2.8	340	386	GCCGCC
-2.7	353	374	CGCC
-2.7	779	795	GGAG
-2.7	92	113	GGCG
-2.7	35	64	GGCUG
-2.6	1010	1037	CAGGG
-2.5	1028	1048	CGAGC
-2.5	830	876	CUAGCUC
-2.5	90	112	GCGG
-2.4	374	417	GGAGGAGGG
-2.4	17	38	AGCC
-2.4	377	426	GGAGGG
-2.3	10	25	GCUG
-2.2	952	970	GUGG
-2.2	62	74	GCC
-2.1	1100	1113	CAGG
-2.1	81	97	GGUG
-2.0	267	305	GUCCGA
-2.0	606	621	GCC
-2.0	730	745	GCC
-2.0	1006	1021	GCC
-2.0	778	793	AGGAGA
-2.0	1013	1024	GGC
-2.0	385	397	GCGAG
-1.9	103	113	GGC
-1.9	380	399	GGGUG
-1.9	95	105	GCC
-1.8	930	943	UUGC
-1.8	828	845	GUCU
-1.7	353	384	CGCCC
-1.7	970	982	CCC
-1.7	633	649	GGC
-1.6	657	681	CUCUG
-1.6	982	1002	GCUG
-1.6	11	35	CUGAG
-1.6	1011	1052	AGGGC
-1.6	725	757	AAAGCG
-1.6	702	737	CCAGC
-1.6	901	931	AAGGUU
-1.6	72	97	GGCG
-1.6	64	84	CACC
-1.5	971	982	CCC
-1.5	962	973	GGG
-1.5	961	972	GGG
-1.5	714	739	UUCUAG
-1.4	374	469	GGAGGAGGGUGG
-1.4	303	342	GGCUC
-1.4	86	96	GCGC
-1.3	988	1023	GUGUCUA
-1.3	309	322	CGC
-1.3	807	819	UCGC
-1.3	160	173	CGC
-1.3	84	107	GAGC
-1.2	758	785	UUUUUUUUUU
-1.2	583	604	GUUUUUUU
-1.2	1028	1041	CGAGC
-1.2	759	785	UUUUUUUUUU
-1.2	568	579	AGCG
-1.1	975	999	GGAAA
-1.1	5	25	GCUG

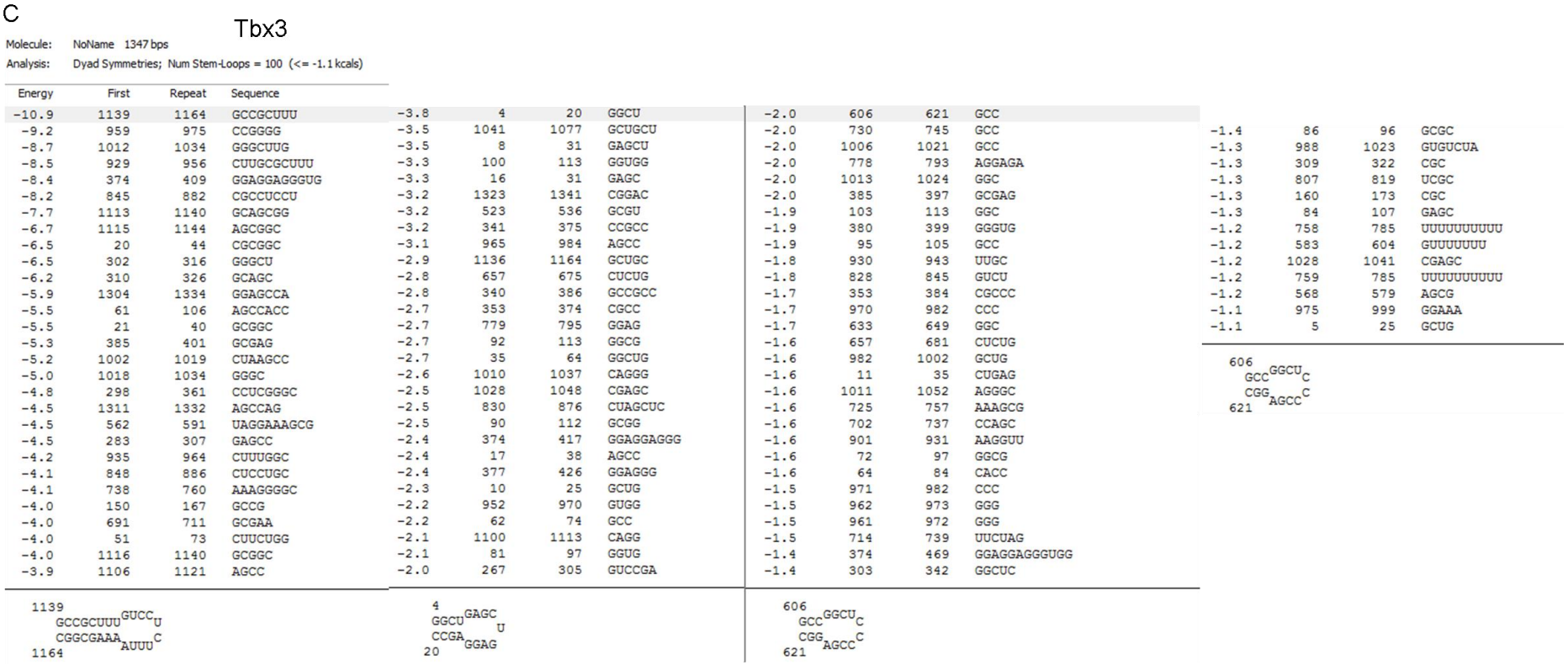


Figure 5.17 Secondary structures in the 5'UTR of Nanog, c-Myc and Tbx3. The number of secondary structures or stem-loops together with the energy required to unwind them in the 5'UTR of *Nanog* (A), *c-Myc* (B) and *Tbx3* (C) is shown. Analysis kindly performed by Benjamin Kumpfmüller.

Translation of specific mRNAs with at least two upstream open reading frames (uORFs) of certain length and position can be increased under stress conditions where the levels of eIF2-ternary complex are low, for example ATF4 and ATF5 (Watatani et al., 2007). This is related to the fact that every round of translation initiation requires binding of the eIF2-ternary complex to the ribosome. In normal conditions the levels of eIF2-ternary complex are high, the majority of ribosomes that finish scanning the uORF1 will get a new eIF2-ternary complex in time to start translation of uORF2 (Figure 5.18 (b)). Consequently, ribosomes that are translating the uORF2 will not be able to translate the ORF of ATF4 or ATF5 because of two reasons. First ribosomes would need backwards scanning, but this is not possible and the second reason is that the uORF2 is too long to permit rescanning. On the other hand, under stress conditions, the eIF2-Ternary complex is low and most ribosomes that finish scanning uORF1 do not get a new eIF2-Ternary complex in time to scan uORF2, but in time to initiate scanning in the ATG of ATF ORF (Figure 5.18 (c)). In this way, ATF4 and ATF5 specific translation is increased under conditions where the cap-depedent or general translation is low.

The 5'UTR of *Nanog* and *Tbx3* was analyzed for the presence of uORFs (Figure 5.19) as this may be a mechanism contributing to the specific increase in translation of their mRNAs. *Nanog* has only one uORF located 59 nucleotides upstream of the Nanog ORF (Figure 5.19 (i)). As mentioned above the presence of at least two uORFs can increase the translation of specific mRNAs. The fact that *Nanog* has only one uORF suggest that translation of *Nanog* is not increased due to presence of uORFs. This is supported by the fact that the uORF of Nanog RNA is not conserved between species (Figure 5.20).

On the other hand, *Tbx3* has four uORFs located 1038, 927, 175 and 83 nucleotides upstream of the Tbx3 ORF respectively (Figure 5.19 (ii)). uORF 1 and 2 are not likely to have an effect on translation of *Tbx3* because they are too far from *Tbx3* ORF. On the other hand, uORF3 and uORF4 are only 175 and 83 nucleotides from the *Tbx3* ORF respectively and they could potentially influence *Tbx3* translation in conditions where the levels of eIF2-ternary complex is low. However, it is unlikely that uORF3 and uORF4 increase translation of *Tbx3* under stress conditions because uORF4 does not overlap with the *Tbx3* ORF (Figure 5.19 (ii)). Although the position

of the uORFs may not increase specific translation of *Tbx3* by themselves, the fact that they are evolutionary conserved (Figure 5.21) suggest that these regions may be important. For example, they may contain sequences for RNA-binding proteins that increase specific translation of *Tbx3*.

