Investigating the mechanisms controlling cell survival during the first steps of embryonic differentiation

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A thesis submitted for the degree of Doctor of Philosophy

June 2016

Abstract

In the mouse and human embryo the onset of differentiation is accompanied by a dramatic series of changes at the level of gene expression, epigenetics, signalling, and metabolism. During this period the epiblast becomes hypersensitive to death stimuli and defective cells are eliminated. Cell competition has been proposed as a quality control mechanism that compares cell fitness levels within a cell population and eliminates those cells that are less fit than their neighbours, whilst promoting the proliferation of the fitter cells.

In *Drosophila*, the transcription factor d*Myc* plays a key role in cell competition. This thesis examines the role of *c-Myc*, the mouse *dMyc* homologue, in mammalian cell competition, and demonstrates that *c-Myc* is key determinant of cell fitness in a number of developmental contexts. We find that in mouse embryonic stem cells (ESCs), and early post-implantation embryos, defective cells decrease c-MYC expression and are eliminated by apoptosis specifically when surrounded by high-MYC expressing neighbours. We also find that defective cardiomyocytes have low c-MYC expression during cardiac regeneration. Furthermore, we show that over-expression of *c-Myc* in ESCs is sufficient to turn cells into super-competitors, therefore capable of eliminating their wild-type neighbours, and that loss of c-*Myc* during early post-implantation development leads to reduced embryo size. Together these results indicate that relative c-MYC levels determine the outcome of cell competition during mammalian embryogenesis.

We have also analysed what triggers cell competition during early development. Metabolomic analysis of wild-type and defective ESCs revealed that upon differentiation (that triggers cell competition), defective ESCs have significantly lower levels of glycolysis, TCA cycle activity and amino-acid synthesis, indicating a lower metabolic activity. These differences in cell metabolism suggest that during early development cell competition is acting as a metabolic selector mechanism, and eliminating those cells that are less metabolically active.

Declaration of originality

The work presented in this thesis is my own and has been done under the supervision of Dr. Tristan Rodriguez, group head of the Molecular Embryology laboratory at the National Heart and Lung Institute, Imperial College London. Any experimental procedures that have been done in collaboration are clearly indicated. Information gained from the work of others is acknowledged within the text and a list of references is provided in the bibliography.

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ACKNOWLEDGEMENTS

Firstly I would like to thank Dr Tristan Rodriguez for convincing me to join the PhD Program of Imperial College. I could not have done it without his guidance, enthusiasm and help throughout my PhD study, in particular his support when I was suffering all sorts of health problems. Tristan, as my supervisor, is responsible of helping me evolve into a good scientist. Even though at times I felt uncertain about his methods, it produced what he thought was the best.

I would like to acknowledge the members of my PhD committee: Prof Eric W-F Lam and Prof Shankar Srinivas, for their encouragement, practical advice and comments from related but also distinct field discussions, which benefit me and this manuscript greatly.

Many thanks to a great bunch of individuals, who have surrounded me all along since the time when I was studying at the Central University of Venezuela (UCV). First of all, my supervisor Dr Abraham Levi-Benshimol and my other good teachers: Andres Carmona, Magda Stojanovich, Thomas Hermoso and “la Chicha”. At the Virology Department at NIMR, Mill Hill (now known as the Crick Institute) where I produced some very interesting results, although I did not manage to finish the work. I would like to thank specially my supervisor then: Sir. John James Skehel FRS, the person whom I admire the most and for giving me a second opportunity; and my gang: Susana de la Luna, Tristan, Juan Pedro, Miguel, Domingos, Rolf, David Stevens, Wharton and have of course, Javier. Later, when I worked at the CSC, MRC, again I was lucky to have been surrounded by many great people: Ines my Darling, Serafi with his exemplary authenticity, Xing Cheng, and Tatiana. But also, Zoe Webster from the Stem Cell Facility.

Effectively, I want to thank all the past and present members of the Rodriguez lab at Imperial College, especially Juanmi, who has actually submitted this manuscript! My other friends: the lovely Lady Farah and her Rene, the optimistic Desmond, Michaela, Veronique and Salomon Narodden. They all managed to show me the bright side of life when I was in despair and they have been really friends during good and bad times.

I obviously could not have written chapter 4 without mentioning the patience and support of Stephen Rothery on the confocal microscope.

A very big thank you goes to Dr Domingos Henrique for very helpful comments on the manuscript at the time when he himself, needed time to comply with his own tight commitments.

I am so grateful to some people who brighten up my days and they don't even know it themselves: Franklin Goldenstein, MacLeod, Gosh Gevashi, Jacqueline, Simon Thom, Luis Levin, Richard Franklin and especially the youngest of them all: Leonardo Franklin!

I cannot avoid thinking and thanking my mother and my fathers. I love you as always. Dear Javier who has unconditionally supported me during these last difficult years of health problems… My adorable Carla and Alberto, the most patient children of all, those who gave me the wings to fly.

Finally, I would like to dedicate this entire dissertation to the person I started with, who is responsible for this end, Dr Tristan Rodriguez.

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Abbreviations

2i	culture media containing two inhibitors: PD0325901 and CHIR99021
4N	tetraploid
ADP	adenosine diphosphate
ADP	adenosine diphosphate
ALK4	BMP receptor type I (ActR-IB)
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
ANR	anterior neural ridge
AP	anterior-posterior
ATP	adenosine triphosphate
AVE	anterior visceral endoderm
bHLHZ	basic helix-loop-helix/leucine zipper
BMP	bone morphogenetic protein
BMP-4	bone morphogenetic protein (sub types; 2, 4, 5, 6,7. 8)
Bmpr1a$^{-/-}$	mutant deficient for bmp receptor type Ia
Brk	brinker
Bst	belly spot and tail mutant
Cdk4	cyclin-dependent kinase 4
C-Myc	c-Myc gene
CytC	cytochrome C
dH_2O	distilled water
Dkk1	Dickkop-related protein 1
DNA	deoxyribonucleic acid
dpc	days post coitum
Dpp	Decapentaplegic
DVE	distal visceral endoderm
Dvl	Dishevelled (Dvl)
DUSPs	dual specificity phosphatases
EB	embryoid body
EMT	epithelial to mesenchymal transition

EG	embryonic germ
EGF	epidermial growth factor
EPC	ectoplacental cone
Epi	epiblast
EpiSCs	epiblast stem cells
ER	estrogen receptors
ERK	extracellular signal–regulated kinases
ERKR2	extracellular signal–regulated kinases receptor 2
ESCs	embryonic stem cells
ETC	electron transport chain
ExE	extra-embryonic ectoderm
FGF	fibroblast growth factor (1-18; 20-23)
FGFRs	fibroblast growth factor receptors (1, 2, 3, 4)
G6PD	glucose 6 phosphate dehydrogenase
GDFs	growth differentiation factor-5 (GDF-5, 6, 7)
GDP	guanosine diphosphate, when bind to RAS, this is inactive
GTP	guanosine triphosphate when binds to RAS, activates it
GSK-3	Glycogen synthase kinase 3
GO	gene ontology
h	hour
Hccs	Holocytochrome C synthase
hESCs	human embryonic stem cells
HIF	hypoxia inducible factor heparin
HSPGs	sulfate proteoglycans
ICM	inner cell mass
iPSCs	induced pluripotent stem cell
JNK	Janus associated tyrosine kinase
LIF	leukaemia inhibitory factor
MAP	mitogen activated protein
MAPK	mitogen activated protein kinase
MAX	myc-associated factor X
MDCK	Madin-Darby canine kidney cells

mESC	mouse embryonic stem cell
min	minute
Miz-1	Myc-interacting zinc finger protein
miRNA	microRNA
mRNA	messenger RNA
MZT	maternal zygotic transition
NP40	nonidet P-40
NaOH	sodium hydroxide
MAPK	mitogen activated protein kinase
mRNA	messenger RNA
O/N	overnight
OM	oligomycin A
OXPHOS	oxidative phosphorylation
PBS	phosphate buffer saline
PCR	polymerase chain reaction
Pdgfra	Platelet-derived growth factor receptor, alpha polypeptide
PFA	paraformaldehyde
PGC	primordial germ cells
PDK1	pyruvate dehydrogenase kinase 1
PI	propidium iodide
PI3K	phosphatidylinositol 3-kinase
PI3P	phosphatidylinositol 3 phosphate
PrE	primitive endoderm
PS	primitive streak
PTEN	phosphatase and tensin homolog
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
RT	room temperature
s	second
SDS	sodium dodecyl sulphate SEM

Sos	Son of Sevenless
STAT	signal transduction and activator of transcription
TAK1	TGF-β-activated kinase
TBS	Tris-buffered saline
TBST	Tris-buffered saline tween
TCA	tricarboxylic acid
TE	trophectoderm
TGF	transforming growth factor
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labelling
VE	visceral endoderm
WISH	whole mount *in situ* hybridization
Wnt	Wingless-related integration site
WT	Wild-type
WISH	whole mount *in situ* hybridization

Chapter 1. INTRODUCTION

1.1 EARLY MOUSE DEVELOPMENT

The *In vivo* mouse studies provide a powerful system to study mammalian genetics and to produce animal models for human diseases. Insights into early mouse embryogenesis have been crucial for analysing the effects of genetic changes and critical for understanding the mechanisms underlying development. The whole process of mouse embryo development is accompanied by a dramatic series of changes at the level of gene expression, epigenetics, signalling and metabolism. These complex processes involve a series of biological activities: cleavage, divisions, compaction, polarisation, cellular differentiation and segregation of developmental lineages. Elucidation of the molecular mechanisms and the dynamic nature that govern the pre- and post-implantation embryo development will not only provide a paradigm for understanding more complex tissue differentiation, but will also allow us to develop and improve new treatments for diseases, as the processes that govern development are often mis-regulated in many pathological conditions.

1.1.1 Pre-implantation embryonic development

The pre-implantation stages of embryogenesis are a highly dynamic period of development that lasts approximately 4.5 days. Pre-implantation development starts at the oviduct with fertilization, a process that involves the fusion of two gametes (haploid cells) to form a diploid one-cell zygote: the highly distinct and differentiated sperm and egg or oocyte. After fertilization, the zygote undergoes a series of cleavage divisions in a stereotypic fashion that is unresponsive to extrinsic perturbation. In contrast to the asymmetric meiotic divisions of the oocyte, the divisions of the early zygote (termed mitotic) are symmetric. At the 8-cell stage (morula) the embryo undergoes compaction and the individual blastomeres establish adherens and, later, tight junctions to increase their cell-cell contact interactions and adhesion. A major driver of this process is the increased expression of the adhesive protein E-Cadherin (Vestweber et al., 1987). Upon compaction the cells on the outside of the embryo acquire apical-basal polarity, with the apical domain facing de external environment and their baso-lateral domain facing the internal compartment (Johnson and Ziomek, 1981, 1983). Those blastomeres found in the inside of the embryo remain apolar. Up until the morula stage the individual blastomeres are totipotent, they still have the potential to give rise to all the cell types in the body; embryonic and extra-embryonic, but from this stage onwards a gradual

restriction in developmental potential occurs.

The segregation of the inner cell mass (ICM) and the trophectoderm (TE) constitutes the first lineage decision during the embryo development. About the 16- to 32 cell stage the cells found on the outside of the embryo will form the TE and those on the inside the ICM (Johnson and McConnell, 2004; Johnson and Ziomek, 1981). The TE cells are considered to be multipotent since they can only differentiate into extra-embryonic structures, the chorion and the embryonic portion of the placenta, two tissues that play essential roles in the correct implantation, patterning and nutrition of the embryo (reviewed by (Cockburn and Rossant, 2010; Stower and Srinivas, 2014). The cells of the ICM, in contrast, will still be able to contribute to the extra-embryonic tissues of the yolk-sac as well as embryonic derivatives.
The segregation of the ICM and TE can also be visualized at the molecular level. At the morula stage (2.5 *days post-coitum -dpc*) the expression of markers of the future TE, such as the caudal-type homeobox protein 2 (*Cdx2*) and *Gata3* and of the future ICM, such as the Pou5fl transcription factor *Oct3/4,* is largely overlapping (Dietrich and Hiiragi, 2007). However, subsequently to this, the cells on the outside will primarily express TE markers and those on the inside ICM markers (Home et al., 2009; Nichols et al., 1998; Niwa et al., 2005; Ralston et al., 2010; Strumpf et al., 2005). Therefore, an important step in the specification of the TE and ICM is the repression of lineage inappropriate gene expression. For example, in *Cdx2* mutant embryos, *Oct4* expression remains in the outside cells, causing a failure of implantation (Ralston and Rossant, 2008). Two signalling pathways that regulate TE versus ICM specification are the Hippo and Notch pathways, as activation of both these pathways is the outside cells is required for correct TE specification (Cockburn et al., 2013; Hirate et al., 2013; Nishioka et al., 2009; Nishioka et al., 2008; Rayon et al., 2014).

From the morula to mid-blastocyst stage (up to ~64 cells, 2.5-3.5 dpc) the mouse embryo develops a cavity, primarily formed by secretion of intracellular vacuoles which when externalized coalesce forming the blastocoel (Calarco and Brown, 1969; Fleming and Pickering, 1985; Garbutt et al., 1987; Manejwala et al., 1989; Motosugi et al., 2005; Wiley and Eglitis, 1981).

At the early 3.5 dpc blastocyst stage (32 cells), Nanog and Gata6, two lineage-specific transcription factors required for epiblast (Epi) and primitive endoderm (PrE) formation, are expressed in the ICM in a "salt and pepper" fashion where their patterns of expression are mutually exclusive (Chazaud et al., 2006). As the PrE differentiates, it expresses factors such as Gata4, Gata6, Sox17 and Pdgfra, whereas the Epi expresses markers such as Nanog, Oct4 and Sox2 (Artus et al., 2010; Artus et al., 2011; Chazaud et al., 2006; Grabarek et al., 2012; Plusa et al., 2008; Schrode et al., 2014; Schrode et al., 2013; Yamanaka et al., 2010). By 4.5 dpc PrE and Epi segregate, with the PrE cells overlying the blastocyst cavity and the Epi cells being enveloped by (Nichols et al., 2009b) the visceral endoderm. During this process, those cells expressing lineage inappropriate gene expression markers or that do not segregate properly are eliminated by apoptosis (Grabarek et al., 2012; Plusa et al., 2008).

The specification of the PrE and Epi lineages appears to be dependent on different responsiveness of these cell populations to FGF signals. Nanog positive Epi cells will repress Gata6 expression and secrete FGF4. Gata6 positive PrE cells, which have higher levels of Fgfr2, will respond to the FGF signals repressing Nanog and activating the transcription of PrE specific genes, such us Sox17 (Bessonnard et al., 2014; Frankenberg et al., 2011; Kang et al., 2013; Nichols et al., 2009b; Schrode et al., 2014; Yamanaka et al., 2010). Gata6 null embryos are not able to develop a primitive endoderm or to respond to FGF signals. Similarly, embryos that lack GRB2, FGF4 or FGFR2, upstream components of the MAPK signalling pathway, do not express Gata6 and therefore also do not form a PrE (Bessonnard et al., 2014; Frankenberg et al., 2011; Kang et al., 2013; Nichols et al., 2009b; Schrode et al., 2014; Yamanaka et al., 2010). In contrast to this, Nanog knockout embryos are not able to develop the Epi and instead, cells form primitive endoderm derivatives (Chambers et al., 2003; Mitsui et al., 2003). On the contrary, overexpression of Nanog was capable of maintaining ESC self-renewal independently of LIF/STAT3 (Chambers et al., 2003; Mitsui et al., 2003; Pan and Thomson, 2007). In summary, once the late blastocyst (from 64 to about 120 cells) is formed it comprises two epithelial layers, the TE and the PrE that enclose the pluripotent cell population of Epi. The TE and PrE are extra embryonic lineages that support the growth of the epiblast into the embryonic ectoderm, which will give rise to the three germ layers during gastrulation.

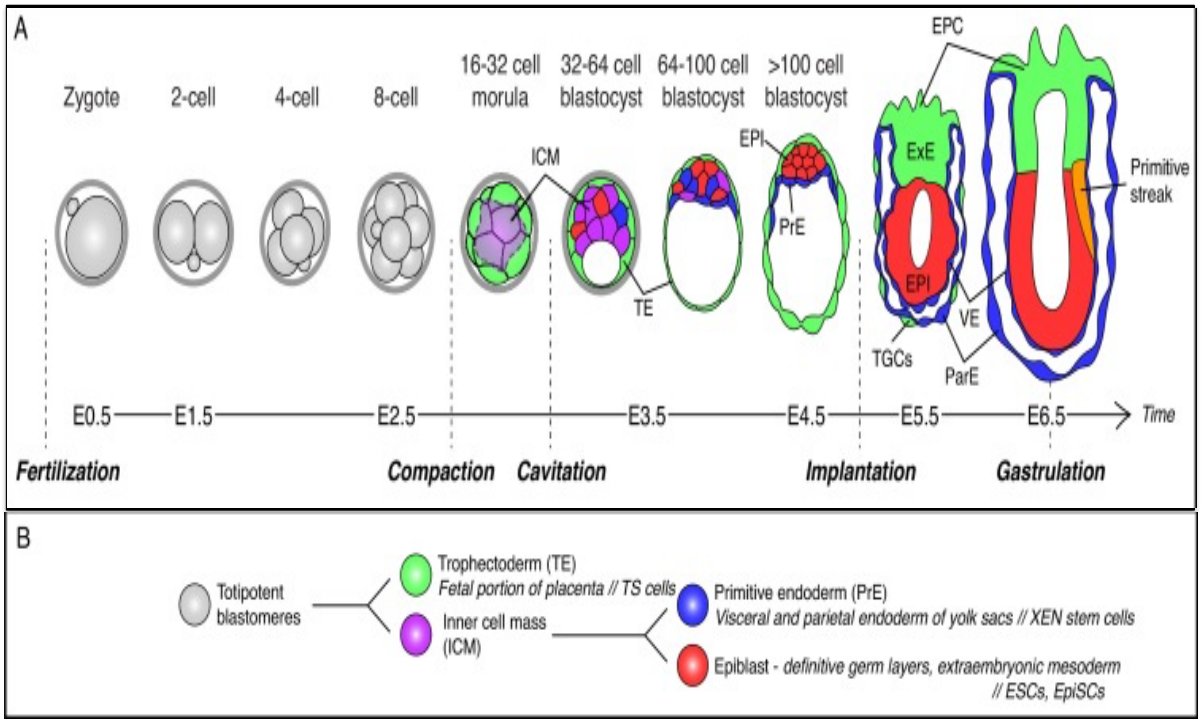


Figure 1: Early stages of mouse development.
A) Timeline indicating subsequent embryonic divisions and embryonic days (E) from zygote and up to gastrulation stage E6.5. B) Model for the gradual formation of embryonic lineages. Initial stochastic and overlapping expression of lineages followed by a gradual switch towards an exclusive expression of these lineages. Colors represent the expression of lineage-specific. Figure adapted from Saiz et al., (2015).

1.1.2 Post-implantation embryonic development

During implantation, around 4.5 and 5 dpc the embryo implants by attachment to the endometrial surface of the uterus, through trophectoderm invasion of the uterine epithelium, provided that the blastocyst and uterus are receptive and have achieved implantation competency. This competency involves a series of complex and coordinated processes for both the blastocyst and the uterus including secretion of signalling molecules such as cytokines, growth factors, transcription factors, lipid mediators and morphogens (reviewed by (Wang and Dey, 2006). The 4.5 dpc blastocyst (see figure 1) is formed by two well defined domains that will undergo a drastic morphological transformation during implantation. The first domain can be considered to include the primitive endoderm, the epiblast and the polar trophectoderm (portion of the TE adjacent to the epiblast). The second domain would include the blastocoel (cavity) enclosed by the rest of the trophectoderm (mural trophectoderm). These two domains can be considered to represent the embryonic- abembryonic axis of the

embryo, with the Epi containing domain representing the embryonic axis (reviewed in Rossant, 2009).

During implantation the polar trophectoderm attaches to the uterine wall and in response to FGF4, starts to proliferate giving rise to the ectoplacental cone (EPC) and to the extra-embryonic ectoderm (ExE) (Tanaka et al., 1998). The growth of extra-embryonic tissues displaces the epiblast that will occupy the blastocoel cavity. During implantation the epiblast cells divide and acquire a columnar epithelial identity and form the egg cylinder that will subsequently cavitate to produce an internal amniotic cavity (reviewed by (Arnold and Robertson, 2009) and (Rossant and Tam, 2009). These morphogenetic movements convert the embryonic axis into a proximal-distal axis, with the ExE situated proximally and the tip of the epiblast distally.

The next step in embryonic development is the conversion of this proximo-distal axis into an anterior-posterior (A-P) one (reviewed in (Arnold and Robertson, 2009) and (Rossant and Tam, 2009). For this, further specialization of other extra-embryonic structures must take place. About the time of implantation, the PrE will give rise to the visceral endoderm (VE) and to the parietal endoderm (PE). Parietal endoderm cells migrate along the mural trophectoderm and together they will form the Reichert's membrane. This membrane will allow nutrient transfer to the embryo as well as prevent invasion of maternal cells. The visceral endoderm will subsequently be divided into an embryonic and an extra-embryonic region, depending on if it is overlaying the embryo or the ExE. The visceral endoderm will contribute to the formation of the yolk sac, a tissue also formed from the extra-embryonic mesoderm. A key step in A-P axis formation is the specification of the anterior visceral endoderm (AVE) that is originated from a subset of cells at the distal tip of the 5.5 dpc embryo (Thomas and Beddington, 1996). The AVE is specified by Nodal signalling but the precise cells that respond to Nodal are determined by repressive signalling from the ExE (Brennan et al., 2001; Rodriguez et al., 2005). These AVE cells express markers, such as *Hex, Cerl, Lefty1, Lhx1* and once it is specified, migrate to the prospective anterior of the embryo through a combination of active migration and epithelial cell migration movements coordinated by the planar cell polarity and Nodal signalling pathways (Srinivas et al., 2004; Trichas et al., 2011; Trichas et al., 2012). The AVE is a signalling centre that has an essential

role determining the anterior-posterior axis of the embryo by facilitating the expression of anterior neural markers in the anterior of the embryos as well as by repressing primitive streak formation in this region (reviewed by (Andoniadou and Martinez-Barbera, 2013; Arnold and Robertson, 2009; Srinivas, 2006). One example for these roles of the AVE is illustrated by the fact that *Lefty1* and *Cer11* mutants develop multiple primitive streaks (Perea-Gomez et al., 2002). Interestingly, Kwon and colleagues have shown that the embryonic visceral endoderm also contributes to the formation of the embryonic gut, providing potential new roles for extra-embryonic signalling centres during embryonic development (Kwon et al., 2008).

1.1.3 Gastrulation and germ layer formation

The organization of the AP axis and the different patterns of gene and protein expression established within the embryo prepare the epiblast for gastrulation, a process in which the three germ layers (ectoderm, mesoderm and endoderm) will be specified. Ectoderm derived cells will form the neural crest, neural tissues and skin. Mesoderm will give rise to muscle, bone, connective tissue, blood and the vascular system. Endoderm derived cells will form the gut an associated organs such as the liver, pancreas.

Gastrulation commences at 6.5 dpc with the formation of the primitive streak in the posterior of the embryo (reviewed by (Arnold and Robertson, 2009). As mentioned above, the position, where the primitive streak begins to form, depends on the absence of repressive AVE signals, and therefore the primitive streak forms opposite the AVE. Nodal signalling plays an important role in primitive streak formation and its regulation is key to establishing where the primitive streak will form as far as possible from Nodal (reviewed by (Arnold and Robertson, 2009). Nodal is expressed throughout the epiblast (Varlet et al., 1997) but the cleavage of the Nodal precursors by extra-cellular convertases secreted by the ExE in combination with the activity of BMP4, which in turn activates Wnt in the proximal posterior epiblast to enhance Nodal expression, and allows for it maximal activity in the proximal epiblast (Beck et al., 2002). This in combination with Nodal antagonism by Left1 and Cerl1 secreted by the AVE restricts primitive streak formation in the posterior epiblast (Perea-Gomez et al., 2002).

During primitive streak specification, Nodal and BMP signalling also acts to promote epiblast pluripotency and represses the expression of neural markers. This is best illustrated by loss of

either BMP or Nodal activity that causes precocious neural differentiation in the epiblast of the post-implantation embryo (Camus et al., 2006; Di-Gregorio et al., 2007). Antagonisms of these BMP and TGFβ signals, first by the AVE and later by the anterior definitive endoderm, lead to anterior neural specification reviewed by (Andoniadou and Martinez-Barbera, 2013).

During gastrulation the cells from the posterior epiblast will undergo an epithelial to mesenchymal transition, delaminate from the epiblast epithelia and ingress through the primitive streak. During this process these cells will be exposed to a combination of signalling inputs that will allow them to acquire the identity of the different mesodermal and endoderm subtypes (reviewed by (Arnold and Robertson, 2009). Once specified, these mesodermal and endodermal cells will migrate to their respective positions in the embryo where they will initiate organogenesis (Viotti et al., 2014).

1.2 PLURIPOTENT STEM CELLS

1.2.1 Embryonic Stem Cells: Isolation and uses

Pluripotency refers to those cells that can give rise to derivatives of the three germ layers: ectoderm, mesoderm and endoderm. Pluripotent stem cells are those that can self-renew while maintaining the potential to differentiate into all embryonic lineages (Jaenisch and Young, 2008). One strategy by which stem cells can accomplish both these tasks is by asymmetric cell division, whereby each stem cell divides to generate one daughter with stem-cell fate (self-renewal) and one daughter that can differentiate. *In vitro* WNT signalling has been shown to drive the asymmetric division of the pluripotent mouse embryonic stem cells (ESCs) (Habib et al., 2013). However, in the early mouse embryo, there is little evidence for asymmetric cell division. Furthermore, given the need to markedly increase cell numbers during early development and the fact that this strategy will not allow stem cells to expand in numbers, there must be additional self-renewal strategies that permit a control of pluripotent stem cell numbers.

ESCs are derived from the ICM of the mid-blastocyst stage of the mouse embryo at embryonic day 3.5 (E3.5) and are transcriptionally and functionally equivalent to the epiblast of the pre-implantation embryo (Boroviak et al., 2014). Mouse ESCs were first isolated 35

years ago (Evans and Kaufman, 1981; Martin, 1981) and the equivalent population from human embryos (hES cells) was isolated 17 years later (Thomson et al., 1998). Due to their stem cell nature, both ESC types can be cultured and propagated in specific media culture conditions for many generations *in vitro* and retain pluripotency and self-renewing properties. However, the culture conditions that allow maintaining these stem cells are different in mouse and human (see below).

The developmental potential of ESCs cells was demonstrated by their ability to re-enter embryogenesis when injected into host blastocysts and to give rise to high-contribution chimeras. Furthermore, in these chimeras the cultured cell genome can be transmitted through the germ line to generate healthy offspring (Morgani et al., 2013; Robertson et al., 1986), suggesting that any changes occurred in culture must be reversible. Furthermore, when ESCs are injected ectopically subcutaneously they are also able to form teratomas, or tumours that contain tissue derivatives from the three germ layers (Solter, 2006; Solter et al., 1970).

If provided with the appropriate stimuli, ESCs can be directed to adopt the fates from the three germ layers, allowing development to be studied at the cellular and molecular level (Murry and Keller, 2008; Nishikawa et al., 2007). Furthermore, this ability also allows for the potential to use ESC derived cell lineages for clinical transplantation, tissue repair, disease modelling, regenerative medicine and drug discovery (Murry and Keller, 2008; Singh et al., 2015). Competence for germ line transmission also means that ESCs can be a useful tool for introducing gene modifications into mice (Robertson et al., 1986). Their facility for clonal expansion enables isolation of relatively rare events, particularly homologous recombination. In 1989, the first mice were generated with a knockout mutation created by gene targeting in ESCs (Thompson et al., 1989). Thus, ESC–based genome engineering rapidly became an ubiquitous technique in mouse genetics (Capecchi, 2005).

1.2.2 States of pluripotency

The conventional way to culture mouse ESCs involves the use of serum and the leukaemia inhibitory factor LIF (Smith et al., 1988). However, in these conditions ESCs exhibit a great degree of heterogeneity and ESCs from certain genetic backgrounds cannot be cultured in serum plus LIF. One major step forward in improving these conditions has been establishing

serum free culture conditions. Here, ESCs can be maintained pluripotent by culturing only with LIF and with small molecule inhibitors for ERK and GSK3beta, known as 2i+LIF (Ying et al., 2008). Under these conditions, ESCs can be maintained in a more homogeneous state and isolated from previously refractory genetic backgrounds (Nichols et al., 2009a; Ying et al., 2008). These observations led the Smith group to propose that in 2i+LIF ESCs are in a ground state of pluripotency that implies robust self-renewal of a biologically homogeneous population of cells, each with unbiased potential to form all somatic cell lineages and germ cells (Silva and Smith, 2008).

The identification of these improved culture conditions has also shed light on the developmental origin of ESCs. The most widely used starting point for derivation of ESCs is from the early blastocyst (3.5 dpc). However, individual ICM cells at this stage are incapable of generating ESC colonies in 2i+LIF and can only do so when supported by feeders, serum, or extracellular matrix (Boroviak et al., 2014). In contrast to this, single cells from the mature blastocyst stage (4.5 dpc) readily form ESC colonies within three to four days in 2i+LIF, with similar efficiency to the clonogenicity of established lines (greater than 50%). Furthermore, studies of transcription factor and signalling markers further confirmed that 2i+Lif ESCs are equivalent to the 4.5 dpc epiblast (Boroviak et al., 2014).

In the post-implantation, embryo cells remain pluripotent (Beddington, 1986); however, their pluripotent identity changes. An indication of this is that ESC–forming ability declines abruptly upon implantation (Boroviak et al., 2014). A second indication of this is that a different type of stem cells can be isolated from post-implantation embryos. Post-implantation epiblast stem cells (EpiSCs) are pluripotent cell lines established by culture of the later stage egg cylinder epiblasts (Brons et al., 2007; Tesar et al., 2007). EpiSCs are cultured without LIF or 2i and instead require FGF and Activin for their self-renewal. Similar to ESCs, EpiSCs can differentiate into cell types of the three germ layers both in vitro and in teratocarcinomas, but they lack the capacity to form chimeras when injected back into the blastocyst (Brons et al., 2007; Tesar et al., 2007). Instead of this, EpiSCs readily contribute to chimeras when injected back into post-implantation embryos (Huang et al., 2012).

Transcriptome profiling and gene transcription studies have shown that EpiSCs are heterogeneous and partially culture-adapted phenotype that resembles the anterior primitive

streak of the mouse embryo (Kojima et al., 2014; Tsakiridis et al., 2014). These, together with the epigenetic differences observed between ESCs and EpiSCs, have led to the suggestion that post-implantation epiblast cells and EpiSCs have exited the ground state of pluripotency and become primed for differentiation (Nichols and Smith, 2009). Interestingly, EpiSCs share defining features with human ESCs (hESCs) such as common mechanisms of epigenetic regulation of transcription as well as culture conditions (Tesar et al., 2007). For these reasons, hESCs are thought to be also primed for differentiation, and support for this idea has come from the recent reversal of hESCs to a state of pluripotency similar to that of the ground state of mouse ESCs (Takashima et al., 2014).

1.2.3 Embryonic germ (EG) cells.

EG formation occurs immediately after dissolution of the näive pluripotency program, when the peri-implantation mouse embryo is setting the stage for egg cylinder formation and subsequent specification of germ cells and gastrulation. In specific culture conditions, primordial germ cells (PGCs) from mouse embryos could convert into proliferative stem cells (Matsui et al., 1992b; Resnick et al., 1992). EG cells can be derived from PGCs from 8.5 to 11.5 dpc embryos and require stem cell factor (SCF), LIF, and FGF as well as a feeder layer for their culture (Matsui et al., 1992a; Resnick et al., 1992). EG cells are pluripotent and are morphologically identical to mouse ESCs and express typical ESC markers, such as SSEA-1 and Oct4, can differentiate *in vitro* into a variety of cell types, and upon blastocyst injection and they can give rise to chimeric mice and contribute to germ cells (Labosky et al., 1994; Matsui et al., 1992a). EG cells can also form teratocarcinomas, but this is likely due to an induced conversion into pluripotent stem cells *in vivo* (Leitch et al., 2013; Stevens, 1983). EG cells retain epigenetic features of PGCs, which are distinct from those of ESCs (Labosky et al., 1994), and in some cases also show loss of genomic imprints (Leitch and Smith, 2013). For these reasons EGs are considered to represent a distinct pluripotent population.

1.3 SIGNALLING PATHWAYS REGULATING PLURIPOTENCY AND DIFFERENTIATION.

1.3.1 LIF/JAK/STAT Pathway

A number of signalling pathways have been suggested to play roles in the regulation of the different pluripotent states. These same pathways also have roles in regulating differentiation, what is therefore important is the balance and combination of signalling inputs, rather than the activity of a single signalling pathway. The cytokine LIF is the first extrinsic factor required to maintain pluripotency and was isolated from conditioned medium that can sustain ESC pluripotency in absence of feeders (Smith et al., 1988; Williams et al., 1988). LIF signals first by binding to its trans-membrane receptor gp130 that leads to subsequent activation of the tyrosine kinase Janus-associated kinases (JAKs) that will in turn activate the signal transducer and activator of transcription 3 (STAT3) (Boeuf et al., 1997; Matsuda et al., 1999; Narazaki et al., 1994; Niwa et al., 1998; Yoshida et al., 1994). Once activated by phosphorylation STAT3 signalling activates downstream pluripotency targets such as Klf4, Gbx2 (Tai, 2013), c-Myc (Cartwright et al., 2005; Hall et al., 2009; Martello et al., 2013; Tai and Ying, 2013; Ye et al., 2013). In the presence of serum, LIF/STAT3 activation alone is sufficient to maintain mouse ESCs pluripotent (Matsuda et al., 1999; Niwa et al., 1998). It is worth mentioning that LIF can also activate phosphatidyl inositol-3 kinase (PI3K) and ERK1/2 signalling cascades (Martello and Smith, 2014), but the activation of these pathways acts as differentiation cues rather than having a role in preventing differentiation (Ying et al., 2008), and this is at least one reason why LIF signalling needs to be counter-balanced by factors in the serum.

1.3.2 FGF/ERK Signalling Pathway

The fibroblast growth factor family is an evolutionally conserved family of secreted proteins, most of them characterized by two binding sites, a secretory sequence at the N-terminus that also serves as the heparan-binding site for cells-surface associated heparin sulfate proteoglycans (HSPGs), and a distinct FGF receptor (FGFR) binding site, that binds to tyrosine kinase receptors. Once a complex between FGFs: FGFRs and HSPGs have been formed, it results in the phosphorylation of tyrosine in the intracellular domain of the FGFR leading to activation of downstream signalling pathways. FGFs are important players in the processes of proliferation and differentiation and are highly conserved, but show greater

diversity in vertebrates. In humans as well as in mice, 22 ligands have been identified – *FGF1-18; FGF20-23* that have a similar and conserved internal core of 28 amino acid residues. The size of FGF proteins ranges from 17 to 34 kDa in vertebrates (reviewed by (Itoh and Ornitz, 2011).

The FGFRs are intracellular tyrosine kinases and are encoded by four *FGFR* genes: FGFR1, FGFR2, FGFR3, FGFR4, that each has a variety of splice variants. The FGFRs consist of three extracellular immunoglobulin-type domains (D1, D2 and D3), a single-span trans-membrane domain and an intracellular split tyrosine kinase domain. This last region can serve for ligand-binding or have auto-inhibitory functions that prevent receptor activation in the absence of FGFs. Modifications of the extracellular ligand binding domain of FGFRs determines the binding affinity of FGFs, HSPGs and other FGFRs and therefore their diversity allows FGFs to activate different signalling pathways (reviewed by (Itoh and Ornitz, 2011).

The Ras/MAP kinase pathway is the most characterized by which FGFs complexes signal. Once the FGF ligand is bound the activated FGFR, it interacts with the FGFR substrate 2 (FRS2), allowing Grb2/Son of Sevenless (Sos) binding. Sos catalyzes the exchange of GDP for GTP on Ras and Ras then activates downstream MAPKs. One branch of these kinases is ERK1/2, that once activated translocates into the nucleus and phosphorylates target transcription factors. In addition to ERK1/2, FGFs can also activate JNK and p38 MAP kinases (reviewed by (Lanner and Rossant, 2010). Other signalling pathways activated by these ligands through FGFRs include the planar cell polarity (PCP) pathway, PLC/Ca^{2+} signalling: the JAK/STAT pathway and the PI3K/AKT pathway.

The FGF signalling pathway is subject to tight regulation at the extracellular and intracellular levels. For example, the 4 secreted extracellular *Sprouty* and the 2 related *Spred* proteins inhibit FGF signalling (Hacohen et al., 1980; Wakioka et al., 2001; Yu et al., 2011). Intracellular modulators of FGF signalling, such as the dual specificity phosphatases (DUSPs) and *Sef,* also inhibit the FGF pathway (reviewed by (Turner and Grose, 2010).

In vivo FGF signalling plays a dynamic role throughout development. From as earlier as specification of the trophoblast, FGFs have been shown to be required for maintenance of

trophoblast cells (Tanaka et al., 1998). Later on, FGF4/FGFR2 has been shown to act as an auto-inductive cue for ICM differentiation and lineage commitment to PrE fate (Arman et al., 1998; Chazaud et al., 2006; Hamilton and Brickman, 2014; Kunath et al., 2007). Furthermore, inhibition of FGF/ERK signalling is one of the pre-requisites for the maintenance of ground-state pluripotency (Ying et al., 2008). In contrast, the post-implantation embryo, FGF/ERK signalling is required for the maintenance of primed pluripotent cells, both for mouse EpiSCs (Brons et al., 2007; Tesar et al., 2007) and human ESCs (Thomson et al., 1998). FGF signalling has also been suggested to have a role in the cavitation of the post-implantation embryo. FGF signalling may also have a role in cavitation, as FGFR1 and FGFR2 mutant ESCs, differentiated as embryoid bodies, fail to cavitate (Esner et al., 2002; Li et al., 2001) and inhibition of the FGF pathway during embryoid body formation also blocks cavitation in a PI3K dependent manner (Chen et al., 2000). Finally, FGF signalling seems to play a role during gastrulation in mesoderm specification and migration, causing mice mutant for FGF4/8 and FGFR1 to display defects in gastrulation (Ciruna and Rossant, 2001; Sun et al., 1999).

1.3.3 WNT Signalling Pathway

The Wnt signalling pathway (Wingless in *Drosophila*) is a family of secreted proteins that plays major roles in development and tissue homeostasis. Wnt signals are transduced in at least two distinct ways: a well-established 'canonical' or Wnt/β-catenin pathway, and a non-canonical pathway that is β-catenin independent. Wnt signals control multiple aspects of development, including proliferation, differentiation, fate specification, polarity, embryogenesis and migration of cells (Logan and Nusse, 2004). In addition to this, mutations that over-activate Wnt signalling are major factors in oncogenesis (Nishisho et al., 1991). 19 Wnt ligands are found in the mouse genome and during canonical signalling they bind to the cell surface receptor Frizzled (Fz) and to its LRP family of co-receptors. Activation of these receptor complexes results in signalling through Dishevelled (Dvl) to inactivate the destruction complex (APC/Axin/CK1/GSK3β), which is responsible for phosphorylating β-catenin and targeting it for ubiquitination and successive proteasome degradation. Inhibition of the destruction complex results in stabilization and nuclear accumulation of β-catenin that will promote its interaction with the TCF/LEF family of transcription factors. The destruction complex kinase responsible for β-catenin phosphorylation is GSK3 and direct inhibition of

GSK3 can mimic Wnt signalling (Patel et al., 2004). However, GSK3 phosphorylates many targets other than β-catenin so its inhibition is predicted to have pleiotropic effects (reviewed in (Doble and Woodgett, 2003).

Wnt acts as a morphogen and in consequence its signalling intensity and duration affects different cell fates. The first expression of Wnt in the embryo is detected in the mouse blastocyst (Kemp et al., 2005), but the first roles of canonical signalling are only described later. For example, mutants in components of Wnt signalling, such as Wnt3, or β-catenin fail to develop a primitive streak and establish a correct anterior-posterior axis (Huelsken et al., 2000; Liu et al., 1999). In contrast to this, mutants lacking negative regulators of the Wnt signalling pathway such as *Dkk1* lack neural structures (Mukhopadhyay et al., 2001), or *Tcf* and *Axin1* develop ectopic axial structures (Merrill et al., 2004; Zeng et al., 1997). Therefore, Wnt signalling plays an essential role in specifying posterior cell fates rather than in the formation of anterior features.

Canonical Wnt signalling has been also implicated in the regulation of ESC pluripotency in mouse and humans. In mouse ESCs, β-catenin has been shown to maintain self-renewal and pluripotency through at least two separate mechanisms, by relieving the Tcf3 mediated repression of pluripotency genes (Wray et al., 2011) and by modulating membrane bound pluripotency factor Oct4 (Faunes et al., 2013). In human ESCs, inhibition of GSK3 activity or activation of β-catenin by Wnt3a has also been implicated in pluripotency (Sato et al., 2004), but the mechanism by which Wnt signalling may act in this system appears much less clear (Merrill, 2012) .

1.3.4 BMP Signalling Pathway

Bone morphogenetic proteins are a family of secreted growth factors belonging to the TGF-β superfamily (Heldin et al., 1997; Shi and Massague, 2003). In mouse, only Bmp2 and Bmp4 have been shown to have roles during early embryonic development, although 20 BMPs ligands have been identified and classified according to their structure and function (Miyazono et al., 2005). BMP2, BMP4 and the *Drosophila* Decapentaplegic (Dpp) gene product form the BMP2/4 subgroup; BMP5, BMP6, BMP7, BMP8, and the *Drosophila gbb-60A* form the OP-1 group; the growth differentiation factor-5 (GDF-5), GDF-6 and GDF-7

belong to the GDF-5 group. The biological functions of BMPs are diverse since they have distinct spatiotemporal expression profiles, post-translational modifications and bind to different receptors with different affinities (Miyazono et al., 2005; Shi and Massague, 2003).

BMPs bind to a heteromeric receptor complex formed by type I and type II receptors. Both types are located at the cell surface where they are required for BMP signal transduction and contain serine/threonine kinase domains in their intracellular portions. The type II receptor kinases are constitutively active; upon ligand binding, hetero-tetrameric complexes comprising two molecules of each receptor type form (Shi and Massague, 2003) and type II receptor kinases trans-phosphorylate the GS (Gly-Ser) domain of type I receptors. Activated type I receptors phosphorylate receptor-regulated SMADs (R-SMADs 1/5/8), which in turn associate with common-partner SMADs (Co-SMAD 4) (Heldin et al., 1997) (The R-SMAD/Co-SMAD) complexes then, translocate into the nucleus where regulate the expression of targeted genes in cooperation with transcription factors, co-activators and co-repressors. Three type I receptors, have been shown to bind BMPs: ACVR1, BMPR1A and BMPR1B (known as ALK2, ALK3 and ALK6 respectively) (Miyazono et al., 2005; Shi and Massague, 2003). These receptors have different and dynamic expression patterns during early mouse development (Roelen et al., 1997). BMPs interact with three distinct type II receptors: BMP type II receptor (BMPR-II), activin type II receptor (ACVR-II) and activin type IIB receptor (ACVR-IIB). The receptor type I *Acvr1,* is expressed from the one-cell to the blastocyst stage, *Bmpr1b* transcripts are detected from the one-cell zygote to the uncompact morula stage, and *Bmpr1a* is present in blastocysts (Roelen et al., 1997) and is the only type I BMP receptor expressed in the epiblast of post-implantation embryos (Mishina et al., 1995). This suggests that different receptor complexes can be formed at different stage of development. *Bmpr2*, *Bmpr1a* and *Bmpr1b* mRNA was also detected in undifferentiated and differentiated embryonal carcinoma and embryonic stem cells (Roelen et al., 1997). BMP signalling can be inhibited both extra-cellularly, for example by NOGGIN or CHORDIN, and intracellularly, for example by SMAD6 or SKI (ten Dijke et al., 2000). These inhibitors help to shape the pattern of BMP signalling during embryogenesis and in the adult.

ID (inhibitors of differentiation) proteins are one of the most crucial targets of BMPs in many cell types (Hollnagel et al., 1999; Lopez-Rovira et al., 2002; Ogata et al., 1993) and may be

responsible for their biological activities (Kowanetz et al., 2004; Nakashima et al., 2001; Norton et al., 1998; Yokota and Mori, 2002). They constitute a family of helix-loop-helix transcription factors that regulate a variety of cellular responses including cell growth, differentiation, tumorigenesis and neoplastic transformation (Norton, 2000), generally acting as positive regulators of cell proliferation and negative regulators of differentiation (Miyazono and Miyazawa, 2002; Norton et al., 1998).