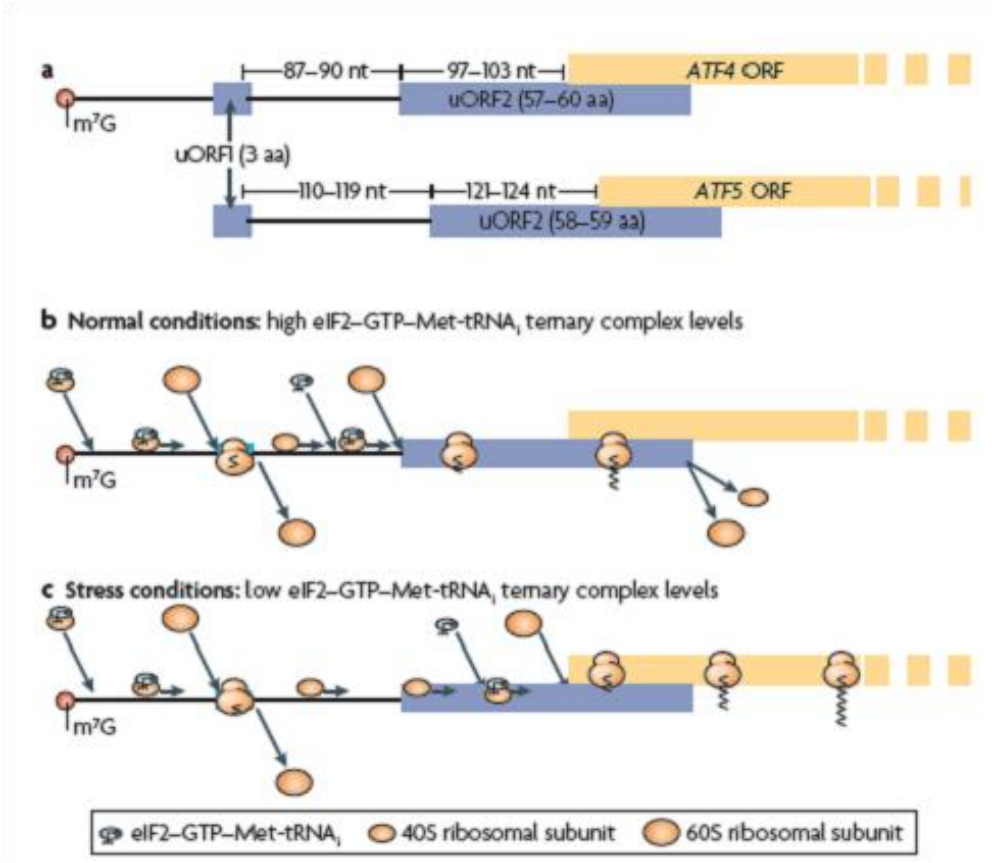


Figure 5.18. Translation of *Atf4* and *Atf5* is regulated by the presence of uORFs. (a) Size, position and spacing of the two uORFs of Atf4 and Atf5 mRNA are shown. (b) In normal conditions, eIF2-Ternary complex is abundant and 40S ribosomal subunits that finished scanning uORF1 get a new eIF2-Ternary complex in time to start scanning the uORF2. As a result, the 40S ribosomal subunits are not able to start scanning the ORF because they can not scan backwards. Under stress conditions (c), where the levels of eIF2-Ternary complex are low, the 40S ribosomal subunits can not acquire a new eIF2-Ternary complex in time to start scanning uORF2 but they do in time to start scanning at the initiation codon of the ORF (Taken from Jackson et al., 2010).

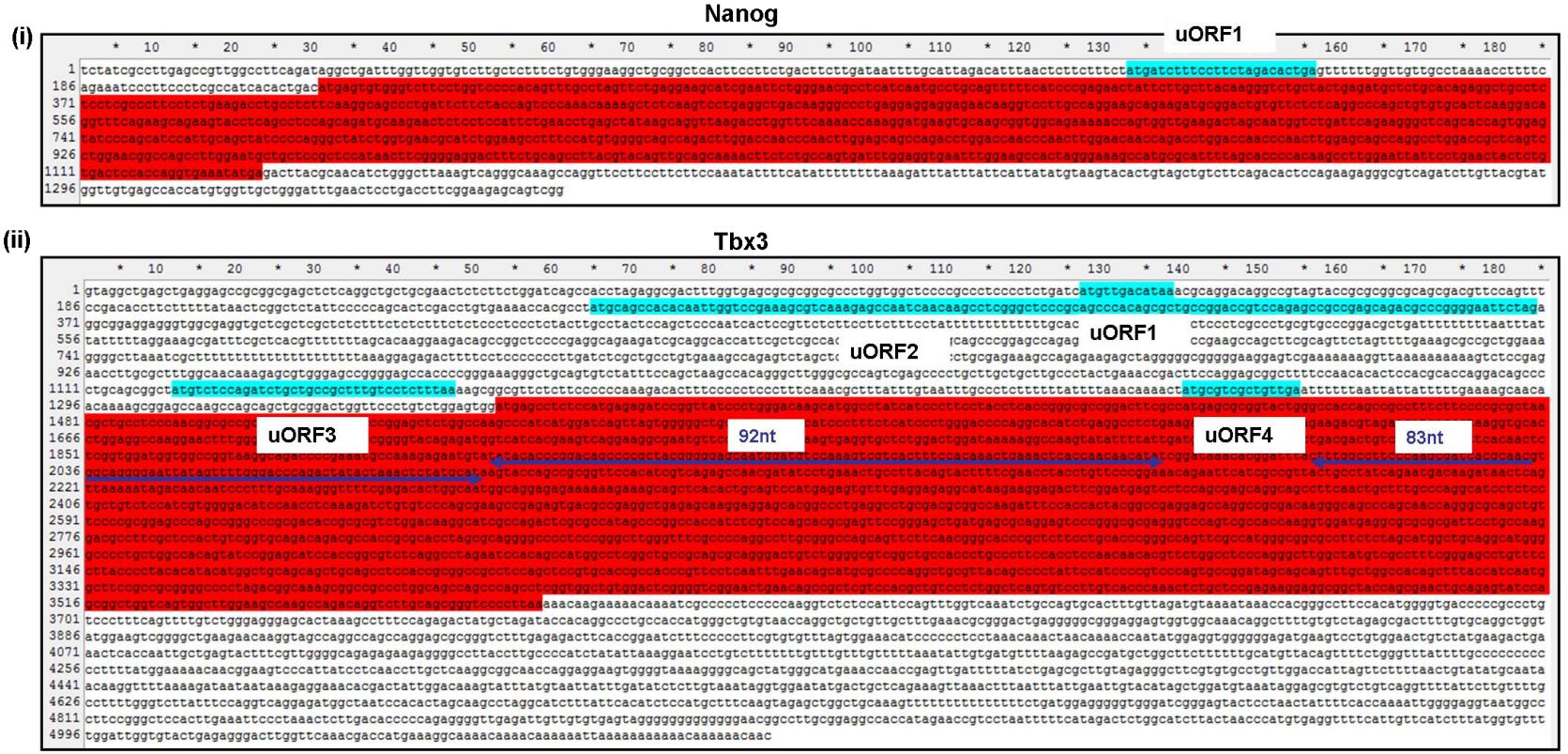


Figure 5.19 Analysis of upstream open reading frames (uORFs) in the 5'UTR of Nanog and Tbx3 RNA. Nanog (i) and Tbx3 (ii) RNA was analysed for the presence of uORFs. The main ORF is highlighted in red and the uORFs are highlighted in blue. (i) Nanog has one uORF 59 nucleotides upstream of the main ORF. (ii) Tbx3 has four uORFs, uORF1 is located 1038 nucleotides upstream of the main ORF, uORF2, uORF3 and uORF4 are located 927, 175 and 83 nucleotides upstream of the main ORF respectively. The distance between uORF1 and uORF2 is 111 nucleotides, between uORF2 and uORF3 is 752 nucleotides, between uORF3 and uORF4 is 92 nucleotides.

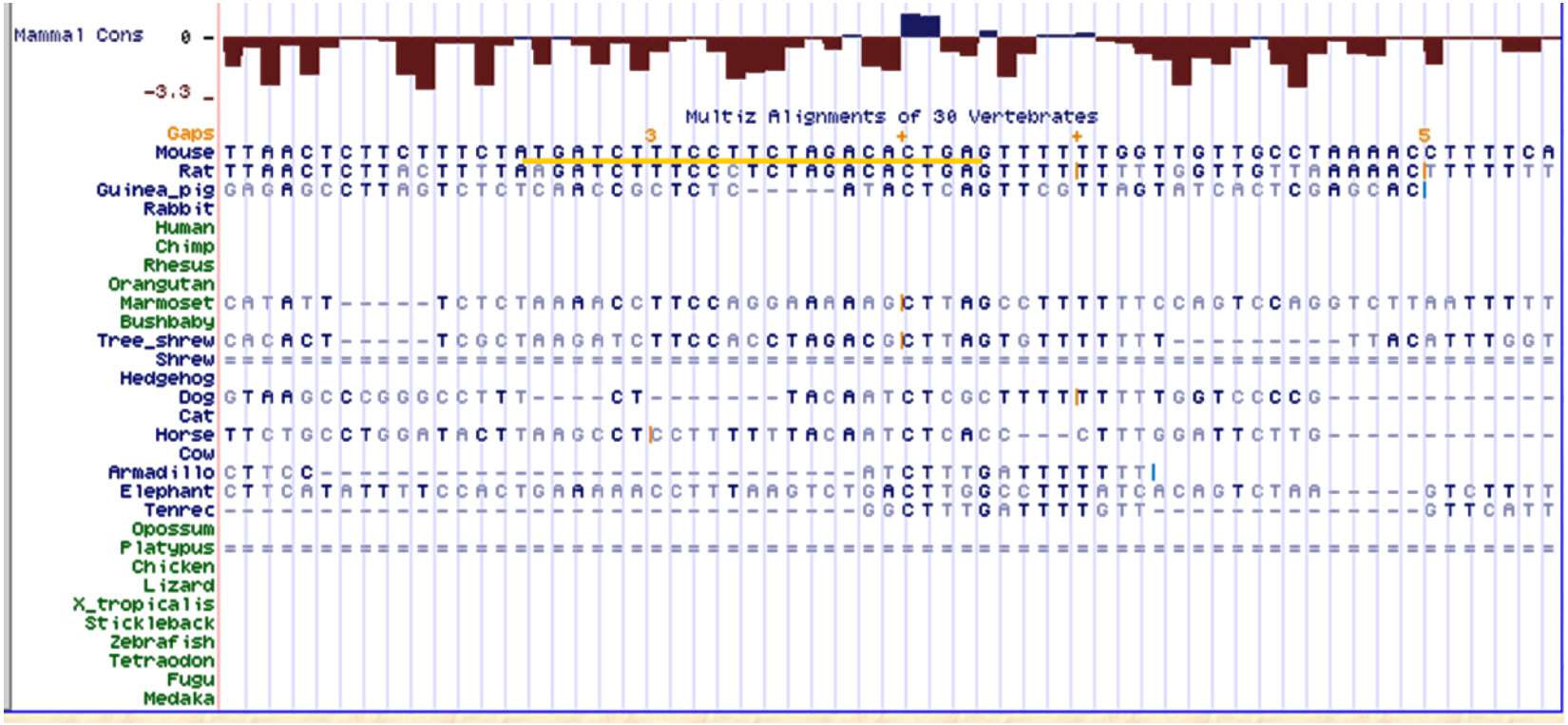


Figure 5.20. Comparison of the uORF of mouse *Nanog* RNA with 30 vertebrate species. The uORF of mouse *Nanog* RNA is underlined in yellow. The bars above *Nanog* mRNA sequence represents consensus between the 30 species compared. Bars above 0 are in blue and represent conservation between species, bars below 0 in brown indicate no conservation.Aligment of the sequences was performed using the University of California Santa Cruz (UCSC) Genome Browser with the help of James Heward.

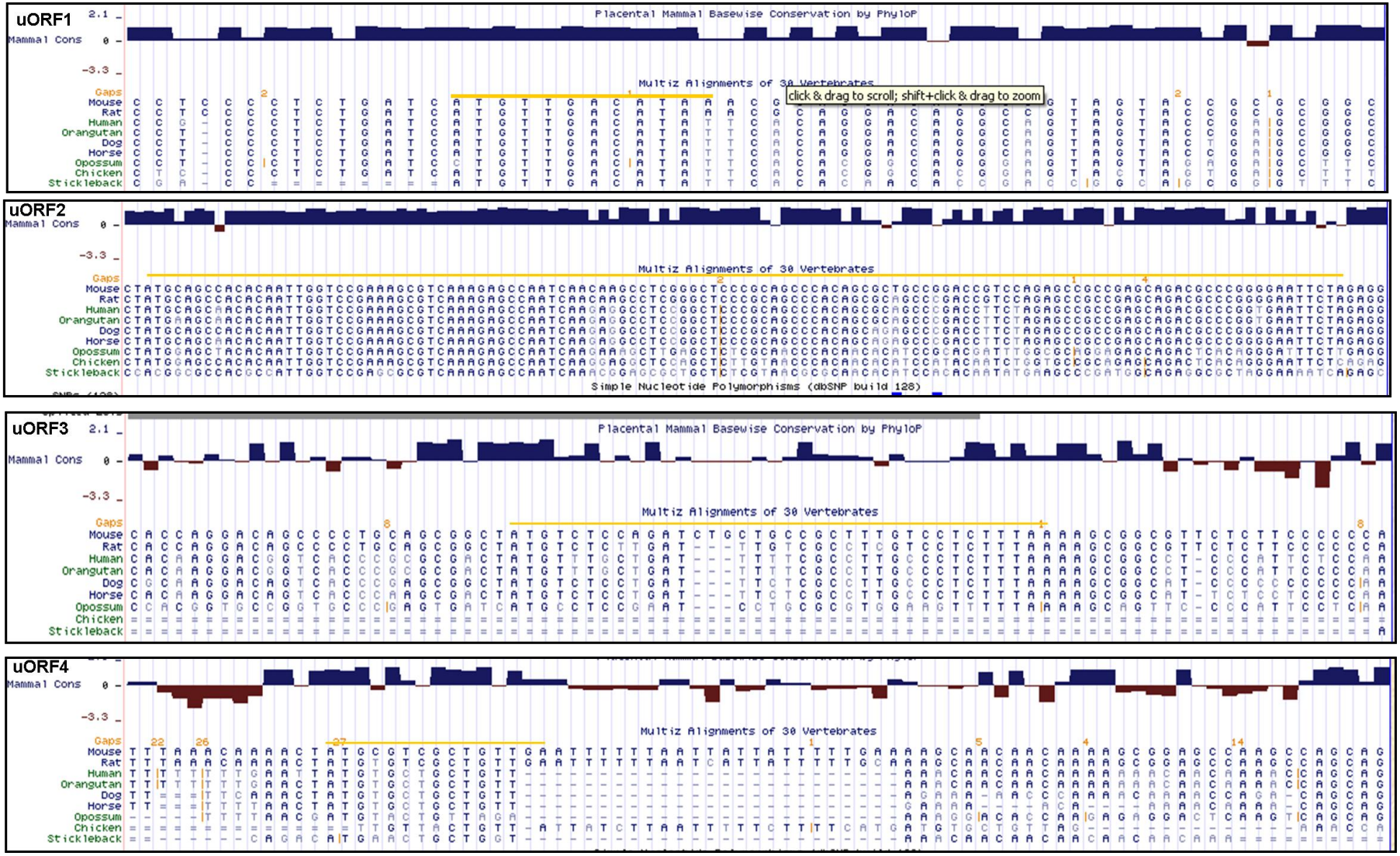

uORF1
Placental Mammal Basewise Conservation by PhyloP
Mammal Cons
Multiz Alignments of 30 Vertebrates
click & drag to scroll; shift+click & drag to zoom
Gaps
Mouse
Rat
Human
Orangutan
Dog
Horse
Opossum
Chicken
Stickleback
uORF2
Multiz Alignments of 30 Vertebrates
Simple Nucleotide Polymorphisms (dbSNP build 128)
uORF3
Placental Mammal Basewise Conservation by PhyloP
Multiz Alignments of 30 Vertebrates
uORF4
Multiz Alignments of 30 Vertebrates

Figure 5.21 Comparison of the uORFs of *Tbx3* mRNA between 30 vertebrate species. The sequence of the uORF1, uORF2, uORF3 and uORF4 of mouse *Tbx3* mRNA were compared with 30 vertebrates species. Bars above 0 are in blue and represent conservation between species; bars below 0 in brown indicate no conservation. uORF1 and uORF2 seem to be more conserved than uORF3 and uORF4. The uORF3 is the uORF that is less conserved between species. Aligment of sequences was performed with the University of California Santa Cruz (UCSC) Genome Browser.

The poly (A) of an mRNA promotes mRNA translation and this is mediated by the association of the poly (A) binding protein (PABP) with the translation initiation factor eIF4G, which interacts with eIF4E and PABP, and circularised the mRNA. This circularisation of the mRNA seems to promote the stabilisation of the translation initiation factors that bind the cap (reviewed in de Moor et al., 2005).

mRNAs with long poly(A) tails (80-500A residues) are often actively translated whereas those with short poly(A) tails (20-50A) are repressed. However, mRNAs with short tails can be polyadenylated and thus actively translated, this is very common during oocyte maturation and early embryo development (Mendez and Richter, 2001). mRNAs subjected to polyadenylation have specific sequences in the 3'UTR including the cytoplasmic polyadenylation element (CPE), which is a U rich element, and the hexanucleotide polyadenylation signal (AAUAAA). CPE binding protein (CPEB) is a protein that binds CPE. Importantly for this study, insulin and progesterone inactivation of GSK-3 leads to activation of Aurora A/Eg2 and phosphorylation of CPEB which in turn recruits polyadenylation specificity factor (CPSF) and CPSF is believed to attract the poly(A) polymerase to the mRNA and adenylation takes place (Sarkissian et al., 2004). Therefore, inhibition of GSK-3 could result in Aurora A activation leading to polyadenylation of *Nanog* and *Tbx3*. Investigating changes in phosphorylation of Aurora A and CPEB would be an indicator of whether polyadenylation occurs following GSK-3 inhibition. If this was the case, the next step would be to investigate the changes in the length of poly (A) following GSK-3 inhibition.

Repression of mRNA translation of specific transcripts can also occur by association of micro RNAs (miRNAs), which are small regulatory RNA molecules, to the 3'UTR. miRNA has complementary base pair to the target mRNAs (Winter et al., 2009). Although there is controversy about how miRNA regulates gene expression, miRNA association to the 3'UTR is thought to result in mRNA degradation or inhibition of translation. The importance of miRNA expression in regulating gene expression is exemplified by the fact that alteration of miRNAs expression is linked to cancer (Esquela-Kerscher and Slack, 2006). For example, c-Myc up-regulation seems to correlate with down-regulation of miRNAs in mouse lymphomas and a number of miRNAs such as Let-7, miR-125b, miR-132 can down-regulate c-Myc

(Bueno et al., 2011). On the other hand, c-Myc can repress some miRNAs including Let-7, mir-15a-16-1, mir-22 and mir-150 (Bueno et al., 2011). miRNAs has also been shown to be important regulators of ESC identity. This is evidence by the fact that knockout of genes involved in maturation of miRNAs such as Dicer or Dgcr8 results in ESC proliferation and differentiation defects (Kanellopoulou et al., 2005; Murchison et al., 2005; Wang et al., 2007). mESCs express the miR-290 and miR-302 clusters (Marson et al., 2008), and their expression is down-regulated as they differentiate. In accordance with a role of these miRNAs in regulating ESC identity, miR-290 and miR-302 can rescue the proliferative and cell cycle defects observed in Dicer and Dgcr8 knockouts ESCs and they are named as embryonic stem cell-cell cycle (ESCC) regulating miRNAs (Sinkkonen et al., 2008; Wang et al., 2008). However, they do not rescue the differentiation defects, which can be rescued by introduction of the Let-7 family of miRNAs (Melton et al., 2010). Let-7 family of miRNAs are expressed at low levels in ESCs and their expression increase as ESCs differentiate where they play a role in the repression of pluripotency transcription factors (Melton et al., 2010). Others miRNAs including miR-134, miR-296, miR-203, miR-200c and miR-183 can down-regulate expression of pluripotency transcription factors (Tay et al., 2008; Wellner et al., 2009).