Although, SMADs are the main transducers of BMP signalling, there is evidence that MAP kinases, particularly p38, acts as an alternative pathway downstream of BMPs. The activation of the MAPK pathway by BMP2/4 is mediated by the activation of TGF-b-activated kinase (TAK1) and subsequent phosphorylation of p38 stress-activated protein kinase to induce apoptosis (Iwasaki et al., 1999; Kimura et al., 2000; Nohe et al., 2004; Shibuya et al., 1998; Yamaguchi et al., 1995). Likewise, inhibitory effects on TAK1 by BMP2 through SMAD 6 and 7 have been also shown (Kimura et al., 2000). Additionally, BMP signalling cross-talks with a variety of signalling pathways in a context-dependent manners, including MAPK, phosphatidylinositol-3 kinase/Akt/Wnt, Hedgehog and Notch (Guo and Wang, 2009).

Analysis of null mutations for Bmp2 and Bmp4 has revealed multiple roles for these proteins during the early post-implantation period, for example, in primordial germ cell (PGC) induction (Lawson et al., 1999), mesoderm and node formation, primitive streak morphogenesis (Fujiwara et al., 2002; Fujiwara et al., 2001; Winnier et al., 1995), extra-embryonic mesoderm and cardiac development (Zhang and Bradley, 1996), left-right patterning (Fujiwara et al., 2001), imbibes premature neural differentiation in mouse embryos (Di-Gregorio et al., 2007). The fact that embryos carrying mutations in the receptors of BMP signalling display phenotypes that are more severe than those of Bmp2 or Bmp4, suggests that functional redundancy exits between BMP proteins (Mishina, 2003).

In mouse ESCs, BMPs have been shown to antagonise neural differentiation and promote differentiation into non-neural fates (Johansson and Wiles, 1995; Tropepe et al., 2001; Ying et al., 2003a; Ying et al., 2003b). However, in combination with LIF, BMP proteins can sustain self-renewal and pluripotency by activation of the ID proteins via the SMAD pathway (Ying et al., 2003a). BMP4 has also been shown to be required for ESC self-renewal by inhibiting the ERK and p38 MAPK pathways (Qi et al., 2004).

1.3.5 NODAL

NODAL is a TGFβ molecule capable of diffusing across large distances. As such, it is able to induce signalling cascades in the surrounding tissues, and this is of particular importance in mesoderm induction and left-right patterning (Robertson, 2014). NODAL precursor ligands are secreted from cells and processed by protein convertases such as PACE4 and FURIN into functional ligands in a complex with NODAL-specific interacting protein CRIPTO at the cell surface (Beck et al., 2002; Ben-Haim et al., 2006; Ding et al., 1998; Gritsman et al., 1999; Mesnard et al., 2006). Similar to BMPs, NODAL signals are transduced via heterodimers of one of two TGFβ serine-threonine kinases receptors: the type I receptor (which can either be the ALK4 (ActR-IB) or the NODAL specific ALK7 isoform) in a complex with type II receptors (which can either be the ActRIIA or ActRIIB isoform)(Shi and Massague, 2003).

Uniquely among TGFβ molecules, NODAL requires the mediation of Epidermal Growth Factor (EGF)- CRIPTO1/FRL-1/Cryptic (EGF-CFC) co-receptors. In mammals there are two known EGF-CFC co-receptors acting *in vivo*: CRIPTO1 and CRYPTIC (Schier and Shen, 2000). Mutations in these genes result in phenotypes similar to loss of NODAL expression. Once activated the serine/threonine kinase heterodimer receptor complex acts to phosphorylate one of two members of the SMAD signal transduction family, SMAD2/3. The phosphorylated SMAD then produces a signalling complex with SMAD4 that is trans located to the nucleus. Finally, at the nucleus the SMAD complex interacts with winged helix transcription factor FOXH1 and the MIXER subclass of homeodomain proteins to induce expression of downstream targets, specific to the tissue activated target genes (Robertson, 2014; Shi and Massague, 2003). NODAL signalling acts as a morphogen, and depending on its concentration and/or signalling duration, different cell fates are specified (Robertson, 2014). Just like BMPs, Nodal can be inhibited at the extracellular level, for example by LEFTY1/2 which act as competitive inhibitors of Nodal binding to its serine/threonine kinase receptors, or at the intra-cellular level, for example by the inhibitory SMAD6/7 that limits availability of the co-receptor SMAD4, or SNOW that represses NODAL dependent targets (Robertson, 2014; Shi and Massague, 2003).

Mutants lacking *Nodal* or its intracellular signal transducer *Smad2* fail to form an anterior-posterior axis or a primitive streak and are embryonic lethal around E8.5 (Brennan et al.,

2001; Conlon et al., 1994; Waldrip et al., 1998). In contrast to *Smad2* mutants, *Smad3* knockout mice are fertile and viable with no overt defects during embryogenesis, but double *Smad2/Smad3* mutants exacerbate the *Smad2* phenotype, indicating that although SMAD2 is the primary signalling transducer during early embryogenesis, both these proteins cooperate to transduce ACTIVIN/NODAl signals (Vincent et al., 2003). Surprisingly, mice mutants lacking the Nodal specific type I receptor *Alk7* are viable and fertile, showing no defects during development, whilst *Alk4*$^{-/-}$ mutants phenocopy *Nodal* knockout mice (Jornvall et al., 2004; Song et al., 1999).

In vitro studies of NODAL signalling have shown that ACTIVIN (another TGFβ that signals through the same pathway but is CRIPTO-independent) signalling is not required for self-renewal or pluripotency of mouse ESCs but may promote BMP signalling by repressing Smad7, which may stimulates BMP signalling to promote self-renewal (Galvin et al., 2010). In contrast to this NODAL/ACTIVIN signalling plays a role in human ESC pluripotency and is required to maintain the self-renewal and pluripotency of later derived EpiSCs and induction of mesoderm and endoderm cell fates (Brons et al., 2007; Tesar et al., 2007; Vallier et al., 2005).

1.4 CELL COMPETITION

1.4.1 What is cell competition?

Cell competition is a type of cell-cell interaction that compares the fitness of a cell with that of its neighbours and it is conserved from *Drosophila* to mammals. During competition and through this relative fitness sensing, those cells less-fit than the surrounding tissues are eliminated, even though they would be viable in a different context. This mechanism was first described in *Drosophila* in the late 1970's. In a series of landmark experiments, Morata and colleagues used the *Drosophila* imaginal wing-disc to study the effect of creating clones that had different growth rates than the surrounding tissue. For this, they analysed cells carrying a heterozygous mutation in one of the *Minute* genes. The *Minute* genes encodes for ribosomal proteins, and although homozygous mutation of *Minute* is lethal, heterozygous animals are viable and display a developmental delay due to the slower proliferation rate of cells. Surprisingly, analysis of mosaic flies found that in contrast to the viability of heterozygous cells (*Minute*$^{+/-}$) in a heterozygous animal, when *Minute*$^{+/-}$ cells are surrounded

by wild-type cells, they undergo apoptosis (Morata and Ripoll, 1975; Moreno et al., 2002). Interestingly, starvation of the mosaic animals, that abolishes the growth differences between the wild-type and *Minute*$^{+/-}$ cell populations, rescues the elimination of the *Minute*$^{+/-}$ cells. Together these observations suggests that some mechanism must exist that can sense differential growth rates within a tissue and induce the elimination of the slower growing population.

The second big conceptual advancement in the field came when the Johnston group as well as Moreno and Bassler showed that cell competition is not necessarily triggered by the slow growth of a defective cell population, but that instead even wild-type cells can be eliminated when surrounded by faster growing neighbours. To prove this, both groups generated clones of cells in the *Drosophila* imaginal disc that over-expressed *d-Myc*, the homologue of the mammalian proto-oncogene *c-Myc*. What they observed was that whilst moderate levels of *d-Myc* over-expression increases the size of the over-expressing clones, the adjacent wild-type cells underwent apoptosis (de la Cova et al., 2004; Moreno and Basler, 2004). The observation, that even normal growing cells can be eliminated by competition implies that in a tissue, cell fitness is not only an absolute value, but that it is also a relative measure dependent on the fitness of neighbouring cells. Since these findings, cell competition has been described in *Drosophila* in a variety of different cell types and induced in many different ways, suggesting that it may act as a fitness sensor and have very broad implications.

The first indication that cell competition may be conserved in mammals came from analysis of belly spot and tail mutant (*Bst*) mice, that carry a heterozygous mutation in the ribosomal protein L24 gene. *Bst*$^{+/-}$ animals display coat colour pigmentation, skeletal and retinal defects due to decreased rates of protein synthesis and proliferation, but survive well until adulthood. In contrast to this, just as occurred in *Drosophila,* when *Bst*$^{+/-}$ cells are mixed with wild-type cells in a chimeric animal, they contribute very poorly to late stage embryos (Oliver et al., 2004). The fact *Bst* mice disrupt a ribosomal protein led to the suggestion that the *Bst* mutation was the mouse equivalent of *Minute*, and that their poor contribution to development was because they were being out-competed by wild-type cells.

Cell competition in mammals was also inferred during rat liver regeneration and in Madin-Darby canine kidney (MDCK) epithelial cells. In the liver, after partial hepatectomy, it was found that when rapidly proliferating foetal liver cells were transplanted into the damaged adult liver, these cells preferentially contributed to the regenerative response, not only due to their increased proliferative ability, but also by inducing the apoptosis of the wild-type host liver cells (Oertel et al., 2006). In MDCK cells, knockdown of VprBP/Mahjong, a protein that interacts with the cell polarity regulator Lgl, induces apoptosis specifically when the knock-down cells are surrounded by wild-type cells (Tamori et al., 2010). Further support for the conservation of cell competition in mammals came from two studies in the early mouse embryo. The first of these studies showed that just as happens in *Drosophila*, if *c-Myc* is over-expressed during the early stages of mouse development in a mosaic fashion, those cells with lower *c-Myc* levels are eliminated by apoptosis (Claveria et al., 2013). The second one found that not only the same occurred during embryonic stem cell (ESC) differentiation, but that additionally in stem cells and in the embryo, cells with a range of defects, including tetraploidy, decreased BMP signalling or defective authophagy, are eliminated specifically when surrounded by wild-type cells (Sancho et al., 2013). These results again highlight the existence of a mechanism that senses relative fitness levels and eliminates the less-fit cell type. Intriguingly, adult mouse cardiomyocytes can be subject to cell competition (Villa Del Campo et al., 2014) and also the replacement of T lymphocytes in the thymus (Martins et al., 2014), indicating that this mechanisms is not restricted to embryonic development, but may operate in a wide variety of contexts.

If we break down the process of cell competition, a minimum of three different steps can be envisaged. First, prospective winner and prospective loser cells must have inherently different fitness levels that will act as a trigger of competition. Second, these cells will need to be able to sense their relative fitness levels. Finally, those cells with lower fitness will activate the apoptotic pathway and the fitter neighbours undergo compensatory proliferation. Here we will summarise what is known about each of these steps.

1.4.2 What are the triggers for cell competition?

When thinking about possible triggers it is worth keeping in mind that it is likely that there are a variety of different modes of cell competition, and that each one of these types of cell

competition has not only different ways of being induced, but also different effector molecules. For the purpose of simplicity, I will primarily focus on those types of cell competition, where the winner cells actively eliminate the loser cells. However, there are other processes that have also been described to fall under the umbrella of cell competition, such as displacement from the niche of loser cells by winner cells in the *Drosophila* testis and ovary (Issigonis et al., 2009; Jin et al., 2008; Rhiner et al., 2009; Sheng et al., 2009) or in the mouse bone marrow (Bondar and Medzhitov, 2010).

1.4.2.1 Growth differences

In the majority of competition experiments described above, the two competing populations have an inherently different growth rate, with the winner cells growing faster than the loser ones. For example, in the *Drosophila* wing-disc *Minute* loser clones grow slower than their wild-type counterparts, and *dMyc* over-expressing winner cells have a higher proliferation status than wild-type cells. For these reasons, one of the first suggested triggers for cell competition has been a differential growth rate between the prospective winner and loser cell populations. However, one thing that is clear is that growth difference alone is not sufficient to induce competition. For example, increasing proliferation in clones of cells in the wing-disc by over-expression of the cell cycle regulators cyclin D and cyclin-dependent kinase 4 (Cdk4), or increasing signalling by mutation of *Pten*, is not sufficient for cells to acquire a winner status when surrounded by slower growing wild-type cells (de la Cova et al., 2004). Conversely, decreasing proliferation by reducing insulin signalling also does not induce competition, as when cells with lower insulin signalling are surrounded by wild-type cells they grow slower but do not undergo apoptosis (Bohni et al., 1999; Verdu et al., 1999). What this suggests is that at least in the context of the *Drosophila* wing-disc, a parameter that regulates growth, rather than growth *per se* may be what is compared during competition (Vivarelli et al., 2012).

An obvious candidate for a parameter regulating growth that could act as a trigger of competition is protein synthesis. The *Minute* and *Bst* mutations disrupt ribosomal proteins and *Myc* is a well-known regulator of protein synthesis (Dang, 2012; Eilers and Eisenman, 2008; Meyer and Penn, 2008). Alternatively, or as well, metabolism that plays a key role in determining the cells proliferation rate, may also act as a trigger of competition. Myc is a well known regulator of glycolysis and regulates metabolism during competition (de la Cova

et al., 2014). Also, in competition experiments using human, mouse and canine immortalised cell models, fast growing lines out-compete slow growing ones. Therefore, differences in energy metabolism are required to drive the elimination of the slow growing clones by the faster growing ones (Penzo-Mendez et al., 2015). It is therefore plausible that the different anabolic properties of prospective winner and loser cells could trigger competition. But how cells may measure their relative protein synthesis or metabolic rates is an intriguing question that will need to be addressed to understand if this is indeed the case.

1.4.2.2 Steep differences in signalling levels

A second group of mutations that induce competition are those that affect signalling pathways. For example steep differences in BMP/Dpp, WNT/Wg, JAK/SAT and Hippo signalling have all been shown to induce cell competition. However, given the important roles of these pathways in the regulation of cell proliferation, it is difficult to disentangle if it is actually differences in signalling levels that trigger competition or the downstream effects of these pathways on growth. As excellent reviews describing how these pathways induce competition have been published elsewhere (Amoyel and Bach, 2014; de Beco et al., 2012), here we will only summarise examples of the roles of these pathways to illustrate how a variety of different signalling inputs can have a similar outcome in triggering of competition.

The first signalling pathway found to regulate competition was the BMP/Dpp pathway. In *Drosophila*, *Minute*$^{+/-}$ mutant clones display lower levels of Dpp signalling that causes *Brk* activation and apoptosis (Moreno et al., 2002), and in mammals pluripotent cells with decreased BMP signalling are also eliminated by cell competition (Sancho et al., 2013).

These findings led to the suggestion that competition for Dpp/BMP may be a common theme, and this possibility will be discussed in more detail below.

Steep differences in WNT/Wg and JAK–STAT signalling also induce competition. For the WNT/Wg pathway, it was demonstrated that in the *Drosophila* wing-disc cells with low signalling levels are eliminated when surrounded by wild-type neighbours. Similarly, cells with *Axin* or *Apc* mutations that over-activate Wg signalling, behave as super-competitors and eliminate the surrounding wild-type neighbours (Suijkerbuijk et al., 2016; Vincent et al., 2011). In these experiments, competition is independent of Myc, but increasing the relative content of ribosomes enhances the ability of *Axin* mutant clones to out-compete their wild-

type neighbours, suggesting that the effects of WNT/Wg signalling and protein synthesis on competition are additive. In the case of the JAK-STAT, cells with deficient signalling are outcompeted in the *Drosophila* wing-disc and eye and sustained activation of this pathway allows cells to out-compete their wild-type neighbours. Furthermore, these effects are independent of Myc, WNT/Wg, BMP/Dpp and Hippo pathway activity as well as of ribosomal biogenesis, suggesting that loser cell elimination induced by differences in JAK-STAT signalling may represent an independent mode of inducing competition (Rodrigues et al., 2012).

A further pathway that when differentially activated between neighbouring cells stimulates competition is the Salvador–Warts–Hippo signalling pathway. In mouse, fibroblast cells with activation of this pathway are eliminated by their wild-type counterparts (Mamada et al., 2015) and in both *Drosophila* and mouse inhibition of the pathway by over-expression of *Yorkie* or *Tead4* leads to cells behaving as super-competitors and eliminating their wild-type neighbours (Mamada et al., 2015; Neto-Silva et al., 2010; Tyler et al., 2007; Ziosi et al., 2010). Myc is a well known target inhibited by Hippo signalling and many of the roles of Hippo in competition appear to be mediated by Myc activation (Menendez et al., 2010; Neto-Silva et al., 2010; Ziosi et al., 2010); however, it also appears likely that Hippo has Myc independent roles as Myc over-expressing clones respect clonal boundaries but the Hippo ones fail to do so.

1.4.2.3 Loss of polarity and disruption of epithelial integrity

A third group of mutations that trigger competition are those that affect cell polarity genes. These genes generally encode proteins that are essential for the maintenance of epithelial apical-basal polarity, such as *Scribble*, *Lethal giant larvae* (*Lgl*) or *Discs Large* (*Dlg*). When these genes are mutated throughout the animal they lead to neoplastic overgrowth and therefore cause tumours that disrupt tissue organization. However, in *Drosophila* and in mammalian MCDK cells, when these genes are mutated in a clonal fashion, and therefore when mutant cells are surrounded by wild-type tissues, they are eliminated by apoptosis (Agrawal et al., 1995; Brumby and Richardson, 2003; Chen et al., 2012; Gateff, 1978; Igaki et al., 2006; Norman et al., 2012; Pagliarini and Xu, 2003; Tamori et al., 2010; Woods and Bryant, 1991). This elimination can be rescued for example when polarity mutant cells are

surrounded by less-fit *Minute*$^{+/-}$ cells, indicating that what is inducing their elimination is their relative fitness level rather than an intrinsic survival defect (Froldi et al., 2010).

Cells with disrupted polarity do not have an inherent lower growth rate than their wild-type counterparts, and for this reason the disruption of cell polarity is thought to represent a distinct mode of competition to that caused by growth rate disparity (Vincent et al., 2013). However, there is some overlap between the pathways that regulate competition induced by growth and/or signalling differences and those that regulate competition induced by loss of polarity. For example, *Lgl* mutant clones have lower expression of *d-Myc* and higher Hippo signalling, reflected by the cytoplasmic localization of *Yorkie*. In these mutant clones, increasing *d-Myc* expression or inducing nuclear *Yorkie* activity can rescue their elimination by wild-type cells and cause neoplastic growth (Froldi et al., 2010; Menendez et al., 2010). Similarly, the competitive elimination of *Scribble* mutant clones can also be rescued by expression of d-Myc (Chen et al., 2012). Interestingly, other oncogenes appear to have a similar effect. Over-expression of Ras^{V12} or Notch causes tissue hyperplasia in *Drosophila* but does not induce cell competition (i.e. the elimination of wild-type surrounding cells). When these oncogenes are activated in polarity deficient clones such as those where *Lgl* or *Scrible* are mutated, they not only rescue their elimination, but also cause unrestricted growth, in a sense transforming them into super-competitors (Brumby and Richardson, 2003; Menendez et al., 2010; Pagliarini and Xu, 2003). However, it is worth noting that these results may also at least be partially explained by clone merging induced by the increased proliferation rate conferred by the oncogene over-expression. This merge of clones would create a microenvironment of polarity deficient cells that would be impervious to competition (Menendez et al., 2010).The fact that a variety of different cues could trigger competition, suggests that there also are likely to be stringent controls that limit the range of the competitive interactions. In this respect it is important to note that cell competition strictly respects compartment boundaries in *Drosophila* and to our knowledge this process has not been demonstrated to occur between cells with different lineage identities. For this reason, lineage has been suggested to represent a barrier that restricts fitness sensing only to cells of the same type (Levayer and Moreno, 2016).

1.4.3 How do cells sense their relative fitness levels?

An intriguing concept at the heart of cell competition is how cells sense their relative fitness levels. A number of theories have been put forward to account for the context-dependent removal of less fit cells and these can be broadly categorised into three distinct mechanisms: competition for growth factors/nutrients, direct cell fitness comparison and mechanical sensing.

1.4.3.1 Competition for growth factors/nutrients

One of the earliest models put forward to explain cell competition was based on the neurotrophic theory, where developing neurons compete for a limited pool of extracellular nutrients or growth factors required for their viability (Raff, 1992). The observation that in the *Drosophila* wing-disc loser cells in both the *Minute*$^{+/-}$ and d-Myc over-expression models of competition have lower levels of Dpp/BMP signalling and that this pathway inhibits apoptotic gene expression in these cells, led to the hypothesis that the Dpp ligand may be limiting and those cells less capable of accessing or transducing its signal would be eliminated by competition (Moreno et al, 2002; Moreno and Basler, 2004). This view was strengthened by the findings that in both these models activation of Dpp signalling in loser cells rescues their elimination (Moreno and Basler, 2004) and that in mammals pluripotent cells with defective BMP signalling are also eliminated by competition (Sancho et al., 2013). However, in *Drosophila* other groups did not find differences in Dpp/BMP signalling in the d-Myc or *Minute*$^{+/-}$ models of competition (de la Cova et al., 2004; Martin et al., 2009) and excess BMP ligands does not rescue the elimination of BMP signalling deficient pluripotent cells during competition (Sancho et al., 2013). Additionally, in *Drosophila* and mammals, cells with defective BMP signalling are not out-competed by other defective cell types, such as *Minute*$^{+/-}$ and autophagy deficient cells (Burke and Basler, 1996; Sancho et al., 2013; Sancho and Rodriguez, 2014), suggesting that these cells are not just competing for BMP/Dpp and that they are either competing for other factors, or other mechanisms must be triggering competition.

1.4.3.2 Fitness comparison mechanisms

An alternative explanation for the context-dependent elimination of less fit cells is that fitness levels can be read and communicated between cells during competition. The first molecular support for this idea came with the discovery of the *Flower* genes, which were proposed to

form a fitness fingerprint that identifies winner from loser cells during competition. *Flower* encodes a trans-membrane calcium channel that in *Drosophila* has three splice isoforms. It was shown that during competition induced by dMyc over-expression, *Minute*$^{+/-}$ mutation or loss of polarity, loser cells up-regulate the Fwe$^{Lose\text{-}A}$ and Fwe$^{Lose\text{-}B}$ isoforms, while the surrounding cells express only the 'ubiquitous' Fwe^{ubi} isoform and that the expression of the loser isoforms is sufficient to induce cell elimination (Merino et al., 2015; Merino et al., 2013; Rhiner et al., 2010). However, it is not clear how during competition the differences in *Flower* isoform expression are established, making it necessary for another sensing step to occur upstream of these isoforms to determine winner and loser status in the first place.

A second molecular explanation for how cells compare their relevant fitness levels has recently been proposed and it draws parallels between the recognition of less-fit cells and the detection of pathogens. The innate immune defence system allows recognition of non-self (e.g. microbial infection) as well as altered-self cells (e.g. viruses and cancer cells)(Song et al., 2012), therefore it presents an attractive possibility as a mechanism to tell apart cells with different fitness levels. In *Drosophila* the conserved Toll and Immune Deficiency signalling pathways mediate innate immunity, but during competition it appears that these pathways are re-wired to effect loser cell elimination. It was found that both in competition induced by d-Myc over-expression and *Minute*$^{+/-}$ mutation, the elimination of loser cells requires activity of Toll related receptors (TRRs) and NFκB homologues, that directly activate pro-apoptotic target genes (Meyer et al., 2014). Interestingly, the combination of TRRs and NFκB genes is different in each of these types of competition (see details bellow), suggesting that this new version of the innate immune pathway has different ways of distinguishing unfit (*Minute*$^{+/-}$), normal (wild-type) and super-fit (d-Myc) cell types.

1.4.3.3 Mechanical sensing

The possibility of fitness sensing by mechanical stress was initially proposed by Shraiman (Shraiman, 2005), who used mathematical modelling to show that in a population of cells with non-uniform growth rates, faster growing cells would compress the slower-growing surrounding cells. The model described that if the mechanical stress inflicted on the slower growing population were sufficiently large, it would be sufficient to activate apoptosis, relieving the overall pressure on the tissue. Since this model was postulated, cell death resulting from mechanical stress has been shown experimentally in a number of contexts. In

the developing *Drosophila* notum, cell crowding is sufficient to cause some cells to delaminate and be 'squeezed out' of the tissue, clearing the way for proliferation of the remaining cells (Marinari et al., 2012). Also in the pupal notum, induction of fast growing clones of cells by activation of the oncogene Ras induces tissue crowding that leads to the delamination and death of the surrounding wild-type cells up to three cell diameters away (Levayer et al., 2016). In both these studies *Flower* was not required for cell elimination, suggesting that mechanical cell competition is a distinct fitness-sensing pathway.

Mechanical stress-induced competition has more recently been described in mammalian MDCK cells (Wagstaff et al., 2016). Here, the elimination of *Scribble* knockdown cells by wild-type cells was found to being induced by compaction-induced stress. The authors observed that in this model, defective cells were hypersensitive to mechanical stress due to a basal level of p53 activation. This meant that when surrounded by wild-type cells they underwent cell compression that activated ROCK and p38, which in turn led to a further elevation of p53 levels and apoptosis. Interestingly, in wild-type MDCK and in mouse tracheal epithelial cells increasing p53 levels alone were sufficient to induce elimination, but only when these cells were surrounded by cells that could not elevate p53 ($p53^{-/-}$ cells), suggesting that p53 is acting as a key sensor of mechanical stress.

Alternative mechanical mechanisms for competition have also been described that do not involve the induction of mechanical stress. For example, in the *Drosophila* imaginal wing disc, during d-Myc induced competition, the elimination of loser cells is preceded by winner–loser cell mixing through cell–cell intercalation (Levayer et al., 2015). This cell mixing was found to be required for loser cell elimination and driven by the differences in tension generated by the relative difference in F-actin levels between winner-loser junctions. Also, extrusion of Ras^{V12} or *v-Src* MDCK cells when surrounded by wild-type cells was observed to occur in a Cdc42 and Rho kinase dependent manner (Hogan et al., 2009; Kajita et al., 2010). Or the engulfment of non-transformed cells by mammalian cancer cells has been suggested to occur due to the mechanical deformability of cancer cells and mediated by RhoA and actin-myosin (Sun et al., 2014). Therefore, in epithelial sheets, there appear to be a variety of different mechanical mechanisms capable of eliminating less-fit cells.

Conceptually, each on of these three sensing mechanisms, including competition for nutrients, comparative fitness sensing and mechanical sensing, all represent very different ways of fitness recognition. These conceptual differences are further highlighted by the fact that some researchers observe that cell competition requires cell-cell contact (Li and Baker, 2007; Portela et al., 2010), while others find that it does not (Sancho et al., 2013; Senoo-Matsuda and Johnston, 2007). It would seem plausible that the mechanism of fitness sensing activated during cell competition would depend on the context, timing and cell type being eliminated, and therefore even within the same tissue different selective pressure may co-exist.

1.4.4 Downstream events

Once cells have sensed their relative fitness levels the next step in competition is the enactment of winner and loser status, therefore the apoptotic elimination of less-fit cells and the compensatory proliferation of the fitter ones. These processes appear intimately related and interdependent, suggesting there are likely a number of feedback mechanisms that act to amplify an initial response. Little is known regarding what directs the compensatory proliferation of winner cells. In contrast to this, some of the triggers of apoptosis in loser cells have been identified, and these include some of the well-established stress response pathways, such as the JNK, NFκB and p53 cascades. As these pathways have been comprehensively studied, here we will focus on explaining those features that are specific to competition. However, analysis of these pathways also make clear that there are not only still important pieces of the puzzle missing, but also a need to understand how the fitness sensing machinery links up with the players already identified.

1.4.4.1 JNK

The JNK pathway was the first pathway to be characterised for its role in mediating competition. JNK signalling was initially described as induced in *Minute*$^{+/-}$ clones when these are surrounded by wild-type cells (Moreno et al., 2002) as well as in wild-type cells during their out-competition by d-Myc over-expressing cells (Moreno and Basler, 2004), and in both these studies inhibition of JNK prevented loser cell being eliminated. However, other groups failed to observe this activation or to see a rescue of competition induced by either of these triggers in a JNK mutant background (de la Cova et al., 2004; Tyler et al., 2007). For these

reasons, JNK has been suggested to be an enhancer of d-Myc/*Minute*$^{+/-}$ induced loser cell elimination, rather than an essential regulator of this process (Vincent et al., 2013).

Much less controversy exists regarding the role of JNK in competition caused by loss of epithelial polarity, where this pathway appears to play multiple roles. In *Drosophila* and canine MDCK cells, JNK signalling is required for loser cell elimination, as its inhibition prevents the apoptotic cell death of these cells (Brumby and Richardson, 2003; Igaki et al., 2006; Pagliarini and Xu, 2003; Tamori et al., 2010; Uhlirova and Bohmann, 2006). Additionally, JNK signalling also plays a role in winner cells during competition induced by loss of polarity. Here, JNK signalling up-regulates expression of the *Drosophila* PDGF/VEGF receptor, PVR, which then activates the phagocytic pathway causing the engulfment of the loser *Scribble* mutant cells (Ohsawa et al., 2011). Finally, a role for JNK in stimulating winner cell compensatory proliferation has also been reported in the adult fly mid-gut, where its activity in *Minute*$^{+/-}$ cells stimulates wild-type proliferation by regulating the expression of this Unpaired-3 (Kolahgar et al., 2015).

1.4.4.2 P53

The roles of p53 in loser cell elimination appear complex and dependent on the mechanism that triggers competition. In *Drosophila*, p53 is required for loser cell elimination during d-Myc-induced competition, possibly at several different steps. First p53 is necessary to sustain the proliferation of d-Myc winners when they are confronted with wild-type cells. During competition, d-Myc increases the glycolytic flux of the winner cells, a step necessary to drive the higher proliferation rates of these cells. In the absence of p53, d-Myc over-expressing cells do not increase glycolysis, and their proliferation levels, accumulate DNA damage and show increased apoptosis. Additionally, and maybe due these defects, these d-Myc over-expressing cells can no longer induce the elimination of their wild-type neighbours. Therefore, it was proposed that either directly or indirectly p53 regulates the signal that directs loser cell elimination (de la Cova et al., 2014). However, the apoptotic death of *Minute*$^{+/-}$ cell during competition was found to be independent of p53, suggesting in this context p53 may not perform such a role (Kale et al., 2015).

The role of p53 in competition induced by loss of polarity also appears to be very different. Under these conditions, p53 is up regulated in response to tissue crowding and this increase

in p53 levels is necessary for loser cell elimination. In canine MDCK cells, *Scribble* knockdown causes above basal levels of p53 and leads these cells to be hypersensitive to tissue crowding. Furthermore, when wild-type cells surround these knockdown cells, mechanical stress further increases p53 levels and induces their elimination. Interestingly, p53 activation is sufficient to make wild-type cells susceptible to mechanical stress, suggesting that p53 is acting in a dose dependent manner, with low levels making cells susceptible to mechanical stress and high levels inducing apoptosis (Wagstaff et al., 2016).

1.4.4.3 Azot

In *Drosophila* competition induced in a variety of ways, including d-Myc over-expression, differences in Wg or JAK/STAT signalling, and loss of *Minute* leads to the up-regulation of Azot in loser cells specifically when they are confronted with fitter-counterparts. Azot is a calcium binding, four EF-hand-containing cytoplasmic protein and evidence for its key role in competition comes from the finding that Azot mutation prevents the elimination of loser cells induced by d-Myc over-expression, or the culling of peripheral photoreceptor neurons in the pupal retina, that is also thought to occur through competition (Merino et al., 2015). These results suggest that Azot is a key target of the fitness sensing machinery; however, the fact that Azot inhibition does not affect the elimination of cells with polarity defects indicates that its function is dependent on the type of trigger that activates competition.

1.4.4.4 NFκB

In *Drosophila,* there are three NFκB homologues, Relish (Rel), Dorsal (Dl) and Dorsal related immunity factor (Dif), and during canonical signalling Rel functions downstream of the innate immunity pathway, while Dl and Dif are activated by the Toll related receptors (TRRs). However, during competition this signalling network appears to be re-wired. When super-competition is induced by dMyc over-expression, loser cell elimination can be prevented by mutation of Rel but not Dl or Dif. In this context, Rel was shown to work downstream of the TRRs 2,3,8 and 9. In contrast to this, when competition is induced by the generation of *Minute*$^{+/-}$ clones, the elimination of mutant cells can be rescued by inhibition of Dl or Dif, that act downstream of TRR 3 and 9. This differential response is also reflected at the level of pro-apoptotic target genes activated by these NFκB factors, as Rel activates Hid, but Dl and Dif appear to act through Reaper (Meyer et al., 2014). Therefore, how competition

is induced will determine not only the subset of NFκB molecules activated but also the type of apoptotic response.

1.4.4.5 Sparc

Sparc is glycoprotein that is part of the osteonectin family. In *Drosophila*, in competition induced by dMyc over-expression, *Minute*$^{+/-}$ mutation or loss of polarity, Sparc was observed to be up-regulated in the loser cells and transiently protect them against apoptotic elimination (Portela et al., 2010). However, using similar models another group did not observe Sparc up-regulation during competition (Rodrigues et al., 2012), suggesting that may be the case for JNK, this protein may play an enhancing rather than an essential role in competition.

Once the apoptotic machinery has been activated in loser cells, a number of different mechanisms have been suggested for the clearance of these dying cells. For example, in the *Drosophila* wing-disc *Minute*$^{+/-}$ mutant cells appear to be engulfed by the neighbouring wild-type cells during competition (Li and Baker, 2007). Loser cell debris was also observed inside wild-type cells during competition induced in mouse pluripotent cells (Claveria et al., 2013; Sancho et al., 2013). Importantly, in the *Drosophila* model inhibition of engulfment by mutation of genes required for this process prevented competition from occurring (Li and Baker, 2007), suggesting multiple levels of feedback between winner and loser cells during competition and that engulfment may be even upstream of the death of loser cells rather than downstream. Engulfment was also observed in the elimination of polarity in mutant cells (Ohsawa et al., 2011), but these observations contrast with others in the *Drosophila* wing-disc that do not observe a requirement for engulfment genes for the elimination of *Minute*$^{+/-}$ mutant cells or polarity deficient cells during competition or for the death of wild-type cells by d-Myc super-competitors (Lolo et al., 2012). Instead in this report, loser cells are extruded from the epithelia and their debris taken up by circulating haemocytes, that are attracted to the site by the secretion by loser cells of tyrosyl tRNA (Casas-Tinto et al., 2015). Extrusion from the epithelia was also observed during the elimination of polarity mutant cells in MCDK (Tamori et al., 2010). It is therefore likely that these different mechanisms of loser cell removal cooperate, and that the timing and type of competition stimulus determines which is activated in each context.

In summary, cell competition implies that cellular fitness is not only a cell-intrinsic property, but is also determined relative to the fitness of neighbouring cells– a cell that is of sub-optimal fitness in one context may be 'super-fit' in the context of a different cell population. This ability of cells to measure and communicate their relative fitness is not only essential for proper development and tissue homeostasis in the adult, but it can also be high jacked by cancer cells to promote their transformation. Over the last few years significant insights have been gained into some of the key parameters that cells can measure during cell competition (i.e. differences in growth, signalling, metabolism or polarity), and into the mechanisms by which cells measure these parameters and enact winner and loser status (e.g. mechanical sensing, Flower code or activation of the innate immune system), but big questions still remain. For example, it will be important to establish at the molecular level how the different triggers of cell competition lead to activation of the different sensing pathways that act during cell competition, and not when winner and loser cells are in a homotypic environment. This information will be key, as it will help establish approaches to therapeutically target competition specifically, without interfering with other cellular surveillance mechanisms.

1.5 AIMS OF THIS INVESTIGATION

The aims of this thesis are several-fold. When the work of this thesis was initiated we did not know if cell competition was conserved in mammals. Therefore, a first important aim was to unravel if this was the case. To address this question, I established a system to over-express *c-Myc* in a conditional manner to analyse if this led to a super-competitor status (an ability to eliminate surrounding wild-type cells). In parallel to this, I generated *in vivo* models of cell competition by analysing the expression of *c-Myc* during early development and the effects of mosaic deletion of *c-Myc* in the embryo. Additionally, I studied the effects of mosaic deletion of the cytochrome C oxidase in the heart, as this deletion had been suggested to lead to phenotypes similar to those described for cell competition. Finally, I analysed the contribution of metabolism as a possible cell fitness parameter that triggers competition. For this, I compared the metabolic profile of winner and loser cells during the onset of differentiation to unravel if these cells showed any distinguishing metabolic features. Together this research will provide invaluable insights into the importance of cell competition as a fitness sensor in mammals.

Chapter 2: MATERIALS AND METHODS

2.1 TISSUE CULTURE METHODS

2.1.1 mESC Lines and culture

E14 mESCs were a gift from Prof. A. Smith (Cambridge University, UK) and E14-GFP mESCs from Dr. Margarida Sancho (MRC, London, UK (Sancho et al., 2013). The *Bmpr1a*$^{-/-}$ FR124 mESC line (University of Southern California, USA (Qi et al., 2004) was given by Dr. Y. Mishina (University of Michigan, USA). cMYC-FR124 mESC lines were generated by electroporation of MYC-ER into the FR124 mESC line. cMYC-E14 ESCs were generated by transfection of MYC-ER into wild-type E14 mESC. In both cell lines, MYC-ER was under the control of the CAG promoter and the construct was a kind gift of S. Dalton ((University of Georgia, USA (Cartwright et al., 2005). All tissue culture reagents were from Gibco (Invitrogen). ESCs were maintained following standard conditions (Cambray et al., 2012).

2.1.2 Lipofection of mESCs

Lipofections, were carried out using Lipofectamine 2000 (Invitrogen) following manufacturer's instructions. Cells were transfected in a 20% confluent 6 well plates that had been seeded the previous day and fed 4 hours before transfection. Subsequently cells were maintained in standard ESC conditions. After 1-2 days antibiotic selection was initiated and colonies were picked after 8 to 10 days.

2.1.3 Electroporation of mESCs

A cell suspension of 3 x 10^7 cells in 1 ml GMEM was mixed with 30 μg of linear DNA and electroporated in a 0.4 cm electroporation cuvette (BioRad) using a BioRad Genepulser electroporation unit. Cells were exposed to two pulses; the first was at 240 volts, and the second at 230 volts, with capacitance set to 500 μF. After the second pulse the cuvette was tapped on the bench and placed on ice for 10 minutes. The cells were then plated into ESC media and selection was initiated after 24 hours.

2.1.4 Cell Competition Assays

After dissociation with Trysin-EDTA, cells was counted and 4x10^5 cells per well were plated into 0.1% (w/v) gelatinised 6 well plates. Cells of each genotype were plated separately or as a mixture of control and transgenic ESCs at either a 50:50 or 40:60 ratios depending on the

cell type. After plating, ESCs were incubated for 4 -5 hours in ESC media without LIF, then washed and kept in ESGRO Basal media (Milipore Coorporation, MA, USA) for the specified times. Media was changed daily thereafter. c-MYC induction was carried out with 4-hydroxytamoxifen (Sigma-Aldrich, MO, USA)

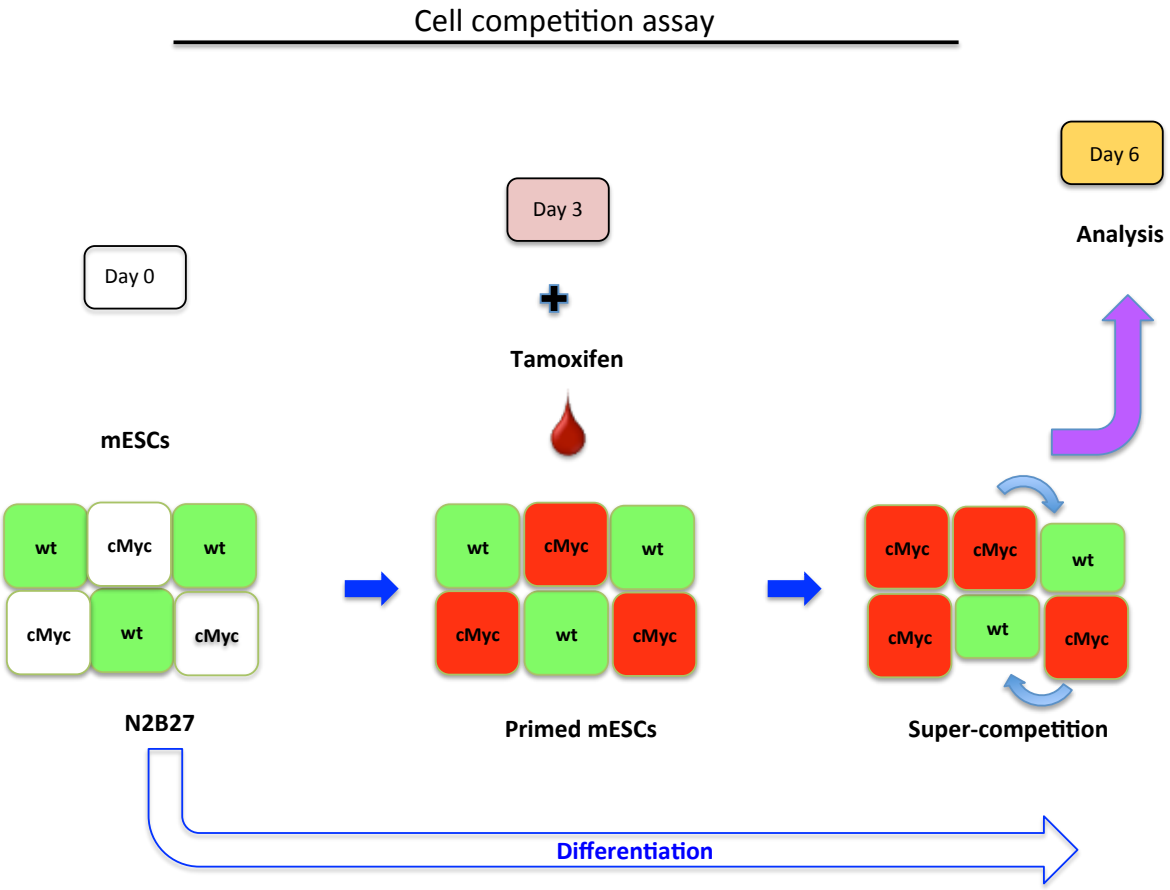


Figure. 2: Cell competition assay for inducible cMyc over-expression in mESCs
Cells require undergoing differentiation before induction with tamoxifen (tam). Higher levels of deregulated Myc provoke wild-type ESC elimination. Over expression of c-Myc was activated by daily systemic administration of tam in N2B27 media for 3 days. After 3 days of sustained overexpression of c-Myc activity, super competition is then analyzed on day 6. Control experiment consisted of N2B27 with methanol during 6 days without tamoxifen induction and, no cell competition was observed, not shown.

2.1.5 Flow Cytommetry analysis (FACs)

FACS analysis was performed using a BD LSR2 cytometer and later on a LSRII Flow Cytometer System (BD Biosciences) and FlowJo software analysis was performed.

For GFP expression analysis in separate and co-cultures, ESCs were washed with PBS, trypsinised, pelleted and resuspended in FACs buffer (3% FCS in PBS). For exclusion of dead cells, cells were stained with propidium iodide (PI). 3 ul of 1mg/ml PI solution (Sigma) was added per 500 ul of cell suspension prior to FACs analysis. PI+ve cells were excluded prior to analysis of GFP expression ratios. Fluorophore expression channels were GFP <525/50 B-A> channel PI <610/20 B-A> channel.

2.2 MOUSE PROCEDURES

All mice used in this investigation were maintained and treated under the Home Office's 'Animals (Scientific Procedures) Act 1986' in the Clinical Biological Services Unit at Imperial College London – Hammersmith Campus, UK.

2.2.1 Mouse Strains and Genotyping

The *cMyc*$^{fx/fx}$ conditional strain was obtained from Dinis Calado at CRUK-London (de Alboran et al., 2001) and was crossed to the *Mox2*-Cre deletor line (Hayashi et al., 2002) to obtain *cMyc*$^{del/+}$*;Mox2*-Cre males that were crossed to *cMyc*$^{fx/fx}$ females. DNA for genotyping was obtained from tail or ear biopsies and lysed for 10 minutes at 100^{o}C before allowing cooling and 100ul of 1M Tris-HCl (pH 8.5) added. Lysis samples were vortexed, spun down and stored at 4^{o}C. PCR was then performed to genotype the strains. Each PCR reaction was made up in 50 μl total volume containing 3-7 μl DNA lysate, 1 unit of Amplitaq, 125 ng of each primer (Sigma, UK), 5 μl 10x buffer and 0.2 mM of each dNTP (All Roche Diagnostics, Germany) made up in dH_2O. PCR was performed on a Peltier thermal cycler (PTC-100™) using the following program: Initial denaturation step at 95°C for 5 min, then 35 amplification cycles of 30 s at 94 °C, 30 secs annealing 58-60 °C, 45 s at 72 °C, followed by a final elongation step of 10 min at 72 °C. The resulting PCR products were size-fractionated by electrophoresis in 1% (w/v) agarose gels containing ethidium bromide and visualised under UV light.