Pluripotency transcription factors including Nanog, Sox2, Oct4, Tcf3 and Klf4, has been shown to positively regulate the expression of ESCC miRNAs, which in turn, seem to control expression of pluripotency transcription factors by repressing their epigenetic silencing. In this respect, miR-290 has been shown to inhibit Rbl2, and thus decrease expression of DNA methyl-transferases (Viswanathan et al., 2008). Moreover, ESCC miRNAs are thought to promote expression of c-Myc and Lin28 indirectly by repressing an unknown factor that would otherwise inhibit Lin28 and c-Myc (Melton et al., 2010). Pluripotency transcription factors also negative regulate the expression of Let-7 family indirectly by promoting expression of the RNA-binding protein Lin28, which can inhibit Let-7 expression (Viswanathan et al., 2008). c-Myc can also positively regulate ESCC miRNAs and inhibit Let-7 family by promoting Lin28 expression (Melton et al., 2010) (Fig 5.22 A). In summary, ESCC miRNAs are thought to support self-renewal and block differentiation whereas Let-7 promotes differentiation.

GSK-3 inhibition could increase specific translation of *Nanog* and *Tbx3* by down-regulating the expression of miRNAs that repress pluripotency transcription factors (Fig 5.22 B). Alternatively, GSK-3 inhibition could increase expression of ESCC miRNAs leading to increase expression of Lin28, which in turn, down-regulates Let-7 miRNAs and consequently relieving Let-7 inhibition of pluripotent targets. Another option would be that GSK-3 directly increases Lin28 (Figure 5.22 B).

Appart from increasing translation of specific transcripts, increase in ESCC miRNAs could lead to increase transcription of pluripotency transcription factors by inhibiting the epigenetic silencing (Sinkkonen et al., 2008).

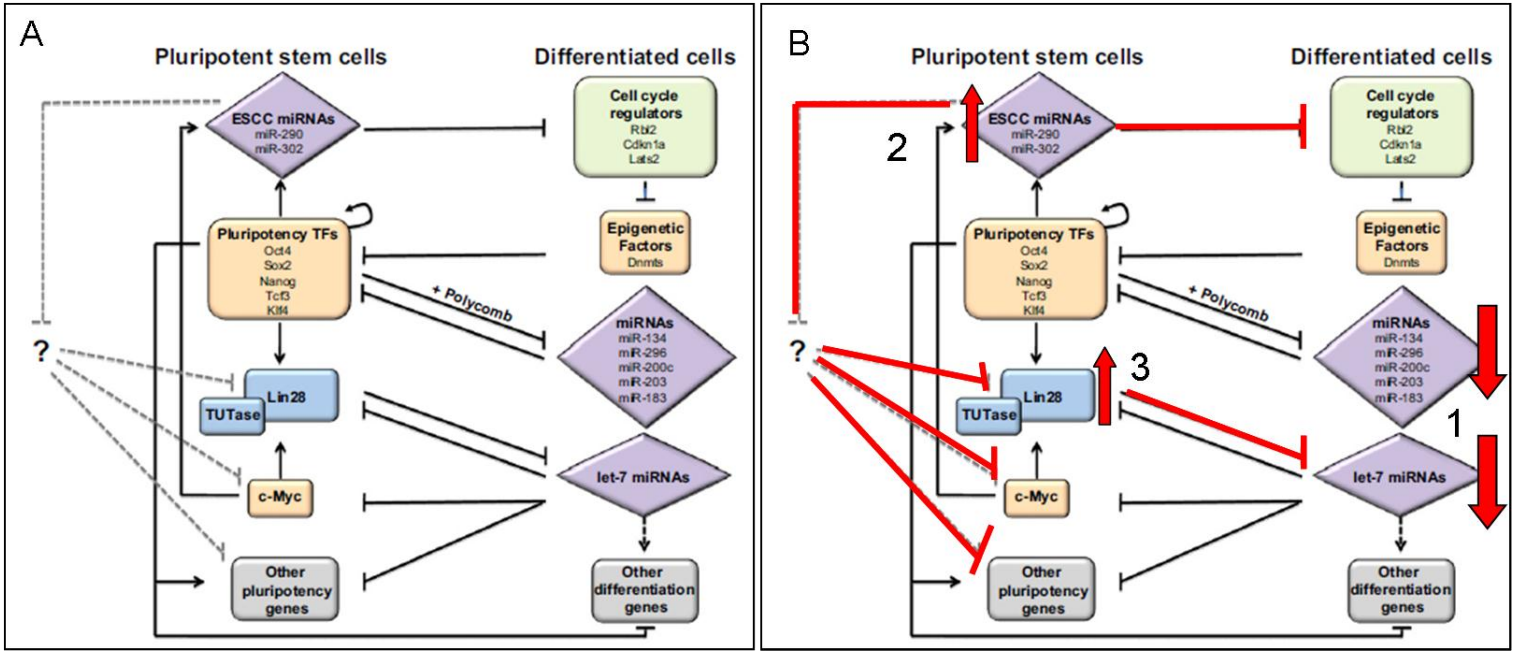


Figure 5.22 Circuit regulating ESC identity. A) Pluripotency transcription factors can promote expression of ESCC miRNAs, which repress an unknown factor that repress Lin28, c-Myc and other pluripotency genes. ESCC also promotes self-renewal by inhibiting epigenetic silencing of pluripotency transcription factors. Let-7 miRNAs repress pluripotency target genes and Lin28 and promote expression of differentiating genes. Other miRNAs including miR-134, miR-296, miR-200c, miR-203 and miR-183 can also repressed pluripotency transcription factors. B) GSK-3 inhibition could contribute to self-renewal in different ways, 1. Down-regulating expression of miRNAs that inhibit pluripotency transcription factrors. 2. Up-regulating expression of ESCC leading to increase Lin28 expression and consequently inhibition of Let-7 miRNAs. 3. Increasing expression of Lin28 and in turn inhibition of Let-7 miRNAs (Modified from Martinez and Gregory, 2010).

Finally, another factor that affects the rate of mRNA translation is the presence of secondary structures in the cap proximal 5'UTR. This is due to the fact that 40S ribosomal subunit binding seems to need a single-stranded RNA and thus unwinding of the secondary structures in the 5' UTR, which is carried out by the eIF4A helicase, is essential for binding of the RNA. Hence, when the levels of the helicase are low, mRNAs that have less secondary structures are expected to be translated at higher rates that those with complex ones. There are several studies supporting this, for example over-expression of eIF4E was shown to increase translation of mRNAs with complex secondary structures (Koromilas et al., 1992). Moreover, dominant-negative eIF4A or inactivation of eIF4B reduced translation of mRNAs with long and structured 5'UTR (Altmann et al., 1995; Svitkin et al., 2001). The present study has shown that phosphorylation of S6K1 at Thr 389 is decreased following GSK-3 inhibition. eIF4B is a downstream target of S6K1 and phosphorylation of eIF4B at Ser422 is likely to be reduced, leading to a decrease in activity. This would affect mRNAs with complex 5'UTR secondary structures and translation of mRNAs with simpler 5'UTR (Figure 5.17), such as Nanog, would increase.

In summary, there are several mechanisms that can contribute to an increase in translation of specific mRNAs (Figure 5.19). *Tbx3*, similar to *c-Myc*, has a complex 5'UTR and its translation could be regulated in a similar fashion. *c-Myc* translation in non-ESC-types has been reported to be achieved by several mechanisms, one of them is IRES and ITAF associated. GRSF1 is one ITAF that increases *c-Myc* translation and interestingly *Tbx3* has binding sites in the 5'UTR for GRSF1. c-Myc can also be regulated by miRNAs and it can itself repress miRNAs. This could be the case also for Tbx3. On the other hand, *Nanog* has much simpler and shorter 5'UTR than *Tbx3* and *c-Myc* and it is unlikely to be translated through IRES-dependent mechanisms. Regulation of Nanog through miRNAs could be possible. *Nanog* translation is very likely to be due to its simple 5'UTR and its reduced requirement for helicase, the activity of which maybe decreased due to a likely decrease in eIF4B. Although *Nanog* does not seem to have binding sites for GRSF1 in its 5'UTR, its translation could be regulated by others RNA-binding proteins. Finally, the translation of *Tbx3* and *Nanog* mRNAs could also be controlled by polyadenylation or uORFs. These possibilities should be further investigated.

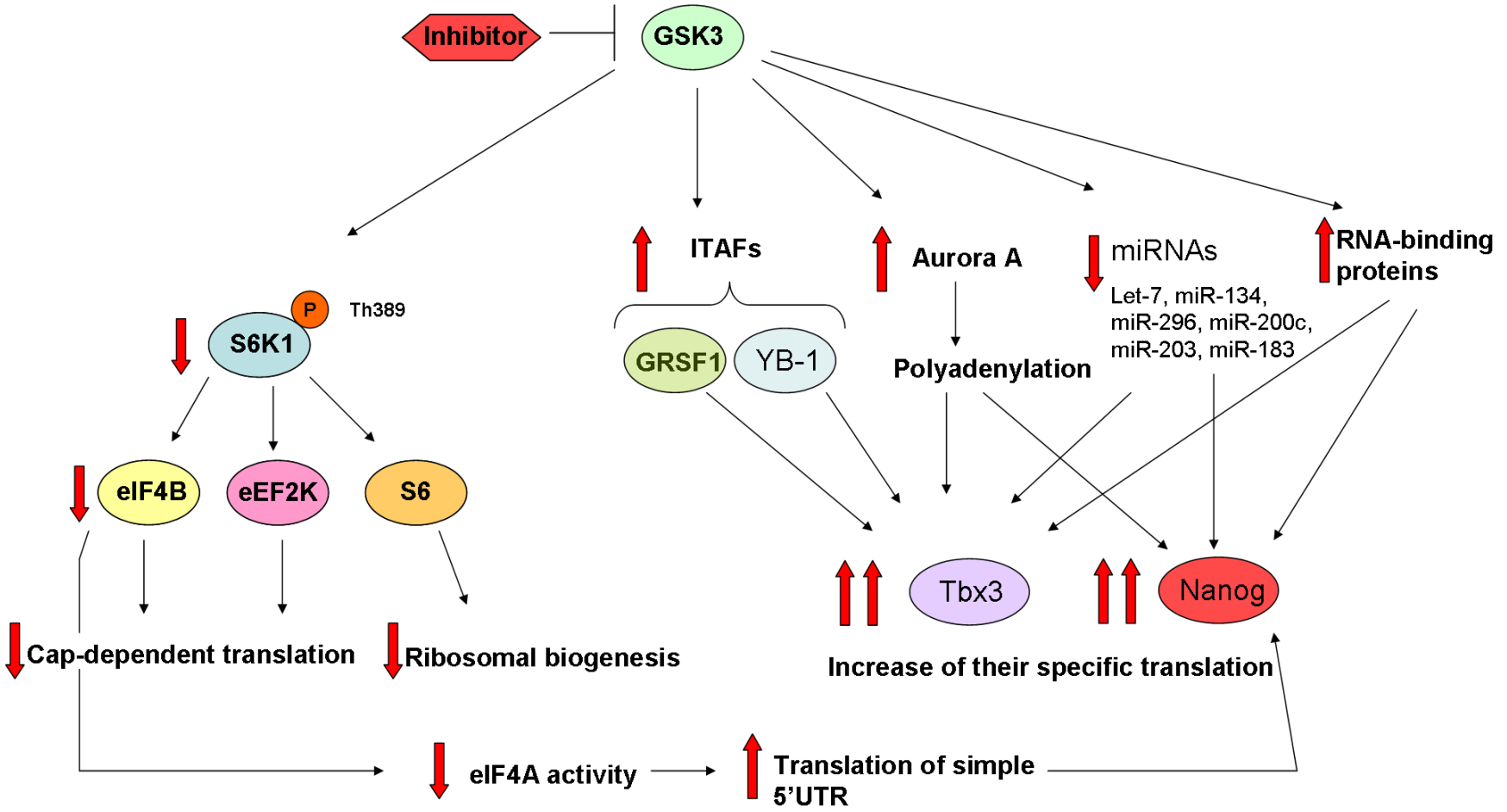

Inhibitor
GSK3
ITAFs
Aurora A
miRNAs
Let-7, miR-134,
miR-296, miR-200c,
miR-203, miR-183
RNA-binding
proteins
P
Th389
S6K1
GRSF1
YB-1
Polyadenylation
eIF4B
eEF2K
S6
Tbx3
Nanog
Cap-dependent translation
Ribosomal biogenesis
Increase of their specific translation
eIF4A activity
Translation of simple
5'UTR

Figure 5.23. GSK-3 inhibition may decrease cap-dependent general translation and increase specific translation of *Tbx3* and *Nanog*. GSK-3 inhibition seems to decrease phosphorylation of Thr389 S6K1 leading to decrease in kinase activity. This is evidence by a decrease in phosphorylation of the downstream target eEF2K. The phosphorylation and in turn the activity of other S6K1 downstream targets including eIF4B and S6 are likely to be decreased and consequently cap-dependent translation and ribosomal biogenesis is decreased. Specific translation of *Tbx3* and *Nanog* could be increased by different mechanisms including increase in ITAFs, GRSF1 and YB-1, or other RNA-binding proteins that may promote *Tbx3* and *Nanog* translation, stabilisation of Aurora A leading to increase polyadenylation and decrease in miRNAs that repress Tbx3 and Nanog. Nanog translation could also be increased because of its simple 5'UTR structure.

6 CHAPTER: GENERAL DISCUSSION AND FUTURE DIRECTIONS

6.1 Final discussion and future directions.

ESCs have two unique and remarkable properties, self-renewal and pluripotency, that together make them very attractive for use in different fields including regenerative medicine, drug development and toxicity screening, as well as a model system to study early development. However, in order for the potential of ESCs to be realised, we must understand the molecular mechanisms controlling their self-renewal, maintenance of pluripotency and their differentiation. There are several pathways regulating mouse ESC self-renewal that are activated by extrinsic stimuli and regulate expression of transcription factors (Boiani and Scholer, 2005). One molecule with a role in mouse ESCs is GSK-3. GSK-3 inhibition was first reported to lead to neuroectoderm differentiation (Ding et al., 2003) and one year later, was shown to maintain self-renewal of ESCs (Sato et al., 2004). After these initial reports, several publications, including our own, have reported that inhibition or deletion of GSK-3 contributes to maintenance of self-renewal (Bone et al., 2009; Doble et al., 2007; Sato et al., 2004; Ying et al., 2008). GSK-3 inhibition was also shown to promote ESC differentiation to mesendoderm lineages (Bakre et al., 2007).

Although ESCs cultured in the presence of GSK-3 inhibitors, BIO or CHIR, have been shown to maintain their pluripotency by contributing to chimeras and generating teratomas containing derivatives of the three germ layers following withdrawal of the inhibitors (Sato et al., 2004; Ying et al., 2008), DKO GSK-3 cells exhibited abnormal differentiation potential in EBs or teratocarcinomas (Doble et al., 2007). Therefore, one of the aims of this study was to investigate whether ESCs treated with novel GSK-3 selective inhibitors, 1m and 1i (Bone et al., 2009), kept their pluripotency following withdrawal of the inhibitors. Another aim was to investigate the effects of GSK-3 inhibition on differentiation. Finally, the mechanism of action by which GSK-3 inhibition contributes to maintenance of self-renewal was also investigated.

6.2 ESCs maintain pluripotency following GSK-3 inhibition.

Prior to this work, we showed that inhibition of GSK-3 with 1i and 1m enhances self-renewal in the presence of LIF and Serum (Bone et al., 2009). However, it was considered essential to ensure that these novel GSK-3 inhibitors were not increasing ESC self-renewal because they were irreversibly blocking the ability of ESCs to differentiate. Expression of pluripotency markers by ESCs grown in the presence of GSK-3 inhibitors and their down-regulation in differentiating conditions suggested that inhibitor-treated ESCs maintained their pluripotency. This was further supported by the ability of ESC to differentiate into the three germ layers, judged on the basis of lineage marker expression. These findings are in agreement with previous reports showing that ESCs maintained their pluripotency after GSK-3 inhibition with BIO or CHIR (Sato et al., 2004; Ying et al., 2008). Further experiments to confirm maintenance of self-renewal following removal of 1m or 1i should include testing the ability of ESC to form teratomas or to contribute to chimeras.

6.3 Inhibition of GSK-3 drives differentiation towards mesendodermal lineages.

Prior to this work, several studies reported that GSK-3 inhibition had an effect on the multi-lineage differentiation of ESCs. Although there was controversy about the effect of GSK-3 inhibition on differentiation of ESCs, most studies agreed that GSK-3 inhibition had a negative effect on neuro-differentation (Aubert et al., 2002). Only one study reported that GSK-3 inhibition promoted neural differentiation (Ding et al., 2003). We observed that GSK-3 inhibition seemed to promote differentiation towards mesendodermal lineages. *Brachyury* (a mesendodermal and early mesodermal marker) expression was up-regulated in the presence of GSK-3 inhibitors, observed by both RT-PCR from EBs-derived RNA and using a *Brachyury*-GFP reporter cell line grown in monolayer differentiating conditions. Hence, our results are in agreement with several reports published before our work commenced (Bakre et al., 2007; Aubert et al., 2002) and two reports published after this study that showed that GSK-3 inhibition promotes non-neural differentiation, as well as blocking neural differentiation (Ying et al., 2008) and promotes mesendoderm differentiation while inhibiting neuroectoderm lineage differentiation

(Thomson et al., 2011). From the present study is not clear whether GSK-3 inhibition also promotes mesoderm and/or endoderm differentiation. *Brachyury* up-regulation could suggest induction of mesoderm but its expression is not restricted to mesoderm since it is also expressed in the mesendoderm and primitive streak. Other mesoderm or endoderm markers, expressed later during embryogenesis, should be used in order to investigate promotion of mesodermal or endodermal differentiation.

The fact that ESC differentiation can be driven towards a specific cell type is of great relevance not only in regenerative medicine for cell therapy but also for toxicity screening of new drugs. Indeed, the small molecule inhibitor, 1m, has been used to direct differentiation of human ESCs into definitive endoderm (Bone et al., 2011). GSK-3 inhibition in human ESCs grown in conditions that maintain self-renewal, promoted differentiation first into primitive streak, then mesendoderm and towards both mesoderm and definitive endoderm. Moreover, the definitive endoderm had the ability to mature into hepatoblast-like cells (Bone et al., 2011).

The fact that GSK-3 inhibition in human ESCs drives differentiation into definitive endoderm contrasts with the effect of GSK-3 inhbition in mESC where it enhances self-renewal. The different outcome of GSK-3 inhibition in mESC and hESC could be due to the fact that they are thought to be derived from two different stages of development. hESC have characteristics more similar to mouse epiblast stem cells (EpiSCs), which are derived from the mouse post-implantation epiblast, than to mouse ESCs, that are derived from the pre-implantation epiblast. Interestingly, mouse pre-implantation epiblast with constitutively active β-catenin develops normally, but after implantation the epiblast expresses Brachyury in the embryo ectoderm layer suggesting that constitutively active β-catenin promotes differentiation into mesodermal lineages (Kemler et al., 2004). If hESC are more like EpiSCs than ESCs, the fact that GSK-3 inhibition promotes differentiation to mesoderm and endoderm is in agreement with promotion of the post-implantation epiblast to mesoderm fate in mouse when β-catenin is constitutively active. Furthermore, although GSK-3 inhibition in self-renewal conditions promotes self-renewal in mouse ESCs, GSK-3 inhibition following LIF withdrawal in mESC also

seems to promote mesendodermal differentiation. This is consistent with the fact that following LIF withdrawal ESCs differentiate, first becoming epiblast stem cells.