°Primers used for genotyping were
MYC-Flox S Forward: GCC CCT GAA TTG CTA GGA AGA CTG
MYC-Flox A Reverse: CCG ACC GGG TCC GAG TCC CTA TT
MYC-Del Forward: TCGCGCCCCTGAATTGCTAGGAA

MYC-Del Reverse: TGCCCAGATAGGGAGCTGTGATACTT

Cre-Forward: CCAGCTAAACATGCTTCATC

Cre-Reverse: CGCTCGACCAGTTTAGTTAC

2.2.2 Embryology methods

Mating pairs were set up in the evening and plugs checked on the following morning. Pregnant females were sacrificed at the embryonic stage required and the uterus was removed. Embryos were then dissected from the uterus in M2 media as previously described (Hogan et al., 1994) and fixed, dehydrated and stored at -20 °C to be used for the according protocol (see Whole Mount in situ hybridization for detailed procedure of fixation and dehydration).

2.2.3 Embryo measurements

All embryos were dissected at 6.5 dpc and their epiblast length measured (from the apex of the egg cylinder the ExE boundary) using measured the Axial software for fluorescent microscope imaging. After these measurements were performed, embryos were genotyped using the protocols described above.

2.3.4 Cryosectioning

Embryos were transferred to an eppendorf tube containing 15% sucrose in PBS solution. This solution was replaced by a 30% solution when embryos reached the bottom of the tube. Embryos were then kept at 4°C until making the blocks. Blocks were made by first removing the 30% sucrose/PBS solution and then soaking the embryos for 30 min at 37 °C in a solution of 7.5% gelatine/15% sucrose in PBS. Wells of a 12-well plate were then half filled with this solution and once it had solidified each embryo was pipetted into a well and orientated. Once each well was completely solidified, the mould was extracted from the plate, cut, stuck to a piece of cardboard with OCT avoiding bubbles and placed in 2-methylbutane kept in dry ice for 1 min. Blocks were stored at -80 °C until sectioned. A Leica cryostat was used to cut the blocks in 8 μm sections. Sections were placed on SuperFrost Plus slides (VWR, West sussex, UK) and kept at -20 °C.

2.3 BIOCHEMICAL MOLECULAR ANALYSIS

2.3.1 Western Blot analysis

Cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris pH8, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1% (w/v) NP-40) containing Complete Mini Protease Inhibitors (Roche). Protein levels were quantified, and after denaturation (10 min at 95 °C), 40 μg of protein was loaded and separated in a 10% (w/v) acrylamide/Tris-HCl gel in glycine/Tris-HCl buffer. Proteins were then transferred onto a polyscreen PVDF membrane (Amersham) using horizontal semi-dry transfer. Membranes were blocked in 0.1u M sodium orthovanadate, 5% (w/v) milk in TBS, 0.1% (v/v) Tween (TBST) for 1 hour before incubation with primary antibody in 5% (w/v) BSA/TBST (or 5% (w/v) milk/TBST for cMYC), overnight at 4 ºC to reduce background. Antibodies and concentrations used are as following: rabbit anti-cMYC (N-262 clone, 1:200), mouse anti-PCNA (1:5000, Santa Cruz). Next, membranes were washed in TBST and subsequently incubated in peroxidase-conjugated secondary antibody (anti-rabbit, 1:2000, from Santa Cruz or anti-mouse 1:5000, from Sigma) in 5% (w/v) milk/TBST for 1 hour at room temperature.

2.3.2 RNA extraction, cDNA synthesis, Real-time qPCR

Total RNA was extracted using the Qiagen kit for RNA extraction as per the manufacturer's instructions. 1.5 μg of total RNA was then reverse transcribed using SuperScript III ReverseTranscriptase system (Invitrogen). PCR conditions were as follow: 95 °C for 15 min, then 40 cycles of 94 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s, followed by plate-read. Quantitative PCR (qRT-PCR) analysis was carried out using an OpticonII™ DNA machine and Opticon Monitor software both from MJ Research Inc. (RT)-PCR primer sets were obtained from Sigma and reactions included Sybr-Green PCR Mastermix (Qiagen), 300 nM primers and 2 μl of diluted cDNA template in a 30 μl reaction volume. Each measurement was performed in triplicate and data normalised according to the expression of selected housekeeping genes. Negative controls included reactions without reverse transcriptase (-RT) to detect residual genomic DNA.

The sequences of each specific set of primers were:

cMyc forward CCTAGTGCTGCATGAGGAGAC

cMyc reverse CCTCATCTTCTTGCTCTTCTTCA

Actin forward CTAAGGCCAACCGTGAAAAG

Actin reverse ACCAGAGGCATACAGGGACA

2.3.3 Immunofluorescence on sections

Cryosections kept at -20 °C were defrosted and brought to room temperature (RT) on the bench for 15 min. Gelatin was removed by incubation of the slides in PBS at 42 ^{o}C followed by three PBS changes for 20 min. Slides were then rinsed in PBST (PBS + 0.1% Triton-X100). Depending on the primary antibody, sections were fixed for further 5 min in 4% PFA at 4 °C and/ or antigen retrieval in 0.1M sodium citrate pH 6.0 for 30 minutes in steamer from cold followed by 30 more minutes in a steamer, in a new pre-warmed solution. Slides were then cooled on bench, washed in PBST and blocked with 10% (v/v) horse serum plus 0.2% (v/v) serum of the animal where the secondary antibody was raised in PBST for 1 hour at RT. Antibodies were diluted according to their specification in blocking solution diluted 1:2 and incubated over night. Slides were washed in PBST three times for 10 min at RT. Slides were then incubated in the dark with secondary antibody, the fluorophores Alexa diluted 1:500 for 40 min to 1 hour at RT. Slides were then washed for 5 minutes in PBST three times, and then one final time only in PBS. Slides were mounted using a slide with Vectashield with DAPI (Vector Laboratories, London UK) and confocal Imaging performed using a Zeiss LSM-780 inverted confocal microscope.

2.3.4 Immunofluorescence on embryos

Immediately after dissection, 6.5 dpc embryos were fixed in fresh 4% PFA in PBS containing 0.1% Tween and 0.1% Triton-X100 for 20 mins at 4C. Embryos were then washed in PBS-T (PBS+0.1% Tween) two times for 5 minutes. Permeabilization was done in 0.5% Triton-X100 dilution in PBS for 20 min at RT. After three washes in PBS-T for 5 minutes, embryos were peroxidise treated by incubation in 3% Peroxidase, 2% BSA (w/v) in PBS for 30 min at RT followed by three washes for 5 minutes each in PBS-T. Primary and secondary antibody staining were then performed as on sections (section 2.3.3).

2.3.5 Whole mount in situ hybridisation (WISH)

WISH was adapted from the standard procedures (Thomas and Beddington, 1996) as follows. After dissection, embryos were fixed on 4% filtered PFA (paraformaldehyde) in PBS at 4 °C

overnight. On the following day, embryos were dehydrated in serial dilutions of methanol in PBS (25, 50, 75 and twice in 100% methanol) 5 minutes in each dilution at room temperature (RT). Dehydrated embryos were kept at -20 °C. Stored embryos were placed in 15 mm net wells (Corning). Depending on the embryonic stage, either 500 µm or 74 µm mesh nets for 12 well plates were used (VWR). Embryos were rehydrated in serial dilutions of methanol in PBS/0.1% Tween (PBT) (75, 50, and 25% 5 min per wash) and twice-in PBT. From here after, 2 ml of solution per well was used. Embryos were bleached in 6% hydrogen peroxide in PBT for 1 hour to stop the activity of endogenous peroxidases at RT and then wash three times in PBT (5 min per wash). Embryos were digested in Proteinase K (10 µg/ml, in PBT) and incubated at RT for different periods depending on their developmental stage (5 min for 6.5 dpc, 6 min for 7.5 dpc, 7.5 min for 8.5 dpc and 10 min for 9.5 dpc). After digestion, embryos were placed in Glycine (2 mg/ml, in PBT) for 5 min to stop proteinase activity and then washed twice in PBT (5 min per wash). Following this, embryos were re-fixed in a solution of 0.2% glutaraldehyde / 4% PFA in PBT at RT for 20 min. This is followed by two 5 min washes in PBT. Embryos were then incubated in pre-hybridisation buffer (50% formamide, 5x SSC, 50 µg/ml heparin sulphate and 0.1% Tween-20 in dH_2O) at 70 °C for 1-2 h and then transferred to the pre-warmed hybridisation buffer (1 mg/ml torula RNA and 1mg/ml Herring sperm DNA in Pre-hybridization buffer) containing the denatured riboprobe. Embryos were incubated O/N at 70 °C. Probes were used at a dilution between 1:50 and 1:200 depending on their approximate concentration.

After hybridisation, embryos were washed two times for 30 min at 70 °C in solution 1, then two times in solution 1:2 for 10 min at 70 °C and finally 3 times in solution 2 for 5 min at RT. These washes are followed by a treatment with RNAse A (100 µg/ml in Solution II, Roche Diagnostics) diluted in solution 2 and incubated for 30 min at 37 °C (2 washes) to remove unbound probe. Embryos were washed once in Solution II and once in Solution III, 5 min per wash at RT after the RNAse A treatment. They were then washed twice in pre-warmed Solution III at 65 °C (30 min per wash) and three times in TBST, 5 min per wash, at RT before being blocked at RT in sheep serum (10% in TBST) for 1-2.5 hrs. After blocking, embryos were incubated with anti-dioxigenin FAB antibody (1:2000, Roche Diagnostics) in Sheep serum (1% in TBST) O/N at 4°C with rocking and washed six times in TBST (1 hour per wash at RT except for the final wash O/N at 4 °C) to remove unbound antibody. Embryos

were then washed three times in NTMT (100 Mm NaCl, 100 mM Tris-HCl pH 9.5, 50 mM $MgCl_2$ and 0.1% Tween-20 made up in dH_2O, 10 min per wash) at RT.

For signal detection, embryos were placed into Repelcote (BDH chemicals, Leith, UK) treated glass embryo dishes (RA Lamb, LE, UK). The staining reaction was then performed with NBT/BCIP solution 20 μL per 1 ml of NTMT (Roche Diagnostics Limited, West Sussex, UK)) at RT in the dark until a clean specific signal is observed. The solution is filtered using a 20 μm pore membrane prior to incubation with the embryos. Once staining had reached the appropriate signal level the embryos were washed with PBT (3 washes, 5 min per wash) to stop the reaction. Embryos were stored at 4 °C until they were photographed, genotyped or used for cryosectioning.

Solutions used: 1- 50% Formamide, 4x SSC pH 4.5, 10% SDS in dH_2O. 2-. 500 mM NaCl, 10 mM Tris-HCl pH 7.5 and 0.1% Tween-20 in dH_2O. 3- 50% Formamide, 2x SSC pH 4.5 in dH_2O. TBST- 137 mM NaCl, 1.34 mM KCl, 12.5 mM Tris-HCl pH 7.5 and 0.1% Tween-20 in dH_2O. NTMT-. 100 mM NaCl, 100 mM Tris-HCl pH 9.5, 50 mM $MgCl_2$ and 0.1% Tween-20 in dH_2O.

2.3.6 Whole mount TUNEL staining

Whole mount TUNEL staining was performed with the ApopTag® Plus Peroxidase In Situ Apoptosis Kit (Merck Millipore, Massachusetts, US). Briefly, dehydrated embryos were rehydrated through methanol gradients, washed in PBT (0.1% Tween-20 in PBS) and treated with proteinase K as for the regular WISH protocol previously described. Following this, they were re-fixed in 4% paraformaldehyde in PBT at RT for 20 minutes. They were then washed 5 times in PBT at RT and fixed in Ethanol/Acetic Acid (2:1) at –20 °C for 20 minutes. A further set of five PBT washes (5 minutes, 0.1% Tween-20) was done at RT before embryos were moved to 1.5 ml Eppendorf tubes, treated twice with equilibration buffer (5 minutes for the first treatment and 1 hour for the second) at 25 °C and incubated overnight at 37 °C in reaction buffer: TdT enzyme (70:30, 0.1% Tween-20). Following incubation embryos were washed 4 times with H_2O: stop buffer (34:1 for 45 minutes) at RT, then transferred back to Net-wells and washed 3 times in PBT (20 min per wash). Following

this immunohistochemistry using an anti-dioxigenin-AP conjugated antibody and signal detection was performed as previously outlined in the WISH protocol.

2.4 METABOLOMIC PROFILING

The metabolic profile was obtained using the Metabolon Platform (Metabolon). Each sample consisted of 5 biological replicates. For each replicate, $1x10^7$ cells were spun down and snap frozen in liquid nitrogen. Samples were analysed by Ultrahigh Performance liquid chromatography-Tandem Mass Spectrometry (UPLC-MS/MS) at 35,000 mass resolution, the raw data files are archived and extracted to follow a Gas Chromatography-Mass Spectrometry (GC-MS) in which, once the samples were dried under vacuum for the minimum of 18 h prior then, they are derived under dried nitrogen using bistrimethyl-silytrifluoroacetamide. At this point, samples were analysed on a fast-scanning single-quadrupole mass spectrometer using electron impact ionization (EI) and operated at unit mass resolving power, the scan range was from 50-750 m/z. The raw data files are archived and extracted using the bioinformatics system that consisted of 4 major components; the laboratory Information Management System (LIMS), the data extraction and peak-identification software, data processing tools for QC and compound identification, and a collection of information interpretation and visualization tools for use by data analyst. The hardware and software foundations for these informatics components were the LAN backbone, and a database server running Oracle 10.2.0.1 Enterprise Edition. Compounds were identified by comparison to library entries of purified standards based on the retention time/index (RI), mass to charge ratio (*m/z)*, and chromatographic data (including MS/MS spectral data) on all molecules present in the library. Samples were normalized to protein content measured by Bradford assay. Statistical significant cut-off: $p \leq 0.05$.

Chapter 3: ROLE OF CMYC IN MOUSE PLURIPOTENT STEM CELL COMPETITION

3.1 INTRODUCTION

The Myc proto-oncogenes encode pleiotropic transcription factors belonging to the family of basic helix-loop-helix/leucine zipper (bHLHZ) proteins. These proteins have an N-terminal transcriptional regulatory domain responsible for many of its protein-protein interactions, and a C-terminal bHLHZ DNA binding domain (Eilers and Eisenman, 2008). The three main Myc family members: c-Myc, N-Myc, and L-Myc are involved in fundamental cellular processes including proliferation, growth, differentiation, apoptosis and tissue homeostasis. Myc proteins have powerful transforming abilities, as when de-regulated and/or over-expressed they contribute to the generation of a variety of human tumours (Dang, 2012). During tumorigenesis, Myc proteins play many roles, including promoting proliferation, inhibition of differentiation, cell growth, the regulation of metabolism, reduction of cell adhesion, and metastasis (Dang, 2012). Myc proteins also play roles in cellular reprogramming illustrated by the fact that Myc is one of the four transcription factors used generate induced pluripotent stem cells (iPSCs) from differentiated cell types (Takahashi and Yamanaka, 2016).

Most characterized functions of c-Myc are when it is a dimer in partnership with the bHLH transcription factor Max, binding to a consensus six-mer motif known as an E-Box (CANNTG) (Blackwood and Eisenman, 1991). Binding of c-Myc/Max to E-boxes occurs near gene promoters and results in the activation of transcription (Dang, 2012). Recently, c-Myc was also found to be a global regulator of RNA Polymerase II (RNA Pol II) transcriptional pause release (Rahl et al., 2010). Transcriptional pausing occurs when additional regulatory steps are required to promote elongation of genes after the transcription has been initiated and c-Myc play a major role in promoting release from pause at its target genes. Thus, the main effect of c-Myc binding to their target gene promoters is to produce higher levels of transcription by promoting RNA Pol II transcriptional pause release. C-Myc seems to also stimulate elongation through direct recruitment of P-TEFb (Dang, 2012). MYC proteins also interact with a diverse range of epigenetic modifiers to promote open chromatin that is accessible to the transcription machinery, and therefore, MYC proteins also directly regulate the expression of specific target genes (Chappell and Dalton, 2013). Finally,

although c-Myc has primarily been implicated in gene activation, it also can have gene repression roles. The mechanism for c-Myc mediated repression is poorly understood, but is thought to involve interaction with the transcriptional repressor Miz-1 or activation of microRNAs (reviewed in (Herkert and Eilers, 2010).

In normal cells, both mRNA and protein expression levels of c-Myc are low and continuously dependent on mitogen signalling such as oncogenic kinases, Ras, or the Wnt/β-catenin pathway. At the translational level the regulation of c-Myc is complex, but one important negative regulator is GSK3β that phosphorylates c-Myc at T58 and mediates its ubiquitin-dependent degradation (Sears et al., 2000; Gregory et al., 2003; Dang, 2012; Gregory et al., 2003)

The use of gene targeting in mice has contributed enormously to our knowledge of the roles of c-Myc proteins during development. Although widely expressed in the murine embryo, *L-myc* knockout mice develop normally, indicating that L-*myc* is dispensable for development (Hatton et al., 1996). In contrast to this, *N-myc*-deficient mice die before 11.5 dpc, displaying neural, heart and lung defects (Charron et al., 1992; Moens et al., 1992; Stanton et al., 1992). Also, conditional knockout of *N-myc* in neural stem and progenitor cells leads to a profound disruption of brain growth due in part to altered neural stem cell cycling (Knoepfler et al., 2002). Finally, embryos with constitutive targeted disruption of *c-Myc* die before 10.5 dpc due to haematopoietic, placental and vascular defects (Davis et al., 1993; Dubois et al., 2008; Trumpp et al., 2001).

Myc proteins have also been demonstrated to play important roles in pluripotency. Myc is highly expressed in undifferentiated mouse ESCs and LIF signalling is required to sustain its expression by suppression of GSK3β activity (Cartwright et al., 2005). Upon differentiation (LIF withdrawal), depletion of c-Myc expression is observed as GSK3β is rapidly activated, leading to c-Myc phosphorylation at T58 and consequently ubiquitin-dependent proteasome degradation. Interesting, Sato and collaborators have shown that inhibition of GSK3β promotes self-renewal of mouse and human ESCs in the absence of LIF or Wnt (Sato et al., 2004) and inhibition of GSK3β and ERK1/2 signalling (2i) is sufficient to maintain mouse and human ESCs in a naïve pluripotent state during long-term culture (Takashima et al., 2014; Ying et al., 2008). This suggests that maintaining c-Myc expression in ESCs is an

important pre-requisite of pluripotency, an idea supported by the fact that constitutive c-Myc can sustain pluripotency in the absence of LIF (Cartwright et al., 2005). Further support for, comes from the observation that deletion of c-Myc and N-Myc induces loss of pluripotency and primitive endoderm differentiation (Smith et al., 2010). Myc proteins promote pluripotency in multiple ways, as indicated above, by acting as a general amplifier of transcription, as well as by regulating the expression of specific target genes and miRNAs (reviewed in (Chappell and Dalton, 2013). For example in ESCs cells grown in serum plus LIF, Myc proteins sustain pluripotency by repressing the primitive endoderm master regulator Gata6 and to contribute to cell-cycle control by regulating the mir-17-92 miRNA cluster (Lin et al., 2009; Smith et al., 2010). Additionally, Myc activity also reversibly controls the biosynthetic and proliferative machinery of naive ESCs, as inhibition of Myc function leads to ESCs entering a state of dormancy similar to embryonic diapause (Scognamiglio et al., 2016).

In the Rodriguez lab, cell competition was shown to occur between differentiating mouse pluripotent stem cells (Sancho et al., 2013). Given the roles of Myc proteins in pluripotency described above and cell competition, discussed in the introduction, I decided to test if c-Myc is mediating cell competition during early mammalian development.

3.2 RESULTS

3.2.1 *Bmpr1a*$^{-/-}$ ESCs are outcompeted by control cells and show decreased levels of c-Myc.

Bone Morphogenic Protein 2 (BMP2) and BMP4 are the mammalian homologues of *Drosophila Dpp*. BMP signalling is required for maintaining pluripotency of mouse epiblast cells in vivo (Di-Gregorio et al., 2007). The Rodriguez lab developed a mammalian model for cell competition using wild-type (WT) and *Bmpr1a* null ESCS. *Bmpr1* is a type I BMP receptor, and although null cells have impaired BMP signalling, they grow at similar rates to control cells. In contrast to this, when *Bmpr1a*$^{-/-}$ ESCs are grown in co-culture with WT ESCs in conditions that induce differentiation, they are out-competed and eliminated by apoptosis (Sancho et al., 2013). Interestingly when *Bmpr1a*$^{-/-}$ and WT ESCs are co-cultured in conditions that maintain pluripotency the mutant cells are not eliminated. This allows comparing the behaviour of wild-type and mutant cells in conditions where no competition occurs (co-culture in pluripotent conditions or in separate culture) to those that induce cell competition (co-culture in differentiating conditions – N2B27). These models have been used in this thesis to further elucidate the mechanism of mammalian cell competition.

In collaboration with Margarida Sancho, we asked whether c-Myc has a role in cell competition in mouse ESCs. To be able to distinguish WT and *Bmpr1a*$^{-/-}$ ESCs we used *Bmpr1a*$^{-/-}$-GFP cells, where the mutant cells express a constitutive GFP. We first analysed the c-Myc protein levels in the control and *Bmpr1a*$^{-/-}$-GFP ES cells in LIF plus foetal calf serum (ESC maintenance conditions) and in FACS sorted separate and co-cultures after 3 and 4 days in differentiation conditions (N2B27). We observed that in separate cultures the levels of c-Myc protein expression dramatically fell in both WT and *Bmpr1a*$^{-/-}$-GFP cells when these were induced to differentiate in N2B27 (Figure 3.1). This is in agreement to previously published results (Cartwright et al., 2005). No difference in the levels of c-Myc expression was observed between these two cell types in either pluripotency or differentiation conditions in separate culture (Figure 3.1). In contrast to this, in co-cultures the levels of c-Myc expression in WT cells were significantly higher at day 3 than those of *Bmpr1a*$^{-/-}$ ESCs or the levels of c-Myc expression in WT cells in separate cultures. These results suggest that c-Myc protein levels are up regulated or stabilised in control cells upon co-culture with *Bmpr1a*$^{-/-}$ ESCs.

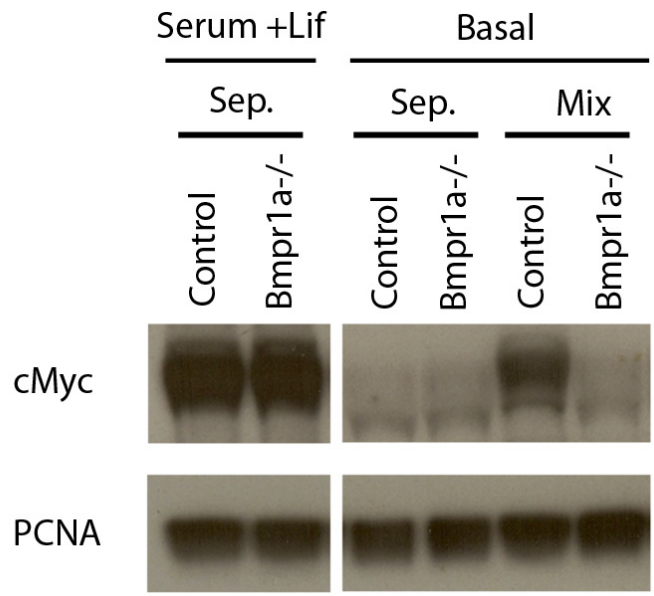


Figure 3.1 Control ES cells show increased cMyc levels when co-cultured with *Bmpr1a*$^{-/-}$ ES cells.
Western blot analysis showing the levels of cMyc expression in control and *Bmpr1a*$^{-/-}$ ES cells when co-cultured (Mix) or grown separately (Sep) showing that cMyc is expressed in control but not *Bmpr1a*$^{-/-}$ ES cells upon co-culture. PCNA was used as loading control.

3.2.2 Generation of *Bmpr1a*$^{-/-}$-c-MycT58A-ER ESCs

Given that *Bmpr1a*$^{-/-}$ ESCs were eliminated specifically when co-cultured with WT cells and exhibited lower levels of c-Myc expression, we decided to test if over-expression of c-Myc in *Bmpr1a*$^{-/-}$ ESCs could rescue this elimination. For this we generated *Bmpr1a*$^{-/-}$ ESCs carrying an inducible c-Myc. *Bmpr1a*$^{-/-}$ ESCs were transfected by lipofection with an expression vector containing the pCAG-c-MycT58A-ER plasmid (kind gift of Dr. Dalton). This plasmid contained a tamoxifen inducible c-Myc driven by the chicken beta-actin minimal promoter. The c-MycT58A mutation was used for these experiments as replacement of Threonine 58 for an Alanine prevents phosphorylation by GSK3β, an event that leads to c-Myc degradation during differentiation (Cartwright et al., 2005). Therefore, this mutation represents a more stable version of c-Myc and should accumulate to higher levels than the non-mutated version of the protein.

We analysed 18 out of 200 clones and in the first instance studied the levels of c-Myc expression by RNA analysis. We found that there was no significant difference between transfected clones and control un-transfected cells (Figure 3.2A). However, when western blot analysis was performed we observed high levels of c-Myc-ER expression in transfected

samples when compared to controls (3. 2B). One possible explanation for the difference in c-Myc levels between the RNA and protein analysis could be that c-Myc negatively auto-regulates its own expression and over-expression of c-Myc-ER could lead to down-regulation of the endogenous c-Myc transcription. Support of this hypothesis is the observation that on the western blots, the endogenous c-Myc band is stronger in the control cells than in the majority of the c-Myc-transfected clones. In contrast to this, as expected, the band for c-MycT58A-ER protein was very strong in transfected clones and absent in un-transfected *Bmpr1a*$^{-/-}$ ESCs. Based on the protein levels of c-MycT58A-ER expression (3. 3.2B), we selected clone 7 for further study.

3.2.3 Analysis of cell competition assays between E14-GFP and *Bmpr1a*$^{-/-}$-c-MycT58A-ER ESCs

To address if over-expression of cMyc can rescue the out-competition of *Bmpr1a*$^{-/-}$ ESCs, we tested the growth abilities of E14-GFP control and *Bmpr1a*$^{-/-}$-cMycT58A-ER ESCs when grown separately or together. We also compared the growth of these cell lines with or without tamoxifen, which should cause nuclear translocation of c-Myc and therefore induce the transcriptional activity of this protein. Cells were cultured separately or together (plated at a 60:40 E14: *Bmpr1a*$^{-/-}$ ratio) for 3 days in N2B27 and the relative proportion of cells of each genotype was measured by FACS and normalised to the original ratio of cells seeded at day 0. We observed the ratios of cells in co-culture and grown separately, either with tamoxifen or without were very similar suggesting that there was little difference in the growth rates of these cells in any condition (for example ratios at day 3 without tamoxifen grown separately=1.00±0.32; co-culture=1.15±0.34 and with tamoxifen grown separately= 0.80±0.24 and co-culture=0.78±0.21; Figure 3.2C-D). This data suggests that unlike what occurs with *Bmpr1a*$^{-/-}$ ESCs cells, *Bmpr1a*$^{-/-}$-c-MycT58A-ER ESCs are not eliminated when co-cultured with control cells. This occurs even without addition of tamoxifen and one possible explanation for this is that the system is leaky and some c-MycT58A-ER does enter the nucleus even without addition of tamoxifen. Further work is required to verify this possibility, but if this was show to be the case it would indicate that c-Myc over-expression is sufficient to rescue the elimination of *Bmpr1a*$^{-/-}$ ESCs and therefore that the down-regulation of c-Myc observed in *Bmpr1a*$^{-/-}$ cells during competition with wild-type cells is functionally important.

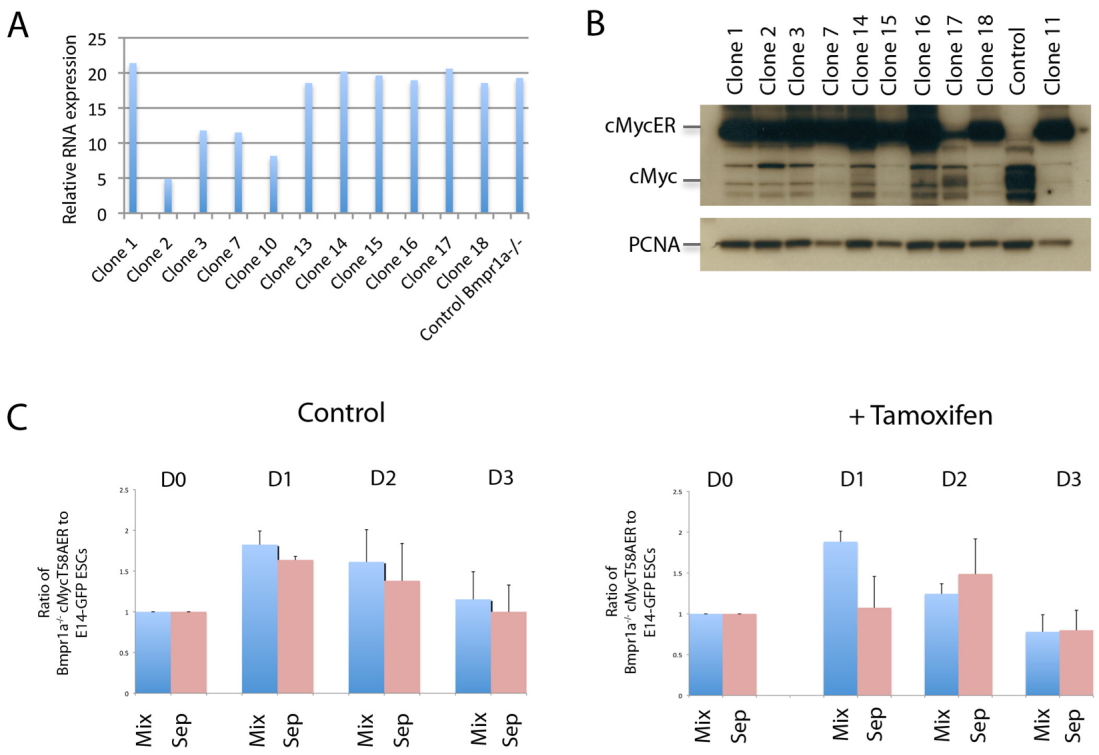


Figure 3.2 *A*nalysis of competition between E14-GFP and *Bmpr1a*$^{-/-}$*-c-MycT58A-ER ESCs.
(A) qRT-PCR analysis of *c-Myc* gene expression in transfected and control (*Bmpr1a*$^{-/-}$) clones. Expression values were normalized to *βActin* expression. Relative RNA expressions of the cMyc-T58A transfected clones are similar to controls. (B) Western blot analysis showed high levels of c-MycT58A-ER expression in transfected samples and not in controls. (C-D) Ratio of *Bmpr1a*$^{-/-}$c-MycT58A-ER to E14 control cells for the following conditions: separate cultures (sep) or co-cultures (Mix), with or without tamoxifen. Data shown as mean +/- SEM (n=2).

3.2.4 Generation of E14-GFP-cMycT58A-ER ESCs

To test if c-Myc over-expression turns cells into super-competitors (capable of eliminating wild-type neighbours) like it does in *Drosophila*, (de la Cova et al., 2004; Moreno and Basler, 2004), we decided to over-express the *c-MycT58A-ER* construct in wild-type ESCs. This would allow us to analyse their behaviour when cultured with untransfected cells before and after tamoxifen administration and therefore to determine if c-Myc over-expression can induce wild-type cell elimination. E14-GFP ESCs were electroporated with the *pCAG-cMycT58A-ER* construct and 18 colonies were taken for RNA and western blot analysis. Similar to what was observed in the analysis of *Bmpr1a*$^{-/-}$cMycT58A-ER cells, no significant difference was observed in the levels of *c-Myc* RNA expression between transfected and non-transfected cells in the majority of clones analysed (Figure 3.3A). One clear exception was

clone 2 that showed higher levels of *c-Myc* RNA. Analysis of c-Myc-ER protein levels revealed that all transfected clones showed c-Myc-ER protein expression, and interestingly clone 2 had lower expression than other clones, for example than clones 1 or 7 (Figure 3.3B). Based on the levels of c-MycT58A-ER expression we selected clone 7 for further study.

3.2.5 Analysis of cell competition assays between E14 and E14-cMyc-T58A-ER ESCs.

To test if over-expression of c-Myc induces competition, E14-c-MycT58A-ER and E14-GFP cells were plated at a 50:50 ratio and co-cultured or grown separately for 4 days in N2B27. FACS analysis was used to determine the relative proportion of cells of each genotype present in the co-cultures. Cells were cultured either without tamoxifen or with tamoxifen from day 0 to day 4, from day 1 to day 4, from day 2 to day 4 or from day 3 to day 4. However, we observed that in all samples analysed the relative ratio of E14-cMycT58A-ER to E14-GFP cells was higher in co-cultured samples compared to when these cell types were grown separately, independently of them being grown with tamoxifen or the duration of the tamoxifen treatment (Figure 3.3C). For example, for untreated co-cultured cells the ratio of E14-c-MycT58A-ER to E14-GFP cells was 2.96±0.122 and when they were grown separately it was 1.33±0.21. When these cells were treated with tamoxifen from day 0 to day 4 this ratio was 3.21±0.75 when the cells were co-cultured and 1.05±0.91 when they were grown separately. These results indicate that E14-cMycT58A-ER ES cells have a competitive advantage with respect to control cells when co-cultured and that addition of tamoxifen made little difference to this behaviour. This suggests that as with the *Bmpr1a*$^{-/-}$-c-MycT58A-ER ESCs, the system may be leaky and some c-MycT58A-ER does enter the nucleus even without addition of tamoxifen. Further work is required to verify this possibility, but as above, if the system were leaky it would indicate that c-Myc over-expressing ESCs can eliminate wild-type cells, and therefore induce cell competition.

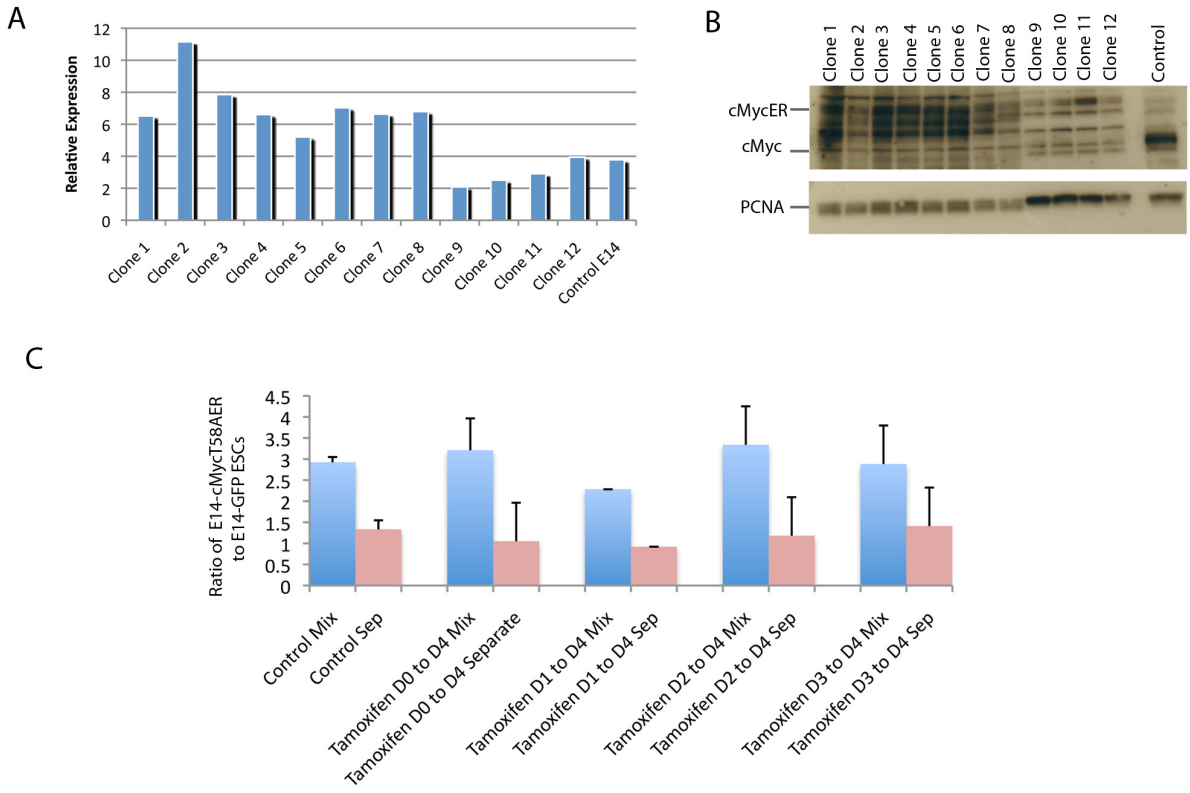


Figure 3.3 Analysis of *E14-GFP-cMycT58A-ER ESC* clones
(A) qRT-PCR analysis of the expression of c-Myc in 12 E14 clones transfected with c-MycT58A-ER and in a control untrasfected clone showing variable RNA expression levels. Expression values were normalised to *βActin*. (B) Western blot analysis showed different levels of c-Myc expression in the E14-cMycT58A-ER transfected clones. PCNA was used as the loading control. (C) Plots of the ratios of E14-cMycT58A-ER to E14-GFP ES cells grown in co-cultures and separate cultures in N2B27 for 4 days with addition of tamoxifen from day 1, from day 2 or from day 3 and analysed on day 4 (tamoxifen D0, D1, D2, D3). Cells grown without tamoxifen for 4 days were used as controls. Ratio of E14-cMycT58A-ER to E14-GFP cells were higher in co-cultured samples than in cultures of cells grown separately, independently of induction with tamoxifen or the duration of the treatment. Data shown as mean +/- SEM (n=3).

3.2.6 Generation of E14-c-Myc-ER ESCs

In an attempt to overcome the leakiness or the increased sensitivity of c-MycT58A we used a construct containing the wild-type c-Myc instead of the cMycT58A mutation, as the wild-type protein would be more sensitive to GSK3b mediated degradation. To achieve this we first lipofected E14 cells with the pCAG-cMycER construct and 32 colonies were taken for western blot analysis. Thirteen samples were analysed and showed different levels of c-Myc-ER protein expression (Figure 4.3A). Analysis of c-Myc levels in E14 and E14 transfected cells revealed clones with high and low levels of c-Myc expression. Clones 12 and 13 were selected as representative of each of these types (Figure 4.3A).

3.2.7 Analysis of E14-c-Myc-ER ESCs

First, we analysed clone 12 that showed high c-Myc protein expression. E14-cMycER and E14-GFP cells were plated at a 50:50 ratio and co-cultured or grown separately. We cultured the cells for 6 days in N2B27, and the relative ratio of control and c-Myc over-expressing cells were determined by FACS analysis at days 4 and 6. We observed that by day 3 c-Myc-ER over-expressing cells cultured in N2B27 showed very tight rounded colony morphology and a significant number of these colonies detached from the plate, suggesting that c-Myc over-expression was altering the adhesive properties of these cells. This fact was likely to distort the ratios obtained by FACS analysis. When these ratios were plotted we found that when cells were co-cultured, the ratio of E14-cMycER to E14-GFP cells was 0.48 at day 4 and 0.71 at day 6 and when these cells were grown separately these ratios were 0.84 and 0.66 (Figure 3.4B). This data indicated that the E14-cMycER cells were at a disadvantage, with respect to the E14-GFP cells even without tamoxifen addition. It is likely that this disadvantage was due the E14-c-Myc-ER cells detaching from the plate, and for this reason we did not pursue these cells any further.

Next we analysed clone 13, this displayed low levels of c-Myc protein expression. We first test if this clone had a competitive advantage over E14-GFP cells without tamoxifen, as occurred with the E14-cMycT58A-ER ESCs. For this, the E14-cMyc-ER and E14-GFP cells were plated at a 50:50 ratio and co-cultured or grown separately for 6 days in N2B27 and the relative ratio of each cell type was determined by FACS analysis at days 4 and 6. When we plotted the ratio of E14-c-MycER to E14-GFP cells, we observed that when these cells were co-cultured this ratio was 1.05 at day 4 and 1.63 at day 6, and when these cells were grown separately the ratio was 0.98 at day 4 and 1.17 at day 6 (Figure 3.4C). This indicates that under these conditions E14-c-MycER and E14-GFP grow at similar rates and when co-cultured for 6 days without tamoxifen the proportion E14-cMycER cells was only slightly higher than the proportion of E14-GFP cells. For this reason this clone was chosen for further analysis.

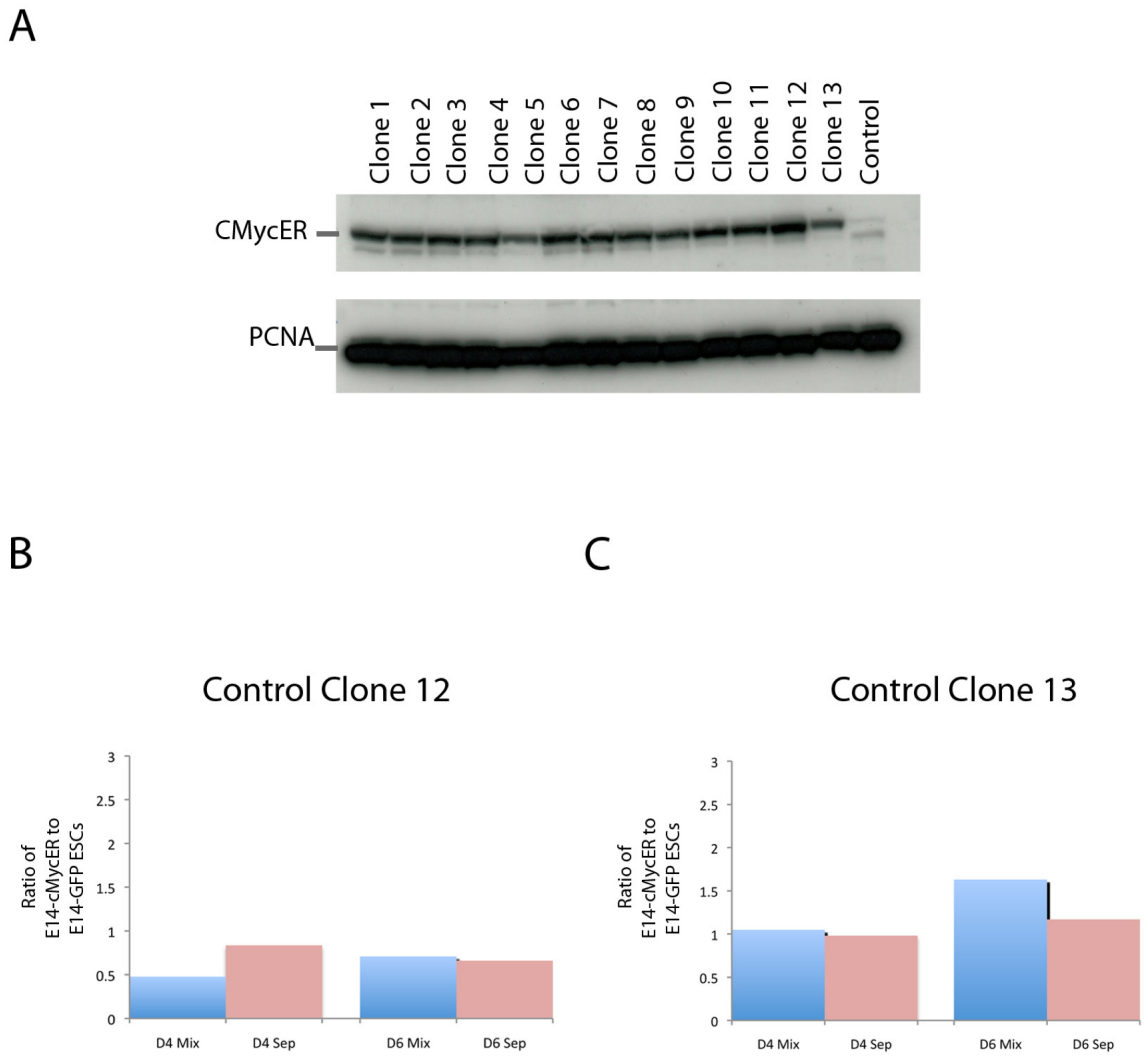


Figure 3.4 Analysis of E14-c-Myc-ER ESCs
(A) Western blot analysis of 12 c-Myc-ER transfected clones and an E14 control ESC line. Clones expressed high and low levels of the transgene (c-Myc-ER). PCNA was used as a loading control and clones 12 and 13 were chosen for further analysis. (B-C) Ratios of c-Myc-ER to E14-GFP ESCs obtained when cells were grown in separate cultures (Sep) and in co-cultures (Mix) for 4 days (D4) and 6 days (D6), for clone 12 (n=1), (B) and for clone 13 (n=1) (C).