Although the effect of GSK-3 inhibition in mESC and hESC is different, mESC can still be used as a model to study differentiation of ESC for later extrapolation in humans.

6.4 Mechanism of action of GSK-3 in mESCs.

Although several reports, including ours (Bone et al., 2009), agreed with the role of GSK-3 in enhancing self-renewal of mESCs, the mechanism of action of GSK-3 in this situation was not fully understood. Some light has been shed recently by several reports suggesting that the effect of GSK-3 inhibition on self-renewal is at least partly due to Wnt/β-catenin regulating expression of the pluripotency network (Wray et al., 2011; Yi et al., 2011). The present study suggests an alternative mechanism that can contribute to enhancement of self-renewal, but which does not contradict the reports mentioned above. We tested the hypothesis that GSK-3 may enhance self-renewal by regulating expression of pluripotency-associated transcription factors including Nanog, Tbx3, c-Myc, Zscan4 and Oct4 in different culture conditions. Some differences were observed regarding the regulation of GSK-3 inhibition of c-Myc and Zscan4 transcription factors in different culture conditions. However, GSK-3 inhibition could regulate expression of Nanog and Tbx3 proteins in all the media conditions tested. In order to investigate the mechanism of action by which GSK-3 regulates these changes, we tested the hypothesis that Nanog and Tbx3 protein up-regulation was due to an increase in mRNA transcription. Interestingly, although Nanog and Tbx3 mRNAs were elevated following GSK-3 inhibition or in GSK-3 DKO cells, these increases were modest in comparison with the increases in protein levels observed, indicating than another mechanism, apart from transcription, was likely to account for the increase in Nanog and Tbx3 proteins. However, changes in Nanog and Tbx3 protein stability were not altered following GSK-3 inhibition in any of the conditions tested, implying changes in stability did not account for the increases observed.

A recent paper suggested that translational control may regulate ESC fate choice (Sampath et al., 2008) and regulation of translation is known to be key during early development and differentiation, where it has a role in fine-tuning gene expression (Mathews et al., 2000). In addition, Nanog protein is downregulated earlier than *Nanog* RNA when cells are treated with the PI3K inhibitor LY294002 and PI3K is known to regulate GSK-3 (Storm et al., 2007). On the basis of our results and these reports, the hypothesis that GSK-3 inhibition increases *Nanog* and *Tbx3* translation was tested. GSK-3 seems to increase *Nanog* translation because Nanog protein re-synthesis was accelerated when GSK-3 was inhibited or in GSK-3 DKO cells and the increase in protein occured without a preceding increase in mRNA. Moreover, the proportion of *Nanog* mRNA bound to polysomes was also higher following GSK-3 inhibition. On the other hand, results obtained with Tbx3 were not conclusive and further experiments should be carried out. In particular, protein re-synthesis experiments should be optimised to look at Tbx3 protein recovery because they were initially optimised to investigate Nanog protein recovery and our data then demonstrated that Tbx3 has a longer half-life than Nanog. Therefore, protein resynthesis experiments for Tbx3 could be optimised by treating the cells for longer with CHX in order to reduce its protein prior CHX washed-out. Tbx3 protein synthesis could also be studied by using radioisotopes. It would also be interesting to investigate *Tbx3* mRNA in protein re-synthesis experiments.

GSK-3 inhibition could potentially increase the expression and translation of other transcription factors that promote self-renewal such as *Sox2* or *Klf4* or decrease translation of transcription factors that repress self-renewal such as *Tcf3*. Moreover, it could also decrease translation of early differentiating markers including *Fgf5*, *Sox1* and *Brachyury* making cells more resistant to differentiation. This should be further investigated for example by investigating proportion of mRNA bound to polysome versus monosome.

The present study suggests that GSK-3 inhibition may contribute to enhancement of self-renewal by increasing translation of *Nanog*, possibly *Tbx3* and potentially other pluripotent transcription factors by a mechanism that could be partly independent of β-catenin-Tcf transcriptional activation. GSK-3 has many downstream effectors including protein synthesis initiation factors, transcriptional regulators and

components of the cell-division cycle (reviewed in Kim et al., 2006; Frame and Cohen 2001; Doble and Woodgett, 2003). Moreover, although two recent reports agree that the major mechanism of GSK-3 inhibition is β-catenin stabilisation and interaction with Tcf3, abrogating its repressive activity on the pluripotency network, they also agreed in that Tcf-independent mechanisms can have a small contribution in the effect of GSK-3 inhibition/Wnt activation (Yi et al., 2011; Wray et al., 2011). Although a recent report proposes that the effect of Wnt signalling is mediated by a Tcf-independent mechanism by which stabilisation of β-catenin binds to Oct4 enhancing its activity (Kelly et al., 2011), Yi *et al.*, did not observed Oct4–β-catenin dependent recruitment to chromatin (Yi et al., 2011). Thus, it could be possible that GSK-3 inhibition acts through an alternative Tcf-independent mechanism, which could be β-catenin dependent or independent.

The present study has shown that GSK-3 inhibition promotes Nanog protein synthesis and translation. However, we have not investigated whether this effect is through a β-catenin-dependent or independent mechanism. There is evidence suggesting that β-catenin-independent mechanisms downstream of GSK-3 may also play a part in maintaining self-renewal (Ying et al., 2008; Wray et al., 2011; Storm et al., 2007; Bechard and Dalton et al., 2009). Thus, it would be very interesting to investigate whether the effect we observed on *Nanog* translation is β-catenin dependent or independent. This could be studied by performing Nanog protein re-synthesis and polysomal experiments in β-catenin null cells, which have recently been generated by several groups (Lyashenko et al., 2011). However, the effect of knocking out β-catenin is also controversial since Lyashenko et al. and Wray et al., showed that null β-catenin still self-renew, while others reports indicate that β-catenin null cells may differentiate to EpiSCs (Anton et al., 2007).

Although the present study has focussed on exploring the possibility that GSK-3 inhibition regulates pluripotency-associated transcription factors at the transcriptional, protein stability and translational levels, it is possible that GSK-3 can also regulate the epigenetic state of these genes. In particular, a recent report proposed that *Nanog* epigenetic silencing in iPSC can be decreased by knockout of *Ezh2*, which is responsible for generating the silencing epigenetic marks H3K27me3

(Margueron and Reinberg, 2011). Interestingly, the outcome of deleting *Ezh2* is very similar to GSK-3 inhibition. *Ezh2* null cells increase the percentage of the high-Nanog population and cells are more resistant to differentiation (Villasante et al., 2011). It could, therefore, be possible that GSK-3 inhibition leads to a decrease of *Ezh2*, leading to a subsequent decrease in *Nanog* epigenetic silencing and increase in self-renewal. It would be interesting to explore this possibility.

To summarise, GSK-3 inhibition has been shown to enhance self-renewal by a β-catenin-dependent mechanisms, which involve inhibition of Tcf-3 and alleviation of its transcriptional repression of the pluripotency network (Wray et al., 2011; Yi et al., 2011) and increase in Tcf1 activity (Yi et al., 2011). We propose that GSK-3 inhibition can also contribute to enhancement of self-renewal by a β-catenin dependent or independent mechanism through an increase in translation of specific pluripotency-associated transcription factors including *Nanog*, maybe *Tbx3* and others, which in turn would feed into the pluripotency network. Finally GSK-3 inhibition could also decrease epigenetic silencing of *Nanog* and other pluripotent transcription factors (Figure 6.1).

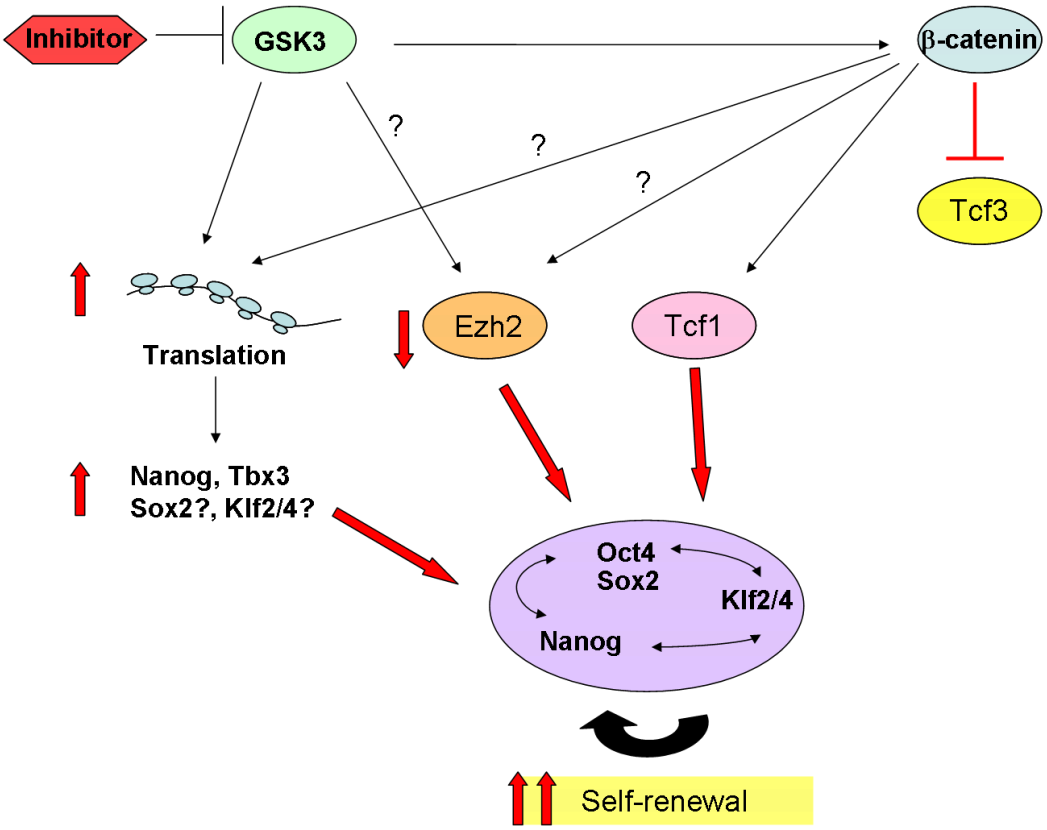


Figure 6.1 GSK-3 proposed mechanisms of action. GSK-3 inhibition can stabilise β-catenin and abrogate Tcf3 repressive activity on the pluripotency network but it could also activate Tcf1 promoting self-renewal. GSK-3 inhibition also leads to an increase in *Nanog* and possibly *Tbx3* translation and maybe others, which in turn would feed into the pluripotency network. GSK-3 inhibition could reduce *Ezh2* reducing epigenetic silencing of pluripotent transcription factors. The increase in translation and decrease in *Ezh2* could be β-catenin dependent or independent.