3.2.8 Analysis of cell competition assays between E14-GFP and E14-c-Myc-ER ESCs.

To address if over-expression of c-Myc leads to cell competition (elimination of wild-type cells) we induced c-Myc-ER activation by culturing the cells in tamoxifen. In order to establish the optimal concentration of tamoxifen that could induce cell competition we tested

4 different tamoxifen concentrations, 850 nM, 500 nM, 100 nM and 10 nM. E14-cMyc-ER and E14-GFP cells were plated at a 50:50 ratio and co-cultured or grown separately with tamoxifen for 6 days in N2B27 and the relative ratio of each cell type was determined by FACS analysis at day 6. We observed that the ratio of E14-cMycER and E14-GFP cells was very similar when cells were co-cultured or grown separately at all tamoxifen concentrations (co-culture ratio= 0.79, 0.47, 0.43 and 0.73; separate ratio 0.73, 0.34, 0.45 and 0.63 for tamoxifen 850 nM, 500 nM, 100nM, 10nM respectively; Figure 5A-D). Similar was observed in the two experiments we performed as controls, therefore without tamoxifen treatment (co-cultured ratio= 0.68 and 1.44; separate ratio= 1.03 and 1.16; Figure 5A-D). This suggested that induction of c-Myc did not lead to cell competition, as the ratio between E14-cMycER and E14-GFP cells was similar when cells were co-cultured or grown separately. However, data from other people in the laboratory suggested that cell competition only occurred once ESCs initiate differentiation. Given that over-expression of c-Myc had been reported to maintain cells in the pluripotent state (Cartwright et al., 2005), a possible explanation for this observation is that c-Myc was unable to induce competition as the E14-cMyc-ER ES cells were still in the ESC state.

To overcome this problem we cultured cells for 3 days in N2B27, that allows for ESCs to exit the pluripotency state and then added tamoxifen for a further 3 days. We again tested 4 different tamoxifen concentrations, 850 nM, 500 nM, 100 nM and 10 nM. E14-c-Myc-ER and E14-GFP cells were plated at a 50:50 ratio and co-cultured or grown separately and the relative ratio of each cell type was determined by FACS analysis at day 6 and normalised to the ratio of the each cell type at the time of tamoxifen addition (day 3). We observed that the proportion of E14-c-Myc-ER was higher than that of E14-GFP cells at the 850 nM and 10 nM tamoxifen concentrations when cells were co-cultured but not when they were grown separately (co-culture ratio 850 nM= 1.10, 500 nM= 0.72, 100 nM= 0.56 and 10 nM= 1.17; separate ratio 850 nM= 0.52, 500 nM= 0.56, 100 nM= 0.51 and 10 nM= 0.65; (Figure 3.5A-D). Therefore, the ratio of E14-cMycER to E14-GFP cells was double when cells were co-cultured compared to when they were grown separately. This suggested that both these conditions induced cell competition. Given that with the 10 nM tamoxifen treatment the cells were generally healthier and that this condition led to less overall levels of dead cells in the media we chose this condition for further studies.

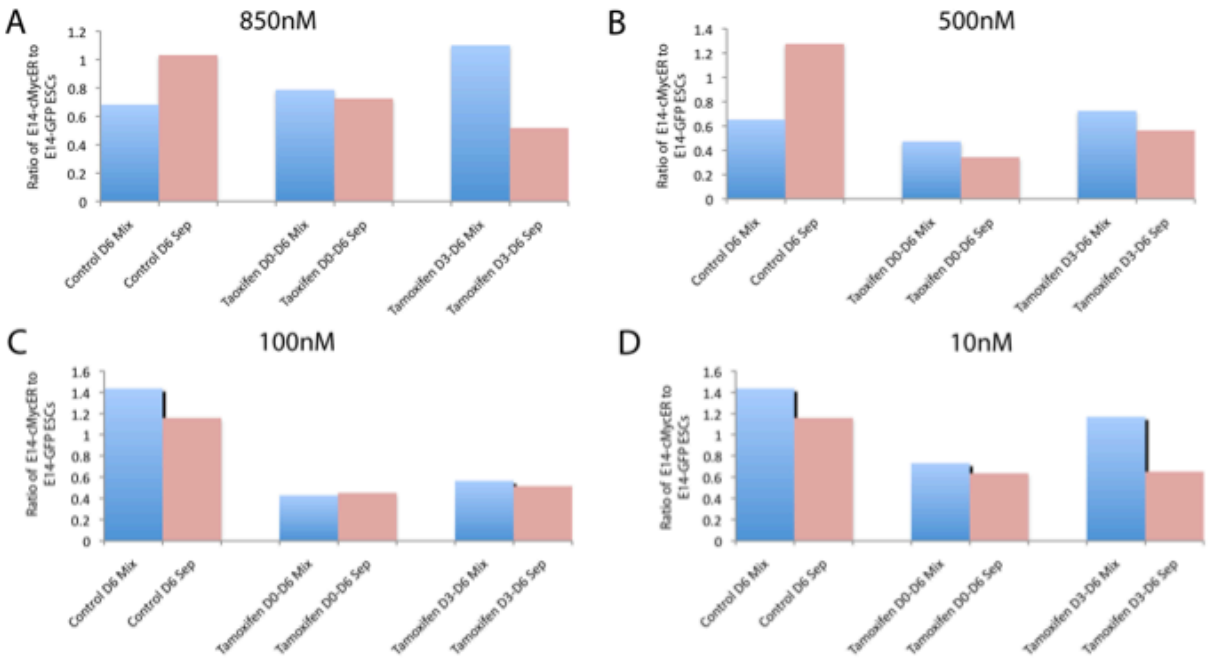


Figure 3.5 Optimization of the conditions for cell competition assays between E14-GFP and E14-c-Myc-ER ESCs (A-D)

Representation of the c-Myc-ER/E14-GFP ratios obtained when cells were grown in separate cultures and in co-cultures for 6 days with 850 nM, 500 nM, 100 nM or 10 nM tamoxifen concentrations. For these experiments tamoxifen was added from day 0 to day 6 (D0-D6) or from day 3 to day 6 (D3-D6). Control cell competition assays were performed without tamoxifen. These experiments showed that whilst c-Myc activation did not provide c-MycER ESCs with a competitive advantage in co-cultures when tamoxifen was administered from day 0, it did provide an advantage when tamoxifen was administered between das 3 to day 6 for the 850 nM and the 10 nM concentrations (n=1).

3.2.9 Over-expression of c-Myc induces cell competition during ESC differentiation

Our studies suggest that when E14-cMyc-ER ESCs are co-cultured with wild-type cells for 3 days in N2B27, and then for a further 3 days in N2B27 plus 10 nM tamoxifen, this induces cell competition (i.e. the elimination of wild-type cells by c-Myc over-expressing cells). To confirm this, we plated E14-cMyc-ER and E14-GFP cells at a 50:50 ratio and co-cultured them or grew them separately for 3 days without tamoxifen and for a further 3 days with 10 nM tamoxifen and studied their growth rates. The relative ratio of each cell type was determined by FACS analysis at day 6 and normalised to the ratio of the each cell type at the time of tamoxifen addition (day 3) and the growth curves of these cell types were also plotted

during this period. Analysis of the ratio of E14-cMyc-ER to E14-GFP cells indicated that there was a significantly higher proportion of E14-cMyc-ER cells when these cells were co-cultured compared to when they were grown separately (co-culture ratio=1.81±0.16; separate ratio=0.52±0.12; $p<0.005$; Figure 6A). When these cells were grown without tamoxifen treatment we observed that there were more E14-cMyc-ER cells in co-culture and less when these cells were grown separately (co-culture ratio=1.37±0.18; separate ratio=0.85±0.13; Figure 6A), suggesting that the system was still to some degree leaky. However when these ratios of tamoxifen treated cells are compared to the ratios of cells without tamoxifen treatment we could observe that these ratios are higher for the tamoxifen co-culture condition and lower for tamoxifen separate condition, indicating that c-Myc over-expression was benefiting cells when these are grown with control cells.

We next analysed the growth curves of E14-cMyc-ER and E14-GFP cells in each condition. For cells grown separately $0.4x10^6$ cells were plated for both E14-cMyc-ER and E14-GFP ESCs. When these cell types were co-cultured a total of $0.4x10^6$ cells was plated, and this number included $0.2x10^6$ E14-c-Myc-ER ESCs and $0.4x10^6$ E14-GFP cells. We observed that without tamoxifen E14-GFP cells grew at similar rates to E14-c-MycER cells in both the separate and co-culture condition (Figure 3.6B). However upon addition of tamoxifen we observed that c-Myc over-expressing cells showed decreased in growth rate compared to E14-GFP cells when grown separately but a higher growth rate than E14-GFP cells when both these cell types were co-cultured (Figure 3.6C). This indicates that cMyc over-expressing cells out-compete control cells when grown together and support the hypothesis that c-Myc can induce cell competition at the onset of ES cell differentiation. Further experiments are required to establish if wild type cells are undergoing proliferation arrest or apoptotic elimination, as occurs with *Bmpr1a*$^{-/-}$ ESCs when they are co-cultured with wild-type ESCs (Sancho et al., 2013).

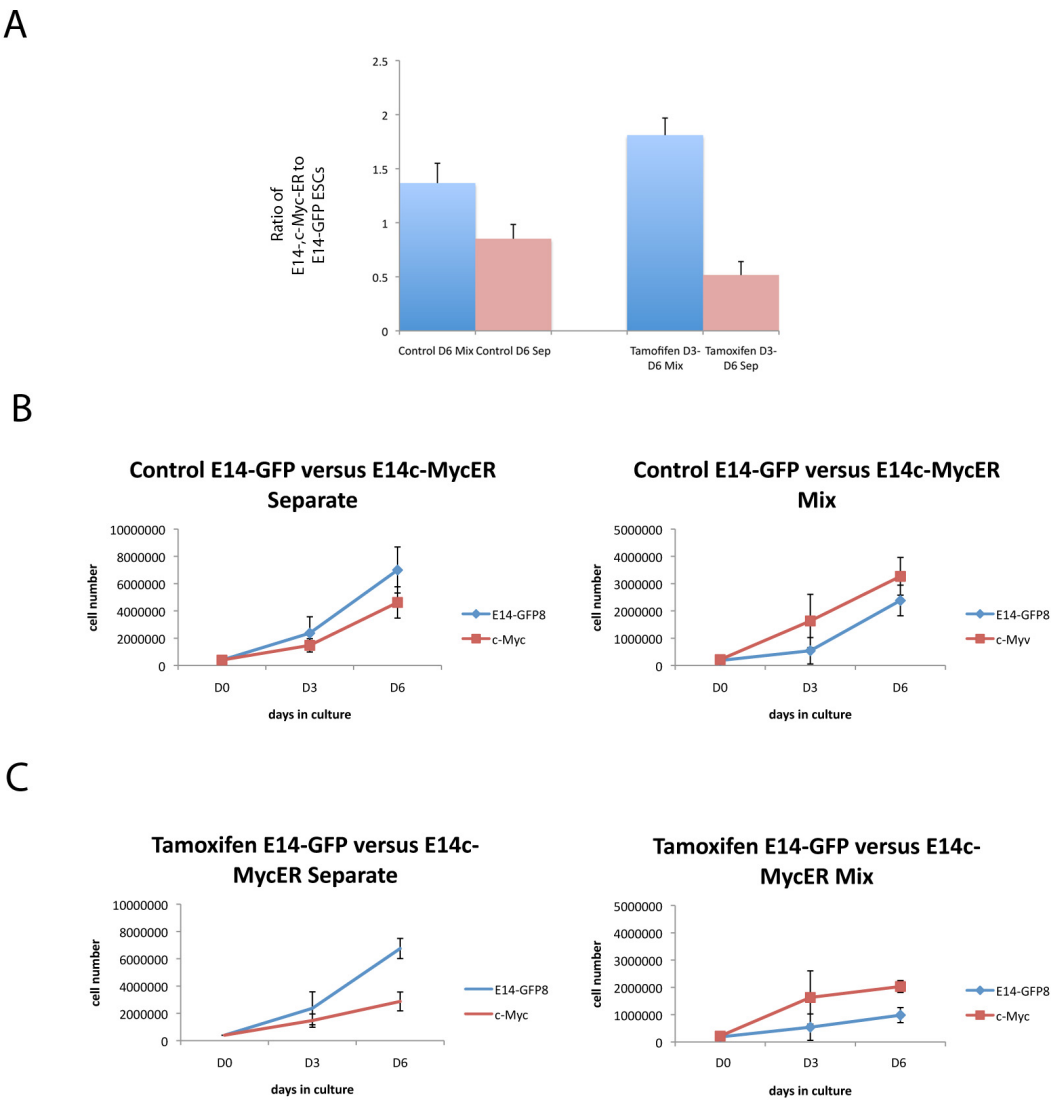


Figure 3.6 Over-expression of c-My-cER induces cell competition during ESC differentiation.
(A) Representation of the c-Myc-ER/E14-GFP ESC ratios obtained when cells were grown in separate cultures or in co-culture for 6 days, without tamoxifen (Control D6) or with tamoxifen added from day 3 to day 6 (Tamoxifen D3-D6). Data shown as mean +/- SEM (n=6) and difference between separate and co-culture ratios after tamoxifen treatment $p<0.005$, student t-test. (B) Growth curves of E14-GFP and c-Myc-ER over-expressing ESCs (E14-c-Myc-ER), when grown separately or in co-culture in N2B27 for 6 days or (C) in N2B27 for 3 days and further 3 days in N2B27 media supplemented with tamoxifen (C). D0, D3 and D6 indicate the length in days of culture. Data shown as mean +/- SEM (n=3). Both the ratios and growth curves indicate that E14-c-Myc-ER ESCs show a competitive advantage when co-cultured with control cells.

3.3 DISCUSSION

Cell competition is a biological phenomenon where the coexistence of two cell populations with different metabolic properties or growth rates, results in growth of the stronger population at the expense of the weaker one (Baker, 2011; Diaz and Moreno, 2005; Johnston, 2009). We hypothesized that cell competition could be a mechanism that ensures that those stem cells that accumulate mutations that do not affect their viability but that could affect their ability to contribute to embryonic development are eliminated via cell competition. To address this question the Rodriguez laboratory has established a model of cell competition based on the co-culture of control and *Bmpr1a*$^{-/-}$ ESCs. In this model the co-culture of *Bmpr1a*$^{-/-}$ ESCs with control cells leads to the elimination by apoptosis of the *Bmpr1a*$^{-/-}$ ESCs. Here we show that during this process the protein levels of the c-Myc oncogene become stabilized in control cells and decreased in *Bmpr1a*$^{-/-}$ ESCs. Furthermore, I have established a system where I can over-express c-Myc in an inducible manner. I find that over-expression of c-Myc during the differentiation of ESCs leads to a growth advantage with respect to control cells only when c-Myc over-expressing and control cells are co-cultured, suggesting that cMyc over-expression induces cells competition (i.e. the elimination of wild-type cells).

3.3.1 c-Myc over-expression induces cell competition

To study the possible role of c-Myc in cell competition we initially attempted to over-express a tamoxifen inducible mutation in c-Myc containing a threonine 58 to alanine substitution. This T58A mutation has been shown to prevent phosphorylation by GSK3b, an event that causes c-Myc degradation during differentiation (Cartwright et al., 2005). Therefore this mutation represents a more stable version of c-Myc than the wild-type protein. This c-MycT58A was transfected into control ESCs to understand if over-expression of c-Myc induces cell competition and into *Bmpr1a*$^{-/-}$ ES cells in attempt to rescue their elimination when co-cultured with control cells. However we observed that over-expression of c-MycT58A in control cells led to growth advantage of c-Myc over-expressing cells in co-culture even without addition of tamoxifen. This growth advantage of c-MycT58A expressing cells was not observed when they were cultured separately. Similarly over-expression of c-MycT58A prevented the out-competition of *Bmpr1a*$^{-/-}$ ES cells by control cells even without the addition of tamoxifen. These results suggest the system is leaky.

Three possible explanations could account for the effects seen in c-MycT58A over-expressing cells. In the first place it is possible that the integration of the transgene carrying c-MycT58A could lead to mutations that cause the growth advantage seen. However we believe this possibility to be unlikely as multiple clones were analysed and all behaved in similar fashion. The second possible explanation is that during cell competition c-Myc functions in the cytoplasm. Tamoxifen induces translocation of the protein fused to ER to the nucleus and therefore it is possible that the c-MycT58A mutation was having an activity independent of the translocation to the nucleus of the c-MycT58A protein. Indeed a cleaved form of cMyc, c-Myc-nick has been proposed to be a driver of cytoplasmic reorganization and differentiation (Conacci-Sorrell et al., 2010). The third possibility is that in over-expressing cells a small amount of c-MycT58A enters the nucleus and that given that the T58A mutation makes this protein less sensitive to degradation, these small levels are sufficient to trigger c-Myc-dependant functions.

To overcome the possible problems caused by the oversensitivity of the c-MycT58A mutation I over-expressed c-Myc-ER without this mutation. I found that when this was done, in the absence of tamoxifen, c-Myc over-expressing cells only had a small growth advantage when co-cultured with control cells. Similarly, c-Myc over-expressing cells did not grow faster than control cells in separate cultures after the addition of tamoxifen. They actually, grew slower than control cells. In contrast to this, addition of tamoxifen to the culture media of co-cultures of c-Myc-ER and control ESCs led to an obvious competitive advantage of the c-Myc expressing cells. In these co-cultures control cells reversed the behaviour that they displayed in separate cultures, and changed from growing exponentially to not increasing in number between days 3 and 6, when tamoxifen was added. This indicates that cMyc over-expression is sufficient to induce cell competition. However, further work is required to understand if c-Myc over-expressing cells induce the elimination or the proliferation arrest of control cells. Also, the signals secreted by c-Myc over-expressing cells to control cells that cause their change in behaviour remain to be elucidated.

3.3.2 Cell competition is dependent on the onset of differentiation

Another interesting question relates to the stage of development at which competition is occurring. The experiments described here suggest that cell competition takes place only

once pluripotent cells have initiated the differentiation program. Evidence for this is that when we induce c-Myc-ER activity with tamoxifen in ESCs, over-expressing cells do not show any growth advantage in co-culture. However when c-Myc-ER over-expressing cells are allowed to initiate differentiation by culture in N2B27 for 3 days (that lacks the factors that maintain ESCs pluripotent (Ying et al., 2003b) and then we induce c-Myc-ER activity with tamoxifen for a further 3 days, we find that over-expressing cells now show a growth advantage in co-culture. C-Myc over-expression has been shown to maintain cells in the pluripotent state (Cartwright et al., 2005), therefore one possible explanation for this finding is that competition can only occur once differentiation has been initiated. Support for this observation are the findings that addition of LIF, inhibition of Erk1/2 signalling and inhibition of Gsk3b activity, events that have all been shown to maintain ESCs in the pluripotent state (Nichols and Smith, 2009), also inhibit cell competition (Sancho et al., 2013). Together these results argue that cell competition is an event that takes places during the first stages of differentiation and is no existent in the naïve pluripotent state.

In conclusion our findings support that cell competition is a mechanism that monitors embryonic cell fitness, specifically during the first stages of differentiation, when cells are subjected to a variety of different insults dues to the big changes they undergo (e.g. transcriptional and epigenetic landscape, metabolism or signalling environment). These insults can cause damage that if were to persist could negatively affect further development or integrity of the germline. In this way cell competition would be important checkpoint acting eliminate those cells that accumulate defects early on in the developmental process. Also, this process of recognition and elimination of vulnerable, miss-patterned or abnormal cells during tissue growth may al o play an important role in organ size control, in tissue homeostasis and stem cell maintenance at later stages of development or in the adult, and it will be intriguing to establish if these roles do exist.

Chapter 4: IN VIVO MODELS OF CELL COMPETITION

4.1 INTRODUCTION

Although primarily described in *Drosophila*, cell competition is also conserved in mammalian systems. For example, *Belly spot and tail* (*Bst*) heterozygote cells, that are defective in the ribosomal protein L24, behave similarly to the *Drosophila Minute*$^{+/-}$ cells. That is they survive well in a heterozygous animal, but contribute poorly to chimeras made of wild-type R26-*lacZ* and *Bst*/+ cells (Oliver et al., 2004). Competitive-like interactions have also been described when rapidly proliferating foetal rat liver cells are injected into the adult liver after partial hepatoctomy. Here, the foetal cells contribute to the regeneration of the liver, not only through their faster proliferation rate, but also by eliminating the wild-type host cells (Oertel et al., 2006). Another example of cell competition in mammalian cells can be found in Martin Canine-Darby Kidney (MCDK) epithelial cells. When MCDK cells that are deficient in the scaffolding protein *Mahjong* are surrounded by wild-type MCDK cells, they are eliminated by extrusion, even though when these mutant cells are surrounded by other mutant cells they survive normally (Tamori et al., 2010).

Cell competition has also been described in the mouse haematopoietic system when haematopoietic stem and progenitor cells are irradiated and introduced with wild-type stem cells and progenitors into lethally irradiated recipients. Here whilst irradiated haematopoietic stem and progenitor cells can repopulate the bone marrow when transplanted as homogenous populations, when they are injected with non-irradiated stem cells and progenitors, the irradiated cells contributed a significantly lower proportion than the non-irradiated cells to the final composition of the haematopoietic system. In this model the outcompeted irradiated cells specifically, when they are in the presence of non-irradiated cells, undergo p53-mediated senescence and as a consequence do not proliferate. Furthermore, deletion or reduction of p53 in the irradiated haematopoietic stem and progenitor cells prevented their senescence and their out-competition by wild-type non-irradiated cells (Bondar and Medzhitov, 2010).

Finally, in the mouse thymus, T lymphocytes develop from precursors that are constantly being replaced by bone-marrow-derived progenitors. This replacement occurs by a cell competition type mechanism, in which ‘old’ bone-marrow progenitors are eliminated by their

‘younger’ counterparts due to their lower responsiveness to IL-7 and therefore presumably their diminished ability to compete for this essential cytokine. However, if this cell competition is prevented and the ‘older’ progenitors are no longer replaced, they will give rise to T-cell acute lymphoblastic leukaemia (T-ALL) (Martins et al., 2014), indicating that in the mouse thymus cell competition plays a role in tumour suppression.

In this chapter I will explore further *in vivo* models of cell competition. I will study if it occurs between embryonic cells within the post-implantation epiblast as well as in the heart. This will help provide evidence for cell competition *in vivo* and under normal physiological conditions.

4.2 RESULTS

4.2.1 Cells with low c-Myc expression are eliminated during early post-implantation development.

In the previous chapter, by using mouse ESCs we have established a model to study mammalian cell competition *in vitro*. This model allows for a detailed molecular and biochemical analysis of the events regulating cell competition. Using this system we found that prior to their elimination by cell competition, cells with defective BMP signalling show lower levels of c-Myc expression than control cells. We also found that over-expression of c-Myc is enough to turn wild-type cells into super-competitors (i.e. they can eliminate their wild-type neighbours). These results suggest that in mouse, c-Myc plays an important role in cell competition, but all these studies are *in vitro* and it is important to establish their physiological relevance by performing *in vivo* studies.

During early post-implantation development, between 5.5 and 6.5 dpc a wave of apoptosis can be observed (Manova et al., 1998; Spruce et al., 2010). These stages of development coincide with when we observe competition to occur between differentiating ESCs (Sancho et al., 2013). To test if the apoptosis observed at 6.5 dpc is due to cell competition I first analysed the levels of c-Myc expression in control embryos at 6.5 dpc and at 7.5 dpc, when little apoptosis is observed (Spruce et al., 2010). Whole-mount in situ hybridisation revealed the *c-Myc* RNA expression was present throughout the entire epiblast and also in the extra embryonic ectoderm (ExE) at 6.5 dpc (Figure 4.1A) but could only be seen in the ExE at 7.5 dpc (Figure 4.1D). Interestingly, the c-Myc expression in the epiblast appeared spotty at 6.5 dpc, suggesting that this gene could be expressed in a mosaic fashion. To confirm this I performed immunohistochemistry. The expression of c-Myc protein showed a similar distribution to that of c-Myc RNA, with expression being present in the epiblast at 6.5 dpc but not 7.5 dpc (Figure 4.1B and E). Furthermore, these experiments also confirmed the mosaic expression of c-Myc in the epiblast at 6.5 dpc (Figure 4.1C). Therefore, c-Myc is expressed in a heterogeneous manner coinciding with the wave of cell death occurring in the post-implantation embryo and absent from the epiblast when levels of death are reduced.

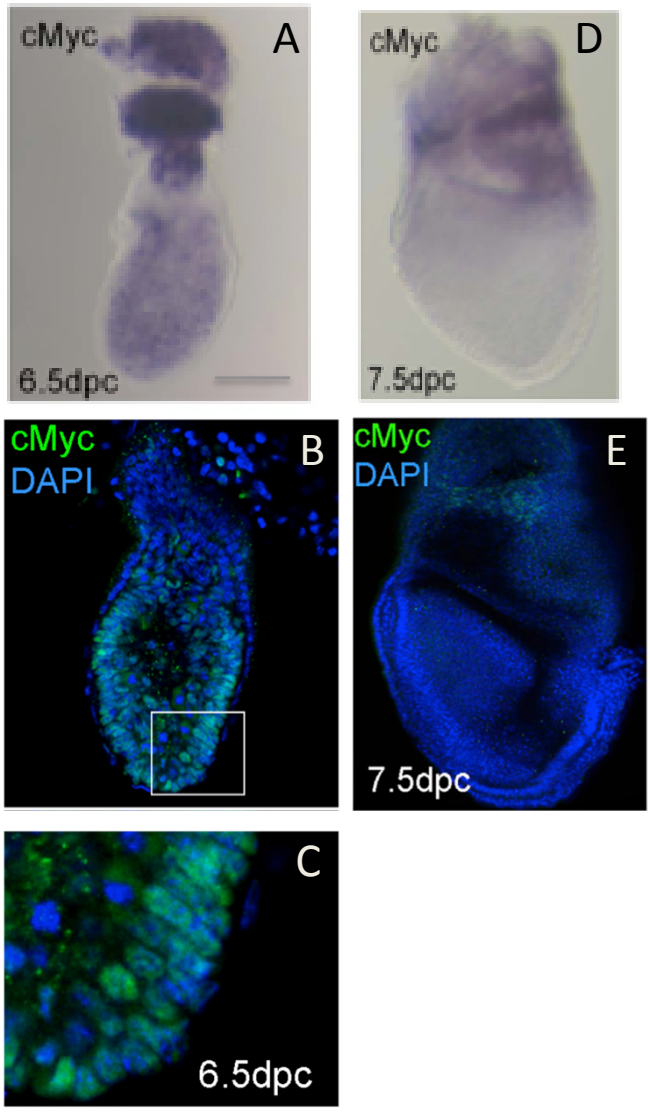


Figure 4.1 Endogenous and heterogeneous expression of c-Myc during the early post-implantation mouse Embryo.

In situ hybridisation showing c-Myc RNA levels in control embryos at 6.5 and 7.5 dpc (A, D). Confocal sections of embryos that have been immunostained showing; a heterogeneous distribution of c-Myc protein in the epiblast at 6.5 dpc (B-C), and not expression in the epiblast at 7.5 dpc (E). Scale bar 75 uM for A and B and 400 um for D and E.

To test if the mosaic c-Myc expression could be indicative of cell competition, I asked if there was a correlation between cells expressing low levels of c-Myc and cell death. Interestingly, double staining for c-Myc protein expression and TUNEL (that detects apoptotic cells) revealed that in the 6.5 dpc epiblast those cells showing low c-Myc expression were those that were preferentially dying (Figure 4.1A). Collectively these results

suggest that cell competition is acting during early post-implantation, but opens the question as to whether those cells eliminated are the defective ones.

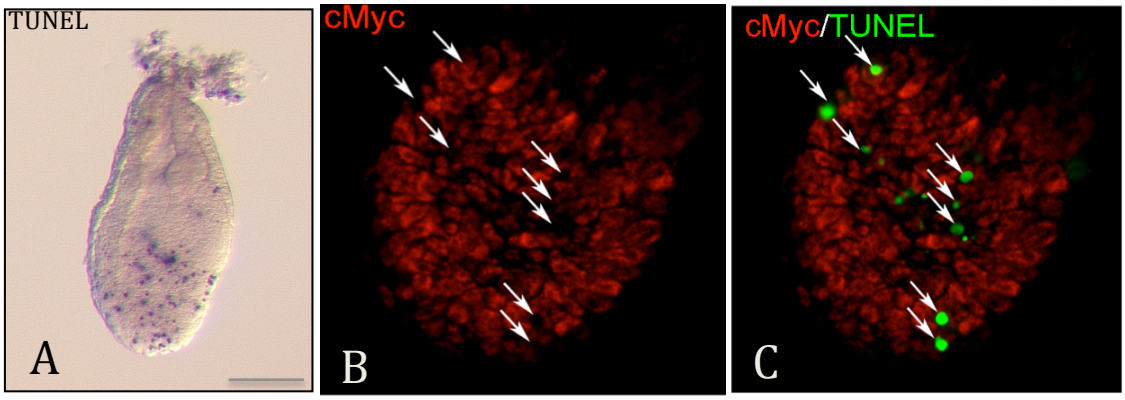


Figure 4.2 Low c Myc expression coincides with dying cells in the 6.5 dpc epiblast.

TUNEL staining of WT mouse embryos at 6.5 dpc, showing cell death levels (A). In red, confocal section sof immunostaining showing heterogenous distribution of c-Myc protein levels in the epiblast at 6.5 dpc (B). Co-staining of c-Myc protein and cell death in green indicates that those cells with low c-Myc expression (arrows) are dying (C). Scale bar 75 uM for A and 150 um for B and C.

To address the question if those cells with low c-Myc expression are defective cells being eliminated during post-implantation development we generated chimaeras using GFP-labeled tetraploid ESCs, that are known to-be out-competed in our in vitro cell competition model (Sancho et al., 2013). Analysis of c-Myc protein levels in the chimeras at 6.5 dpc revealed a striking lower c-Myc expression in the majority of tetraploid cells (Figure 4.3B, arrows). Next, we analysed if these tetraploid cells were dying by analysing cleaved-caspase-3 staining, a late apoptotic marker. We observed co-localization between GFP and cleaved-caspase-3 (Figure 4.3D and F) in the vast majority of tetraploid cells Figure 4.3E). These results indicate that in the embryo those cells that are the less fit are eliminated by Caspase dependent apoptosis and through a mechanism reminiscent to that of cell competition.

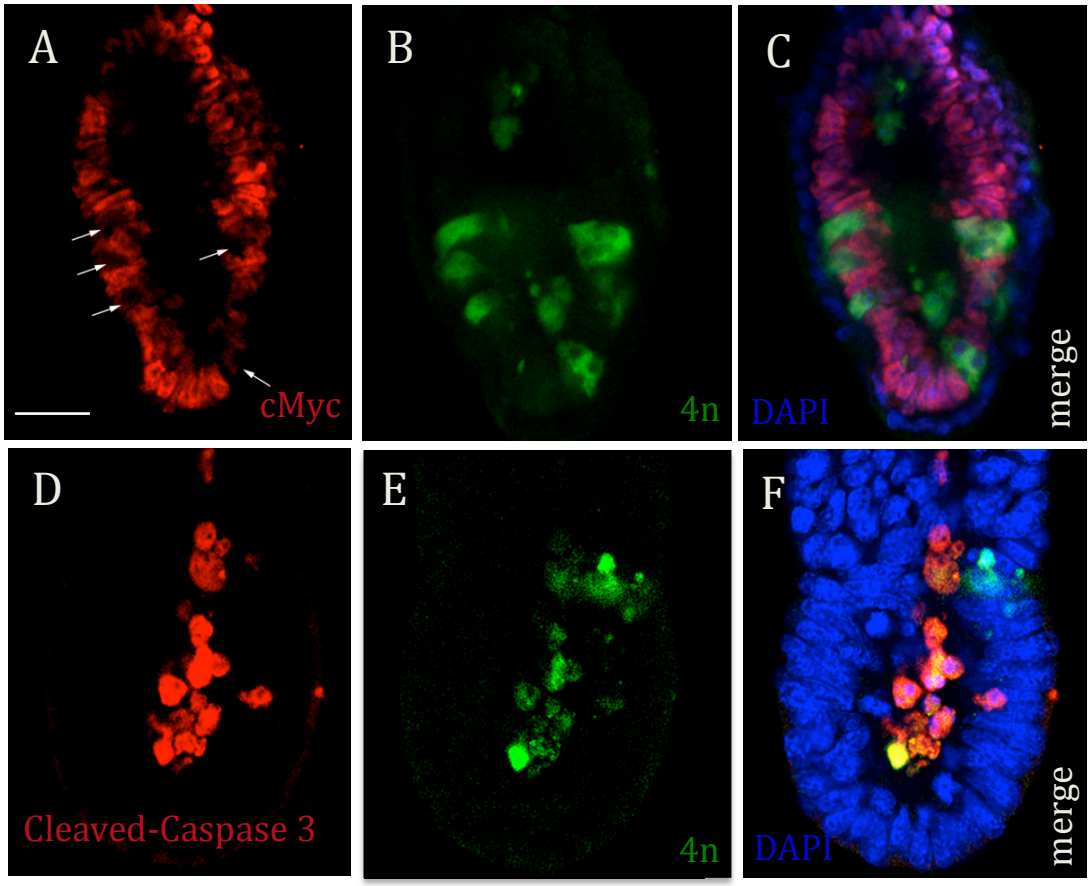


Figure 4.3 Tetraploid cells are eliminated by cell competition *in vivo*.
GFP-labelled tetraploid cell chimeras (n=10) showing low levels of cMyc expression (A-C) and increased in cell death (D-F). Immunofluorescence of; c-Myc (A). Cleaved Caspase 3 (D). Tetraploid-GFP cells are marked in green and DAPI marks the nuclei blue. Scale bar 100 um.

4.1.2 c-Myc regulates embryo growth during early mouse development

The results presented so far in the thesis suggest that c-Myc is a key mediator of the elimination of defective cells during cell competition. Given that loser cells decrease c-Myc prior to being eliminated in ESCs and tetraploid chimeras, I next asked if loss of c-Myc is sufficient to lead to apoptosis. For this, I analysed the effects of deleting *c-Myc*. As c-Myc is required in embryonic and extra-embryonic tissues (Dubois et al., 2008), I first generated mice where *c-Myc* is specifically deleted from the epiblast. For this, we crossed conditionally targeted mice carrying the *c-Myc* floxed allele (de Alboran et al., 2001) with Mox2Cre mice, carrying an epiblast-specific Cre-recombinase (Tallquist and Soriano, 2000). Embryos from inter-crosses were dissected at 6.5 dpc, when we observe cell competition to occur. Inspection of these embryos revealed that about a quarter of embryos were smaller than the

rest of their littermates. For this reason, I proceeded to perform immunohistochemistry for c-Myc protein expression, measure and then genotype all embryos. Comparison of those embryos with c-Myc protein expression in the epiblast and low or no c-Myc expression in the epiblast revealed that the mutant embryos were identifiably smaller than their littermate controls (Figure 4.4A). Measurements were then carried out of the epiblast length, that is the distance between the distal tip of the embryo and the embryonic and extra-embryonic boundary. When the epiblast length of wild-type and *c-Myc*$^{+/fx}$*; Mox2Cre* embryos was compared, I found that the epiblast length of the mutant embryos was significantly smaller (143.5±28.6um versus 288.2±24.8um; p<0.005; Figure 4.4B). These results indicate that c-Myc is an essential regulator of embryonic growth during post-implantation development.

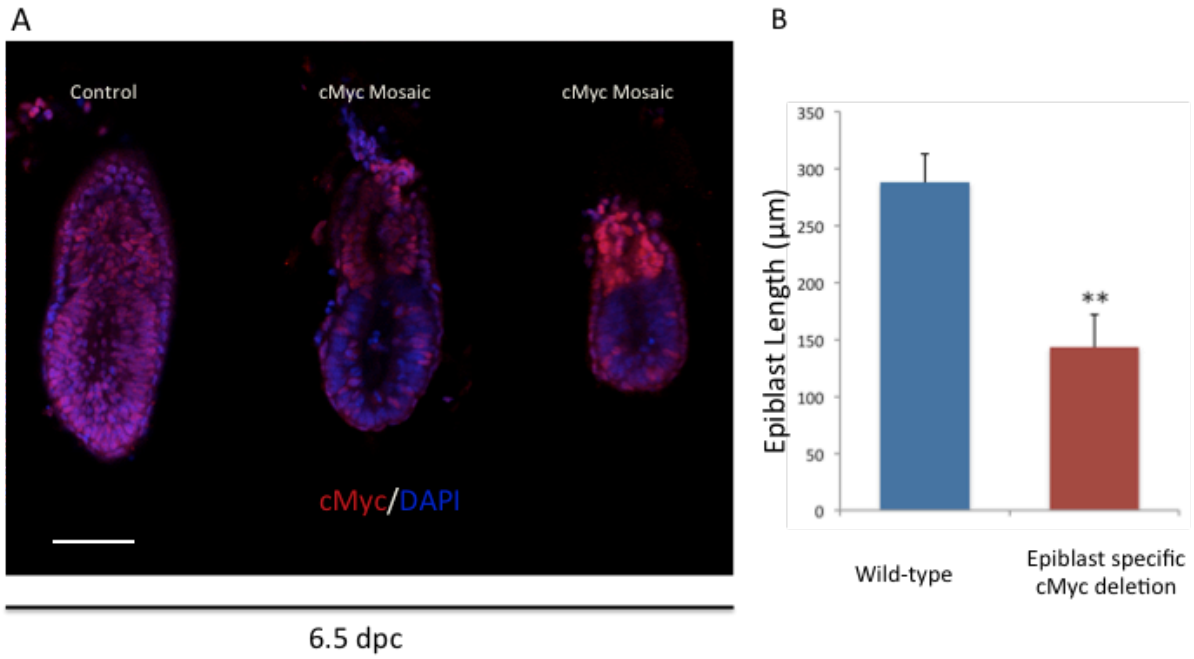


Figure 4.4 Deletion of *c-Myc* specifically in the epiblast leads to a reduced embryonic growth.
C-Myc protein expression (A) and epiblast length (B) indicate a growth defect in *c-Myc*$^{+/fx}$*; Mox2Cre* embryos. In red c-Myc protein expression levels measured by immunofluorescence and in blue nuclear DAPI (A). Growth was estimated by measuring the epiblast using an Axion software fluorescent imaging microscope. Mutant embryos n= 6 embryos; Student t-test **P <0.005. Scale bar is 250 um.

Loser cells during cell competition have low c-Myc expression (Figures 4.2 and 4.3) and are eliminated by apoptosis (Sancho et al., 2013). For this reason we compared the levels of cell death between *c-Myc*$^{+/fx}$*; Mox2Cre* and control embryos by performing TUNEL assays. Embryos were also co-stained for c-Myc protein expression to confirm *c-Myc* deletion in the epiblast. Surprisingly, in spite of the smaller size of the embryos, the number of TUNEL positive cells in *c-Myc*$^{+/fx}$*; Mox2Cre* and control embryos was very similar (Figure 4.5), indicating that loss of *c-Myc* does not lead to apoptosis. These results suggest that c-Myc is regulating embryonic growth not by preventing apoptosis but rather by sustaining proliferation in the epiblast. Careful analysis of proliferation is required to verify this result.

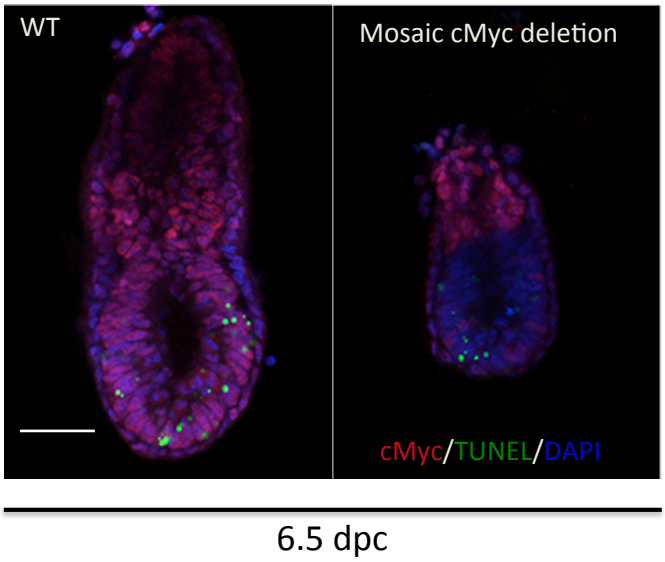


Figure 4.5 Mosaic c-Myc expression leads to reduced embryonic growth and is not due to increased death.
Confocal images showing c-Myc expression levels (red) and cell death (green = TUNEL) in *c-Myc*$^{+/fx}$*; Mox2Cre* and control embryos. In blue, DAPI nuclei staining. n=6. Scale bar is 150 um.

The Mox-2-Cre mouse line deletes in a mosaic fashion (Hayashi et al., 2002), and we observed some c-Myc positive cells in *c-Myc*$^{+/fx}$*; Mox2Cre* embryos (Figures 4.4A and 4.5). As mosaic c-Myc expression induces cell competition (Chapter 3), we wanted to analyse if

the growth arrest of mutant embryos was due to this mosaic c-Myc expression. To test this we analysed epiblast size in null embryos resulting from the cross of two mice that are heterozygous for the c-Myc mutant allele (Trumpp et al., 2001). Embryos at 6.5dpc were stained for c-Myc protein expression, had their epiblast length measured and were genotyped. Interestingly, the *c-Myc* null also displayed a smaller epiblast length than controls (141.3±14.5um versus 187.9±9.0um; $p<0.05$; Figure 4.6), indicating that the reduced epiblast size is not caused by mosaic c-Myc expression. It is worth noting that the epiblast size of control embryos in this experiment was smaller than the epiblast size of the control embryos used for the analysis of the *c-Myc*$^{+/fx}$*; Mox2Cre* embryos (187.9±9.0um versus 288.2±24.8um). One likely explanation for this difference was that the genetic background of these animals was different, with the null mutation being a mixture of C57BL6 and 129SvEv and the *c-Myc*$^{+/fx}$*; Mox2Cre* embryos having a similar mixture but have been backcrossed a couple more times to C57BL6.

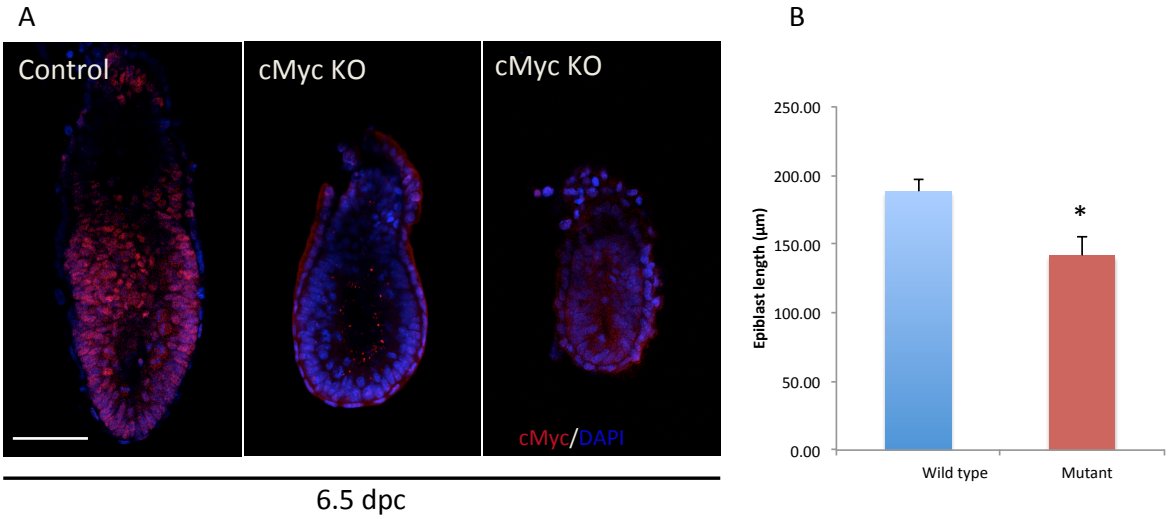


Figure 4. 6. c-Myc null embryos show a growth impairment during early post-implantation development. In red c-Myc protein expression levels measured by immunofluorescence in control and null 6.5dpc embryos. Blue represent DAPI nuclear staining (A). Epiblast length of null embryos is significantly smaller than the epiblast length of controls (B). Student t-test *P <0.05, n=6. Scale bar is 150 um.

4.1.3 Studying the role of cell competition in the heart

As a parallel approach to analysing the role of cell competition in the early post-implantation embryo, I attempted to establish a model of cell competition in the developing heart, a tissue

where cell competition has been shown to occur (Claveria et al., 2013). Foetal heart regenerative capacity can compensate for a loss of up to 50% of heart cells (Drenckhahn et al., 2008). Here, the effects of deleting Holocytochrome C synthase (Hccs) in a mosaic manner specifically in the heart were analysed. *Hccs* is an X-linked gene and therefore males carrying an *Hccs* mutant allele will be hemizygous for the mutation. In contrast to this, due to random X inactivation, heterozygous *Hccs* females ($Hccs^{+/-}$) will initially have hearts that have a 50:50 composition of wild-type and mutant cells. What this study found was that whilst *Hccs* males displayed mid-gestational lethality, at mid-gestation females had the expected 50:50 ratio of wild-type and mutant cells, but by birth the majority of mutant cells had been lost. In spite of this loss, the hearts of $Hccs^{+/-}$ mutant mice were the normal size, indicating that wild-type cells had undergone compensatory proliferation to make up for the loss of mutant cells. These features are very reminiscent of cell competition, and for this reason we investigated if these cells could be subjected to this type of mechanism.

As a first step we wanted to verify if we could distinguish control and *Hccs* mutant cells in the hearts of $Hccs^{+/-}$ females. Cytochrome C is synthesized by Hccs and therefore should be absent in *Hccs* mutant cells. Cryostat sections of 14.5dpc hearts were analysed for Cytochrome C expression and clear patches of negative staining could be observed (Figure 4. 7C-E). Therefore staining for Cytochrome C can be used to distinguish *Hccs* mutant and control cells in $Hccs^{+/-}$ hearts.

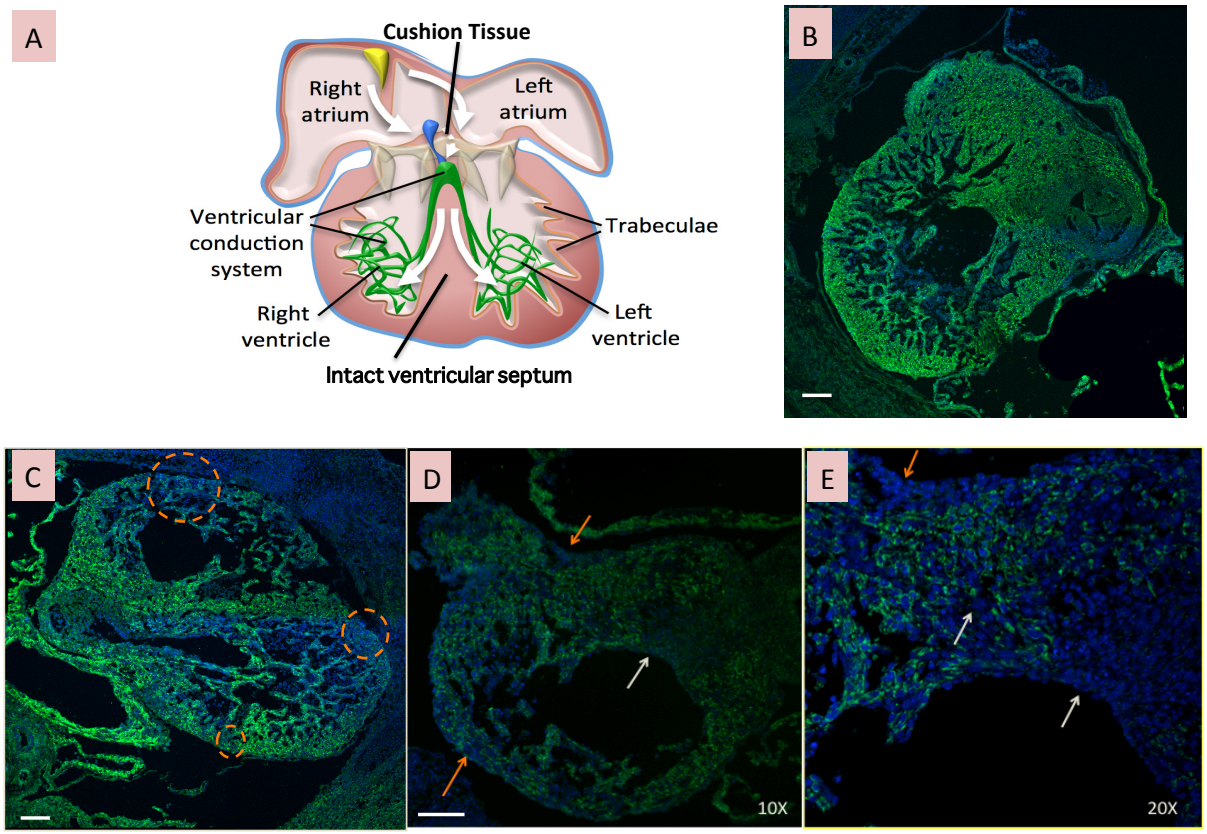


Figure 4.7 Heterozygous *Hccs* female hearts show mosaic Cytochrome C expression.
Cryosection of 14.5 dpc mouse heart (A), schematic representation of the main structures of the heart (B). Immuno-cytochemistry for Cytochrome C reveals clear patches of negative cells in a 14.dpc *Hccs*$^{+/-}$ female heart (C-E). In blue DAPI nuclear stain. B) Distribution and expression of Cytochrome C on WT is expressed throughout the whole heart. C) But small patches of not Cytochrome C expression (doted circles) throughout the heart of Hccs KO female heart. D) Heart sites of high and low proliferation rates indicated with orange and white arrows respectively. E) No expression of cytochrome C on the walls toward the trabecular myocardium as shown by orange arrows. White arrows indicate the negative staining not considerate for the studies. Scale bar is 100 um.

Next, we analysed if *Hccs* mutant cells were being subjected to cell competition. For this we analysed c-Myc expression in *Hccs*$^{+/-}$ female hearts by co-staining for Cytochrome C and c-Myc. When this was done, we found that a high proportion of Cytochrome C negative cells also lacked c-Myc expression (Figure 4.8). However, it is worth noting that although c-Myc expression should have been nuclear, we could only detect cytoplasmic expression. This was a problem with other antibodies used with this immuno-staining protocol and will be commented on below. In spite of this, these results suggest that those cardiomyocytes that are lost from *Hccs*$^{+/-}$ female hearts may be subject to cell competition induced by differences in c-Myc expression.

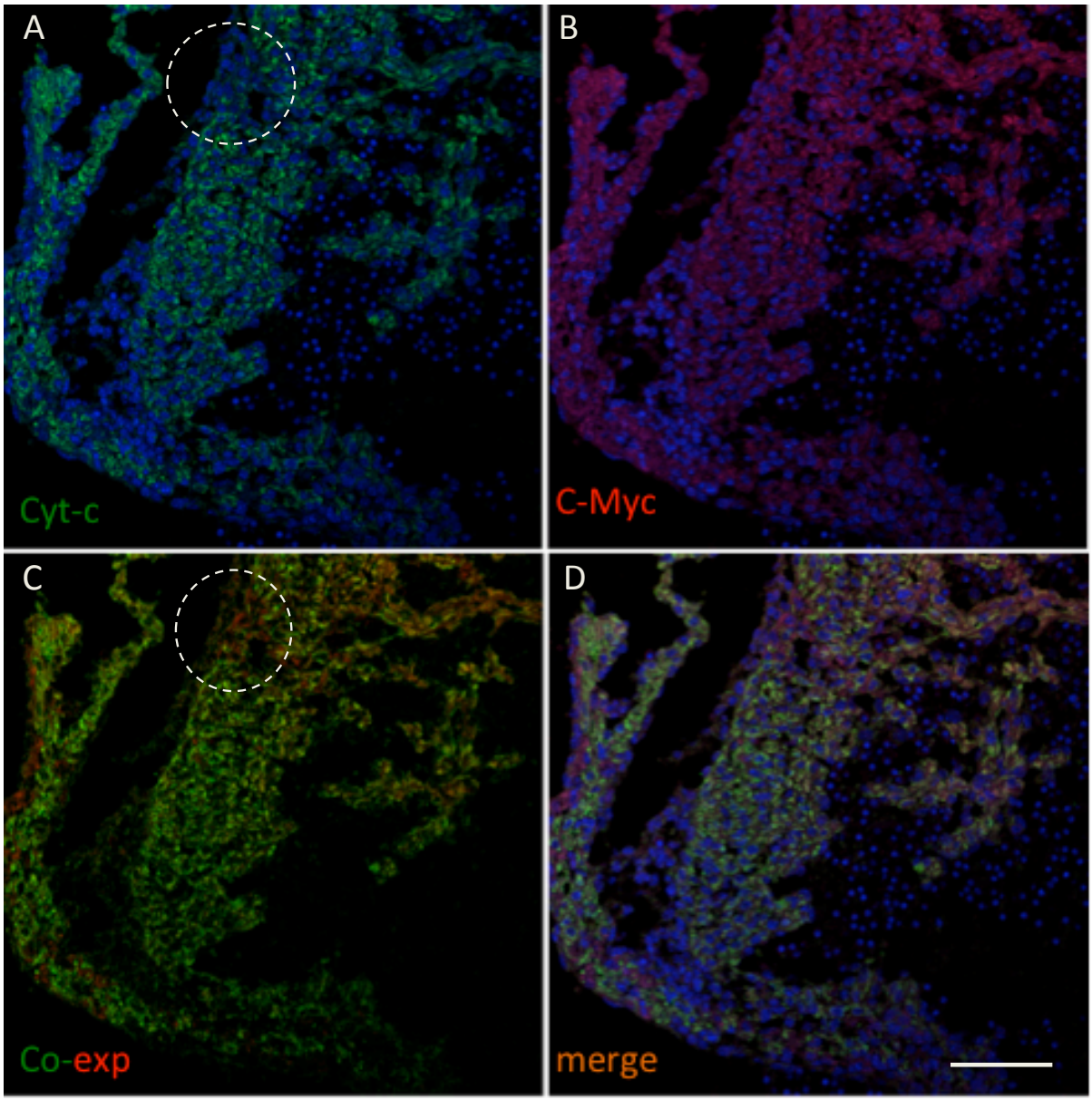
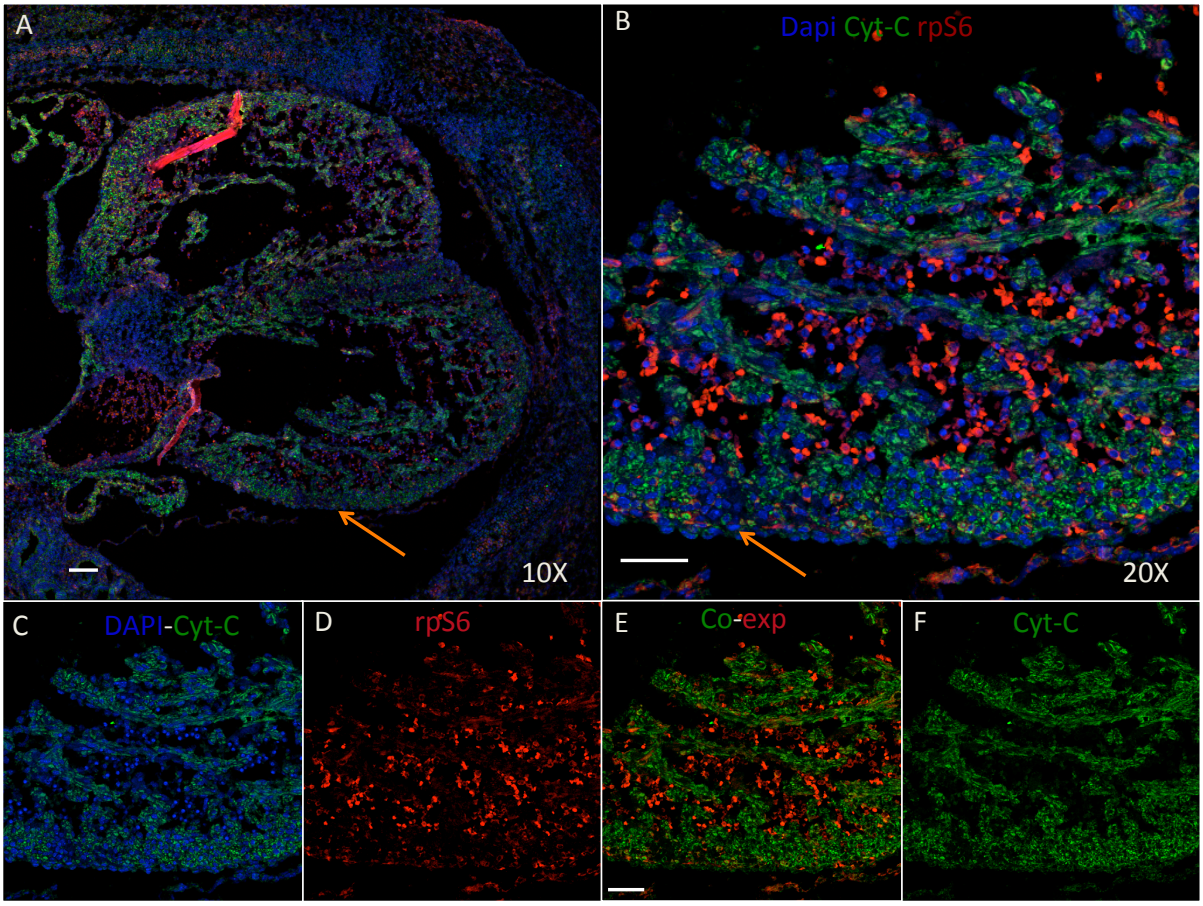


Figure 4.8. C-Myc expression is low in *Hccs* mutant cells.Cryosection of 14.5dpc Hccs KO heart.
C-Myc (red) and Cytochrome C (green) immunostaining showing those cells in the heart that lack cytochrome C also have low c-Myc expression (dotted circle). In blue DAPI nuclear stain. N=3. Scale bar is 100 um.

Preliminary results in our lab showed that during cell competition loser cells decrease mTOR signalling and increase p53 expression. Furthermore, either increasing mTOR signalling in defective cells or inhibiting p53, both rescues their elimination (unpublished results). For this reason we first investigated mTOR signalling in *Hccs*$^{+/-}$ female hearts by analysing rpS6 phosphorylation, that gives an indication of mTOR activity. Analysis of the p-rpS6 immuno-

fluorescence in *Hccs*$^{+/-}$ female hearts revealed a low level of p-rpS6 expression throughout the myocardium with no difference being observed between Cytochrome C expressing and non-expressing cells (Figure 4.9A, B). In contrast to this, blood cells showed high levels of p-rpS6 expression. This suggests that mTOR is not differentially activated during the out-competition of *Hccs* mutant cells.



igure 4.9 Low level of p-rpS6 expression is observed during cardiac regeneration.
Immuno-histochemistry for p-rpS6 (red) and Cytochrome C (green) reveals that the cardiomyocytes at 14.5 dpc express low levels of p-rpS6, irrespective of them being *Hccs* mutant or control cells in an *Hccs*$^{+/-}$ female heart (A-B). In blue are DAPI-stained nuclei. The orange arrow indicates the part of the heart that is amplified in the lower panels. n=3. Scale bar is 100 um.

I next analysed p53 expression. In the case of p53, areas of high and low expression could be clearly detected (Figure 4.10A-E). However, just as was the case for p-rpS6, no difference in p53 expression was observed between Cytochrome C positive and negative cells, suggesting

that p53 does not mediate the out-competition of *Hccs* mutant cells in the *Hccs*$^{+/-}$ female heart. Also, just as was the case for c-Myc, p53 expression could only be detected in the cytoplasm. This suggests that the immuno-staining protocol used for co-staining with Cytochrome C was not allowing permeabilization and access of the cMyc antibody into the nucleus. Also the nature of the c-Myc antibody used as shown by the nuclei stain when using Nkx2.5 antibody in a 14.5 dpc Hccs heart (fig. 8). Further experiments are required to confirm that these are indeed the case.

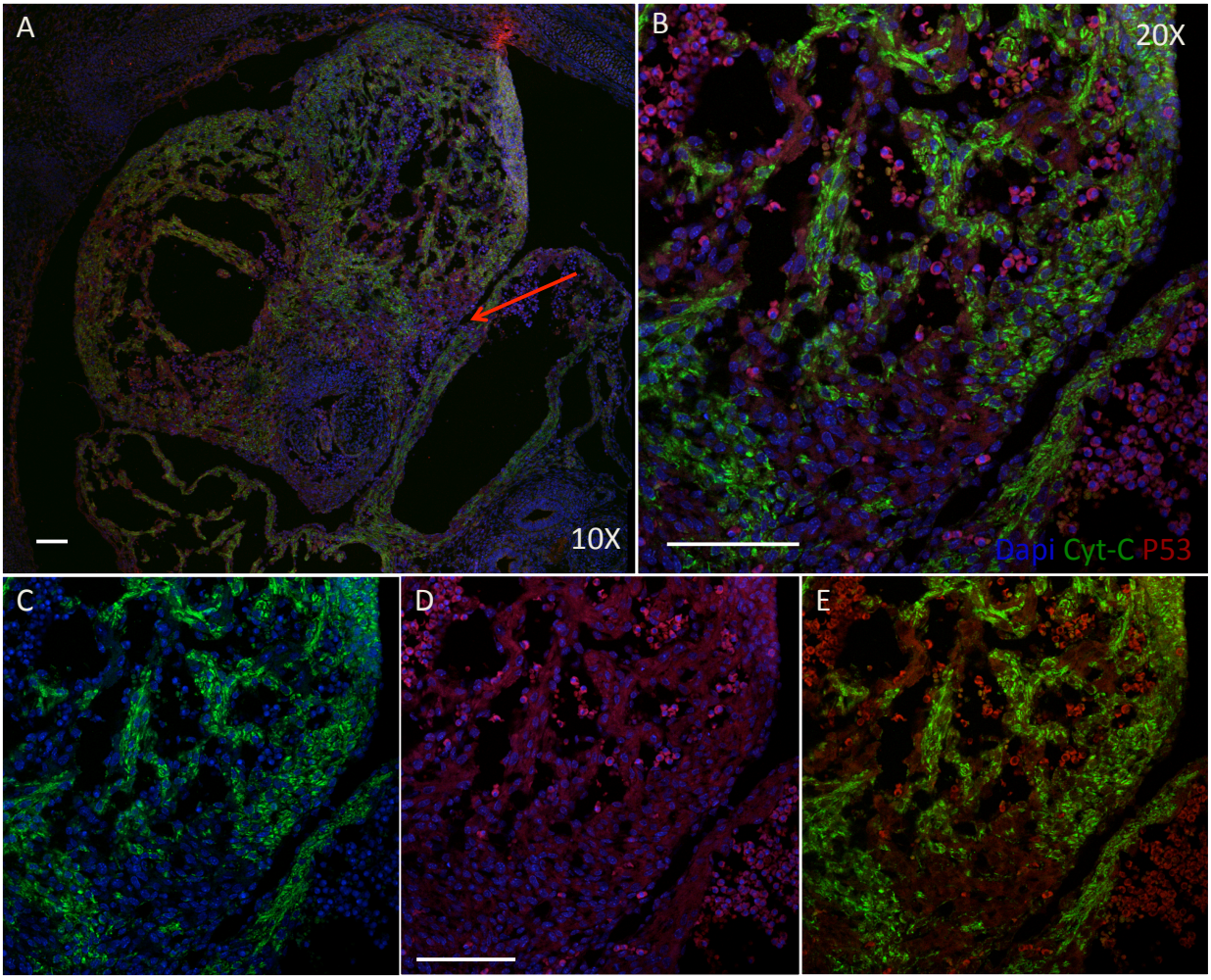


Figure 4.10 *Hccs*$^{+/-}$ female hearts do not show differential p53 protein expression.
Immunocytochemistry staining for Cytochrome C (green) and p53 (red) proteins on a 14.5dpc *Hccs*$^{+/-}$ female heart showing no differential p53 expression between Cytochrome C expressing and non-expressing cells (A-D). In blue are DAPI-stained nuclei. N=3. Scale bar is 100 um.

4.3 DISCUSSION

Cell competition has been proposed as a type of quality control mechanism that eliminates unfit cells (Amoyel and Bach, 2014). Cell competition has been primarily studied in *Drosophila*, and here I provide evidence for its conservation in mammals. For this I use two models to study cell competition: the early mouse embryo and the developing mouse heart. In both we find that loser cells have low levels of c-Myc expression, an event that in the case of the early embryo is sufficient to lead to growth arrest. These results highlight the importance of cell competition as an evolutionary conserved fitness sensing mechanism acting in a variety of different developmental contexts.

In previous work from the lab (Sancho et al., 2013) and Chapter 3 I demonstrated that during the first steps of differentiation unfit cells are eliminated by cell competition. We found that when wild-type and *Bmpr1a*-null mouse ESC are cultured together, the mutant ESCs are eliminated. In *Drosophila* c-Myc has been shown to be a key mediator of cell competition, as cells that over-express c-Myc eliminate wild-type surrounding cells (de la Cova et al., 2004; Moreno and Basler, 2004). Also, in different modes of competition in *Drosophila*, loser cells decrease c-Myc prior to their elimination (reviewed by (Amoyel and Bach, 2014). We found that prior to their elimination, *Bmpr1a*-null ESCs show lower c-Myc expression. Interestingly, we found that these observations were paralleled *in vivo*. In the post-implantation epiblast, a wave of apoptosis occurs at 6.5 dpc (Manova et al., 1998; Spruce et al., 2010). Here, we found that at this stage c-Myc is expressed in heterogeneous fashion throughout the epiblast. Furthermore, we observed that those cells in the epiblast with low c-Myc levels were undergoing apoptosis, suggesting that they were being eliminated by cell competition. For this reason we set out to functionally test this assumption in two ways.

First we analysed the fate of a defective cell type that we had shown to be eliminated by competition *in vitro*. We chose tetraploid cells, as these are known not to contribute to embryonic tissues in chimeras composed of diploid and tetraploid cells (Eakin and Behringer, 2003). In contrast to this, tetraploid embryos survive until late gestation, and even birth in some instances (Henery et al., 1992; Kaufman, 1991; Kaufman and Webb, 1990; Snow, 1973, 1975). Here, we observed that in chimeras with diploid cells, tetraploid cells show a lack of c-Myc expression and then die by apoptosis. The low level of c-Myc cells in chimeras

is similar to that shown by these cells when they are in co-culture with diploid ESCs, and contrasts with the robust c-Myc expression of tetraploid cells when they are in a homogeneous culture, either in the ESC state or during differentiation (Sancho et al., 2013). Therefore, together these results indicate that in the embryo, cell competition is a mechanism that can recognise abnormal cells and eliminate them and that during this process, these abnormal cells decrease c-Myc expression.

We next assessed what are the effects of lowering c-Myc expression in the post-implantation epiblast. For this, we used a conditional knockout (KO) technique and analysed the phenotype at 6.5 dpc, the same stage of development when c-Myc is expressed in a heterogeneous manner *in vivo*. We found that deleting c-Myc specifically from the epiblast led to embryos that are phenotypically smaller than their littermates. Similar result was observed when analysing full KO embryos, although there were slight differences between the size of the control embryos in both these experiments. This was possibly due to differences in the genetic background, as the mice used for the conditional KO experiment had been back-crossed a couple of times more onto a C57BL6 background. In spite of these differences, the size of the mutant embryos was significantly lower in both experiments.

Interestingly, the growth arrest observed in c-Myc mutant embryos was not due to the cells lacking c-Myc dying, as mutant embryos did not show higher levels of apoptosis than controls. These results suggest that the growth inhibition triggered by loss of c-*Myc* is most likely due to a proliferation arrest. C-Myc plays a variety of roles in the control of proliferation, including the regulation of RNA Polymerase II transcriptional pause release (Rahl et al., 2010), stimulation of transcriptional elongation (Dang, 2012) and interaction with epigenetic modifiers to promote an open chromatin status that is accessible to the transcription machinery (Chappell and Dalton, 2013). C-Myc also plays important roles in the regulation of protein synthesis, splicing and the regulation of the cell cycle (Dang, 2012). Therefore, it is likely that c-Myc impacts early embryonic growth in multiple ways. These results are in agreement with the recent finding that in the naïve pluripotent state c-Myc controls transcription, splicing and protein synthesis and that when both c-Myc and N-Myc are mutated the cells enter a state of dormancy that is similar to diapause (Scognamiglio et al., 2016). It is worth noting that *c-Myc* mutant embryos have been reported to survive until

12.5dpc and can gastrulate properly, as they have a normal germ layer specification (Dubois et al., 2008). This suggests that either c-Myc mutant embryos recover from their growth delay or we have uncovered a more severe c-Myc mutant phenotype, possible due to a genetic background effect.

Our results indicate that c-Myc mutant cells undergo growth arrest but not apoptosis. This contrasts with the fate of loser cells eliminated during competition that die by apoptosis and indicates that down-regulation of c-Myc is not sufficient to drive loser cell elimination. This suggests that downstream of c-Myc further sensing mechanisms must occur leading to the signalling of cells with low levels of c-Myc or that the proliferation arrest of low c-Myc cells sensitizes them to a death signal secreted by winner cells. Further experiments are required to distinguish between these possibilities.

Finally, our work also shows that cell competition in mammals is not restricted to stem cell populations in the early embryo, but is also likely to operate in a wide variety of other contexts, including the heart. It had previously been shown that during heart development, cells over-expressing c-Myc replace cells wild-type cells in a phenotypically silent manner and through cell competition (Villa Del Campo et al., 2014). Similarly, wild-type cells replace cells defective for Holocytochrome C synthase function during the development of the heart (Drenckhahn et al., 2008). This replacement was shown to occur through a regenerative response of the wild-type cells that increase their proliferation rate to compensate for the loss of mutant cells. In this way, in spite of losing 50% of its cells, the heart can retain normal morphology and function. Here, we show that during this regenerative process, the defective cells decrease c-Myc expression, suggesting that a sensing mechanism similar to cell competition, can detect the loss of functional heart cells and enhance the proliferation of healthy cells to ensure that normal heart function is preserved even after injury. It will be fascinating to unravel if the molecular mechanisms regulating cell competition in the early embryo also regulate this regenerative response, and if this response is restricted to embryonic development or can be activated in the adult.

Chapter 5: METABOLIC PROFILING OF THE CHANGES THAT OCCUR DURING PLURIPOTENT STEM CELL DIFFERENTIATION

5.1 INTRODUCTION

During early mammalian development energy production depends upon factors such as nutrient availability and uptake, as well as O_2 tension. In the cell, ATP is produced to comply with the energy demands primarily through of glycolysis and oxidative phosphorylation (OXPHOS). The relative reliance of the cell on each of these processes varies between developmental stages. Glycolysis is the enzymatic conversion of glucose to pyruvate, which generates 2 net ATP molecules per molecule of glucose (Figure 5.1). Cells that depend mainly on glycolysis for ATP production further convert pyruvate to lactate, which is secreted. By contrast, cells in oxygen-rich environments preferentially use pyruvate to drive OXPHOS and ATP production. OXPHOS generates an average of 34 additional ATP molecules per glucose molecule, by oxidizing pyruvate to acetyl-CoA in the mitochondrial tricarboxylic acid (TCA) cycle (Figure 5.2) (reviewed in (Teslaa and Teitell, 2015).

Both, in the mouse and in the human embryo, the first stage of differentiation involves the transition from a naïve state of pluripotency to a state where cells are still pluripotent, but primed for differentiation (Nichols and Smith, 2009). This transition is thought to occur between the pre-implantation and post-implantation stages of development (Figure 5.3). Early mouse embryo pre-implantation cleavage stage embryos cannot utilise glucose, and their energy is derived entirely from aerobic respiration. In contrast to this, from the late pre-implantation stage to early post-implantation stages both OXPHOS and glycolysis are involved in ATP generation (Houghton et al., 1996). These studies also showed that in post implantation embryos, glucose utilization increases 5-10 fold. In agreement with these observations, comparative studies of the metabolic status of mouse ESCs, EpiSCs and hESCs have demonstrated that while mESCs depend both on oxidative phosphorylation and glycolysis for their energy generation, EpiSCs and hESCs have a glycolytic metabolism (Zhou et al., 2012).

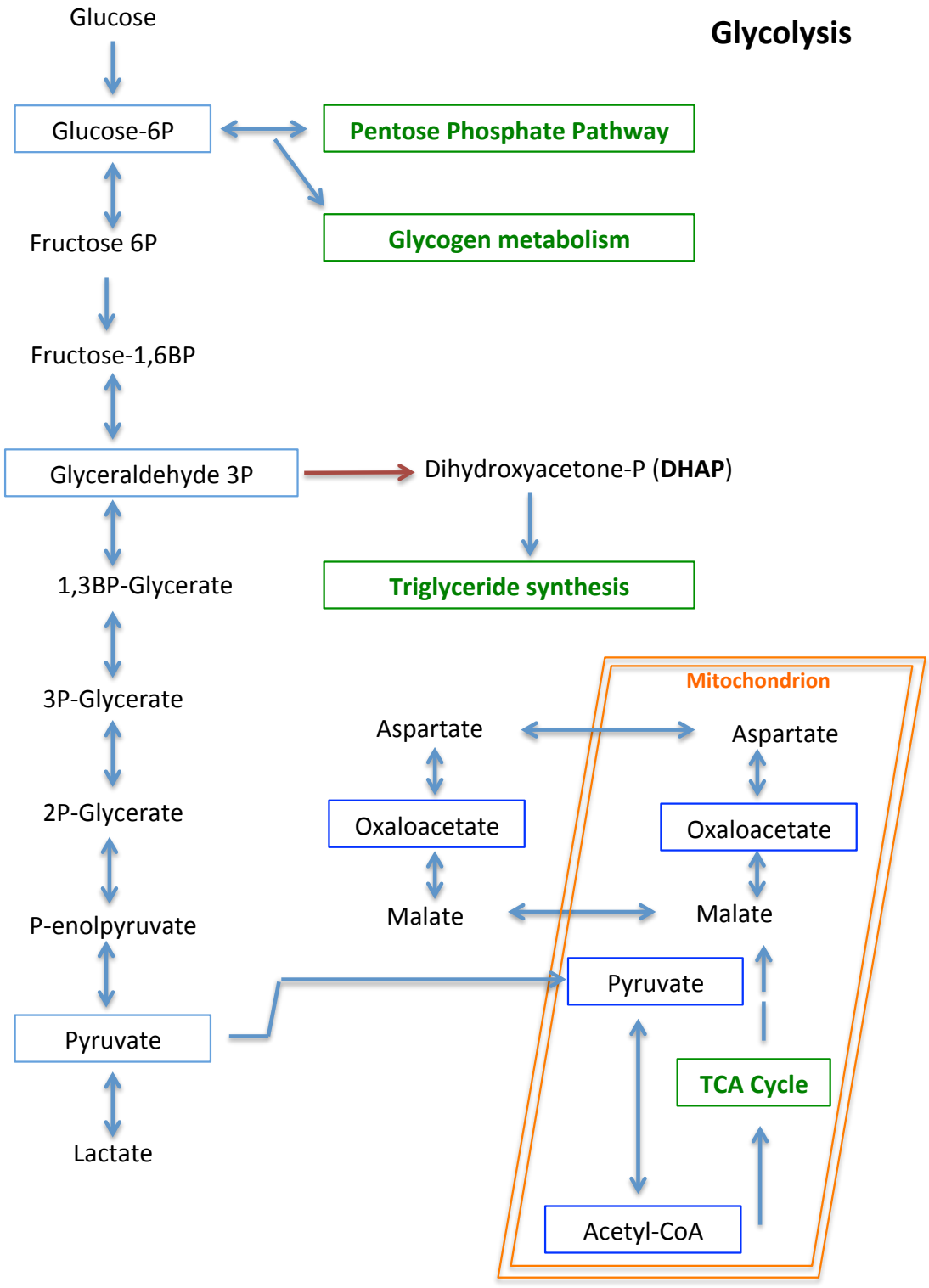


Figure 5.1. Glycolysis: Schematic representation of the metabolic route, its precursors products and relationship with other metabolic pathways. Adapted from Wikipedia

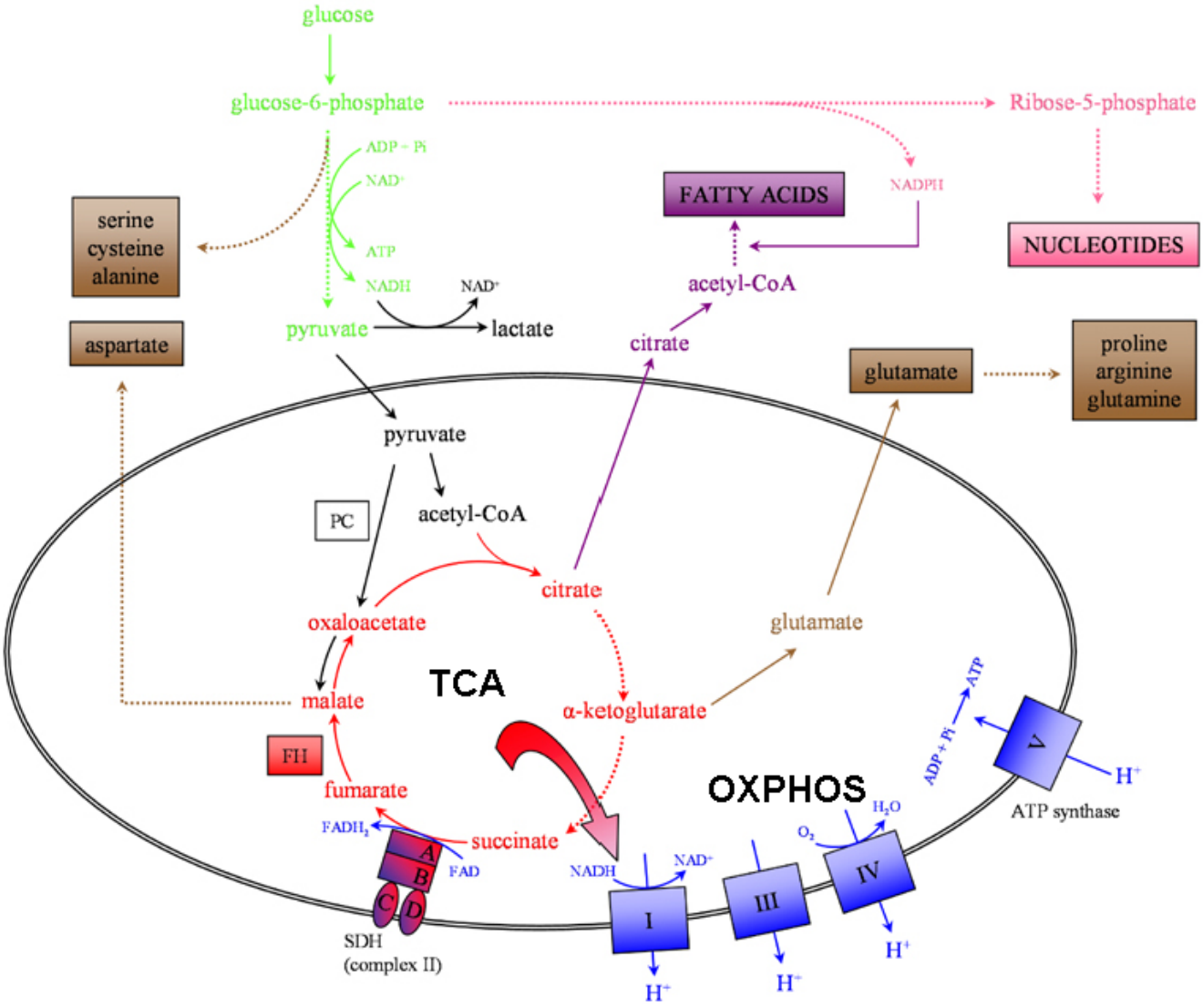


Figure 5.2. Schematic representations of the TCA cycle and its precursor products and relationship with other pathways, including OXPHOS
Adapted from (King et al., 2006).

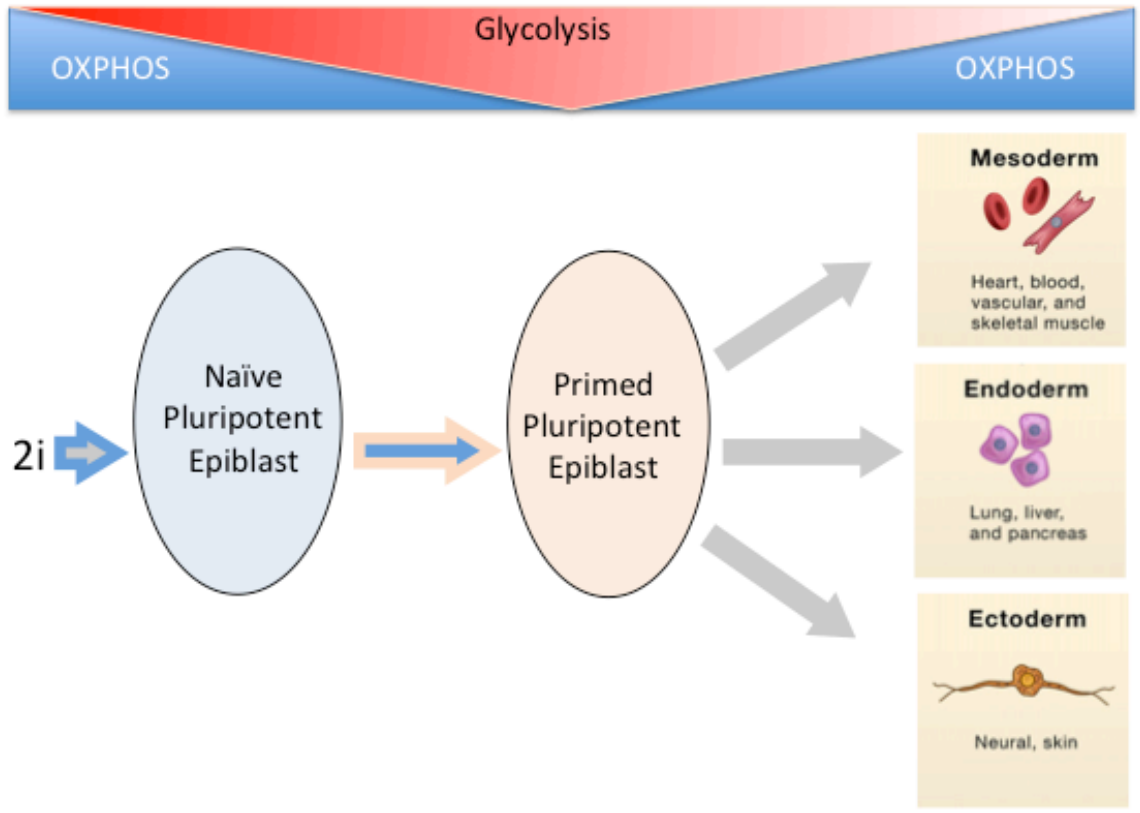


Figure 5.3. Metabolic changes upon differentiation of mESCs .
Schematic representation of different stages of pluripotency and the metabolism changes between the naïve and primed pluripotent state**.** In culture: 2i when ESCs are in a naïve pluripotent state and rely on glycolysis and OXPHOS for their metabolism. In the primed pluripotent state they rely in glycolysis and as they continue to differentiate they switch back to OXPHOS metabolism. Adapted from: (Folmes C.D. and Terzic A. (2014) and Murry, C.E., and Keller, G. (2008).

One of the most conserved fitness differences between winners and loser cells that triggers cell competition is growth differences (see introduction). Given that metabolism is a major regulator of proliferation, we predict that metabolic differences could be one of the common triggers of cell competition. To address if winner and loser cells have different metabolism we have analysed the metabolic profile of winner and loser cells as they initiate differentiation (when cell competition is induced).

5.2 RESULTS

5.2.1 Metabolic profiling of wild-type and defective cells during differentiation

Previous work from the lab identified that cell competition is triggered specifically by the onset of differentiation, and if differentiation is blocked, elimination of loser cells is prevented (Sancho et al., 2013). This suggests that an event associated with the onset of differentiation may be occurring differentially between winner and loser cells. As described above, a key change that occurs as cells exit pluripotency is a metabolic shift. Cells in the naïve pluripotent state, represented by ESCs, have a bivalent metabolism (glycolysis and OXPHOS) and those in the primed state of pluripotency, represented by EpiSCs, rely only on glycolysis for their energy. Here, I hypothesize that defective looser cells may be less efficient at performing this metabolic change and this may lead to their competitive disadvantage. To analyse if this is the case we compared the metabolic profile of wild-type ESCs to that of two defective cell types eliminated by competition, *Bmpr1*$^{-/-}$ and tetraploid (4N) ESCs.

Cell competition is induced by culturing cells for 3 days in N2B27, that allows cells to exit the naïve pluripotent state and reach an EpiSCs-like state (Sancho et al., 2013). We therefore collected cell pellets from these three cell types cultured under two conditions, in ESC media (that prevents competition) and for 3 days in N2B27 (that induces competition). Pellets from 5 independent experiments for each condition were analysed by Metabolon by a combination of Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS) and Gas Chromatography-Mass Spectroscopy (GC-MS). These studies detected 442 metabolites. Metabolon then performed normalization to Bradford protein concentrations and we compared the levels of expression of each metabolite between the three cell types analysed using the mView software provided by the company. Welch's two-sample t-test was used to identify biochemical that differed significantly between experimental groups. For ease of interpretation of the results I have grouped metabolites according to the metabolic pathway they belong to, and those showing the largest consistent differences between wild-type and defective cells are discussed bellow. In ESC media no significant differences were

observed in the metabolism of WT, *Bmpr1*$^{-/-}$ and 4N ESCs. For this reason we concentrated our analysis on how metabolism changes in these cells upon differentiation.

5.2.2 Glycolysis

Previous studies have shown that when cells transit from the naïve to the primed pluripotent state they increase glycolysis (Zhou et al., 2012). To analyze if this also occurs when ESCs are differentiated for 3 days in N2B27, I compared the levels of expression of key metabolites of the glycolytic pathway between wild-type ESCs cultured in ESC conditions and wild-type ESCs cultured for 3 days in N2B27 (Figure 5.4). When this was done an increase in the expression of a number of intermediates of the glycolytic pathway was observed in cells cultured in N2B27 compared to ESC media. For example, expression of; glucose-6-phosphate, 3-phosphogylcerate, phosphophenol pyruvate were all found to be increased, although these differences were not statistically significant. In contrast to this the expression of glucose and fructose-6-phosphate were decreased. The decrease in glucose could indicate that in N2B27 the ECSs are consuming more of this metabolite. One reason for it is that N2B27 has the same amount of glucose as ESC media, and therefore if the activity of the glycolytic pathway is higher in N2B27 this would consume more of the available glucose. A possible explanation for the decrease in fructose-6-phosphate levels is that, the Pentose Phosphate Pathway (PPP) also produces this metabolite, and the activity of the PPP may be lower in N2B27. This possibility will be analysed further on in this chapter.

The end products of the glycolytic pathway are those metabolites used by other pathways that do not have further roles in glycolysis. When these are analysed an increase in levels of pyruvate and lactate were observed between N2B27 and ESC media, while the amino-acid alanine and the lipid precursor dihydroxyacetone phosphate (DHAP) were decreased. As pyruvate is used by the TCA cycle, these results are consistent with the activity of the TCA being lower upon differentiation (Zhou et al., 2012) and with a switch to the use of glycolysis for the generation of lactate during this process. Therefore, although these results need to be complemented with steady flux analyses, together they suggest that upon differentiation in N2B27, wild-type ESCs de-couple glycolysis from the TCA cycle and rely on glycolysis alone for their energy production.

Next I compared the levels of metabolites from the glycolytic pathway between wild-type, *Bmpr1*$^{-/-}$ and 4n ESCs cultured for 3 days in N2B27 to analyse if the defective cell types are also able to perform the switch to glycolysis. Interestingly, I observed a statically significant decrease in the levels of glucose, 3-phosphogylcerate, phosphophenol pyruvate,
Alanine, pyruvate and lactate in both the *Bmpr1*$^{-/-}$ and 4n ESCs. This clearly suggests that the defective cells analysed have either a lower glycolytic activity or are using the glycolysis to feed the TCA cycle. This possibility will be explored further on in this chapter. In contrast to these changes the levels of DHAP are increased in defective cells in N2B27, suggesting that *Bmpr1*$^{-/-}$ and 4n ESCs may be using glycolysis for lipid generation in N2B27. This will also be explored later on. In conclusion, these results indicate that upon differentiation defective cells do not switch to a glycolytic metabolism as efficiently as wild-type cells do, and this may be the reason they are less competitive than wild-type cells in N2B27.