6.5 Are general translation rates affected by GSK-3 inhibition?

The present study has shown that GSK-3 inhibition seems to promote translation of *Nanog* and also possibly *Tbx3* and we tested the hypothesis that the increase in mRNA translation observed in pluripotency-associated transcription factors following GSK-3 inhibition was due to an increase in general (cap-dependent) translation. Changes in general translation following ESC differentiation into EBs have previously been reported (Sampath et al., 2008). GSK-3 downstream of PI3K has been reported to regulate translation by phosphorylating Ser539 of eIF2Bε in non-ESC types (Welsh et al., 1997; Welsh et al., 1998); therefore we tested the hypothesis that inhibition of GSK-3 leads to a decrease in phosphorylation of Ser539eIF2Bε and an increase in translation. However, changes in phosphorylation of Ser539 following GSK-3 inhibition or in GSK-3 DKO cells were not observed, suggesting that cap-dependent translation was not affected. Nevertheless, there are other regulators of translation that could be affected upon GSK-3 inhibition and we next investigated possible changes in phoshorylation of Ser51 eIF2α because a decrease in phosphorylation of Ser51 of eIF2α was suggested following 24hour treatment with the GSK-3 inhibitor 1i in a Kinexus antibody microarray previously performed (Bone et al., 2009). A decrease in phosphorylation of Ser51 eIF2α would increase translation initiation of most RNAs (Day and Tuite, 1998; Goss et al., 1984). Phosphorylation of Ser51 eIF2α was not dramatically affected following GSK-3 inhibition suggesting that cap-dependent translation is not affected. However, Wnt signalling, through inhibition of GSK-3, has been implicated in indirect regulation of mTOR through TSC2 (Goss et al., 1984; Inoki et al., 2006) and we tested whether GSK-3 inhibition increases cap-dependent translation through stimulation of mTOR activity. Changes in phosphorylation of the mTOR downstream effectors 4E-BP1 and S6K1 were investigated following GSK-3 inhibition to assess mTOR activity. mTOR activity did not seem to increase following GSK-3 inhibition as phosphorylation of Thr389 on S6K1 declined and preliminary results suggest that phosphorylation of 4E-BP1 is not dramatically altered. Moreover, phosphorylation of mTOR itself at Ser2481, the autoregulatory phosphorylation site that reflects mTOR catalytic activity (Soliman et al., 2010), was modestly increased following inhibition of GSK-3 indicating that mTOR activity may be slightly increased. Although the decrease in phosphorylation of Thr389 of S6K1 would suggest a decrease in mTOR

activity, Thr389 can be phosphorylated by other kinases apart from mTOR including PDK1 and Akt/PKB and S6K1 can also autophosphorylate itself, raising the possibility that GSK-3 inhibition decreases the activity of PDK1 and Akt/PKB, and subsequently phosphorylation of S6K1. Phosphorylation of Akt at Ser473 did not change following GSK-3 inhibition (results not shown) indicating that Akt/PKB activation is not altered. However, phosphorylation of Akt at Th308 should also be examined as it is also needed for full activation. A decrease in PDK1 activity should be investigated because it could be possible that there is a feedback regulatory loop between PDK-1 and GSK-3. The ability of PDK-1 to phosphorylate and activate downstream effectors such as Akt/PKB relies on its recruitment to the plasma membrane through a pleckstrin homology (PH) domain that binds the intracellular second messengers $PI(3,4)P_2$ and $PI(3,4,5)P_3$, which are products of activated class I PI3Ks (Anderson et al., 1998; Klippel et al., 1997; Vanhaesebroeck and Alessi, 2000) Hence, an experimental approach to study whether activation of PDK-1 decreases following GSK-3 inhibition would be to check whether its levels are decreased at the plasma membrane.

Another possible explanation for the decrease in S6K1 Thr389 phosphorylation could be that GSK-3 is directly phosphorylating Thr389 S6K1. Although GSK-3 is not known to regulate S6K1, S6K1 can phosphorylate GSK-3 under certain conditions (Zhang et al., 2006a). It could, therefore, be possible, similar to what I proposed for PDK1, that there is a feedback regulatory mechanism whereby GSK-3 phosphorylates S6K1. The ability of GSK-3 to directly phosphorylate Thr389 S6K1 could be tested by performing an *in vitro* kinase assay.

This study suggest that GSK-3 inhibition may decrease the cap-dependent translation and although this is the opposite what was expected, since Nanog and Tbx3 translation seem to be increased following GSK-3 inhibition, a decrease in general translation would be in accordance with the work of Sampath *et al.,* because they observed an increase in general translation during ESC differentiation (Sampath et al., 2008). Therefore, it is reasonable to think that an enhancement of self-renewal observed following GSK-3 inhibition could lead to a decrease in general translation. Electron microscopy could be used to further study a possible decrease in general translation, for example a decrease in general translation would lead to a decrease in

the content of Golgi apparatus and rough endoplasmic reticulum because they are involved in protein synthesis. Consequently, the cytoplasmic volume would also decrease. Sampath et al., used electron microscopy to investigate these types of changes as ESCs differentiate (Sampath et al., 2008).

The fact that translation of other genes, including *Oct4* and *Cyclin D1*, are not increased following GSK-3 inhibition, suggests that the increase in translation observed with *Nanog* and *Tbx3* is specific. Translation of specific mRNA transcripts, without an increase in general translation or in conditions where the cap-dependent translation is compromised, can occur via a number of different mechanisms.

Specific translation of *Tbx3* could be through increases in IRES translation, which can be regulated by proteins that bind the internal initiation site and are named IRES trans-acting factors (ITAFS). It could be possible that GSK-3 inhibition increases the levels of some ITAFs. Of relevance to this study is the fact that the ITAF, guanine-rich RNA sequence binding factor 1 (GRSF-1), which promotes translation of target genes (Kash et al., 2002; Park et al., 1999), was identified as a Wnt/β-catenin downstream target (Lickert et al., 2005). This raises the possibility that GSK-3 inhibition, which mimics Wnt activation, leads to up-regulation of GRSF-1 promoting translation of mRNA targets. Interestingly, Tbx3 has a binding site for GRSF-1 in its 5'UTR and thus Tbx3 may be a GRSF-1 target. GRSF-1 together with YB-1 (Y-box binding protein) and P54nrb were identified as ITAFs that associate with the IRES of c-Myc by affinity chromatography (Cobbold et al., 2008). It could, therefore, be possible that YB-1 and p54nrb also associate to Tbx3 promoting its translation. An indication of whether GRSF1, YB-1 or p54nrb could increase translation of *Tbx3* would be to check whether their levels are elevated following GSK-3 inhibition. However, this would only be an indication, ultimately affinity chromatography or co-immunoprecipitation studies should be carried out to check whether these proteins or others are associated with Tbx3. On the other hand, *Nanog* mRNA does not have GRSF1 binding sites and the 5'UTR is much simpler than those of *c-Myc* and *Tbx3* so it is unlikely to be translated through IRES because IRES are highly structured (Komar and Hatzoglou, 2005). However, its translation could be regulated by other mechanisms such as the presence of structural features or regulatory sequences in the 5′ or 3'UTR of its mRNA. (Gray and Wickens, 1998).

This hypothesis could be experimentally tested by replacing the 5' or 3'UTR of a gene, whose translation does not change following GSK-3 inhibition, for the 5' or 3'UTR of *Nanog*. It would be interesting to determine not only if the 5' or 3'UTR of *Nanog* and *Tbx3* increase specific translation but also which UTR is responsible for the increase because this will help to elucidate the mechanism whereby translation is increased. There are mechanisms that regulate translation which are specifically associated to the 5'UTR and some to the 3'UTR.

The 3'UTR contains specific sequences for binding of miRNAs and also binding of cytoplasmic adenylation element (CPE) and thus they control translation of specific transcripts through repression of their mRNA translation or polyadenylation. On the other hand, features that increase translation of specific mRNAs in the 5'UTR include the presence of upstream open reading frames, the presence of secondary structures, RNA-binding proteins and as mentioned above the presence of IRES. The hypothesis that GSK-3 inhibition regulates translation of specific transcripts by controlling one or several of these mechanisms could be tested experimentally.