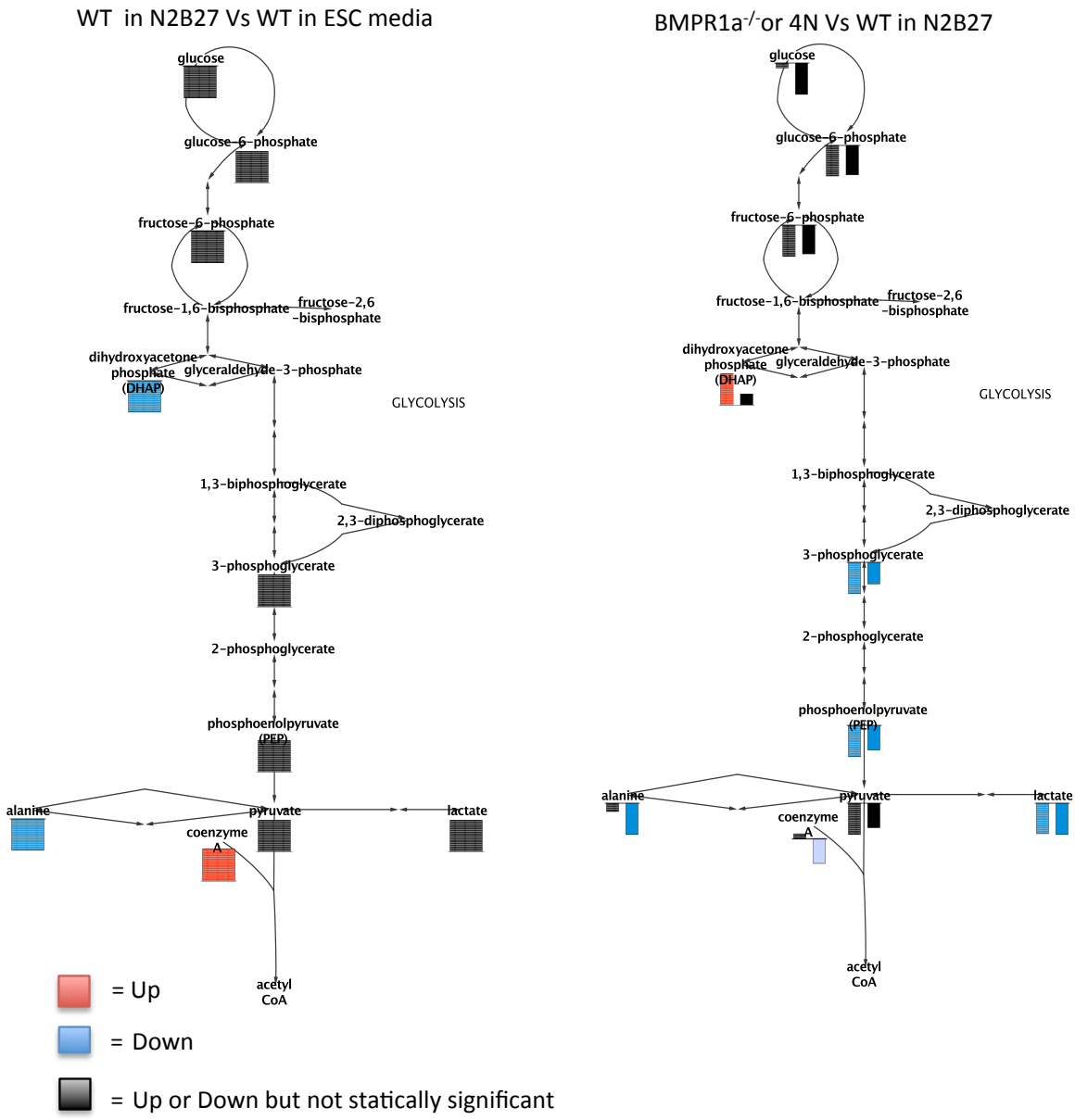


Figure 5.4A. Upon differentiation, loser cells have lower glycolytic levels than winner cells.
Left panel: WT ESCs cultured in ESC media compared to WT ESCs cultured for 3 days in N2B27 media showing increased levels of glycolytic metabolites in N2B27. During differentiation wild-type cells have decreased DHAP and alanine levels and increased coA levels.
Right panel: Comparison of the levels of metabolites involved in glycolysis during differentiation of WT and mutant ESCs (BMPR1a null on the left and 4N on the right). Defective cells have decreased levels of certain glycolytic metabolitessuch as 3-phophoglycerate, phosphoenolpyruvate, lactate and alanine. DHAP is up regulated in BMP defective cells. Co-A is down regulated in 4n cells**.**

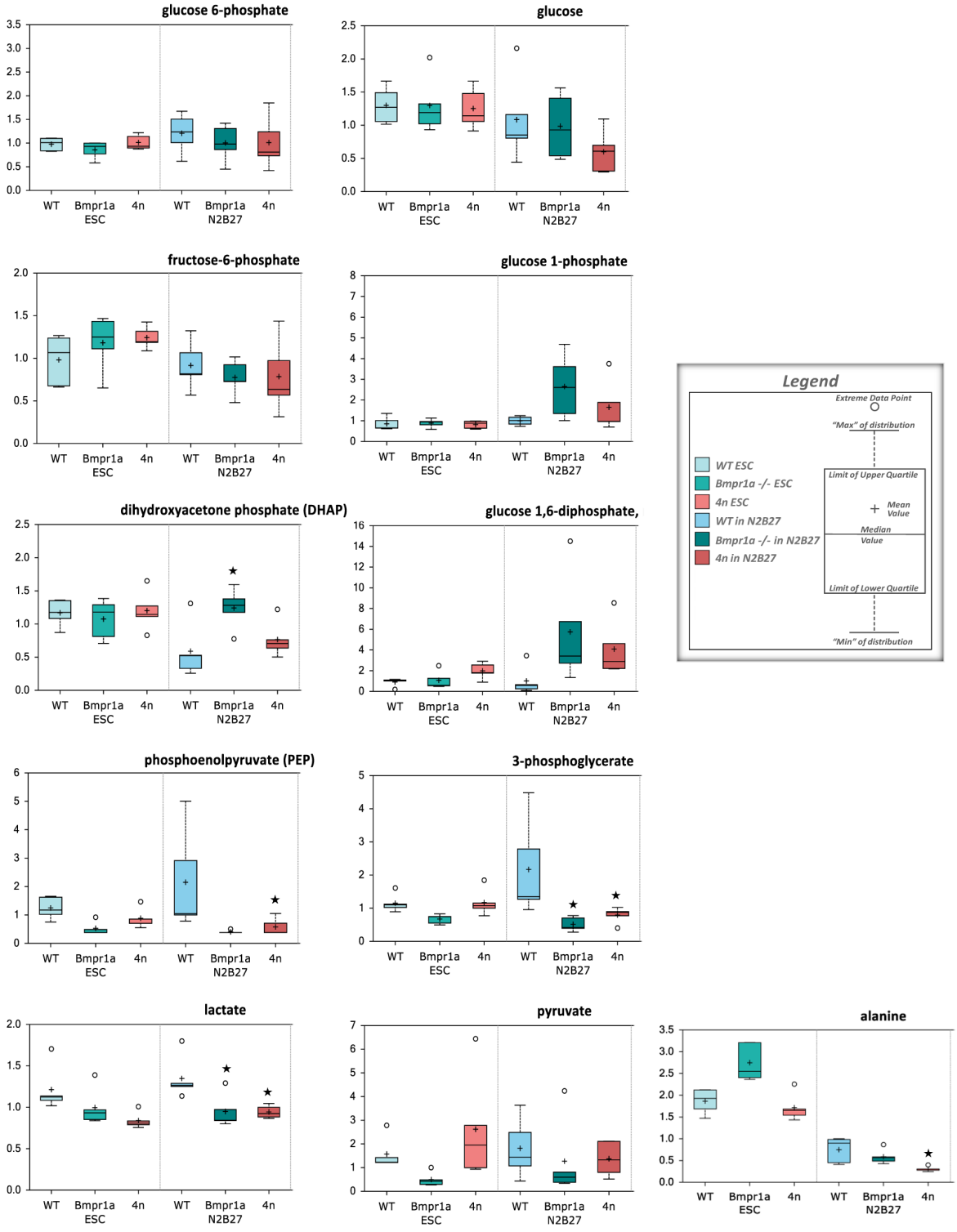


Figure 5.4B. Upon differentiation, loser cells have lower glycolytic levels than winner cells.
Comparison of metabolites involved in glycolysis during differentiation of WT and mutant ESCs (BMPR1a null and 4N) showed that defective cells have decreased levels of certain glycolytic metabolites such as 3-phophoglycerate, phosphoenolpyruvate, lactate and alanine. DHAP is up regulated in BMP defective cells. Star = *$P \leq 0.05$ Welch's two-sample *t*-test

5.2.3 The TCA cycle.

The comparison of glycolytic metabolites between cells cultured in ESC media and N2B27 suggested that upon differentiation glycolysis becomes de-coupled from the TCA cycle. For this reason we compared the levels of key metabolites of the TCA cycle pathway between wild-type ESCs cultured in ESC conditions and wild-type ESCs cultured for 3 days in N2B27 (Figure 5.5A). When this was done a decrease in the expression of all the intermediates of the TCA pathway was observed in cells cultured in N2B27 compared to ESC media. For example, expressions of Fumarate, Citrate and Malate were all found being decreased in N2B27, although these differences were not statistically significant in the case of the expressions of succinate and alpha-ketoglutarate. These results are consistent with cells switching from glycolysis to OXPHOS upon differentiation (Zhou et al., 2012).

Next I compared the levels of metabolites from the TCA pathway between wild-type, *Bmpr1*$^{-/-}$ and 4n ESCs cultured for 3 days in N2B27 to analyse if defective cells also have a lower TCA upon differentiation (Figure 5.5B). Interestingly, with the exception of citrate that was increased, a decrease in the levels of the rest of TCA cycle metabolites was observed in defective cells. For example 2-phophoenolpyruvate and 2-hydroxyglutarate were decreased in BMP-defective and 4n cells and aspartate, malate, fumarate and succinate were decreased in 4n cells. This clearly suggests that the defective cells analysed display an even lower TCA cycle activity than wild-type cells, and therefore suggesting a lower OXPHOS activity. This, together with the analysis of glycolytic metabolites, suggests than upon differentiation, defective cells have a lower metabolism than wild-type cells. Steady state analysis is required to confirm these results.

Figure 5.5A TCA Cycle

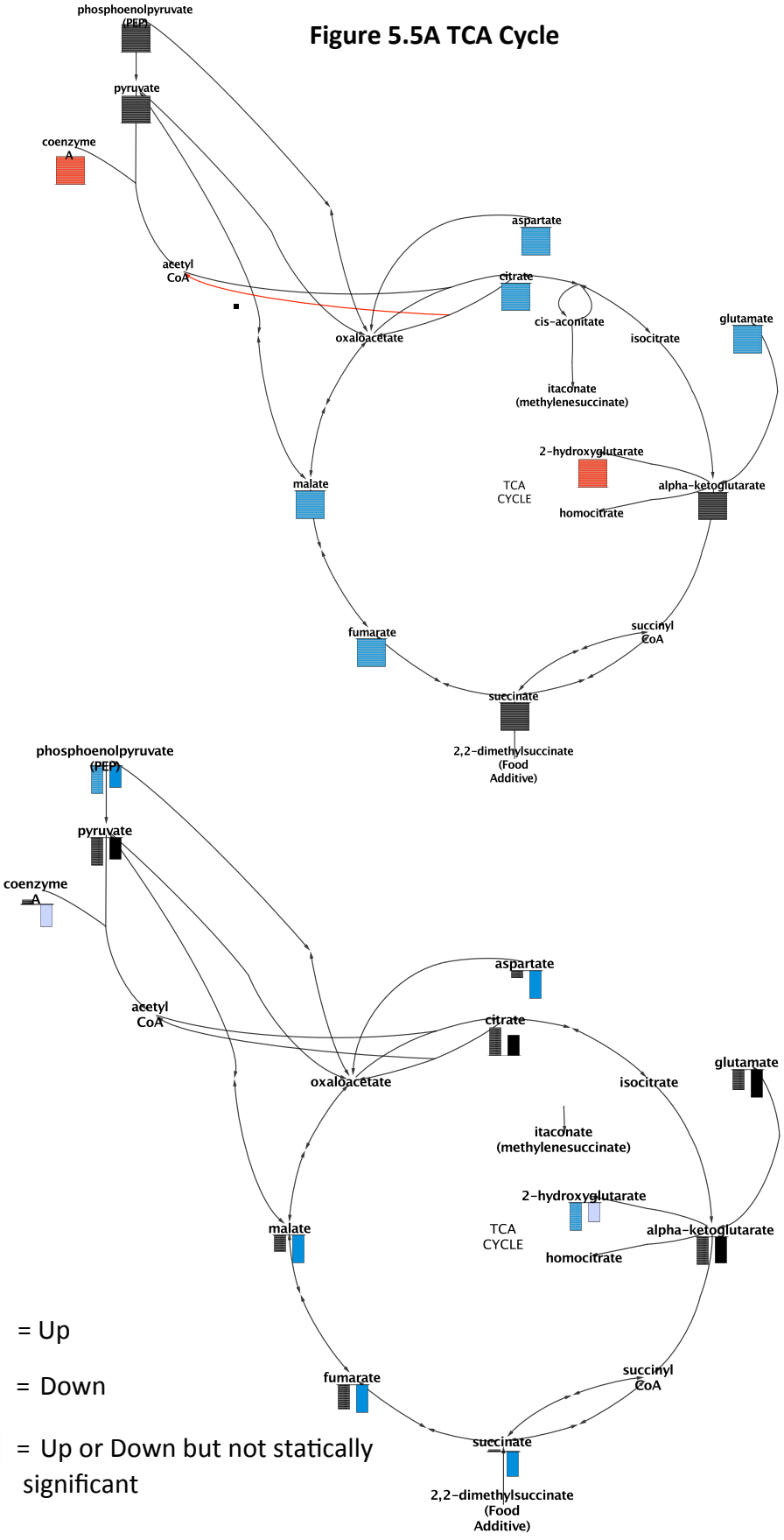


Figure 5.5A Lower levels of TCA cycle metabolites in defective cells upon differentiation.
A) WT cells cultured for 3 days in N2B27 show lower TCA activity than WT cells cultured in ESC media. For example aspartate, citrate, glutamate, malate and fumarate are lower in wild-type cells and co-A and 2-hydroxyglutarate are increased in WT cells.
B) Metabolite comparison between WT, *Bmpr1a*$^{-/-}$ (left bar) and 4N (right bar) ESCs in N2B27 reveals that the defective cells have lower levels of TCA metabolites. For example 2-phophoenolpyruvate and 2-hydroxyglutarate were decreased in BMP-defective and 4n cells and aspartate, malate, fumarate and succinate were decreased in 4n cells.

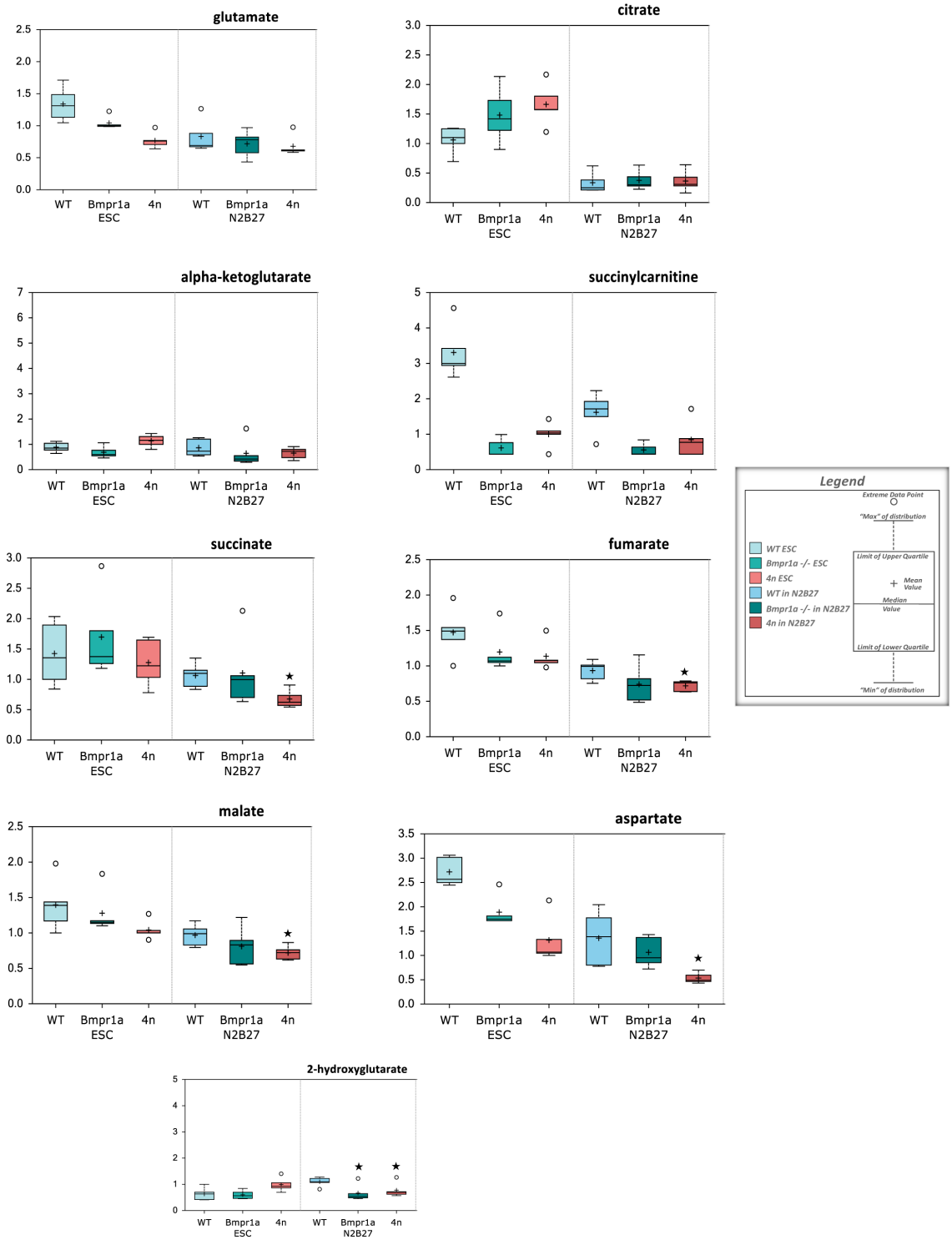


Figure 5.5B lower levels of TCA cycle metabolites in defective cells upon differentiation.
Metabolite comparison between WT, *Bmpr1a*$^{-/-}$ and 4N ESCs in N2B27 reveals that the defective cells have lower levels of TCA metabolites. For example 2-phophoenolpyruvate and 2-hydroxyglutarate were decreased in BMP-defective and 4n cells and aspartate, malate, fumarate and succinate were decreased in 4n cells. Star = $p \leq 0.05$ Welch's two-sample *t*-test

5.2.4 The Pentose Phosphate Pathway (PPP)

Glycolysis feeds into the PPP pathway, and as in mutants glycolysis was lower, I also wanted to study if the PPP was affected. I first analysed how PPP activity changes upon differentiation. For this I compared the levels of expression of key metabolites in the PPP between wild-type ESCs cultured in ESC conditions and wild-type ESCs cultured for 3 days in N2B27 (Figure 5.6A). A decrease in the levels of all intermediates of the PPP analysed was observed in cells cultured in N2B27 compared to ESC media. This suggests that although glycolysis is increased upon differentiation, PPP activity is decreased. One possible reason for this is that differentiating cells may be concentrating their glycolytic activity on the generation of ATP rather than on feeding into other pathways as the TCA cycle or PPP.

Next, I compared the levels of metabolites from the PPP between wild-type, *Bmpr1*$^{-/-}$ and 4N ESCs cultured for 3 days in N2B27 (Figure 5.6B). Intriguingly, we observed, a statistically significant increase in the levels of a number of PPP metabolites, including ribose 5-phosphate, ribose 1-phosphate and ribitol in *Bmpr1*$^{-/-}$ and 4N ESCs. In contrast to this erythritol, sedoheptulose-7-phosphate and 5-phosphoribosyl-1-pyrophosphate (PRPP) were decreased in defective cells. Given that PRPP is generated from ribose 5-phosphate, which in turn, is formed from ribitol and/or ribose 1-phosphate, this suggests that defective cells may have a block in PRPP formation. PRPP is a precursor for both the *de novo* synthesis and salvage of purine and pyrimidine nucleotides. Suggesting that the defective cells could have a defect in nucleotide metabolism as a consequence a defect in DNA synthesis and hence to proliferation. This possibility will need to be investigated further. In addition to producing precursors for nucleotide synthesis, the PPP produces NADPH via the oxidation of glucose-6-phosphate and 6-phosphoglutanate. Glucose-6-phosphate is lower in both defective cell types and 6-phosphoglutanate is lower in *Bmpr1*$^{-/-}$ and higher in 4N ESCs. Given the importance of NADPH in maintaining the oxidising-reducing balance (REDOX) in the cell, it will therefore also be important to analyse if defective cells have changes is REDOX potential.

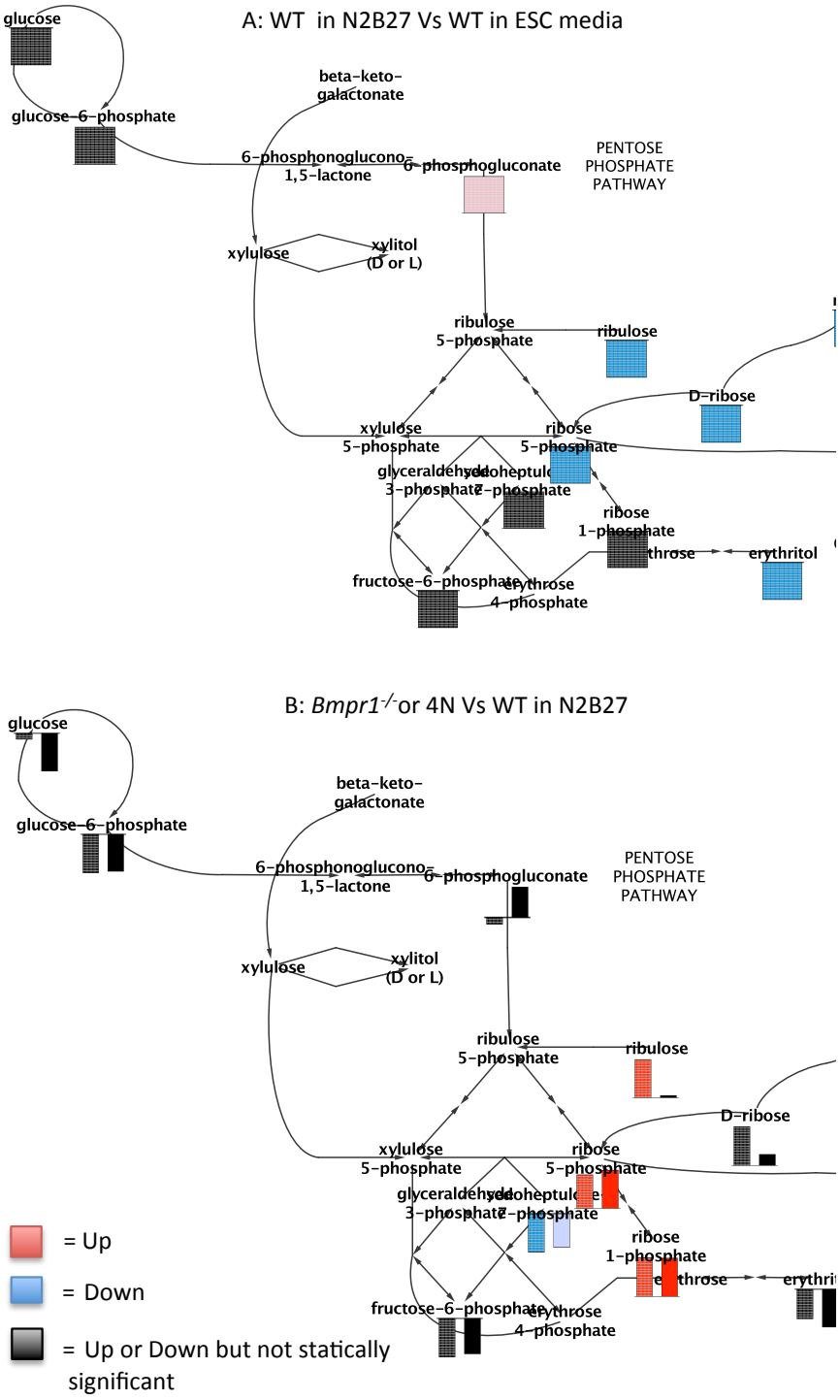


Figure 5.6A Analysis of the metabolites involved in the Penthose Phosphate Pathway.
A) During differentiation WT cells show lower levels of PPP metabolites such as ribulose, D-ribose, ribose-5-phosphate and ribitol. In contrast to this phospho-gluconate is decreased. B) Metabolite comparison between *Bmpr1a*$^{-/-}$ (left) and 4N (right) ESCs in N2B27 indicates that the activity of the PPP pathway is different between WT and defective cells during differentiation. In defective cells ribose-5-phosphate, ribitol and ribose

phosphateerythrose are increased and ribulose is increased in BMP-defective cells. PRPP and glyceraldehyde heptulose are decreased in both defective cell types.

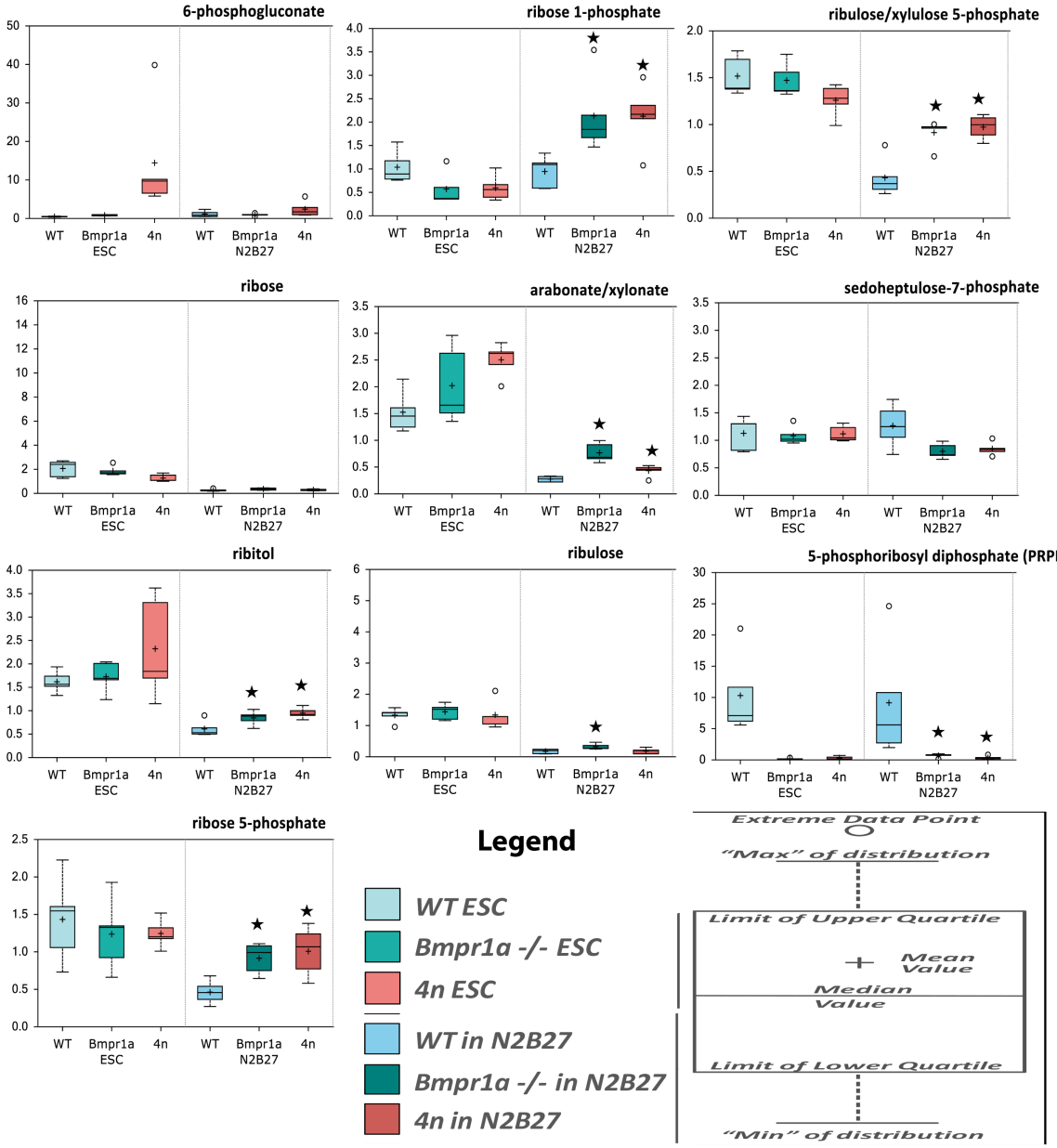


Figure 5.6B Analysis of the metabolites involved in the Penthose Phosphate Pathway.
Metabolite comparison between *Bmpr1a*$^{-/-}$ and 4N ESCs in N2B27 indicates that the activity of the PPP pathway is different between WT and defective cells during differentiation. In defective cells ribose-5-phosphate, ribitol and ribose 1-phosphateerythrose are increased and ribulose is increased in BMP-defective cells. PRPP and glyceraldehyde heptulose are decreased in both defective cell types. Star = $p \leq 0.05$ Welch's two-sample *t*-test.

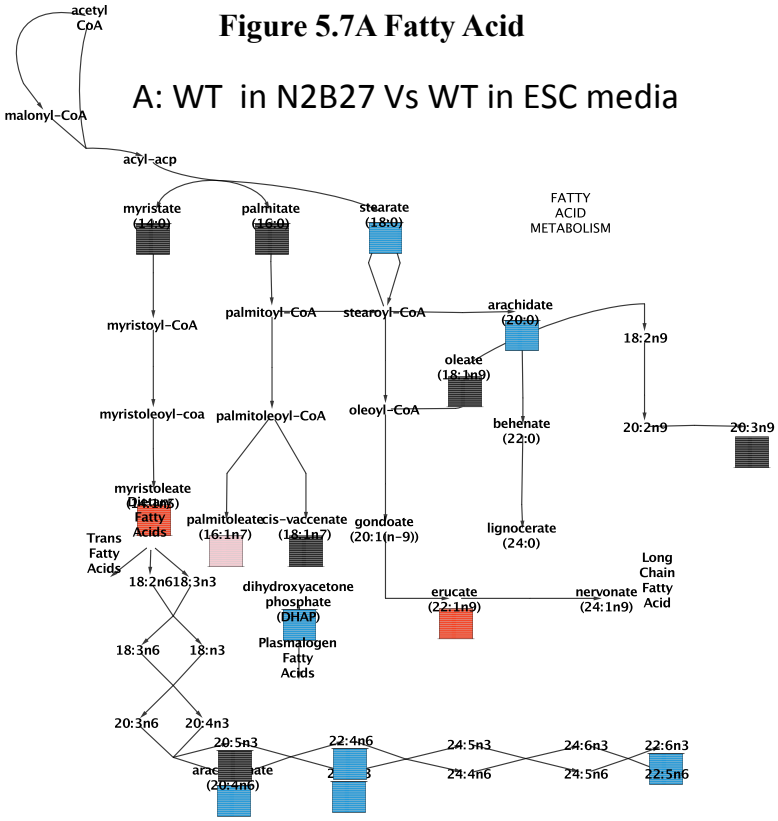
5.2.5 Lipid metabolism

Comparison of the glycolytic levels between WT and defective cells in N2B27 revealed that the defective ones had higher levels of dihydroxyacetone phosphate (DHAP) that is precursor for triglyceride (TG) synthesis (Figure 5.4). For this reason we investigated fatty acid metabolism in these cells. First we analysed how fatty acid metabolism changes between wild-type cells during the onset of differentiation. For this, we compared the levels of expression of key metabolites in the fatty acid metabolism pathway between wild-type ESCs cultured in ESC conditions and for 3 days in N2B27. When this was done, we observed a decrease in the expression of the majority of fatty acid metabolites in N2B27 (Figure 5.7A). For example, expression of all saturated fatty acids, such as stearate, arachidate, myristate and palmitate were decreased, although the last two were not significantly. Similarly some polyunsaturated fatty acids were also decreased in N2B27 in a statistically significant manner (e.g. arachidonate). In contrast to this, other polyunsaturated fatty acids were increased (e.g. mysristoleate and erucate). The end products of the fatty acid pathway and glycerol are used for TG synthesis. DHAP that is formed during glycolysis can be converted to glycerol and the resulting glyceraldehyde then undergoes phosphorylation to glyceraldehyde-3-phosphate for TG synthesis. DHAP was decreased upon differentiation, but our metabolic analysis did not detect TGs, and for this reason it was impossible for us to verify how they change upon differentiation.

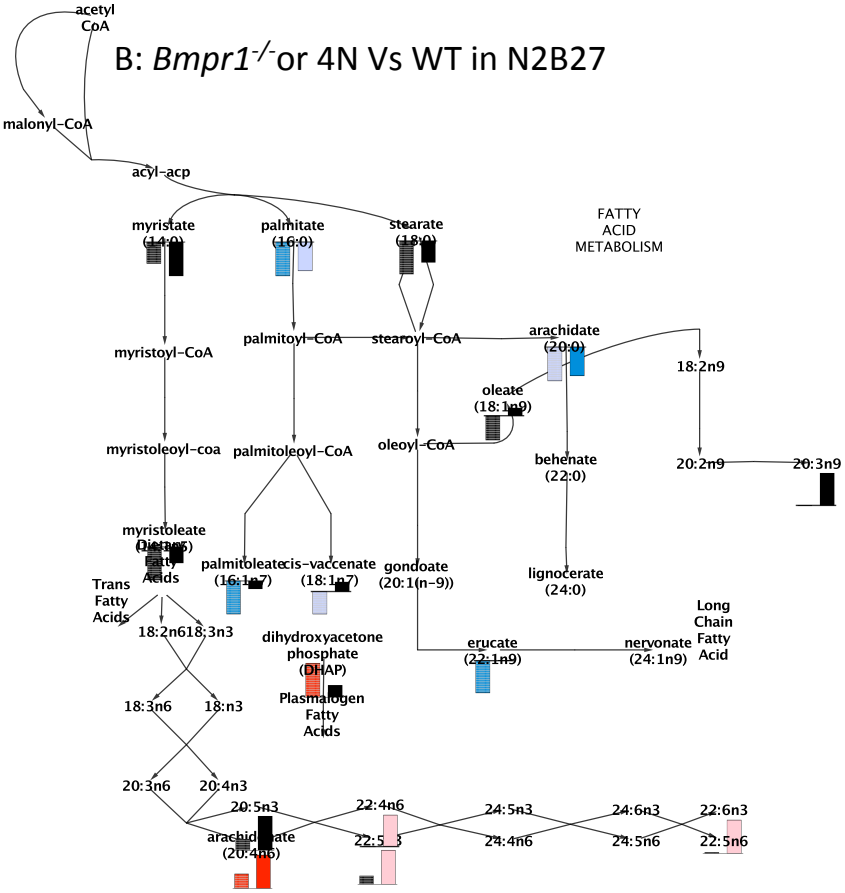
Next we compared the levels of fatty acid metabolites between wild-type, *Bmpr1*$^{-/-}$ and 4n ESCs cultured for 3 days in N2B27. We only observed a decrease in the levels palmitate and an increase in aracnidate in both defective cell types in N2B27. As we were looking for metabolites changed in both defective cell types in N2B27, as this would be suggestive of a role in triggering competition, these results suggest that lipid metabolism may not be a major contributor to the trigger of competition. We could not detect TGs in our study and therefore we were unable to determine the direct consequences of the DHAP increase, but these results suggest that defective cells do not have a generalised increase in fatty acid metabolism. Further work is required to determine the implications of those fatty acids that change in expression in defective cells compared to controls.

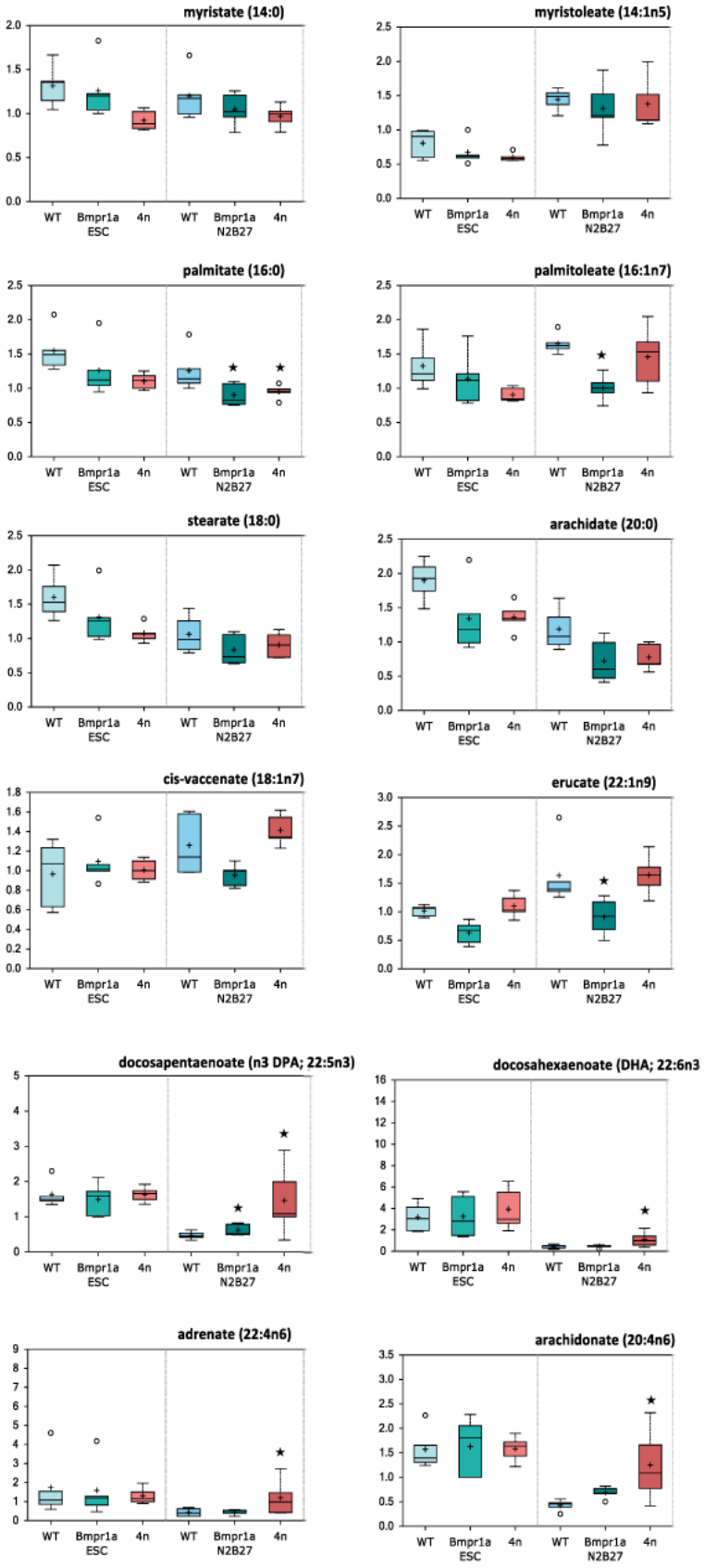
Figure 5.7A Fatty Acid

A: WT in N2B27 Vs WT in ESC media



B: *Bmpr1*$^{-/-}$ or 4N Vs WT in N2B27





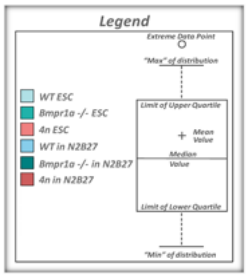


Figure 5.7B Fatty acid metabolism during differentiation.

Figure 5.7A Fatty acid metabolism during differentiation.
A) Changes in fatty acid metabolites in WT cells upon differentiation. During differentiation stereate, arachidate, DHAP, 20:4n6, 22:4n6, 22:4n3 and 22:5n levels are decreased while myristoleate and erucate are increased. B**)** In *Bmpr1*$^{-/-}$ and 4n ESCs when compared to WT cells upon differentiation there is a decrease in palmitate are arachidate and an increase in 20:4n6. In BMP defective cells palmitoleatesis, vaccinate and erucate are decreased and DHAP is increased. In 4n cells 22:5n3, 22:4n6 and 22:5n6 are increased.

Figure 5.7B Fatty acid metabolism during differentiation.
In *Bmpr1*$^{-/-}$ and 4n ESCs when compared to WT cells upon differentiation there is a decrease in palmitate are arachidate and an increase in 20:4n6. In BMP defective cells palmitoleatesis, vaccinate and erucate are decreased and DHAP is increased. In 4n cells 22:5n3, 22:4n6 and 22:5n6 are increased. Star = $p \leq 0.05$ Welch's two-sample *t*-test.

5.2.6 Amino acid levels

One of the important products of glycolysis and the TCA cycle are amino acids. Given the lower activity of both these pathways in defective cells in N2B27, we next analysed amino-acid levels in these cells. We observed that whilst amino acid levels were not significantly different between WT, *Bmpr1*$^{-/-}$ and 4N cells in ESC media, in N2B27 a number of amino acids were found at lower levels than in control cells. For example: arginine, alanine, lysine, proline and threonine (Figure 5.8). These results suggest that defective cells have reduced their amino acid metabolism.

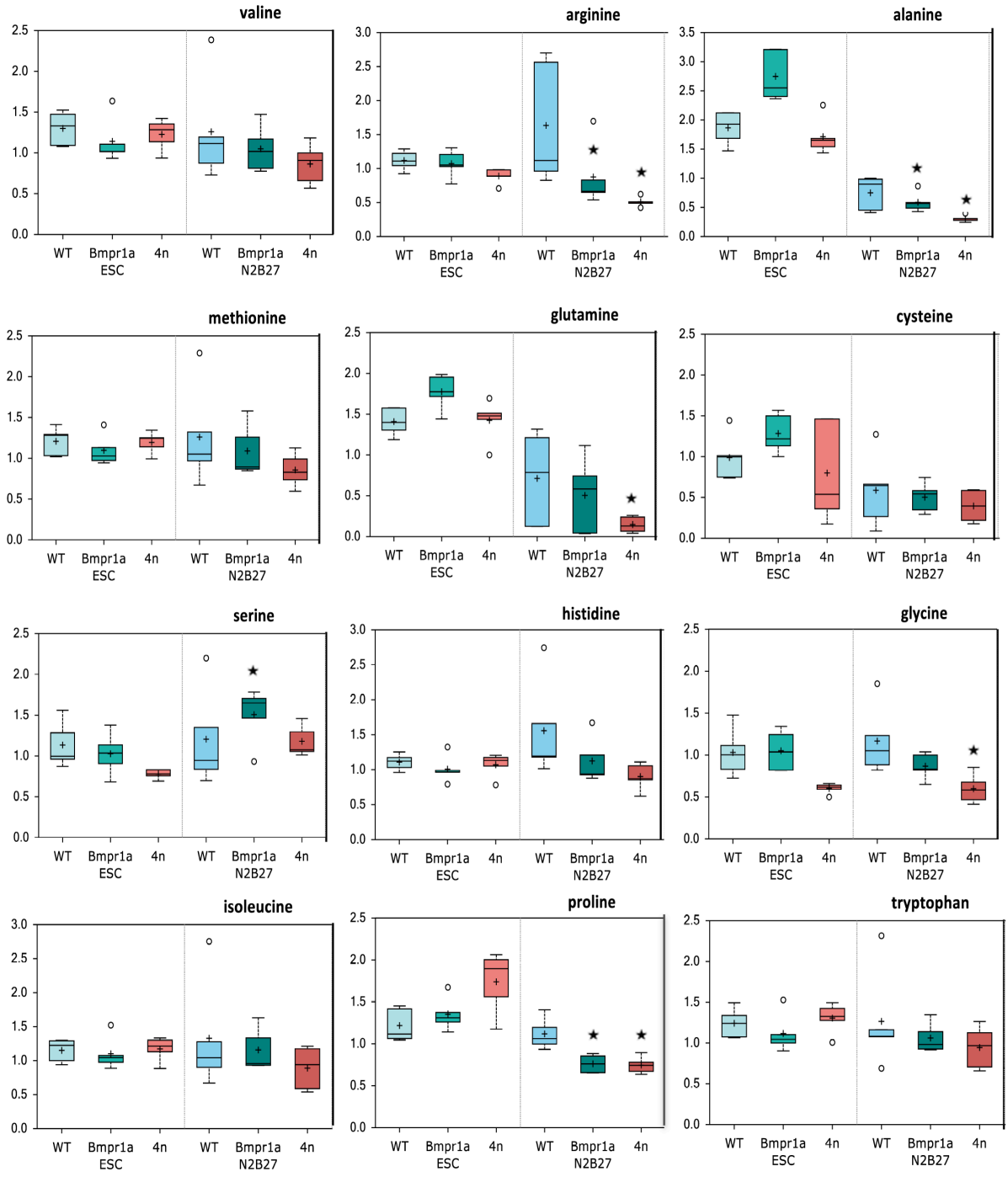


Figure 5.8-A Amino Acid levels overview of WT, *Bmpr1*$^{-/-}$ and 4N ESCs.

Analysis of amino acid levels in WT and defective cells reveals that a significant number show decreased levels in defective cells. Samples are grouped according to the media culture condition and are displayed as box plots, 12/20 amino acids. See legend and rest of the amino acids in fig. 5.8-B. Star = *$P \leq 0.05$ Welch's two-sample *t*-test.