To begin with, whether GSK-3 inhibition leads to mRNA polyadenylation could be investigated as follows. Initially, *Nanog* and *Tbx3* should be checked for the presence of the cytoplasmic polyadenylation element (CPE), which is a U-rich element and the hexanucleotide polyadenylation signal (AAUAAA). CPE binding protein (CPEB) binds CPE. Insulin and progesterone inactivation of GSK-3 leads to activation of Aurora A/Eg2 and phosphorylation of CPEB which in turn recruits polyadenylation specificity factor (CPSF) and CPSF is believed to attract the poly(A) polymerase to the mRNA and adenylation takes place (Sarkissian et al., 2004). Therefore, inhibition of GSK-3 could result in Aurora A activation leading to polyadenylation of *Nanog*, *Tbx3* and maybe others transcripts. If for example *Nanog* has a CPE, the next step would be to investigate changes in phosphorylation of Aurora A and CPEB following GSK-3 inhibition. If GSK-3 inhibition leads to activation of Aurora and subsequent activation of CPEB, the next step would be to investigate the changes in the length of poly (A) following GSK-3 inhibition.

GSK-3 inhibition could also lead to down-regulation of miRNAs that repress translation of *Nanog*, *Tbx3* and maybe others. In particular, it could decrease the

expression of Let-7 family of miRNAs that seem to repress pluripotency transcription factors (Melton et al., 2010). A decrease in Let-7 family following GSK-3 inhibition should be investigated. Alternatively, GSK-3 inhibition could increase expression of embryonic stem cell-cell cycle (ESCC) miRNAs, which promote the expression of transcription factors by repressing their epigenetic silencing. In this respect, miR-290 has been shown to inhibit Rbl2, and thus decrease expression of DNA methyl-transferases (Viswanathan et al., 2008). Increase of ESCC miRNAs also leads to increase in the RNA-binding protein Lin28, which inhibits Let-7 miRNAs. Finally, GSK-3 inhibition could also directly increase the levels of Lin28. All these possibilities could be investigated by looking at the levels of miRNAs and Lin28.

Translation of specific mRNAs with at least two upstream open reading frames (uORFs) of certain length and position can be increased under stress conditions where the levels of eIF2-ternary complex are low, for example ATF4 and ATF5 (Watatani et al., 2007). The 5'UTR of *Nanog* and *Tbx3* was analyzed for the presence of uORFs. The fact that *Nanog* only has one uORF, which is not conserved between species suggest that *Nanog* translation is not increased due to the presence of uORFs. On the other hand, the position of *Tbx3* uORFs suggests that *Tbx3* uORFs may not influence *Tbx3* translation. However, *Tbx3* uORFs seem to be evolutionary conserved raising the possibility that they may contain important regulatory regions such as sites for RNA-binding proteins.

Finally, the presence of secondary structures in the 5'UTR of an mRNA decreases its translation as binding to the 40S ribosomal subunit seems to need a single-stranded RNA and the eIF4A helicase is involved in unwinding secondary structures. Hence, if the levels of the helicase are low, mRNAs that have less secondary structures are expected to be translated at higher rates that those with complex ones. There are several studies supporting this (Altmann et al., 1995; Koromilas et al., 1992; Svitkin et al., 2001). Relevant to this work, inactivation of eIF4B reduced translation of mRNAs with long and structured 5'UTR (Altmann et al., 1995; Svitkin et al., 2001) and the present study has shown that phosphorylation of S6K1 at Thr 389 is decreased following GSK-3 inhibition. eIF4B is a downstream target of S6K1 and phosphorylation of eIF4B at Ser422 is likely to be reduced, leading to a decrease in

activity. This would affect mRNAs with complex 5'UTR secondary structures and translation of mRNAs with simpler 5'UTR (Figure 5.17), such as *Nanog*, would increase. Phosphorylation of eIF4B following GSK-3 inhibition should be investigated.

To summarise, there are several mechanisms that could regulate specific translation of *Nanog*, *Tbx3* and potentially other pluripotency transcription factors. Tbx3 translation could be specifically up-regulated upon GSK-3 inhibition through IRES and ITAFs, for example, GRSF1, YB1 and p54nrb. Although *Nanog* mRNA is unlikely to be regulated by IRES-dependent means, other RNA-binding proteins could associate with *Nanog* mRNA increasing its translation. Both *Nanog* and *Tbx3* translation could be regulated by polyadenylation, down-regulation of miRNAs or up-regulation of the RNA-binding protein Lin28. Finally *Nanog* translation could be increased due to its simple 5'UTR structure. All this possibilities could be experimentally tested.

6.6 CONCLUSIONS

The present study supports a role for GSK-3 inhibition in specifically regulating translation of *Nanog*, possibly *Tbx3* and potentially other transcription factors. This would be in accordance with recent reports that indicate that Tcf-independent mechanisms can contribute to the increase in self-renewal following GSK-3 inhibition (Wray et al., 2011; Yi et al., 2011). It is not known whether the increase in translation is β-catenin dependent or independent and this could be addressed using the β-catenin null cells. Another future direction should be to investigate whether other pluripotency-associated transcription factors are also regulated at the translational level. Near future experiments should focus in elucidating the mechanisms whereby *Nanog* translation is specifically increased. Finally, it would be interesting to explore whether GSK-3 inhibition decreases epigenetic silencing of *Nanog* and other pluripotent transcription factors. Figure 6.2 summarise mechanisms that could contribute to enhancement of self-renewal upon GSK-3 inhibition.

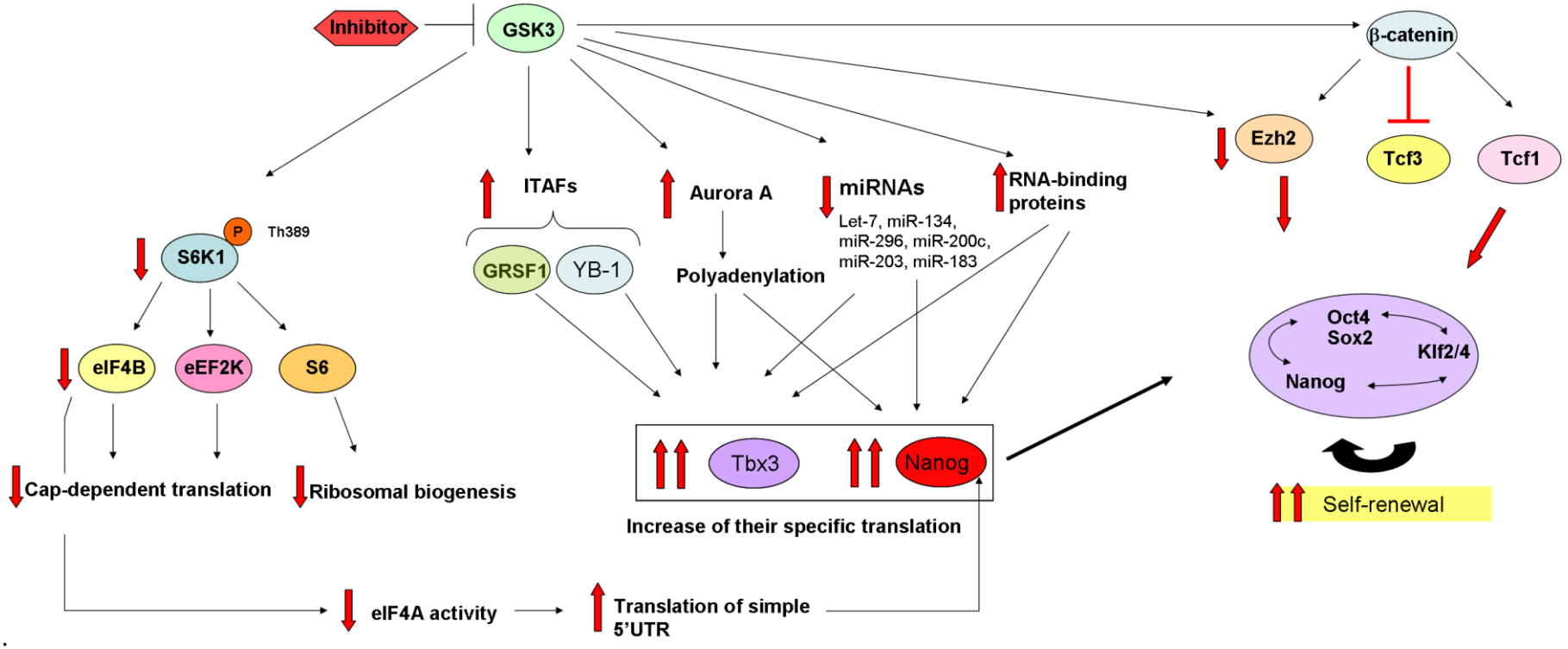

Inhibitor
GSK3
β-catenin
Ezh2
Tcf3
Tcf1
ITAFs
Aurora A
miRNAs
Let-7, miR-134,
miR-296, miR-200c,
miR-203, miR-183
RNA-binding
proteins
P
Th389
S6K1
GRSF1
YB-1
Polyadenylation
eIF4B
eEF2K
S6
Oct4
Sox2
Klf2/4
Nanog
Tbx3
Nanog
Cap-dependent translation
Ribosomal biogenesis
Increase of their specific translation
Self-renewal
eIF4A activity
Translation of simple
5'UTR

Figure 6.2. Mechanism of action of GSK-3 in mESC. GSK-3 inhibition can stabilise β-catenin and abrogate Tcf3 repressive activity on the pluripotency network but it could also activate Tcf1 promoting self-renewal. GSK-3 inhibition also leads to an increase in *Nanog* and possibly *Tbx3* translation and maybe others, which in turn would feed into the pluripotency network. The increase in translation could be due to specific mechanisms including ITAFs, polyadenylation, down-regulation of miRNAs, increase in RNA-binding proteins and possible decreases in cap-dependent translation facilitating increase in translation of mRNA with simple 5'UTR such as *Nanog*. GSK-3 inhibition could reduce Ezh2 reducing epigenetic silencing of pluripotent transcription factors.

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