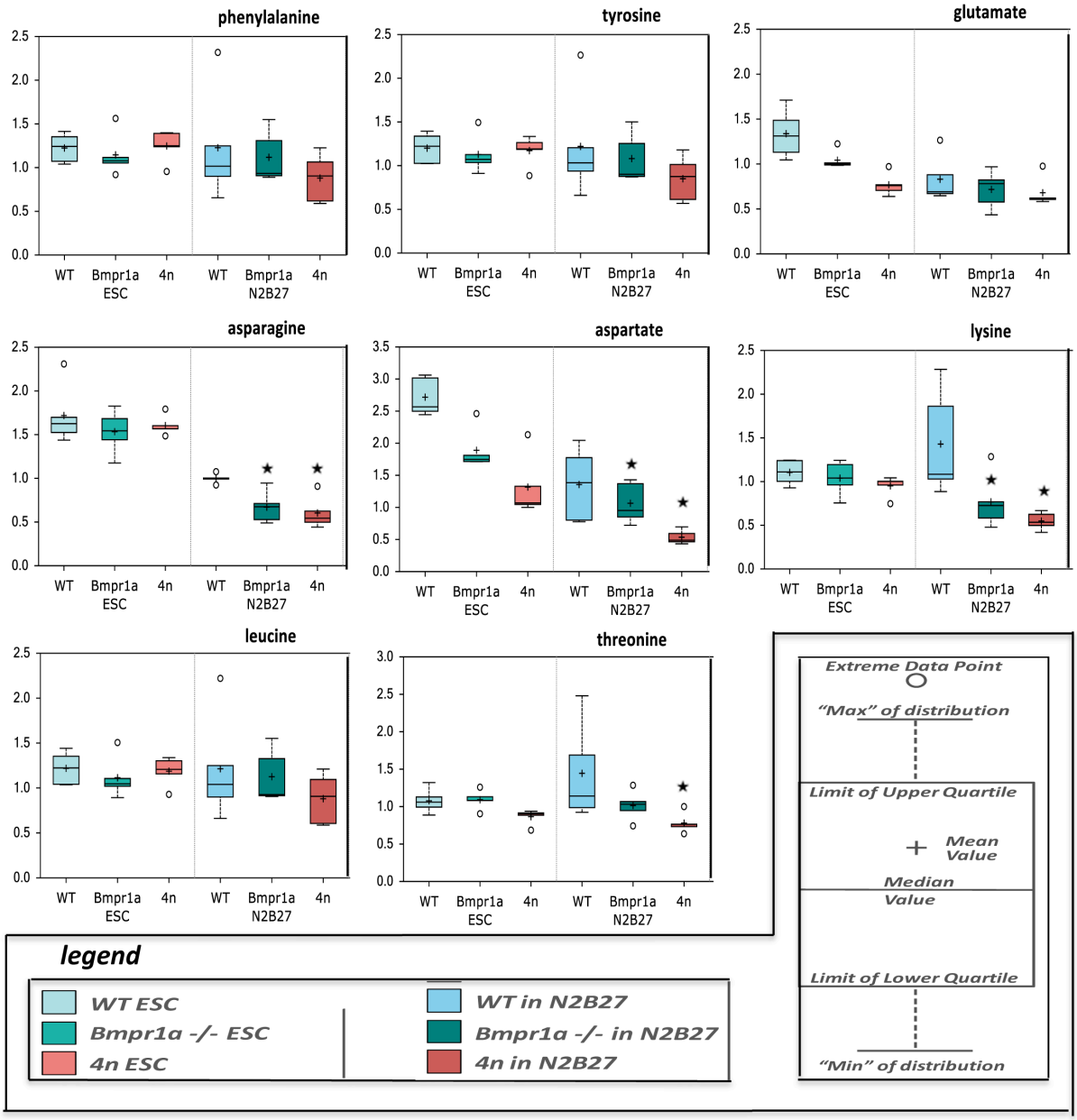


Figure 5.8-B Amino acid metabolism, overview of WT, *Bmpr1*$^{-/-}$ and 4N ESCs.
Analysis of amino acid levels in WT and defective cells reveal that a significant number show decreased levels in defective cells. Samples are grouped according to the media culture condition and are displayed as box plots, 8/20 amino acids. Defective cells show lower level of amino acid synthesis in almost all the 20 s analysed. Star = *$P \leq 0.05$ Welch's two-sample *t*-test.

5.3 DISCUSSION

Cell competition is a fitness sensor that can be triggered in a number of ways, including growth differences, differences in signalling levels and polarity mismatch (Amoyel and Bach, 2014; Vincent et al., 2013). One of the things that drew our attention about all defective cell types eliminated by cell competition between mouse ESCs, is that they always proliferated slower than WT cells. This suggests that what may be triggering cell competition here is growth differences. A number of parameters control cell proliferation, but amongst them metabolism plays a fundamental role, as it provides the energy and macromolecules for this process. For this reason, we hypothesized that metabolic differences may underlie the competitive disadvantage of defective cells during cell competition in the early mouse embryo. Therefore we compared the metabolic profile of WT, *Bmpr1*$^{-/-}$ and 4N ESCs cultured in ESC conditions (where cell competition is not observed), and in N2B27 (where cell competition is induced). In the first instance we have performed our metabolic profiling on cells cultured in separate conditions, as we predicted that those parameters that determine cell fitness must be cell-intrinsic, and therefore independent of the confrontation of winner and loser cells. Once fit and unfit status has been established, cells must communicate their fitness levels.

Our metabolic profiling of WT and defective cells revealed no significant differences between these cells types in ESC culture conditions, that fits with the lack of competition in this culture condition. In contrast to this both *Bmpr1*$^{-/-}$ and 4N show very different metabolic profiles in N2B27, suggesting these metabolic differences could be the underlying cause of defective cell elimination during cell competition. We found that upon differentiation defective cells have lower glycolysis level feeding into the TCA cycle activity, as well as block in PRPP synthesis and an accumulation of ribonucleotide precursors. The TCA cycle and glycolysis are also used to generate amino acids. Additionally, we observed lower levels of amino acids in defective cells in N2B27. Upon the onset of differentiation pluripotent cells switch to a highly glycolytic state (Zhou et al., 2012), most likely required to sustain the increase in proliferation that occurs during this process (Snow, 1976; Stuckey et al., 2011). Our results suggest that defective cells are unable to perform this switch efficiently, and this may explain why they are out-competed by wild-type cells specifically upon the onset of differentiation.

A number of causes could be the primary metabolic defect that leads to the competitive disadvantage of loser cells when they are confronted with wild-type cells. For example lower glycolysis and TCA cycle activity would lead to lower levels of ATP generation. The lower PRPP level, which is used in the formation of purines and pirimidines, indicates these cells may also have lower nucleotide for DNA synthesis. The changes in TCA and pentose phosphate pathway activity could also alter the REDOX potential of the cells. The lower amino acid levels could also be responsible for the loser status of defective cells, as amino acid sensing is in important cellular function and low amino-acid levels have important signalling outcomes (Efeyan et al., 2012). Amino acid levels will also impact on protein synthesis, and as discussed in the introduction, differences in protein synthesis have been suggested as a trigger for cell competition (Vivarelli et al., 2012). It is worth noting that work from other people in the lab have found that specifically when WT and defective cells are confronted with each other, the defective cells show decreased mTOR activity, and that increasing mTOR signalling in loser cells during cell competition can rescue their elimination (Sarah Bowling and Tristan Rodriguez, unpublished results). mTOR is an essential nutrient sensor and can be modulated by many of the factors that could be affected in defective cells during cell competition, including ATP levels, amino acid levels or REDOX potential (Laplante and Sabatini, 2012). Further work is required to disentangle specifically which metabolic component is the key factor that is responsible for the loser status of *Bmpr1*$^{-/-}$ and 4N when they are confronted with WT cells.

Our results suggest that cell competition could be acting as a metabolic selector mechanism. Ensuring that the most metabolically fit cells contribute preferentially to development, and eliminating metabolically sub-optimal cells during tissue homeostasis, has obvious evolutionary advantages. In these contexts, cell competition would contribute to the metabolic refinement of the tissue. Metabolic selection would also allow competition to remove cells with mitochondrial defects that do not directly compromise viability, but that could be a substrate for further transforming mutations (Ohsawa et al., 2012). Furthermore, during cancer, tumours cells take advantage of metabolic selection to promote their transformation (Chang et al., 2015; Flavahan et al., 2013), and this together with the fact that wild-type but metabolically distinct cell lines can compete with each other (Penzo-Mendez et al., 2015), and that metabolic differences underlie the competitive advantage of d-Myc

overexpressing cells (de la Cova et al., 2014), support the hypothesis that competition may indeed have a role as a metabolic selector mechanism.

Chapter 6: GENERAL DISCUSSION

Cell competition has been primarily studied in *Drosophila* and has been described as a fitness sensor that eliminates cells deemed to be less fit than their neighbours (Amoyel and Bach, 2014). Here we show it performs similar roles in mouse ESCs, the early mouse embryo and during development of the heart. One of the most remarkable conclusions that can be drawn from the study of cell competition is that cellular fitness is not only a cell-intrinsic property, but it is also relative to the fitness of neighbouring cells. Thus, cells that are of sub-optimal fitness in one context may be 'super-fit' in the context of a different cell population. For example, in ESCs, wild-type cells eliminate cells with defective BMP signalling or tetraploid cells but are eliminated by the fitter *c-Myc* over-expressing cells. These findings, together with the observation that during cell competition in ESCs and the embryo the loser cells have lower levels of c-MYC, suggests that the relative levels of c-MYC is what determines the winner or loser status during cell competition. This role appears to be evolutionarily conserved as dMYC plays similar roles in the *Drosophila* imaginal wing-disc (de la Cova et al., 2004; Moreno and Basler, 2004).

Originally cell competition was described in developing epithelia (Amoyel and Bach, 2014; Vincent et al., 2013) and here we show it has a role in mouse ESCs and in the developing mouse embryo. Cell competition has also been described in other stem cell populations, for example in the ovary stem cell niches in *Drosophila*, where it plays a role in regulating stem cell selection, maintenance and tissue repopulation (Rhiner et al., 2009), or in the mouse thymus during the substitution of thymus-resident progenitors ('old') by bone-marrow-derived ('young') progenitors (Martins et al., 2014). Conversely, cell competition has also been demonstrated in *Drosophila* post-mitotic tissues, such as follicular epithelium, where aberrant but viable cells are eliminated through cell competition and the resulting loss of local tissue volume triggers sporadic cellular hypertrophy (Tamori and Deng, 2013), or in adult mouse cardiomyocytes, where *c-Myc* over-expressing cells replace wild-type cells (Villa Del Campo et al., 2014). Together, these results indicate that cell competition acts as a cell quality control mechanism to assure maximum tissue fitness by eliminating those suboptimal cells that are not eliminated by the cell-intrinsic stress mechanisms in a wide variety of contexts, including the embryo and adult.

6.1 What determines cell fitness during cell competition in the early mouse embryo?

In the embryo, just before gastrulation takes place, there is a window of time when the cells responsible for the differentiation into the three germ layers, and therefore into all somatic cells of the adult organism including the germ-line, are in a state of primed pluripotency. During this primed pluripotency period, cells display a characteristic rapid proliferation rate. (Snow, 1977; Stuckey et al., 2011). This proliferation rate is faster than that of naïve pluripotent cells and creates the potential for the accumulation of mutations that even if not lethal, could compromise the fitness of the future embryo or germ-line. Coinciding with this period or primed pluripotency, epiblast cells have been shown to change their apoptotic response and become hypersensitive to cell death signals (Heyer et al., 2000; Pernaute et al., 2014), suggesting that embryonic fitness may be actively monitored at this stage of development.

In agreement with possibility that the cell competition is triggered specifically in the primed pluripotent state are a number of observations that indicate that cell competition in the early mouse embryo is differentiation-dependent. First, in chapter 3 in the attempt to study the possible role of the oncogene c-*Myc* in cell competition, I found that over-expressing *c-Myc* was enough to induce cell competition in mouse ESCs, but this phenomenon was only possible once the cells had initiated differentiation. Additionally, LIF, ERK inhibition or GSK3beta inhibition, that all block naïve pluripotent cells from initiating differentiation, also block cell competition in ESCs (Sancho et al., 2013). Therefore cell competition appears to be linked to the onset of differentiation. One reason for this may come from our findings in chapter 5. Here defective cells only showed metabolic defects upon differentiation, when pluripotent cells undergo a switch to a highly glycolytic metabolism (Zhou et al., 2012), and the defective cells we analysed were not capable of performing this switch. This suggests that metabolic differences may be the trigger of cell competition during early mouse development.

6.2 What selection mechanisms distinguish fit from unfit pluripotent cells?

The existence of different modes of cell competition is due not only to the great variety of triggers and effector molecules involved in this process, but also to the different mechanisms used by cells to compare their fitness levels. Several mechanisms by which cells sense their

relative fitness have been proposed. Mechanical induced competition was first described in *Drosophila* (Shraiman, 2005), and subsequently demonstrated in a variety of contexts. For example when the compression induced by the faster growing cells causes the delamination of the slowing growing cells from the epithelia (Marinari et al., 2012). Also, it is evident when the elimination of loser cells is preceded by winner– loser cell mixing through cell–cell intercalation (Levayer et al., 2015). Finally, mechanical stress-induced competition has been shown where MDCK cells mutant for the cell polarity gene *Scribble* are eliminated by apoptosis triggered by the increase in p53 expression triggered by mechanical stress (Wagstaff et al., 2016). Due to the nature of the forces involved, these forms of mechanical cell competition occur on epithelia sheets.

Other sensing mechanisms of cell competition involve direct cell fitness comparisons, where the win/lose decision is determined by the relative differences in expression of the three splice isoforms of the *Flower* gene (fwe^{ubi}, $fwe^{Lose\text{-}A}$, and $fwe^{Lose\text{-}B}$). During cell competition the cells with reduced fitness express the isoforms fwe^{ubi}, $fwe^{Lose\text{-}A}$ and this leads to them being eliminated and replaced by winners cells expressing only the fwe^{ubi} isoform (Rhiner et al., 2010). A further fitness sensing model involves the innate immune defense system, that is responsible of detecting cells by differentiating self from non-self (e.g. microbial infection) as well as self from altered-self cells (e.g. viruses and cancer cells) (Song et al., 2012). During competition it was shown that the activation of the Toll related receptors (TRRs) and NFkB homologues directly activate pro-apoptotic target genes that are necessary for loser cell elimination (Meyer et al., 2014). These sensing mechanism all present very different ways of fitness recognition, confirming that cell competition is a phenomenon that is context-dependent and with its precise mechanism determined by the type of trigger and stage of development where it occurs.

In the early mouse embryo we find that the mechanism that differentiates fit from unfit cells appears to be different. As mentioned above, we find that a key fitness difference between wild-type and defective cells is their metabolism, with loser cells having lower glycolysis, TCA cycle activity and amino acid levels. The mTOR is a metabolic sensor required to achieve the high levels of protein synthesis necessary for cell cycle entry (Laplante and Sabatini, 2012). Work from other members in the lab showed that defective ESCs in co-

culture with wild-type ESCs exhibit low mTOR activity and that this decrease in mTOR occurred only when these cell types were co-cultured specifically at the onset of differentiation. Furthermore, they found that increasing mTOR in loser cells was sufficient to rescue loser cell elimination. This suggests mTOR is a key regulator of cell competition and that during early mouse development cell competition may be eliminating those cells that are metabolically less active. Furthermore, the low amino acid levels observed in the loser (mutants) compare to the winner (wild-type) cells raises the possibility that the fitness selection mechanism acting in the early embryo may be a competition for nutrients and that the nutrients that cells may be competing for amino acids.

6.3 What are the roles of cell competition in the early embryo*?*

An important question is what are the physiological roles of cell competition during early embryogenesis. The experiments described here suggest two possible roles. A first possible role is the elimination of unfit or abnormal cells before they contribute to further development or the germ-line. The fact that we find that a number of defective cell types are eliminated by competition at the onset of differentiation strongly supports this possible role. The second possible role for cell competition in the early mammalian embryo could be maintaining or regulation embryo size.

One type of defective cells eliminated by cell competition are cells with defective ploidy, and indeed cell competition has been suggested to have evolved as a mechanism to eliminate aneuploid cells (Baker, 2011; Vincent et al., 2013). This possibility is particularly relevant when we think about early mammalian development. Mitotic errors during mammalian pre-implantation development are surprisingly common. In humans, 78% of pre-implantation embryos analysed are karyotypically abnormal, and most commonly these embryos show mosaic aneuploidy (73% of embryos), rather than karyotypic abnormalities in all cells of the conceptus (van Echten-Arends et al., 2011). In contrast to this the level of aneuploidy at later stages of gestation is only 1-2% (Kalousek et al., 1991). The pre-implantation embryos analysed were primary leftover embryos from *in vitro* fertilization (IVF), therefore it is conceivable that a proportion would be non-viable. However, in the exceptional cases where mosaic aneuploid embryos have been used for IVF they have given perfectly normal and

healthy babies (Greco et al., 2015), indicating that some clearance mechanism must exist to eliminate aneuploid/aberrant ploidy cells during early mammalian embryogenesis.
Cells with defective ploidy, such as some aneuploidy types or tetraploid cells survive normally until very late in gestation or birth when the whole embryo is of the same genotype (Henery et al., 1992; Kaufman, 1991; Kaufman and Webb, 1990; Siegel and Amon, 2012; Snow, 1973, 1975). In contrast to this aneuploid and tetraploid cells are efficiently eliminated from embryonic tissues in chimeras with diploid cells (Bolton et al., 2016; Eakin et al., 2005; Tarkowski et al., 1977). This suggests that the death of these cells is unlikely to be solely through cell-intrinsic mechanisms, a possibility strengthened by the demonstration that the elimination of tetraploid cells in chimeras is induced specifically by their wild-type neighbours, and therefore occurring through a competition-type mechanism (Sancho et al., 2013).

The second possible role for cell competition is that it could regulate embryo size during early mammalian development. Cell competition has already been suggested as a mechanism for organ size regulation (Penzo-Mendez and Stanger, 2014); however, at least in *Drosophila*, the evidence for this role is contradictory (Martin et al., 2009). In mammals, the embryo needs to reach a certain cell number before gastrulation can initiate (Snow, 1977), and cell competition is one possible mechanism that could act to fine-tune cell numbers as gastrulation approaches. C-MYC is a key regulator of cellular growth by controlling of *de novo* ribosome biogenesis, transcription and metabolism (Dang, 2012) and the ability of c-MYC to regulate proliferation is key to its role in cell competition. In the early embryo, during cell competition the relative levels of c-MYC are essential to determine winner and loser status. In this system, as well as in the developing heart, the replacement of loser cells by the winners occurs in a phenotypically silent manner (Claveria et al., 2013; Drenckhahn et al., 2008; Villa Del Campo et al., 2014). This silent replacement indicates that cell competition acts to maintain embryo and organ size even when up to 50% of the cells are lost (Drenckhahn et al., 2008). Our findings that loss of *c-Myc* compromises embryo growth indicates that when cell competition is defective embryo size is affected and strengthens the argument that cell competition has a role in the regulation of embryo or organ size.

In conclusion, cell competition is a fitness sensor mechanism that is likely to play important roles in the elimination of defective cells during embryogenesis and the regulation of embryo and organ size during development. Our data indicates that c-MYC plays an essential role in this process as the levels of expression of this protein determine which cells will be eliminated and which will undergo compensatory proliferation. Although it is not clear yet how cells recognise their relative fitness levels our data indicates that differences in metabolism may play a role. Next it will be important to establish at the molecular level how differences in metabolism act on different sensing pathways during cell competition. This information will be key, as it will help establish approaches to therapeutically target competition specifically, without interfering with other cellular surveillance mechanisms.

7. REFERENCES

Agrawal, N., Kango, M., Mishra, A., and Sinha, P. (1995). Neoplastic transformation and aberrant cell-cell interactions in genetic mosaics of lethal(2)giant larvae (lgl), a tumor suppressor gene of Drosophila. Dev Biol *172*, 218-229.

Amoyel, M., and Bach, E.A. (2014). Cell competition: how to eliminate your neighbours. Development *141*, 988-1000.

Andoniadou, C.L., and Martinez-Barbera, J.P. (2013). Developmental mechanisms directing early anterior forebrain specification in vertebrates. Cell Mol Life Sci *70*, 3739-3752.

Arman, E., Haffner-Krausz, R., Chen, Y., Heath, J.K., and Lonai, P. (1998). Targeted disruption of fibroblast growth factor (FGF) receptor 2 suggests a role for FGF signalling in pregastrulation mammalian development. Proc Natl Acad Sci U S A *95*, 5082-5087.

Arnold, S.J., and Robertson, E.J. (2009). Making a commitment: cell lineage allocation and axis patterning in the early mouse embryo. Nat Rev Mol Cell Biol *10*, 91-103.

Artus, J., Panthier, J.J., and Hadjantonakis, A.K. (2010). A role for PDGF signalling in expansion of the extra-embryonic endoderm lineage of the mouse blastocyst. Development *137*, 3361-3372.

Artus, J., Piliszek, A., and Hadjantonakis, A.K. (2011). The primitive endoderm lineage of the mouse blastocyst: sequential transcription factor activation and regulation of differentiation by Sox17. Dev Biol *350*, 393-404.

Baker, N.E. (2011). Cell competition. Curr Biol *21*, R11-15.

Beck, S., Le Good, J.A., Guzman, M., Ben Haim, N., Roy, K., Beermann, F., and Constam, D.B. (2002). Extraembryonic proteases regulate Nodal signalling during gastrulation. Nat Cell Biol *4*, 981-985.

Beddington, R.S.P. (1986). Analysis of tissue fate and prospective potency in the egg cylinder. In Experimental Approaches to Mammalian Embryonic Development, J. Rossant, and R.A. Pedersen, eds. (New York, Cambridge University Press), pp. 121-147.

Ben-Haim, N., Lu, C., Guzman-Ayala, M., Pescatore, L., Mesnard, D., Bischofberger, M., Naef, F., Robertson, E.J., and Constam, D.B. (2006). The nodal precursor acting via activin receptors induces mesoderm by maintaining a source of its convertases and BMP4. Dev Cell *11*, 313-323.

Bessonnard, S., De Mot, L., Gonze, D., Barriol, M., Dennis, C., Goldbeter, A., Dupont, G., and Chazaud, C. (2014). Gata6, Nanog and Erk signalling control cell fate in the inner cell mass through a tristable regulatory network. Development *141*, 3637-3648.

Blackwood, E.M., and Eisenman, R.N. (1991). Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc. Science *251*, 1211-1217.

Boeuf, H., Hauss, C., Graeve, F.D., Baran, N., and Kedinger, C. (1997). Leukemia inhibitory factor-dependent transcriptional activation in embryonic stem cells. J Cell Biol *138*, 1207-1217.

Bohni, R., Riesgo-Escovar, J., Oldham, S., Brogiolo, W., Stocker, H., Andruss, B.F., Beckingham, K., and Hafen, E. (1999). Autonomous control of cell and organ size by CHICO, a Drosophila homolog of vertebrate IRS1-4. Cell *97*, 865-875.

Bolton, H., Graham, S.J., Van der Aa, N., Kumar, P., Theunis, K., Fernandez Gallardo, E., Voet, T., and Zernicka-Goetz, M. (2016). Mouse model of chromosome mosaicism reveals lineage-specific depletion of aneuploid cells and normal developmental potential. Nat Commun *7*, 11165.

Bondar, T., and Medzhitov, R. (2010). p53-mediated hematopoietic stem and progenitor cell competition. Cell Stem Cell *6*, 309-322.
Boroviak, T., Loos, R., Bertone, P., Smith, A., and Nichols, J. (2014). The ability of inner-cell-mass cells to self-renew as embryonic stem cells is acquired following epiblast specification. Nat Cell Biol *16*, 516-528.
Brennan, J., Lu, C.C., Norris, D.P., Rodriguez, T.A., Beddington, R.S., and Robertson, E.J. (2001). Nodal signalling in the epiblast patterns the early mouse embryo. Nature *411*, 965-969.
Brons, I.G., Smithers, L.E., Trotter, M.W., Rugg-Gunn, P., Sun, B., Chuva de Sousa Lopes, S.M., Howlett, S.K., Clarkson, A., Ahrlund-Richter, L., Pedersen, R.A., *et al.* (2007). Derivation of pluripotent epiblast stem cells from mammalian embryos. Nature *448*, 191-195.
Brumby, A.M., and Richardson, H.E. (2003). scribble mutants cooperate with oncogenic Ras or Notch to cause neoplastic overgrowth in Drosophila. EMBO J *22*, 5769-5779.
Burke, R., and Basler, K. (1996). Dpp receptors are autonomously required for cell proliferation in the entire developing Drosophila wing. Development *122*, 2261-2269.
Calarco, P.G., and Brown, E.H. (1969). An ultrastructural and cytological study of preimplantation development of the mouse. J Exp Zool *171*, 253-283.
Cambray, S., Arber, C., Little, G., Dougalis, A.G., de Paola, V., Ungless, M.A., Li, M., and Rodriguez, T.A. (2012). Activin induces cortical interneuron identity and differentiation in embryonic stem cell-derived telencephalic neural precursors. Nat Commun *3*, 841.
Camus, A., Perea-Gomez, A., Moreau, A., and Collignon, J. (2006). Absence of Nodal signalling promotes precocious neural differentiation in the mouse embryo. Dev Biol *295*, 743-755.
Capecchi, M.R. (2005). Gene targeting in mice: functional analysis of the mammalian genome for the twenty-first century. Nat Rev Genet *6*, 507-512.
Cartwright, P., McLean, C., Sheppard, A., Rivett, D., Jones, K., and Dalton, S. (2005). LIF/STAT3 controls ES cell self-renewal and pluripotency by a Myc-dependent mechanism. Development *132*, 885-896.
Casas-Tinto, S., Lolo, F.N., and Moreno, E. (2015). Active JNK-dependent secretion of Drosophila Tyrosyl-tRNA synthetase by loser cells recruits haemocytes during cell competition. Nat Commun *6*, 10022.
Chambers, I., Colby, D., Robertson, M., Nichols, J., Lee, S., Tweedie, S., and Smith, A. (2003). Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell *113*, 643-655.
Chang, C.H., Qiu, J., O'Sullivan, D., Buck, M.D., Noguchi, T., Curtis, J.D., Chen, Q., Gindin, M., Gubin, M.M., van der Windt, G.J., *et al.* (2015). Metabolic Competition in the Tumor Microenvironment Is a Driver of Cancer Progression. Cell *162*, 1229-1241.
Chappell, J., and Dalton, S. (2013). Roles for MYC in the establishment and maintenance of pluripotency. Cold Spring Harb Perspect Med *3*, a014381.
Charron, J., Malynn, B.A., Fisher, P., Stewart, V., Jeannotte, L., Goff, S.P., Robertson, E.J., and Alt, F.W. (1992). Embryonic lethality in mice homozygous for a targeted disruption of the N-myc gene. Genes Dev *6*, 2248-2257.
Chazaud, C., Yamanaka, Y., Pawson, T., and Rossant, J. (2006). Early lineage segregation between epiblast and primitive endoderm in mouse blastocysts through the Grb2-MAPK pathway. Dev Cell *10*, 615-624.

Chen, C.L., Schroeder, M.C., Kango-Singh, M., Tao, C., and Halder, G. (2012). Tumor suppression by cell competition through regulation of the Hippo pathway. Proc Natl Acad Sci U S A *109*, 484-489.
Chen, Y., Li, X., Eswarakumar, V.P., Seger, R., and Lonai, P. (2000). Fibroblast growth factor (FGF) signalling through PI 3-kinase and Akt/PKB is required for embryoid body differentiation. Oncogene *19*, 3750-3756.
Ciruna, B., and Rossant, J. (2001). FGF signalling regulates mesoderm cell fate specification and morphogenetic movement at the primitive streak. Dev Cell *1*, 37-49.
Claveria, C., Giovinazzo, G., Sierra, R., and Torres, M. (2013). Myc-driven endogenous cell competition in the early mammalian embryo. Nature *500*, 39-44.
Cockburn, K., Biechele, S., Garner, J., and Rossant, J. (2013). The Hippo pathway member Nf2 is required for inner cell mass specification. Current Biology.
Cockburn, K., and Rossant, J. (2010). Making the blastocyst: lessons from the mouse. J Clin Invest *120*, 995-1003.
Conacci-Sorrell, M., Ngouenet, C., and Eisenman, R.N. (2010). Myc-nick: a cytoplasmic cleavage product of Myc that promotes alpha-tubulin acetylation and cell differentiation. Cell *142*, 480-493.
Conlon, F.L., Lyons, K.M., Takaesu, N., Barth, K.S., Kispert, A., Herrmann, B., and Robertson, E.J. (1994). A primary requirement for nodal in the formation and maintenance of the primitive streak in the mouse. Development *120*, 1919-1928.
Dang, C.V. (2012). MYC on the path to cancer. Cell *149*, 22-35.
Davis, A.C., Wims, M., Spotts, G.D., Hann, S.R., and Bradley, A. (1993). A null c-myc mutation causes lethality before 10.5 days of gestation in homozygotes and reduced fertility in heterozygous female mice. Genes Dev *7*, 671-682.
de Alboran, I.M., O'Hagan, R.C., Gartner, F., Malynn, B., Davidson, L., Rickert, R., Rajewsky, K., DePinho, R.A., and Alt, F.W. (2001). Analysis of C-MYC function in normal cells via conditional gene-targeted mutation. Immunity *14*, 45-55.
de Beco, S., Ziosi, M., and Johnston, L.A. (2012). New frontiers in cell competitiondagger. Dev Dyn *241*, 831-841.
de la Cova, C., Abril, M., Bellosta, P., Gallant, P., and Johnston, L.A. (2004). Drosophila myc regulates organ size by inducing cell competition. Cell *117*, 107-116.
de la Cova, C., Senoo-Matsuda, N., Ziosi, M., Wu, C., Bellosta, P., Quinzii, C.M., and **Johnston, L.A.** (2014). Supercompetitor Status of Drosophila Myc Cells Requires p53 as a Fitness Sensor to Reprogram Metabolism and Promote Viability. Cell Metabolism *19*, 1-14.
Di-Gregorio, A., Sancho, M., Stuckey, D.W., Crompton, L.A., Godwin, J., Mishina, Y., and Rodriguez, T.A. (2007). BMP signalling inhibits premature neural differentiation in the mouse embryo. Development *134*, 3359-3369.
Diaz, B., and Moreno, E. (2005). The competitive nature of cells. Exp Cell Res *306*, 317-322.
Dietrich, J.E., and Hiiragi, T. (2007). Stochastic patterning in the mouse pre-implantation embryo. Development *134*, 4219-4231.
Ding, J., Yang, L., Yan, Y.T., Chen, A., Desai, N., Wynshaw-Boris, A., and Shen, M.M. (1998). Cripto is required for correct orientation of the anterior-posterior axis in the mouse embryo. Nature *395*, 702-707.
Doble, B.W., and Woodgett, J.R. (2003). GSK-3: tricks of the trade for a multi-tasking kinase. J Cell Sci *116*, 1175-1186.

Drenckhahn, J.D., Schwarz, Q.P., Gray, S., Laskowski, A., Kiriazis, H., Ming, Z., Harvey, R.P., Du, X.J., Thorburn, D.R., and Cox, T.C. (2008). Compensatory growth of healthy cardiac cells in the presence of diseased cells restores tissue homeostasis during heart development. Dev Cell *15*, 521-533.
Dubois, N.C., Adolphe, C., Ehninger, A., Wang, R.A., Robertson, E.J., and Trumpp, A. (2008). Placental rescue reveals a sole requirement for c-Myc in embryonic erythroblast survival and hematopoietic stem cell function. Development *135*, 2455-2465.
Eakin, G.S., and Behringer, R.R. (2003). Tetraploid development in the mouse. Dev Dynam *228*, 751-766.
Eakin, G.S., Hadjantonakis, A.K., Papaioannou, V.E., and Behringer, R.R. (2005). Developmental potential and behavior of tetraploid cells in the mouse embryo. Developmental Biology *288*, 150-159.
Efeyan, A., Zoncu, R., and Sabatini, D.M. (2012). Amino acids and mTORC1: from lysosomes to disease. Trends Mol Med *18*, 524-533.
Eilers, M., and Eisenman, R.N. (2008). Myc's broad reach. Genes Dev *22*, 2755-2766.
Esner, M., Pachernik, J., Hampl, A., and Dvorak, P. (2002). Targeted disruption of fibroblast growth factor receptor-1 blocks maturation of visceral endoderm and cavitation in mouse embryoid bodies. Int J Dev Biol *46*, 817-825.
Evans, M.J., and Kaufman, M.H. (1981). Establishment in culture of pluripotential cells from mouse embryos. Nature *292*, 154-155.
Faunes, F., Hayward, P., Descalzo, S.M., Chatterjee, S.S., Balayo, T., Trott, J., Christoforou, A., Ferrer-Vaquer, A., Hadjantonakis, A.K., Dasgupta, R.*, et al.* (2013). A membrane-associated beta-catenin/Oct4 complex correlates with ground-state pluripotency in mouse embryonic stem cells. Development *140*, 1171-1183.
Flavahan, W.A., Wu, Q., Hitomi, M., Rahim, N., Kim, Y., Sloan, A.E., Weil, R.J., Nakano, I., Sarkaria, J.N., Stringer, B.W.*, et al.* (2013). Brain tumor initiating cells adapt to restricted nutrition through preferential glucose uptake. Nat Neurosci *16*, 1373-1382.
Fleming, T.P., and Pickering, S.J. (1985). Maturation and polarization of the endocytotic system in outside blastomeres during mouse preimplantation development. J Embryol Exp Morphol *89*, 175-208.
Folmes C.D. and Terzic Andre, (2014). Metabolic determinants of embryonic development and stem cell fate
Frankenberg, S., Gerbe, F., Bessonnard, S., Belville, C., Pouchin, P., Bardot, O., and Chazaud, C. (2011). Primitive endoderm differentiates via a three-step mechanism involving Nanog and RTK signalling. Dev Cell *21*, 1005-1013.
Froldi, F., Ziosi, M., Garoia, F., Pession, A., Grzeschik, N.A., Bellosta, P., Strand, D., Richardson, H.E., and Grifoni, D. (2010). The lethal giant larvae tumour suppressor mutation requires dMyc oncoprotein to promote clonal malignancy. BMC Biol *8*, 33.
Fujiwara, T., Dehart, D.B., Sulik, K.K., and Hogan, B.L. (2002). Distinct requirements for extra-embryonic and embryonic bone morphogenetic protein 4 in the formation of the node and primitive streak and coordination of left-right asymmetry in the mouse. Development *129*, 4685-4696.
Fujiwara, T., Dunn, N.R., and Hogan, B.L. (2001). Bone morphogenetic protein 4 in the extraembryonic mesoderm is required for allantois development and the localization and survival of primordial germ cells in the mouse. Proc Natl Acad Sci U S A *98*, 13739-13744.

Galvin, K.E., Travis, E.D., Yee, D., Magnuson, T., and Vivian, J.L. (2010). Nodal signalling regulates the bone morphogenic protein pluripotency pathway in mouse embryonic stem cells. J Biol Chem *285*, 19747-19756.
Garbutt, C.L., Chisholm, J.C., and Johnson, M.H. (1987). The establishment of the embryonic-abembryonic axis in the mouse embryo. Development *100*, 125-134.
Gateff, E. (1978). Malignant neoplasms of genetic origin in Drosophila melanogaster. Science *200*, 1448-1459.
Grabarek, J.B., Zyzynska, K., Saiz, N., Piliszek, A., Frankenberg, S., Nichols, J., Hadjantonakis, A.K., and Plusa, B. (2012). Differential plasticity of epiblast and primitive endoderm precursors within the ICM of the early mouse embryo. Development *139*, 129-139.
Greco, E., Minasi, M.G., and Fiorentino, F. (2015). Healthy Babies after Intrauterine Transfer of Mosaic Aneuploid Blastocysts. N Engl J Med *373*, 2089-2090.
Gregory, M.A., Qi, Y., and Hann, S.R. (2003). Phosphorylation by glycogen synthase kinase-3 controls c-myc proteolysis and subnuclear localization. J Biol Chem *278*, 51606-51612.
Gritsman, K., Zhang, J., Cheng, S., Heckscher, E., Talbot, W.S., and Schier, A.F. (1999). The EGF-CFC protein one-eyed pinhead is essential for nodal signalling. Cell *97*, 121-132.
Guo, X., and Wang, X.F. (2009). Signalling cross-talk between TGF-beta/BMP and other pathways. Cell Res *19*, 71-88.
Habib, S.J., Chen, B.C., Tsai, F.C., Anastassiadis, K., Meyer, T., Betzig, E., and Nusse, R. (2013). A localized Wnt signal orients asymmetric stem cell division in vitro. Science *339*, 1445-1448.
Hacohen, N., Kramer, S., Sutherland, D., Hiromi, Y., and Krasnow, M.A. (1998). sprouty encodes a novel antagonist of FGF signalling that patterns apical branching of the Drosophila airways. Cell *92*, 253-263.
Hall, J., Guo, G., Wray, J., Eyres, I., Nichols, J., Grotewold, L., Morfopoulou, S., Humphreys, P., Mansfield, W., Walker, R., *et al.* (2009). Oct4 and LIF/Stat3 additively induce Kruppel factors to sustain embryonic stem cell self-renewal. Cell Stem Cell *5*, 597-609.
Hamilton, W.B., and Brickman, J.M. (2014). Erk signalling suppresses embryonic stem cell self-renewal to specify endoderm. Cell Rep *9*, 2056-2070.
Hatton, K.S., Mahon, K., Chin, L., Chiu, F.C., Lee, H.W., Peng, D., Morgenbesser, S.D., Horner, J., and DePinho, R.A. (1996). Expression and activity of L-Myc in normal mouse development. Mol Cell Biol *16*, 1794-1804.
Hayashi, S., Lewis, P., Pevny, L., and McMahon, A.P. (2002). Efficient gene modulation in mouse epiblast using a Sox2Cre transgenic mouse strain. Mech Dev *119 Suppl 1*, S97-S101.
Heldin, C.H., Miyazono, K., and ten Dijke, P. (1997). TGF-beta signalling from cell membrane to nucleus through SMAD proteins. Nature *390*, 465-471.
Henery, C.C., Bard, J.B.L., and Kaufman, M.H. (1992). Tetraploidy in mice, embryonic cell number, and the grain of the developmental map. Dev Biol *152*, 233-241.
Herkert, B., and Eilers, M. (2010). Transcriptional repression: the dark side of myc. Genes Cancer *1*, 580-586.
Heyer, B.S., MacAuley, A., Behrendtsen, O., and Werb, Z. (2000). Hypersensitivity to DNA damage leads to increased apoptosis during early mouse development. Genes Dev *14*, 2072-2084.

Hirate, Y., Hirahara, S., Inoue, K., Suzuki, A., Alarcon, V.B., Akimoto, K., Hirai, T., Hara, T., Adachi, M., Chida, K.*, et al.* (2013). Polarity-dependent distribution of angiomotin localizes Hippo signalling in preimplantation embryos. Current Biology.
Hogan, B., Beddington, R., Constantini, F., and Lacy, E. (1994). Manipulating the mouse embryo (Cold Sprin harbor. New York, Cold Spring Harbor Laboratory Press).
Hogan, C., Dupre-Crochet, S., Norman, M., Kajita, M., Zimmermann, C., Pelling, A.E., Piddini, E., Baena-Lopez, L.A., Vincent, J.P., Itoh, Y.*, et al.* (2009). Characterization of the interface between normal and transformed epithelial cells. Nat Cell Biol *11*, 460-467.
Hollnagel, A., Oehlmann, V., Heymer, J., Ruther, U., and Nordheim, A. (1999). Id genes are direct targets of bone morphogenetic protein induction in embryonic stem cells. J Biol Chem *274*, 19838-19845.
Home, P., Ray, S., Dutta, D., Bronshteyn, I., Larson, M., and Paul, S. (2009). GATA3 is selectively expressed in the trophectoderm of peri-implantation embryo and directly regulates Cdx2 gene expression. J Biol Chem *284*, 28729-28737.
Houghton, F.D., Thompson, J.G., Kennedy, C.J., and Leese, H.J. (1996). Oxygen consumption and energy metabolism of the early mouse embryo. Mol Reprod Dev *44*, 476-485.
Huang, Y., Osorno, R., Tsakiridis, A., and Wilson, V. (2012). In Vivo differentiation potential of epiblast stem cells revealed by chimeric embryo formation. Cell Rep *2*, 1571-1578.
Huelsken, J., Vogel, R., Brinkmann, V., Erdmann, B., Birchmeier, C., and Birchmeier, W. (2000). Requirement for beta-catenin in anterior-posterior axis formation in mice. J Cell Biol *148*, 567-578.
Igaki, T., Pagliarini, R.A., and Xu, T. (2006). Loss of cell polarity drives tumor growth and invasion through JNK activation in Drosophila. Curr Biol *16*, 1139-1146.
Issigonis, M., Tulina, N., de Cuevas, M., Brawley, C., Sandler, L., and Matunis, E. (2009). JAK-STAT signal inhibition regulates competition in the Drosophila testis stem cell niche. Science *326*, 153-156.
Itoh, N., and Ornitz, D.M. (2011). Fibroblast growth factors: from molecular evolution to roles in development, metabolism and disease. J Biochem *149*, 121-130.
Iwasaki, S., Iguchi, M., Watanabe, K., Hoshino, R., Tsujimoto, M., and Kohno, M. (1999). Specific activation of the p38 mitogen-activated protein kinase signalling pathway and induction of neurite outgrowth in PC12 cells by bone morphogenetic protein-2. J Biol Chem *274*, 26503-26510.
Jaenisch, R., and Young, R. (2008). Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming. Cell *132*, 567-582.
Jin, Z., Kirilly, D., Weng, C., Kawase, E., Song, X., Smith, S., Schwartz, J., and Xie, T. (2008). Differentiation-defective stem cells outcompete normal stem cells for niche occupancy in the Drosophila ovary. Cell Stem Cell *2*, 39-49.
Johansson, B.M., and Wiles, M.V. (1995). Evidence for involvement of activin A and bone morphogenetic protein 4 in mammalian mesoderm and hematopoietic development. Mol Cell Biol *15*, 141-151.
Johnson, M.H., and McConnell, J.M. (2004). Lineage allocation and cell polarity during mouse embryogenesis. Semin Cell Dev Biol *15*, 583-597.
Johnson, M.H., and Ziomek, C.A. (1981). The foundation of two distinct cell lineages within the mouse morula. Cell *24*, 71-80.

Johnson, M.H., and Ziomek, C.A. (1983). Cell interactions influence the fate of mouse blastomeres undergoing the transition from the 16- to the 32-cell stage. Dev Biol *95*, 211-218.
Johnston, L.A. (2009). Competitive interactions between cells: death, growth, and geography. Science *324*, 1679-1682.
Jornvall, H., Reissmann, E., Andersson, O., Mehrkash, M., and Ibanez, C.F. (2004). ALK7, a receptor for nodal, is dispensable for embryogenesis and left-right patterning in the mouse. Mol Cell Biol *24*, 9383-9389.
Kajita, M., Hogan, C., Harris, A.R., Dupre-Crochet, S., Itasaki, N., Kawakami, K., Charras, G., Tada, M., and Fujita, Y. (2010). Interaction with surrounding normal epithelial cells influences signalling pathways and behaviour of Src-transformed cells. J Cell Sci *123*, 171-180.
Kale, A., Li, W., Lee, C.H., and Baker, N.E. (2015). Apoptotic mechanisms during competition of ribosomal protein mutant cells: roles of the initiator caspases Dronc and Dream/Strica. Cell Death Differ *22*, 1300-1312.
Kalousek, D.K., Howard-Peebles, P.N., Olson, S.B., Barrett, I.J., Dorfmann, A., Black, S.H., Schulman, J.D., and Wilson, R.D. (1991). Confirmation of CVS mosaicism in term placentae and high frequency of intrauterine growth retardation association with confined placental mosaicism. Prenat Diagn *11*, 743-750.
Kang, M., Piliszek, A., Artus, J., and Hadjantonakis, A.K. (2013). FGF4 is required for lineage restriction and salt-and-pepper distribution of primitive endoderm factors but not their initial expression in the mouse. Development *140*, 267-279.
Kaufman, M.H. (1991). Histochemical identification of primordial germ cells and differentiation of the gonads in homozygous tetraploid mouse embryos. J Anat *179*, 169-181.
Kaufman, M.H., and Webb, S. (1990). Postimplnataton development of tetraploid mouse embryos produced by electrofusion. Development *110*, 1121-1132.
Kemp, C., Willems, E., Abdo, S., Lambiv, L., and Leyns, L. (2005). Expression of all Wnt genes and their secreted antagonists during mouse blastocyst and postimplantation development. Dev Dyn *233*, 1064-1075.
Kimura, N., Matsuo, R., Shibuya, H., Nakashima, K., and Taga, T. (2000). BMP2-induced apoptosis is mediated by activation of the TAK1-p38 kinase pathway that is negatively regulated by Smad6. J Biol Chem *275*, 17647-17652.
King, A., Selak, M.A., and Gottlieb, E. (2006). Succinate dehydrogenase and fumarate hydratase: linking mitochondrial dysfunction and cancer. Oncogene *25*, 4675-4682.
Knoepfler, P.S., Cheng, P.F., and Eisenman, R.N. (2002). N-myc is essential during neurogenesis for the rapid expansion of progenitor cell populations and the inhibition of neuronal differentiation. Genes Dev *16*, 2699-2712.
Kojima, Y., Kaufman-Francis, K., Studdert, J.B., Steiner, K.A., Power, M.D., Loebel, D.A., Jones, V., Hor, A., de Alencastro, G., Logan, G.J., *et al.* (2014). The transcriptional and functional properties of mouse epiblast stem cells resemble the anterior primitive streak. Cell Stem Cell *14*, 107-120.
Kolahgar, G., Suijkerbuijk, S.J., Kucinski, I., Poirier, E.Z., Mansour, S., Simons, B.D., and Piddini, E. (2015). Cell Competition Modifies Adult Stem Cell and Tissue Population Dynamics in a JAK-STAT-Dependent Manner. Dev Cell *34*, 297-309.
Kowanetz, M., Valcourt, U., Bergstrom, R., Heldin, C.H., and Moustakas, A. (2004). Id2 and Id3 define the potency of cell proliferation and differentiation responses to transforming growth factor beta and bone morphogenetic protein. Mol Cell Biol *24*, 4241-4254.

Kunath, T., Saba-El-Leil, M.K., Almousailleakh, M., Wray, J., Meloche, S., and Smith, A. (2007). FGF stimulation of the Erk1/2 signalling cascade triggers transition of pluripotent embryonic stem cells from self-renewal to lineage commitment. Development *134*, 2895-2902.
Kwon, G.S., Viotti, M., and Hadjantonakis, A.K. (2008). The endoderm of the mouse embryo arises by dynamic widespread intercalation of embryonic and extraembryonic lineages. Dev Cell *15*, 509-520.
Labosky, P.A., Barlow, D.P., and Hogan, B.L. (1994). Mouse embryonic germ (EG) cell lines: transmission through the germline and differences in the methylation imprint of insulin-like growth factor 2 receptor (Igf2r) gene compared with embryonic stem (ES) cell lines. Development *120*, 3197-3204.
Lanner, F., and Rossant, J. (2010). The role of FGF/Erk signalling in pluripotent cells. Development *137*, 3351-3360.
Laplante, M., and Sabatini, D.M. (2012). mTOR signalling in growth control and disease. Cell *149*, 274-293.
Lawson, K.A., Dunn, N.R., Roelen, B.A., Zeinstra, L.M., Davis, A.M., Wright, C.V., Korving, J.P., and Hogan, B.L. (1999). Bmp4 is required for the generation of primordial germ cells in the mouse embryo. Genes Dev *13*, 424-436.
Leitch, H.G., Nichols, J., Humphreys, P., Mulas, C., Martello, G., Lee, C., Jones, K., Surani, M.A., and Smith, A. (2013). Rebuilding pluripotency from primordial germ cells. Stem Cell Reports *1*, 66-78.
Leitch, H.G., and Smith, A. (2013). The mammalian germline as a pluripotency cycle. Development *140*, 2495-2501.
Levayer, R., Dupont, C., and Moreno, E. (2016). Tissue Crowding Induces Caspase-Dependent Competition for Space. Curr Biol *26*, 670-677.
Levayer, R., Hauert, B., and Moreno, E. (2015). Cell mixing induced by myc is required for competitive tissue invasion and destruction. Nature *524*, 476-480.
Levayer, R., and Moreno, E. (2016). How to be in a good shape? The influence of clone morphology on cell competition. Commun Integr Biol *9*, e1102806.
Li, W., and Baker, N.E. (2007). Engulfment is required for cell competition. Cell *129*, 1215-1225.
Li, X., Chen, Y., Scheele, S., Arman, E., Haffner-Krausz, R., Ekblom, P., and Lonai, P. (2001). Fibroblast growth factor signalling and basement membrane assembly are connected during epithelial morphogenesis of the embryoid body. J Cell Biol *153*, 811-822.
Lin, C.H., Jackson, A.L., Guo, J., Linsley, P.S., and Eisenman, R.N. (2009). Myc-regulated microRNAs attenuate embryonic stem cell differentiation. EMBO J *28*, 3157-3170.
Liu, P., Wakamiya, M., Shea, M.J., Albrecht, U., Behringer, R.R., and Bradley, A. (1999). Requirement for Wnt3 in vertebrate axis formation. Nat Genet *22*, 361-365.
Logan, C.Y., and Nusse, R. (2004). The Wnt signalling pathway in development and disease. Annu Rev Cell Dev Biol *20*, 781-810.
Lolo, F.N., Casas-Tinto, S., and Moreno, E. (2012). Cell competition time line: winners kill losers, which are extruded and engulfed by hemocytes. Cell Rep *2*, 526-539.
Lopez-Rovira, T., Chalaux, E., Massague, J., Rosa, J.L., and Ventura, F. (2002). Direct binding of Smad1 and Smad4 to two distinct motifs mediates bone morphogenetic protein-specific transcriptional activation of Id1 gene. J Biol Chem *277*, 3176-3185.

Mamada, H., Sato, T., Ota, M., and Sasaki, H. (2015). Cell competition in mouse NIH3T3 embryonic fibroblasts is controlled by the activity of Tead family proteins and Myc. J Cell Sci *128*, 790-803.
Manejwala, F.M., Cragoe, E.J., Jr., and Schultz, R.M. (1989). Blastocoel expansion in the preimplantation mouse embryo: role of extracellular sodium and chloride and possible apical routes of their entry. Dev Biol *133*, 210-220.
Manova, K., Tomihara-Newberger, C., Wang, S., Godelman, A., Kalantry, S., Witty-Blease, K., De Leon, V., Chen, W.S., Lacy, E., and Bachvarova, R.F. (1998). Apoptosis in mouse embryos: elevated levels in pregastrulae and in the distal anterior region of gastrulae of normal and mutant mice. Dev Dyn *213*, 293-308.
Marinari, E., Mehonic, A., Curran, S., Gale, J., Duke, T., and Baum, B. (2012). Live-cell delamination counterbalances epithelial growth to limit tissue overcrowding. Nature *484*, 542-545.
Martello, G., Bertone, P., and Smith, A. (2013). Identification of the missing pluripotency mediator downstream of leukaemia inhibitory factor. EMBO J *32*, 2561-2574.
Martello, G., and Smith, A. (2014). The nature of embryonic stem cells. Annu Rev Cell Dev Biol *30*, 647-675.
Martin, F.A., Herrera, S.C., and Morata, G. (2009). Cell competition, growth and size control in the Drosophila wing imaginal disc. Development *136*, 3747-3756.
Martin, G.R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acd Sci, USA *78*, 7634-7638.
Martins, V.C., Busch, K., Juraeva, D., Blum, C., Ludwig, C., Rasche, V., Lasitschka, F., Mastitsky, S.E., Brors, B., Hielscher, T., *et al.* (2014). Cell competition is a tumour suppressor mechanism in the thymus. Nature *509*, 465-470.
Matsuda, T., Nakamura, T., Nakao, K., Arai, T., Katsuki, M., Heike, T., and Yokota, T. (1999). STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells. Embo J *18*, 4261-4269.
Matsui, Y., Zsebo, K., and Hogan, B.L. (1992a). Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. Cell *70*, 841-847.
Matsui, Y., Zsebo, K., and Hogan, B.L.M. (1992b). Derivation of pluripotent embryonic stem cells from murine primordial germ cells in culture. Cell *70*, 841-847.
Menendez, J., Perez-Garijo, A., Calleja, M., and Morata, G. (2010). A tumor-suppressing mechanism in Drosophila involving cell competition and the Hippo pathway. Proc Natl Acad Sci U S A *107*, 14651-14656.
Merino, M.M., Rhiner, C., Lopez-Gay, J.M., Buechel, D., Hauert, B., and Moreno, E. (2015). Elimination of unfit cells maintains tissue health and prolongs lifespan. Cell *160*, 461-476.
Merino, M.M., Rhiner, C., Portela, M., and Moreno, E. (2013). "Fitness fingerprints" mediate physiological culling of unwanted neurons in Drosophila. Curr Biol *23*, 1300-1309.
Merrill, B.J. (2012). Wnt pathway regulation of embryonic stem cell self-renewal. Cold Spring Harb Perspect Biol *4*, a007971.
Merrill, B.J., Pasolli, H.A., Polak, L., Rendl, M., Garcia-Garcia, M.J., Anderson, K.V., and Fuchs, E. (2004). Tcf3: a transcriptional regulator of axis induction in the early embryo. Development *131*, 263-274.

Mesnard, D., Guzman-Ayala, M., and Constam, D.B. (2006). Nodal specifies embryonic visceral endoderm and sustains pluripotent cells in the epiblast before overt axial patterning. Development *133*, 2497-2505.
Meyer, S.N., Amoyel, M., Bergantinos, C., de la Cova, C., Schertel, C., Basler, K., and Johnston, L.A. (2014). An ancient defense system eliminates unfit cells from developing tissues during cell competition. Science *346*, 1258236.
Mishina, Y. (2003). Function of bone morphogenetic protein signalling during mouse development. Front Biosci *8*, d855-869.
Mishina, Y., Suzuki, A., Ueno, N., and Behringer, R.R. (1995). Bmpr encodes a type I bone morphogenetic protein receptor that is essential for gastrulation during mouse embryogenesis. Genes Dev *9*, 3027-3037.
Mitsui, K., Tokuzawa, Y., Itoh, H., Segawa, K., Murakami, M., Takahashi, K., Maruyama, **M., Maeda, M., and Yamanaka, S.** (2003). The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell *113*, 631-642.
Miyazono, K., Maeda, S., and Imamura, T. (2005). BMP receptor signalling: transcriptional targets, regulation of signals, and signalling cross-talk. Cytokine Growth Factor Rev *16*, 251-263.
Miyazono, K., and Miyazawa, K. (2002). Id: a target of BMP signalling. Sci STKE *2002*, PE40.
Moens, C.B., Auerbach, A.B., Conlon, R.A., Joyner, A.L., and Rossant, J. (1992). A targeted mutation reveals a role for N-myc in branching morphogenesis in the embryonic mouse lung. Genes Dev *6*, 691-704.
Morata, G., and Ripoll, P. (1975). Minutes: mutants of drosophila autonomously affecting cell division rate. Dev Biol *42*, 211-221.
Moreno, E., and Basler, K. (2004). dMyc transforms cells into super-competitors. Cell *117*, 117-129.
Moreno, E., Basler, K., and Morata, G. (2002). Cells compete for decapentaplegic survival factor to prevent apoptosis in Drosophila wing development. Nature *416*, 755-759.
Morgani, S.M., Canham, M.A., Nichols, J., Sharov, A.A., Migueles, R.P., Ko, M.S., and Brickman, J.M. (2013). Totipotent embryonic stem cells arise in ground-state culture conditions. Cell Rep *3*, 1945-1957.
Motosugi, N., Bauer, T., Polanski, Z., Solter, D., and Hiiragi, T. (2005). Polarity of the mouse embryo is established at blastocyst and is not prepatterned. Genes Dev *19*, 1081-1092.
Mukhopadhyay, M., Shtrom, S., Rodriguez-Esteban, C., Chen, L., Tsukui, T., Gomer, L., Dorward, D.W., Glinka, A., Grinberg, A., Huang, S.P., *et al.* (2001). Dickkopf1 is required for embryonic head induction and limb morphogenesis in the mouse. Dev Cell *1*, 423-434.
Murry, C.E., and Keller, G. (2008). Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development. Cell *132*, 661-680.
Nakashima, K., Takizawa, T., Ochiai, W., Yanagisawa, M., Hisatsune, T., Nakafuku, M., Miyazono, K., Kishimoto, T., Kageyama, R., and Taga, T. (2001). BMP2-mediated alteration in the developmental pathway of fetal mouse brain cells from neurogenesis to astrocytogenesis. Proc Natl Acad Sci U S A *98*, 5868-5873.
Narazaki, M., Witthuhn, B.A., Yoshida, K., Silvennoinen, O., Yasukawa, K., Ihle, J.N., Kishimoto, T., and Taga, T. (1994). Activation of JAK2 kinase mediated by the interleukin 6 signal transducer gp130. Proc Natl Acad Sci U S A *91*, 2285-2289.

Neto-Silva, R.M., de Beco, S., and Johnston, L.A. (2010). Evidence for a growth-stabilizing regulatory feedback mechanism between Myc and Yorkie, the Drosophila homolog of Yap. Dev Cell *19*, 507-520.
Nichols, J., Jones, K., Phillips, J.M., Newland, S.A., Roode, M., Mansfield, W., Smith, A., and Cooke, A. (2009a). Validated germline-competent embryonic stem cell lines from nonobese diabetic mice. Nat Med *15*, 814-818.
Nichols, J., Silva, J., Roode, M., and Smith, A. (2009b). Suppression of Erk signalling promotes ground state pluripotency in the mouse embryo. Development *136*, 3215-3222.
Nichols, J., and Smith, A. (2009). Naive and primed pluripotent states. Cell Stem Cell *4*, 487-492.
Nichols, J., Zevnik, B., Anastassiadis, K., Niwa, H., Klewe-Nebenius, D., Chambers, I., Scholer, H., and Smith, A. (1998). Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell *95*, 379-391.
Nishikawa, S., Jakt, L.M., and Era, T. (2007). Embryonic stem-cell culture as a tool for developmental cell biology. Nat Rev Mol Cell Biol *8*, 502-507.
Nishioka, N., Inoue, K., Adachi, K., Kiyonari, H., Ota, M., Ralston, A., Yabuta, N., Hirahara, S., Stephenson, R.O., Ogonuki, N.*, et al.* (2009). The Hippo signalling pathway components Lats and Yap pattern Tead4 activity to distinguish mouse trophectoderm from inner cell mass. Dev Cell *16*, 398-410.
Nishioka, N., Yamamoto, S., Kiyonari, H., Sato, H., Sawada, A., Ota, M., Nakao, K., and Sasaki, H. (2008). Tead4 is required for specification of trophectoderm in pre-implantation mouse embryos. Mech Dev *125*, 270-283.
Nishisho, I., Nakamura, Y., Miyoshi, Y., Miki, Y., Ando, H., Horii, A., Koyama, K., Utsunomiya, J., Baba, S., Hedge, P.*, et al.* (1991). Mutations of Chromosome-5q21 Genes in Fap and Colorectal-Cancer Patients. Science *253*, 665-669.
Niwa, H., Burdon, T., Chambers, I., and Smith, A. (1998). Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. Genes Dev *12*, 2048-2060.
Niwa, H., Toyooka, Y., Shimosato, D., Strumpf, D., Takahashi, K., Yagi, R., and Rossant, J. (2005). Interaction between Oct3/4 and Cdx2 determines trophectoderm differentiation. Cell *123*, 917-929.
Nohe, A., Keating, E., Knaus, P., and Petersen, N.O. (2004). Signal transduction of bone morphogenetic protein receptors. Cell Signal *16*, 291-299.
Norman, M., Wisniewska, K.A., Lawrenson, K., Garcia-Miranda, P., Tada, M., Kajita, M., Mano, H., Ishikawa, S., Ikegawa, M., Shimada, T.*, et al.* (2012). Loss of Scribble causes cell competition in mammalian cells. J Cell Sci *125*, 59-66.
Norton, J.D. (2000). ID helix-loop-helix proteins in cell growth, differentiation and tumorigenesis. J Cell Sci *113 (Pt 22)*, 3897-3905.
Norton, J.D., Deed, R.W., Craggs, G., and Sablitzky, F. (1998). Id helix-loop-helix proteins in cell growth and differentiation. Trends Cell Biol *8*, 58-65.
Oertel, M., Menthena, A., Dabeva, M.D., and Shafritz, D.A. (2006). Cell competition leads to a high level of normal liver reconstitution by transplanted fetal liver stem/progenitor cells. Gastroenterology *130*, 507-520; quiz 590.
Ogata, T., Wozney, J.M., Benezra, R., and Noda, M. (1993). Bone morphogenetic protein 2 transiently enhances expression of a gene, Id (inhibitor of differentiation), encoding a helix-loop-helix molecule in osteoblast-like cells. Proc Natl Acad Sci U S A *90*, 9219-9222.

Ohsawa, S., Sato, Y., Enomoto, M., Nakamura, M., Betsumiya, A., and Igaki, T. (2012). Mitochondrial defect drives non-autonomous tumour progression through Hippo signalling in Drosophila. Nature *490*, 547-551.
Ohsawa, S., Sugimura, K., Takino, K., Xu, T., Miyawaki, A., and Igaki, T. (2011). Elimination of oncogenic neighbors by JNK-mediated engulfment in Drosophila. Dev Cell *20*, 315-328.
Oliver, E.R., Saunders, T.L., Tarle, S.A., and Glaser, T. (2004). Ribosomal protein L24 defect in belly spot and tail (Bst), a mouse Minute. Development *131*, 3907-3920.
Pagliarini, R.A., and Xu, T. (2003). A genetic screen in Drosophila for metastatic behavior. Science *302*, 1227-1231.
Patel, S., Doble, B., and Woodgett, J.R. (2004). Glycogen synthase kinase-3 in insulin and Wnt signalling: a double-edged sword? Biochem Soc Trans *32*, 803-808.
Penzo-Mendez, A.I., Chen, Y.J., Li, J., Witze, E.S., and Stanger, B.Z. (2015). Spontaneous Cell Competition in Immortalized Mammalian Cell Lines. PLoS One *10*, e0132437.
Penzo-Mendez, A.I., and Stanger, B.Z. (2014). Cell competition in vertebrate organ size regulation. Wiley Interdiscip Rev Dev Biol *3*, 419-427.
Perea-Gomez, A., Vella, F.D., Shawlot, W., Oulad-Abdelghani, M., Chazaud, C., Meno, C., Pfister, V., Chen, L., Robertson, E., Hamada, H., *et al.* (2002). Nodal antagonists in the anterior visceral endoderm prevent the formation of multiple primitive streaks. Dev Cell *3*, 745-756.
Pernaute, B., Spruce, T., Smith, K.M., Sanchez-Nieto, J.M., Cobb, B.S., and T.A., R. (2014). MicroRNAs control the apoptotic threshold in primed pluripotent stem cells through regulation of BIM. Genes & Development *28*, 1873-1878.
Plusa, B., Piliszek, A., Frankenberg, S., Artus, J., and Hadjantonakis, A.K. (2008). Distinct sequential cell behaviours direct primitive endoderm formation in the mouse blastocyst. Development *135*, 3081-3091.
Portela, M., Casas-Tinto, S., Rhiner, C., Lopez-Gay, J.M., Dominguez, O., Soldini, D., and Moreno, E. (2010). Drosophila SPARC is a self-protective signal expressed by loser cells during cell competition. Dev Cell *19*, 562-573.
Qi, X., Li, T.G., Hao, J., Hu, J., Wang, J., Simmons, H., Miura, S., Mishina, Y., and Zhao, G.Q. (2004). BMP4 supports self-renewal of embryonic stem cells by inhibiting mitogen-activated protein kinase pathways. Proc Natl Acad Sci U S A *101*, 6027-6032.
Rahl, P.B., Lin, C.Y., Seila, A.C., Flynn, R.A., McCuine, S., Burge, C.B., Sharp, P.A., and Young, R.A. (2010). c-Myc regulates transcriptional pause release. Cell *141*, 432-445.
Ralston, A., Cox, B.J., Nishioka, N., Sasaki, H., Chea, E., Rugg-Gunn, P., Guo, G., Robson, P., Draper, J.S., and Rossant, J. (2010). Gata3 regulates trophoblast development downstream of Tead4 and in parallel to Cdx2. Development *137*, 395-403.
Ralston, A., and Rossant, J. (2008). Cdx2 acts downstream of cell polarization to cell-autonomously promote trophectoderm fate in the early mouse embryo. Dev Biol *313*, 614-629.
Rayon, T., Menchero, S., Nieto, A., Xenopoulos, P., Crespo, M., Cockburn, K., Canon, S., Sasaki, H., Hadjantonakis, A.K., de la Pompa, J.L., *et al.* (2014). Notch and hippo converge on Cdx2 to specify the trophectoderm lineage in the mouse blastocyst. Dev Cell *30*, 410-422.
Resnick, J.L., Bixler, L.S., Cheng, L., and Donovan, P.J. (1992). Long-term proliferation of mouse primordial germ cells in culture. Nature *359*, 550-551.

Rhiner, C., Diaz, B., Portela, M., Poyatos, J.F., Fernandez-Ruiz, I., Lopez-Gay, J.M., Gerlitz, O., and Moreno, E. (2009). Persistent competition among stem cells and their daughters in the Drosophila ovary germline niche. Development *136*, 995-1006.
Rhiner, C., Lopez-Gay, J.M., Soldini, D., Casas-Tinto, S., Martin, F.A., Lombardia, L., and **Moreno, E.** (2010). Flower forms an extracellular code that reveals the fitness of a cell to its neighbors in Drosophila. Dev Cell *18*, 985-998.
Robertson, E.J. (2014). Dose-dependent Nodal/Smad signals pattern the early mouse embryo. Semin Cell Dev Biol *32*, 73-79.
Robertson, E.J., Bradley, A., Kuehn, M., and Evans, M. (1986). Germ-line transmission of genes introduced into cultured pluripotential cells by retroviral vectors. Nature *323*, 445-448.
Rodrigues, A.B., Zoranovic, T., Ayala-Camargo, A., Grewal, S., Reyes-Robles, T., Krasny, M., Wu, D.C., Johnston, L.A., and Bach, E.A. (2012). Activated STAT regulates growth and induces competitive interactions independently of Myc, Yorkie, Wingless and ribosome biogenesis. Development *139*, 4051-4061.
Rodriguez, T.A., Srinivas, S., Clements, M.P., Smith, J.C., and Beddington, R.S. (2005). Induction and migration of the anterior visceral endoderm is regulated by the extra-embryonic ectoderm. Development *132*, 2513-2520.
Roelen, B.A., Goumans, M.J., van Rooijen, M.A., and Mummery, C.L. (1997). Differential expression of BMP receptors in early mouse development. Int J Dev Biol *41*, 541-549.
Rossant, J., and Tam, P.P. (2009). Blastocyst lineage formation, early embryonic asymmetries and axis patterning in the mouse. Development *136*, 701-713.
Saiz Nestor, Berenika Plusa, Anna-Katerina Hadjantonakis (2015) Single cells get together: High-resolution approaches to study the dynamics of early mouse development
Sancho, M., Di-Gregorio, A., George, N., Pozzi, S., Sanchez, J.M., Pernaute, B., and Rodriguez, T.A. (2013). Competitive interactions eliminate unfit embryonic stem cells at the onset of differentiation. Dev Cell *26*, 19-30.
Sancho, M., and Rodriguez, T.A. (2014). Selecting for fitness in mammalian development. Cell Cycle *13*, 9-10.
Sato, N., Meijer, L., Skaltsounis, L., Greengard, P., and Brivanlou, A.H. (2004). Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signalling by a pharmacological GSK-3-specific inhibitor. Nat Med *10*, 55-63.
Schier, A.F., and Shen, M.M. (2000). Nodal signalling in vertebrate development. Nature *403*, 385-389.
Schrode, N., Saiz, N., Di Talia, S., and Hadjantonakis, A.K. (2014). GATA6 levels modulate primitive endoderm cell fate choice and timing in the mouse blastocyst. Dev Cell *29*, 454-467.
Schrode, N., Xenopoulos, P., Piliszek, A., Frankenberg, S., Plusa, B., and Hadjantonakis, A.K. (2013). Anatomy of a blastocyst: cell behaviors driving cell fate choice and morphogenesis in the early mouse embryo. Genesis *51*, 219-233.
Scognamiglio, R., Cabezas-Wallscheid, N., Thier, M.C., Altamura, S., Reyes, A., Prendergast, A.M., Baumgartner, D., Carnevalli, L.S., Atzberger, A., Haas, S.*, et al.* (2016). Myc Depletion Induces a Pluripotent Dormant State Mimicking Diapause. Cell *164*, 668-680.

Sears, R., Nuckolls, F., Haura, E., Taya, Y., Tamai, K., and Nevins, J.R. (2000). Multiple Ras-dependent phosphorylation pathways regulate Myc protein stability. Genes Dev *14*, 2501-2514.
Senoo-Matsuda, N., and Johnston, L.A. (2007). Soluble factors mediate competitive and cooperative interactions between cells expressing different levels of Drosophila Myc. Proc Natl Acad Sci U S A *104*, 18543-18548.
Sheng, X.R., Brawley, C.M., and Matunis, E.L. (2009). Dedifferentiating spermatogonia outcompete somatic stem cells for niche occupancy in the Drosophila testis. Cell Stem Cell *5*, 191-203.
Shi, Y., and Massague, J. (2003). Mechanisms of TGF-beta signalling from cell membrane to the nucleus. Cell *113*, 685-700.
Shibuya, H., Iwata, H., Masuyama, N., Gotoh, Y., Yamaguchi, K., Irie, K., Matsumoto, K., Nishida, E., and Ueno, N. (1998). Role of TAK1 and TAB1 in BMP signalling in early Xenopus development. Embo J *17*, 1019-1028.
Shraiman, B.I. (2005). Mechanical feedback as a possible regulator of tissue growth. Proc Natl Acad Sci U S A *102*, 3318-3323.
Siegel, J.J., and Amon, A. (2012). New insights into the troubles of aneuploidy. Annu Rev Cell Dev Biol *28*, 189-214.
Silva J. and Smith A. (2008). Capturing Pluripotency. Cell *132*, 532-536
Singh, V.K., Kalsan, M., Kumar, N., Saini, A., and Chandra, R. (2015). Induced pluripotent stem cells: applications in regenerative medicine, disease modeling, and drug discovery. Front Cell Dev Biol *3*, 2.
Smith, A.G., Heath, J.K., Donaldson, D.D., Wong, G.G., Moreau, J., Stahl, M., and Rogers, D. (1988). Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. Nature *336*, 688-690.
Smith, K.N., Singh, A.M., and Dalton, S. (2010). Myc represses primitive endoderm differentiation in pluripotent stem cells. Cell Stem Cell *7*, 343-354.
Snow, M.H. (1973). Tetraploid mouse embryos produced by cytochalasin B during cleavage. Nature *244*, 513-515.
Snow, M.H. (1975). Embryonic development of tetraploid mice during the second half of gestation. J Embryol Exp Morphol *34*, 707-721.
Snow, M.H.L. (1976). Embryo growth during the immediate postimplantation period. In Embryogenesis in Mammals CIBA Foundation Symposium 40 (new series) (Amsterdam, Elsevier), pp. 53-70.
Snow, M.H.L. (1977). Gastrulation in the mouse: growth and regionalization of the epiblast. J Embryol exp Morph *42*, 293-303.
Solter, D. (2006). From teratocarcinomas to embryonic stem cells and beyond: a history of embryonic stem cell research. Nat Rev Genet *7*, 319-327.
Solter, D., Skreb, N., and Damjanov, I. (1970). Extrauterine growth of mouse egg-cylinders reults in malignant teratoma. Nature *227*, 503-504.
Song, J., Oh, S.P., Schrewe, H., Nomura, M., Lei, H., Okano, M., Gridley, T., and Li, E. (1999). The type II activin receptors are essential for egg cylinder growth, gastrulation, and rostral head development in mice. Dev Biol *213*, 157-169.
Song, X., Jin, P., Qin, S., Chen, L., and Ma, F. (2012). The evolution and origin of animal Toll-like receptor signalling pathway revealed by network-level molecular evolutionary analyses. PLoS One *7*, e51657.

Spruce, T., Pernaute, B., Di-Gregorio, A., Cobb, B.S., Merkenschlager, M., Manzanares, M., and Rodriguez, T.A. (2010). An early developmental role for miRNAs in the maintenance of extraembryonic stem cells in the mouse embryo. Dev Cell *19*, 207-219.
Srinivas, S. (2006). The anterior visceral endoderm-turning heads. Genesis *44*, 565-572.
Srinivas, S., Rodriguez, T., Clements, M., Smith, J.C., and Beddington, R.S. (2004). Active cell migration drives the unilateral movements of the anterior visceral endoderm. Development *131*, 1157-1164.
Stanton, B.R., Perkins, A.S., Tessarollo, L., Sassoon, D.A., and Parada, L.F. (1992). Loss of N-myc function results in embryonic lethality and failure of the epithelial component of the embryo to develop. Genes Dev *6*, 2235-2247.
Stevens, L.C. (1983). Testicular, ovarian amd embryo-derived teratomas. Cancer Surveys *2*, 75-91.
Stower, M.J., and Srinivas, S. (2014). Heading forwards: anterior visceral endoderm migration in patterning the mouse embryo. Philos Trans R Soc Lond B Biol Sci *369*.
Strumpf, D., Mao, C.A., Yamanaka, Y., Ralston, A., Chawengsaksophak, K., Beck, F., and Rossant, J. (2005). Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst. Development *132*, 2093-2102.
Stuckey, D.W., Clements, M., Di-Gregorio, A., Senner, C.E., Le Tissier, P., Srinivas, S., and Rodriguez, T.A. (2011). Coordination of cell proliferation and anterior-posterior axis establishment in the mouse embryo. Development *138*, 1521-1530.
Suijkerbuijk, S.J., Kolahgar, G., Kucinski, I., and Piddini, E. (2016). Cell Competition Drives the Growth of Intestinal Adenomas in Drosophila. Curr Biol *26*, 428-438.
Sun, Q., Luo, T., Ren, Y., Florey, O., Shirasawa, S., Sasazuki, T., Robinson, D.N., and Overholtzer, M. (2014). Competition between human cells by entosis. Cell Res *24*, 1299-1310.
Sun, X., Meyers, E.N., Lewandoski, M., and Martin, G.R. (1999). Targeted disruption of Fgf8 causes failure of cell migration in the gastrulating mouse embryo. Genes Dev *13*, 1834-1846.
Tai, C.I., and Ying, Q.L. (2013). Gbx2, a LIF/Stat3 target, promotes reprogramming to and retention of the pluripotent ground state. J Cell Sci *126*, 1093-1098.
Takahashi, K., and Yamanaka, S. (2016). A decade of transcription factor-mediated reprogramming to pluripotency. Nat Rev Mol Cell Biol *17*, 183-193.
Takashima, Y., Guo, G., Loos, R., Nichols, J., Ficz, G., Krueger, F., Oxley, D., Santos, F., Clarke, J., Mansfield, W., *et al.* (2014). Resetting transcription factor control circuitry toward ground-state pluripotency in human. Cell *158*, 1254-1269.
Tallquist, M.D., and Soriano, P. (2000). Epiblast-restricted Cre expression in MORE **mice: a** tool to distinguish embryonic vs. extra-embryonic gene function. Genesis *26*, 113-115.
Tamori, Y., Bialucha, C.U., Tian, A.G., Kajita, M., Huang, Y.C., Norman, M., Harrison, N., **Poulton, J., Ivanovitch, K., Disch, L., *et al.*** (2010). Involvement of Lgl and Mahjong/VprBP in cell competition. PLoS Biol *8*, e1000422.
Tamori, Y., and Deng, W.M. (2013). Tissue repair through cell competition and compensatory cellular hypertrophy in postmitotic epithelia. Dev Cell *25*, 350-363.
Tanaka, S., Kunath, T., Hadjantonakis, A.K., Nagy, A., and Rossant, J. (1998). Promotion of trophoblast stem cell proliferation by FGF4. Science *282*, 2072-2075.
Tarkowski, A.K., Witkowska, A., and Opas, J. (1977). Development of cytochalasin in B-induced tetraploid and diploid/tetraploid mosaic mouse embryos. J Embryol Exp Morphol *41*, 47-64.

ten Dijke, P., Miyazono, K., and Heldin, C.H. (2000). Signalling inputs converge on nuclear effectors in TGF-beta signalling. Trends Biochem Sci *25*, 64-70.
Tesar, P.J., Chenoweth, J.G., Brook, F.A., Davies, T.J., Evans, E.P., Mack, D.L., Gardner, **R.L., and McKay, R.D.** (2007). New cell lines from mouse epiblast share defining features with human embryonic stem cells. Nature *448*, 196-199.
Teslaa, T., and Teitell, M.A. (2015). Pluripotent stem cell energy metabolism: an update. EMBO J *34*, 138-153.
Thomas, P., and Beddington, R. (1996). Anterior primitive endoderm may be responsible for patterning the anterior neural plate in the mouse embryo. Curr Biol *6*, 1487-1496.
Thompson, S., Clarke, A.R., Pow, A.M., Hooper, M.L., and Melton, D.W. (1989). Germline transmission and expression of a corrected HPRT gene produced by gene targeting in embryonic stem cells. Cell *56*, 313-321.
Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., and Jones, J.M. (1998). Embryonic stem cell lines derived from human blastocysts. Science *282*, 1145-1147.
Trichas, G., Joyce, B., Crompton, L.A., Wilkins, V., Clements, M., Tada, M., Rodriguez, T.A., and Srinivas, S. (2011). Nodal dependent differential localisation of dishevelled-2 demarcates regions of differing cell behaviour in the visceral endoderm. PLoS Biol *9*, e1001019.
Trichas, G., Smith, A.M., White, N., Wilkins, V., Watanabe, T., Moore, A., Joyce, B., Sugnaseelan, J., Rodriguez, T.A., Kay, D., *et al.* (2012). Multi-cellular rosettes in the mouse visceral endoderm facilitate the ordered migration of anterior visceral endoderm cells. PLoS Biol *10*, e1001256.
Tropepe, V., Hitoshi, S., Sirard, C., Mak, T.W., Rossant, J., and van der Kooy, D. (2001). Direct neural fate specification from embryonic stem cells: a primitive mammalian neural stem cell stage acquired through a default mechanism. Neuron *30*, 65-78.
Trumpp, A., Refaeli, Y., Oskarsson, T., Gasser, S., Murphy, M., Martin, G.R., and Bishop, J.M. (2001). c-Myc regulates mammalian body size by controlling cell number but not cell size. Nature *414*, 768-773.
Tsakiridis, A., Huang, Y., Blin, G., Skylaki, S., Wymeersch, F., Osorno, R., Economou, C., Karagianni, E., Zhao, S., Lowell, S., *et al.* (2014). Distinct Wnt-driven primitive streak-like populations reflect in vivo lineage precursors. Development *141*, 1209-1221.
Turner, N., and Grose, R. (2010). Fibroblast growth factor signalling: from development to cancer. Nat Rev Cancer *10*, 116-129.
Tyler, D.M., Li, W., Zhuo, N., Pellock, B., and Baker, N.E. (2007). Genes affecting cell competition in Drosophila. Genetics *175*, 643-657.
Uhlirova, M., and Bohmann, D. (2006). JNK- and Fos-regulated Mmp1 expression cooperates with Ras to induce invasive tumors in Drosophila. EMBO J *25*, 5294-5304.
Vallier, L., Alexander, M., and Pedersen, R.A. (2005). Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells. J Cell Sci *118*, 4495-4509.
van Echten-Arends, J., Mastenbroek, S., Sikkema-Raddatz, B., Korevaar, J.C., Heineman, M.J., van der Veen, F., and Repping, S. (2011). Chromosomal mosaicism in human preimplantation embryos: a systematic review. Hum Reprod Update *17*, 620-627.
Varlet, I., Collignon, J., Norris, D.P., and Robertson, E.J. (1997). Nodal signalling and axis formation in the mouse. Cold Spring Harb Symp Quant Biol *62*, 105-113.

Verdu, J., Buratovich, M.A., Wilder, E.L., and Birnbaum, M.J. (1999). Cell-autonomous regulation of cell and organ growth in Drosophila by Akt/PKB. Nat Cell Biol *1*, 500-506.
Vestweber, D., Gossler, A., Boller, K., and Kemler, R. (1987). Expression and distribution of cell adhesion molecule uvomorulin in mouse preimplantation embryos. Dev Biol *124*, 451-456.
Villa Del Campo, C., Claveria, C., Sierra, R., and Torres, M. (2014). Cell competition promotes phenotypically silent cardiomyocyte replacement in the Mammalian heart. Cell Rep *8*, 1741-1751.
Vincent, J.P., Fletcher, A.G., and Baena-Lopez, L.A. (2013). Mechanisms and mechanics of cell competition in epithelia. Nat Rev Mol Cell Biol *14*, 581-591.
Vincent, J.P., Kolahgar, G., Gagliardi, M., and Piddini, E. (2011). Steep differences in wingless signalling trigger Myc-independent competitive cell interactions. Dev Cell *21*, 366-374.
Vincent, S.D., Dunn, N.R., Hayashi, S., Norris, D.P., and Robertson, E.J. (2003). Cell fate decisions within the mouse organizer are governed by graded Nodal signals. Genes Dev *17*, 1646-1662.
Viotti, M., Nowotschin, S., and Hadjantonakis, A.K. (2014). SOX17 links gut endoderm morphogenesis and germ layer segregation. Nat Cell Biol *16*, 1146-1156.
Vivarelli, S., Wagstaff, L., and Piddini, E. (2012). Cell wars: regulation of cell survival and proliferation by cell competition. Essays Biochem *53*, 69-82.
Wagstaff, L., Goschorska, M., Kozyrska, K., Duclos, G., Kucinski, I., Chessel, A., Hampton-O'Neil, L., Bradshaw, C.R., Allen, G.E., Rawlins, E.L., *et al.* (2016). Mechanical cell competition kills cells via induction of lethal p53 levels. Nat Commun *7*, 11373.
Wakioka, T., Sasaki, A., Kato, R., Shouda, T., Matsumoto, A., Miyoshi, K., Tsuneoka, M., Komiya, S., Baron, R., and Yoshimura, A. (2001). Spred is a Sprouty-related suppressor of Ras signalling. Nature *412*, 647-651.
Waldrip, W.R., Bikoff, E.K., Hoodless, P.A., Wrana, J.L., and Robertson, E.J. (1998). Smad2 signalling in extraembryonic tissues determines anterior-posterior polarity of the early mouse embryo. Cell *92*, 797-808.
Wang, H., and Dey, S.K. (2006). Roadmap to embryo implantation: clues from mouse models. Nat Rev Genet *7*, 185-199.
Wiley, L.M., and Eglitis, M.A. (1981). Cell surface and cytoskeletal elements: cavitation in the mouse preimplantation embryo. Dev Biol *86*, 493-501.
Williams, R.L., Hilton, D.J., Pease, S., Willson, T.A., Stewart, C.L., Gearing, D.P., Wagner, E.F., Metcalf, D., Nicola, N.A., and Gough, N.M. (1988). Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. Nature *336*, 684-687.
Winnier, G., Blessing, M., Labosky, P.A., and Hogan, B.L. (1995). Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. Genes Dev *9*, 2105-2116.
Woods, D.F., and Bryant, P.J. (1991). The discs-large tumor suppressor gene of Drosophila encodes a guanylate kinase homolog localized at septate junctions. Cell *66*, 451-464.
Wray, J., Kalkan, T., Gomez-Lopez, S., Eckardt, D., Cook, A., Kemler, R., and Smith, A. (2011). Inhibition of glycogen synthase kinase-3 alleviates Tcf3 repression of the pluripotency network and increases embryonic stem cell resistance to differentiation. Nat Cell Biol *13*, 838-845.

Yamaguchi, K., Shirakabe, K., Shibuya, H., Irie, K., Oishi, I., Ueno, N., Taniguchi, T., Nishida, E., and Matsumoto, K. (1995). Identification of a member of the MAPKKK family as a potential mediator of TGF-beta signal transduction. Science *270*, 2008-2011.
Yamanaka, Y., Lanner, F., and Rossant, J. FGF signal-dependent segregation of primitive endoderm and epiblast in the mouse blastocyst. Development *137*, 715-724.
Yamanaka, Y., Lanner, F., and Rossant, J. (2010). FGF signal-dependent segregation of primitive endoderm and epiblast in the mouse blastocyst. Development *137*, 715-724.
Ye, S., Li, P., Tong, C., and Ying, Q.L. (2013). Embryonic stem cell self-renewal pathways converge on the transcription factor Tfcp2l1. EMBO J *32*, 2548-2560.
Ying, Q.L., Nichols, J., Chambers, I., and Smith, A. (2003a). BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. Cell *115*, 281-292.
Ying, Q.L., Stavridis, M., Griffiths, D., Li, M., and Smith, A. (2003b). Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. Nat Biotechnol *21*, 183-186.
Ying, Q.L., Wray, J., Nichols, J., Batlle-Morera, L., Doble, B., Woodgett, J., Cohen, P., and Smith, A. (2008). The ground state of embryonic stem cell self-renewal. Nature *453*, 519-523.
Yokota, Y., and Mori, S. (2002). Role of Id family proteins in growth control. J Cell Physiol *190*, 21-28.
Yoshida, K., Chambers, I., Nichols, J., Smith, A., Saito, M., Yasukawa, K., Shoyab, M., Taga, T., and Kishimoto, T. (1994). Maintenance of the pluripotential phenotype of embryonic stem cells through direct activation of gp130 signalling pathways. Mech Dev *45*, 163-171.
Yu, T., Yaguchi, Y., Echevarria, D., Martinez, S., and Basson, M.A. (2011). Sprouty genes prevent excessive FGF signalling in multiple cell types throughout development of the cerebellum. Development *138*, 2957-2968.
Zeng, L., Fagotto, F., Zhang, T., Hsu, W., Vasicek, T.J., Perry, W.L., 3rd, Lee, J.J., Tilghman, S.M., Gumbiner, B.M., and Costantini, F. (1997). The mouse Fused locus encodes Axin, an inhibitor of the Wnt signalling pathway that regulates embryonic axis formation. Cell *90*, 181-192.
Zhang, H., and Bradley, A. (1996). Mice deficient for BMP2 are nonviable and have defects in amnion/chorion and cardiac development. Development *122*, 2977-2986.
Zhou, W., Choi, M., Margineantu, D., Margaretha, L., Hesson, J., Cavanaugh, C., Blau, C.A., Horwitz, M.S., Hockenbery, D., Ware, C., *et al.* (2012). HIF1alpha induced switch from bivalent to exclusively glycolytic metabolism during ESC-to-EpiSC/hESC transition. EMBO J *31*, 2103-2116.
Ziosi, M., Baena-Lopez, L.A., Grifoni, D., Froldi, F., Pession, A., Garoia, F., Trotta, V., Bellosta, P., and Cavicchi, S. (2010). dMyc functions downstream of Yorkie to promote the supercompetitive behavior of hippo pathway mutant cells. PLoS Genet *6*, e1001140.

8. APPENDIX

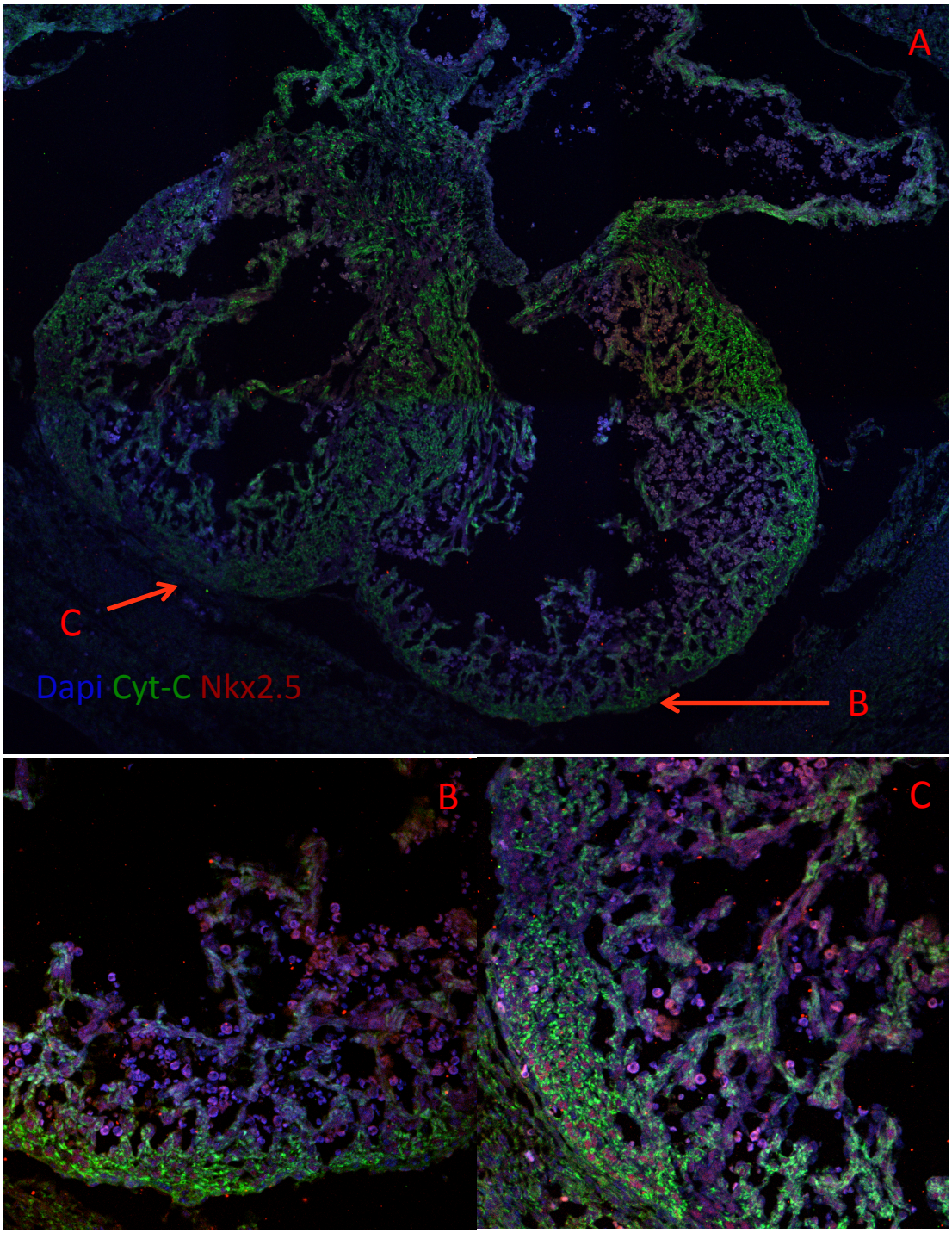


Figure 8 Nkx2.5 Immuno-staining pattern of Nkx2.5 in a Hccs heart at 14.5 dpc.
A) Confocal image of a whole heart including part of the atria at magnification 10X' green indicates cytochrome C, red is Nkx2.5 stained and blue is DAPI-stained nuclei. BC) 20x magnification showing nuclei stain of Nkx2.5 heart